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Wolf feeding ecology in a multi-ungulate system – investigating the effect of individual predator traits and abundance of co-occurring species

Ph.D. Candidate: Cecilia Di Bernardi

Tutor: Prof. Paolo Ciucci, Prof. Luigi Boitani Department of Biology and Biotechnologies "Charles Darwin", University of Rome La Sapienza, Rome, Italy

Co-Tutor: Assoc. Prof. Camilla Wikenros, Dr. Mikael Åkesson, Assoc. Prof. Håkan Sand Grimsö Wildlife Research Station, Department of Ecology, Swedish University of Agricultural Sciences, Sweden

Coordinator: Prof. Giorgio Manzi

Front page illustration: Cecilia Di Bernardi

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2. ABSTRACT

Predation patterns by large carnivores are influenced by a combination of physical, behavioural, and environmental factors. With the recolonization of these apex predators reaching vast parts of their former ranges, there is a need to better understand carnivores' ecology and effect on prey populations in human dominated landscapes. Data on individual traits of large carnivores are difficult to retrieve and even more to associate with individual feeding behaviour. Alongside cluster checks of GPS-collared wolves, the growing field of faecal DNA-based diet analysis in combination with individual genotyping has the potential to increase the feasibility of large-scale analyses of food use related to individual predator traits. However, the validation of prey DNA detection protocols is still lagging behind the methodological advances.

In this thesis, the development and empirical validation of a molecular method for prey DNA detection was followed by the analysis of intrinsic and extrinsic factors affecting feeding ecology of wolves (*Canis lupus*). The method used nanofluidic array technology and a set of 80 multiple species-specific markers to detect DNA of 17 target prey from wolf scats. Through controlled feeding experiments with captive wolves, we estimated method sensitivity and accordingly calibrated the thresholds to reliably define a positive prey detection. The application of this methodology to the Scandinavian wolf population revealed variability at the landscape level in the use of the two main prey species, moose (*Alces alces*) and roe deer (*Capreolus capreolus*), indicating a dietary response of wolves to changes in wild ungulates relative abundance. In addition, GPS-data showed how scavenging constituted only a minor proportion of wolves' feeding behaviour, related to season and with moderate support to bear and human density. By taking advantage of the long-term wolf monitoring, we showed that wolf feeding patterns (i.e. prey use and extent of scavenging) were affected by social status,

sex, and level of inbreeding. These innovative patterns underline the relevance of considering predator individual traits when studying carnivore feeding ecology.

The development and validation of our molecular method highlighted the overlooked relevance of assessing method sensitivity and including it in the evaluation of optimal thresholds for binary detection of prey species in predator scats. As the molecular method can be easily customized to different ecological settings, it may be further developed and applied to other areas and large carnivores. The knowledge gained in this study has the potential to help understanding the impact of recolonizing wolf populations on prey communities and inform the adaptive management of such predator and prey species living in a landscape highly managed by humans.

3. GENERAL INTRODUCTION

3.1. Large carnivore feeding ecology and extrinsic and intrinsic drivers

Large carnivores have been recolonizing vast parts of their former ranges in Europe (Chapron et al., 2014). Locally, the return of apex predators into areas with multiple prey species can affect the composition and population dynamics of wild ungulate communities (Linnell et al., 2020). In addition, the recolonized landscape is heavily modified by humans, and their presence and activities can affect predator and prey species behaviour through a broad range of anthropogenic factors, from directly affecting their survival through hunting or lethal control to the provision of anthropogenic food subsidies (Dorresteijn et al., 2015; Lodberg-Holm et al., 2019; Penteriani et al., 2018). Therefore, knowledge on carnivore feeding ecology in anthropized systems can help disentangle predator-prey relationships and inform conservation and management of both the predator and prey populations (Gervasi et al., 2012; Newsome et al., 2016).

Feeding ecology of large carnivores is influenced by a combination of physical, behavioural and environmental factors associated with both the prey and predator species (Becker et al., 2008; Bojarska & Selva, 2012). Despite the differential dietary strategies characterizing each large carnivore species, the abundance and vulnerability of prey play a key role in determining predation patterns (Heurich et al., 2016; Mattisson et al., 2016; Niedziałkowska et al., 2019). Prey vulnerability (i.e., the accessibility of individual prey to single predators) varies among prey species due to their body size and defensive behaviour (Garrott et al., 2007; Tallian et al., 2017), as well as within the same species for the different sex, age and nutritional conditions (Kunkel et al., 2004). Intrinsic factors of individual predators also affect feeding strategies of carnivores. Different dietary patterns and hunting success have been observed in relation to sex in wolf, lynx and wolverine (Gustavsen, 2006;

Mattisson et al., 2014; Sand et al., 2006; Sunde & Kvam, 1997), but also in relation to body conditions, age and social status of individual wolves and bears (Imbert et al., 2016; MacNulty et al., 2009; Ordiz et al., 2020; Zimmermann et al., 2015). Seasonality and environmental extrinsic factors such as climatic conditions and landscape heterogeneity can influence both prey vulnerability and predator behaviour (Bojarska & Selva, 2012; Kauffman et al., 2007; Mejlgaard et al., 2013). Additionally, the co-occurrence of other large carnivores can reduce the access to resources through interference and exploitation competition (Ordiz et al., 2020; Tallian et al., 2022). In combination with the aforementioned factors, humans affect the availability of prey to carnivores by hunting the prey populations but also affecting their use of space and risk perception (Kuijper et al., 2016; Linnell et al., 2020).

3.2. Methods to associate feeding behaviour with individual predator traits

Data on individual traits of predators are difficult to collect, and associating this information with individual feeding behaviour is even more challenging. In field studies, GPS-collars deployed on live-captured predators have long been used to investigate feeding behaviour and determine diet composition based on identification of kill or scavenging sites through cluster checks (Ciucci et al., 2020; Krofel et al., 2013; Sand et al., 2005). Substantial field effort is required to avoid potential bias against small prey species that have shorter handling times and leave few traces on kill sites (Bacon et al., 2011; Vogt et al., 2018). GPS cluster checking also allows to associate feeding behaviour with individual predator traits, even though this approach is often limited by sample size (but see Ordiz et al., 2020; Sand et al., 2006).

Another well-established method to assess predators' diet is by traditional scat-analysis (i.e., the macroscopic identification of undigested food remain in predator scats) (Klare et al., 2011); yet, while this technique makes it more feasible to study the diet of hundreds to thousands of individuals, it is time consuming and may suffer from several technical and

interpretational challenges (Ciucci et al., 1996; Spaulding et al., 2000). The growing field of faecal DNA-based diet analysis has become a viable alternative to traditional scat analysis, with higher detection rate of prey and higher taxonomic resolution with more reliable separation of closely related taxa (Mumma et al., 2016; Nørgaard et al., 2021; Shores et al., 2015). Combining genetic analyses of scats, for both genotyping individual predators and detecting the DNA of consumed prey, has the potential to increase the feasibility of large-scale diet analyses investigating the effect of individual predator traits on feeding ecology (Monterroso et al., 2019).

However, the application of such an approach to ecological research has lagged behind the rapid advances of the corresponding methodological advances (Alberdi et al., 2019; Pompanon et al., 2012). Variation in detection probability can occur between target species (Broadhurst et al., 2021; Bylemans et al., 2019). Validation and calibration of DNA-based methods involves the optimization of both specificity (i.e., true negative rate) and sensitivity (i. e., true positive rate). In species detection studies through DNA, priority is usually given to the former to avoid false positive detections, assessing primer specificity *in silico* using databases of barcode sequence and *in vitro* with high quality DNA reference samples (Di Bernardi et al., 2021; Ficetola et al., 2010). Sensitivity is instead rarely investigated, with the substantial risk to overlook false negatives and their confounding effects (Darling & Mahon, 2011). The assessment of sensitivity is even more challenging as the expected presence of a species in a predator scat is needed to estimate its detectability. Such validation can be dealt with by analysing samples using two or more complementary methods, such as DNA-based methods and macroscopic scat analysis (Nørgaard et al., 2021). The limitation of this kind of comparative approaches, however, is that sensitivity is not tested against an error-free method. An alternative more reliable approach is to use captive animals whose diet is composed of

known prey species (Schattanek et al., 2021). Although logistically complex, this approach allows to reliably estimate sensitivity, as well as the factors affecting it.

3.3. The wolf as model species

Wolves are generalist-opportunistic predators and their feeding behaviour is the result of several interacting forces (Becker et al., 2008). Across their range, wolves mostly consume wild ungulates, responding to their vulnerability and relative abundance (Mech & Peterson, 2003). A minor part of their diet can be composed by smaller prey species and plants (Newsome et al., 2016). Wolves can also consume livestock or other anthropogenic foods such as slaughter remains and garbage, mostly in association with low availability of wild ungulates and high levels of human presence (Zlatanova et al., 2014). Humans, in combination with other factors affecting both wolves and their prey, can indeed alter wolf feeding ecology and increase the degree of scavenging by introducing supplementary sources of food in the landscape (Ciucci et al., 2020; Pereira et al., 2014). In such conditions, the ecological role of wolves as an apex predator may be attenuated and predation may lack top-down regulating processes (Kuijper et al., 2016). Knowledge on predator-prey interactions in anthropogenic landscapes is therefore pivotal to understand the ecological functionality of large carnivores in systems altered by humans, and the wolf represents an ideal model species for such investigations.

