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## Diet of the raccoon dog, an invasive mesopredator, during the breeding season of declining waterbird populations

Elina Tuomikoski<sup>\*</sup>, Vesa Selonen, Kaisa Merimaa, Toni Laaksonen

Department of Biology, University of Turku, Turku FI-20014, Finland

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### ABSTRACT

Invasive predators are one of the leading causes of global biodiversity loss, threatening local ecosystems through predation and spreading diseases. In Europe, the most common and widespread invasive mesopredator is the raccoon dog (*Nyctereutes procyonoides*). Raccoon dog is an opportunistic omnivore listed as a species of European Union concern, as they are suspected of harming ground-nesting waterbird populations. However, the actual extent of risks caused by raccoon dog predation has been disputed, partly due to methodological constraints in detecting all prey in the diet. Here, we studied the animal components of raccoon dog diet using DNA metabarcoding, a powerful tool to detect even the smallest traces of ingested food items. We sequenced genes 12S rRNA and COI from raccoon dog scat samples collected during the breeding season of birds close to water bodies in southern Finland. We also examined whether the occurrence of different dietary items would depend on the habitat around the latrine. We found that frogs were the most common vertebrate food item in raccoon dog diet during spring. The proportion of detected bird material was not higher compared to earlier studies, despite the potential to detect DNA originating from eggs. The proportion of fish in the diet increased with increasing water area around the latrine, but no other relationships between diet and habitat were found. We conclude that even though raccoon dogs predate on waterbirds' and other ground nesting birds' nests, they do not seem to be specialized on it in our study areas. However, declining waterbird populations and, in particular, local amphibian populations could be negatively impacted by raccoon dog predation.

### 1. Introduction

Invasive predators cause significant ecological and economic damage worldwide (IUCN, 2000; Salo et al., 2007; Doherty et al., 2016; Pyšek et al., 2020, but see García et al., 2014; Sax et al., 2022). They predate on native species, compete for resources with them and alter local ecosystems (Pyšek et al., 2020). It is especially problematic when invasive species create additional threat for endangered species or even are the cause of the endangerment (Doherty et al., 2016). For example, populations of ground-nesting birds such as many waterbirds have dramatically decreased in many parts of Europe in just few decades (McMahon et al., 2020), which has been linked to increasing mesopredator populations including invasive predators (Dahl and Åhlén, 2019; Nummi et al., 2019; Koshev et al., 2020; McMahon et al., 2020; Pöysä and Linkola, 2021; Jaatinen et al., 2022).

The most common and widespread invasive mesopredator in Europe is the raccoon dog (*Nyctereutes procyonoides*). In the European

<sup>\*</sup> Corresponding author.

E-mail address: [elaielt@utu.fi](mailto:elaielt@utu.fi) (E. Tuomikoski).

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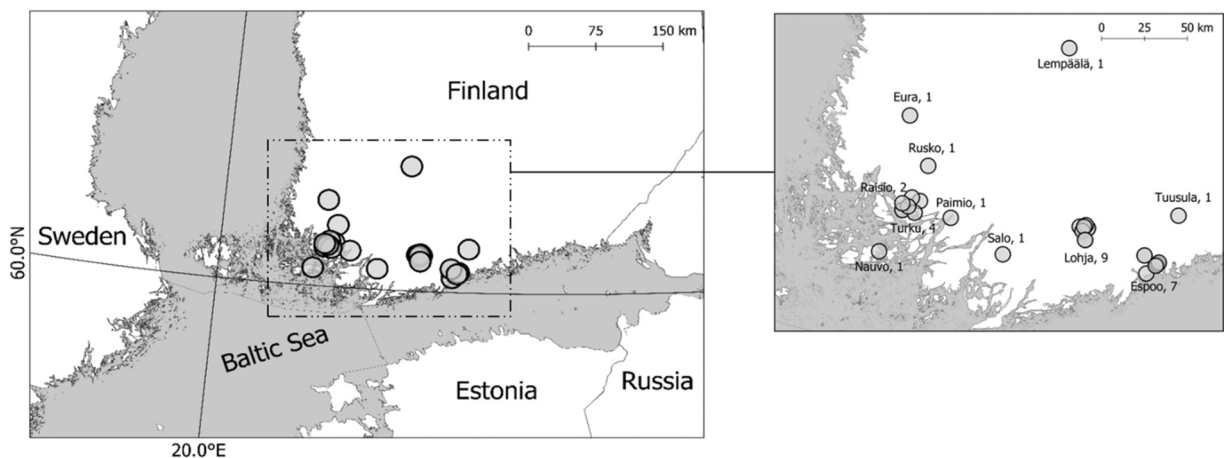
Union (EU), it is listed as a species of Union concern (EU, 2017/1263; European Commission, 2020). Originally from East-Asia, raccoon dogs spread to Europe via introductions to western Russia and fur farming (Kauhala and Kowalczyk, 2011). They are opportunistic, omnivorous and highly adaptive mammals that have successfully established permanent populations in eastern and central parts of the continent, and continue to spread (Sidorovich et al., 2008; Kauhala and Kowalczyk, 2011). Raccoon dogs are suspected of causing significant harm, e.g. through predation on native fauna and by spreading diseases like rabies and echinococcosis (Kauhala and Kowalczyk, 2011; Pyšek et al., 2020). In particular, raccoon dogs are suspected to be the reason for strong declines of some ground-nesting waterbird species through nest predation (Väänänen et al., 2007; Dahl and Åhlen, 2019; Holopainen et al., 2021; Jaatinen et al., 2022) and to have a negative effect on amphibian populations (Kauhala, 2009). In some cases, non-native species can have a neutral or even positive effect on the ecosystem (Norbury et al., 2013; García et al., 2014; Sax et al., 2022). However, to our knowledge, there is no evidence yet for possible neutral or positive effects of the raccoon dogs in alien ecosystems.

