Running Head: DNA metabarcoding of degraded wolf scats

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

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1   **Abstract**

2   DNA metabarcoding has become a powerful technique for identifying species and

3   profiling biodiversity with the potential to improve efficiency, reveal rare prey species, and

4   correct mistaken identification error in diet studies. However, the extent to which molecular

5   approaches agree with traditional approaches is unknown for many species. Here, we compare

6   diets from wolf scats profiled using both mechanical sorting and metabarcoding of amplified

7   vertebrate DNA sequences. Our objectives were: (1) compare findings from mechanical sorting

8   and metabarcoding as a method of diet profiling and (2) use results to better understand diets of

9   wolves on Prince of Wales Island, a population of conservation concern. We predicted

10   metabarcoding would reveal both higher diversity of prey and identify rare species that are

11   overlooked with mechanical sorting. We also posited that the relative contribution of Sitka black-

12   tailed deer (*Odocoileus hemionus sitkensis*) and beaver (*Castor canadensis*) would be

13   overestimated with mechanical sorting methods because of the failure to account for the full diet

14   diversity of these wolves. We found that there was substantial overlap in the diets revealed using

15   both methods, indicating that deer, beaver, and black bear (*Ursus americanus*) were the primary

16   prey species. However, there was a large discrepancy in the occurrence of beaver in scats (54%

17   and 24% from mechanical sorting and metabarcoding, respectively) explained by the high rate of

18   false positives with mechanical sorting methods. Metabarcoding revealed more diet diversity

19   than mechanical sorting, thus supporting our initial predictions. Prince of Wales Island wolves

20   appear to have a more diverse diet with greater occurrence of rare species than previously

21   described including 14 prey species that contribute to wolf diet. Metabarcoding is an effective

22   method for profiling carnivore diet and enhances our knowledge concerning the full diversity of

23   wolf diets, even in the extremely wet conditions of southeast Alaska, which can lead to DNA

24   degradation. Given the increasingly efficient and cost-effective nature of collecting eDNA, we

25   recommend incorporating these molecular methods into field-based projects to further examine

26   questions related to increased use of alternate prey coinciding with changes in abundance of

27   primary prey and habitat alteration.

28

29   **Keywords:** *Canis lupus*, eDNA, noninvasive sampling, temperate rainforest, Prince of Wales

30   Island, Alaska, metabarcoding, diets, wolves, scats

31

32   **Introduction**

33   Animal scats are a vital tool for answering scientific questions related to animal behavior,

34   diet, and species interactions. Traditionally, scat-based diet analysis has relied upon the

35   mechanical processing and sorting of scat remains. This typically includes processing a scat to

36   remove fecal material followed by meticulous sorting and identification of remaining hair and

37   hard parts. However, diet analysis with mechanical sorting of scats has well-known biases (Lake

38   et al. 2003); rare species or species that lack non-digestible hard parts are often overlooked or

39   misidentified. In addition, some species are challenging to distinguish based on bone fragments

40   or hair samples leading to additional misidentification. This is often the case for large mammals

41   that consume prey tissue rather than whole individuals such that diagnostic hard parts like teeth

42   and bones are frequently absent in scats. Metabarcoding of fecal DNA presents a new alternative

43   method for diet analysis (Shehzad et al. 2012, De Barba et al. 2014, Kartzinel et al. 2015,

44   McInnes et al. 2017, Eriksson et al. 2019). The DNA metabarcoding workflow includes

45   extracting DNA from environmental samples, DNA amplification using 'universal' primers

46   (Binladen et al. 2007), and mass-parallel sequencing of amplified product using next generation

47   sequencing technologies. This process allows DNA barcodes from multiple species in a bulk

48   sample to be sequenced simultaneously for an efficient and thorough profile of species present

49   within an environmental sample (Valentini et al. 2009).

50   The utility of metabarcoding for informing important management objectives, where

51   accurate taxonomic assignment and detection is paramount, is uncertain because unlike

52   mechanical sorting (1) it is unknown how quality of inference from metabarcoding depends on

53   acquiring relatively fresh scats with minimally degraded DNA, which can be challenging for rare

54   taxa, and (2) it is not yet clear if the relative read abundance from metabarcoding can yield

55   quantitative information that approximates the volume or biomass arising from each prey

56   species. The degree to which relative read abundance (RRA) from DNA metabarcoding is

57   correlated with the relative biomass of each prey species is a subject of substantial debate

58   (Deagle et al. 2018, Pinol et al. 2018). Limited empirical research validating RRA against

59   estimated biomass or volume from mechanical sorting informs this debate (Soininen et al. 2009,

60   Thomas et al. 2017), although no studies have done so with terrestrial carnivores. Pinol et al.

61   (2018) argued that metabarcoding results can only be interpreted quantitatively if amplification

62   of DNA through PCR with universal primers is avoided because different amplification

63   efficiencies among species can lead to poor representation of original biomass proportions.

64   While this is often true for invertebrates, for which primer mismatch is common (Krehenwinkel

65   et al. 2017), our 12S mtDNA primers rarely contains basepair mismatches for vertebrates and

66   contain no mismatches for the taxa considered here (Appendix S1: Fig. S1). In addition, recent

67   evidence suggests that as long as primer efficiency is high (no mismatches), the proportion of

68   sequences arising from each species in metabarcoding (RRA) can produce semi-quantitative

69   results (Kartzinel et al. 2015, Thomas et al. 2016, Krehenwinkel et al. 2017, Deagle et al. 2018).

4

70   This could allow metabarcoding to approximate relative biomass or volume information similar

71   to that produced by mechanical sorting of hard parts as well as frequency of occurrence

72   (proportion of scats that contain each species). Nevertheless, the degree to which degraded scats

73   yield suitable inference comparable to mechanical sorting is not currently well-understood

74   because of a lack of formal comparisons between metabarcoding and mechanical sorting (Deagle

75   et al. 2018, Pinol et al. 2018).

76   To provide this methods comparison, we focused on the Alexander Archipelago wolf

77   (*Canis lupus ligoni*) as a case-study. The Alexander Archipelago wolf has been repeatedly

78   petitioned for listing as threatened under the U.S. Endangered Species Act (ESA). These wolves

79   occur in relative geographic isolation in southeast Alaska, where continued pressure from habitat

80   loss, population decline of their primary prey, and wolf harvesting have raised concern about the

81   future of the population. Wolf population estimates at regional scales in southeast Alaska have

82   been based on expected Sitka black-tailed deer (*Odocoileus hemionus sitkensis*) abundance under

83   the assumption that wolves are closely tied to the abundance of their primary prey. This is

84   evident in the most recent ESA species status review where deer habitat quality metrics were

85   used to project wolf abundance (U.S. Fish and Wildlife Service 2015).

86   The wolves on Prince of Wales Island (POW) (Fig. 1) were of particular concern in the

87   most recent assessment (2015) because in addition to high levels of wolf harvest, POW has the

88   highest rate of old-growth logging in southeast Alaska (Albert and Schoen 2013, Person and

89   Brinkman 2017). Deer populations are predicted to decline as old-growth forests with palatable

90   understory forbs and shrubs are converted into dense, even-aged, closed canopy forests (Alaback

91   1982, Schoen et al. 1988, Person et al. 1996, Farmer and Kirchhoff 2007, Gilbert et al. 2016,

92   Person and Brinkman 2017, Porter 2018) that are strongly avoided by deer (Wallmo and Schoen

5

93   1980, Kohira and Rexstad 1997, Gilbert et al. 2017). Deer were the most frequently occurring

94   prey species for the Alexander Archipelago wolf based on previous research conducted on POW

95   (Kohira 1995, Person et al. 1996, Kohira and Rexstad 1997). However, mechanical sorting of

96   wolf scats has revealed other prey in significant quantities (Kohira 1995), and coastal wolves in

97   this region can consume substantial quantities of salmon seasonally and other marine resources

98   (Szepanski et al. 1999, Darimont et al. 2003, 2004, 2008a, Lafferty et al. 2014), suggesting that

99   wolf population abundance may also be dictated by the availability of prey other than deer.

100   Consequently, refining knowledge regarding the diet of wolves in the system has important

101   implications for wolf management, potential ESA considerations, and forest management in

102   southeast Alaska.

