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Master's Thesis

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DNA Metabarcoding Analyses of Non-Native Raccoon Dog Diet Inside and Outside Nature Conservation Areas in Denmark

Using invasive and non-invasive sampling techniques to link diet- and habitat selection of an exotic canid

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PREFACE

This research thesis was initially spurred on by a shared interest between my advisors and me in heightening the ecological understanding of the much-debated non-native raccoon dog (*Nyctereutes procyonoides*), who during the previous years has become a regular feature in the Danish national news. The project initially started as a diet selection study inferred from fecal content only, but quickly morphed into a study based on a combination of sample types when the opportunity to include guts from culled raccoon dogs became an option. Over time, the project grew to include preparations of a third sample type (intestines) and a pilot metabarcoding study, whose results were presented at a scientific conference. Due to space limitations, however, the results of the pilot study are not included in the main body of this thesis (but see Appendix, Fig. A.1 for brief results), yet its methods and those associated with preparing intestines are provided with the hopes that they may inspire future studies.

This thesis is presented in two parts of which Part I contains detailed background information on raccoon dogs, techniques associated with the metabarcoding analyses for the primary metabarcoding and the pilot study, the statistical and analytical tools used throughout the thesis, and some thoughts on interpretations and limitations of our data. Part II contains a journal-style manuscript, in which succinct introduction and method sections allow readers to interpret the primary results and subsequent discussion by themselves.

Field data analyzed in this project was collected by hunters, the Danish Nature Agency, and LP during the year of 2018.

Louisa Pless
October 18, 2019

"Climb mountains not so the world can see you, but so you can see the world"
David McCullough Jr.

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ABSTRACT

Exotic predators are commonly believed to have negative impacts on native fauna, yet supporting facts are often sparse. This renders detailed descriptions of diet selection and feeding habits imperative for understanding the ecological impacts of exotic species, particularly those with a wide dietary breadth. Here, we use DNA metabarcoding analyses to assess vertebrate diet selection of non-native, omnivorous raccoon dogs (*Nyctereutes procyonoides*) collected across Jutland, Denmark and compare it to diet selection within two nature conservation areas (NCA). We use generalized linear models to predict diet selection in response to habitat and temporal variables and note differences between Jutland and NCAs. We also compare our findings with other raccoon dog diet studies conducted in Denmark and briefly discuss advantages associated with non-invasive sampling techniques.

Gut samples from 44 euthanized raccoon dogs collected across Jutland showed that birds and mammals were the most diverse and frequently consumed taxonomic classes constituting a combined 72.4% of all vertebrate read counts, while Toads *sp. (*Bufo *sp.*) was the single most frequently detected dietary item found in 45.5% of gut samples. Within NCAs, fecal samples from nine latrines indicated that amphibians were the most frequently consumed taxonomic class detected in 59% of all samples with Moor frog (*Rana arvalis*) being the single most frequent and abundant food item detected in 55.8% of all samples. Generalized linear models showed that avian and mammalian consumption was correlated with drier habitats, while amphibian consumption was correlated with wet habitat types both inside and outside of NCAs, but with distinct differences between the study regions.

The potential frequent consumption of amphibians detected in this study suggests a need for additional knowledge and we recommend that future studies look into possible negative impacts on native amphibians by an exotic omnivore.

Key Words: Alien species, diet preference, environmental DNA, modified CO1, nature preserves, generalized linear models

RESUMÉ

Ikke-hjemmehørende rovdyr antages tit for at have negative indvirkninger på hjemmehørende fauna, omend der kun eksisterer få underbyggede studier. Det gør detaljerede beskrivelser af fødepræferencer og adfærd vigtige for at kunne klarlægge økologiske indvirkninger af eksotiske arter, særligt dem med et bredt fødevalg. I dette studie bruger vi DNA metabarcoding analyser til at undersøge vertebrat fødeemner af ikke-hjemmehørende mårhunde (*Nyctereutes procyonoides*), indsamlet i hele Jylland, Danmark, og sammenligner det med fødevalg indenfor to beskyttede naturområder. Vi bruger generalized linear modeller til at forudsige fødeselektion som respons til habitat- og tidsvariabler og nævner forskelle mellem Jylland og beskyttede naturområder. Derudover sammenholder vi vores resultater med andre mårhunde føde studier udført i Danmark og diskuterer kort fordele forbundet med non-invasive indsamlingsteknikker.

Maveprøver fra 44 aflivede mårhunde, indsamlet bredt i hele Jylland, viste at fugle og pattedyr udgjorde de mest artsrige og hyppigst fortærede taksonomiske klasser, som tilsammen udgjorde 72.4% af alle vertebrat sekvensantal, mens Skrubtudse *sp*. (Bufo *sp*.) var det hyppigst forekommende individuelle fødeemne fundet i 45.5% af alle maveprøver. Indenfor beskyttede naturområder indikerede fækalieprøver fra ni latriner, at padder var den hyppigst fortæret taksonomiske klasse, fundet i 59% af alle prøver, med Spidssnudet frø (*Rana arvalis*) som det hyppigst forekommende og største andel konsumeret individuelle fødeemne fundet i 55.8% af alle fækalieprøver.

Generalized linear modeller viste at fortærelse af fugle og pattedyr var korreleret med tørre habitater, mens fortærelse af padder var korreleret med våde habitatstyper både indenfor og udenfor beskyttede naturområder, omend med tydelige forskelle mellem indsamlingsområderne.

Den potentielt hyppigt forekommende fortærelse af padder, påvist i dette studie, indikerer et behov for yderligere viden og vi anbefaler, at fremtidige studier undersøger potentielle negative indvirkninger på hjemmehørende padder forårsaget af et ikke-hjemmehørende, altædende dyr.

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AccuGENE	AccuGENE molecular biology graded water
AIC	Akaike information criterion
AVJF	Aage V Jensen Naturfond
BLOCKERS	Oligonucleotide blockers
COI	Cytochrome c oxidase 1
CPTT	Counts per ten thousand
ΔAIC	Delta AIC
DMI	Danmarks Meteorologiske Institut
eDNA	Environmental DNA
FO	Relative frequency of occurrence
GIS	Geographic Information System
GLM	Generalized Linear Models
GLMM	Generalized Linear Mixed Effect Models
HTS	High throughput sequencing
LAND COVER	Habitat types
LRT	Likelihood ratio test
MDS	Multidimensional scaling
METABARCODING	DNA metabarcoding
NCBI	National Center for Biotechnology Information
NCA	Nature conservation areas
NST	Naturstyrelsen
OTU	Operational taxonomic unit
PCR	Polymerase chain reaction
PopArt	Population Analysis with Reticulate Trees
QUBIT	Qubit dsDNA HS Buffer
RA	Overall relative abundance
RD	Raccoon dog

Part I

SYNOPSIS

1. BACKGROUND

1.1 Raccoon dog ecology

The opportunistic and omnivorous non-native raccoon dog (hereafter RD) (*Nyctereutes procyonoides*) , first detected in Denmark in 1980 (Mikkelsen et al. 2016; Nørgaard 2017), is native to East Asia where its natural range includes southeastern Siberia, China, northern Indo-China, Japan, and Manchuria (Baltrūnaitė, 2006; Sutor et al. 2010). Between 1929 and 1955, an estimated 9100 RDs were deliberately introduced multiple times to the European part of the former Soviet Union for enrichment of the fur trade (Kauhala and Kowalczyk 2011). The species has since spread throughout eastern, central, and northern Europe (Fig. 1) and has, as of 2008, established free-living populations in Denmark (Kauhala and Kowalczyk 2011; Rømer et al. 2015).

RDs are a medium sized (3-11 kg), predominantly nocturnal canid (Saeki et al. 2007; Nørgaard et al. 2014; Sheard & Pedersen 2012) with high behavioral, dietary, and ecological plasticities (Saeki et al. 2007; Kauhala & Kowalczyk 2011). It displays opportunistic, omnivorous feeding habits that change in response to temporal and spatial availability of dietary items (Elmeros et al. 2018), which includes small mammals, birds, amphibians, invertebrates, crops, fruit, and carrion (Miljøstyrelsen 2010; Sutor et al. 2010; Kauhala & Kowalczyk 2011; Mikkelsen et al. 2016; Elmeros et al. 2018; Takatsuki et al. 2018). Habitat preferences include wet habitats with abundant undergrowth, reed beds, mixed and deciduous forests, fields, lakes, shore lines, and gardens (Drygala et al. 2008c; Kauhala & Kowalczyk 2011; Kauhala & Ihalainen 2014; Elmeros et al. 2018). As the only canid (Nørgaard et al. 2014), RDs are facultative hibernators (Pagh & Chriél 2017) that retreat to dens when temperatures are low, day length decreases, and snow cover increases (Kauhala et al. 2007; Kauhala & Kowalczyk 2011). In Denmark, however, RDs appear to remain active throughout the year (Sheard & Pedersen 2012) likely due to a generally mild winter climate (Danmarks Meteorologiske Institut 2019).

Reproductive rates are high with females maturing sexually between 8-12 months (Miljøstyrelsen 2010, 2019a; Kauhala & Kowalczyk 2011) and having average litter sizes of 8-10 pups (Helle & Kauhala 1995; Kauhala et al. 2010). A recent study (Buxbom 2017) suggests reproductive outputs are higher in Denmark with litter sizes averaging 11 pups, likely in response

to the temperate climate (Helle & Kauhala 1995; Kauhala & Kowalczyk 2011; Danmarks Meteorologiske Institut 2019) and abundant food availabilities (Sutor et al. 2010). First year mortality is 70% ± 20% (Drygala et al. 2010), but rates of 89% have been estimated north of Denmark (Kauhala & Kowalczyk 2011).

RD's home ranges have been found to vary from 0.93 km^2 reported from southern Finland (Kauhala et al. 2010) to 5.0 km^2 reported from Poland (Jędrzejewska & Jędrzejewski 1998), while average home ranges in Denmark have been estimated at 4.04 km^2 (Pagh 2016). Individuals are non-territorial (Drygala et al. 2008a, b; Miljøstyrelsen 2010; Sutor & Schwarz 2012; Drygala & Zoller 2013) and multiple pairs can be observed feeding in close proximity or even from the same food source (Ikeda 1984; NST 2018, unpublished). The species is highly monogamous (Kleiman 1977; Helle & Kauhala 1995; Nørgaard et al. 2014) and usually travels in pairs accompanied by pups of the year for a few months following den emergence in June (Ikeda 1984; Kauhala et al. 2007). This social structure may explain why RDs build latrines, utilized by multiple individuals, since latrines are known to serve as communication centers amongst conspecifics and as landmarks that enable orientation (Ikeda 1984; Yamamoto 1984; Roper et al. 1993). Latrines may thus contain fecal matter from different individuals (Pagh & Chriél 2017) with fecal samples deposited along a temporal gradient (Ikeda 1984; Yamamoto 1984).

Natural predators of RDs include wolves (*Canis lupus*), lynx (*Lynx lynx*), Golden eagle (*Aquila chrysaetos*), and Eurasian Eagle owl (*Bubo bubo*) (Miljøstyrelsen 2010), while intraguild predation by red fox (*Vulpes vulpes*) and European badger (*Meles meles*) does occur (Kauhala and Kowalczyk 2011; NST 2018, unpublished). Conversely, RDs have been observed feeding on red fox pups (NST 2018, unpublished) and have been known to outcompete red fox and badger from dens and other hiding places (Kauhala & Salonen 2012). More commonly though, is co-inhabitation between badger and RD in badger setts, suggesting badger facilitation of RD establishment as setts provide refuge against predation and low temperatures (Kauhala & Kowalczyk 2011). Interspecific competition and commensalism are not addressed in this study but should be considered in future studies.

1.2 Exotic or invasive

Whether a species is invasive or simply non-native depends on both the impact and evolutionary history of the species. Invasion biology classifies species present outside of their past or present natural ranges due to human facilitation as non-native (exotic, alien, colonizing etc.), while species with disproportionate ecological or economic impacts, or who spread aggressively, are classified as invasive regardless of their native or non-native statuses (Lockwood et al. 2013; Simberloff et al. 2013). However, studies indicate that non-native species are more likely to negatively impact novel environments than native species and are 40 times more likely to be designated as invasive (Simberloff et al. 2013).

Successful colonizers often share traits that increase the likelihood of successful establishment including high reproductive output and dispersal abilities, generalist feeding behaviors, and high propagule pressures that allow for increased genetic variation commonly lost during establishment events (Lockwood et al. 2013). RDs with their high recruitment rates, flexible diet and habitat requirements, numerous introduction events, and broad climatic tolerances have enabled them to establish and spread rapidly from their first release in the Soviet Union into multiple European countries (Fig. 1) at a rate of 40 km/year (Kauhala & Kowalczyk 2011). The species is now considered one of the most successful exotic carnivores in Europe (Kauhala & Kowalczyk 2011) and is listed as invasive by the European Union (European Environmental Agency 2012), despite few findings documenting disproportionally negative impacts of RDs in their introduced range (Baltrūnaitė 2006, 2010; Drygala et al. 2000; Kauhala & Kowalczyk 2011; Elmeros et al. 2018).

1.3 Raccoon dogs in Denmark

RD escapees from captive stocks were first recorded in Denmark in 1980 and spotted sporadically until frequent detections by 2008 led to the conclusion that the species had established free-living, reproducing populations in Jutland (Mikkelsen et al. 2016; Nørgaard et al. 2014, 2017; Rømer et al. 2015). Based on the number of dead RDs collected by the Danish Nature Agency (Naturstyrelsen, hereafter NST) (Fig. 2; NST 2019, unpublished), the population appears to be increasing exponentially and is expected to reach an estimated carrying capacity of 30000 individuals (Rømer et al. 2015).

As part of the "Raccoon Dog Action Plan" developed by the Danish Environmental Agency (Miljøstyrelsen) to limit the species' expansion and prevent colonization of southern Sweden

(Miljøstyrelsen 2010; Svart, pers. comm.), RDs can be hunted throughout the year. Despite these efforts, the species is now found throughout Jutland and occasionally on Funen where individuals have been culled in 2018 and 2019 (NST 2019, unpublished). RDs are considered a threat to native amphibians and ground-nesting birds, partially due to the species' readiness to swim which may enable it to reach islands not frequented by native predators (Miljøstyrelsen 2010; Dahl & Åhlén 2018). Several studies (Vulla et al. 2009; Sutor et al. 2010) have found a positive correlation between increased latitude and increased carnivory towards birds, suggesting that RDs could pose a threat to avian species in Scandinavia. More recently, the species has been documented as an active nest predator of sensitive species, adding an apparent additive rather than compensatory predation pressure onto local native prey populations (Dahl & Åhlen 2018; Salewski & Schmidt 2019). However, to date the vast majority of studies show no decline in neither prey nor native predator (badger and red fox) populations (Elmeros et al. 2018; Kauhala & Auniola 2001), indicating that RDs fit a trophic niche between red fox and badger and that prey populations have yet to sustain negative impacts caused by RD colonization (Kauhala & Kowalczyk 2011; Mikkelsen et al. 2016; Elmeros et al. 2018).

1.4 DNA barcoding, metabarcoding, and environmental DNA

DNA barcoding methods are used to link a DNA sequence to a species (Valentini et al. 2008; Coissac et al. 2012). The barcode consists of a short and taxonomically informative DNA region (sequence) flanked by two conserved regions that serve as anchors for either universal or group-specific primers needed for polymerase chain reactions (hereafter PCR) (Taberlet et al. 2018). Standard barcode primers for animals consists of a fragment of the cytochrome oxidase I (hereafter COI) gene found in mitochondrial DNA (Hebert et al. 2003). After binding to the target region, the primers enable PCR amplifications which produces millions of copies of the target region (Taberlet et al. 2018). Following amplification, PCR products are sequenced and subsequently matched with a reference database for identification of the organism to species level (Valentini et al. 2008). The central idea behind barcoding is the ability to amplify species' diagnostic areas of the genome (i.e. sequences) rather than sequencing the whole genome thereby reducing associated costs (Taberlet et al. 2018). Since all organisms shed DNA into their environment (Willerslev et al. 2003), barcoding can be used to detect indiscernible DNA

remnants from a variety of organisms in a multitude of materials (Hebert et al. 2003; Valentini et al. 2008).

The term DNA metabarcoding is used when DNA from more than one organism is identified from the same sample using DNA barcoding methods (Coissac et al. 2012). DNA metabarcoding combines the principles of DNA barcoding with high throughput sequencing (hereafter HTS), a technology that allows for massive parallel sequencing of amplicons and identification of a broad range of species contained in a sample (Bohmann et al. 2014; Nielsen et al. 2018; Robeson et al. 2018).

The term environmental DNA (hereafter eDNA) metabarcoding refers to metabarcoding techniques used to extract DNA from environmental samples such as water, soil, sediment, or feces (Taberlet et al. 2012a, b, 2018; Bohmann et al. 2014). Such samples usually contain DNA from different organisms characterized by a complex mixture of genomic DNA with different levels of degradation (Taberlet et al. 2012a, 2018; Bohmann et al. 2014).

Here gut samples are considered DNA samples, while fecal samples are considered eDNA samples; collectively the method used to analyze both types is referred to as metabarcoding.

1.5 Invasive and non-invasive sampling techniques

In ecology, and most other fields, time, ethics, and financial restrictions often dictate the scope and possibilities of potential research projects. Techniques that allow researchers to save money and time when collecting and analyzing data while still obtaining accurate, conclusive, and ethically acceptable results are therefore desirable. The ability to implement non-invasive sampling techniques to analyze and monitor wildlife populations are becoming ever more feasible with continued improvements in eDNA metabarcoding techniques (Valiére et al. 2003; Valentini et al. 2009).

Here we used invasive and non-invasive sampling techniques to collect gut and fecal samples respectively. The former required RDs to be located and euthanized by licensed people, followed by gut removals by skilled personnel and subsequent gut content sampling and homogenization prior to DNA extractions. Fecal samples, on the contrary, were collected by a single person, required no special equipment, training, or direct encounters between animal and collector, and no sample preparations prior to DNA extractions.

1.6 Read counts as a proxy for abundance

High throughput sequencing provides occurrence data as well as read counts for targeted organisms. Whether read counts can serve as a proxy for relative biomass consumed (i.e. overall relative abundance (hereafter RA) of taxa based on read counts) is still debated (Deagle et al. 2019), as both biological and technical biases affect recovery rates of barcode markers from different taxa during HTS (Amend et al. 2010). While a direct translation of counts into abundance does not appear valid (Deagle et al. 2019), many studies suggest that read counts do work as a semi-quantitative proxy for prey abundance within individual samples and can be used to compare read count ratios between samples (Farrell et al. 2000; Deagle et al. 2006, 2009, 2019; Deagle & Tollit 2007; Murray et al. 2011; Nichols et al. 2016). As such, read counts contain valuable ecological information and, in this study, are interpreted as a rough estimate of the RA or biomass of food items, albeit with a wide margin of error. Read counts analyzed here do, however, only reflect RD diet within a limited time period ranging from early March to late September.