In Northern Europe, raccoon dogs inhabit varying habitats, preferring small-scaled landscape mosaic of meadows and fields, small mixed forests, gardens (Kauhala et al., 2010) and wetlands (Toivonen, 2023). They mostly move around the nest within a territory sized on average 1 km<sup>2</sup> (Kauhala and Kowalczyk, 2011; Toivonen, 2023). Available habitat may determine the diet of raccoon dogs (Sidorovich et al., 2008; Osaki et al., 2019), but detailed studies on the effects of habitat on diet at a larger scale are lacking. Raccoon dog diet can be studied through examining fecal samples from latrines, the favored spots of raccoon dogs to defecate. They are usually situated near the nest, and by time their excrements form a pile of feces. Pairs can use the same latrines.

The real impact of raccoon dogs on their prey species populations has remained unclear (Kauhala and Kowalczyk, 2011). Their impact on ground-nesting birds has been studied through predator removal studies, the results of which have been contradictory (e.g. no impact: Kauhala, 2004, impact: Väänänen et al., 2007). Dietary analyses performed mainly as morphological analyses of scat or stomach contents (Kauhala et al., 1993; Sidorovich et al., 2008; Sutor et al., 2010; Elmeros et al., 2018) have indicated that raccoon dog diets contain a wide range of prey species that consist mainly of invertebrates, plants like berries and fruits, and small mammals (Kauhala et al., 1993; Sutor et al., 2010). In previous studies bird remains have been found in variable amounts, occurring in 8–41 % of samples (Sutor et al., 2010; Elmeros et al., 2018), passerines being the most common order (Kauhala, 2009; Sutor et al., 2010; Elmeros et al., 2018). Raccoon dogs are considered not to hunt actively on bigger prey like adult game birds but to scavenge them (Kauhala, 2009; Sutor et al., 2010). Egg shells are rarely (< 9 %) found in diet analyses (Kauhala et al., 1993; Sutor et al., 2010), even though raccoon dogs are known to actively scare brooding hens (Dahl and Åhlen, 2019) and to predate on natural and artificial nests (Dahl and Åhlen, 2019; Holopainen et al., 2021; Selonen et al., 2022). However, raccoon dogs usually only lick the egg content without consuming egg shells (Dahl and Åhlen, 2019), leaving no visible traces in stomach or scat. Frogspawn is another food type that is missed in morphological analyses of scats. It is also possible that human waste food leaves no morphological traces, when it does not contain indigestible parts like hair or bones.

The inability to identify easily digestible and hardly visible food items is one of the general limitations of morphological scat analyses. As an alternative method that should enable detecting also eaten eggs and frogspawn, DNA metabarcoding of scat content is increasingly used for wildlife diet analyses (Vesterinen et al., 2016; Buglione et al., 2020; Woo et al., 2022). To our knowledge, DNA metabarcoding has been used only once before in a raccoon dog diet study, in its natural habitat in Korea (Woo et al., 2022). There is thus a need to conduct DNA metabarcoding studies also in the areas where the raccoon dog is invading and suspected to cause harm to local fauna. Molecular methods are necessary to reliably assess the frequency of nest predation or possible prevalence of frogspawn as a dietary component.

We investigated the diet of the raccoon dog during the nesting period of waterbirds, to focus on the role of raccoon dogs as nest predator in spring, using DNA metabarcoding from fecal samples. Spring is also the breeding season of many other animals, like amphibians, making them vulnerable to predation (e.g. frogspawn is easy prey). We expected that raccoon dog diet would prove as



**Fig. 1.** Map of locations of latrines ( $n = 29$ ) in Southern Finland used by raccoon dogs from where fecal samples ( $n = 92$ ) were collected in May 2022. Numbers after collection place names indicate the number of latrines found in each area.

omnivorous as in previous studies (e.g. Kauhala et al., 1993; Sutor et al., 2010; Woo et al., 2022), but that the proportion of bird material could be higher than in previous diet studies conducted via visual examination if it consists largely of eggs. We further examined how diet was associated with habitat and predicted that more particular habitat type within the area of average territory leads to an increase in food items associated with that habitat type in raccoon dog diet.