103   Here we provide the first formal comparison of carnivore diet analysis from mechanical

104   sorting and DNA metabarcoding using opportunistically collected scats across an assumed

105   degradation spectrum in a temperate rainforest which is hostile to DNA preservation. We

106   examined whether metabarcoding revealed a more diverse wolf diet than mechanical sorting,

107   achieved increased taxonomic precision, and identified infrequently consumed prey species. We

108   included both scats appearing highly degraded and those appearing fresh and assessed whether

109   age of scats or biases introduced during the molecular processing affected the diet profile shown

110   by metabarcoding. We additionally analyzed in detail Alexander Archipelago wolf diets with a

111   particular focus on Prince of Wales Island to determine the prey profile of wolves and their

112   dependence on deer.

113

114   **Materials and Methods**

115   *Study area and field collection*

6

116   Southeast Alaska lies within the Alexander Archipelago composed of over 2,000 named

117   islands (Fig. 1) (Cook et al. 2006). This region receives between 130 – 400 cm of precipitation

118   annually (Shanley et al. 2015) thus making it particularly inhospitable to the preservation of

119   DNA in exposed environmental samples. The mainland is buttressed by the rugged Coast

120   Mountains and extensive temperate rainforests at lower elevations. As a result of natural

121   fragmentation and isolation, the North Pacific coast region supports many endemic plant and

122   animal lineages, particularly on Prince of Wales Island, the largest island in the archipelago

123   (Cook et al. 2006, MacDonald and Cook 2007, Smith 2016). Most of the forested area is within

124   the Tongass National Forest managed by the U.S. Forest Service. This ecosystem hosts a

125   diversity of mammals including iconic species such as Sitka black-tailed deer (*Odocoileus*

126   *hemionus sitkensis*), American black bear (*Ursus americanus*), North American beaver (*Castor*

127   *canadensis*), American marten (*Martes americanus*), mountain goat (*Oreamnos americanus*),

128   Steller sea lion (*Eumetopias jubatus*), harbor seal (*Phoca vitulina*), and moose (*Alces alces*).

129   Species distribution and assemblages vary among island and mainland areas of this region.

130   We collected wolf scats along wolf travel routes, near den sites, and on secondary roads

131   during planned scat collection surveys during October 2014 - December 2015 (Fig. 1). We

132   collected wolf scats primarily on Prince of Wales Island (55° 46'45.9480" N; 132° 49' 4.7748"

133   W) (n = 145), but also opportunistically collected samples in other mainland and island systems

134   (n = 38). We estimated the age (fresh [<3 months] and old [>3 months]) of scat based on

135   appearance, time since last site visit (Ciucci et al. 1996), and exposure time considering that scats

136   decompose rapidly in rainforest environments (Wallmo et al. 1962, Ciucci et al. 1996, Darimont

137   et al. 2008b) (Fig. 2). Collected wolf scats were stored in plastic bags, labeled with location,

138   date, and perceived age of scat, and then frozen (-20°C). Frozen scats were shipped to Oregon

139   State University for sample preparation and analysis.

140

141   *Mechanical sorting*

142   We stored a subsample of each scat for later molecular analysis (sterilized forceps and

143   razors were used to collect a sample from the middle section of each scat to minimize wolf DNA

144   (Stenglein et al. 2010)), and then placed each scat in a mesh bag (1/8") and soaked it in water for

145   48 hours in a mason jar. We power-washed the scat to remove as much remaining fecal matter as

146   possible. The remaining contents (i.e., hair, bones, other hard parts) were put in a labeled paper

147   bag and dried in an oven (at approximately 50°C) for at least five days. We weighed the

148   processed scat material (hair, bone, scale, feather, etc.) and mechanically homogenized and

149   sorted the remains by hand. On average, the fine-scale sorting took 3.6 hours per scat. We

150   examined hairs under a microscope and compared to hair samples from the Alaska Fur ID

151   project (Carrlee and Horelick 2011). We made slide mounts using clear nail polish to examine

152   scale pattern and medulla diameter in order to identify species. Following identification, the slide

153   was labeled with the species name and sample of origin. This exhaustive, fine-scale sorting (Fig.

154   3) ensured that even rare species could be identified. Along with species identification, we

155   estimated the volume of each prey species as a proportion of estimated hard parts for a species in

156   relation to all hard parts in an individual scat.

157

158   *Molecular analysis*

159   Using the stored subsamples from each scat, we extracted DNA from each sample using

160   the Qiagen DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) with slight modifications as

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161   follows: 500 ul Buffer ATL, 50 ul Proteinase K, and 1.0 mm Zirconia/Silica beads (BioSpec

162   Products, Bartlesville, OK) were added to the 1.7 ml tube containing the scat. Samples were

163   vortexed for 10 minutes at maximum speed prior to incubation at 56°C for 4-6 hours. The DNA

164   was eluted in a total volume of 100 ul. A negative control was extracted with each round

165   (approximately 17 samples) of DNA extraction to identify possible cross contamination.

166   Following DNA extraction, each sample was amplified in three separate reactions using

167   the primer pair 12SV5F/12SV5R (Riaz et al. 2011). We used the forward primer

168   (TTAGATACCCCACTATGC) as Riaz et al. (2011) but modified the first base pair of the

169   reverse primer (YAGAACAGGCTCCTCTAG) to allow broader binding of vertebrate targets.

170   These primers target approximately 100 base pairs in the 12S region of the vertebrate

171   mitochondrial genome. The initial PCR was carried out using AmpliTaq Gold 360 Master Mix

172   (Life Technologies, Carlsbad, CA). To label samples for multiplexing, we used 384 unique 8 bp

173   dual matching indexes on the forward and reverse primers to eliminate contamination due to tag

174   jumping by filtering reads that did not have identical indexes, and we included 3 bp of random

175   nucleotides on the 5'end to increase sequence diversity and prevent degradation of indexes

176   during subsequent blunt-ending and ligation steps. PCR reactions were carried out in a volume of

177   20 ul with 10 ul AmpliTaq Gold 360 Master Mix for a final concentration of 1x, 5 ul of forward

178   and reverse primers for a final concentration of 0.25 uM, 3 ul of water, and 2 ul of DNA

179   template. PCR cycling included initial denaturing at 95°C for 10 minutes, followed by 40 cycles

180   of 95°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds, with a final extension at

181   72°C for 7 minutes.

182   After the initial PCR, all PCR amplicons were cleaned using PCRClean DX solid-phase

183   reversible immobilization magnetic beads (Aline Biosciences, Woburn, MA). Each PCR reaction

9

184   was quantified using Accublue High Sensitivity dsDNA Quantitation kit (Biotium, Fremont, CA)

185   and normalized to 6 ng/ul. Each group of 384 PCR products was then pooled into a single

186   library, for a total of 3 libraries. Individual libraries were then tagged with an additional 6 base

187   pair identifying index using the NEBnext Ultra II DNA Library Prep kit (New England Biolabs,

188   Ipswich, MA). Pooled samples were analyzed on a Bioanalyzer to confirm fragment size. The

189   libraries were then sequenced on one lane of Illumina HiSeq 3000 2 x 150 bp PE at the Center

190   for Genome Research and Biocomputing at Oregon State University.

191

192   *Sequence analysis*

193   Raw sequence reads were analyzed using a bioinformatics pipeline designed to trim and

194   sort the sequence reads according to scat sample identification. An outline of the bioinformatic

195   process is as follows: (1) raw reads were paired using PEAR (Zhang et al. 2013); (2) followed by

196   demultiplexing using 8 basepair index sequences unique to each sample (mismatches discarded);

197   (3) lastly, sequences from each sample were clustered by 100% similarity and taxonomically

198   assigned using BLAST against 12S vertebrate sequences in GenBank and from a custom 12S

199   database.