1.7 Ecological focus

This study contains multiple aspects broadly divided up into ecology and genetics. While the methods used to obtain our data were rooted in metabarcoding and associated bioinformatic data handling, the results and discussion described herein focus on the ecological aspects and interpretations. We acknowledge that our interpretations can be biased by differences in DNA quality due to uneven preservation quality of the samples (i.e. guts were kept frozen from the time of death of the animal, while fecal samples were exposed to environmental degradation for an unknown time period), yet many of the methods used in this study have previously been successfully applied to biological samples characterized by highly degraded DNA of varying quality (e.g. ancient samples; Pääbo 1989; Willerslev & Cooper 2005; Choi et al. 2015) and are thus considered appropriate. Additional methodological aspects may also have biased our data interpretations, including uneven sequencing depths, preferential amplification of selected species, and generation of chimeric PCR products of multiple species origin, (Valentini et al. 2008; Taberlet et al. 2018).

2. METHODS

Note: Relevant method descriptions contained in this section are repeated verbatim in Part II.

2.1 Study area

RDs were euthanized across Jutland by NST and local hunters, while fecal samples were collected from two different nature conservation areas (hereafter NCA) in Jutland (Fig. 3). The NCAs belong to Aage V. Jensen's Fond (hereafter AVJF) and are Filsø (23.28 km^2), located in the mid-western part of Jutland (55.703090, 8,224129), and Lille Vildmose (55.21 km^2), located southeast of Aalborg in the north-eastern part of Jutland (56.881681, 10.202946) (Fig. 3). Both NCAs provide important bird and amphibian habitat and are co-inhabited by RDs (AVJF managers, pers. comm.).

2.2 Field methods

RDs were culled by hunters and NST (n = 43) throughout 2018, collected as road kill (n = 1), or found dead (n = 1) (NST 2018, unpublished). Methods used to locate RDs consisted of tracking the individuals with detection dogs, digging them out of dens, trapping or luring individuals into bait stations, tracking Judas animals (i.e. gps collared RDs used to reveal the location of conspecifics; Miljøstyrelsen 2010) to locate their mates, and opportunistically spotting RDs (NST 2018, unpublished). Each time RDs were located, the animals were euthanized, except for judas animals who were most often released (NST 2018, unpublished). Euthanized individuals were kept at -20° C until being transferred to the National Veterinary Institute at the Technical University of Denmark where guts and intestines were removed and stored for the purpose of scientific studies (Chriél, pers. comm.).

Fecal samples were collected during September and October 2018 by LP who spent 3 days in each location hiking around the areas and visually scanning the ground for scat. Once detected, scat samples were placed in 50 ml Falcon tubes, marked with a unique identification code, and the location was recorded on a GPS unit (Garmin eTrex 30). Samples were stored at -20° C until DNA extractions commenced.

Latrines were located through the aide of AVJ managers' knowledge of existing latrines (n = 4) and the study area and by locating latrines through intense ground surveys (n = 5). All latrines were located in relatively dry areas such as mixed forests or mixed grassland with shrubs,

consistent with existing knowledge (Ikeda 1984; Baltrünaité 2006; Kauhala & Salonen 2012; Kauhala and Ihalainen 2014), always near the base of a tree/shrub, and with a max. distance of 540 m to a freshwater source (min. 77 m) (ESRI 2011).

2.3 Preparation of gut and intestine content for DNA extraction

Guts and intestines from 45 RDs that fit specific selection criteria were collected and prepared for metabarcoding analyses in multiple steps. Selection criteria were: (i) individual had not been baited, (ii) both intestines and stomachs were present, (iii) culled in a) June and Maj, b) April and July, and c) the rest of the year. We initially focused on individuals euthanized during the bird breeding season (May-June), but since only 26 samples fitted that criteria, the eradication period was extended to include individuals killed between March 6 – September 18, 2018.

Gut and intestine content samplings were conducted at the Zoological Museum at the University of Copenhagen. Stomach content was sampled by cutting open the stomach and filling a 50 ml Falcon tube with content. When stomachs were large (1.5+ grapefruit sized) (Fig. 4) and/or had highly heterogeneous content, two Falcon tubes were collected and DNA was subsequently extracted from both samples. Before removing gut content, stomach liquid content was pipetted into 1.5 ml Eppendorf tubes when possible. However, since a later study determined inconsistent DNA degradation rates in stomach liquid between different taxonomic classes (Bahlke 2019) the stomach liquid was excluded from further analyses. All samples were stored at -20° C until DNA extractions commenced.

Intestine content was sampled from the small intestines, large intestines and rectum by squeezing the content from approximately 15 cm of the small intestine and rectum into separate 50 ml Falcon tubes. Large intestines were cut open and content from an approximately 4 x 4 cm area was squeezed into 50 ml Falcon tubes. All samples were stored at -20° C. Intestine samples were initially collected with the aim of analyzing their microbiome and create a link between guts and intestines, but due to time restrictions no intestine samples were included in the following DNA analyses.

2.4 Homogenizing gut samples

Gut content homogenization and all remaining lab work was conducted under laminar flow hoods at the Section for GeoGenetics, University of Copenhagen, Denmark. To account for

heterogeneity, the contents of each gut was weighed and mixed with AccuGENE molecular biology graded water (hereafter AccuGENE) at a 1:1 ratio to enable content blending with a stick blender until content was homogenous (Fig. 5, 6). Two (2 ml) samples from each blended gut content was taken aside for DNA extractions, except when guts were very small (n = 6) or very large (n = 1) in which case one or four samples, respectively, were taken. No empty guts were examined. Blending and homogenization was conducted to ensure a sample was representative of all content contained in the stomach from which the sample originated.

To prevent contamination between individual guts, the stick blender was cleaned between each gut by being scrubbed in running water, rinsed in a bucket of water, immersed for 5 min in a 5% bleach solution, rinsed in a second solution of 5% bleach, followed by a rinse in autoclaved water and a 5 min submersion in a second batch of autoclaved water. Finally, the blender was left to dry before being used for the next sample. After every 5th sample, all water and bleach solutions were changed and controls of the autoclaved water were taken, as were controls of the autoclaved water after the last gut had been processed. All controls were included in subsequent PCRs to monitor for contamination.

2.5 DNA methods

2.5.1 Extractions

DNA extractions followed two protocols, QiAamp PowerFecal (Qiagen 2018) and DNeasy Blood & Tissue (Qiagen 2019) per the manufacturer's directions for fecal and stomach content respectively. The latter protocol was modified at the last step by eluding DNA with 2 x 30 ul Al buffer instead of 200 ul and a 5 minutes incubation at 56° C instead of no incubation. These modifications were done to increase DNA concentrations.

2.5.2 Sanger sequencing of fecal samples

To verify that fecal samples collected from latrines were deposited by RDs and not badger or otter (*Lutra canadensis*), the only other latrine-building carnivores that occur in Denmark, three subsamples from each latrine were amplified with cytochrome B primers (CanidC1 5'AATGACCAACATTCGAAA 3'(Paxinos et al. 1997); HCarn200 5'ATTCAGCCRTARTTA CGTC 3' (Bidlack et al. 2007)). These primers, produced by Integrated DNA Technologies, were chosen because they target a conserved region within the carnivore genome that still contains

enough variation to allow for species level assignment and because of the extensive coverage of mammals in their reference database (Nowak et al. 2014).

DNA concentrations of individual samples were quantified with the Qubit dsDNA HS buffer (hereafter Qubit) (Invitrogen) prior to each PCR run and samples were diluted 1:10 or 1:100 when DNA concentrations were too high. Identical PCR reagents and thermocycling conditions were used for all samples amplified with CytB with a single reaction per sample. Each PCR was performed in a total volume of 25µl that consisted of 2.5µl 10X PCR Gold buffer (Thermo Fisher Scientific), 2.5µl GOLD $MgCl_2$ (25 mM, Thermo Fisher Scientific), 0.2µl dNTPs (25 mM, Invitrogen), 0.2µl AmpliTaq GOLD polymerase (5U/µl, Thermo Fisher Scientific), 1µl forward primer (10 µM), 1µl reverse primer (10 µM), 1 µl Bovine Serum Albumin (20 mg/ml, New England Biolabs Inc.) 13.6 µl AccuGENE, and 3 µl RD fecal DNA. Thermocycling was conducted in Applied Biosystems 2720 Thermal Cycler machines with an initial denaturing step at 95° C for 10 min followed by 38 cycles of 95° C for 45 s, 54° C for 30 s, 72° C for 60 s, 1 cycle of 72° C for 5 min, and a hold at 4° C. For all PCR reactions, negative extraction and PCR controls, in which AccuGENE replaced extracted DNA, were included to monitor for contamination, while a positive PCR control with DNA known to amplify was included to ensure PCR reactions worked.

Following the PCR, a 50 bp DNA ladder along with 5 µl of each PCR product combined with 2 µl GelRed Nucleic Acid Gel Stain (Biotium) were loaded into wells in a 2% agarose gel and electrophoresed for 30 minutes at 140 V and 350 mA. Reaction products were subsequently visualized on a UV light platform and amplification success was determined based on the presence of bands at the expected fragment length.

PCR products (234 bp; Nowak et al. 2014) were sent to Macrogen Europe BV for Sanger sequencing and the results were subsequently verified by blasting against the National Center for Biotechnology Information (hereafter NCBI; NCBI 2019) database and matching the sequences to species level based on a >98% identity threshold. All samples (n = 3) from each of eight latrines matched RD, while the ninth latrine was inconclusive due to unsuccessful sequencing. The latter was eventually matched to RD based on the metabarcoding results, allowing for all fecal samples to be included in the data analysis.

2.5.3 Metabarcoding 18S primers, pilot study

A subsample of ten gut (two samples from each of five guts) and ten fecal (five samples from each of two latrines) samples were initially sequenced with individually tagged 18S eukaryotic primers TAReuk454FWD1- 5'CCAGCASCYGCGGTAATTCC3'; TAReukREV3- 5'ACTTTCGTTCTT GATYRA 3' (390 bp; Stoeck et al. 2010) to (i) ensure the entire procedure from amplification to successful sequencing worked, (ii) look for overall patterns of eukaryotic food items, and (iii) produce preliminary results needed for a poster presentation at a conference. 18S primers were chosen because they were in stock but were discontinued for the following and primary metabarcode analysis due the primers' lack of vertebrate specificity and inability to assign amplified DNA sequences to species level. Data from the pilot study was not included in the primary metabarcode analysis.

PCR reagents and thermocycling equipment used for all samples amplified with18S primers were identical to those described above, except for the addition of 1.5 µl tagged forward primers (10 µM), 1.5 µL tagged reverse primers (10 µM), and 2 µl DNA. Thermocycling conditions were 95° C for 7 min followed by 15 cycles of 95° C for 30 s, 53° C for 30 s, 72° C for 45 s, 20 cycles of 95° C for 30 s, 48° C for 30 s, 72° C for 45 s, 1 cycle of 72 ° C for 10 min, and a hold at 4° C. Contamination precautions, negative controls and positive PCR controls were included for all reactions as described above. Detailed results from the pilot study are not presented in this thesis (for brief results, see Appendix, Fig. A.1).

2.5.4 Sample pooling and Illumina library preparations, pilot study

Illumina library construction, conducted in this study, required all samples to be pooled. To ensure that a representative proportion of DNA from each sample was added to the pool, the PCR-amplified DNA was assigned according to the strength of each sample's gel band. These bands had previously been compared to a set of reference bands determined from Qubit DNA measurements of a subset of the samples. Strong intensity bands were assigned at 2.5µL, medium strength bands at 5µL, and low intensity bands at 7.5µL. The pooled samples were subsequently purified with MinElute (Qiagen) and the final DNA concentration was measured using Qubit. The latter was done to ensure the required >250 ng input of DNA into each library would be assigned correctly.

2.5.5 Illumina library build and MiSeq sequencing of 18S primers, pilot study

Single-indexed libraries with pooled samples were built with the Illumina TruSeq DNA PCR-Free Library Preparation kit (Illumina) per the manufacturer's directions. One library containing 26 samples with a DNA input of 400ng and one blank library were pooled and purified with MinElute (Qiagen), followed by an additional purification with Beckman Coulter Agencourt AMPure XP (1.5 bead ratio; Beckman Coulter) to remove primer dimers. DNA concentrations and specific lengths of the targeted sequences were measured using the Bioanalyzer (Agilent Technologies 2100 Bioanalyzer) with the Agilent High Sensitivity DNA kit, before the pooled libraries were submitted to the National High Throughput DNA Sequencing Centre, Section for GeoGenetics, Copenhagen, Denmark, for paired-end sequencing on a single MiSeq flowcell using a v3 300 cycle kit. Prior to sequencing, Illumina checked library quality following MiSeq recommendations.

2.5.6 Metabarcoding with modified COI primers

For the primary metabarcoding analyses conducted on the total number of samples (n = 251), tagged modified COI primers (Mod_RepCOI_F 5'-TNTTYTCMACYAACCACAAAGA-3'; VertCOI_7216_R 5'-CARAAGCTYATGTTRTTYA TDCG-3' (Reeves et al. 2018)) were chosen for their ability to amplify PCR products to species level from a taxonomically diverse range of vertebrates, production of relatively short amplicons (244 bp) well suited for commonly degraded fecal-derived DNA sequences (Deagle et al. 2006), and the highest taxonomic coverage in reference databases of all species identification markers (Kress et al. 2015; Reeves et al. 2018). No RD oligonucleotide blockers (oligonucleotide modified a the 3' end to avoid polymerase extension; Shehzad et al. 2012; Pompanon et al. 2012) (hereafter blockers)) were used. PCR, library build, and sequencing procedures followed the same protocols as described for the 18S primers, but with thermocycling conditions of: 95° C for 5 min followed by 40 cycles of 95° C for 30 s, 48.5° C for 30 s, 72° C for 60 s, 1 cycle of 72° C for 7 min, and a hold at 4° C.

2.5.7 Illumina library build and MiSeq sequencing of modified COI primers

Four libraries, each containing 72 - 73 samples and a DNA input of 400 ng, and one blank library were prepared and pooled according to the above described procedures.

2.6 Bioinformatics

All bioinformatic procedures were conducted by Dr. Tobias G. Frøslev.

2.7 Geographic Information System

All Geographic Information System (GIS) work was conducted in ArcMap 10.5.1 (ESRI 2011). All samples with corresponding gps locations (9 latrines, 41 guts) were imported into ArcMap and overlayed with Basemap (Levin et al. 2012), a raster file containing 34 georeferenced habitat types (hereafter land cover) within 10x10 m grid cells covering all of Denmark. Land covers were subsequently used as predictor variables in statistical models. To focus on areas deemed suitable for RD habitation, we excluded all anthropogenic land covers (except agriculture), which left 15 land covers that, for simplicity, were combined into eight unique land covers (Table 1). Buffers (r = 1000 m) were created around each sample using the "Buffer" tool, while the amount (m^2) of individual land covers found within each buffer were extracted with the "Intersect" tool (Fig. 7). The buffer radius was chosen so as to best capture the area in which a RD may have foraged, based on estimated home range sizes of 4.04 km^2 (Pagh 2016), while still keeping the buffers small enough to be representative of the area in which a sample (gut or latrine) had been collected. Samples were subsequently paired with the amount (m^2) of each land cover found within their buffer through a "Spatial Join", after which the percentage of each land cover within each buffer was calculated and used as predictor variables in candidate generalized linear models (hereafter GLM) and generalized linear mixed effect models (hereafter GLMM). Additional predictor variables included "distance (m) from each sample to the nearest freshwater source" calculated with the "Near" tool and "time of death" ((i.e. the date a RD was culled, available only for gut samples).

To estimate general habitat selection across Jutland (i.e. the land covers in which RDs were culled), the total amount of each land cover present in all of Jutland was extracted with the "Extract by Mask" tool and compared to the amount of each corresponding land cover averaged across all 41 gut buffers. Latrine locations were not included in the general habitat selection analysis.

2.8 Statistical analyses

Statistical analyses were performed in R version 3.5.2 (R Core Team, 2018) using the lme4 (Bates et al. 2015) and ggplot2 (Wickham 2016) packages. In ecology, GLMs are frequently used to

analyze how the odds of a dependent variable change in response to an incremental increase in the predictor variable which allows researchers to predict outcomes in dependent variables based on predictor variables.

Here, GLMs and GLMMs, an extension of GLMs that allows for mixed effects to be incorporated, were built to predict the presence of amphibians, birds, and mammals in gut and fecal samples, respectively, in response to amount (%) of land covers in buffers surrounding the samples, distance (m) to freshwater, and time of death of the individual (only available for guts). Latrine was included as a random variable in the GLMMs to correct for the nestedness of fecal samples within latrines. Fish and reptiles were excluded from the modelling analyses due to their low FOs (maximum 11.4% and 6.8%, respectively, Table 3 & 5).

Predictor variables consisted of eight land covers (agriculture, bog, coast, forest, freshwater, grassland, heather, wetland (Table 1)), distance (m) to freshwater, and time of death (expressed as Julian date where day 1 = earliest day a gut sample was collected), while taxonomic classes (amphibian, bird, mammal) served as binomial response variables (0 = absent, 1 = present). Coast and agriculture were excluded as predictor variables from the GLMMs since all latrines were located in inland NCAs where agriculture and coast were not present.

Candidate models around individual predictor variables were built and analyzed using the Likelihood Ratio Test (hereafter LRT), a method used to compare the likelihood of a model by determining the contribution of individual variables when they are either included in or excluded from the model (Bolker et al. 2008). Akaike information criterion (hereafter AIC) values, an information-theoretic approach that ranks models based on measures of the models' expected predictive powers (Akaike 1973; Burnham and Anderson 2002; Stephens et al. 2005; Bolker et al. 2008), associated with the LRT analyses were subsequently used to rank models and define the model with the smallest delta AIC value (hereafter ΔAIC) as the top model and hence the model with the best fit to the data (Akaike 1973).

Models were kept simple with just one predictor variable to ensure correct interpretations of their outcomes. While this may have led to few of the models achieving a high level of support (Table $7_{a1\text{-}c2}$, Table $8_{a1\text{-}c2}$), the models are an important step in linking habitat with diet selection and provide information around which to shape future management strategies. However, further studies should consider building more complex models to capture more of the variation associated with habitat- and diet selection.

To account for differences in sequencing depth, the relative abundance of the sample specific operational taxonomic unit (hereafter OTU) was calculated using the sample specific read count for each OTU normalized by the total read count per sample.

$$\text{Sample\_relative\_abundance}_{i,j} = \text{count}_{i,j} / \text{total\_read\_count}_j$$

i = 1, 2, …, n

j = 1, 2, …, m

n = number of identified OTUs

m = number of samples

The overall RA of OTUs, in fecal and gut samples respectively, was calculated using the sum of the sample_relative_abundance divided by the number of samples.

$$\text{Relative\_abundance (RA)} = \sum_{j=1}^{m} \left(\text{sample\_relative\_abundance}_{i,j}/\text{m}\right)$$

Relative frequency of occurrence (hereafter FO) was calculated as percentage of occurrence in gut and fecal samples, respectively. Differences in the number of OTUs detected in gut and fecal samples were analyzed with a nonparametric Wilcoxon test as this test is less sensitive to non-normally distributed data than regular t-tests.

