Comparison of mechanical sorting and DNA metabarcoding for diet analysis with fresh and degraded wolf scats

AIMEE L. MASSEY,† GRETCHEN H. ROFFLER,‡ TESSA VERMEUL, JENNIFER M. ALLEN, and TAAL LEVI

1Department of Fisheries and Wildlife, Oregon State University, Corvallis, Oregon 97331 USA
2Alaska Department of Fish and Game, Division of Wildlife Conservation, P.O. Box 110024, Juneau, Alaska 99811 USA

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Abstract. DNA metabarcoding has become a powerful technique for identifying the species present in a bulk environmental sample. The application of DNA metabarcoding to wildlife diet analysis is a particularly promising tool for exploring trophic interactions. The extent to which molecular approaches agree with traditional approaches, and how this varies with the quality of field-collected scats, is unknown. Here, we compare diets from wolf scats profiled using both mechanical sorting and metabarcoding of amplified vertebrate DNA sequences. Our objectives were to (1) compare findings from mechanical sorting and metabarcoding as a method of diet profiling and (2) use results to better understand diets of wolves on Prince of Wales Island, a population of conservation concern. We predicted metabarcoding would reveal both higher diversity of prey and identify rare species that are overlooked with mechanical sorting. We found that there was substantial overlap in the diets revealed using both methods, indicating that deer, beaver, and black bear were the primary prey species, but metabarcoding revealed a more diverse diet with greater occurrence of rare species. However, there was a large discrepancy in the occurrence of beaver in scats (52% and 25% from mechanical sorting and metabarcoding, respectively) explained by the high rate of false positives with mechanical sorting methods. While the number of wolf sequence reads for fresh scats was nearly eight times higher than in degraded scats, neither the number of prey sequence reads nor the quantity of DNA to be sequenced varied between fresh and degraded scats suggesting that metabarcoding is sensitive enough to determine prey assemblages in degraded scats. Even using scats from extremely wet conditions hostile to DNA preservation, we found that metabarcoding was more effective than mechanical sorting in describing diet.

Key words: Alaska; Canis lupus; diet analysis; eDNA; metabarcoding; noninvasive sampling; Prince of Wales Island; scat; temperate rain forest; wolves.

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† E-mail: aimeelmassey@gmail.com

INTRODUCTION

Diet analysis of carnivores plays a pivotal role in ecological study and wildlife management as it can help elucidate the magnitude and direction of trophic interactions. Animal scats are the most widely used tool for answering scientific questions related to animal diet. Traditionally, scat-based diet analysis has relied upon the mechanical processing and sorting of scat remains (Lockie 1959, Leopold and Krausman 1986, Spaulding et al. 2000, Klare et al. 2011). This typically requires removing fecal material followed by meticulous sorting and identification...
of remaining hair and hard parts. However, diet analysis with mechanical sorting of scats has well-known biases (Reynolds and Aebischer 1991, Ciucci et al. 1996, Lake et al. 2003); in particular, rare species or species that lack non-digestible hard parts are often overlooked or misidentified (Spaulding et al. 2000). In addition, some species are challenging to distinguish based on hard parts leading to additional misidentification (Zeale et al. 2011).

Metabarcoding of fecal DNA presents an alternative method for diet analysis (Shehbaz et al. 2012, De Barba et al. 2014, Kartzinel et al. 2015, Eriksson et al. 2019, Thuo et al. 2020, McInnes et al. 2017). The DNA metabarcoding workflow includes extracting DNA from environmental samples, DNA amplification using universal primers (Binladen et al. 2007), and mass-parallel sequencing of amplified product using next-generation sequencing technologies. This process allows DNA barcodes from multiple species in a bulk sample to be sequenced simultaneously for a profile of the species present within an environmental sample (Valentini et al. 2009).