3.4. The Scandinavian wolf population

The Scandinavian wolf population offers the opportunity to increase knowledge on the interacting extrinsic and intrinsic drivers of the feeding ecology of an opportunistic apex predator living in a multi-prey system. Since the recolonization of Scandinavia by wolves in the early 1980's, the wolf distribution has mainly covered areas where moose (*Alces alces*) and roe deer (*Capreolus capreolus*) have been the main prey (Sand et al., 2008, 2016; Zimmermann et al., 2015). However, wolves are expanding southwards into areas with increasing abundance

of roe deer and other ungulates (red deer (*Cervus elaphus*), fallow deer (*Dama dama),* wild boar (*Sus scrofa*)), experiencing a shift in the composition of available wild ungulate prey species. The monitoring of the Scandinavian wolf population has been conducted each winter since 1998 with the aim to estimate the number of pairs, packs, and reproduction events (Åkesson et al., 2022). Monitoring techniques comprise a combination of snow tracking, identification of individual wolves from DNA samples collected both non-invasively (scats, urine) and invasively (hair, blood from captured and dead wolves), and GPS-tracking of collared individuals (Åkesson et al., 2022). Additionally, a near complete pedigree of the population has been reconstructed based on the genetic identification and parental assignment of reproductive pairs in the population (Åkesson et al., 2016). Such an intensive monitoring of the wolf population results in a detailed knowledge on wolf individuals. On the side of the long-term wolf monitoring, knowledge on the abundance of the highly managed wild ungulate populations and on other large carnivores co-occurring with wolves is available, making Scandinavia suitable for conducting the present study.

3.5. Scope of the Ph.D. project and synopsis of the chapters

The overall scope of the Ph.D. project has been to investigate the feeding ecology of wolves in human-dominated landscapes with multiple co-occurring prey species. The thesis consists of four sections, two of which are methodological chapters aiming at developing and validating a molecular method for binary detection of prey in wolf scats. The third and fourth chapters aimed instead at examining the effect of intrinsic and extrinsic factors on patterns of prey use and on the extent of scavenging, using the DNA-based method and GPS-technology.

3.5.1. Chapter 1. Development of a molecular method to detect prey DNA in wolf scats

In the first chapter, we developed a molecular method using nanofluidic array technology with species-specific markers on the mitochondrial *cyt b* gene for identification of 17 target prey in

wolf scats. As a practical example, the detection procedure was applied on a set of 80 wolf scats collected in Sweden. The final panel consisted of 80 assays that in major part amplified specifically with reference tissue samples, with a minimum of four markers tested per target species. When applied to wolf scats, the success in determining at least one prey species from the scats ranged from 44% to 92% depending on the number of amplifying markers required to obtain a positive call in a scat. This study presented a promising non-invasive, fast and cost-efficient tool for ecological studies on wolves and highlighted the need to evaluate the optimal number of markers for sensitive target species detection.

3.5.2. Chapter 2. Molecular method validation through experimental feeding trials

The second chapter presents the experimental validation of the molecular method previously developed in chapter 1. Feeding trials with captive wolves were conducted to 1) quantitatively evaluate the method sensitivity by comparing true positives and false negatives estimates, and 2) assess how sensitivity was affected by the number of available markers, chosen threshold, prey species, and feeding regime. Using 371 scat samples from wolves fed with a single-prey diet, a variation in method sensitivity was observed among the six ungulate prey species, ranging from 0.45 to 0.95. Sensitivity was favoured using multiple markers per species and a relatively low threshold number of amplifying markers required to give a positive call. The results highlight the relevance of feeding experiments to optimally calibrate the relative thresholds to define a positive detection and investigate occurrence and extent of biases in sensitivity.

3.5.3. Chapter 3. Wolf prey use in a multi-ungulate system

In the third chapter, wolf prey use was examined by applying the molecular method for prey detection developed in chapter 1 and validated in chapter 2 to wolf scats collected in Sweden. The objectives were to i) describe prey use through the proportional occurrence of 17 target

prey in wolf scats, and ii) examine how feeding patterns on the main prey, moose and roe deer, were influenced by the social status (solitary, pair, pack), sex, level of inbreeding of individual wolves, and by the relative abundance of moose, roe deer and alternative wild ungulate prey species. The results showed that consumption of moose and roe deer was affected by changes in their relative abundance as well as to the abundance of alternative wild ungulates. By associating scat samples to individual wolves' genotypes, we were able to reveal that social status, sex, and inbreeding coefficient of individual wolves affected patterns of consumption. These findings supported the influence of ungulate prey abundance and underlined the relevance of considering predator individual traits when investigating feeding patterns.

3.5.4. Chapter 4. Intrinsic and extrinsic factors explaining the extent of scavenging by wolves

The last chapter reported the analysis of patterns of predation versus scavenging by wolves in Scandinavia. Through cluster checks of GPS-collared wolves, we determined the cause of death of different carcasses utilized by wolves. We then i) estimated the proportion of consumption time spent at scavenged versus wolf-killed carcasses, and ii) examined how the proportion of consumption time spent scavenging was related to the social affiliation of adult wolves (solitary, pack) and their level of inbreeding, the density of their primary prey species (moose), the density of their main competitor (brown bear), human density and season. We revealed that scavenging only accounted for 6-15% of wolves' overall consumption time and varied seasonally. Solitary wolves and a higher level of inbreeding of the adult female and male were associated with increased scavenging, while we found only moderate support for an effect of bear and human densities. Despite wolves are known to frequently scavenge when given the opportunity, the results show that wolves in Scandinavia mainly consumed wolf-killed wild ungulates despite a large amount of biomass from hunter harvest remains during specific time periods.

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CHAPTER 1

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Multiple species-specific molecular markers using nanofluidic array as a tool to detect prey DNA from carnivore scats

Cecilia Di Bernardi[1,2] | Camilla Wikenros[2] | Eva Hedmark[2] | Luigi Boitani[1] | Paolo Ciucci[1] | Håkan Sand[2] | Mikael Åkesson[2]

[1]Department of Biology and Biotechnologies "Charles Darwin", University of Rome La Sapienza, Rome, Italy

[2]Grimsö Wildlife Research Station, Department of Ecology, Swedish University of Agricultural Sciences, Riddarhyttan, Sweden

Correspondence
Cecilia Di Bernardi, Department of Biology and Biotechnologies "Charles Darwin", University of Rome La Sapienza, Viale dell'Università 32, 00185 Rome, Italy.
Email: cecilia.dibernardi@uniroma1.it

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Abstract

Large carnivore feeding ecology plays a crucial role for management and conservation for predators and their prey. One of the keys to this kind of research is to identify the species composition in the predator diet, for example, prey determination from scat content. DNA-based methods applied to detect prey in predators' scats are viable alternatives to traditional macroscopic approaches, showing an increased reliability and higher prey detection rate. Here, we developed a molecular method for prey species identification in wolf (*Canis lupus*) scats using multiple species-specific marker loci on the cytochrome *b* gene for 18 target species. The final panel consisted of 80 assays, with a minimum of four markers per target species, and that amplified specifically when using a high-throughput Nanofluidic array technology (Fluidigm Inc.). As a practical example, we applied the method to identify target prey species DNA in 80 wolf scats collected in Sweden. Depending on the number of amplifying markers required to obtain a positive species call in a scat, the success in determining at least one prey species from the scats ranged from 44% to 92%. Although we highlight the need to evaluate the optimal number of markers for sensitive target species detection, the developed method is a fast and cost-efficient tool for prey identification in wolf scats and it also has the potential to be further developed and applied to other areas and large carnivores as well.

KEYWORDS

Canis lupus, carnivore, cytochrome *b* gene, diet assessment, prey identification, species detection

1 | INTRODUCTION

Understanding of species' feeding ecology is of critical importance when studying species interactions such as predator–prey dynamics (Symondson, 2002), and it can be a crucial tool to inform management and conservation (Newsome et al., 2016; Xiong et al., 2017). For large carnivores, collecting dietary information is difficult, since they are elusive and move over large areas (Kéry et al., 2011; Shehzad et al., 2012). In the field, GPS-collars on predators have long been used to investigate predatory behavior and determine diet composition based on identification of kill sites through cluster checks (Peterson & Ciucci, 2003; Sand et al., 2005). One drawback of this approach is the potential bias against small prey species that require shorter handling times and leave few traces on kill sites

(Bacon et al., 2011; Knopff et al., 2009; Webb et al., 2008). Scat analysis is a well established and frequently used methodology to characterize the diet of carnivores (Klare et al., 2011), with the advantage of being a noninvasive approach. Compared to GPS data, it is also more affordable when applied over large spatial and temporal scales. However, macroscopic scat analysis can present technical and interpretational challenges, such as various sources of bias in detecting and quantifying prey types and relative occurrence (Ciucci et al., 1996; Klare et al., 2011; Spaulding et al., 2000).

DNA-based detection of prey from predators' scats or guts has become a viable alternative for the analysis of food habits among invertebrate and vertebrate organisms (King et al., 2008; Pompanon et al., 2012; Traugott et al., 2021; Valentini et al., 2009). When compared with traditional morphological/macroscopic techniques, DNA analyses of scats have become more and more reliable due to a markedly higher prey detection rate (Casper et al., 2007; Mumma et al., 2016; Shores et al., 2015), a reduced observer bias (Shores et al., 2015), and a higher taxonomic resolution with more reliable separation of closely related taxa (Gosselin et al., 2017; Nørgaard et al., 2021; Shores et al., 2015). The predominant DNA region used for species discrimination in taxonomic and phylogenetic studies is the mitochondrial DNA (Simon et al., 2006), which, compared to the nuclear DNA, presents gene sequences with little intraspecific variability but provides adequate interspecific variation (Yang et al., 2014). Moreover, since the mitochondrial genome is normally represented in many more copies per cell than the nuclear genome, it has a greater chance of being amplified with PCR when samples contain few cells or degraded DNA (Yang et al., 2014). Within the mitochondrial genome, the cytochrome *b* (cyt *b*) gene is a suitable gene for species identification, being accurate in separating species and reconstructing phylogeny (Tobe et al., 2009, 2010).