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Part II

Raccoon Dogs – Using Novel Techniques to Assess Diet Selection of an Exotic Canid in Denmark

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1. INTRODUCTION

Despite an explosive growth in studies analyzing the effects of biological invasions (e.g. Mack et al. 2000; Richardson & Pyšek 2008; Vilá et al. 2010; Richardson & Ricciardi 2013; Dueñas et al. 2018), actual impacts of many colonizing species remain largely unknown (Kulhanek et al. 2011; Larson et al. 2013; Simberloff et al. 2013; Jeschke et al. 2014). Some studies suggest that colonizers have minor impacts in their introduced ranges (Sax et al. 2002; Gurevitch & Padilla 2004; Stohlgren et al. 2008), while others argue that non-native species cause significant impacts to established ecosystems and greatly reduce biodiversity (Wilcove et al. 1998, Clavero & García-Berthou 2005; Clavero et al. 2009). Such equivocal results indicate a need for additional knowledge and render detailed diet and feeding behavior studies of exotic species imperative, particularly for species with wide dietary preferences (Ballari & García 2014).

To answer the seemingly simple question: "what does this species eat?" requires accurate diet information that can be challenging to obtain due to the complex tasks of observing consumption events and directly identifying food items from stomach content (Pompanon et al. 2012). Traditional diet analyses are often limited to detecting recently consumed food as highly digested items, or items without easily discernible body parts, are difficult to accurately identify (Ballari & García 2014; Schley & Roper 2003; Valentini et al. 2008).

DNA metabarcoding (hereafter metabarcoding) has successfully been applied to infer diet selection for fauna with complex feeding behaviors (Valentini et al. 2008; Robeson et al. 2018) since molecular methods generally provide high taxonomic prey resolution and because DNA from morphologically indiscernible items remain traceable for extended periods of time (Nichols et al. 2016). The advent of high throughput sequencing technologies that allow for simultaneous screenings of a broad diversity of taxa within a sample (Valentini et al. 2008; Bohmann et al. 2014; Nielsen et al. 2018; Robeson et al. 2018) make metabarcoding a useful method with which to obtain deeper insight into the diet of omnivorous, elusive species (De Barba et al. 2014), such as the non-native raccoon dog (hereafter RD) (*Nyctereutes procyonoides*).

Originating in East Asia, the highly adaptable, opportunistic and generalist RD was deliberately introduced to the Soviet Union (Fig. 1) in the 1920s from where it spread into Denmark and, as of 2008, established free-living, reproducing populations (Kauhala & Kowalczyk 2011; Rømer et al. 2015; Nørgaard 2017). The species is classified as invasive by the European Union (European

Environmental Agency 2012), who in 2010 provided a LIFE+ grant to support transnational eradication efforts between Sweden, Denmark, and Finland which included culling RDs and erecting a warning system through which citizens could report RD sightings (Miljøstyrelsen 2010). Despite these efforts, the species has established across northern Europe (Drygala et al. 2008b; Kauhala & Kowalczyk 2011) including on the Jutland peninsular, Denmark (Sunde & Elmeros 2016). Recent detections of individuals on islands east of Jutland (NST 2018, 2019, unpublished) indicate the population is expanding eastward.

RDs are considered a threat to native fauna, particularly ground-nesting birds (Dahl & Åhlén 2018; Salewski & Schmidt 2019) and amphibians, because of the species' broad feeding habits coupled with its readiness to swim and hence ability to access areas not frequented by native predators, primarily Red fox (*Vulpes vulpes*) and European badger (*Meles meles*) (Miljøstyrelsen 2010). Its diet includes birds, amphibians, small mammals, carrion, invertebrates, crops, and fruit (Miljøstyrelsen 2010; Sutor et al. 2010; Kauhala and Kowalczyk 2011; Drygala & Zoller 2013; Mikkelsen et al. 2016; Elmeros et al. 2018; Takatsuki et al. 2018), which are predominantly hunted or foraged for in wet habitats with understory vegetation, reed beds, crop land, mixed and deciduous forests, lakes, gardens, and shore lines (Drygala et al. 2008a; Kauhala and Kowaczyk 2011; Kauhala & Ihalainen 2014; Pagh & Chriél 2017; Elmeros et al. 2018).

In Denmark, RDs have estimated home ranges of 4.04 km^2 (Pagh 2016) that are frequently shared by multiple individuals as RDs are non-territorial and highly monogamous animals who travel in pairs accompanied by pups of the year for a few months following den emergence in June (Ikeda 1984; Kauhala et al. 2007; Drygala et al. 2008a). Their social nature may explain why RDs build latrines, consisting of multiple scats from one or more individuals (Ikeda et al. 1984; Yamamoto 1984; Roper et al. 1986, 1993; Kauhala & Salonen 2012; Pagh & Chriél 2017), as latrines are thought to serve as communication centers amongst conspecifics (Ikeda 1984; Yamamoto 1984).

A recent study (Buxbom 2017) found that RDs have slightly larger litter sizes in Denmark with a mean of 11 pups relative to the 8-10 pups per litter found in surrounding countries (Helle & Kauhala 1995; Kowalczyk et al. 2009; Miljøstyrelsen 2010). Increased fecundity may be a response to abundant food sources (Sutor et al. 2010) and a generally mild Danish climate (Danmarks Meteorologiske Institut 2019) that allows RDs to forego hibernation (Sheard & Pedersen 2012) and maintain better body condition throughout the year (Canale et al. 2016). The

high reproductive output coupled with their flexible feeding habits, elusive nature, and great dispersal abilities have enabled the Danish population to increase (estimated from the exponential increase in dead RDs collected by the Danish Nature Agency (Naturstyrelsen, (hereafter NST); Fig. 2) (Nørgaard et al. 2014). To date, RDs have rarely been linked to direct impacts on native populations (Kauhala & Auniola 2001, Elmeros 2018), yet as the population in Denmark expands from its current size to its estimated carrying capacity of 30000 individuals (Rømer et al. 2015), potential impacts could arise and, at that point, be difficult to contain.

Understanding direct impacts of exotic species are particularly important in areas where damage potentials could be high. The two Danish nature conservation areas (hereafter NCA), Filsø and Lille Vildmose (Fig. 3), harbor some of the highest biodiversities nationally and provide important breeding habitat for amphibians and rare and ground-nesting birds (AVJF 2019a, b). Both areas have been invaded by RDs (AVJF managers, pers. comm.), yet little information exists about the species' impacts inside NCAs. The current management action consists of culling RDs, but this method is resource consuming and appears inadequate at curbing population growth (Fig. 2). Should preventative conservation strategies become necessary, an alternative approach could include efforts to directly protect native prey against potential RD predation. To do this, researchers need to understand predation vulnerability of specific taxa in response to ecosystem features, such as habitat types (hereafter land cover), as land covers are known to drive predator-prey interactions through differing resource and shelter provisions (Friman et al. 2008).

Generalized linear models (hereafter GLM) and generalized linear mixed effect models (hereafter GLMM) are tools with which researchers can design preventative management actions by linking habitat- and diet selection and directly model taxonomic consumption in response to individual land covers. Such models thus enable optimization of future management strategies by predicting the land covers in which focal prey are particularly vulnerable to consumption and hence where protection efforts should be maximized.

In this study, we conducted metabarcoding analyses to analyze RD diet collected with invasive and non-invasive sampling techniques. Specifically, we (i) examined diet selection and potential differences between NCAs and non-preserved areas, (ii) related habitat to diet selection, (iii) compared our findings to other RD diet studies conducted in Denmark, and (iv) discussed some of the benefits associated with non-invasive sampling techniques.

Gut content from 45 individuals culled across mainland Jutland was analyzed and used to assess general RD diet, while fecal samples collected from nine latrines located within two NCAs were analyzed and used for a more direct evaluation of diet selection inside preserved areas. We built sets of candidate models using GLMs and GLMMs to analyze relationships between habitat- and diet selection inside and outside NCAs and briefly compared the models to general habitat selection calculated from gps locations associated with culled RDs. Additionally, we related our findings to other RD diet analyses conducted in Denmark and discussed the potentials associated with cheap, non-invasive, and easily reproducible sampling techniques.

To our knowledge, this is the first exclusive metabarcoding study of RD diet in Europe. As such, it may provide novel information regarding predation on native taxa by an exotic canid due to a greater level of species identification rates commonly associated with metabarcoding techniques (Mumma et al. 2015; Oja et al. 2017; Robeson et al. 2018).

2. METHODS

2.1 Study area

RDs were euthanized across Jutland, Denmark by NST and local hunters, while fecal samples were collected from two different NCAs in Jutland. The NCAs belong to Aage V. Jensen's Fond (hereafter AVJF) and are Filsø (23.28 km^2), located in the mid-western part of Jutland (55.703090, 8,224129) and Lille Vildmose (55.21 km^2), located southeast of Aalborg in the north-eastern part of Jutland (56.881681, 10.202946) (Fig. 3). Both NCAs provide important bird and amphibian habitat and are co-inhabited by RDs (AVJF managers, pers. comm.).

2.2 Field methods

RDs were culled by hunters and NST (n = 43) throughout 2018, collected as road kill (n = 1), or found dead (n = 1) (NST 2018, unpublished). Euthanized individuals were kept at -20° C until being transferred to the National Veterinary Institute at the Technical University of Denmark where guts were removed and stored for the purpose of scientific studies (Chriél, pers. comm.).

Fecal samples were collected during September and October 2018 by LP who spent 3 days in each location hiking around the NCAs and visually scanning the ground for latrines. Five latrines were located through visual scans, while four were located through the aid of AVJF managers' knowledge of existing latrines. Once detected, fecal samples were placed in 50 ml Falcon tubes, marked with a unique identification code, and the location was recorded on a GPS unit (Garmin eTrex 30). Samples were stored at -20° C until DNA extractions commenced. All latrines were located in relatively dry areas including mixed forests or mixed grassland with shrubs, consistent with existing knowledge (Ikeda 1984; Baltrünaité 2006; Kauhala & Salonen 2012; Kauhala and Ihalainen 2014), always near the base of a tree/shrub, and with a max. distance of 540 m to a freshwater source (min. 77 m) (ESRI 2011).

2.3 Preparation of gut and intestine content for DNA extraction

Guts from 45 RDs that fit specific selection criteria were collected and prepared for metabarcoding analyses in multiple steps. Selection criteria were: (i) individual had not been baited, (ii) culled in a) June and Maj, b) April and July, and c) the rest of the year. We initially focused on individuals euthanized during the bird breeding season (May-June), but to increase our sample size we extended the eradication period to include individuals culled between March 6 – September 18, 2018. Gut content samplings were conducted at the Zoological Museum at the University of Copenhagen. Stomach content was sampled by cutting open the stomach and filling a 50 ml Falcon tube with content. When stomachs were large (1.5+ grapefruit sized) (Fig. 4) and/or had highly heterogeneous content, two Falcon tubes were collected and DNA was subsequently extracted from both samples. All samples were stored at -20° C until DNA extractions commenced.

2.4 Homogenizing gut samples

Gut content homogenization and all remaining lab work was conducted under laminar flow hoods at the Section for GeoGenetics, University of Copenhagen, Denmark. To account for heterogeneity, the contents of each gut was weighed and mixed with AccuGENE molecular biology graded water (hereafter AccuGENE) at a 1:1 ratio to enable content blending with a stick blender until content was homogenous (Fig. 5 & 6). Two (2 ml) samples from each blended gut content was taken aside for DNA extractions, except when guts were very small (n = 6) or very large (n = 1) in which case one or four samples, respectively, were taken. No empty guts were

examined. Blending and homogenization was conducted to ensure a sample was representative of all content contained in the stomach from which the sample originated.

To prevent contamination between individual guts, the stick blender was cleaned between each gut by being scrubbed in running water, rinsed in a bucket of water, immersed for 5 min in a 5% bleach solution, rinsed in a second solution of 5% bleach, followed by a rinse in autoclaved water and a 5 min submersion in a second batch of autoclaved water. Finally, the blender was left to dry before being used for the next sample. For every fifth sample, all water and bleach solutions were changed, while controls of the autoclaved water were taken for every fifth sample and after the last gut had been processed. All controls were subsequently included in polymerase chain reactions (hereafter PCR) to monitor for contamination.

2.5 DNA methods

2.5.1 Extractions

DNA extractions followed two protocols, QiAamp PowerFecal (Qiagen 2018) and DNeasy Blood & Tissue (Qiagen 2019) per the manufacturer's directions for fecal and stomach content respectively. To increase DNA concentrations, the latter protocol was modified at the last step by eluding DNA with 2 x 30 ul Al buffer instead of 200 ul and a 5 minutes incubation at 56° C instead of no incubation.

2.5.2 Sanger sequencing of fecal samples

To verify that fecal samples collected from latrines were deposited by RDs, three subsamples from each latrine were amplified with carnivore specific cytochrome B primers (Nowak et al. 2014) (CanidC1 5'AATGACCAACATTCGAAA 3' (Paxinos et al. 1997); HCarn200 5'ATTCAG CCRTARTTAA CGTC 3' (Bidlack et al. 2007)). Each PCR was performed in a total volume of 25µl that contained 2.5µl 10X PCR Gold buffer (Thermo Fisher Scientific), 2.5µl GOLD $MgCl_2$ (25 mM, Thermo Fisher Scientific), 0.2µl dNTPs (25 mM, Invitrogen), 0.2µl AmpliTaq GOLD polymerase (5U/µl, Thermo Fisher Scientific), 1µl forward primer (10 µM), 1µl reverse primer (10 µM), 1 µl Bovine Serum Albumin (20 mg/ml; New England Biolabs Inc.) 13.6 µl AccuGENE, and 3 µl fecal DNA. The thermocycling program used an initial denaturing step at 95° C for 10 min followed by 38 cycles of 95° C for 45 s, 54° C for 30 s, 72° C for 60 s, 1 cycle of 72° C for 5

min, and a hold at 4° C. One extraction blank and two PCR controls (positive and negative), were included in all amplifications to check for contamination.

Following the PCR, a 50 bp DNA ladder along with 5µl of each PCR product combined with 2µl GelRed Nucleic Acid Gel Stain (Biotium) were loaded into wells in a 2% agarose gel and electrophoresed for 30 minutes at 140 V and 350 mA. Reaction products were visualized on a UV light platform and amplification success was determined based on the presence of bands at the expected fragment length.

PCR products (234 bp; Nowak et al. 2014) were sent to Macrogen Europe BV for Sanger sequencing and the results were subsequently verified by blasting against the National Center for Biotechnology Information (hereafter NCBI; NCBI 2019) database and matching the sequences to species level based on a >98% identity threshold. All samples (n = 3) from each of eight latrines matched RD. The ninth latrine was initially sequenced unsuccessfully, but was matched to RD during the later metabarcoding analyses allowing for all fecal samples to be included in the data analysis.

2.5.3 Metabarcoding with modified COI primers

For the metabarcoding analyses conducted on the total number of samples (n = 251), tagged modified cytochrome c oxidase subunit 1 (hereafter COI) primers (Mod_RepCOI_F 5'-TNTTY TCMACYAACCACAAAGA-3'; VertCOI_7216_R 5'- CARAAGCTYATGTTRTTYATDCG -3' (Reeves et al. 2018)) were chosen for their ability to amplify PCR products to species level from a taxonomically diverse range of vertebrates, production of relatively short amplicons (244 bp) well suited for commonly degraded fecal-derived DNA sequences (Deagle et al. 2006), and the highest taxonomic coverage in reference databases of all species identification markers (Kress et al. 2015; Reeves et al. 2018). No RD oligonucleotide blockers (hereafter blockers) were used. PCR reagents and thermocycling equipment were identical to those described above, except for the addition of 1.5 µl tagged forward primers (10 µM), 1.5 µL tagged reverse primers (10 µM), and 2 µl DNA. Thermocycling conditions were: 95° C for 5 min followed by 40 cycles of 95° C for 30 s, 48.5° C for 30 s, 72° C for 60 s, 1 cycle of 72° C for 7 min, and a hold at 4° C.

2.5.4 Illumina library build and MiSeq sequencing of modified COI primers

Single-indexed libraries with pooled samples were built with the Illumina TruSeq DNA PCR-Free Library Preparation kit (Illumina) per the manufacturer's directions. Four libraries, each containing 72 -73 samples and a DNA input of 400 ng, and one blank library were pooled and purified with MinElute (Qiagen), followed by an additional purification with Beckman Coulter Agencourt AMPure XP (1.5 bead ratio; Beckman Coulter) for primer dimer removal. DNA concentrations and specific lengths of the targeted sequences were measured using the Bioanalyzer (Agilent Technologies 2100 Bioanalyzer) with the Agilent High Sensitivity DNA kit, before the pooled libraries were submitted to the National High Throughput DNA Sequencing Centre, Section for GeoGenetics, Copenhagen, Denmark, for paired-end sequencing on a single MiSeq flowcell using a v3 300 cycle kit. Prior to sequencing, Illumina checked library quality following MiSeq recommendations.

2.6 Bioinformatics

All bioinformatic procedures were conducted by Dr. Tobias G. Frøslev.

2.7 Geographic Information System

All Geographic Information System (GIS) work was conducted in ArcMap 10.5.1 (ESRI 2011). All samples with corresponding gps locations (9 latrines, 41 stomachs) were imported into ArcMap and overlayed with Basemap (Levin et al. 2012), a raster file containing 34 georeferenced land covers within 10x10 m grid cells covering all of Denmark. Land covers were subsequently used as predictor variables in statistical models. To focus on areas deemed suitable for RD habitation, we excluded all anthropogenic land covers (except agriculture), which left 15 land covers that, for simplicity, were combined into eight unique land covers (Table 1). Buffers (r = 1000 m) were created around each sample and the percentage of each land cover within each buffer was extracted (Fig. 7). The buffer radius was chosen so as to best capture the area in which the animal may have foraged, based on estimated home range sizes of 4.04 km^2 (Pagh 2016), while still keeping the buffers small enough to be representative of the area in which a sample (gut or latrine) had been collected. The amount (%) of each land cover contained within each buffer was subsequently used as predictor variables in candidate GLMs and GLMMs. Additional predictor variables included

distance (m) from each sample to the nearest freshwater source" and "time of death" (i.e. the date a RD was culled).

To estimate general habitat selection across Jutland (i.e. the land covers in which RDs were culled), the total amount of each land cover present in all of Jutland was extracted and compared to the amount of each corresponding land cover averaged across all 41 gut buffers. Latrine locations were not included in this analysis.

2.8 Statistical analyses

Statistical analyses were performed in R version 3.5.2 (R Core Team, 2018) using the lme4 (Bates et al. 2015) and ggplot2 (Wickham 2016) packages.