200   Similar to the step-wise methods used by De Barba et al. (2014), a series of filtering and

201   quality control measures were carried out on taxonomically assigned sequences. We initially

202   removed sequences that were identified to be *Canis* spp. and contaminants based on read counts

203   in no-template controls (which contained primarily human contamination). We then removed

204   sample replicates that failed to amplify during PCR which included sample replicates with fewer

205   than a total of 400 sequence reads. We compared taxonomic assignments with known fauna of

206   southeast Alaska (MacDonald and Cook 2007) to replace non-regional species identified with

10

207   BLAST with closely-related regional taxa. We then excluded prey items occurring in fewer than

208   2 of 3 PCR replicates. Finally, we combined those sample replicates that amplified so that

209   sequence reads were totaled for each species within a sample and over the entire sample and

210   eliminated sequences that appeared in less than 1% of the total reads for an individual sample.

211

212   *Age of scats*

213   Prior to processing, we observed marked differences between the appearance and quality

214   of scats (Fig. 2). We performed t-tests to determine whether the perceived age of a scat made

215   during field collection correlated with either the average quantity of DNA (ng/ul) in a sample

216   (measured post normalization using Accublue High Sensitivity dsDNA Quantitation kit

217   (Biotium, Fremont, CA)), the total number of sequence reads in a sample including the wolf

218   defecator, or the total number of sequence reads excluding wolf.

219

220   *Frequency of occurrence*

221   We used both frequency of occurrence (FOO) and metrics of relative abundance (see

222   below) to describe the occurrence of prey in wolf diet. FOO was calculated to determine which

223   prey species were present and how often they were present based on the number of samples. For

224   mechanical sorting methods, a species was present if there was evidence (including trace

225   elements) of a prey species (e.g., hair, bone, scales, etc.) within a scat sample. FOO was then

226   calculated as the proportion of scats in which a prey species occurred. For metabarcoding, a

227   species occurrence was determined by whether sequence reads for a particular species were

228   found in an individual scat after quality control measures. We compared FOO from mechanical

229   sorting and metabarcoding using the subset of scats analyzed by both methods (n = 104), but we

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230   additionally present diet analysis from all scats collected on Princes of Wales Island and close

231   surrounding islands (n = 118 metabarcoding; n = 98 mechanical sorting) to describe diet on

232   POW.

233   To analyze discrepancies between species present in samples with mechanical sorting and

234   not found with metabarcoding, we used generalized logistic regression with logit link to explore

235   whether false positives from mechanical sorting or false negatives generated from metabarcoding

236   best explained the absence of species. Statistical analyses were conducted in the R statistical

237   program using the 'stats' package (R Core Team 2018). We reasoned that false negatives could

238   arise if scats contained poor quality DNA or sequencing depth was insufficient. We therefore fit

239   three separate logistic regression models using average DNA quantity per sample (across the

240   three replicates PCRs), total number of sequence reads prior to quality control and including

241   wolf sequence reads, and total number of sequences reads post quality control and not including

242   wolf sequences reads as univariate predictors in each model. In our analysis, zeroes were defined

243   as an absence in metabarcoding where mechanical sorting had indicated an occurrence of a

244   particular species in a sample; one indicated where metabarcoding was in agreement with

245   occurrence found in mechanical sorting. Therefore, positive coefficients imply an increasing rate

246   of proper assignment as DNA quality or sequencing depth increases. The absence of such an

247   effect would suggest that mismatch between metabarcoding and mechanical sorting is unlikely to

248   be due to false negatives by metabarcoding.

249

250   *Relative abundance*

251   To test whether metabarcoding and mechanical sorting yield similar metrics for relative

252   abundance of a prey species within a scat, we compared percent estimated volume from

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253   mechanical sorting with the relative read abundance (RRA) from metabarcoding. RRA for each

254   species i was calculated as

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

257

258   where $n_{i,k}$ is the number of sequences of prey species i in sample k, S is the total number of

259   samples, and T is the total number of species. We compared estimated volume of a prey species

260   from mechanical sorting with RRA from metabarcoding using simple linear regression (R Core

261   Team 2018).

262   For both the frequency of occurrence and relative abundance analyses we additionally

263   revisited results from scats with mismatches from metabarcoding and mechanical sorting to

264   assess whether metabarcoding found many sequence reads of an alternative species that was

265   incorrectly assigned by mechanical sorting and was thus likely a false positive.

266

267   **Results**

268   *Age of scats*

269   Purportedly fresh scats contained significantly more total sequence reads on average

270   ($\mu_{fresh} = 269,514 \pm 173,902$) compared to the total number of reads from degraded wolf scats

271   ($\mu_{degraded} = 200,378 \pm 135,646$) (t = 2.09, df = 85, p-value = 0.039). Likewise, fresh scats ($\mu_{fresh} =$

272    $139,939 \pm 135,858$) had significantly more wolf sequence reads than degraded scats ($\mu_{degraded} =$

273    $52,411 \pm 75,531$) (t = 3.80, df = 73.73, p-value < 0.001). However, we found no significant

274   difference between degraded and fresh scats when considering only reads from prey items

275   (excluding any wolf DNA reads), although degraded scats yielded a greater average number of

13

276   non-wolf reads per sample than fresh scats ($\mu_{degraded}$ = 147,966 ± 125,223; μ_{fresh} = 129,575 ±

277   124,124; t = -0.69, df = 84.18, p-value = 0.49) (Fig. 4). Fresh scats had a higher average DNA

278   quantity post PCR (ng/ul; $\mu_{degraded}$ = 4.12 ± 1.97; μ_{fresh} = 4.55 ± 2.20) but the difference was not

279   statistically significant (t = 0.97, df = 85.93, p-value = 0.33).

280

281   *Comparing wolf diet by mechanical sorting and metabarcoding – frequency of occurrence*

282   We compared wolf diet from 104 scat samples that were analyzed with both mechanical

283   sorting and metabarcoding. Metabarcoding revealed a number of rare species that were not found

284   using mechanical sorting methods and thus revealed greater dietary diversity (Fig. 5). Species

285   that were found with metabarcoding methods but were absent when using mechanical sorting

286   methods include: duck (*Anas* spp.), dusky grouse (*Bonasa umbellus*), elk (*Cervus elaphus*), raven

287   (*Corvus* species), Northern collared lemming (*Dicrostonyx groenlandicus*), Steller sea lion

288   (*Eumetopias jubatus*), American marten (*Martes americana*), and American red squirrel

289   (*Tamiasciurus hudsonicus*). Mechanical methods identified moose (*Alces alces*) in a single scat

290   where metabarcoding did not, although moose was identified by metabarcoding in this particular

291   scat prior to quality filtering.

292   Frequency of occurrence (FOO) (Fig. 5) results were similar with both methods.

293   However, there was substantial discrepancy between the primary prey species (Sitka black-tailed

294   deer) and the secondary prey species (beaver) between metabarcoding and mechanical

295   occurrence results. The occurrence of deer was greater in the mechanical sorting results

296   (FOO_{mech} = 0.962) compared to metabarcoding results (FOO_{MB} = 0.8) and the occurrence of

297   beaver was twice as frequent in the mechanical sorting (FOO_{mech} = 0.519) results compared to

298   metabarcoding (FOO_{MB} = 0.236).

14

299   Logistic regression to assess mismatch between metabarcoding and mechanical sorting

300   revealed that neither average DNA quantity, total sequence reads, nor total sequence reads of

301   prey (i.e. excluding wolf) were associated with failing to detect species that were identified by

302   metabarcoding (Table 1). However, contrary to predictions, increasing number of prey sequence

303   reads (i.e. excluding wolves) was associated with increasing mismatch with beaver occurrences

304   detected by mechanical sorting (p = 0.025), which suggests that the error was due to

305   misassignment by mechanical sorting rather than by metabarcoding. Thirty-two of the 59 beaver

306   occurrences had disagreement between mechanical sorting and metabarcoding results. Notes and

307   hair slides taken during mechanical sorting showed that 18 of the 32 mismatches could be

308   attributed to false positives generated from mechanical sorting. In addition, a substantial number

309   of definitive deer occurrences (i.e. high relative read abundance for deer) were mistakenly

310   assigned to beaver by mechanical sorting (Fig. 6), further suggesting that mismatch between

311   methods was due to misassignment by mechanical sorting.