When investigating generalist species or predators with unknown diets, universal primers followed by DNA sequencing have been frequently used in both vertebrates (De Barba et al., 2014; Jarman et al., 2013; Shutt et al., 2020; Šturm et al., 2021) and invertebrates (Pons, 2006; Symondson, 2002). Metabarcoding with next-generation sequencing allows for high-throughput identification of several species by simultaneously sequencing DNA from multiple species in environmental samples (eDNA; Francioli et al., 2021; Taberlet et al., 2012). In prey detection with generic primers, the amplifiable host DNA can however largely outnumber the presence of prey DNA (Krehenwinkel et al., 2017), and strategies to prevent host DNA amplification may be necessary, for example, by using predator-specific blocking primers (Krehenwinkel et al., 2019; Shi et al., 2021; Vestheim & Jarman, 2008). When the diet is characterized by a limited number of prey species, and there is a priori knowledge of the animal's diet, multiplex PCR assays and DNA barcoding with species-/group-specific primers have been used, mostly in invertebrates (Harper et al., 2005; King et al., 2010, 2011; Staudacher et al., 2016) but also in mammals and birds (Casper et al., 2007; Deagle et al., 2007; Shores et al., 2015). Diagnostic PCR methods using species-specific primers often involve relatively low cost per sample and are well suited for scats that contain multiple prey species, as detectability of a species in principal does not depend on the relative quantity of DNA from other species (Rubbmark et al., 2019).

The advances of nanotechnology and the multiplexing approach have improved the speed and efficiency compared to the more conventional PCR setups by reduced reaction volumes, number of pipetting steps, and a multiplexed preparation of DNA templates (Gorgannezhad et al., 2019; Wang et al., 2009). In particular, the use of Nanofluidic array technology (Fluidigm Inc.), which allows for multiplexing and high-throughput analysis of small quantities of DNA, has proven to be useful for determining ungulate species from browsed twigs (Nichols & Spong, 2017) and blood samples (Blåhed et al., 2018), and for detecting pathogen species in ticks (Michelet et al., 2014). Moreover, nanofluidic array technology has also increased the efficiency of species and individual identification using single nucleotide polymorphisms (SNPs) of predator species from scat samples (Förster et al., 2018; Kraus et al., 2015; Von Thaden et al., 2017). Whereas this technology has increasingly been used as diagnostic tool for species detection, there is poor knowledge on its applicability to detect and identify prey DNA from predator scats using diagnostic molecular markers. The cost of using this technology in the year 2021 was ca 20 €/sample including DNA extraction. This aspect, together with the high sensitivity of detection when amplifying very short DNA fragment lengths (Broquet et al., 2007), potentially makes nanofluidic array technology a good contender to, for example, metabarcoding with NGS and conventional sequencing (Tercel et al., 2021) for detecting prey species from large sample sizes for ecological studies.

The aim of our study was to develop a molecular method using nanofluidic array technology with species-specific molecular markers on the mitochondrial cyt *b* gene, for prey species identification in wolf (*Canis lupus*) scats for 14 potential prey species and four other carnivores in Scandinavia. Here, wild ungulates such as moose (*Alces alces*) and roe deer (*Capreolus capreolus*) represent the bulk of wolves' diet (Sand et al., 2005, 2008). However, an expansion of the Scandinavian wolf population into habitats having multiple prey species, such as wild boar (*Sus scrofa*), red deer (*Cervus elaphus*), and fallow deer (*Dama dama*), would likely affect the predation ecology of wolves. As an example of the method applicability, we used our prey species detection procedure on a set of wolf scats collected within the genetic monitoring of the Scandinavian wolf population (Åkesson et al., 2016; Liberg et al., 2012).

2 | MATERIAL AND METHODS

2.1 | Development of molecular markers and target specificity test

We developed species-specific molecular markers for 18 target species (moose, red deer, fallow deer, roe deer, wild boar, reindeer (*Rangifer rangifer*), sheep (*Ovis orientalis*), cattle (*Bos taurus*), European badger (*Meles meles*), Eurasian beaver (*Castor fiber*),

European hare (*Lepus europeus*), mountain hare (*Lepus timidus*), Western capercaillie (*Tetrao urogallus*), black grouse (*Lyrurus tetrix*), brown bear (*Ursus arctos*), Eurasian lynx (*Lynx lynx*), wolverine (*Gulo gulo*), and red fox (*Vulpes vulpes*). The target species were selected among known prey species and also from allopatric medium-sized and large carnivore species to wolves in northern Europe (Chapron et al., 2014; Gade-Jørgensen & Stagegaard, 2000; Nowak et al., 2011; Sand et al., 2008). The species-specific markers were developed using sequences of the cytochrome *b* (cyt *b*) gene in the mitochondrial DNA from the 18 target species, and wolf and dog (*Canis familiaris*; 1–25 sequences/species) found in GenBank on the NCBI website (http://www.ncbi.nlm.nih.gov/; Appendix S1). After aligning the sequences in Geneious Prime 2019.0.4 (Biomatters, Ltd.), we screened visually and identified species-specific cyt *b* target DNA sites (loci; Appendix S2) that showed no conspecific variation and were highly diagnostic in relation to all target species, dogs and wolves.

We aimed at increasing marker species specificity in two ways. First, we had a strong preference for markers with fully diagnostic nucleotide at the 3′ end of at least one of primer-pairs. At three occasions, two different wild ungulate species carried the same nucleotide at the 3′ end and in no cases was it the same nucleotide as those in the wolves and dogs. Second, when designing assays using Fluidigm's custom assay design criteria we added the instruction to increase target specificity by placing locus-specific primers in regions that appeared conserved among conspecifics while differentiated in relation to the other target species, wolves and dogs. The aim was to use the Fluidigm EP1™ system to detect presence or absence of the target specific DNA. For this, we used assays (SNPtype™ assays, Fluidigm Corp.) with one reverse primer and two forward primers with identical annealing sequences but with different SNPtype™ (Fluidigm Corp.) tail sequences for HEX and FAM fluorescence (SNPtype™-HEX and SNPtype™-FAM). This enabled us to use either the FAM or HEX signal to quantify the amplification intensity (see below). We did not include dogs among the target species as we did not succeed in developing markers that specifically amplified and separated dogs and wolves. Moreover, there was already a potential risk of a negative bias against wolf scats containing DNA from dogs, since these scats are difficult to link to individual wolves, due to the overlap in allelic composition among the two species.

We developed 207 assays (Appendix S3) with a minimum of four assays and different loci for each target species. Multiple assays for the same locus and species were occasionally developed when we found nonsufficient separation in amplification intensity between specific and nonspecific species, but we finally kept one assay per locus. We aimed to develop assays for at least four loci per target species in order to (a) increase the chance of detecting the target species in the event of some markers not amplifying due to low DNA quantity, (b) account for the possibility that we missed intraspecific variation that prohibits amplification for some marker, and (c) increase the target specificity in the case of markers not being fully diagnostic in relation to other species. For most species, we tested more than four loci and we continued to use those that showed the best separation between specific and nonspecific reference tissue samples.

The molecular markers were tested for target specificity with 2–5 tissue samples for each target species (≥3 samples for the wild and domestic ungulates), using specimens provided by the Swedish Museum of Natural History. The tissue samples were geographically distributed throughout Sweden in the attempt to cover any spatial intraspecific variability in sequences of the target species. Additionally, samples from wolves ($n = 3$), bank voles (*Myodes glareolus*; $n = 5$), and a negative control (water) were also included in the run. All the markers were tested against all the tissue samples. For each marker, a two-sample *t*-test was conducted between the amplification intensity of specific and nonspecific samples. Because of multiple testing, we adjusted the p-values using the BY approach (Benjamini & Yekutieli, 2001). Additionally, the frequency of overlap was measured as the proportion of nonspecific samples overlapping in amplification intensity with the minimum amplification intensity of the specific reference tissue samples. Since the four fallow deer markers were tested with a one-sample *t*-test (only one specific sample was finally available for statistical analysis), we additionally ran a two-sample *t*-test and estimated the frequency of overlap from a rerun. Out of the 207 molecular markers developed and tested, we selected a final panel of 80 markers with the largest separation between specific and nonspecific reference tissue samples, maintaining a minimum of four markers for each target species (Figure 1, Appendix S3).

2.2 | Molecular analysis and PCR optimization

DNA was extracted from the tissue samples using standard phenol/chloroform-isoamylalcohol extraction, and DNA was quantified using NanoDrop™ 2000 Spectrophotometer. The prepared DNA (10 ng/μl) was amplified with PCR and visualized with fluorescence detection using a Fluidigm® 96.96 Dynamic Integrated Fluidic Circuit (IFC) Array, according to the manufacturer's instructions (http://www.fluidigm.com). To avoid cross-contamination, the PCR setup was done in a hood prepared with ultraviolet (UV) light exposure. Each Fluidigm plate enabled the PCR amplification of 96 assays on 96 samples simultaneously, and the standard procedure recommended by Fluidigm was modified by excluding the specific target amplification and increasing the starting temperature of the touch-down cycle of 1°C (65–60°C with 1°C decrease between cycles). Both modifications reduced the amplification intensity of nonspecific samples and therefore increased the specificity of our molecular markers.