## 2. Materials and methods

### 2.1. Study areas and sampling

Latrines were found with the help of a trained detection dog and human searchers. In total 92 fecal samples were collected and analyzed from 29 latrines distributed over a large spatial scale in Southern Finland (Fig. 1) during May 2022. Latrines were visited weekly from one to five times and 1–4 samples were collected per visit. Every sample was taken from a different excrement. Sample collection was targeted on the freshest looking scats, evaluated by appearance and reference photos from previous visits. Sampling effort was thus targeted on scats that were 0–8 days old, with the freshest samples clearly being only a few hours old. Collection places were in Lohja (9), Espoo (7), Turku (4), Raisio (2), Salo (1), Nauvo (1), Paimio (1), Tuusula (1), Rusko (1), Lempäälä (1) and Eura (1) (Fig. 1). All sites were in a close proximity to a waterbody such as a lake or a river and most of them belong to the Helmi habitats program led by the Ministry of the Environment of Finland. Helmi-habitats are biodiversity rich wetlands and lakes which are under protection or restoration. After collection, samples were stored in  $-20^{\circ}\text{C}$  until DNA extractions.

There are only two mesopredators in Finland that produce latrines (i.e. a site of regular defecation where faeces accumulate), raccoon dog and badger (*Meles meles*). The latrines differ so that the latrines of badger are in a shallow formation dug on the ground, while those of raccoon dogs are produced on bare ground, making identification of latrines possible. Furthermore, we searched and collected latrines from sites, where we knew raccoon dogs were present, but where badgers had not been observed. Thus, the likelihood of collecting badger faeces was small. Finally, we confirmed that all samples were of raccoon dog origin by DNA sequencing.

### 2.2. DNA metabarcoding

The animal components of the diet were detected through DNA metabarcoding. The vertebrate mitochondrially encoded 12S ribosomal RNA gene (12S rRNA) and a universal animal gene cytochrome c oxidase subunit I (COI) were sequenced in separate analyses ( $n = 92$  and  $n = 88$ ). DNA analysis company BioName (<https://bioname.fi/>) carried out the molecular workflow as turnkey service from sample to the bioinformatics and final data. All raw nucleotide sequence data is made available in the Sequence Read Archive (SRA) database under the BioProject accession PRJNA109030.

For DNA extractions, 100 mg of each sample was used per analysis. DNA extraction was done using Macherey-Nagel Stool Kit (ref 740472.250, Düren, Germany) following the manual version November 2016/Rev. 02. Two microlitres of DNA extract was used per sample in polymerase chain reaction (PCR).

The vertebrate gene 12S rRNA was amplified by duplicate using the universal vertebrate primer pair 12SV5 (Riaz et al., 2011). The universal animal COI locus was amplified by duplicate using primer pairs Leray and fwh2 (Geller et al., 2013; Leray et al., 2013; Vamos et al., 2017). Amplicon library diversity was increased by using each primer as two different versions, so that they included so called heterogeneity spacers between the linkertag and the actual locus-specific oligo. Two different blocking oligos were used in 12S and COI analyses to block the amplification of raccoon dog DNA: a blocking oligo Sup-Blk-Lerfwh (blocking oligonucleotide: GACTGTG-TACCCCCATTAGCTGGCAACCTA) was designed by BioName specifically to be used with Leray and fwh2 primers in COI analysis, and RacBlk was used in 12S analysis following Woo et al., (2022). In 12S analysis the PCR reaction setup followed Kankaanpää et al., (2020) and the cycling conditions followed Woo et al., (2022). The cycling conditions for COI analysis were as follows: 5 min in  $95^{\circ}\text{C}$ , then 30 cycles of 30 sec in  $95^{\circ}\text{C}$ , 30 sec in  $48^{\circ}\text{C}$  and 2 min  $72^{\circ}\text{C}$ , ending with 10 min in  $72^{\circ}\text{C}$ . In a 10  $\mu\text{l}$  reaction, BioName used 6000 nM of blocking primer with 300 nM of forward and reverse primers each in COI analysis.

Next Generation Sequencing (NGS) library was prepared both times following Vesterinen et al. (2016) with modifications. 12S sequencing was done using an Illumina MiSeq v3 PE  $2\times 300$  run, and COI sequencing was done using an Illumina NovaSeq6000 SP FlowCell v1.5 PE  $2\times 250$  run (Illumina Inc., San Diego, California, USA). PhiX control library by the Turku Centre for Biotechnology, Turku, Finland was included in both sequences runs.

Bioinformatics pipeline followed Kaunisto et al., (2020). For 12S dataset, the length of merged reads after primer trimming was 80–100 bp. For COI dataset, the minimum length after primer trimming was 196 bp. Accepted error rate for primers was 20 % in both analyses. After primer trimming reads were collapsed into unique sequences (singletons removed), after which chimeras were removed (unique reads denoised) and reads were clustered into zero-radius OTUs (ZOTUs).