312

313   *Comparing wolf diet by mechanical sorting and metabarcoding – relative read abundance*

314   There was minimal discrepancy between RRA of primary prey species (metabarcoding)

315   and their estimated volume in scats (mechanical sorting); the difference between RRA and

316   estimated volume for deer was 2% (RRA_{deer} = 68.3%; estimated $volume_{deer}$ = 66.3%) and for

317   beaver it was less than 7% (RRA_{beaver} = 14.1%; estimated $volume_{beaver}$ = 20.5%). For the rarer

318   species, we found a close association (within 2%) between the RRA and the estimated volume

319   for that species.

320   The estimated volume from mechanical sorting was positively correlated with RRA of

321   deer (β = 0.53; R^2 = 0.26; $p < 0.01$, n = 87), beaver (β = 0.57; R^2 = 0.28; $p < 0.01$, n = 25), and

15

322   black bear ($\beta = 0.80$; $R^2 = 0.28$; $p = 0.17$, n = 6) (Fig. 6), supporting a positive but variable

323   relationship between the volume of parts of a particular species found in the physical scat and the

324   proportion of DNA sequence reads for that species. However, substantial variability is likely due

325   to species misidentification by mechanical sorting such as deer falsely identified as beaver (Fig.

326   6).

327

328   *Prince of Wales*

329   Metabarcoding of scats found only within Prince of Wales Island (POW) (Fig. 7)

330   revealed 14 species (Supplementary table) including Sitka black-tailed deer ($FOO_{MB\_POW} =$

331   0.852), beaver ($FOO_{MB\_POW} = 0.231$), and black bear ($FOO_{MB\_POW} = 0.157$) were the most

332   common prey items (Fig. 7). Other common prey species were salmon (*Oncorhynchus* spp.)

333   ($FOO_{MB\_POW} = 0.056$), American marten (*Martes americana*) ($FOO_{MB\_POW} = 0.046$), North

334   American river otter (*Lontra canadensis*) ($FOO_{MB\_POW} = 0.037$), and bald eagle (*Haliaeetus*

335   *leucocephalus*) ($FOO_{MB\_POW} = 0.019$). Additional prey items in less than 1% of scats include

336   American red squirrel (*Tamiasciurus hudsonicus*), deermouse (*Peromyscus* spp.), vole (*Myodes*

337   and *Microtus* spp.), dusky grouse (*Bonasa umbellus*), duck (*Anas* spp.), and unidentified bird

338   species.

339   Mechanical sorting revealed a total of 10 prey species (Fig. 7), including harbor seal

340   which was not found with metabarcoding for the POW samples. However, it should be noted that

341   for this sample, mechanical sorting estimated only 2% harbor seal and metabarcoding instead

342   found otter, which could have been mistaken for harbor seal during sorting. Deer (FOO_{mech_POW}

343    $= 0.969$) and beaver ($FOO_{mech\_POW} = 0.561$) (the two primary prey species) showed greater FOO

344   compared to metabarcoding, although mechanical sorting did not show any American marten

16

345   and had a lower FOO of salmon species compared to the metabarcoding results. There was also

346   substantial occurrence of material from unknown species in the mechanical results (FOO_{mech_POW}

347   = 0.163) that is not seen with metabarcoding.

348

349   **Discussion**

350   DNA metabarcoding has emerged as a novel method for diet analysis because of the

351   ability to reveal rare or difficult to identify species (Shehzad et al. 2012, De Barba et al. 2014,

352   Berry et al. 2015, Srivathsan et al. 2015, Kartzinel et al. 2015, McInnes et al. 2017, Buglione et

353   al. 2018). However, substantial uncertainty remains as to whether inference from mechanical

354   sorting and DNA metabarcoding produce comparable results, particularly if scats are of

355   uncertain age and quality. Our results suggest that excluding purportedly degraded scats from

356   DNA metabarcoding analyses does not improve inference about diet. Perceived fresh scats

357   contained on average a greater number of reads per scat when including wolf sequence reads, but

358   there was no significant difference in the average number of reads between fresh and degraded

359   scats when only including reads from prey species (Fig. 4). The average quantity of DNA was

360   also not significantly different between fresh and degraded scats; this is likely because fresh scats

361   contained more fecal material relative to hair and bone, and total DNA quantity per sample is

362   normalized prior to sequencing such that abundant wolf DNA leads to dilution of prey DNA.

363   Many degraded scats were primarily clusters of hair and bone that were washed of fecal material.

364   Importantly, these results suggest that metabarcoding is sensitive enough to determine prey

365   assemblages in degraded scats and thus scat collection and processing should not be predicated

366   upon perceived scat quality.

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367   FOO and RRA metrics were qualitatively similar among methods. RRA of each species

368   was significantly correlated with estimated volume determined with mechanical sorting ($p_{all} <$

369   0.01, $p_{deer} < 0.01$, $p_{beaver} < 0.01$, $p_{bear} = 0.16$) suggesting that RRA can be a reasonable proxy for

370   volume of prey species obtained from mechanical sorting (which in turn could be used to

371   estimate relative biomass using biomass equations that correct for body size (Weaver 1993)).

372   Both mechanical sorting and metabarcoding agreed that Sitka black-tailed deer was the primary

373   prey item, followed by beaver, and then black bear as suggested by previous research in this

374   region (Kohira, 1995; Kohira & Rexstad, 1997) (Fig. 5). However, both deer and beaver

375   occurred substantially more frequently in mechanically sorted scats than in metabarcoded scats.

376   The divergence between the two methods examined in our study was more substantial for beaver

377   which were identified mechanically in 52% of scats while only detected by metabarcoding in

378   24%.

379   We closely examined scats that were mismatched (i.e. the prey species was found in a

380   scat during mechanical sorting but not found in the same scat with DNA metabarcoding) with a

381   focus on beaver to assess whether mismatches were due to false positives produced from

382   mechanical sorting or false negatives produced from metabarcoding. Eighteen of the 32

383   mismatched samples show evidence of false positive resulting from mechanical sorting. In these

384   scats, beaver was thought to be present, but notes during sorting specified uncertainty that these

385   small amounts of unknown hair samples could also be attributed to deer or black bear. In fact, we

386   found that in all mismatched beaver samples metabarcoding showed a high RRA of deer and

387   mechanical sorting found low volume of deer, strongly suggesting that mechanical sorting mis-

388   assigned deer hair to beaver as the primary prey species in that scat (highlighted in Fig. 6). Our

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389 logistic regression analysis additionally suggests that these errors resulted from mis-assignment

390 by mechanical sorting rather than metabarcoding (Table 1).

391   Why do we see these potential false positives generated from mechanical sorting? One

392 explanation is that relying on mechanical sorting of scats results in the overestimation of primary

393 prey species (i.e. deer and beaver) due to search image bias. Mechanical sorting can lead to

394 mislabeling difficult to identify parts as common species rather than an infrequently detected

395 species because the researcher is accustomed to encountering the common prey species. The

396 pronounced difference seen in beaver FOO could also be attributed to the difficulty in

397 distinguishing between beaver and guard hair from other species such as deer and black bear

398 (Fig. 8).

399   The remaining 14 of the 32 beaver mismatches were attributed to false negatives

400 generated by metabarcoding; we concluded this because beaver was verified to have occurred in

401 mechanical sorting but was absent from metabarcoding results. However, for 10 of these scats

402 beaver occurred in the metabarcoding results prior to quality filtering that removed prey that

403 occurred in fewer than 2 of 3 PCR replicates and with fewer that 1% of the total reads

404 (importantly, beaver was nearly absent from our negative controls), which had the effect of

405 underestimating prey items that occurred in only a small portion of a scat. It is important to note

406 that our conservative quality filtering thresholds following De Barba et al. (2014) led to some of

407 these false negatives at the expense of false positives. Thus, it is imperative to explicitly reason

408 through quality control protocols to balance false positives and false negatives when using

409 bioinformatically-generated metabarcoding data.

410   There was also divergence in the detection of rare species among methods. Although

411 metabarcoding revealed several clear false negatives, this was substantially more common with

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412   mechanical sorting where 8 species in final metabarcoding results were not found by mechanical

413   sorting for the same subset of samples. In particular, American marten, Northern collared

414   lemming, and a number of bird species were missing from mechanical sorting but evident in the

415   metabarcoding results. This conclusion supports our initial prediction that metabarcoding would

416   be more advantageous in identifying rare species.