Accurate taxonomic assignment and detection are paramount for informing management objectives. Thus, the utility of metabarcoding has been uncertain because unlike mechanical sorting (1) it is not yet clear whether the relative read abundance (the number of DNA sequence reads for a species in a sample divided by the total number of DNA sequence reads in that sample) from metabarcoding can yield quantitative information that approximates the volume or biomass arising from each prey species and (2) it is unknown how quality of inference from metabarcoding depends on acquiring relatively fresh scats with minimally degraded DNA, which can be challenging for rare taxa. The degree to which relative read abundance (RRA) from DNA metabarcoding is correlated with the relative biomass of each prey species is a subject of substantial debate (Deagle et al. 2019, Pinol et al. 2019). Limited empirical research validating RRA against estimated biomass or volume from mechanical sorting has been informative (Soininen et al. 2009, Thomas et al. 2017), although no studies have done so with terrestrial carnivores. Pinol et al. (2019) argued that metabarcoding results can only be interpreted quantitatively if amplification of DNA through PCR with universal primers is avoided because different amplification efficiencies among species can lead to poor representation of original biomass proportions. However, some evidence suggests that as long as primer efficiency is high (no mismatches), the proportion of sequences arising from each species in metabarcoding (RRA) can produce semi-quantitative results (Thomas et al. 2016, Krehenwinkel et al. 2017, Deagle et al. 2019). This could allow metabarcoding to approximate relative biomass or volume information similar to that produced by mechanical sorting of hard parts as well as frequency of occurrence (proportion of scats that contain each species). Nevertheless, the degree to which degraded scats yield suitable inference comparable to mechanical sorting is not currently well understood because of a lack of formal comparisons between metabarcoding and mechanical sorting (Deagle et al. 2019, Pinol et al. 2019).

To provide this methods comparison, we focused on the Alexander Archipelago wolf (Canis lupus ligoni) as a case study. The Alexander Archipelago wolf is a subspecies of the gray wolf (Canis lupus) and has been repeatedly petitioned for listing as threatened under the U.S. Endangered Species Act (ESA). The wolves on Prince of Wales Island (POW; Fig. 1) were of particular concern in the most recent assessment (2015) because they occur in relative geographic isolation and face continued pressure from high wolf harvest. In addition to high levels of wolf harvest, POW has the highest rate of old-growth logging in Southeast Alaska (Albert and Schoen 2013, Person and Brinkman 2013). The rate of clear-cut logging in this region peaked during the late 1980s and 1990s and while this rate has slowed in recent years, a total of nearly 30% of old-growth forests have been logged on POW (U.S. Fish and Wildlife Service 2015). Deer populations are predicted to decline as old-growth forests with palatable understory forbs and shrubs are converted into dense, even-aged, closed-canopy forests (Alaback 1982, Schoen et al. 1988, Person 1996, Farmer and Kirchhoff 2007, Gilbert et al. 2015, Person and Brinkman 2013, Porter 2018) that are strongly avoided by deer (Wallmo and Schoen 1980, Kohira and Rextad 1997, Gilbert et al. 2017). Given that Sitka black-tailed deer (Odocoileus hemionus sitkensis) are the primary prey of wolves in the southern islands of
Southeast Alaska (Kohira 1995, Person 1996, Kohira and Rextad 1997, Roffler et al. 2021), the predicted declines of deer have raised concerns about the future of the wolf population in this region with the assumption that wolf abundance is closely linked to that of deer. This is evident in the most recent ESA species status review where deer habitat quality metrics were used to project wolf abundance at a regional scale (U.S. Fish and Wildlife Service 2015).

Wolf populations are predicted to decline more severely under scenarios where alternate prey are less available necessitating wolves rely primarily on deer (Gilbert et al. 2015). However, mechanical sorting of wolf scats has revealed other prey in significant quantities (Kohira 1995),

![Study area map showing Alexander Archipelago in Southeast Alaska. Red and yellow points represent individual wolf scat collection sites (scats were collected from 2014 to 2015). Most scats collections were concentrated on Prince of Wales Island and surrounding islands (yellow points).]
and salmon are seasonally available during late summer and fall and other marine resources year-round (Szepanski et al. 1999, Darimont et al. 2003, 2004, Darimont et al., 2008a, Lafferty et al. 2014), suggesting that wolf population abundance may also be dictated by the availability of prey other than deer. Consequently, refining knowledge regarding the diet of wolves in the system has important implications for wolf management, potential ESA considerations, and forest management in Southeast Alaska.

Here, we provide a formal comparison of carnivore diet analysis from mechanical sorting and DNA metabarcoding using opportunistically collected scats across a perceived degradation spectrum in a temperate rain forest hostile to DNA preservation. Using scats that have each been processed with both mechanical sorting and DNA metabarcoding methods, we examine whether metabarcoding reveals a more diverse wolf diet, achieves increased taxonomic precision, and identifies infrequently consumed prey species. We include both scats appearing highly degraded and those appearing fresh and assess whether the degradation of scats affects the diet profile shown by metabarcoding. Finally, we use DNA metabarcoding to provide a current diet profile for wolves on Prince of Wales Island.