Data on fluorescence intensity were obtained from the Fluidigm EP1™. The reported fluorescence signal, relative to the passive reference ROX™ dye, reflects the DNA amplification intensity (Kubista et al., 2006; Whitcombe et al., 1999). Since we used two fluorescence dyes on the same target locus, we got two measures of amplification intensity, I_F and I_H, respectively, representing the amplification intensity of SNPtype-FAM and SNPtype-HEX amplicons. To account for the overlap of amplification intensity between nonspecific and specific samples, which was occasionally observed in one of the two

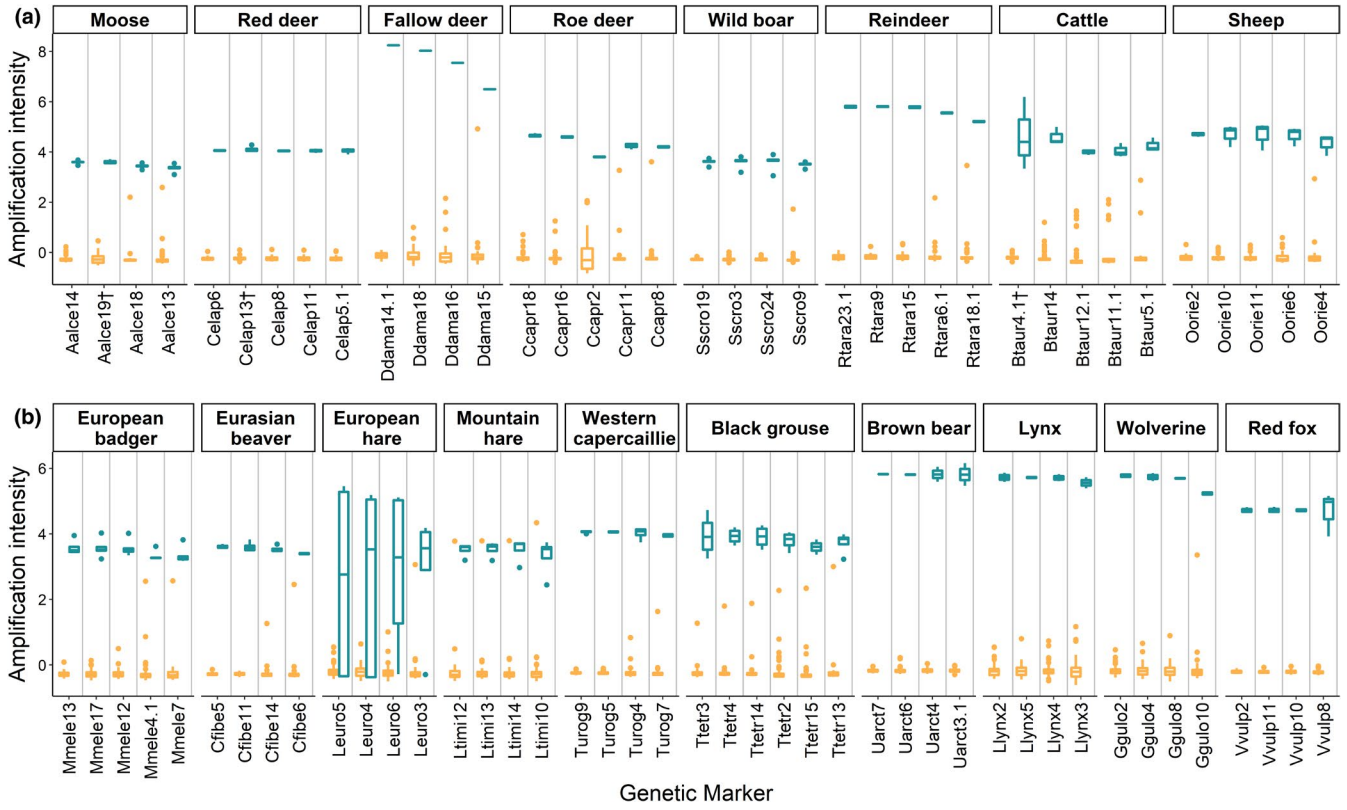

FIGURE 1 Amplification intensity for the specific (turquoise) and nonspecific (yellow) reference tissue samples analyzed on 80 different markers for the identification of (a) wild and domestic ungulates (moose, red deer, fallow deer, roe deer, wild boar, reindeer, cattle, sheep) and (b) smaller prey species (European badger, Eurasian beaver, European hare, mountain hare, Western capercaillie, black grouse) and large-sized and medium-sized carnivores (brown bear, Eurasian lynx, wolverine, red fox) A negative control (water) was used for each marker. The markers are arranged within each species based on the frequency of overlap and additionally on the distance between the minimum specific sample and the maximum nonspecific sample (from left to right, increasing frequency of overlap and decreasing distance). The amplification intensities were standardized for visual purposes. †For 3 markers, we illustrate the amplification intensity from a rerun

dyes of a marker, we systematically extrapolated the amplification intensity of the reference samples based on the frequency of overlap of both dyes. Frequency of overlap was measured for each dye as the proportion of nonspecific samples overlapping in amplification intensity with the minimum intensity of the specific reference tissue samples. If both dyes showed no overlap, one of the two was randomly picked; if only one dye had a null frequency of overlap, it was picked against the dye with frequency of overlap >0; if both dyes had frequency of overlap >0, the dye with lower frequency of overlap was picked. Occasionally, the ROX-signal for some samples was near absent, possibly due to the occurrence of dirt particles in the samples that hindered the solution to flow in the IFC. As this appeared to affect the relative intensity of the calls, we omitted samples identified as outliers with regard to ROX intensity.

2.3 | Wolf scat samples and target species determination

To exemplify the applicability of our method, we examined the occurrence of the target species in 80 wolf scat samples collected in Sweden between 2009 and 2018 (Appendix S6) during the yearly monitoring (October–March) of the Scandinavian wolf population (Åkesson et al., 2016; Liberg et al., 2012). The DNA was extracted within each monitoring period, using QIAamp DNA Stool Kit (Qiagen) or ISOLATE Faecal DNA Kit (Bioline). The presence of wolf-specific DNA and the identity of the wolf were determined in accordance with the methods described in Åkesson et al. (2016).

We used thresholds for getting a binary detection for a prey species in each scat, where the intensity of 0.2 (value indicating low amplification intensity) and the intensities of nonspecific reference tissues from the run were used as baseline in each marker. Any sample showing intensities below the baseline was regarded as not amplifying. The sensitivity of using a minimum of 1, 2, 3, or 4 markers with a positive call (out of the total of used markers) for detecting the target species DNA was tested and compared (Appendix S7). For each such scenario, all the possible combinations of markers were checked, and the target species DNA was deemed as present in a sample when at least one combination showed amplification intensities above all markers' baseline levels. This was done separately for the 18 target species. All statistical analyses were conducted in R version 3.6.0 (R Core Team, 2018).

3 | RESULTS

3.1 | Molecular markers

Amplification intensity of reference tissue samples for the target species varied from 0.02 to 1.31, with an average intensity of 0.93 (range 0.02–1.31) for specific samples and 0.08 (range 0.00–1.13) for nonspecific samples (Figure 1). The 80 selected markers all showed amplification for the specific samples, and the majority (n = 77) showed significantly higher intensity of specific than nonspecific samples ($p \leq 0.05$, Appendix S4, Figure 1). After the correction for multiple testing, 70 out of the 80 markers had a significant separation (Appendix S4) and we hereafter refer to the adjusted p-values. One moose marker (Aalce19) showed an overlap between specific and nonspecific samples with a higher intensity of the nonspecific samples compared to the specific samples (t = 3.83, p = 0.027, frequency of overlap = 1, Appendix S4). The 80 markers had an average frequency of overlap of 0.05 ± 0.02 (mean ± SE), with the majority (n = 71) having no overlap (Appendix S4). A sample from a morphologically determined European hare consistently amplified with markers for mountain hare, indicating that this individual had hybrid origin. After a rerun of the 11 markers with nonsignificant (n = 10) or negative difference between specific and nonspecific sample intensities (n = 1), we found that two out of 11 (Aalce19, Llynx2) showed significant separation with no overlap (Appendix S5). The other nine markers showed a nonsignificant separation in the rerun as well, with frequency of overlap >0.22 for the European hare markers and no overlap for the remaining markers (Appendix S5). For fallow deer, both the one-sample t-test and the two-sample t-test from a rerun resulted in significant separation and the frequency of overlap was zero for all four markers in both runs (Appendices S4 and S5).

For all target species, the final panel included at least four markers available for species identification, while five markers were available for red deer, roe deer, reindeer, sheep, cattle, and European badger, and six markers for black grouse (Figure 1, Appendix S3). The negative control never amplified with any of the 80 selected markers.

3.2 | Application to wolf scat samples

Setting the thresholds to reach full specificity for each target species, we detected the presence of DNA from at least one target species in 73 (92%), 53 (67%), 43 (54%), and 35 scats (44%) when minimum one, two, three, and four amplifying markers were set as threshold, respectively (Figure 2). In each scenario, the remaining samples did not meet the criteria for species detection. Out of the 80 wolf scat samples analyzed, one was invalidated due to outlier ROX intensity. The average number of detected species per scat sample was, respectively, 1.7 (range 1–7), 1.1 (range 1–4), 1.1 (range 1–2), and 1.1 (range 1–2) when one, two, three, and four amplifying markers were set as threshold. In total, 16 different target species were identified, comprising wild ungulates (moose, red deer, fallow deer, roe deer, wild boar), domestic and semi-domestic animals (reindeer, cattle, sheep), small prey species (European badger, European hare, mountain hare, Western capercaillie, black grouse), and other carnivores (Eurasian lynx, wolverine, red fox).