417

418   *POW wolf diet – policy and management*

419   The issue of what wolves eat and how much is an important question in southeast Alaska

420   and in particular on Prince of Wales Island where there are concerns about the long-term

421   viability of wolves given the trophic linkage between wolves, Sitka black-tailed deer, and old-

422   growth forest. The population of Sitka black-tailed deer is expected to decline in this region with

423   continued logging of old-growth forests (Person and Brinkman 2017). Given this, wolf

424   populations are predicted to decline and these declines are most significant under scenarios

425   where wolves rely heavily on deer in the future (Gilbert et al. 2016).

426   Our study shows the promise of eDNA and metabarcoding methods to examine wolf diet

427   diversity and diet changes. Comparing our results with previous work indicated that the

428   occurrence of the primary prey species (Sitka black-tailed deer) is comparable on POW; Person

429   et al. (1996) reported a >90% occurrence while we report 85.2% occurrence using DNA

430   metabarcoding and 96.9% occurrence using mechanical sorting. However, the occurrence of

431   beaver is greater compared to previous work; the frequency of occurrence of beaver was 13.7%

432   (Person et al. 1996) and 31% (Kohira and Rexstad 1997), whereas we report 23.1% occurrence

433   using DNA metabarcoding and 56.1% using mechanical sorting. These previous studies found

434   that aside from Sitka black-tailed deer, beaver, and black bear, the only significant other prey

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435   were small mustelid species, river otter, and fish. Our results show a diverse diet with 14 total

436   prey species identified from mechanical sorting that contribute to wolf diet on POW (Fig. 7),

437   which more closely resembles the diversity found by Darimont et al. (2004) in their study of

438   wolf diet using scats along the coastal region in British Columbia. Importantly, our findings

439   suggest that metabarcoding was able to reveal the breadth of Alexander Archipelago wolf diet

440   diversity more accurately than mechanical sorting. (24 vs. 14 refer to Appendix S1: Table S1).

441   Continued diet analysis using metabarcoding of wolf scats found on POW could reveal

442   whether this increase in diversity is due to the increased power in the method used

443   (metabarcoding vs. mechanical sorting), or if wolves are beginning to exhibit increased

444   opportunistic predation on species other than Sitka black-tailed deer. Given that we also found

445   greater dietary diversity using mechanical sorting compared to results using the same methods

446   from the mid-1990's points towards a potential dietary shift in wolves on POW (Kohira and

447   Rexstad 1997). The rate of clear-cut logging in this region peaked during the late 1980's and

448   1990's and while this rate has slowed in recent years, a total of nearly 30% of old-growth forests

449   have been logged on POW (U.S. Fish and Wildlife Service 2015). Because young-growth stands

450   older than 25 years are the least productive in terms of deer forage (U.S. Fish and Wildlife

451   Service 2015), the effects of potential deer abundance decline on wolf populations are only just

452   being realized. As well-known diet generalists, it remains to be seen whether wolves on POW

453   are resilient to landscape-level ecological changes expected from old-growth logging.

454   Metabarcoding has revealed a more diverse and precise diet for wolves on POW and in

455   southeast Alaska, potentially pointing towards these wolves making greater use of alternate prey.

456   In general, DNA metabarcoding can be used as a tool to reliably describe diet for other carnivore

457   species. Even in a hostile environment for the preservation of eDNA, we have shown that DNA

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458   metabarcoding is an effective and powerful method for describing carnivore diet. Diet analysis

459   remains one of the most important avenues of wildlife study as it is a necessary component of

460   understanding species interactions, predator-prey dynamics, and the biodiversity of systems. This

461   nuanced profiling of diet is especially important as vulnerable wildlife populations face

462   continued habitat loss and degradation, and thus we can use changes in diet can as potential

463   indicators of environmental health.

464

465   **Acknowledgements**

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617 **Table 1:** Summary statistics for all generalized logistic regression models. Predictor variables

618 include average DNA quantity per sample (avg DNA quant), total number of sequence reads

619 prior to quality control and including wolf sequence reads (reads with wolf), and total number of

620 sequences reads post quality control and not including wolf sequences reads (reads no wolf).

621 Models were tested against all mechanically sorted samples that had a positive occurrence for a

622 species and against all mechanically sorted samples that had a positive occurrence for beaver.

623

Model	Estimate	SE	z value	Pr($>|z|$)
spp.presence ~ avg DNA quantity	0.13	0.14	0.966	0.33
spp.presence ~ total reads with wolf	4.16e-07	1.5e-06	0.273	0.79
spp.presence ~ total reads no wolf	-1.5e-07	1.63e-06	-0.095	0.93
beaver.presence ~ avg DNA quantity	-0.034	0.139	-0.25	0.81
beaver.presence ~ total reads with wolf	4.4e-07	1.78e-06	0.25	0.81
beaver.presence ~ total reads no wolf	-5.85e-06	2.61e-06	-2.24	0.025*

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632 **Figure Legends**

633 **Figure 1**: Study area map showing Alexander Archipelago in Southeast Alaska. Red and yellow

634 points represent individual scat collection sites. Most scats collections were concentrated on

635 Prince of Wales Island (yellow points).

636 **Figure 2**: Examples of wolf scats collected in southeast Alaska near Prince of Wales Island.

637 Left-sided panels (a, c, and e) are examples of fresh scats (< 3 months old) and the right-sided

638 panels (b, d, f) are examples of old/degraded scats (> 3 months old). Age was determined by the

639 collector; scats were collected throughout 2014 – 2015.

640 **Figure 3**: Photographs depicting examples of fine-scale mechanical sorting results of prey

641 species in wolf scats collected in Southeast Alaska, 2014-2015. Starting from the top left panel

642 and moving clockwise, species shown are salmon, black bear, bald eagle, harbor seal, and

643 sculpin.

644 **Figure 4**: Boxplots depicting the total number of reads and the DNA quantity (measured post

645 normalization) for scat samples binned by the age of the scat.

646 **Figure 5**: Diet summary from analysis of wolf scats (Southeast Alaska, 2014-2015) using (a)

647 metabarcoding methods and (b) mechanical sorting methods. For the diet trees, each branch and

648 terminal node represent a prey species identified in the wolf scats with the size and color of the

649 branch showing the number of occurrences of that prey species. Frequency of Occurrence and

650 RRA and estimated volume are compared.

651 **Figure 6**: Correlation between relative read abundance data for metabarcoding methods and

652 estimated volume for mechanical sorting methods by scat sample for the three most prevalent

653 prey species from wolf scats, Southeast Alaska, 2014-2015. Estimated volume is measured as the

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654   proportion of a prey species consumed per scat relative to the whole scat. RRA is the relative

655   read abundance. Data points highlighted in brown show samples where deer was thought to be

656   mistakenly identified as beaver in mechanical sorting.

657   **Figure 7**: Diet diversity, frequency of occurrence (FOO), and RRA and estimated volume found

658   with a) metabarcoding results and b) mechanical results for scats found on Prince of Wales

659   Island, Alaska.

660   **Figure 8**: Panel of hair samples. The top row shows examples of guard hairs from the Alaska

661   Fur ID project of Sitka black-tailed deer, beaver, and black bear. The bottom row shows

662   examples of scale pattern from scale casts from the Alaska Fur ID project of Sitka black-tailed

663   deer, beaver, and black bear (left to right). The last panel in each row is an example of a difficult

664   to identify hair and scale pattern from a wolf scat sample, Southeast Alaska 2014-2015.