GLMs and GLMMs were built to predict the presence of amphibians, birds, and mammals in gut and fecal samples, respectively, in response to amount (%) of land covers in buffers surrounding the samples, distance (m) to freshwater, and time of death of the individual (only available for gut samples). Latrine was included as a random variable in the GLMMs to correct for the nestedness of fecal samples within latrines. Fish and reptiles were excluded from the modelling analyses due to their low FOs (maximum 11.4% and 6.8%, respectively, Table 3 & 5).

Predictor variables consisted of eight land covers (agriculture, bog, coast, forest, freshwater, grassland, heather, wetland (Table 1)), distance (m) to freshwater, and time of death (expressed as Julian date where day 1 = earliest day a gut sample was collected), while taxonomic classes (amphibian, bird, mammal) served as binomial response variables (0 = absent, 1 = present). Coast and agriculture were excluded as predictor variables from the GLMMs since all latrines were located inland in NCAs where agriculture and coast were not present.

Candidate models around each predictor variable were built and analyzed using the Likelihood Ratio Test (hereafter LRT), while Akaike information criterion (hereafter AIC) values associated with the LRT analyses were used to rank models and define the model with the smallest delta AIC value (hereafter ΔAIC) as having the best fit to the data (Akaike 1973). Models were kept simple with just one predictor variable to ensure correct interpretations of their outcomes. While this may have led to few of the models achieving a high level of support (Table $7_{a1\text{-}c2}$, Table$8_{a1\text{-}c2}$), the models are an important step in linking habitat with diet selection and provide information around which to shape future management strategies. However, further studies should consider building more complex models to capture more of the variation associated with habitat and diet selections.

To account for differences in sequencing depth, the relative abundance of the sample specific operational taxonomic unit (hereafter OTU) was calculated using the sample specific read count for each OTU normalized by the total read count per sample.

$$\text{Sample\_relative\_abundance}_{i,j} = \text{read\_count}_{i,j} / \text{total\_read\_count}_{j}$$

i = 1, 2, …, n

j = 1, 2, …, m

n = number of identified OTUs

m = number of samples

The overall RA of OTUs, in fecal and gut samples respectively, was calculated using the sum of the sample_relative_abundance divided by the number of samples.

$$\text{Relative\_abundance (RA)} = \sum_{i=1}^{m} \left(\text{sample\_relative\_abundance}_{i,j}/\text{m}\right)$$

Relative frequency of occurrence (hereafter FO) was calculated as percentage of occurrence of food items, in gut and fecal samples, respectively. Differences in the number of OTUs detected in gut and fecal samples were analyzed with a nonparametric Wilcoxon test as this test is less sensitive to non-normally distributed data than regular t-tests.

3. RESULTS

A total of 251 samples (89 samples from 45 guts and 162 fecal samples from nine latrines) were sequenced resulting in $1x10^7$ reads. These were divided up on 1733 OTUs and an average sequencing depth of $4x10^4$ reads per sample. To reduce potential stochastic effects associated with extraction, sampling, and PCR runs, gut replicates were combined leaving a total of 45 unique gut samples. One gut and 67 fecal samples only contained RD reads and were discarded from further analyses leaving a total of 44 guts and 95 fecal samples. Only vertebrate OTUs were included in

the analysis and only observations with read counts ≥ 10/sample were analyzed, as anything less was considered background noise (secondary predation, sequencing errors, contamination (De Barba et al. 2014; Taberlet et al. 2018; Deagle et al. 2019)). DNA sequences from Chondrichthyes, cartilaginous fish, were present in a few samples, but were discarded as those fish do not occur in this part of the world (Møller, pers. comm.) and were likely assigned in error.

Of the remaining $6.5x10^6$ reads, 23.7% ($1.3x10^6$ from the gut data, $3.0x10^5$ from the fecal data) belonged to prey vertebrates (the remaining 76.3% were assigned to non-vertebrates) with sequences from five taxonomic classes, Amphibia (amphibian), Aves (bird), Mammalia (mammal), Actinopteri (fish), and Reptilia (reptile). In total, 72 OTUs were detected; however, after combining prey haplotypes (n = 7) into one, as they were not of interest to this study, and discarding observations considered to be background noise, 53 unique OTUs remained (43 in the gut data, 26 in the fecal data, 16 in common). These were assigned to the following taxonomic levels: species = 42, genus = 3, family = 3, order = 5 (Table 2). For the remaining analyses, gut and fecal data were analyzed separately.

3.1 General diet across Jutland

The 44 gut samples contained a total of 17 different avian OTUs, 17 mammals, four piscean, three amphibians, and two reptiles making birds and mammals the most diverse food source selected for (Fig. 8). Mammals were the most frequently observed taxonomic class occurring in 75.0% (33 samples) of all samples with an RA of 34.9%, while birds were the most abundantly detected class (RA of 37.5%) and an FO of 65.9% (29 samples) (Table 3). Amphibians had an FO of 45.5% (20 samples) with an RA of 24%, while fish and reptiles occurred in 11.4% and 6.8% of all samples, respectively, both with RAs of less than 3.5% (Table 3).

The single most frequently detected, and most abundant, OTU in the gut data was Toad *sp.* (no exact match but likely *Bufo bufo*) with an FO of 45.5% (20 samples) and an RA of 22% ($9.9x10^4$ reads), followed by Common shrew (*Sorex araneus*) and Bat *sp.* (Chiroptera *sp.*) both with FOs of 22.7% (ten samples) and RAs of 7.1% and 5.2% respectively (Table 4). Domestic chicken (*Gallus gallus*) was the most frequently consumed avian species detected in nine samples (FO 20.5%) with an RA of 6.4%, followed by Woodcreepers (Dendrocolaptidae *sp.*) with an FO of 15.9% (seven samples) and an RA of 2.6% (Table 4). The most abundantly consumed bird was Ring-necked pheasant (*Phasianus colchicus*) with an RA of 6.5%.

Overall, mammals made up seven of the ten most frequently detected species, four of which were ungulates, but with Common shrew as the most abundantly consumed mammal (Table 4). The top ten most frequently occurring food items constituted 63.0% of the total RA.

3.2 Diet inside nature conservation areas

The 95 fecal samples contained a total of ten mammalian, seven avian, four amphibian, four piscean, and one reptilian OTUs making mammals the most diverse prey class, followed by birds (Fig. 8). Amphibians were the most frequently occurring, and abundant, taxonomic class detected in 56 samples (FO 59%) and accounting for 63.9% of the total RA followed by mammals, detected in 27 samples (FO 28.4%) with an RA of 23.9% (Table 5). Birds were identified in seven samples (FO 7.4%) and accounting for 8.0% of the total RA, while fish and reptiles occurred in 5.3% and 1.1% of all samples, respectively, both with RAs below 3.5% (Table 5).

The single most frequently detected species was Moor frog occurring in 55.8% (53 samples) of all samples with an RA of 60.1%, followed by Red deer (*Cervus elaphus*) and Toad *sp.* both detected in ten samples (FO 10.5%) and with RAs of 11.2% and 3.0%, respectively (Table 6). The second most frequently consumed mammal was European mole (*Talpa europaea*) occurring in four samples (FO 4.2%) with an RA of 4.2% (Table 6), while the most frequently occurring avian species were Wood pigeon (*Columba palumbus*) and Common starling (*Sturnus vulgaris*) both with an FO of 1.1% (one sample) and RAs of 5% and 0.3%, respectively (Table 6).

The top ten most frequently consumed species were mammalian (5 species), three of which were small mammals (Table 6). Overall, the top ten most frequent food items detected in fecal samples accounted for 90.6% of the total RA.

3.3 Linking habitat and diet selection with GLMs and GLMMs

Based on gut data, amphibian consumption was best explained by wetland (ΔAIC = 0, df = 2, w_i = 0.335; Table 7_{a1}) where increased proportions of wetland decreased the likelihood of amphibians having been consumed (Table 7_{a2}, Fig. 9_a). The top model for bird consumption included an inverse relationship with grassland (ΔAIC = 0, df = 2, w_i = 0.609; Table 7_{b1}) so that increased proportions of grassland decreased the likelihood of birds having been consumed (Table 7_{b2}, Fig. 9_b). Mammalian consumption was best described by agriculture (ΔAIC = 0, df = 2, w_i = 0.568; Table 7_{c1}); here increased proportions in agricultural land cover correlated with increased mammalian

consumption (Table 7_{c2}, Fig. 9_c). No seasonal effect was detected on the response variables, hence Julian date was not part of any of the top models.

Based on fecal data, the top model describing amphibian consumption contained bog as a predictor (ΔAIC = 0, df = 3, w_i = 0.744; Table 8_{a1}) showing that proportional increases of bog increased the likelihood of amphibians having been consumed (Table 8_{a2}, Fig. 10_a). The top model for avian consumption included a positive correlation with heather (ΔAIC = 0, df = 3, w_i = 0.284; Table $8_{b1, b2}$, Fig. 10_b), while mammalian consumption correlated positively with grassland (ΔAIC = 0, df = 3, w_i = 0.448; Table $8_{c1, c2}$, Fig. 10_c). Thus, proportional increases in the amounts of heather and grassland, increased the likelihood that birds and mammals, respectively, had been consumed.

3.4 Comparing gut and fecal samples

Gut samples contained a maximum number of eight OTUs in a single sample, while fecal samples contained a maximum of five OTUs. The highest number of avian OTUs found in a single sample (gut) was four, while a maximum number of mammalian OTUs detected in a single sample (gut) was six. For amphibians this number was three (fecal), while fish and reptiles both were detected at a maximum of one (gut and fecal both) (Table 9).

The number of OTUs detected in fecal samples was significantly lower than in gut samples (W = 2076, p-value < 0.00001; Fig. 11 & 12) with fecal samples containing a mean of 1.32 OTUs (95% CI [1.1, 1.54]) and guts containing a mean of 2.94 OTUs (95% CI [2.52, 3.36]). Species level assignment was 86.0% for guts and 57.7% for fecal samples, while the genus level was 90.7% and 65.45% for guts and feces, respectively. At the family level, 97.7% of OTUs detected in gut samples had been assigned, while 76.9% had been assigned in fecal samples. At the order level, assignment level was 100% for both sample types (Table 10).

Species detected in gut samples and latrines in both NCAs included Moor frog, Toad *sp*., Red deer, Domestic pig (*Sus scrofa*), and Bat *sp*. (Table 2).

Prior to any filtration of the raw data, we detected a significant, systematic higher proportion of non-vertebrate read counts in fecal samples than in gut samples ($t(95) = 1.985$, $p < 0.00001$), which caused a greater systematic loss of counts from fecal samples post-filtration.

3.5 Red Listed species

Two species listed as vulnerable on the Danish "Red List" (Aarhus Universitet, 2019) were detected in low numbers. Northern pintail (*Anas acuta*) was found in one gut with an RA of 0.002%, while European hare (*Lepus europaeus*) was found in two guts, collected in separate locations, with an RA of 0.03% and 0.33%, respectively.

3.6 General habitat selection

Based on a comparison between the average amount (%) of individual land covers calculated across buffers surrounding the gut samples and the total amount available of each corresponding land cover in all of mainland Jutland, RDs select for bog, coast, freshwater, and wetland (Fig. 13).

4. DISCUSSION

In this study, we analyzed fecal and gut content to assess diet selection of non-native RDs inside and outside NCAs and found that 53 unique vertebrate food items had been consumed. We built statistical models to correlate habitat selection with consumption of amphibians, birds, and mammals in order to evaluate the land covers in which consumption of specific taxonomic classes had occurred most frequently. We also tested whether fecal samples collected with minimal impact and resource requirements could be used to assess diet selection in NCAs and briefly contrasted our results to other RD diet studies conducted in Denmark.

RDs analyzed in this study had consumed a wide breadth of prey including taxa from each of the five vertebrate classes commonly found in Denmark, thus confirming their omnivorous feeding habits (Miljøstyrelsen 2010; Sutor et al. 2010; Kauhala and Kowalczyk 2011; Mikkelsen et al. 2016; Elmeros et al. 2018, Takatsuki et al. 2018). We observed a greater number of OTUs across Jutland than inside NCAs (43 versus 26) possibly due to Jutland, with its nearly 400 times greater landmass and subsequent increased habitat richness (ESRI 2011), harboring greater biodiversity than NCAs, which RDs may have exploited. Biased amplification rates between gut and fecal samples, whose DNA had underwent different degradation rates, may also have affected these results as advanced degradation of fecal samples may have caused fecal content to be less informative than gut content. Differences between study sites and sample types, paired with

differences in sample sizes (44 guts versus nine latrines), suggest that, in this study, guts provided a more inclusive diet composition than fecal samples.

Despite stringent efforts to exclude baited animals from our study, RDs examined here may have fed at bait stations or scavenged on mammalian or avian carcasses prior to being collected causing an artificial inflation of particularly mammalian DNA detections. We assume that most ungulates detected in this study (Red deer, Domestic cow (*Bos taurus*), Domestic sheep (*Ovis aries*), Domestic pig), and possibly Domestic chicken were not primary prey, but instead consumed as carrion or ingested at bait stations (commonly baited with cat food, cheese, or roadkill (NST 2018, unpublished)). However, potential bait items were retained in the analyses as we could not exclude them from a natural diet selection process (e.g. carrion, fawns, eggs, chicks), which would render them ecological relevant. It is also possible that RDs were preferentially culled on or near certain land covers, such as agriculture due to ease of access for hunters or because hunters encountered RDs while hunting for other prey (NST 2019, unpublished), which would skew our data interpretation. Uneven sampling efforts and their associated considerations apply across study sites to all samples and land covers analyzed in this study.

Barring biased sampling efforts and amplification rates, the combined species richness detected across gut and fecal samples corresponds well with the number of vertebrate species occurring in Denmark, although challenges associated with RDs locating or catching individual food items may also be reflected. Across all samples, birds constituted the most diverse class (20 OTUs), likely reflecting the high avian species richness found in Denmark where nearly 400 species either reside, migrate through, or overwinter (Miljøstyrelsen 2019a). Conversely, Mammalia was the second most diverse class detected across gut and fecal samples (18 OTUs) even though just 50 mammalian species occur in Denmark (Miljøstyrelsen 2019b). The near identical number of avian and mammalian OTUs, despite the eightfold difference in their species richnesses, suggests that a greater proportion of mammalian species were susceptible to RD consumption, relative to avian species, either through active predation or as carrion or bait. Amphibians constituted the second to least species rich class (5 OTUs) correlating with the low number of amphibian species in Denmark (n = 14; Miljøstyrelsen 2019c). Only seven piscean OTUs were detected across both sample types, despite Actinopteri being a diverse taxonomic class with 258 species (marine and freshwater) occurring in Denmark (Miljøstyrelsen 2019d). This low richness, coupled with a low FO of fish across samples, may reflect the difficulties associated with catching rapid moving, aquatic species.

Reptilia was the least species rich class detected across samples which corresponds with the low number of reptilian species native to Denmark (n = 5; Miljøstyrelsen 2019e).

4.1 Amphibian consumption

Amphibian consumption occurred frequently in both study areas (FO 45.5% across Jutland; FO 59.0% inside NCAs) and with a high RA inside NCAs where 63.9% of all read counts stemmed from amphibians compared to 24% outside NCAs (Table 3 & 5). Amphibians thus appear to be a regular prey across both study sites, but particularly inside NCAs. It is important to note that fecal samples may not be independent of each other (i.e. the number of RDs that defecated in a latrine is unknown) which could skew our data interpretation. Yet, the presence of amphibian sequences in eight of nine latrines (Table 2) implies that RDs consume amphibians across both NCAs, while the age difference between latrines with some appearing inactive and old, while others were clearly active (i.e. fresh scat was deposited each day during the sampling period), indicates that amphibians were consumed over an extended time period, likely while amphibians were active (spring migration – post-breeding (late summer); Popescu et al. 2012).

Moor frog was the single most frequent (FO 55.8%) and abundant (RA 60.1%) (Table 6) of all consumed food items inside NCAs, suggesting that Moor frogs may be an important food source for RDs in NCAs. The age difference between latrines (old versus fresh) and the great distance between latrine sites (three latrines were located across multiple km^2 in Western Jutland (Filsø), six latrines were located across multiple km^2 in Eastern Jutland (Lille Vildmose)) indicate that Moor frog was consumed throughout different seasons and across NCAs, rather than during short intervals in confined areas. This level of potential Moor frog consumption has not previously been reported and, while the species is neither classified as vulnerable nor threatened (Aarhus Universitet 2019), its protected status in Denmark (Miljøstyrelsen 2019f) suggests a need for future studies designed to analyze this topic.

Across Jutland, amphibians (primarily Toad *sp*.) were detected in nearly half of all gut samples (20 of 44, Table 2, 3, 4), demonstrating that amphibians also provide a steady food base in less protected areas, albeit in much lower quantities (RA 24.0%; Table 3). The relatively low RA indicate that these items may have been encountered as individuals rather than knots, consistent with the behavior of amphibians who are generally solitary except during breeding seasons (Orloff 2011). Amphibian populations are potentially also sparser outside NCAs due to the taxon's

sensitivity to habitat degradation and chemical pollution (Cushman 2006; Orloff 2011), factors commonly associated with anthropogenic land covers. RDs may thus rely more heavily on alternative and more diversified prey bases outside NCAs, which corresponds with the higher number of OTUs detected outside NCAs (Table 2, Fig. 8).

In both study sites, amphibian predation was best explained by moist land covers (bog and wetland), albeit with opposite effects. Across Jutland the best predictor, wetland (Table 7_{a1}) was negatively correlated with amphibian consumption (Table $7_{a2,}$ Fig. 9_{a}) indicating that predation may have occurred during the upland life stage of amphibians (Cushman 2006; Orloff 2011; Popescu et al. 2012). This is supported by the remaining candidate models, of which none contained predictors associated with wet land covers (Table 7_{a1}) and correlates with the low RA detected in gut samples, possibly as a result of solitary predation events. However, since no temporal effect was detected in any of our models, we cannot evaluate this link further.

Within NCAs, however, bog, followed by wetland, was positively correlated with amphibian consumption (Table $8_{a1, b2}$, Fig. 10_{a}). This suggests that adults, and likely tadpoles and spawn, were consumed, at least partially, during the aquatic breeding season when amphibians congregate in knots (Cushman 2006) and thus amount to an abundant food source for potential predators. This association fits with the high RAs detected in fecal samples and is well in line with existing literature which unanimously link RDs to wet areas (Drygala et al. 2008a; Kauhala and Kowaczyk 2011; Kauhala & Ihalainen 2014; Pagh & Chriél 2017; Elmeros et al. 2018) and thus in close proximity to amphibian breeding sites (Popescu et al. 2012).