The resulting 12S sequence variants were matched to taxa using GenBank nt database with local BLAST (Altschul et al., 1990) and assigned to the taxa by naïve Lowest Common Ancestor-assignment algorithm using software MEGAN6 (Huson et al., 2016) (min score 100.0, top percent 5.0). The COI sequence variants were assigned to taxa using a custom database with SINTAX (Edgar, 2010) in VSEARCH (Rognes et al., 2016) and BOLD (Ratnasingham and Hebert, 2007) identification engine with a custom script (Vesterinen et al., 2020). All reads per each taxon were summarized within a sample.

At this point, the reads that were assigned into “Nyctereutes”, order “Primates”, or family “Canidae” were filtered away from the 12S data. The tag-jumping rate was 0.5 % to ensure thorough filtering. The non-target taxa (Bacteria, Algae etc.) were filtered from both datasets, as was taxa with read count less than 2. Four samples were filtered out from the COI dataset due to not enough read counts, leaving 88 samples into the final COI dataset.

Despite the blocking primer, samples contained more raccoon dog DNA than DNA of any other canid ensuring that the latrines were actually raccoon dog latrines, and not badger's, which latrines look similar.

### 2.3. Analysis

Food items were categorized into food groups mimicking grouping in earlier studies and based on their presumed origin and/or significance in raccoon dog diet (Table 1, Supplement Table 1) (Sutor et al., 2010). For example, carrion and small mammals are distinguished based on the fact that raccoon dogs do not predate on large animals like deer, but can predate on smaller mammals like voles. Waterbirds are categorized as its own group apart from all other birds due to the focus of the study. *Sus scrofa* is classified as food waste, as the chances of a raccoon dog to come across a carcass of a wild boar in Finland are small. When grouped, different taxa were not taken into consideration separately, meaning that if a sample had DNA reads e.g. from taxa assigned as roe deer and from taxa assigned as white-tailed deer, the observation for the sample to contain carrion was 1 (= contains remains belonging to carrion).

The frequency of occurrence (% FO, the proportion of samples containing at least one food item belonging to a specific food group), relative frequency of occurrence (% RFO, the proportion of the food group in the diet) and relative read abundance (% RRA, the average percentage of reads of a given food group among positive samples) were used to summarize raccoon dog fecal sample content. Occurrence data is considered a conservative method and is thus easily comparable to earlier diet studies (Deagle et al., 2019). The con is that it may overestimate the proportion of small dietary groups in the diet. RRA, on the other hand, is more recently but also increasingly used approach, and it has been proposed to provide better estimates of population level diet (Deagle et al., 2019). However, whereas occurrence summaries are able to moderate methodological DNA read count biases, RRA summaries are not. To include both ways of incorporating the data, both occurrence and proportional DNA count data were used to summarize diet consistency in this study. We also calculated the trophic diversity of vertebrate food items in latrines with Shannon diversity index ( $H = -\sum \text{Pi}(\ln\text{Pi})$ ) using latrine specific RFO values.

For occurrence counts, DNA reads were transformed into binomial data, with any DNA read turned into 1 (1 = taxa found, 0 = taxa not found). The DNA reads varied from 2 to 105,635 (biggest DNA count was assigned for moose, *Alces alces*). For RRA the DNA reads were proportionalised per sample, and then averaged per food group.

To test whether habitat had an effect on vertebrate occurrence in raccoon dogs' diet, we used generalized linear mixed models (glmer from package lm4 in R). Occurrence of food group in the sample was the dependent variable (binomial model) and habitat variables (the surface area of urban areas, fields and waterbodies within 1 km<sup>2</sup> around the latrine) were the independent variables. Latrine ID was set as a random effect. Habitat class forest was excluded from the analyses due to strong correlation with agricultural fields (Spearman correlation  $-0.53$ ,  $p < 0.001$ ) and urban areas (Spearman correlation  $-0.51$ ,  $p < 0.001$ ). Habitats were measured around latrines around an area of 1 km<sup>2</sup> circle ( $r = 0,564$  km) and habitats are based on the Corine 2018 data using QGIS 3.16. The habitat types were used as proportions of the area they covered. Habitat class water bodies includes wetlands, treeless bogs, rivers, lakes and sea. Data analysis was made with R 4.1.3.

## 3. Results

### 3.1. Sequencing statistics

After sequencing, the raw sequence output was 5,367,612 reads (~56 000 per sample,  $n = 92$ ) and 1,486,435 reads (~15 400 per sample,  $n = 88$ ) for 12S and for COI runs, respectively. After all bioinformatics steps, the 12S sequenced samples contained on average 17,920 final reads and 1–10 taxa (median = 4.0, average  $\pm$  SD =  $4.2 \pm 2.1$ ) per sample. In turn, COI sequenced samples contained on average 4141 final reads and 1–10 taxa per sample (median = 6.0, average  $\pm$  SD =  $7.2 \pm 4.5$ ). 77 and 399 ZOTUs were assigned to genus level per run, respectively. We were only able to identify a few vertebrate species through COI sequencing. However, it seemed to work well with invertebrate DNA.