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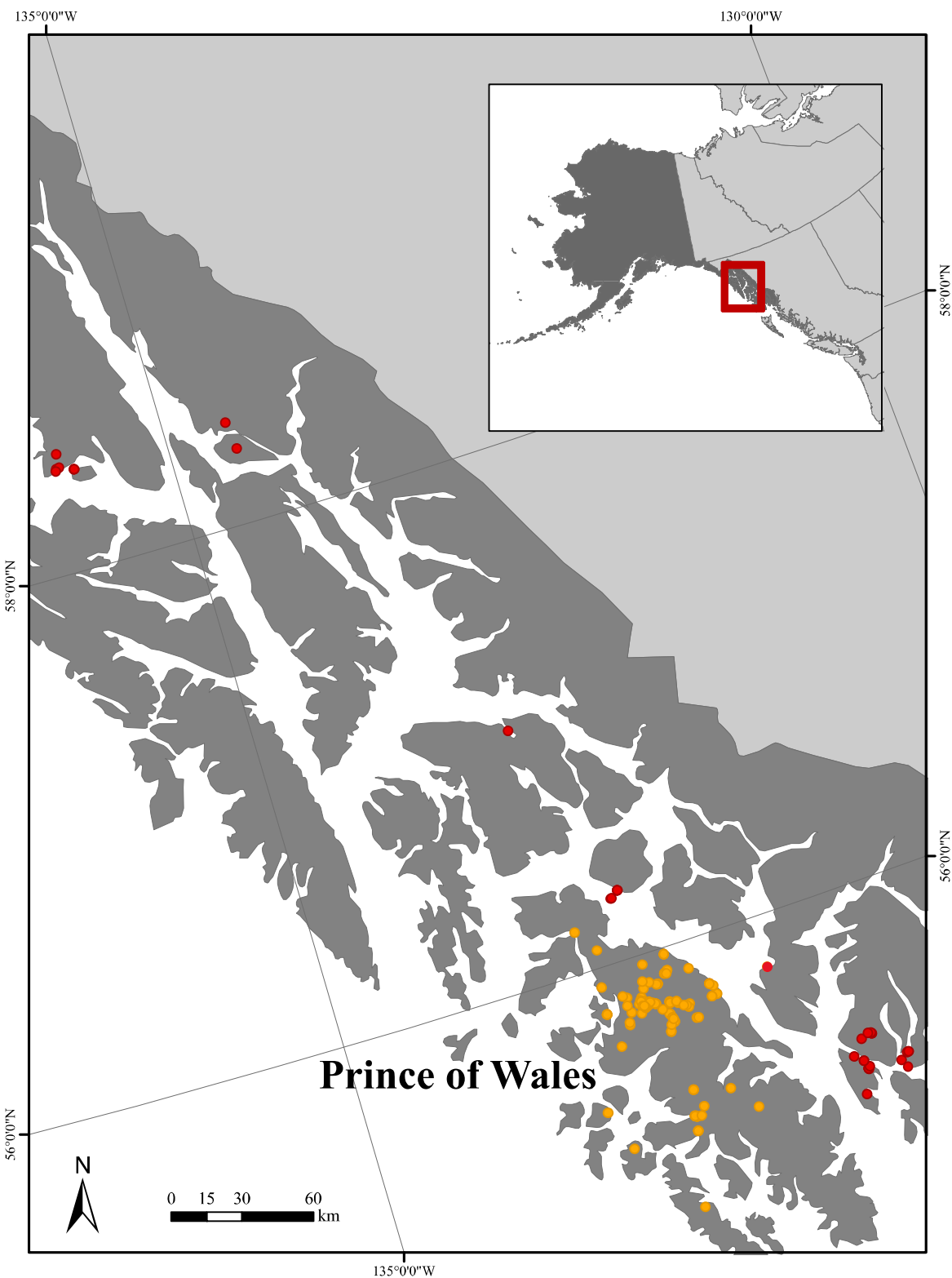
678   **Figure 2**
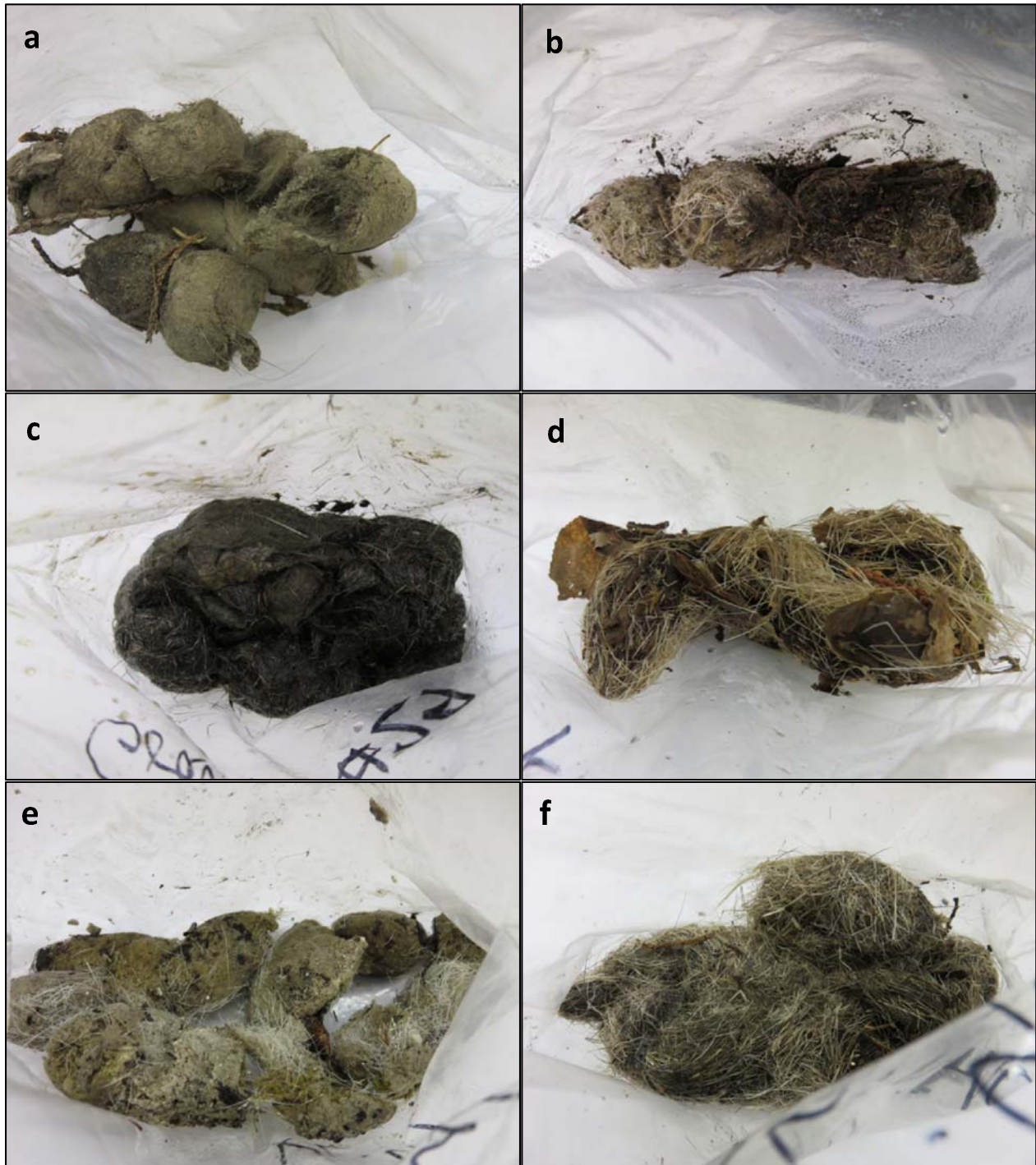
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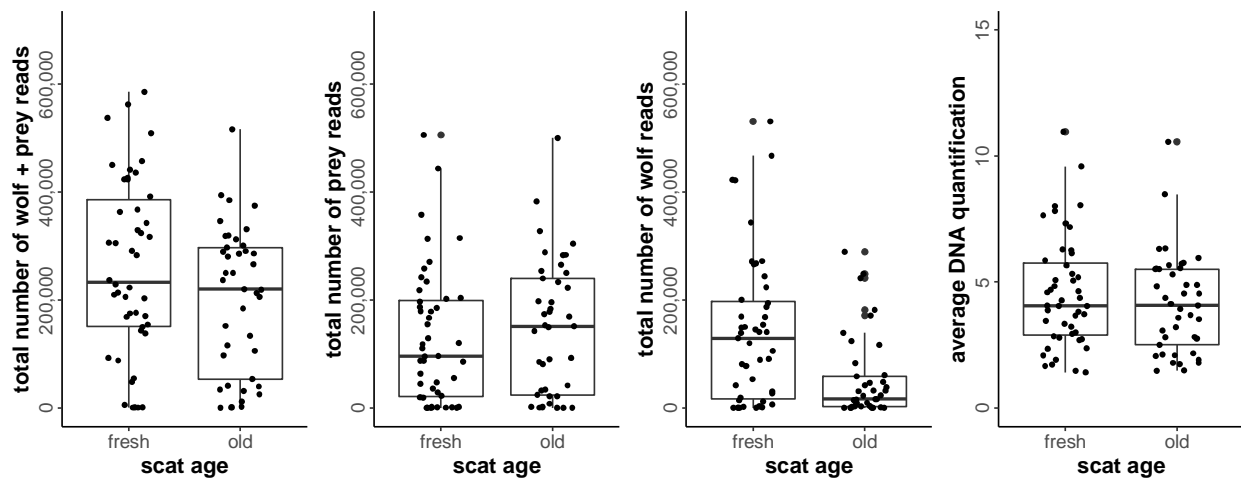
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682   **Figure 3**
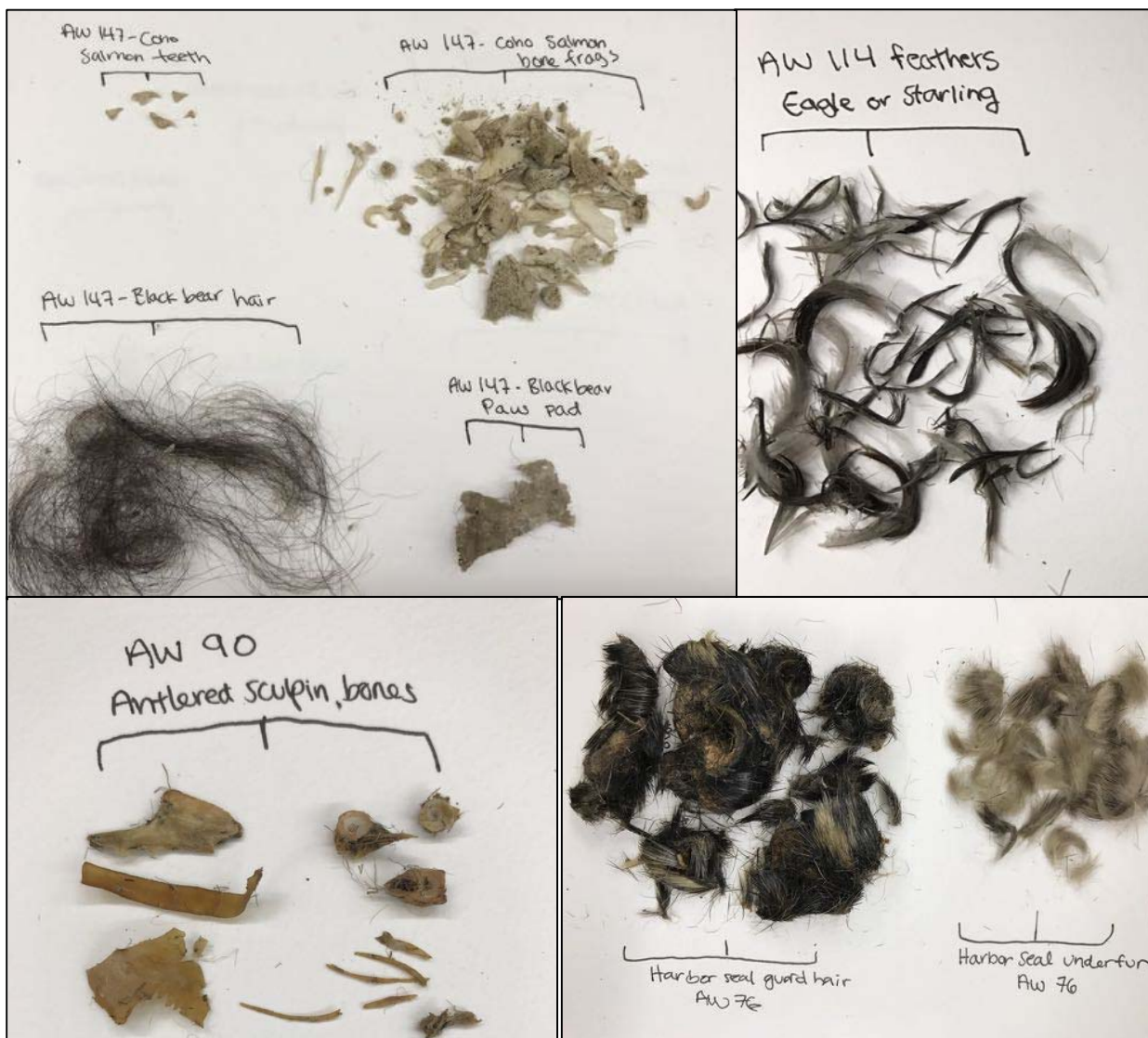
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690   **Figure 4**