Materials and Methods

Study area and field collection

Southeast Alaska lies within the Alexander Archipelago composed of over 2000 named islands (Fig. 1; Cook et al. 2006). This region receives between 130 and 400 cm of precipitation annually (Shanley et al. 2015) thus making it particularly inhospitable to the preservation of DNA in exposed environmental samples. The mainland is buttressed by the rugged Coast Mountains and extensive temperate rain forests at lower elevations. As a result of natural fragmentation and isolation, the North Pacific coast region supports many endemic plant and animal lineages, particularly on Prince of Wales Island, the largest island in the archipelago (Cook et al. 2006, MacDonald and Cook 2007, Smith et al. 2016). Most of the forested area is within the Tongass National Forest managed by the U.S. Forest Service. This ecosystem hosts a diversity of mammals including iconic species such as Sitka black-tailed deer (O. hemionus sitkensis), American black bear (Ursus americanus), North American beaver (Castor canadensis), American marten (Martes americana), mountain goat (Oreamnos americanus), Steller sea lion (Eumetopias jubatus), harbor seal (Phoca vitulina), and moose (Alces alces). Species distribution and assemblages vary among island and mainland areas of this region.

Wolf scats were collected along wolf travel routes, near den sites, and on secondary roads by Alaska Department of Fish and Game personnel during planned scat collection surveys from October 2014 to December 2015. Scats were primarily collected on Prince of Wales Island (55°46‘45.9480″ N, 132°49‘4.7748″ W), but also opportunistically collected from other mainland and island systems for a total of 183 scats collected (of which 145 scats were collected from GMU 2Z [POW and surrounding islands], Fig. 1). Scats were separated by field personnel into two categories: fresh and degraded (Appendix S1: Fig. S1). Whether scats were categorized as fresh or degraded was determined at the time of collection based on appearance, time since last site visit (Ciucci et al. 1996), and exposure time considering that scats decompose rapidly in rain forest environments (Wallmo et al. 1962, Ciucci et al. 1996, Darimont et al. 2008b). Collected wolf scats were stored in plastic bags, labeled with location, date, and either a fresh or degraded status, and then frozen (−20°C). Frozen scats were shipped to Oregon State University for sample preparation and analysis.

Mechanical sorting

Of the 183 total scats, 129 were processed using mechanical sorting methods. We stored a subsample of each scat for later molecular analysis (sterilized forceps and razors were used to collect a sample from the middle section of each scat to minimize wolf DNA; Stenglein et al. 2010), and then placed each scat in a mesh bag (1/8″) and soaked it in water for 48 h in a mason jar. We power-washed the scat to remove as much remaining fecal matter as possible. The remaining contents (i.e., hair, bones, other hard parts) were dried at 50°C. We weighed the processed scat material (hair, bone, scale, feather, etc.) and mechanically homogenized and sorted the remains by hand. On average, the fine-scale sorting took 3.6 h per scat. We examined hairs
under a microscope and compared with hair samples from the Alaska Fur ID project (Carrlee and Horelick 2011). We made slide mounts using clear nail polish to examine scale pattern and medulla diameter in order to identify species. Following identification, the slide was labeled with the species name and sample of origin. We attempted to identify all other hard parts present in the scats (feathers, bones, teeth) using reference specimens found online for species typical to Southeast Alaska (iDigBio.org; Matsunaga et al. 2013, Page et al. 2015). This exhaustive fin-scale sorting (Appendix S1: Fig. S2) was used to increase the probability that rare species present in a scat could be identified. We estimated the volume of each prey species as a proportion of estimated hard parts for a species in relation to all hard parts in an individual scat.

**Molecular analysis**

All 183 scats were analyzed using molecular methods. Using the stored subsamples from each scat, we extracted DNA from each sample (200–500 mg) using the Qiagen DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) with slight modifications as follows: 500 μL Buffer ATL, 50 μL Proteinase K, and 1.0 mm Zirconia/Silica beads (BioSpec Products, Bartlesville, Oklahoma, USA) were added to the 1.7-mL tube containing the scat subsample. Samples were vortexed for 10 min at maximum speed prior to incubation at 56°C for 4–6 h. The DNA was eluted in a total volume of 100 μL. A negative control was extracted with each round (approximately 17 samples) of DNA extraction to identify possible cross-contamination.