4.2 Bird consumption

Across Jutland, birds were the most abundant and second most frequently occurring taxonomic class (Table 3), suggesting this taxon provides a significant food source. A total of 37.5% of all detected sequences were avian with most (36.7%) belonging to Galliformes (primarily Ring-necked pheasant), followed by Wood pigeon, Common blackbird (*Turdus merula*), Mute swan (*Cygnus olor*), and Woodcreeper *sp*. Much of this abundance stemmed from chick predation as five stomachs contained 2-4 intact passerine chicks with associated high avian read counts, but for the remainder, the source (e.g. primary prey, eggs, or carrion) of avian DNA could not be determined. Active predation by RDs on adult birds seems questionable, as the agility of birds likely serves as a defense against a somewhat lumbersome predator, yet nest predation may be

significant and requires more studies. Here, the intact chicks all belonged to tree nesting species (Common blackbird, Wood pigeon, and Rook (*Corvus frugilegus*)). Since each of the five guts contained more than one chick, consumption may not be a result of a single chick falling out of a nest but potentially from a nest blowing down, leaving all the chicks vulnerable to predation, or from RDs accessing arboreal nests. The literature does not indicate whether RDs can climb, but captive RDs have been known to climb out from enclosures (fence height ≈ 1.6 m; Aqua animal caretakers, pers. comm.). Whether that indicates an ability for climbing bushes and particularly trees seems unlikely, but should be analyzed in future studies. Larger birds (Mute swan, Gray Heron (*Ardea cinerea*)) detected in high abundances were likely consumed as eggs, chicks, or carrion as the size of such adult birds presumably deters active predation by RDs.

Avian DNA in fecal samples was detected infrequently and in low abundances (FO 7.4%, RA 8.0%; Table 5), suggesting that little bird biomass had been consumed inside NCAs or that our analyses failed to detect avian sequences. Bahlke (2019) found that metabarcoding analyses (12S primers) did not detect avian DNA in stomach liquid from RDs, despite visual confirmations of bird presence in the stomachs, and suggested that avian DNA may be sensitive to acidic gut fluids and degrade rapidly. In this study, however, we detected high FOs and RAs of avian sequences in gut samples which seem to counter Bahlke's explanation and render further studies on the subject necessary.

Across Jutland, bird consumption was negatively correlated with grassland (Table $7_{b1, b2}$, Fig. 9_b), indicating that birds were not a primary prey in or near grassland. However, upon examining the data, it appears that one outlier shifts the correlation from positive to negative, which may illustrate a need for additional models to better analyze this relationship.

Within NCAs, bird consumption was best explained by increasing amounts of heather (Table $8_{b1, b2}$, Fig. 10_b), an important land cover for both breeding and migrating birds (Dansk Ornitologisk Forening 2019; Miljøstyrelsen 2019a). However, this model received the lowest support (*wi* = 0.28, Table 8_{b1}) of all the models, indicating that the link between land cover and bird consumption has not been fully explained. In general, bird consumption may be difficult to link to one specific land cover given the high mobility of birds that allows them to frequent different habitats in short periods of time. Additionally, the small sample sizes associated with each sample type (41 guts, nine latrines) likely decreased the explanatory power of our models.

4.3 Mammalian consumption

Mammalian OTUs were the most frequently detected (FO 75%; Table 3) in all gut samples, indicating that mammals may be the most frequently consumed vertebrate class across Jutland, well in line with the findings of existing literature (Kauhala and Kowalczyk 2011; Mikkelsen et al. 2016; Elmeros et al. 2018). Mammals were also the second most abundantly consumed vertebrate class primarily driven by ungulates (Table 4) whose larger body sizes, if encountered as prey, carrion, or at bait stations, likely offer bigger meals than smaller mammals would. Yet, small mammals (insectivores, bats, rodents; Table 2) constituted 8 of the remaining 17 mammalian OTUs with Common shrew and bats being the most frequently observed of all mammals (Table 4). While bats were presumably eaten as carrion as their aerial lifestyle makes them an unlikely prey, the remaining small mammals may have been encountered as primary prey. Around 62% of Jutland consists of agriculture (Normander et al. 2009) which RDs are known to frequent particularly to feed on maize and rapeseed fields (Drygala 2008a, 2013; NST 2018, 2019, unpublished), but potentially also to prey on high rodent and insectivorous populations commonly associated with hedgerows and edges in and along crop fields (Hole et al. 2005; Witmer et al. 2007; Witmer & Proulx 2010). While our analysis of habitat selection across Jutland did not indicate active selection for crop land (Fig. 13), a high proportion of gut samples were collected in or near agriculture (NST 2018, unpublished), which correspondingly was the land cover that best explained mammalian consumption outside of NCAs (Table $7_{c1, c2}$). The GLM indicated that mammalian consumption increased in response to increases in agricultural land cover (Fig. 9_c), suggesting that agriculture may hold a dual attraction for RDs.

Inside NCAs, mammals were the most diverse taxonomic class appearing in 28.4% of all samples (Table 5), indicating that mammals constituted a regular prey, if less so than outside NCAs. Five of the ten most frequently consumed species detected in fecal samples were mammalian (Table 6), but after excluding the most abundant mammal, Red deer (RA 11.2%; Table 6), the remaining mammals were detected in such low proportions (RA 0.4% - 4.2%; Table 6) to make their contribution to the overall energy requirement of RDs appear inadequate and suggest that alternative food sources sustained RDs inside NCAs.

Mammalian consumption in NCAs was positively correlated with grassland (Table $8_{c1, c2}$, Fig. 10_c), possibly driven by ungulates and high rodent populations usually associated with this land cover (Davidson et al. 2010; Meisingset et al. 2013). The general habitat selection outside NCAs

indicated that RDs used grassland in proportion to its availability (Fig. 13), suggesting neither active selection for nor avoidance of grassland. If RDs in NCAs exhibited a similar behavior, it indirectly supports the notion that small mammals where not the primary food items inside NCAs.

4.4 Fish and reptile consumption

Fish and reptiles were detected sporadically (Table 2) and, save for Atlantic salmon (*Salmo salar*), at low read counts (max RA 3.3% and 1.1% respectively; Table 3 & 5)). For fish, the low diversity, low FO, and low RA could reflect challenges associated with catching fish, making them a rare food item. The presence of both marine and freshwater fish (Table 2) does, however, indicate that RDs prey or scavenge in both coastal and freshwater areas or potentially from garbage cans. If RDs feed on human trash, the initial discarding of cartilaginous fish from our analyses may have been erroneous as these fish too could have been scavenged from human compost or trash. Conversely, piscean OTUs may also have been consumed as a biproduct if RDs feed on crops enriched with fish fertilizer commonly used by organic farmers (López-Mosquera et al. 2011), however this aspect is not discussed here.

Reptilian prey was near absent from this study possibly due to disjunct activity patterns between predator and prey as RDs are primarily nocturnal, while reptiles are active during the day (Kauhala et al. 1998). We assume that reptilian sequences detected in this study mainly stem from roadkill rather than actual predation events.

4.5 Consumption of Red Listed species

Sensitive species did not appear to have been actively selected for by RDs, yet consumption of Red Listed species did occur which suggests a need for future studies designed to understand potential impacts on such species.

Sequences from two vulnerable species identified in this study, Northern pintail (detected in one gut) and Eurasian hare (detected in two guts), both matched 100% when blasted against the NCBI database (NCBI 2019), yet their low RAs (N. pintail 0.002%; E. hare 0.33% and 0.03%, respectively) suggest the samples were incompletely sequenced, the DNA content was of poor quality, or that just a minor part of each species had been consumed, possibly as carrion. Alternatively, low RAs could indicate the food items were ingested at an earlier stage and that most content had been expelled in fecal samples not collected for this study. Regardless, the

possibility of either species succumbing to RD predation seems unlikely as both are highly agile prey that would be expected to escape most predation attempts by RDs.

4.6 Invasive and non-invasive sampling outcomes

We expected gut samples to return better diet data than fecal samples due to gut content being less degraded pre-data collection and optimally stored post-data collection. In fact, fecal matter may yield data on par with data retrieved from guts of culled individuals, if the systematically higher loss of read counts from fecal samples, occurring during data filtration, is offset by greater sequencing efforts.

Here, sequencing efforts were similar between all samples and guts did return more vertebrate OTUs and reads, both overall and per sample (Table 9, Fig. 11 & 12), suggesting that diet selection was better estimated from gut content. Particularly avian and mammalian OTUs had higher detection rates in guts, while amphibian, piscean, and reptilian OTUs were detected approximately on par between sample types (Table 2). Taxonomic assignment level was more precise in gut samples where only six (14%) OTUs remained unassigned at species level compared to 11 (42%) in fecal samples (Table 10). These differences may be due to better DNA quality in gut samples and subsequent more successful amplification rates, while the higher number of OTU detections also may reflect differences in species richness between the two study areas. Gut samples had been collected from a presumably more biodiverse area than fecal samples, as Jutland is nearly 400 times larger than Filsø and Lille Vildmose combined (78.5 km^2) (ESRI 2011; AVJF 2019a, 2019b). Conversely, NCAs would be expected to have a greater species richness per unit area than non-protected areas which, in Denmark, are dominated by urban and monocultural land covers (Normander et al. 2009). However, this relationship was not analyzed in the study at hand.

When using metabarcoding analyses to analyze dietary samples, blockers are commonly used to prevent amplification of host DNA which otherwise may mask prey sequences (Deagle et al. 2006, 2009; Shehzad et al. 2012; Pompanon et al. 2012). In this study, we did not use RD blockers during PCRs, which may have reduced the proportion of non-host sequences retrieved from both sample types. Fecal samples may additionally have been disproportionally affected due to their generally higher level of degradation (Zaidi et al. 1999; Jarman et al. 2002; Taberlet et al. 2018) and because feces routinely contain few food fragments whose amplification can easily be missed

during extractions (Shehzad et al. 2012). These differences make it difficult to compare the performance of the sampling methods applied in our study and render additional studies necessary.

Yet, despite the absence of RD blockers, fecal samples returned sequences from all five vertebrate classes, well in line with gut data, and revealed taxa not detected in gut samples (Table 2). Amphibian sequences were particularly well identified in fecal content, occurring more than twice as often as any other vertebrate class, and a previously unknown high proportion of Moor frog consumption may have been documented. One major advantage of fecal content is the ability to monitor diet selection within a specified area and, for species who defecate in latrines, the ability to monitor diet selection over an extended time period and even from the same individual. Gut analyses, in contrast, are limited to items consumed within the maximum food retention time which varies greatly between species (Roswag et al. 2012). Gut sampling comes with the added drawback of being resource and time consuming, as licensed people are required to cull the animal and remove and prepare guts for content samplings. Combining the minimal resource requirement per unit effort spent on collecting and preparing fecal samples with the ability to sample over time make non-invasive sampling techniques an appealing, cheap, and easily reproducible method with which to analyze the diet of omnivorous species.

While blocking host DNA during amplification procedures may increase non-host sequence detection rates, it concurrently prevents researchers from answering host-specific questions. Since we did not reduce RD sequence amplifications, we were able to determine the number of haplotypes associated with individual samples in our study. We used the NCBI database and the bioinformatics software, Geneious 2019.0 (Geneious 2019) to import RD sequences into the Population Analysis with Reticulate Trees (PopArt; Bandelt et al. 1999) program and determined that only one haplotype could be assigned (see Appendix, section A.1-A.2, Table A.1 and Fig. A.4).

4.7 Comparisons with other raccoon dog diet analyses

We compared our gut results to two macroscopic and one mixed (macroscopic and metabarcoding (12S primer)) studies of gut content from RDs culled in Denmark. We detected more OTUs and assigned a greater percentage (86%) to species level then the remaining studies (Table 2) where species assignments ranged from 7.1% (Mikkelsen et al. 2016) to 17.0% (Bahlke 2019) in the macroscopic studies (vertebrates only, egg shells included; Mikkelsen et al. 2016; Elmeros et al.

2018; Bahlke 2019), and 57.1% in the 12S metabarcoding study (Bahlke 2019). The tendency for metabarcoding analyses to reveal greater taxonomic coverage and assignment precision has previously been documented (Nichols et al. 2016; Frøslev et al. 2019) and may, in part, be due to the wider time frame in which DNA is identifiable relative to morphological studies where identification of items turned indiscernible due to rapid and differential digestion rates can be challenging (Tollit et al. 2003; Nichols et al. 2016). In our study, the increased coverage and precision was likely also a result of the vertebrate specific primers we used, known for their ability to successfully assign food items to species level (Reeves et al. 2018). Had we screened for all eukaryotic food items, in line with the remaining studies, we would likely have lost some species resolution or been required to use a hierarchical barcoding approach in which primers designed for barcodes with broad taxonomic coverage but low resolution, are used in conjunction with primers specific to a lower taxonomic group (Moszczynska et al. 2009; Pompanon et al. 2012). This valid approach would, however, have demanded additional resources and increased lab work complexity.

The FO of each taxonomic class was higher in our study, except for Amphibia which was detected more frequently in the 12S metabarcoding study (Bahlke 2019; Table 11). Increased taxonomic resolution and broader discovery rates suggest that metabarcoding may be superior to macroscopy if dietary breadth and monitoring for invasive, rare, threatened, or endangered species are of interest. Conversely, macroscopic assessments may be more accurate when estimating biomass consumption as the estimates are based on content weight, as opposed to sequence counts from DNA samples (Nichols et al. 2016; Deagle et al. 2019). Morphologic analyses also allow researchers to assess life stage (egg, young, adult) of undigested dietary items, an aspect that is available when prepping gut content for DNA extractions, but rarely when working with fecal DNA. An additional benefit of macroscopic analyses is that the full stomach content is analyzed, whereas DNA relies on a small amount from each sample (25 mg in this study), which can greatly reduce or bias the outcomes of the latter. These issues can be overcome (e.g. by homogenizing samples) but require additional measurements. We believe that gut content homogenizations conducted in this study ameliorated potential biases (based on the clustering of gut replicates; see Appendix, Fig. A.2), but it added a time and resource consuming step to an already complex lab protocol.

One similarity between Bahlke's (2019) macroscopic and our metabarcoding analyses was a tendency for samples to contain just one or a few food items. In our study, fecal and gut samples contained an average of 1.32 and 2.94 OTUs, respectively, with many samples containing just one OTU (Fig. 11) A stark difference between Bahlke's (2019) metabarcoding study and ours was a complete lack of avian OTU detections in the former, despite avian content being present, while a diverse group of birds were frequently and abundantly detected in our study. Differences in primer specificities between the studies may explain this disparity or alternatively, as Bahlke (2019) suggests, avian DNA may have been disproportionally degraded in acidic gut liquid and thus failed to amplify. Since gut data in this study was based on solids rather than liquids, our results would not have been affected in a similar manner.

Relative to the comparable studies, our metabarcoding analysis returned more detailed information regarding vertebrate food items, but this was expected as our study was designed for vertebrate specificity. Differences in study designs seem to preclude further comparisons, but metabarcoding and morphological methods overall appear as complementary techniques that, if used in conjunction, could add a synergy to studies aimed at deciphering complex ecological relationships.

5. CONCLUSIONS

Exotic RDs consume a wide variety of vertebrates present within their invaded range, creating a need for additional knowledge about their role in the Danish landscape. This study highlights the benefits associated with metabarcoding techniques when inferring diets of elusive species, particularly if dietary breadth and species-specific questions are of interest. Invasively and non-invasively collected samples both provided useful diet information, but each also came with unique drawbacks. Gut samples yielded more dietary information, yet the effort expended on collecting and preparing gut samples far exceed those of fecal samples. Fecal content, on the contrary, provided less diet information, yet this shortcoming could likely be amended through greater sequencing efforts. Overall, metabarcoding may be superior to macroscopic analyses when prey

species identification is of interest as the former generally provides greater level of taxonomic annotations and are not dependent on visual cues from food items.

The apparent frequent consumption of amphibians across study sites and seasons suggests that focus should be on analyzing potential impacts of RDs on amphibians, particularly during the latter's breeding season when adults congregate near freshwater sources, making them and their spawn prone to predation. Researchers, who seek to understand such relationships could successfully combine metabarcoding techniques with temporal sampling methods that enable diet items from within a season to be identified to the desired taxonomic level. To this avail, non-invasive fecal sampling methods may be useful, particularly for species who defecate in latrines, as latrines contain a build-up of fecal matter that presents researchers with the unique ability to monitor diet across time and even to look back in time. Locating latrines may be challenging, but certainly possible, and once located, could be sampled on a regular basis allowing for a continuous monitoring interval.

Should future preventive management strategies be required, researchers could successfully build GLMs and their derivatives around variables of interest to optimize conservation efforts. Models could target consumption of taxonomic classes, as done in this study, but also species-specific consumption given a large enough sample size. To be more informative, such models should be built around larger data sets and have increased complexity in order to best capture the intricate relationships that so often characterize the diet selection of exotic, elusive omnivores.

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6. FIGURES

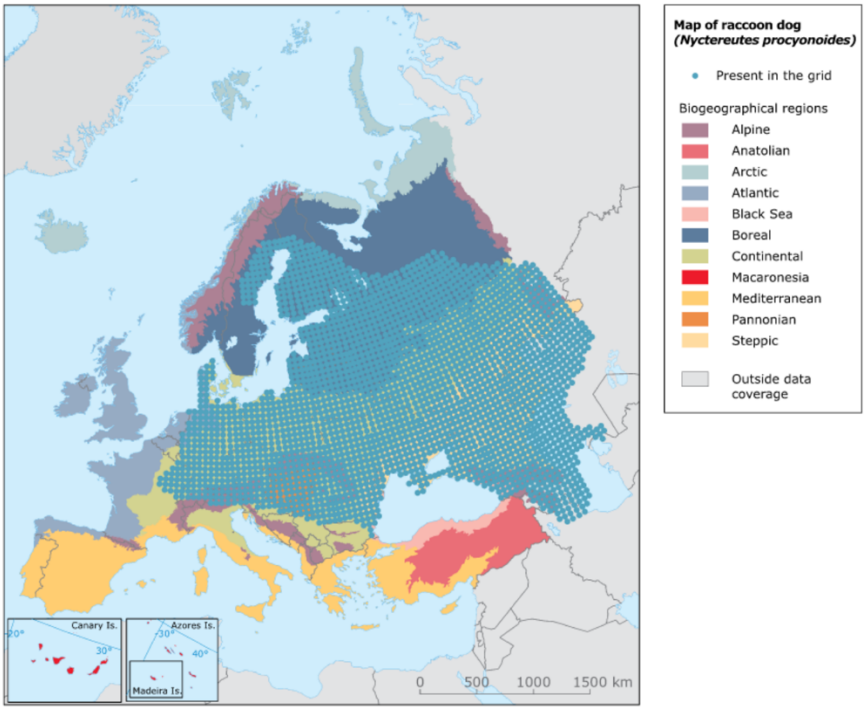

Figure 1. RD presence in its introduced range. Primary introductions occurred in the European part of the former Soviet Union.
Source: EEA 2012, based on Kauhala & Kowalczyk 2011.