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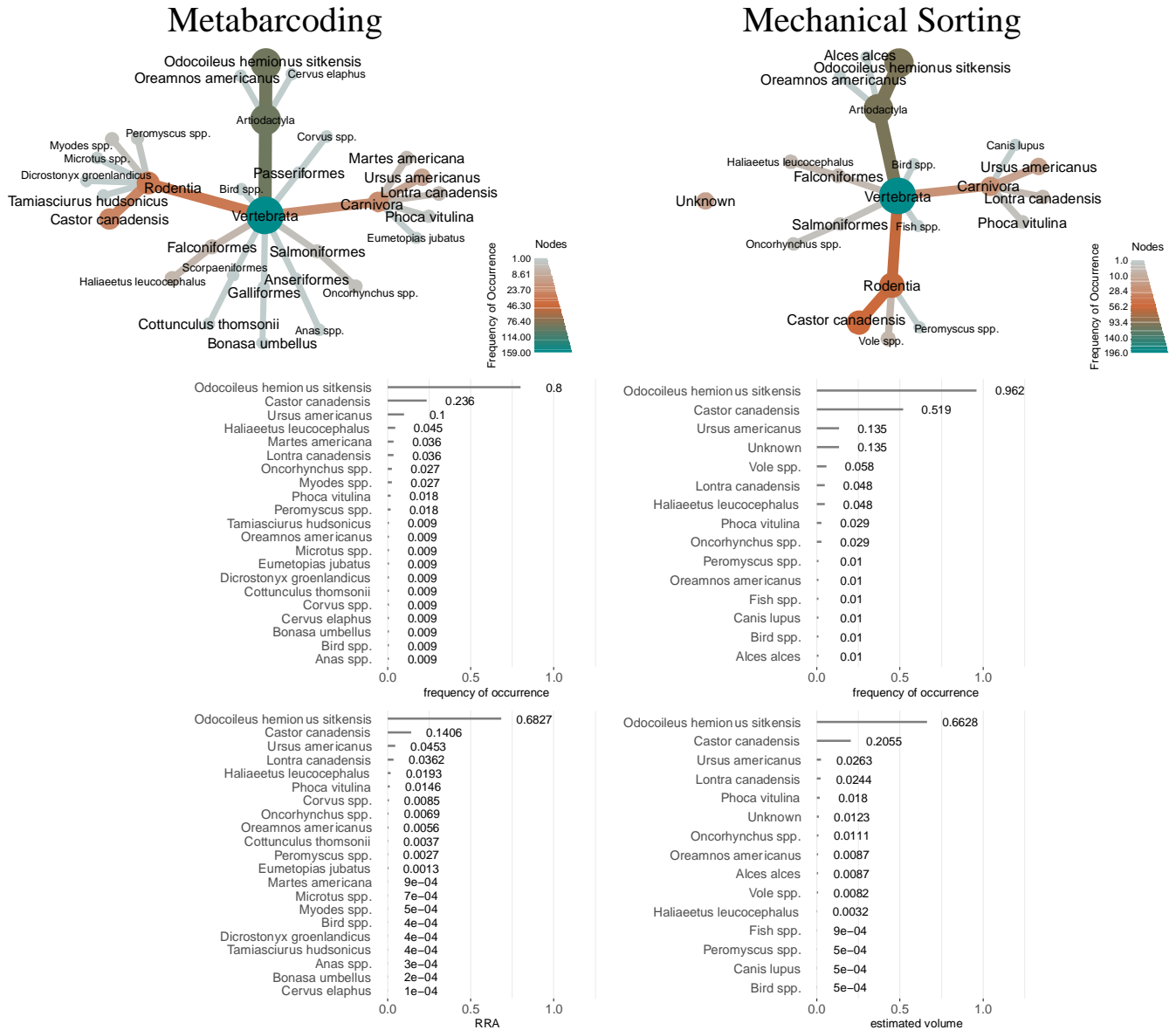
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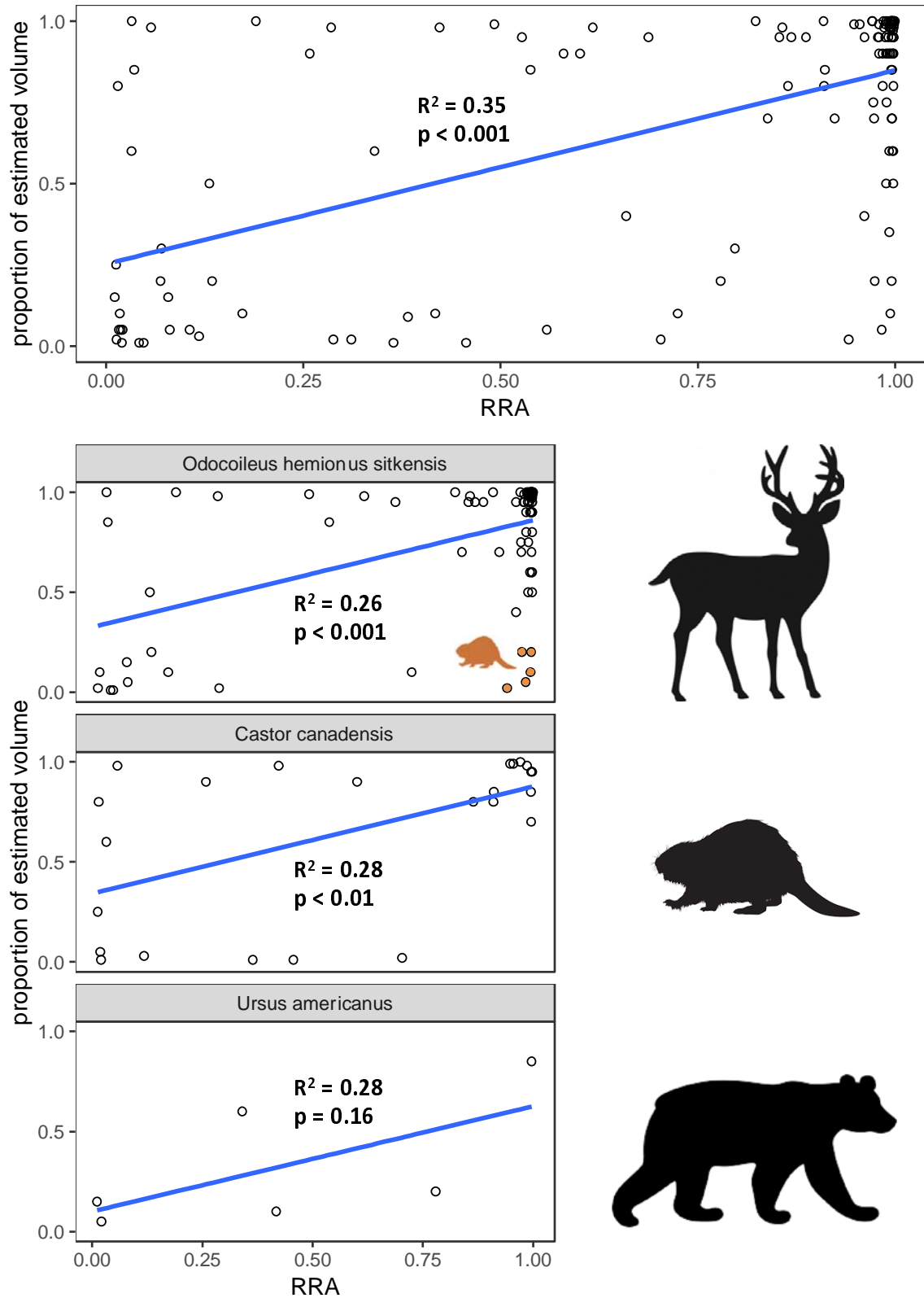699