Following DNA extraction, each sample was amplified in three separate reactions using a minor modification to the primer pair 12Sv5F/12Sv5R (Riaz et al. 2011). We used the reverse primer 12Sv5R (TTAGATACCCCACTATGC) as Riaz et al. (2011) and a modified version (we change the thymine to a degenerate base shown underlined) of the forward primer 12Sv5F to allow for broader binding of vertebrate targets (YAGAACAGGCTCTCCTTAG). These primers target approximately 100 base pairs in the 12S region of the vertebrate mitochondrial genome. These commonly used 12S mtDNA primers rarely contain base pair (bp) mismatches for vertebrates and contain no mismatches for the prey taxa considered here (Appendix S1: Fig. S3). The initial PCR was carried out using AmpliTaq Gold 360 Master Mix (Life Technologies, Carlsbad, California, USA). To label samples for multiplexing, we used 384 unique 8 bp dual matching indexes on the forward and reverse primers to eliminate contamination due to tag jumping by filtering reads that did not have identical indexes, and we included 3 bp of random nucleotides on the 5’ end to increase sequence diversity and prevent degradation of indexes during subsequent blunt-ending and ligation steps. PCRs were carried out in a volume of 20 μL with 10 μL AmpliTaq Gold 360 Master Mix for a final concentration of 1X, 5 μL of forward and reverse primers for a final concentration of 0.25 μM, 3 μL of water, and 2 μL of DNA template. PCR cycling included initial denaturing at 95°C for 10 min, followed by 40 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 7 min.

After the initial PCR, all PCR amplicons were cleaned using PCRClean DX solid-phase reversible immobilization magnetic beads (Aline Biosciences, Woburn, Massachusetts, USA). Each PCR was quantified using Accublue High Sensitivity dsDNA Quantitation kit (Biotium, Fremont, California, USA) and normalized to 6 ng/μL. Each group of 384 PCR products was then pooled into a single library, for a total of three libraries. Individual libraries were then tagged with an additional 6 bp identifying index using the NEBNext Ultra II DNA Library Prep kit (New England Biolabs, Ipswich, Massachusetts, USA). Pooled samples were analyzed on a Bioanalyzer to confirm fragment size. The libraries were then sequenced on one lane of Illumina HiSeq 3000 2 × 150 bp PE at the Center for Genome Research and Biocomputing at Oregon State University.

**Sequence analysis**

Raw sequence reads were analyzed using a bioinformatics pipeline (see Appendix S1: Text S1 for details) designed to trim and sort the sequence reads according to scat sample identification. An outline of the bioinformatic process is as follows: (1) raw reads were paired using PEAR (Paired-End reAd merger; Zhang et al. 2014); (2) followed by demultiplexing using 8 bp index sequences unique to each sample PCR replicate (mismatches discarded); (3) lastly,
sequences from each sample were clustered by 100% similarity and taxonomically assigned using BLAST (basic local alignment search tool) against 12S vertebrate sequences in GenBank and from a custom 12S database that contains sequences of some Pacific Northwest birds and small mammals not yet present in GenBank.

Similar to the step-wise methods used by De Barba et al. (2014), a series of filtering and quality control measures were carried out on taxonomically assigned sequences. We initially removed sequences that were identified to be Canis spp. and contaminants based on read counts in no-template controls (which contained primarily human contamination). We then removed sample replicates that clearly failed to amplify during PCR which included sample replicates with fewer than a total of 400 sequence reads. We compared taxonomic assignments with known fauna of Southeast Alaska (MacDonald and Cook 2007) to replace non-regional species identified with BLAST with closely related regional taxa. We then excluded prey items occurring in fewer than two of the three PCR replicates. Finally, we combined those sample replicates that amplified so that sequence reads were totaled for each species within a sample and over the entire sample and eliminated sequences that appeared in less than 1% of the total reads for an individual sample.

**Data analyses**

All statistical analyses were conducted in R (R Core Team 2019). There were marked differences between the appearance and quality of scats (Appendix S1: Fig. S1) based on the perceived freshness (classified as either fresh or degraded) of each scat determined by scat collectors during fieldwork. Because fresh scats yield higher genotyping success, researchers might assume that fresh scats are also needed for quality metabarcoding results. To test whether this assumption holds true for diet analysis using metabarcoding, we performed Mann-Whitney tests to determine whether fresh and degraded scats yielded a different number of total wolf sequence reads, total prey sequence reads, or average quantity of DNA (ng/μL) in a sample (measured post normalization using Accublue High Sensitivity dsDNA Quantitation kit; Biotium, Fremont, California, USA). We used both frequency of occurrence (FOO) and metrics of relative abundance (see below) to describe the occurrence of prey in wolf diet. FOO was calculated to determine which prey species were present and how often they were present based on the number of samples. For mechanical sorting methods, a species was present if there was evidence (including trace elements) of a prey species (e.g., hair, bone, scales, etc.) within a scat sample. FOO was then calculated as the proportion of scats in which a prey species occurred. For metabarcoding, a species occurrence was determined by whether sequence reads for a particular species were found in an individual scat after quality control measures. To visualize and compare the diversity revealed by metabarcoding and mechanical sorting, we constructed heat trees based on taxonomy using the metacoder package in R (Foster et al. 2017). FOO determined the size and color of each node with larger and darker nodes indicating species with higher occurrence and lighter and smaller nodes indicating species with lesser occurrence.