### 3.2. Vertebrates in the raccoon dog fecal samples

Amphibians and reptiles were the most frequently (FO and RFO) found vertebrate food group occurring in almost 70 % of samples,

**Table 1**

Food groups and food items classified into them in order of incidence where taxa are listed. See Supplement Table 1 for more taxa specific information. \*There was only one occurrence assigned as Ardeidae, which is most likely a predated egg, as the DNA read count was very low.

Food group	Food items
Amphibians and reptiles	Frogs, toads, newts, slowworms and (common) lizards
Carrion	White-tailed deer, roe deer, moose, unassigned Cervidae, hares and felines
Food waste	Pork (pig), chicken, salmon, red deer and beef (cow)
Small mammals	Small rodents like voles, mice, squirrels and shrews
Fish	Fish found in Finnish water bodies like cyprinids and perches
Waterbirds	All Anseriformes, family Laridae, common snipe and a grey heron/Eurasian bittern*
Other birds	Doves and pigeons, passerines, unassigned Phasianidae, Eurasian woodcock and a woodpecker

followed by carrion and food waste (Fig. 2), as detected through sequencing the vertebrate gene 12S rRNA. When measured as relative read abundance (RRA), amphibians and reptiles were the most abundant food group, followed by carrion and fish (Fig. 2). Waterbird DNA was found in 20 % of the samples, and made up the smallest part of the diet as a food group when examining the occurrence (RFO 8 %, Fig. 2). The RRA for waterbirds was higher than RRA for food waste, small mammals and other birds (Fig. 2). It is noteworthy that nearly half of the samples (9) containing waterbird DNA were found from one particular latrine in Koroinen, Turku. In total, samples with waterbird DNA were found from 13 latrines out of 29. The Shannon's diversity index for vertebrate food items in latrines was on average  $1.2 \pm 0.5$ .

The most frequently occurring species were common frog (*Rana temporaria*), moor frog (*Rana arvalis*) and pig (*Sus scrofa*) found in 44, 30 and 26 samples from 92, respectively. See Supplement Table 1 for all species occurring in the data and their occurrences. Most DNA reads were attained from common frog (510 108 DNA read counts), moor frog (325 225 read counts) and moose (*Alces alces*, 113 253 read counts).

Habitat did not have any obvious effects on occurrence of vertebrate food groups, except for positive association between occurrence of fish and proportion of water bodies within an area of a circle of 1 km<sup>2</sup> around the latrine (Table 2).

### 3.3. Invertebrates in the raccoon dog fecal samples

The two most frequent invertebrate taxa found in raccoon dog scat samples through COI gene sequencing were Nematoda (mostly class Chromadorea) (FO 86 %) and Arachnida (FO 84 %), found in almost all samples (Fig. 3). Nematoda are not assumed to be part of raccoon dogs' diets, but mainly to be secondary prey and consisting of species living in feces and soil.

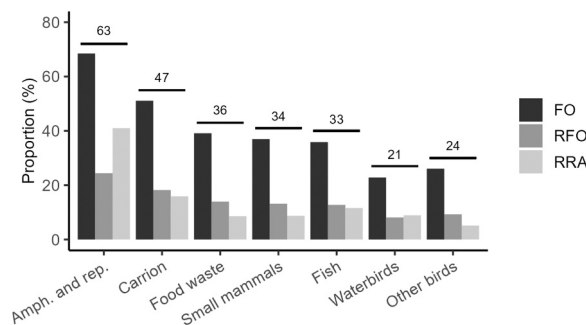
Canine gut parasite *Toxocara canis* DNA was found in two samples from two different latrines. Strongylida parasites (mammal gut parasites) were found from 25 samples out of 88. *Echinococcus multilocularis*, a parasite causing a disease called echinococcosis which is dangerous to humans, was not found (has not been found in Finland yet). The detected DNA sequences of *Toxocara canis* and Strongylida parasites are visible in the Supplement.

## 4. Discussion

We studied the diet of the invasive raccoon dog with DNA barcoding during the most important breeding season of waterbirds and amphibians in the proximity of wide range of water bodies and in different habitat compositions. Frogs were found to be the most common vertebrate food item during the study period, as they occurred in more than half of the samples. Waterbird DNA was found in 20 % of examined samples, which is similar or less than in previous studies of bird occurrence in raccoon dog diet (Kauhala et al., 1993; Drygala et al., 2014; Elmeros et al., 2018). Thus, we detected less waterbirds than could be expected, given that we used a more powerful tool, DNA barcoding, during the breeding season of waterbirds, for detecting potential DNA traces from eggs. Habitat proportion around the latrine did not affect the occurrence of food groups, except for occurrence of fish, which unsurprisingly was affected positively by the proportion of water bodies near the latrine.