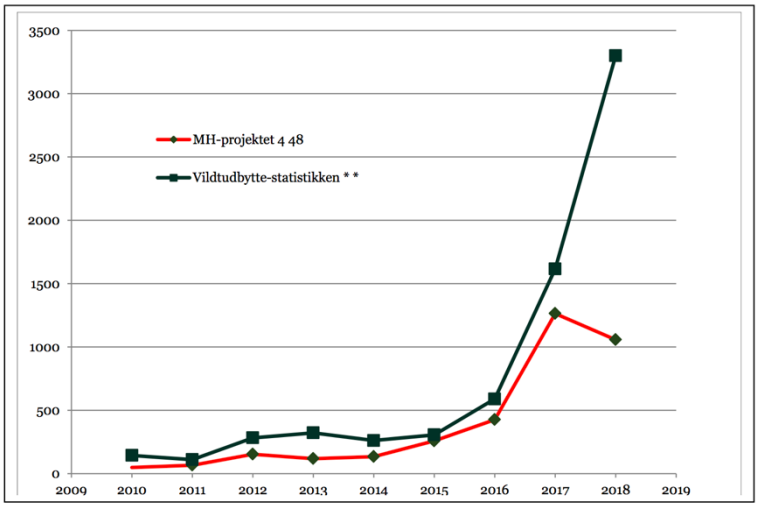

Figure 2. Number of dead RDs collected by NST from 2010 – 2018.
Source: NST 2019, unpublished

Figure 3. Map of Jutland, Denmark showing sample locations. Star = latrine, circle = gut.

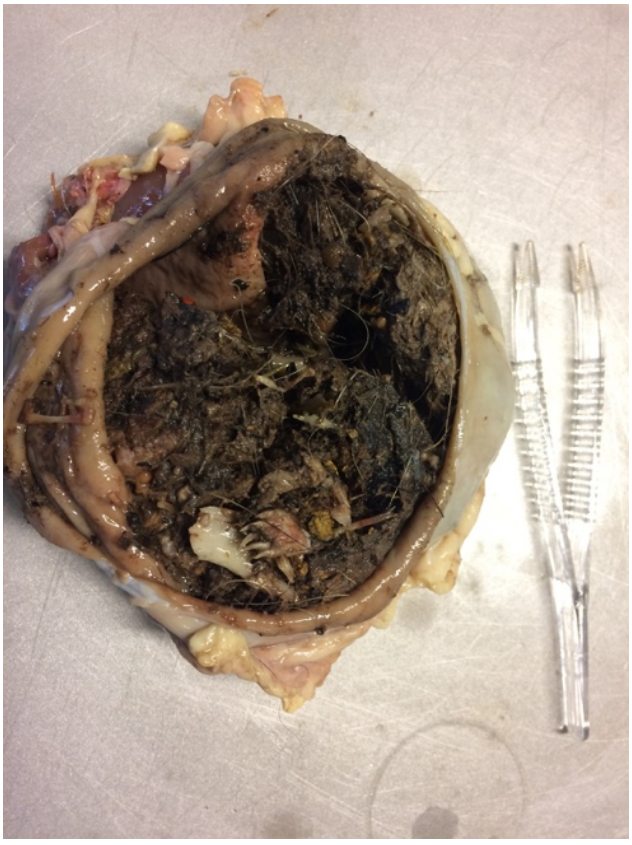

Figure 4. RD gut sampling

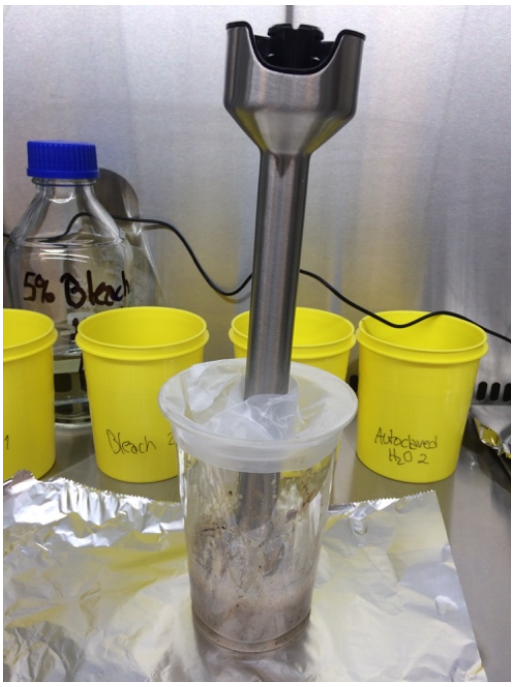

Figure 5. Blending gut content to create homogenous samples from which DNA was extracted.

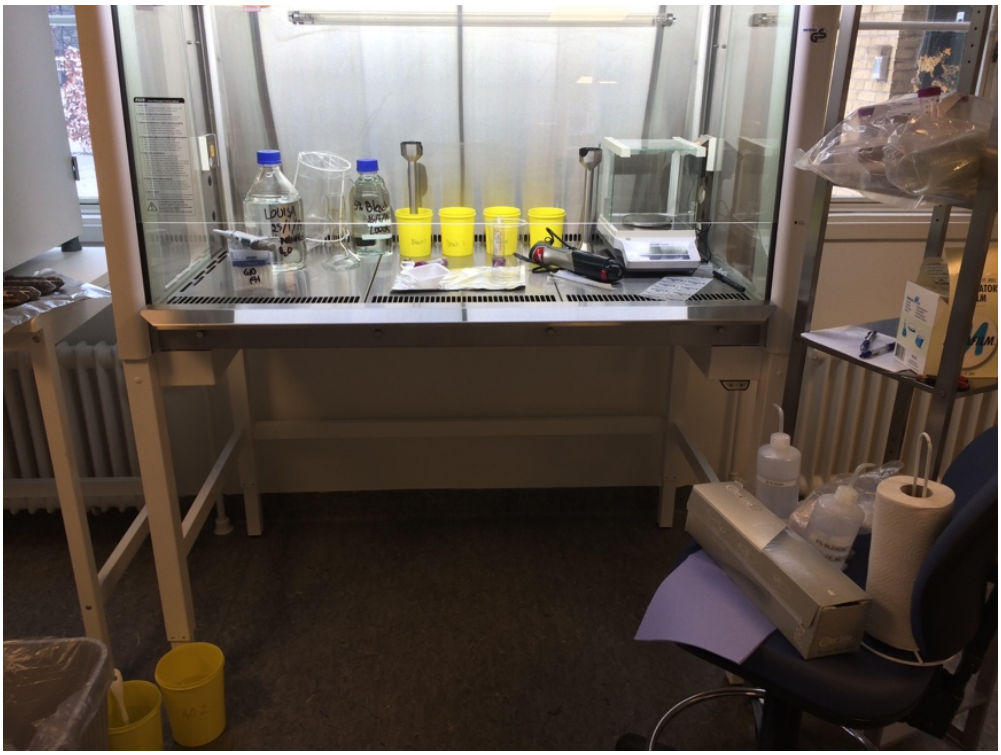

Figure 6. Setup for gut blending and sterilization procedures. Between each gut, the blender was scrubbed in running water, dipped into a bucket of water, followed by insertions into two 5% bleach solutions and two autoclaved water solutions. Lastly, the blender was set to dry before being used for the next gut.

Table 1. Fifteen different habitat types extracted from Basemap (Levin et al. 2012) were combined into eight habitat types, collectively referred to as "land cover". Each land cover was used as a predictor variable in GLMs and GLMMs. "Classification" indicates the habitat types that are contained within each land cover variable.

Land Cover	Classification
Agriculture	Agriculture Intensive
	Agriculture Extensive
	Agriculture Undefined
Bog	Bog
Coast	Coast
	Sea
	Coastal Meadow
	Dune Sand
Forest	Forest
Freshwater	Lake
	Stream
Grassland	Dry Grassland
Heather	Heather
Wetland	Wetland
	Wet Meadow

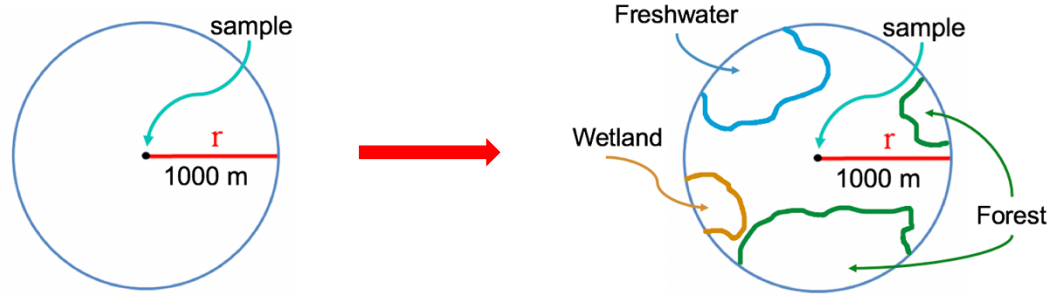

Figure 7. Individual land covers present within each buffer was extracted in ArcGIS and amount (%) was calculated.

Table 2. Food items detected in gut and fecal samples. Number in parenthesis indicates the number of times that food item was found in either latrines or guts. * Latrines (n = 3) from Filsø; ** Latrines (n = 6) from Lille Vildmose; *** Guts (n = 44) from across Jutland; ✦ RD vertebrate food items identified in macroscopic studies in Denmark (Mikkelsen et al. 2016, Elmeros et al. 2018, Bahlke 2019). ★ RD food items identified with 12S primers (Bahlke 2019). † Food items NOT detected in this study

Class	Species	English Name	Filsø*	Lille Vildmose**	Gut***	Morphological✦	12S★
Actinopteri	Clupea harengus	Atlantic herring	-	-	x (1/44)		
Actinopteri	Esox lucius	Northern pike	-	-	x (1/44)		
Actinopteri	Oncorhynchus mykiss	Rainbow trout	-	-	x (1/44)		
Actinopteri	Salmo salar	Atlantic salmon	-	-	x (2/44)		
Actinopteri	Characiformes *sp.*	Ray-finned fish *sp.*	x (1/3)	-	-		
Actinopteri	Cypriniformes *sp.*	Ray-finned fish *sp.*	x (2/3)	x (1/6)	-		
Actinopteri	Zoarces *sp.*	Eelpouts *sp.*	x (1/3)	-	-		
Amphibia	Bufo bufo	Common toad					x†
Amphibia	Lissotriton vulgaris	Smooth newt					x†
Amphibia	Rana arvalis	Moor frog	x (3/3)	x (5/6)	x (3/44)		
Amphibia	Rana temporaria	European common frog	-	x (1/3)			x
Amphibia	Triturus cristatus	Northern crested newt	-	-	x (1/44)		
Amphibia	Anura *sp.*	Frog *sp.*	x (1/3)	-	-	x	
Amphibia	Bufo *sp.*	Toads *sp.*	x (2/3)	x (3/6)	x (20/44)	x	
Aves	Alauda arvensis	Eurasian skylark	x (1/3)	-	-		
Aves	Anas acuta	Northern pintail	-	-	x (1/44)		
Aves	Anas platyrhynchos	Mallard	-	-	-	x†	
Aves	Ardea cinerea	Grey heron	-	-	x (1/44)		
Aves	Cairina moschata	Muscovy duck	-	-	x (3/44)		
Aves	Chroicocephalus ridibundus	Black-headed gull	-	-	x (1/44)		
Aves	Columba palumbus	Wood pigeon	-	x (1/6)	x (5/44)		
Aves	Corvus frugilegus	Rook	-	-	x (1/44)		
Aves	Cygnus olor	Mute swan	-	-	x (2/44)		
Aves	Erithacus rubecula	European robin	-	-	x (3/44)		
Aves	Gallus gallus	Domestic chicken	-	-	x (9/44)		
Aves	Meleagris gallopavo	Turkey	-	-	x (2/44)		
Aves	Perdix perdix	Gray Partridge	-	-	-	x†	
Aves	Phasianus colchicus	Ring-necked pheasant	-	-	x (5/44)	x	
Aves	Pyrrhula pyrrhula	Eurasian bullfinch	-	-	x (1/44)		
Aves	Rallus aquaticus	Water rail	x (1/3)	-	-	x	
Aves	Regulus regulus	Goldcrest	x (1/3)	-	-		
Aves	Scolopax rusticola	Eurasian woodcock	-	-	-	x†	
Aves	Sturnus vulgaris	Common starling	x (1/3)	-	x (1/44)		
Aves	Turdus merula	Common blackbird	-	-	x (4/44)	x	
Aves	Turdus philomelos	Song thrush	x (1/3)	-	x (2/44)		
Aves	Cygnus *sp.*	Swan *sp.*	-	-	x (2/44)		
Aves	Dendrocolaptidae *sp.*	Woodcreepers *sp.*	x (2/3)	-	x (7/44)		
Mammalia	Apodemus flavicollis	Yellow-necked mouse	-	-	x (1/44)	x	
Mammalia	Arvicola amphibius	Water vole	-	-	-	x†	
Mammalia	Bos taurus	Domestic cow	-	x (1/6)	x (11/44)		
Mammalia	Capreolus capreolus	European roe deer	-	-	x (6/44)	x	x
Mammalia	Cervus elaphus	Red deer	x (1/3)	x (1/6)	x (6/44)	x	
Mammalia	Erinaceus europaeu	European hedgehog	-	-	-	x†	
Mammalia	Lepus europaeus	European hare	-	-	x (2/44)	x	
Mammalia	Meles meles	European badger	-	x (1/6)	x (1/44)		
Mammalia	Micromys minutus	Harvest mouse					x†
Mammalia	Myodes glareolus	Bank vole	-	-	x (2/44)	x	
Mammalia	Neomys fodiens	Eurasian water shrew	-	-	x (1/44)	x	
Mammalia	Ovis aries	Domestic sheep	-	-	x (1/44)		
Mammalia	Rattus norvegicus	Brown rat	-	-	x (1/44)	x	
Mammalia	Sorex araneus	Common shrew	-	x (2/6)	x (10/44)	x	x
Mammalia	Sorex minutus	Eurasian pygmy shrew	x (1/3)	-	x (3/44)	x	
Mammalia	Sus scrofa	Domestic pig	x (1/3)	x (1/6)	x (6/44)		
Mammalia	Talpa europaea	European mole	x (2/3)	-	-	x	
Mammalia	Vulpes vulpes	Red fox	-	-	x (2/44)		
Mammalia	Canidae *sp.*	Canids *sp.*	x (1/3)	-	x (8/44)		
Mammalia	Chiroptera *sp.*	Bat *sp.*	x (3/3)	x (1/6)	x (10/44)		
Mammalia	Muridae *sp.*	Murids *sp.*	-	x (1/3)	x (1/44)	x	
Reptilia	Anguis fragilis	Slowworm	-	-	x (2/44)	x	
Reptilia	Natrix natrix	Grass snake	-	-	-	x†	
Reptilia	Zootoca vivipara	Eurasian lizard	-	-	x (1/44)		x
Reptilia	Squamata *sp.*	Squamates	x (1/3)	-	-		

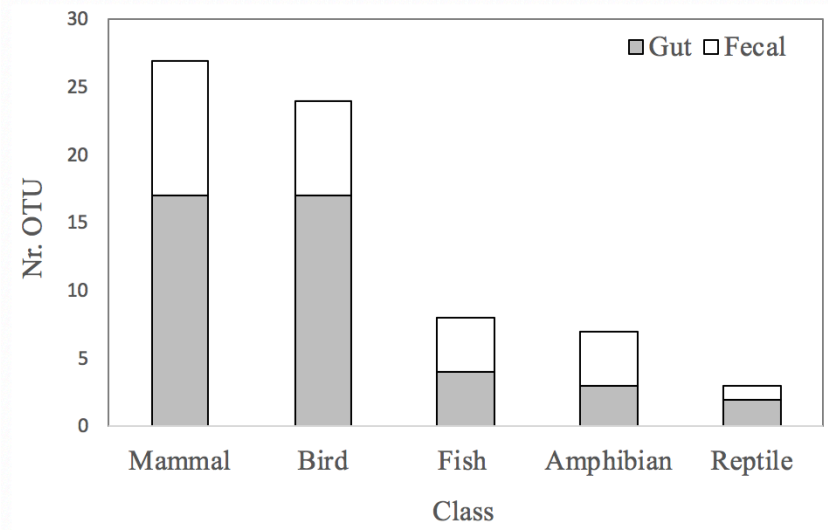

Figure 8. Diversity of taxonomic prey classes detected in gut and fecal samples

Table 3. Number of OTUs, FO, and RA detected in gut samples. FO = percentage of occurrence of food items; RA = sum of sample_relative_abundance divided by the number of samples.

Class	Nr. OTU	FO (%)	RA (%)
Mammal	17	75.0	34.9
Bird	17	65.9	37.5
Amphibian	3	45.5	24.0
Fish	4	11.4	3.3
Reptile	2	6.8	0.2

Table 4. Top ten food items detected most frequently in gut samples. FO = percentage of occurrence of food item; RA = sum of sample_relative_abundance divided by the number of samples.

Species	Common name	FO (%)	RA (%)
Bufo *sp.*	Toads *sp.*	45.5	22.0
Sorex araneus	Common shrew	22.7	7.1
Chiroptera *sp.*	Bat *sp.*	22.7	5.2
Gallus gallus	Domestic chicken	20.5	6.4
Bos taurus	Domestic cow	20.5	3.3
Canidae *sp*	Canids *sp.*	18.2	1.9
Dendrocolaptidae *sp.*	Woodcreepers *sp.*	15.9	2.6
Cervus elaphus	Red deer	13.6	9.0
Capreolus capreolus	European Roe deer	13.6	4.7
Sus scrofa	Domestic pig	13.6	0.2

Table 5. Number of OTUs, RA and FO detected in fecal samples. FO = percentage of occurrence of food item; RA = sum of sample_relative_abundance divided by the number of samples.

Class	Nr. OTU	FO (%)	RA (%)
Amphibian	4	59.0	63.9
Mammal	10	28.4	23.9
Bird	7	7.4	8.0
Fish	4	5.3	3.2
Reptile	1	1.1	1.1

Table 6. Top ten food items detected most frequently in fecal samples. FO = percentage of occurrence of food item; RA = sum of sample_relative_abundance divided by the number of samples.

Species	Common name	FO (%)	RA (%)
Rana arvalis	Moor frog	55.8	60.1
Cervus elaphus	Red deer	10.5	11.2
Bufo *sp.*	Toads *sp.*	10.5	3.0
Talpa europaea	European mole	4.2	4.2
Muridae *sp*	Murids *sp.*	3.2	0.4
Sus scrofa	Domestic pig	2.1	4.1
Sorex araneus	Common shrew	2.1	2.2
Rana temporaria	European common frog	2.1	0.03
Columba palumbus	Wood pigeon	1.1	5.0
Sturnus vulgaris	Common starling	1.1	0.3

Table 7a_1-c_1. Generalized linear models based on gut data. Model selection output for the top three models that best described the relationship between habitat and consumption of each of three taxonomic prey classes (from top: amphibian, bird, mammal). Predictor variables include land covers (%) (Table 1) and distance to freshwater (m). All models were evaluated based on their LRT values, while *Wi* (weight of the model) indicates the support for each model.