To test whether metabarcoding and mechanical sorting yield similar metrics for relative abundance of a prey species within a scat, we compared estimated volume (the proportion of hard parts from a prey species per scat relative to the whole scat) from mechanical sorting with the relative read abundance (RRA) from metabarcoding using simple linear regression. RRA for each species $i$ was calculated as

$$RRA_i = \frac{1}{S} \sum_{k=1}^{S} \frac{n_{i,k}}{\sum_{i=1}^{T} n_{i,k}}$$

where $n_{i,k}$ is the number of sequences of prey species $i$ in sample $k$, $S$ is the total number of samples, and $T$ is the total number of species. Because the assumption of normally distributed residuals is challenging to meet with a response variable bounded between 0 and 1, we additionally conducted linear regression with logit-transformed data for improved parameter estimation but present the untransformed data in the main text to facilitate a straightforward presentation of the observed data.

To explore mismatches in species assignments between the methods, we took two approaches. First, to analyze discrepancies between species present in samples with mechanical sorting and
not found with metabarcoding, we used logistic regression with a logit link to explore whether false positives from mechanical sorting or false negatives generated from metabarcoding best explained the absence of species. We reasoned that false negatives could arise from metabarcoding if scats contained poor-quality DNA or if sequencing depth was insufficient. We therefore fit three separate logistic regression models using average DNA quantity per sample (across the three replicates PCRs), total number of sequence reads prior to quality control and including wolf sequence reads, and total number of sequences reads post-quality control and not including wolf sequences reads as univariate predictors in each model. We performed these three models for all species included and then again for just beaver (due to the large difference in beaver frequency of occurrence results between mechanical sorting and metabarcoding). In our analysis, zeroes were defined as an absence in metabarcoding where mechanical sorting had indicated an occurrence of a particular species in a sample; one indicated that metabarcoding was in agreement with occurrence found in mechanical sorting. Therefore, positive coefficients imply an increasing rate of proper assignment as DNA quality or sequencing depth increases. The absence of such an effect would suggest that mismatch between metabarcoding and mechanical sorting is not due to poor-quality DNA but rather more likely due to false positives by mechanical sorting.

Secondly, we closely examined outlier scats resulting from the linear regressions of RRA (metabarcoding) and estimated volume (mechanical sorting) that showed the greatest disagreement between the abundance measures. This indicated whether metabarcoding clearly identified a species distinct to what was identified by mechanical sorting, and whether such misidentifications fit a general pattern (e.g., biases toward assigning difficult to identify hair as common species). The outlier scats for this analysis were defined by scats with a difference of two standard deviations above the mean difference between RRA and estimated volume proportions.

Prince of Wales Island

145 of the total 183 scats were collected on Princes of Wales and surrounding islands (GMU 2Z; highlighted in yellow on Fig. 1 and referred to as POW hereafter). To look at wolf diet diversity only on POW, we used DNA metabarcoding results from these POW scats (n = 108; reduced from 145 after quality control measures). Similar to the comparison analysis, we calculated FOO and RRA for each species to compare to previous diet studies on POW.

RESULTS

The number of paired sequence reads was 44,041,331 for the entire sample data set (183 scats). The average read depth per PCR replicate was 80,221 reads with wolf reads included. After quality control steps, the final metabarcoding data set used for diet analysis had 25,598,803 sequence reads from 138 samples with an average read depth per PCR replicate of 48,688 reads. A random subset of the 183 total scats (n = 129) were processed and sorted using mechanical methods. Prior to subsetting the data for the comparison analysis, metabarcoding revealed a total of 24 taxa while mechanical sorting revealed 14 taxa (refer to Appendix S1: Table S1).

Fresh vs degraded scats

Mann-Whitney tests revealed no significant difference between the number of prey sequence reads for fresh vs degraded scats (Fig. 2; median_degraded = 151,305; median_fresh = 95,886; W = 881; P = 0.50). There was also no significant difference in the DNA quantity post-PCR for fresh and degraded scats (Fig. 2; median_degraded = 4.072 ng/µL; median_fresh = 4.06 ng/µL; W = 1050; P = 0.47). However, the number of wolf reads for fresh scats was nearly eight times higher than in degraded scats (Fig. 2; median_degraded = 16,717; median_fresh = 129,010; W = 1346; P < 0.01).