### 4.1. Possible threats to native fauna through predation - amphibians and waterbirds in raccoon dog diet

We found a relatively low occurrence of waterbird DNA in the diet of raccoon dog during the breeding season of these birds (Fig. 2). This supports the conclusions that raccoon dog does not specialize on or consume large amounts of bird eggs in nature (Sutor et al., 2010; Mulder, 2012). The proportion of waterbird DNA occurrences from the vertebrate diet in May was only 8 %, similar to the RRA of waterbird sequences. This observed proportion of waterbirds in the diet drops even lower, when invertebrates and plants are included in the comparison. In addition, most waterbird DNA belonged to bird order Anserinae (geese and swans; Supplement Table 1). This DNA is likely to originate rather from migrating than from nesting birds, because geese migrating to the Arctic were very abundant in some of our study sites during the data collection. It is furthermore likely that this Anserinae DNA originates from carcasses, because

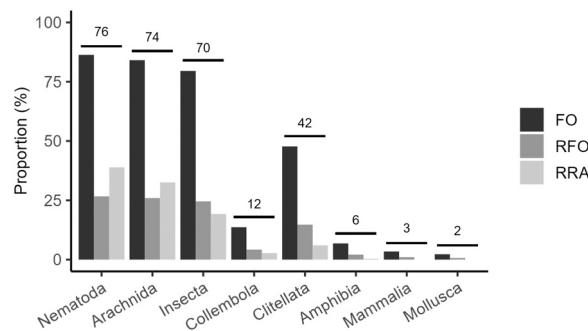


**Fig. 2.** Frequency of occurrence (% FO), relative frequency of occurrence (% RFO) and relative read abundance (% RRA) of different vertebrate food groups measured from raccoon dogs' fecal samples (n = 92) after DNA metabarcoding and 12S rRNA gene amplification. Number of occurrence of each food group is depicted above corresponding bars. See Supplement Table 1 for taxa-specific categorisation of food groups.

**Table 2**

The probability (occurrence) of a food group to be detected in fecal samples of raccoon dogs near water bodies in southern Finland in May in relation to habitat proportions around the latrine. For definitions of food groups, see Table 1 or Supplement Table 1. Estimate is given with  $\pm$  S.E.

Prey item	Effect	Estimate	$\chi^2$	P
Amphibians and reptiles	Intercept	1.11 $\pm$ 0.94		
	Urban	-3.81 $\pm$ 2.42	2.47	0.12
	Field	2.42 $\pm$ 2.11	1.31	0.25
Carrion	Water bodies	-1.29 $\pm$ 2.00	0.42	0.52
	Intercept	0.54 $\pm$ 0.83		
	Urban	-3.09 $\pm$ 2.20	1.96	0.16
Food waste	Field	0.59 $\pm$ 1.76	0.11	0.74
	Water bodies	-1.07 $\pm$ 1.79	0.36	0.55
	Intercept	0.04 $\pm$ 0.72		
Small mammals	Urban	1.29 $\pm$ 1.82	0.50	0.48
	Field	-0.82 $\pm$ 1.46	0.31	0.58
	Water bodies	-2.73 $\pm$ 1.78	2.36	0.13
Fish	Intercept	-0.26 $\pm$ 0.67		
	Field	0.99 $\pm$ 1.68	0.34	0.56
	Water bodies	-3.52 $\pm$ 2.01	3.09	0.08
Waterbirds	Intercept	-5.31 $\pm$ 1.86		
	Urban	6.44 $\pm$ 3.40	3.58	0.06
	Field	4.64 $\pm$ 3.04	2.33	0.13
Waterbirds	Water bodies	11.39 $\pm$ 4.28	7.08	<b>0.01</b>
	Intercept	-2.39 $\pm$ 1.10		
	Urban	3.68 $\pm$ 2.63	1.94	0.16
Waterbirds	Field	-0.35 $\pm$ 2.49	0.02	0.89
	Water bodies	1.36 $\pm$ 2.35	0.34	0.56



**Fig. 3.** Frequency of occurrence FO (%), RFO (%) and RRA (%) of animals found in raccoon dog fecal samples (n = 88) after DNA metabarcoding and COI gene amplification. Number of occurrence of each food group is depicted above corresponding bars.

raccoon dogs are not known to predate on fully grown geese. However, some Anserinae DNA could originate from eggs, such as those of whooper swan. DNA belonging to families Anas (2 occurrences) and Ardeidae (1 occurrence), and DNA assigned to a wader species *Gallinago gallinago* (2 occurrences) were detected, indicating that some waterbirds of conservation interest may also have been consumed.

The role of nest predation by raccoon dogs on waterbird populations is a debated topic. Nest predation does occur, so to some extent raccoon dogs do affect the decreasing waterbird populations (Holopainen et al., 2021; Dahl and Åhlen, 2019). It is also important to note, that even if nest predation is not a significant food resource for raccoon dogs, when raccoon dog populations are dense, even occasional nest predation events can cause significant harm to vulnerable prey populations (Väänänen et al., 2007). In addition, if the waterbird nests are rare in the landscape, they obviously cannot be an abundant part of raccoon dog diet. It is also likely that there is variation in nest predation rate in different regions. For example, in Finnish archipelago raccoon dogs may be a bigger threat to waterbirds than in the inland study areas of the current study (Kauhala and Auniola, 2001; Jaatinen et al., 2022). Nest predation rates may also vary seasonally, for example in a study by Brzeziński et al. (2024) the raccoon dog was observed to predate on artificial nests mostly in July, but not in May, which is when this study was performed. Egg content and frogspawn are also food items that may be difficult to trace even molecularly, as they are likely prone to methodological biases (delicate DNA templates) and may produce small DNA read amounts. In order to detect also the smallest DNA traces, the DNA count threshold was kept low in this study.