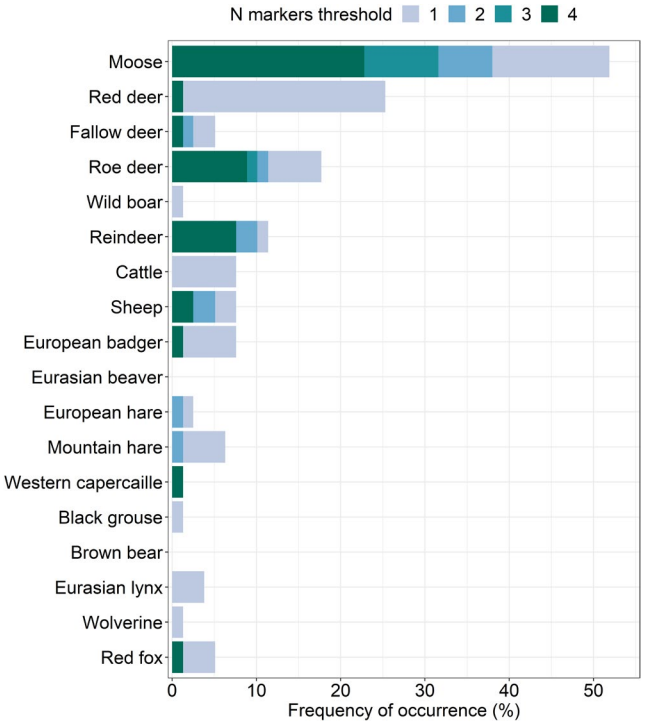

FIGURE 2 Prey diversity observed in the diet of wolves from scats (n = 79) collected in Sweden (2009–2018), depending on the threshold minimum number of amplifying markers required to detect the target species in a scat sample. The frequency of occurrence was measured as the percentage of scat samples with the detected target species out of the total number of samples analyzed. For the two hare species, the result illustrates the maternal lineages of the two species, while potential hybrid status of the detected hares was not known

4 | DISCUSSION

We developed a molecular method to detect prey species DNA in wolf scats by using multiple diagnostic molecular markers that amplified specifically when tested with high-quality DNA, that is, tissue samples, from 18 target species. After setting thresholds that maximized specificity for a binary species detection, the application of the method to a sample of genetically verified wolf scats collected in the field was tested and resulted in the amplification of 16 species. While this study was not meant to make a comparative assessment between the nanofluidic array approach and traditional scat analysis techniques (i.e., hand separation), our aim was to develop a practical and efficient technique to identify prey species from predators' scats and assess its performance.

The final panel contained 80 molecular markers with a minimum of four markers on different target loci of cyt b for each target species. Although the focal species we considered are wild ungulates,

which make up the bulk of the diet in the Scandinavian wolf population (Sand et al., 2005, 2008), our panel also offers the possibility to detect smaller prey species that are less likely to be found by GPS technology due to their smaller amount of biomass (Sand et al., 2005). Markers for detecting other medium-sized (red fox) and large (lynx, wolverine, brown bear) carnivores occurring in the study area were also included in the panel, and hold the potential for providing information on the interaction between wolves and other carnivores. However, is worth recommending that the presence of DNA from other carnivores does not necessarily indicate intraguild predation, as these species may be prone to contaminate scats with their DNA through territorial marking (Wikenros et al., 2017).

Among the 80 molecular markers we used, significant separation and low frequency of overlap generally indicated a good marker performance in discerning the target species. The hare markers successfully separated hares from the other target species, but the two different hare species were not always distinctly separated. Indeed, the consistent amplification of a morphologically determined European hare sample with mountain hare markers is likely due to hybridization, as the European hare and mountain hare hybridize in the wild (Jansson et al., 2007). We therefore caution against the distinction between the two hare species with only mitochondrial markers, but encourage to maintain the two developed marker sets separated in order to keep the distinction of maternal lineages. Beside the hare markers, for the few other cases of nonspecific samples with amplification intensity similar or higher than specific samples, a possible explanation could be that the nontarget species carried intraspecific variation that overlapped with the target species but was missing in the reference sequences used in this study (Appendix S1). Here, we took into account the nonspecific amplification by setting threshold intensities resulting in full specificity in relation to the range of tested reference tissue samples. This was done separately for the four scenarios, using a minimum of 1, 2, 3, or 4 markers with a positive call (out of the total of used markers) to determine the presence of target species DNA. When applying our molecular method to wolf scats, we obtained different DNA detection rates depending on the minimum number of markers required. The percentage of scat samples with presence of at least one target species was 92% when using a threshold of one marker, while it was 67% when using a threshold of two markers, therefore confirming the amplification on at least two independent loci. If we prioritize the sensitivity of our detection procedure and set a threshold of only one marker to detect a species, we minimize the occurrence of false negatives (type II error). However, despite that we developed markers as diagnostic as possible and set thresholds with full specificity in relation to the reference samples analyzed, the intraspecific locus variation in the wild may not have been fully represented among the animals in this study. As this can potentially lead to the risk of false positives (type I error), caution should therefore be taken with regard to using too few markers for a diagnostic species determination. In line with the principles of replication and multiple tubes approach (Ficetola et al., 2015; Taberlet et al., 1996), requiring more than one amplifying marker out of the used set of species-specific markers may thus be a way to ensure the quality of target species determination.

Our diagnostic method with species-specific markers adds to the more frequent studies using DNA from carnivore scats to identify prey (Hacker et al., 2021; Quéméré et al., 2021; Roffler et al., 2021; Shi et al., 2021; Smith et al., 2018; Xiong et al., 2017). These studies primarily used DNA metabarcoding, which produces a vast amount of valuable information but sometimes also needs consideration of potential bias sources in key steps in the data handling process, lack of reference databases of barcodes for many prey species, but also intensive laboratory procedures and considerable bioinformatics training (Hacker et al., 2021; Tercel et al., 2021; Zinger et al., 2019). The relatively simple molecular method developed here, applying the nanofluidic array technology to detect prey DNA, represents a promising and valid alternative to other methods. However, although the use of multiple markers has previously been shown to increase the species detection success (Zhang et al., 2018), further validation of our method by using scats with known content would provide further insights into the method sensitivity and which thresholds to use. The latter is indeed a critical step faced in other molecular approaches as well, including the setting of thresholds to discard sequences with next-generation sequencing (Darling & Mahon, 2011; Taberlet et al., 2012).

As observed from our sample of wolf scats collected in Sweden, other studies using either traditional scat analysis and DNA approaches have shown that the majority of wolf scats contain on average one prey per scat sample (Ciucci et al., 2018; Shores et al., 2015). In addition, the occurrence of prey species detected in our study is in line with previous and ongoing research on the diet of the Scandinavian wolf population conducted using GPS technology. Specifically, moose and roe deer compose the bulk of the wolf diet in Scandinavia, but also predation on domestic animals (i.e., sheep and cattle) is evidenced (Karlsson & Johansson, 2010; Sand et al., 2005, 2008, 2016; Zimmermann et al., 2015). The position of the scats with detected large ungulate wild prey species, that is, moose, roe deer, red deer, and wild boar, fitted well within the species' distribution range (Linnell et al., 2020). The detection of reindeer in wolf scats only occurred among scats collected within the reindeer husbandry area, in the northern part of Sweden, where wolf attacks on semi-domestic reindeer are documented (Sand et al., 2019). Detections of fallow deer, red deer, and wild boar were found only in scats from the southern part of Sweden. This is where these species are known to occur, but there currently is little knowledge about their importance as prey for wolves. With the recent expansion of the wolf population into the southern parts of Sweden (Svensson et al., 2021), our method will therefore be a useful tool in investigating the potential changes in prey use of wolves and its effect on ungulate populations. Consumption of smaller prey has previously been documented by GPS technology as constituting a small percentage of wolves' diet in Scandinavia (Sand et al., 2008; Zimmermann et al., 2015). However, GPS technology is likely underestimating the contribution of small prey, and DNA identification can contribute to better estimates of the frequency of small prey consumption by wolves. Moreover,

the implementation of the molecular method to a broader sample of scats will increase our knowledge on wolf diet in areas that are difficult, or not prioritized, to cover with GPS-collared wolves, for example, southern Sweden. The molecular method will therefore serve as a valuable complement to the current GPS technology used to investigate wolf predation.

Applications of our molecular method to the management of wolves' main prey species include providing information about wolf prey consumption over large spatial and temporal scales. Knowledge of area specific wolf prey consumption, especially with multiple ungulate prey species, is important information for management when deciding on hunting quotas of ungulates. Additionally, this method can provide information on the use of domestic animals and thus help to set levels of compensation in areas with free-ranging domestic animals (e.g., sheep in Norway, semi-domestic reindeer in the reindeer husbandry area in Sweden).

We conclude that the method we developed, suitable for high-throughput analysis of scat samples on up to 96 markers and 96 samples simultaneously, represents a promising noninvasive, fast, and cost-efficient DNA-based tool for ecological studies on wolves. As this method can be easily adapted to new situations and customized to fit regional demands with new prey species, it has the potential to be further developed and applied to other areas and other large carnivores as well.

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CONFLICT OF INTEREST

None declared.

AUTHOR CONTRIBUTION

Cecilia Di Bernardi: Conceptualization (lead); Data curation (lead); Formal analysis (lead); Investigation (lead); Methodology (lead); Writing-original draft (lead); Writing-review & editing (lead). **Camilla Wikenros:** Conceptualization (supporting); Funding acquisition (equal); Methodology (supporting); Project administration (lead); Supervision (equal); Writing-original draft (supporting); Writing-review & editing (equal). **Eva Hedmark:** Conceptualization (supporting); Methodology (supporting); Supervision (supporting); Writing-original draft (supporting). **Luigi Boitani:** Conceptualization (supporting); Methodology (supporting); Supervision (equal); Writing-original draft (supporting); Writing-review & editing (supporting). **Paolo Ciucci:** Conceptualization (supporting); Funding acquisition (equal); Methodology (supporting); Supervision (equal); Writing-original draft (supporting); Writing-review & editing (equal). **Håkan Sand:** Conceptualization (supporting); Funding acquisition (equal); Methodology (supporting); Supervision (equal); Writing-original draft (supporting); Writing-review & editing (supporting). **Mikael Åkesson:** Conceptualization (lead); Data curation (supporting); Formal analysis (supporting); Funding acquisition (equal); Methodology (supporting); Supervision (equal); Writing-original draft (supporting); Writing-review & editing (equal).