Comparing wolf diet by mechanical sorting and metabarcoding

We compared wolf diet from the 104 scat samples that were analyzed with both mechanical sorting and metabarcoding. Metabarcoding revealed a number of rare species that were not found using mechanical sorting methods and thus revealed greater dietary diversity (Fig. 3). Species that were found with metabarcoding methods but were absent when using mechanical sorting methods include the following: duck...
(Anas spp.), dusky grouse (Bonasa umbellus), elk (Cervus elaphus), raven (Corvus corax), Northern collared lemming (Dicrostonyx groenlandicus), sea otter (Enhydra lutris), Steller sea lion (E. jubatus), American marten (Martes americana), and American red squirrel (Tamiasciurus hudsonicus). Mechanical methods identified moose (A. alces) in a single scat where metabarcoding did not, although moose was identified by metabarcoding in this particular scat prior to quality filtering. We also found evidence of wolf hard parts in one scat sample suggesting part of a wolf was digested by the wolf defecator, which could result from a wolf killing another wolf during territorial defense (Marhenke 1971, Cassidy et al. 2015).
Frequency of occurrence (FOO; Figs. 3 and 4) results were qualitatively similar among both methods. However, there was substantial discrepancy between the primary prey species (Sitka black-tailed deer) and the secondary prey species (beaver) between metabarcoding and mechanical occurrence results. The occurrence of deer was greater in the mechanical sorting results (FOO_{mech} = 0.96) compared to metabarcoding results (FOO_{MB} = 0.85; Fig. 4), and the occurrence of beaver was twice as frequent in the mechanical sorting (FOO_{mech} = 0.52) results compared with metabarcoding (FOO_{MB} = 0.25; Fig. 4).

There was minimal discrepancy between RRA of primary prey species (metabarcoding) and their estimated volume in scats (mechanical sorting; Fig. 4); the difference between RRA and estimated volume for deer was 3% (RRA_{deer} = 69.2%; estimated volume_{deer} = 66.3%) and for beaver it was less than 6% (RRA_{beaver} = 14.8%; estimated volume_{beaver} = 20.6%). For the rarer species, we found a close association (within 2%) between the RRA and the estimated volume for that species.

The estimated volume from mechanical sorting showed a positive but variable correlation with RRA of deer ($\beta = 0.54; R^2 = 0.28; P < 0.001, n = 87$), beaver ($\beta = 0.52; R^2 = 0.29; P < 0.01, n = 25$), and all other species ($\beta = 0.66; R^2 = 0.50; P < 0.001, n = 21$; Fig. 5; see Appendix S1: Fig. S4 for logit-transformed regressions). Further investigation of the outliers revealed that the substantial variability was likely due to species misidentification by mechanical sorting such as deer hair falsely identified as beaver (highlighted in Fig. 5 and see below). With outliers removed, the linear relationship between estimated volume and RRA became stronger and with more variance explained for both deer ($\beta = 0.79; R^2 = 0.62; P < 0.001, n = 79$) and beaver ($\beta = 0.73; R^2 = 0.48; P < 0.001, n = 23$; Fig. 5). However, much of this relationship is driven by samples with very low or very high RRA, and there were few intermediate points with both moderate RRA and percent volume.

**Mismatches between methods**

Logistic regressions to assess mismatches between metabarcoding and mechanical sorting revealed that neither average DNA quantity, total sequence reads, nor total sequence reads of prey (i.e., excluding wolf) were associated with failing to detect species that were identified by metabarcoding (Table 1). However, contrary to predictions, increasing number of prey sequence reads (i.e., excluding wolves) was associated with increasing mismatch with beaver occurrences detected by mechanical sorting ($P = 0.019$). Because a stronger signal in the metabarcoding data set was more likely to be associated with mismatch among the two methods, this mismatch is very likely caused by misassignment by mechanical sorting for beaver rather than by metabarcoding. Closer inspection revealed that a substantial number of definitive deer occurrences (i.e., high relative read abundance for deer) were mistakenly assigned to beaver by mechanical sorting (Fig. 5), further suggesting that mismatch between methods was due to misassignment by mechanical sorting.

**Prince of Wales Island**

Metabarcoding of scats found only within POW ($n = 108$; Fig. 6) revealed 15 species with Sitka black-tailed deer (FOO = 0.85; RRA = 0.74), beaver (FOO = 0.23; RRA = 0.15), and black bear (FOO = 0.16; RRA = 0.06) as the most common prey items (Fig. 6). Other common prey species were salmon (Oncorhynchus spp.; FOO = 0.056; RRA = 0.03) and North American river otter (Lontra canadensis; FOO = 0.037; RRA = 0.03). Less frequent or abundant prey species include marten (Martes americana), bald eagle (Haliaeetus leucocephalus), American red squirrel (Tamiasciurus hudsonicus), deermouse (Peromyscus spp.), vole (Microtus spp.), bald eagle (Haliaeetus leucocephalus), American red squirrel (Tamiasciurus hudsonicus), deermouse (Peromyscus spp.), vole (Microtus spp.), and unidentified bird species.