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707 **Figure 5**

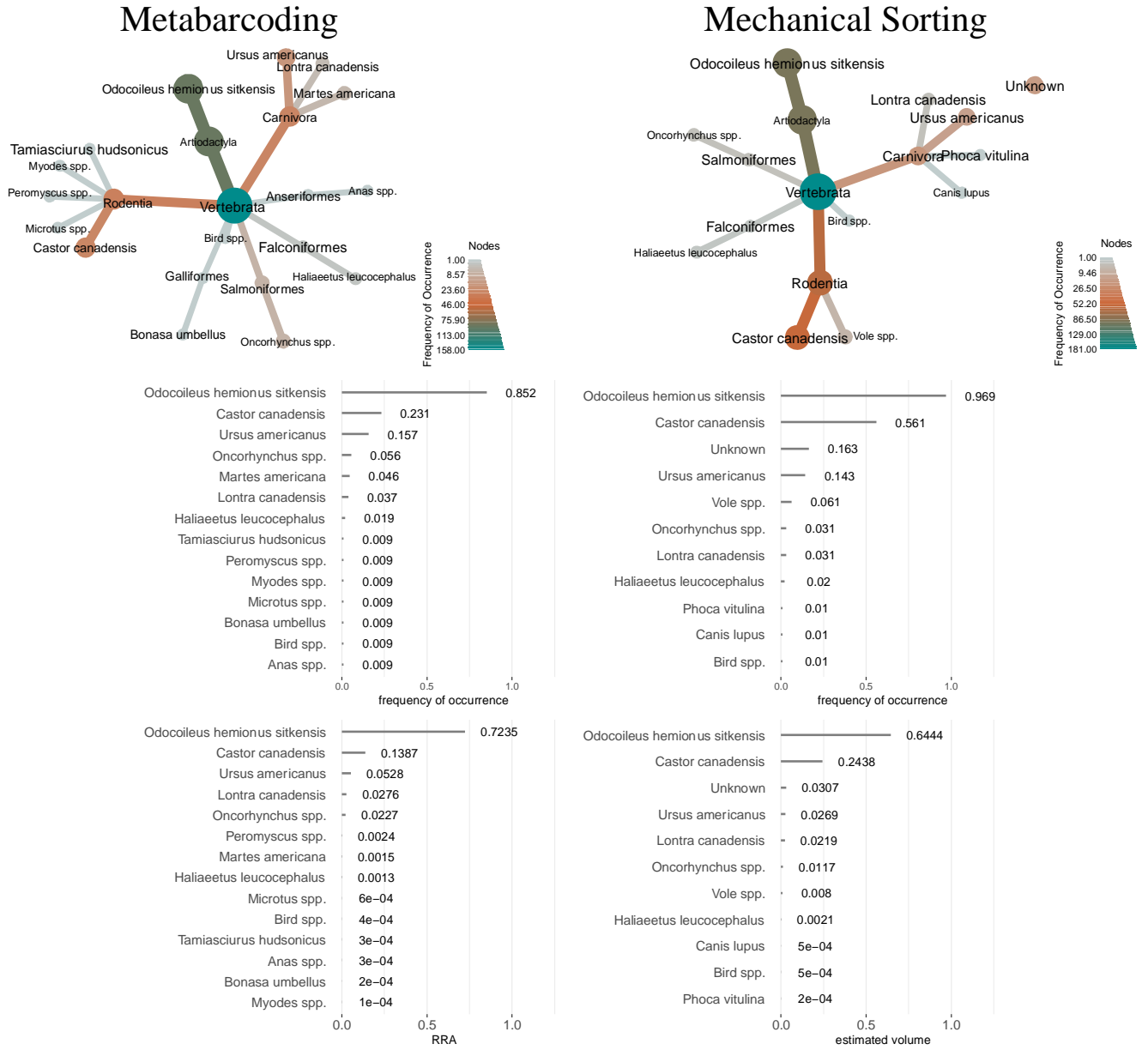714 **Figure 6**

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716 **Figure 7**

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