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the Supplemental Information of this article, available from the Zenodo Digital Repository DOI https://doi.org/10.5281/zenodo.5066742. Specifically, the NCBI accession numbers of DNA reference sequences, and their literature citations where applicable, are available in Appendix S1, while the DNA sequence information of the developed molecular markers is available in Appendices S2 and S3.

ORCID

Cecilia Di Bernardi https://orcid.org/0000-0002-1171-1516
Camilla Wikenros https://orcid.org/0000-0002-2825-8834
Luigi Boitani https://orcid.org/0000-0003-1382-7800
Paolo Ciucci https://orcid.org/0000-0002-0994-3422
Håkan Sand https://orcid.org/0000-0002-2646-022X
Mikael Åkesson https://orcid.org/0000-0002-4325-8840

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Supplemental Information for CHAPTER 1:

Multiple species-specific molecular markers using nanofluidic array as a tool to detect prey DNA from carnivore scats

Di Bernardi Cecilia, Camilla Wikenros, Eva Hedmark, Luigi Boitani, Paolo Ciucci, Håkan Sand, Mikael Åkesson

Questions to: cecilia.dibernardi@uniroma1.it

Description of Supplemental Information

Table S1, Table S2, Table S3, Table S4, and Table S5 can be found in the excel file in the digital deposit at https://zenodo.org/record/5066742#.YuEZMXZBzq4, while Table S6 and Figure S1 can be found below.

Table S1. Reference sequences from GenBank, with accession number for the 18 target species. The list of literature reference for the published sequences can be found in the table as well.

Table S2. Target position, allele of the target species, and allele of the non-target species for 207 developed genetic markers for 18 target species.

Footnote for Table S2:
† The target position refers to the moose reference sequence AJ000026 (1140 bp). For the two forest bird species, Western capercaillie and black grouse, the position refers to the black grouse reference sequence EF571183 (1143 bp), followed by the corresponding homological position in the moose reference sequence AJ000026 within brackets.

Table S3. Allele, sequence with target position, STA sequence, LSP sequences (locus specific sequence), and ASP sequence (allele specific sequence) of 207 developed genetic markers for 18 target species. The sequences contain IUPAC ambiguity codes, indicating where there is variation within species. One column indicates if the assay is included in the final selection of 80 markers and anther column indicates the four best markers per species (based on t test,

frequency of overlap, and the distance between the minimum specific sample and the maximum non-specific sample).

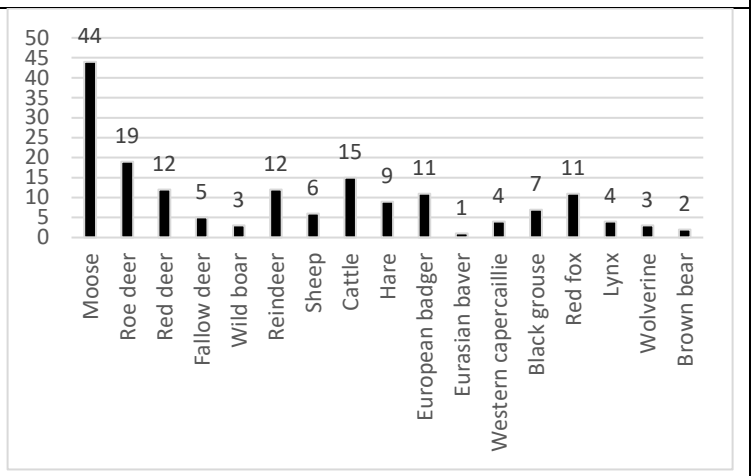
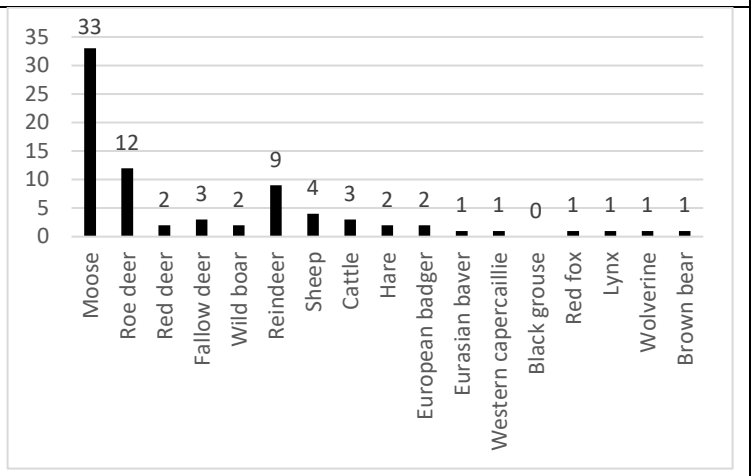
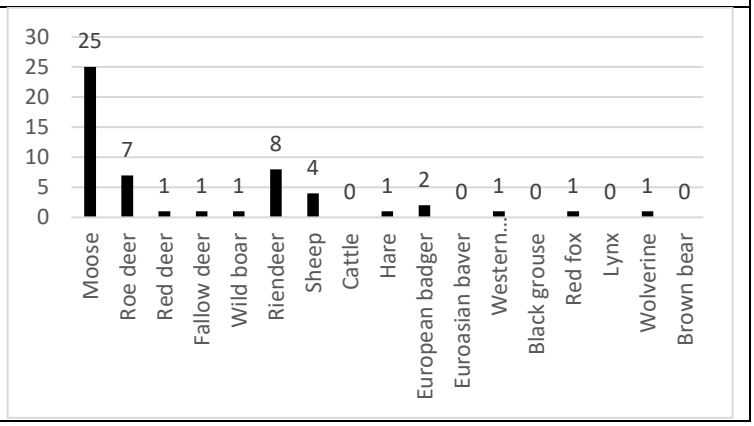
Footnote for Table S3:
† The Specific target amplification (STA) was excluded during the protocol optimization and not used in the final presented protocol.
‡ We used two sets of identical ASP primer sequences but with different fluorescence.

Table S4. Results from two-sample t-test for the 80 selected genetic markers. Assay name, t estimate, p value, p value adjusted with BY correction method for multiple testing (Benjamini & Yekutieli, 2001), average and standard deviation of non-specific and specific reference tissue samples, and the frequency of overlap, which is the proportion of non-specific reference tissue samples that overlap with the minimum of the specific reference tissue samples. For the four fallow deer markers we conducted instead a one-sample t-test since we had only one specific sample available, following the ROX outlier filtering.

Footnote for Table S4:
† When i) the adjusted p value was non-significant (n = 10), or ii) the test gave a positive t estimate (which means higher intensity of the non-specific reference tissues compared to the specific reference tissues therefore no separation) (n = 1), or iii) we could only run a one-sample t-test because only one specific reference tissue was available (n = 4), we additionally ran a two-sample t-test and estimated the frequency of overlap from a different run (results are in Table S5).

Table S5. Results from additional two-sample t-test and estimate of frequency of overlap on a different run for 15 genetic markers.

Table S6. Detection of target species in wolf scats (n = 79) collected in Sweden, depending on the threshold set, i.e. the minimum number of amplifying markers required to detect the target species in a scat sample.

Threshold	Amplification success	N species detected	Average (range) of N species detected/scat	N scats detecting each species
1 marker	94%	17	2 (0 - 14)	
2 markers	72%	16	1 (0 - 13)	
3 markers	57%	12	0.7 (0 - 6)	