Raccoon dogs spend time in wetlands in spring (Toivonen, 2023), for which the main reason has been assumed to be predation on waterbird eggs and chicks. Our results do not support this claim and indicate that waterbird population numbers in inland ecosystems might not be impacted as heavily by the presence of raccoon dogs as previously thought. However, also amphibian populations are in decline in many parts of Europe (IUCN, 2009). They were the most important vertebrate food group for raccoon dogs in spring and, therefore, local amphibian populations could be impacted negatively by the raccoon dog predation, as suggested earlier by Kauhala

(2009). The main threats to amphibian declines globally are related to loss of wetland habitats (IUCN, 2009). The extra predation on top of that might create problems for declining amphibian populations.

We combined amphibian and reptilian species into one food group in our study, but this group consisted mainly of common frogs and moor frogs (Supplement Table 1). It is notable that the latter is protected by the EU habitats directive (Council Directive 92/43/EEC). Thus, raccoon dogs might at least locally cause extra risks for the protected moor frog populations. Interestingly, we also located two samples of *Pelophylax* (water frog group) in our data. These frogs are invasive in Finland and actually the site where *Pelophylax* was consumed is a site where these invasive species are abundant in Finland.

#### 4.2. Comparison of diet to earlier studies

The results in this study are somewhat similar to results in earlier studies (Kauhala et al., 1993; Sutor et al., 2010). In previous studies invertebrates, small mammals, amphibians and carcasses are frequently occurring food groups in raccoon dog diet in Europe (Kauhala et al., 1993; Elmeros et al., 2018). Small rodents have been reported previously in high numbers in raccoon dog diet, but in our data their occurrence was relatively low (Fig. 2). This is likely due to low vole population density in Southern Finland in spring 2022 (Luke, 2022), as the raccoon dog diet may vary with the availability of small rodents (Kauhala et al., 1993). The availability of small rodents has been proposed to even affect the proportion of birds in the raccoon dog diet (Kauhala, 2009). In our study, however, the low occurrence of small rodents was not reflected in high bird occurrence. Also, diversity of food items observed using Shannon index was comparable with earlier mesopredator diet studies (Hisano et al., 2022; Lanszki et al., 2023), being somewhat higher than for raccoon dogs in their native range in Japan (Osaki et al., 2019) and lower than previously calculated in Finland (Kauhala and Auniola, 2001).

Carcasses are an important food source for raccoon dogs (Kauhala, 2009). The occurrence and proportion of carcasses in raccoon dog diet is probably even higher than estimated here or in earlier studies, as some animals not classified as carcasses (like some birds) may be found dead by raccoon dogs (Kauhala, 2009). In winter and early spring carcasses are an especially important food source for not only raccoon dogs, but also to native mesopredators the red fox and the badger. Therefore, some competition for this food resource is possible (Kauhala, 2009). However, even though raccoon dog diet overlaps with the diet of native mesopredators, it seems unlikely that significant competition for food resources occur, as the diet range is wider for the previous (Elmeros et al., 2018). If there is no competition for carcasses between native species and the raccoon dog, scavenging on carrion could be considered an ecosystem service.

Food waste was the third most frequently occurring food group, including all non-wild DNA: pork, chicken, beef, red deer and salmon (Fig. 2, Supplement Table 1). It can be assumed that raccoon dogs get access to these food items anthropogenically, for example from garbage. Food items like pork and chicken have been reported in raccoon dog diet also previously (Kauhala et al., 1993). Due to efficient waste management in Finland food waste is likely from waste containers or garden composts accessible to raccoon dogs near human settlements, not from large amounts of waste in nature. High occurrence of food waste and carcasses imply that human-provisioned food sources are important for raccoon dogs. Some carcasses can also be classified as an anthropogenic food source, as hunters leave them behind and some are road kills. Thus, it seems clear that raccoon dogs benefit from human presence and based on habitat use analysis of GPS-tracked raccoon dogs they sometimes prefer close proximity to human settlements (Toivonen, 2023).

Canids, including raccoon dogs, are known to consume faeces of other species (Waggershauser et al., 2022; Kauhala, 2009). Faeces of domestic dog are relatively common and calorific, and therefore would be an easy source of extra nutrition (Waggershauser et al., 2022). Dog food often contains chicken and pork, separately or simultaneously, which could impact their occurrence in our data (simultaneously present in high number of samples). Thus, non-wild animal DNA may partly be secondary prey. As all canid DNA was filtered out before analysis, we could not check the occurrence of domestic dog DNA in raccoon dog faecal samples, though it was present. The relatively low RRA of food waste also points to a secondary source rather than primary (primary sources are predicted to be DNA-richer), though not excluding food waste as a real source of food (Fig. 2).