**Discussion**

Our results suggest that excluding purportedly degraded scats from DNA metabarcoding analyses does not substantively improve inference about carnivore diet. While perceived fresh scats contained a much greater number of reads per scat when including wolf sequence reads, which we surmise was due to wolf DNA concentrated in the outer scat and fecal matrix that washed away and degraded with exposure and time, there was no significant difference in the number of prey sequence reads between perceived fresh
Fig. 4. Diverging bar plots displaying the (A) frequency of occurrence (FOO) of each species according to
and degraded scats. There was also no significant difference in the average DNA quantity; this is likely because fresh scats contained more fecal material relative to hair and bone, and total DNA quantity per sample is normalized prior to sequencing such that abundant wolf DNA leads to dilution of prey DNA. Importantly, these results suggest that metabarcoding is sensitive enough to determine prey assemblages in degraded scats, and thus scat collection and processing should not be predicated upon perceived scat quality.

Using both fresh and degraded scats, we found that FOO and RRA metrics were qualitatively similar among methods. Both mechanical sorting and metabarcoding agreed that Sitka black-tailed deer was the primary prey item, followed by beaver, and then black bear as suggested by previous research in this region (Kohira 1995, Kohira and Rextad 1997). However, we also found patterns of disagreement among the two methods. Both deer and beaver occurred substantially more frequently in mechanically sorted scats than in metabarcoded scats, and there was divergence in the detection of rare species with false negatives of rarer species more common with mechanical sorting.

We hypothesized that mismatches in species assignment between the methods could be due to low-quality DNA sequencing results from metabarcoding. However, low read count and DNA quantity were unrelated to the probability of mismatching species assignment. Instead, in the case of beaver identified by mechanical sorting, we found that mismatch was actually more likely in scats with high read numbers. In those scats with the greatest difference between RRA and estimated volume proportions, we found high RRA for deer by metabarcoding despite a high relative volume of beaver identified with mechanical sorting. Our explanation for the potential false positives of beaver generated from mechanical sorting is that relying on mechanical sorting of scats results in the overestimation of certain species due to observer bias. The previous study has found the mechanical sorting of scat remains can result in erroneous or biased results (Reynolds and Aebischer 1991, Spaulding et al. 2000). Spaulding et al. (2000) specifically found that observer bias was present in mechanical sorting results of wolf scats primarily attributed to differences in training provided to the observers and preexisting biases concerning the feeding ecology of wolves. We take this a step further and theorize that mechanical sorting can lead to a pattern of repeatedly mislabeling difficult to identify parts as one species (usually a commonly occurring species) rather than the true species due to biases propagated from early, erroneous assignments. A re-examination of the extensive notes taken by the trained personnel during the mechanical sorting process affirms this and revealed a pattern of uncertainty. In many of the mismatching scats, beaver was thought to be present but that the assignment was questionable, and notes from the sorters asserted that the unknown hair(s) could also be attributed to deer or black bear (the similarity between degraded beaver, deer, and black bear hair is shown in Appendix S1: Figure S5).

In addition to false positives of beaver in mechanical sorting, we also found evidence of potential false negatives in mechanical sorting. We hypothesize that this is due to lack of hard parts or difficult to distinguish hard parts for some of the rarer species. Given (1) the high confidence in metabarcoding efficacy from previous studies that used mock communities (Ford et al. 2016, Thomas et al. 2016, Deagle et al. 2019) and (2) evidence from previous work that found disagreement in diet resolution between mechanical sorting of scat and DNA barcoding methods (Newmaster et al. 2013, Granquist et al. 2018), we conclude that mechanical sorting was the primary source of false assignment.

It is important to note that we did find evidence of a number of false negatives generated by metabarcoding where beaver was clearly
Fig. 5. (A) Correlation between relative read abundance (RRA) data for metabarcoding methods and estimated volume proportions for deer, beaver, and all other species. (B) Comparison of estimated volume and RRA for various samples, with species assignments and differences highlighted.
verified by mechanical sorting. In these cases, beaver tended to occur in the metabarcoding results but at low levels that were filtered during the analysis phase because they contained fewer than 1% of the total reads in the sample. This occurred when beaver was a minor prey item in a scat, presumably because substantially more DNA amplified from the primary component of the scat. Thus, it is imperative to explicitly select quality control protocols to balance false positives and false negatives when using bioinformatically generated metabarcoding data.