Table 7a_2-c_2. The top model was chosen based on the lowest ΔAIC (the scaled value of AIC). The Estimate indicates an either positive or negative correlation between land cover and consumption of the taxonomic class.

Figure 9a-c. The top model (based on gut data) for each taxonomic class is illustrated with the land cover (%) on the x-axis and consumption (presence/absence) of the taxonomic class on the y-axis.

a_1)

Model	ΔAIC	df	*wi*	Resid. Dev
amphibian ~ wetland	0	2	0.335	53.8
amphibian ~ heather	2.0	2	0.122	55.8
amphibian ~ agriculture	2.2	2	0.110	56.5

a_2)

Model	Variable	Estimate	SE	LRT
amphibian ~ wetland	intercept	0.441	0.433	
	wetland	-0.129	0.085	3.058

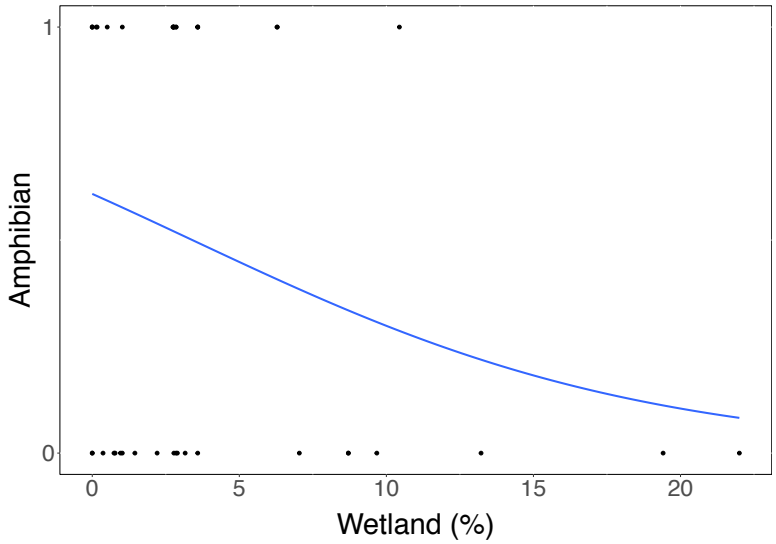

Figure 9a. The model that best described amphibian consumption shows an inverse correlation with wetland. Based on gut data.

b_1)

Model	ΔAIC	df	*wi*	Resid. Dev
bird ~ grassland	0	2	0.609	47.2
bird ~ bog	3.9	2	0.085	51.2
bird ~ agriculture	4.3	2	0.07	51.6

b_2)

Model	Variable	Estimate	SE	LRT
bird ~ grassland	intercept	1.0538	0.398	
	grassland	-1.096	0.656	5.396

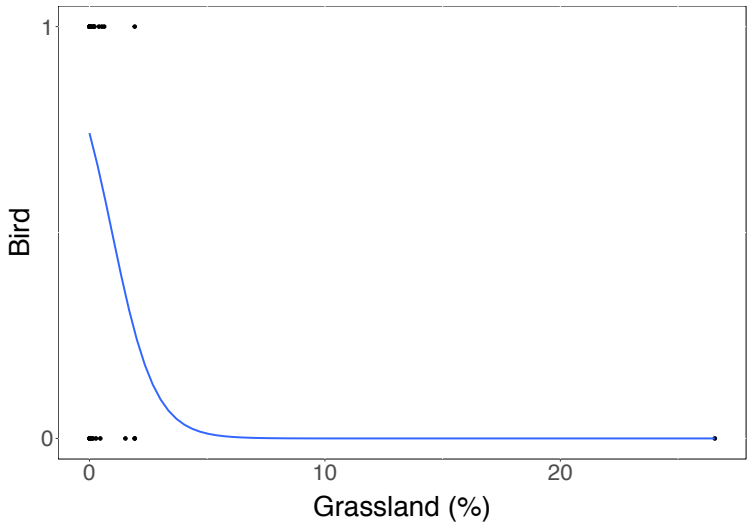

Figure 9b. The model that best describes bird consumption shows a negative correlation with grassland. Based on gut data.

c_1)

Model	ΔAIC	df	*wi*	Resid. Dev
mammal ~ agriculture	0	2	0.568	39.5
mammal ~ freshwater	3.2	2	0.114	42.7
mammal ~ grassland	3.3	2	0.112	42.7

c_2)

Model	Variable	Estimate	SE	LRT
mammal ~ agriculture	intercept	1.485	0.481	
	agriculture	0.382	0.509	6.069

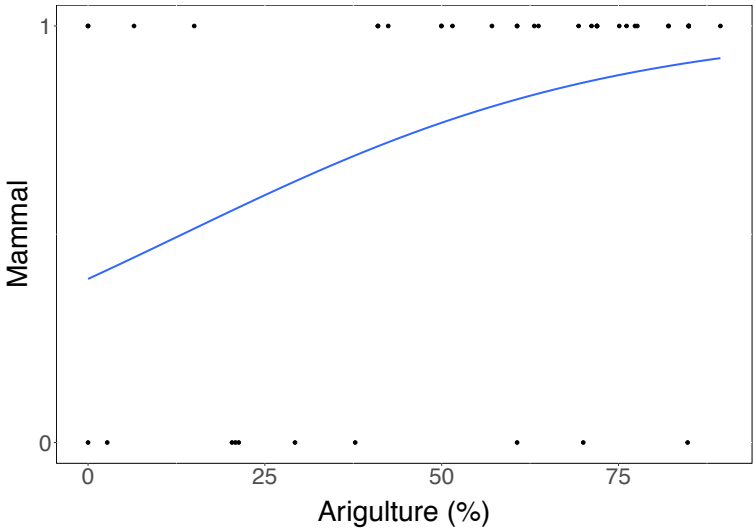

Figure 9c. The top model for mammalian consumption shows a positive correlation with agriculture. Based on gut data.

Table 8a_1-c_1. Generalized linear mixed effect models based on fecal data with latrine as a random variable. Model selection output for the top three models that best described the relationship between habitat and consumption of each of three taxonomic prey classes (from top: amphibian, bird, mammal). Predictor variables include land covers (%) (Table 1) and distance to freshwater (m). All models were evaluated based on their LRT values, while *Wi* (weight of the model) indicates the support for each model.

Table 8a_2-c_2. The top model was chosen based on the lowest ΔAIC (the scaled value of AIC). The Estimate indicates an either positive or negative correlation between land cover and consumption of the taxonomic class.

Figure 10a-c. The top model (based on fecal data) for each taxonomic class is illustrated with the land cover (%) on the x-axis and consumption (presence/absence) of the taxonomic class on the y-axis.

a_1)

Model	ΔAIC	df	*wi*	Resid. Dev
amphibian ~ bog + (1|latrine)	0	3	0.744	82.8
amphibian ~ wetland + (1|latrine)	3.5	3	0.127	70.6
amphibian ~ forest + (1|latrine)	5.4	3	0.049	71.7

a_2)

Model	Variable	Estimate	SE	LRT
amphibian ~ bog + (1|latrine)	intercept	-2.087	0.549	
	bog	0.126	0.026	8.804

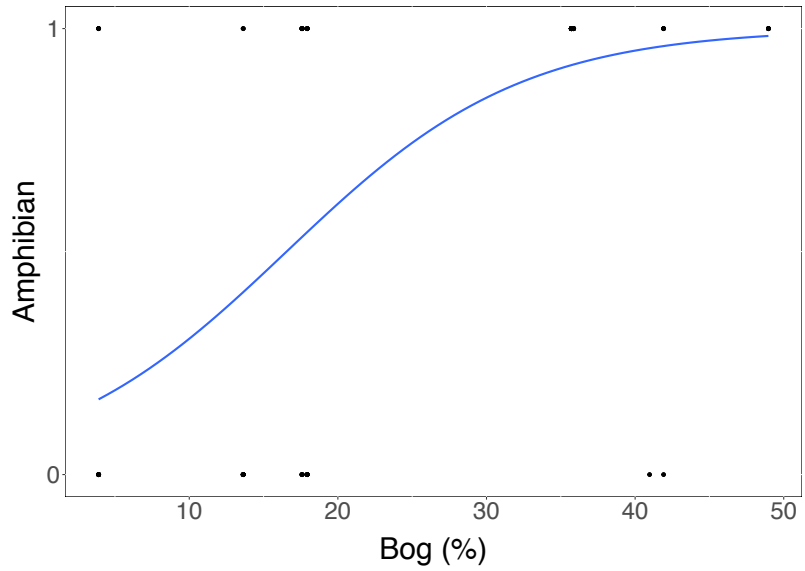

Figure 10a. The model that best describes amphibian consumption includes a positive correlation with bog. Based on fecal data.

b_1)

Model	ΔAIC	df	*wi*	Resid. Dev
bird ~ heather + (1|latrine)	0	3	0.284	84.2
bird ~ forest + (1|latrine)	0.6	3	0.212	84.8
bird ~ freshwater + (1|latrine)	1	3	0.176	81.9

b_2)

Model	Variable	Estimate	SE	LRT
bird ~ heather + (1|latrine)	intercept	-2.072	0.4020	
	heather	0.086	0.0390	3.103

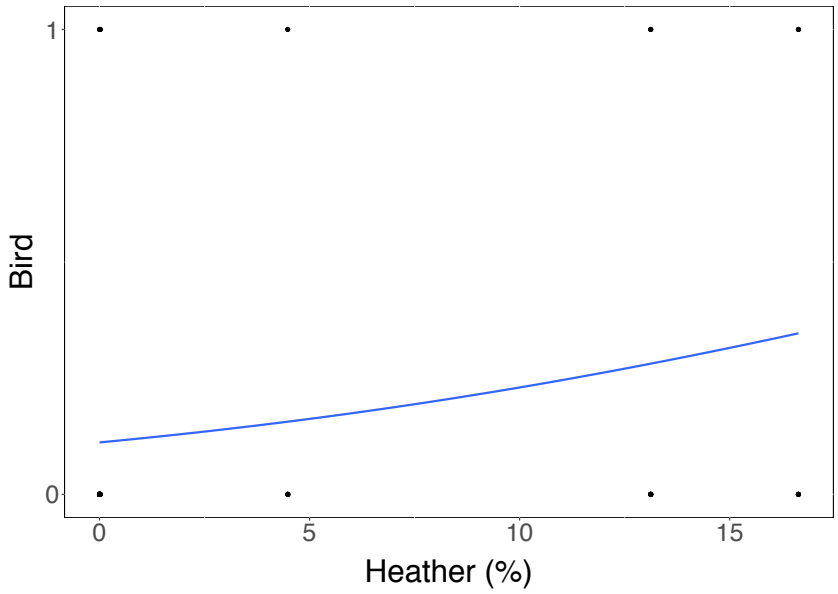

Figure 10b. The model that best describes bird consumption contains a positive correlation with heather. Based on fecal data.

c_1)

Model	ΔAIC	df	*wi*	Resid. Dev
mammal ~ grassland + (1|latrine)	0	3	0.448	110.4
mammal ~ bog + (1|latrine)	2.5	3	0.127	109.9
mammal ~ heather + (1|latrine)	2.7	3	0.114	109.8

c_2)

Model	Variable	Estimate	SE	LRT
mammal ~ grassland + (1|latrine)	intercept	-0.061	0.351	
	grassland	0.157	0.087	3.153

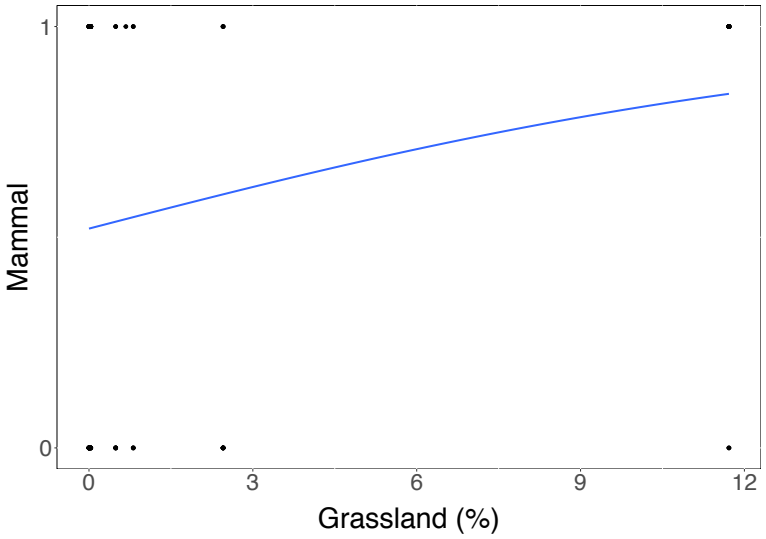

Figure 10c. The model that best describes mammalian consumption contains a positive correlation with grassland. Based on fecal data.

Table 9. The maximum number of OTUs detected in individual sample types.

Max nr. OTU	Gut	Fecal
Mammal	6	3
Bird	4	2
Amphibian	2	3
Fish	1	1
Reptile	1	1
Per sample	8	5

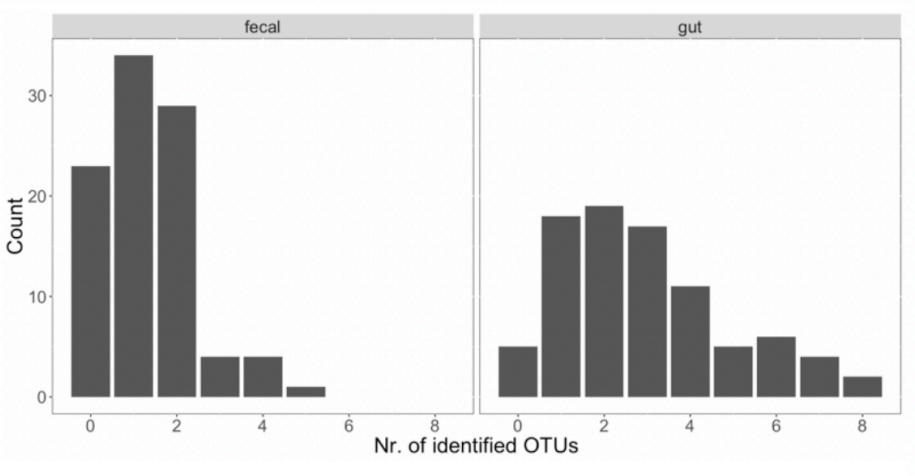

Figure 11. Number of OTUs identified in fecal and gut samples.

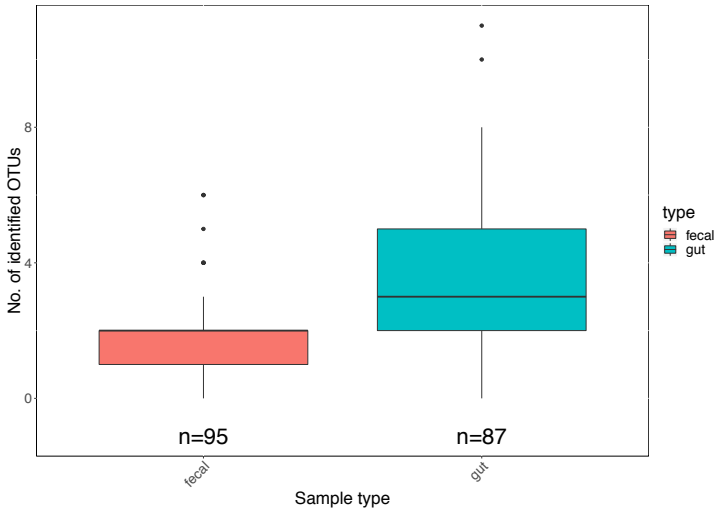

Figure 12. Gut samples contained a significantly higher number of OTUs than fecal samples ($W = 2076$, $p < 0.0001$) (based on gut replicates).

Table 10. Percent OTUs assigned to specified level in gut and fecal samples.

Assigned OTUs (%)		
Taxonomic level	Gut	Fecal
Species	86.0	57.7
Genus	90.7	65.4
Family	97.7	76.9
Order	0.0	0.0

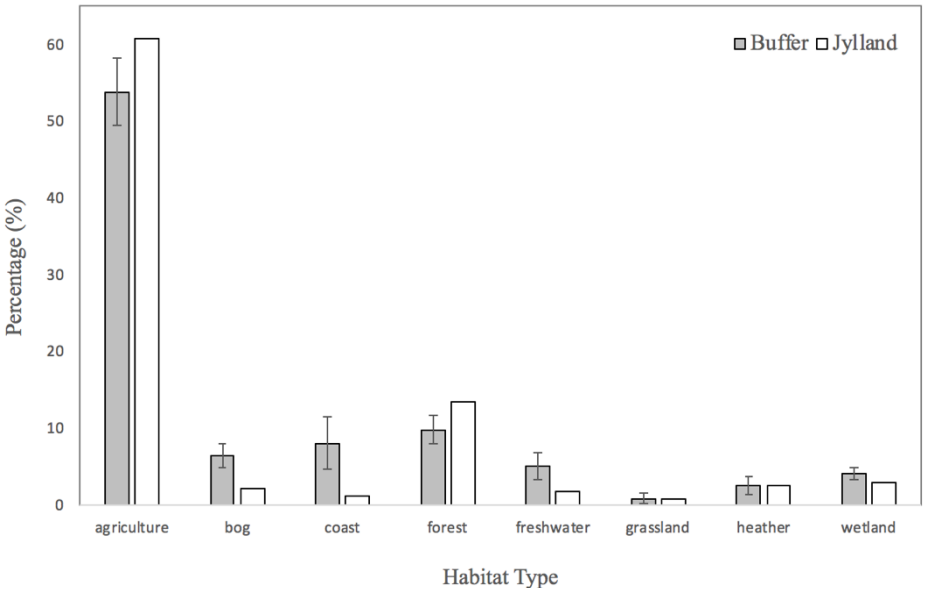

Figure 13. Comparing the mean proportion (%) of individual land covers found within gut sample buffers to the availability (%) of each corresponding land cover on the Jutland peninsular indicates that RDs select for bog, coast, freshwater, and wetland. Standard error bars included.

Table 11. Comparison of FO between morphological studies and metabarcoding studies. 12S primers were used by Bahlke (2019) on 24 gut samples, Modified COI primers were used on 45 gut samples in this study. Bold numbers indicate the highest FO detected across studies. No direct comparison for mammals was made due to differences in how mammalian FOs were recorded.