Earlier studies have found that invertebrates can be the most frequently occurring animal food group of raccoon dogs in spring (Fig. 3; Sutor et al., 2010; Drygala et al., 2014). In our data spiders were the most abundant insect taxa. Unfortunately, we cannot compare the 12S rRNA gene and COI data, the previous indicating the consumed vertebrates and the latter mainly invertebrates. The reason why COI did not find vertebrates remains unclear, but this also means that we are unable to evaluate how much invertebrates and vertebrates were consumed in relation to each other. In addition, it is known that the raccoon dog diet consists much of plant material, like berries in autumn (Kauhala, 2009), but plant material was not studied here.

#### 4.3. Habitat analysis

Habitat type around the latrine did not affect the occurrence of most food groups. This suggests that raccoon dogs likely forage for food within a wide area and the habitats around the latrine only poorly reflects the habitats used for foraging (Table 2). For example, larger proportion of urban area around a latrine did not have a significant positive effect on the occurrence of food waste, despite that non-wild food items like salmon and chicken are clearly an anthropogenic food source (Table 2). This may be due to the habitat class urban being a poor predictor of the availability of food waste that may come from waste produced in single households or food waste left at recreation areas visited by humans.

The only habitat type that had a significant effect on a food group was habitat class water bodies, which had a positive effect on the occurrence of fish. Sidorovich et al., (2008) found the proportion of water bodies to positively correlate with fish too, as with birds

(they looked at the proportion of lakes and open grassy marshes, which were included in water bodies category here). In raccoon dog's native area in Korea, its animal diet consists largely of aquatic vertebrates (Woo et al., 2022), indicating water bodies to be important for foraging. It was expected for water bodies to have a stronger effect of the occurrence of food groups here too, especially on waterbird occurrence.

#### 4.4. The limitations of DNA metabarcoding in studying the diet of raccoon dog

DNA metabarcoding is a widely used method to study animal diets and trophic interactions. However, it has some limitations/downsides. First, the detectability of DNA in fecal samples may be influenced by the gut passage time (= prey digestibility, variance in time different tissues get digested, e.g. egg content vs. meat), environmental conditions affecting the stability of DNA in the fecal sample, the time passed between excretion and sampling, and laboratory steps like PCR (Deagle et al., 2010). This is usually "corrected" by transforming DNA reads into binomial data, making any DNA observation equal. Another downside of DNA metabarcoding is that primers will amplify all DNA with the target gene, despite it being exogenous or accidentally consumed, like a worm with a fruit (Tercel et al., 2021). Thirdly, primary and secondary prey are not possible to tell apart. Primary prey is taxa that is intentionally eaten and which belongs to the diet. Secondary prey is taxa eaten by the target prey of studied animal. Coprophagy can also be classified as secondary prey.

For the above reasons, DNA metabarcoding can be tricky when studying omnivore diets (De Barba et al., 2014). To deal with that, Tercel et al., (2021) propose to run a post-hoc probabilistic co-occurrence analysis to test if taxa are simultaneously present in samples, in order to help to recognize secondary/accidental consumption. In our data, for example, some invertebrates detected may have been originally consumed by amphibians that then were eaten by the raccoon dog (there was a trend on this direction in our data, but it is clear that raccoon dogs also directly eat invertebrates). In general, small number of taxa per sample make dietary counts more accurate (Deagle et al., 2019), and here the mean number of food taxa per sample for 12S analyses was not high (average  $\pm$  SD:  $4.16 \pm 2.14$ ). Finally, we cannot recommend COI sequencing with the primers used in this study for analyses aiming to sequence vertebrate DNA, because it poorly detected the vertebrates from our samples (more critique regarding COI sequencing expressed in e.g. Deagle et al., 2014).

## 5. Conclusion

We conclude that DNA metabarcoding did not detect more traces of vulnerable waterbirds in the diet of invasive raccoon dogs than did earlier diet analyses that were poor in detecting traces of e.g., egg content in the diet. Thus, the raccoon dog does not seem to be specialized on nest predation of waterbirds in our inland study areas in southern Finland. This finding is encouraging for waterbird conservation even though it might suggest that other factors (native bird nest predators like the red fox and crows, eutrophication) contributing to declining waterbird populations play a bigger role than previously has been assumed or, alternatively, our study areas are already deprived of waterbirds. It is also clear that raccoon dog as an omnivorous opportunist eats easily accessible food (Kauhala 2009; Osaki et al., 2019), including eggs of all ground nesting bird species, when it detects them, playing a role as a predator. However, our study pinpoints that amphibians may be in a risk due to dietary preferences of the raccoon dog, which currently is one of the most successful invasive mesopredators in Europe. It is advisable to take actions to mitigate possible impacts on native species.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.gecco.2024.e02917](https://doi.org/10.1016/j.gecco.2024.e02917).

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