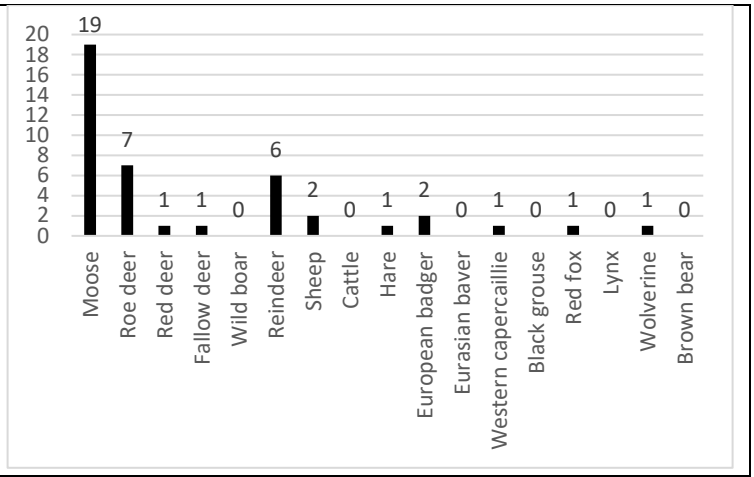
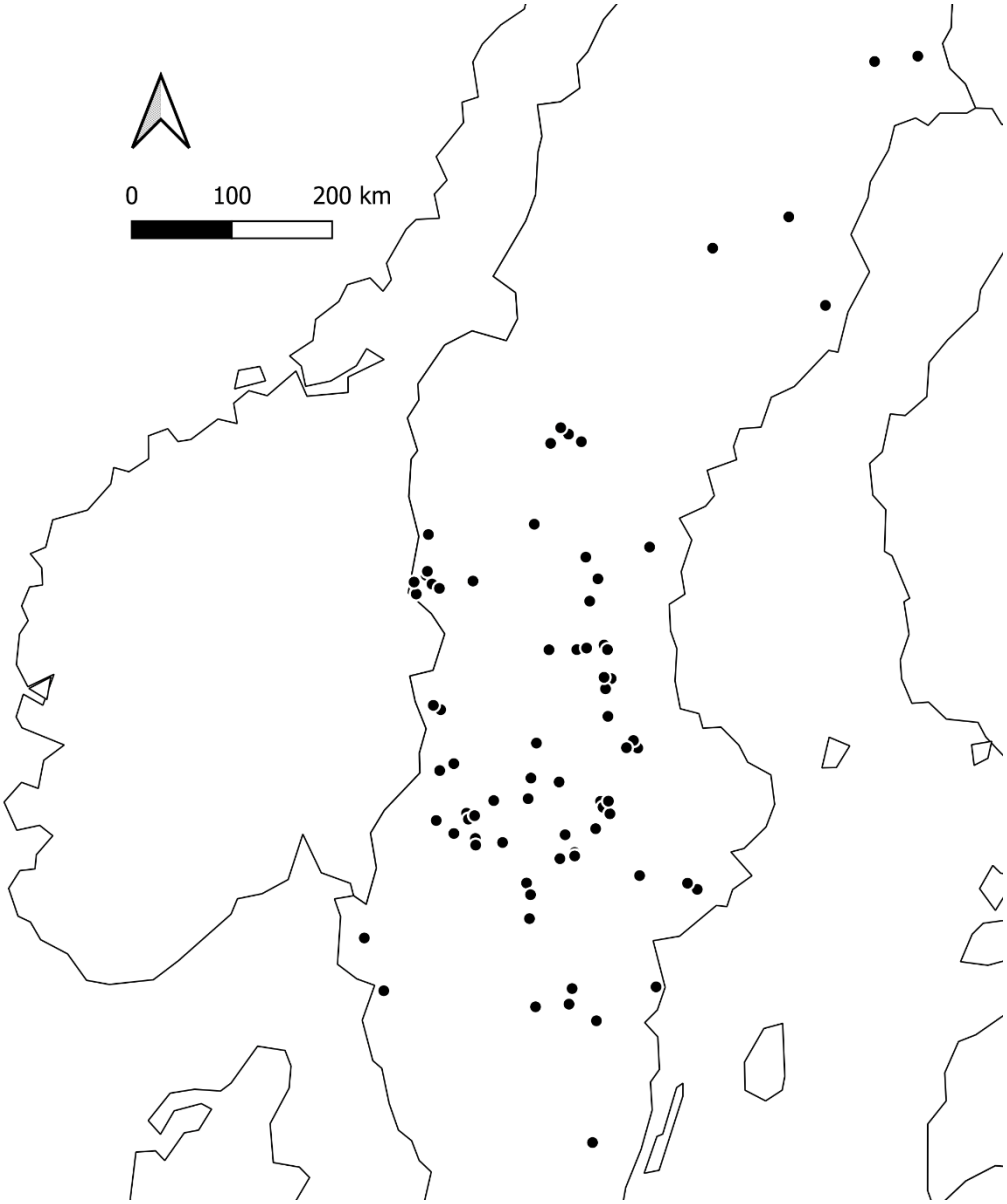
4 markers	47%	11	0.5 (0 - 4)	

Figure S1. Collection site for wolf scats (n = 80) during winters (1 Oct to 31 March) of 2009-2018 in Sweden within the monitoring of the Scandinavian wolf population.



CHAPTER 2

Experimental feeding validates nanofluidic array technology for DNA detection of ungulate prey in wolf scats

Cecilia Di Bernardi[1, 2], Camilla Wikenros[2], Paolo Ciucci[1], Luigi Boitani[1], Håkan Sand[2], Mikael Åkesson[2]

Affiliations:

1 Department of Biology and Biotechnologies "Charles Darwin", University of Rome La Sapienza, Viale dell'Università 32, 00185, Rome, Italy

2 Grimsö Wildlife Research Station, Department of Ecology, Swedish University of Agricultural Sciences, 739 93 Riddarhyttan, Sweden

Corresponding author: Cecilia Di Bernardi, cecilia.dibernardi@uniroma1.it

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Abstract

The study of carnivore diet is a key component to enhance knowledge on the ecology of predators and their effect on prey populations. Although molecular approaches to detect prey species DNA in carnivore scats are improving, the validation of their accuracy, a prerequisite for reliable applications within ecological frameworks, is still lagging behind the methodological advances. Indeed, variation in detection probability among prey species can occur, representing a potentially insidious source of bias in food-habit studies of carnivores. Calibration of DNA-based methods involves the optimization of both specificity and sensitivity

and, while priority is usually given to the former to avoid false positive detections, sensitivity is rarely investigated and false negatives may therefore be overlooked.

We conducted feeding trials with captive wolves (*Canis lupus*) in order to validate a nanofluidic array technology recently developed for detection of multiple prey species in scats. Using 371 scat samples from wolves fed with a single-prey diet, the method sensitivity varied for the six ungulate target prey species, ranging between 0.45 and 0.95. The method sensitivity increased using multiple markers per species and a relatively low threshold number of amplifying markers required to give a positive call. Yet, at least two markers should be used in order to avoid false positives. Acknowledging sources of bias in sensitivity to reliably interpret results of DNA-based dietary methods, our study highlights the relevance of feeding experiments to optimally calibrate the relative thresholds to define a positive detection and investigate occurrence and extent of biases in sensitivity.

Key words: *Canis lupus*, diet analysis, DNA, false negatives, feeding experiment, sensitivity

1. Introduction

Knowledge of carnivores' feeding ecology is important for the understanding of their effect on prey population size and demography (Gervasi et al., 2012; Wallach et al., 2017). Except for a few observational studies (Smith et al., 2020; Vucetich et al., 2002), predation is generally investigated based on signs left on the ground after the predation event, such as remains from animals killed by carnivores equipped with a GPS-collar. Additionally, faeces can be collected and macroscopically or molecularly analysed for prey content (Mech & Boitani, 2003). In particular, the investigation of carnivore food-habits through molecular detection of prey DNA from predator faeces has received increasing attention, with the development of several

methods that enable high taxonomic resolution (Quéméré et al., 2021; Roffler et al., 2021; Shi et al., 2021). However, the implementation of DNA-based methods into ecological frameworks has lagged behind the methodological advances (Alberdi et al., 2019; Pompanon et al., 2012). The major challenge, using molecular species detection, is that estimates of species composition may be affected by variation in detection probability between target species, which is more or less pronounced depending on the initial abundance of DNA in the sample and proportional differences in abundance of DNA from different species (Broadhurst et al., 2021; Bylemans et al., 2018, 2019). The overall effect of the heterogeneity in detection probability among prey species needs to be tested and accounted for before a new method can be applied to reliably depict a carnivore diet (Broadhurst et al., 2021; Bylemans et al., 2018).

False positives (erroneous detection of a prey species absent in the sample) and false negatives (missed detection of a species present in the sample) are errors that can cause under- or overestimation of a given prey species in the diet (Darling & Mahon, 2011; Lahoz-Monfort et al., 2016; MacKenzie et al., 2002). Two key estimates that measure the magnitude of such errors are specificity (true negative rate), which represents the capability to distinguish the target prey DNA from the background noise, and sensitivity (true positive rate), which is the ability to detect the target prey DNA when occurring in the sample (Darling & Mahon, 2011; Glas et al., 2003; Symondson, 2002). In the case of DNA-based methods, specificity can be improved in the method development stage by e.g. assessing primer specificity *in silico* using databases of barcode sequence and *in vitro* with high quality DNA reference samples including positive and negative controls (Di Bernardi et al., 2021; Ficetola et al., 2010; Shores et al., 2015). Specificity can also be assessed by sequencing PCR products to test for target amplification (King et al., 2008; Michelet et al., 2014), and maximized using a multitube approach with a confirmed detection in several sample replicates (Taberlet et al., 1996). Method sensitivity is unlikely to be complete due to the low amount and/or poor quality of

74 DNA resulting from prey remains after digestion (Pompanon et al., 2005; Symondson, 2002).
75 Sensitivity can be improved by a careful design of DNA primers, which are firmly dependent
76 on the richness of available reference sequence databases (Gibson et al., 2014). Attempts to
77 increase sensitivity have been done by pooling sequences of PCR replicates employing a
78 multiplexing strategy with multiple universal primer sets targeting the same taxonomic group
79 but amplifying several loci (Alberdi et al., 2018; De Barba et al., 2014), or multiple primer sets
80 amplifying the same locus (Gibson et al., 2014). The evaluation of the molecular methods'
81 sensitivity for diet studies can be dealt by concurrently analyzing samples using a
82 complementary method, such as traditional macroscopic identification (Deagle et al., 2009;
83 Nørgaard et al., 2021; Tollit et al., 2009). The limitation of comparative approaches is that
84 estimates of sensitivity can only be made if tested against an error-free method, a condition
85 which is difficult to achieve in practice. An alternative approach to measure detection
86 probability of target prey DNA from scats is to study captive animals fed with a known diet.
87 Although this approach may be time consuming and logistically complex, it provides
88 experimentally reliable estimates of method sensitivity and factors affecting it (Pompanon et
89 al., 2012; Schattanek et al., 2021).
90 Usually, there is a trade-off between sensitivity and specificity, where conservative
91 approaches that seek to minimize the probability of false positives also risk to increase the
92 probability of false negatives, thus reducing the method sensitivity (Clare et al., 2016; Darling
93 & Mahon, 2011; Ficetola et al., 2016). The definition of a binary detection using molecular
94 methods is often not straightforward and requires the use of cut-offs, which are generally fixed,
95 arbitrarily defined and conservatively chosen to avoid erroneous detections (Darling & Mahon,
96 2011; Divoll et al., 2018; Pompanon et al., 2012). However, the application of such fixed
97 thresholds can have the downsides of missing rare food components and resulting in low
98 taxonomic assignment success (Alberdi et al., 2018; Divoll et al., 2018). Recent guidelines