	Frequency of Occurrence (%)				
	Mikkelsen et al. 2016	Elmeros et al. 2018	Bahlke 2019	12S	COI
Bird	46.1	41.1	40.0	0.0	**65.9**
Mammal	40.2 (Rodents) 23.5 (Insectivores) 10.8 (Ungulates) 8.8 (Other)	45.0 (Rodents) 22.8 (Insectivores) 9.4 (Ungulates) 5.9 (Other)	40.0 (Rodents) 18.0 (Insectivores) 4.0 (Ungulates) 12.0 (Other)	62.5	75.0
Amphibian	44.1	35.6	32.0	**50.0**	45.5
Fish	3.9	3.5	4.0	0.0	**11.4**
Reptile	1.0	2.0	4.0	6.3	**6.8**

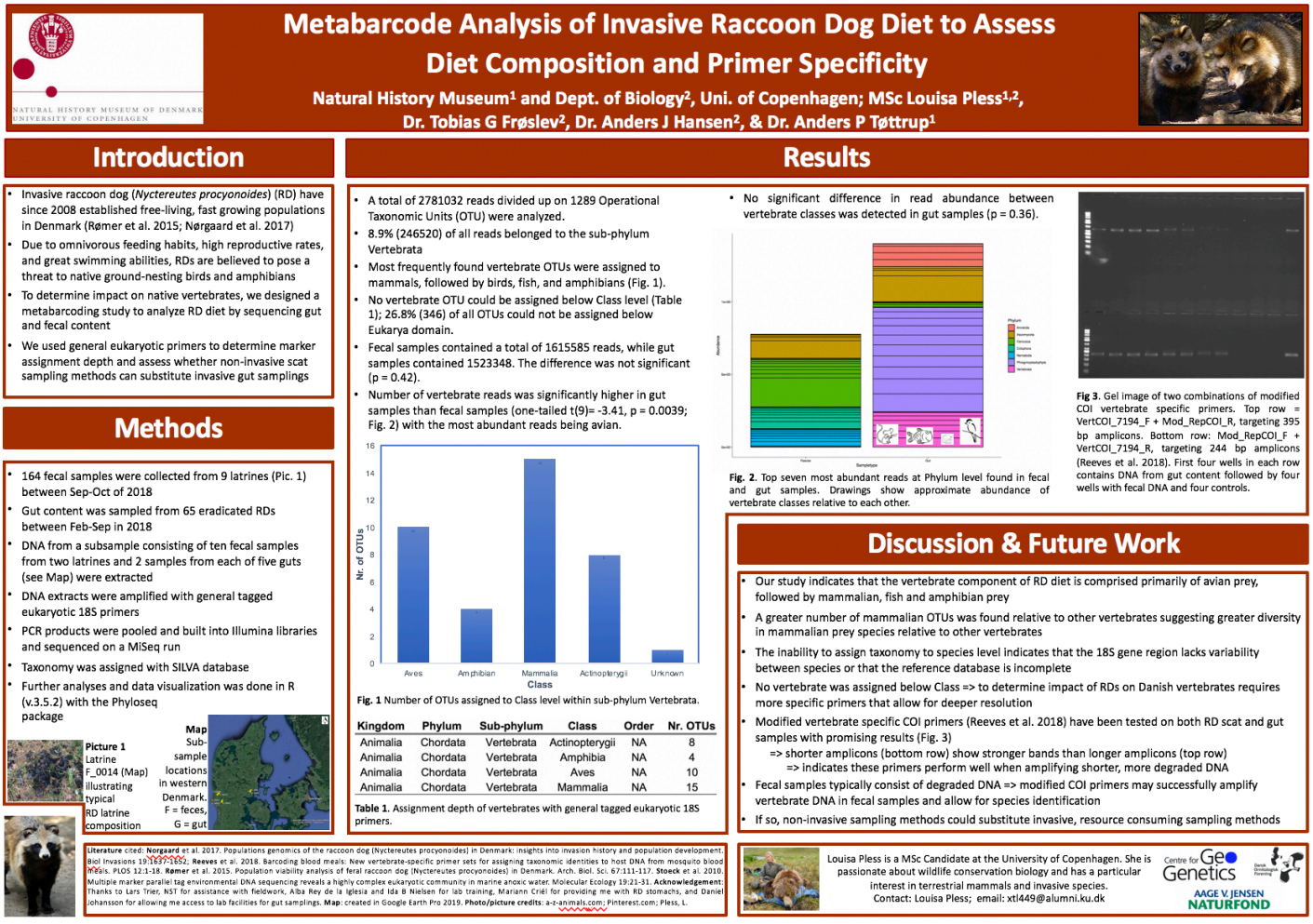

Figure A.1. General tagged eukaryotic 18S primers (390 bp; Stoeck et al. 2010) were used in a pilot study designed to analyze diet composition and test primer specificity from a subset of RD samples (ten fecal- and ten gut samples). The results showed that mammals were the most diversely consumed vertebrate taxa, while avian RA was the highest. No OTUs could be assigned below Class level, indicating that 18S primers would not be appropriate if species identification of dietary items was the objective. The results of this pilot study were presented at the "Student Conference on Conservation Science", 2018, at the University of Cambridge, UK.

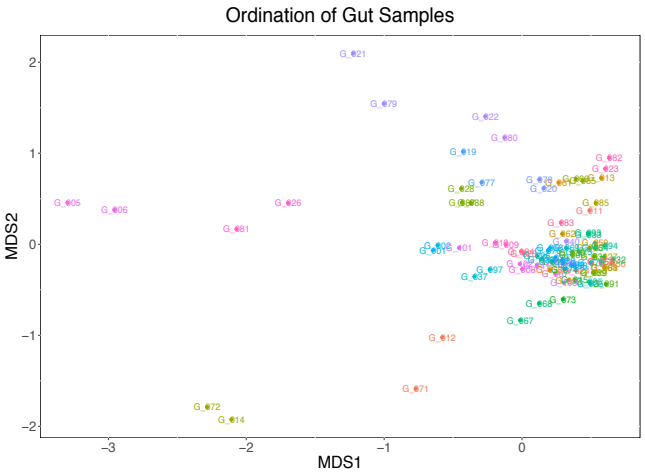

Figure A.2. Multidimensional scaling of gut replicates colored according to individual guts.

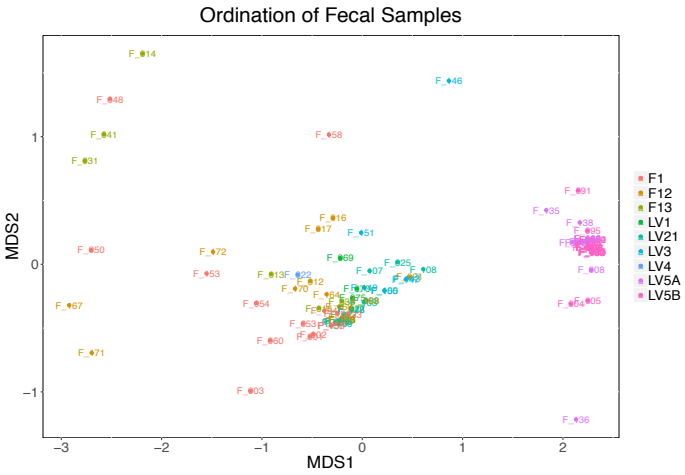

Figure A.3. Multidimensional scaling of fecal samples colored according to latrines.

A.1 Haplotype determination and origin

The raw, unclustered sequencing data from 251 samples contained 11 unique RD sequences. Ten of the sequences appeared only sporadically in a fraction of samples, at very low read counts, and always in the presence of one unique sequence (TATTTGGGGCATGGGCCG GCATAGTAGGC ACTGCCTTGAGCCTCCTTATTCGAGCCGAATTAGGTCAGCCTGGCACCCTATTGGGA GACGACCAAATTTATAATGTTGTCGTAACTGCCCATGCTTTCGTGATAATCTTCTTCA TGGTTATACCCATTATAATTGGAGGGTTCGGAAATTGACTGGTTCCACTGATGATCG GTGCCCCAGACATAGCATTTCCC), which appeared at high read counts in 87% of all samples. To distinguish the actual number of haplotypes from haplotypes that may have been assigned erroneously, we calculated the proportion with which a RD sequence appeared in a sample and compared it to all other sequences. Sequences, whose read counts were a fraction of another sequence found within the same sample were considered as noise or PCR or sequencing errors (Coissac et al. 2012; Taberlet et al. 2018) and were discarded from the haplotype analysis. This left one sequence indicating that only one haplotype was detected within all the samples.

To determine the origin of this haplotype, the sequence was compared to 12 other CO1 or whole genome RD sequences downloaded from the NCBI's database (NCBI 2019) by aligning and trimming all sequences in Geneious 2019.0 (Geneious 2019) and importing the sequences into the Population Analysis with Reticulate Trees (PopArt; Bandelt et al. 1999) software for haplotype

identification and visualization. PopArt groups DNA fragments into different haplotypes based on sequence similarity with a single nucleotide difference placing sequences in different haplotype groups.

A.2 Haplotype results

RD sequences found in all 251 samples matched 11 different haplotypes, however one sequence (haplotype 11) appeared in 87% of all samples, was always present when other RD sequences occurred, and always had a read count of a factor 100 or more than the remaining sequences. That sequence was thus considered to be the only true haplotype detected in the data. Comparing this sequence with 12 COI or whole genome sequences downloaded from NCBI (NCBI 2019) showed the haplotype from this study was identical to 8 other sequences (Table A.1 & Fig. A.4) that all aligned most closely with the Asian haplotype.

Table A.1. RD haplotype origin. Twelve RD CO1 and whole genome sequences recorded in NCBI were compared to the RD sequence found in this study (sequence 11). Only three nucleotides differentiate these haplotypes from one another.

Node Label	Matching Sequences	Nr. Matching Sequences
KP992969	KP992969.1	0
KP992976	KP992976.1	0
	KP992974.1	
	KP992976.1	
	11	
KP99297...	KP992972.1	8
	KP992971.1	
	NC_013700.1	
	GU256221.1	
	MG256392.1	
	KP992973.1	
KP99297...	KP992970.1	3
	KF709435.1	

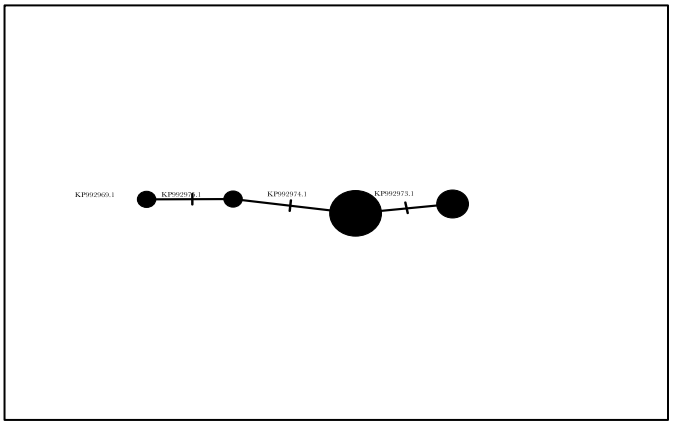

Fig. A.4. Grouping of 13 RD CO1 sequences consisting of 12 sequences from NCBI and the single haplotype found in this study. Size of circles indicates the number of identical haplotypes, while cross lines indicate one nucleotide difference between surrounding circles. The Danish haplotype falls within the greater circle.

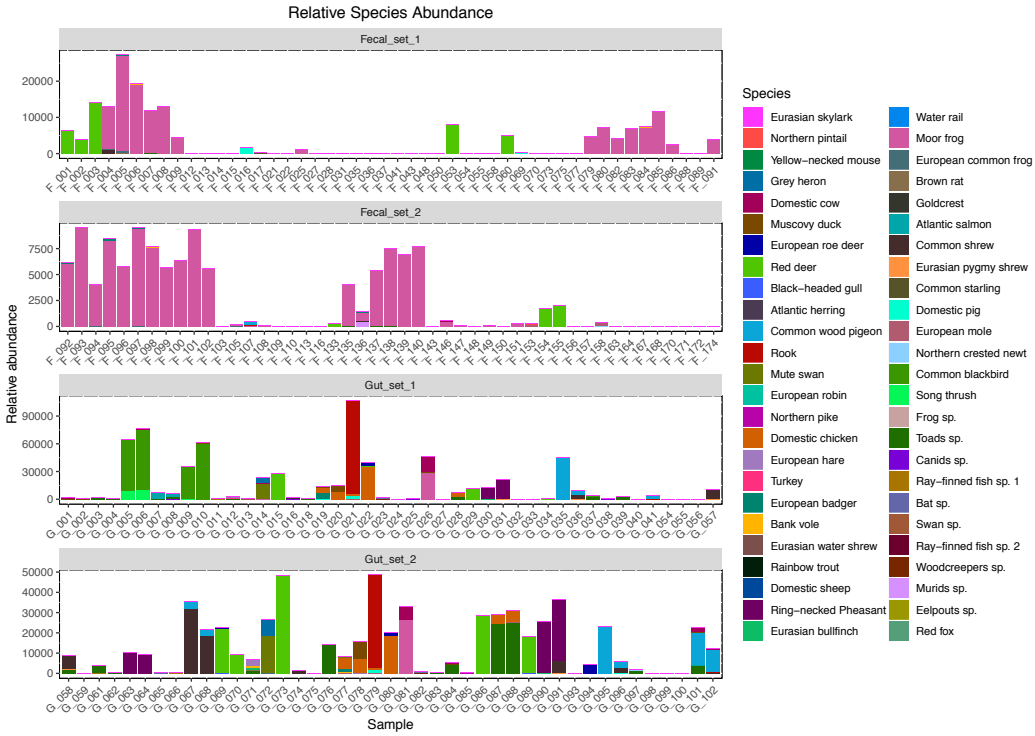

Figure A.5. Relative abundance of each prey item. F = fecal samples, G = gut replicates.

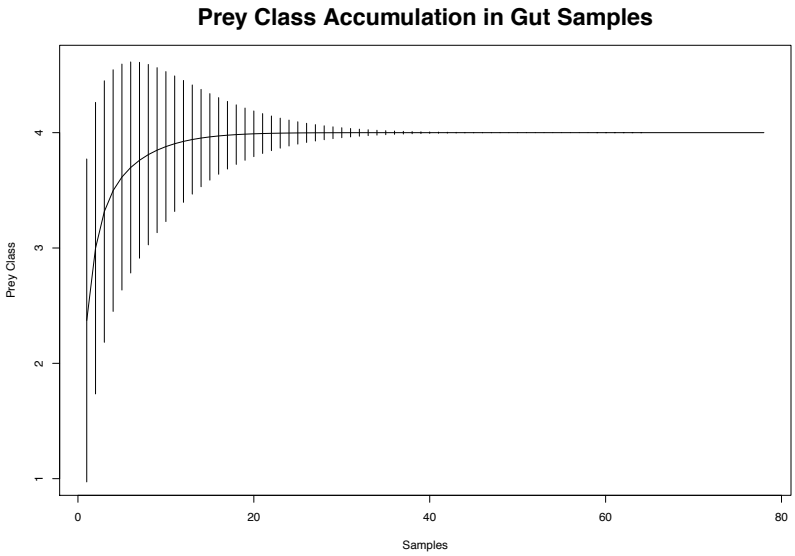

Figure A.6. Food item accumulation curve. Gut analysis requires approximately 35 samples before all four vertebrate classes have been detected.

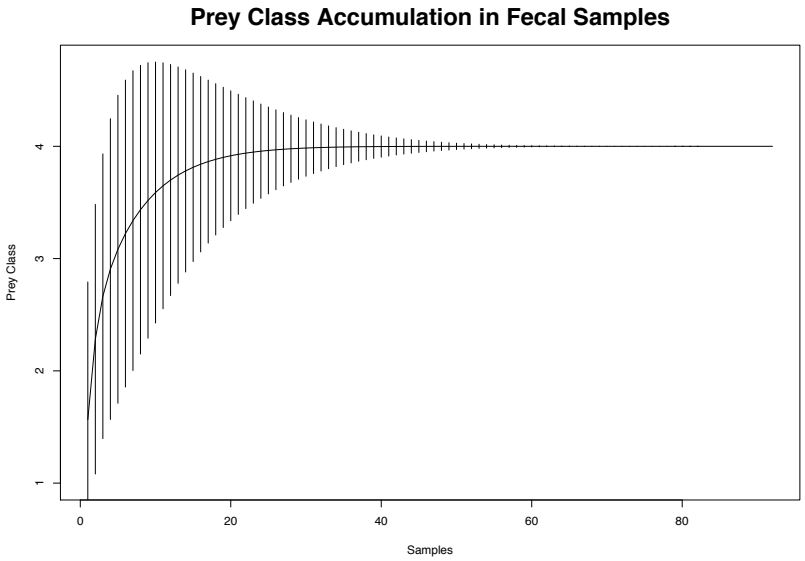

Figure A.7. Food item accumulation curve. Fecal analysis requires approximately 55 samples before all four vertebrate classes have been detected.

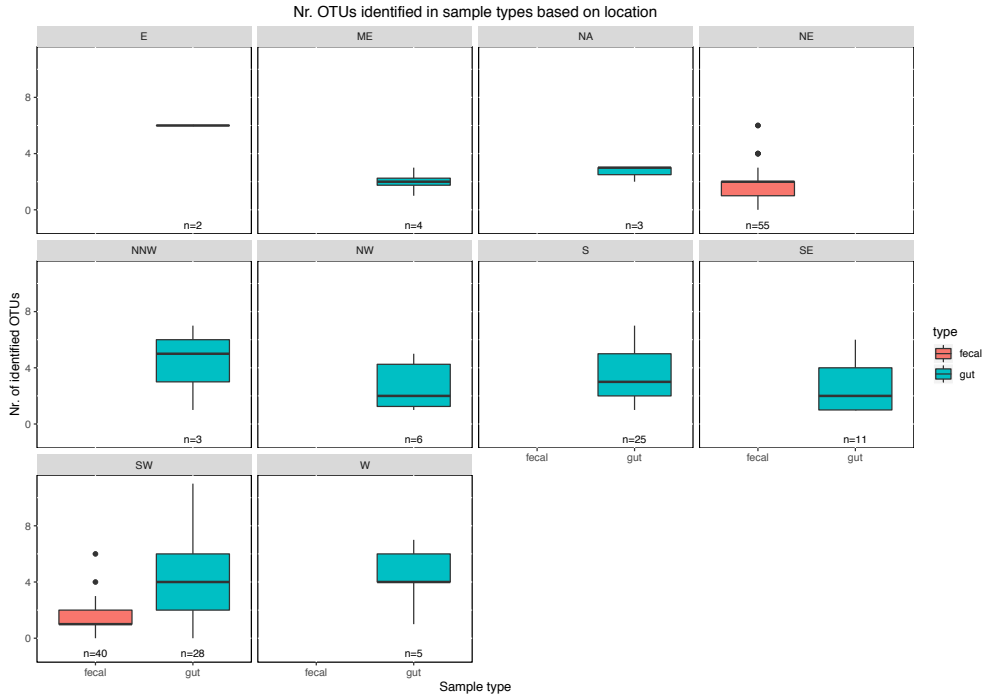

Figure A.8. The number of OTUs found in gut and fecal samples in different locations across Jutland.

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