We observed a highly variable but positive relationship between the RRA of a species and the percent volume recorded in a scat. Because wolves typically prey on large-bodied mammals, most scats contained no or many reads of a particular prey item. These values near 0 or 1 drove a substantial portion of the relationship as we observed minimal data with intermediate values of both RRA and percent volume. However, it is still unclear whether RRA or percent volume provides a better indication of the proportion of actual energetic intake because metabarcoding, but not mechanical sorting, can detect the presence of animal tissue without the presence of hard parts. This is an important consideration for large carnivores that generally consume prey tissue rather than whole individuals such that diagnostic hard parts such as teeth and bones are frequently absent in scats. In addition, RRA is less influenced than frequency of occurrence by species that appear in small proportions within scats. This in conjunction with false positives introduced by mechanical sorting likely explains the high beaver FOO in mechanical sorting and bolsters the argument for including RRA results in addition to FOO results for minimizing this bias. As a result, we recommend that practitioners of DNA metabarcoding use RRA as a

Table 1. Summary statistics for all logistic regression models of all mechanically sorted wolf scat samples.

| Model                        | β       | SE     | P       |
|------------------------------|---------|--------|---------|
| species.match ~ avg DNA quantity| 0.13    | 0.14   | 0.33    |
| species.match ~ total reads with wolf| 4.17e−07 | 1.53e−06 | 0.78    |
| species.match ~ total reads no wolf| 1.6e−07  | 1.72e−06 | 0.93    |
| beaver.match ~ avg DNA quantity| −0.034  | 0.14   | 0.81    |
| beaver.match ~ total reads with wolf| 4.4e−07  | 1.78e−06 | 0.81    |
| beaver.match ~ total reads no wolf| −6.34e−06 | 2.7e−06  | 0.019*  |

Notes: The two response variables indicate where metabarcoding agreed with a species occurrence found by mechanical sorting (=1) and where metabarcoding did not agree with a species occurrence found by sorting (=0) (species.match considers all species occurrences whereas beaver.match only considers beaver occurrence data). Predictor variables include average DNA quantity per sample (avg DNA quant), total number of sequence reads prior to quality control and including wolf sequence reads (reads with wolf), and total number of sequence reads post-quality control and not including wolf sequence reads (reads no wolf). The only significant predictor of mismatches between methods was the increasing number of prey sequence reads (i.e., excluding wolves) which was associated with the increasing mismatch of beaver occurrences (P = 0.019). Scats were collected from southeast Alaska, 2014–2015. Significant P values are shown in bold.
standard metric for carnivore diet analysis rather than only frequency of occurrence.

**POW wolf diet—policy and management**

Using metabarcoding, we found similar frequencies of occurrence of the primary and secondary prey species (Sitka black-tailed deer and beaver) on POW compared with previous studies (Person 1996, Kohira and Rexstad 1997). Kohira and Rexstad’s prior study (1997) found that aside from Sitka black-tailed deer, beaver, and black bear, the only other significant prey were small mustelid species, river otter, and fish. In contrast, our metabarcoding results find a more diverse diet with 15 total prey species in the wolf diet on POW, which more closely resembles the diversity found by Darimont et al. (2004) in their study of wolf diet using scats along the coastal region of British Columbia.

The issue of what wolves eat and how much is an important question in Southeast Alaska and in particular on Prince of Wales Island where the populations of Sitka black-tailed deer and wolves are expected to decline with continued logging of old-growth forests (Person and Brinkman 2013). Because young-growth stands older than 25 yr are the least productive in terms of deer forage (Person et al. 2009), the potential effects of deer abundance decline on wolf populations are only just being realized. It remains to be seen whether wolves on POW will have sufficient dietary plasticity to be resilient to landscape-level ecological changes expected from old-growth logging.

Our results demonstrate that DNA metabarcoding is a powerful method for characterizing carnivore diets even when scats are not pre-selected for quality. This was true even in the temperate rain forest of Southeast Alaska, which is particularly hostile to the preservation of DNA. Diet analysis has been a key avenue of wildlife research to improve understanding of...
species interactions, predator–prey dynamics, and the biodiversity of systems. However, diet analysis with mechanical sorting has many challenges that make it difficult to capture the true breadth and details of wildlife diet. Given our extensive comparison between these methods, we conclude DNA metabarcoding provides an efficient, effective, and accessible option for carnivore diet analysis.

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