highlight the relevance of adjusting the detection procedure by basing thresholds on empirical data rather than relying on standard and fixed settings in order to attain detections better fitted to the actual sample and to each specific situation (Alberdi et al., 2018; De Barba et al., 2014). For instance, empirical cut-offs can be set based on baselines relative to reference negative control samples included in the same PCR run (Di Bernardi et al., 2021). This was done for a molecular method developed to simultaneously detect 18 target prey species in wolf (*Canis lupus*) scats through species-specific molecular markers on the mitochondrial *cyt b* gene using a high-throughput Nanofluidic array technology (Di Bernardi et al., 2021). Target detection was based on four markers per species, where thresholds were tailored to each marker in each run to obtain full specificity in relation to the non-specific reference tissues from the run. The definition of a binary detection was determined by the cut-off minimum number of markers with confirmed detection, and detection rate among 79 scats from wild wolves with unknown diet ranged between 44% and 92% depending on the chosen cut-off (Di Bernardi et al., 2021). Even though this pilot study indicated cut-off dependent variation in sensitivity, ultimately scats with known content are needed to evaluate the method sensitivity for the different target prey species and to find the optimal cut-off. In developing new DNA-based approaches, the process of setting cut-offs that weighs sensitivity against specificity is therefore a critical step and should ideally be systematically and empirically validated before their implementation in ecological studies (Alberdi et al., 2018; Chivers et al., 2014; Richardson et al., 2017). However, despite the growing number of molecular methods used to investigate diet in both vertebrates and invertebrates, there are relatively few studies that have experimentally validated the molecular method performance (bats: Galan et al., 2018; Schattanek et al., 2021; bears: De Barba et al., 2014; birds: Oehm et al., 2011; pinnipends: Deagle et al., 2010; Deagle & Tollit, 2007; cheetah: Thuo et al., 2019).

We conducted feeding trials with captive wolves provided with a known diet in order to validate the molecular method developed by Di Bernardi et al. (2021) to detect prey in wolf scats using nanofluidic array technology and species-specific markers. We quantitatively evaluated the method sensitivity by comparing true positives and false negatives estimates, and assessed how sensitivity was affected by the number of available markers, chosen threshold, prey species, and feeding regime (i.e., entire carcass or only meat).

2. Material and Methods

2.1 Feeding trials and sample collection

To obtain fecal samples from wolves fed with a known diet, we conducted a total of 11 feeding trials with captive wolves at a zoo (Järvzoo) in Sweden, during October–November 2019, February–March 2020, and April 2021. In total 12 wolves (2 adults, 3 subadults, 7 pups) were housed in an enclosure of 2500 m^2 with dirt bare sandy ground, with scots pine trees (*Pinus sylvestris*), and scattered bushes of birch (*Betula spp.*) and willow (*Salix spp.*). In each trial, the wolves were fed a single prey species, either moose (*Alces alces*), red deer (*Cervus elaphus*), fallow deer (*Dama dama*), roe deer (*Capreolus capreolus*), wild boar (*Sus scrofa*), or reindeer (*Rangifer rangifer*). These ungulates are available in the wolf breeding range in Scandinavia although moose and roe deer are the main prey (Sand et al., 2008, 2016; Zimmermann et al., 2015). We conducted two sets of trials, one in which wolves were fed only meat (hereafter meat trials) and another one in which wolves were offered whole, degutted carcasses (hereafter carcass trials). This was done for all species except roe deer, which was only offered in a carcass trial.

A fasting period preceded each feeding trial to make sure the digestive tract was empty (*cf.* Floyd et al., 1978; Van Dijk et al., 2007; Weaver, 1993). To avoid erroneous collection of scats from previous feeding events, all scats were removed 24 hours before scat collection from

148 a restricted area of the enclosure (1250 m^2 designated for scat collection). Additionally, the
149 whole enclosure was cleaned from scats, prey remains, and food caches, prior to the first trial
150 for the meat trials. Cleaning was also done between each carcass trial. This was done in order
151 to minimize the risk of wolves feeding on hidden bones from previous trials.

152 Each meat trial started with 2 days of fasting followed by 3 days of feeding. Starting
153 from the second day of feeding, we conducted 3 days of scat collection targeting a minimum
154 of 30 scats. To reduce the risk of DNA detection being affected by feeding on food caches
155 between carcass trials, the fasting period was extended to 3 days, but interrupted by one day
156 of feeding with dog pellet (based on chicken) between the 2nd and 3rd day of fasting (*cf.* Floyd
157 et al., 1978). Scat samples were collected, individually bagged in plastic bags, and immediately
158 frozen at – 18 ºC. From all collected scat samples, a subset (range 30-65) was randomly selected
159 within each trial and used for molecular analysis. A tissue sample was collected from each
160 ungulate carcass used in the trials and stored in an 95% ethanol solution until DNA extraction.
161

162 **2.2 DNA extraction and molecular analysis for prey detection**

163 DNA was extracted from wolf scat samples using QIAamp DNA Stool Kit (Qiagen) in
164 accordance with the manufacturer's instructions and from tissue samples using standard
165 phenol/chloroform-isoamylalcohol extraction. The tissue samples included i) fresh tissue
166 samples from the carcasses given to the wolves during the feeding trials, ii) reference tissues
167 for the 18 different target species, including moose, roe deer, red deer, fallow deer, wild boar,
168 reindeer, sheep (*Ovis orientalis*), cattle (*Bos taurus*), European badger (*Meles meles*), beaver
169 (*Castor fiber*), European hare (*Lepus europeus*), mountain hare (*Lepus timidus*), Western
170 capercaillie (*Tetrao urogallus*), black grouse (*Lyrurus tetrix*), bear (*Ursus arctos*), lynx (*Lynx*
171 *lynx*), wolverine (*Gulo gulo*) and red fox (*Vulpes vulpes*). The tissue samples were all collected
172 from animals that had died in Sweden and were provided by the Swedish Museum of Natural

History. The laboratory work was conducted following contamination prevention procedures, as the use of pipettes with filter tips and the physical separation of pre-PCR and post-PCR activities.

The prepared DNA from scat and tissue samples was amplified with a PCR in a 96.96 Dynamic Integrated Fluidic Circuit Array plate and visualized with fluorescence detection using the EP1^{TM} system (Fluidigm Inc.), according to the manufacturer's protocol. Each Fluidigm plate contained 96 molecular markers and 96 samples. The 96 markers consisted of a minimum of 4 species-specific markers for each of the 18 target species, built on species specific loci on the cytochrome b gene (Di Bernardi et al., 2021). All target species were provided with at least four markers, while five markers were available for red deer, roe deer, reindeer, sheep, cattle, and European badger, and six markers for black grouse. In each run, we analysed DNA extracted from scat samples and DNA from 18 reference tissue samples as positive control, one wolf tissue, and one water sample as negative control. All the tissue samples from the ungulate carcasses were analysed to make sure that the animal tissues correctly amplified with the specific markers. Following the protocol described in Di Bernardi et al. (2021), the reference tissue samples were used in each run to set non-arbitrary thresholds for each marker for binary prey detection to be target species specific in relation to the tissues from non-specific reference species. Finally, to get a binary detection for a prey species in each scat sample, a threshold defining the minimum number of species-specific markers with a positive call (indicating amplification) is required to determine the presence of DNA from a target species.

2.3 Method performance

To find out a proper threshold to determine the presence of a target species we quantitatively evaluated the performance of the molecular method in detecting the target prey species by

198 measuring sensitivity, that is the proportion of true positives on the total sum of true positives
199 and false negatives. For instance, a scat collected during a trial with moose that rendered moose
200 DNA was a true positive, whereas failure to detect moose DNA in that sample corresponded
201 to a false negative. Sensitivity was estimated separately for each threshold (minimum number
202 of species-specific markers with positive call), for the six target prey species, and separately
203 for the meat and carcass feeding trials. The 95% binomial confidence interval for sensitivity
204 was calculated with the R package *binom* (Dorai-Raj, 2014). We estimated the method
205 accuracy as the sum of true positives and true negatives on the total of samples. For instance,
206 a true positive for roe deer would be the correct detection of roe deer DNA from a scat collected
207 in a roe deer trial, while a true negative for roe deer would be the correct non-detection of roe
208 deer DNA from a scat collected in a moose trial.
209
210 **2.4 Statistical analyses**
211 We fitted generalized linear models (GLM) with binomial distribution to estimate the effect on
212 sensitivity (response variable) of the prey target species, the threshold used, and the feeding
213 regime (meat or carcass), included as categorical. The interaction between feeding regime and
214 species was included to investigate potential differences in sensitivity of the method when
215 providing the two distinct feeding regimes for the different target prey species. The interaction
216 between feeding regime and threshold was included to get unique coefficients for the carcass
217 feeding regime for the different species, as this is the main focus given its resemblance to the
218 feeding conditions in the wild. We used the sample-size corrected Akaike information criterion
219 (AICc) to compare the candidate models (Bartón, 2013).
220 We tested scenarios where a lower number of markers was available per species, to
221 assess the effect on sensitivity of the number of available tested markers. We used generalized
222 mixed models (GLMM) with binomial distribution, with available markers and thresholds

included as fixed factors, and target species as a random factor. All statistical analyses were conducted in R, using the package *stats* for GLMs and *lme4* for GLMMs (Bates et al., 2015; R Core Team, 2021).

3. Results

3.1 Feeding trials

During the 11 feeding trials (5 meat trials, 6 carcass trials), a total of 613 wolf scats were collected (32–113 scats per trial) (Appendix S1). Out of the subset of 381 samples analysed, 10 of these were invalidated through the detection protocol, as they were identified as outliers with regards to the signal of the passive reference dye ROX (see Di Bernardi et al. (2021)). A final sample size of 371 scats was included in the analyses (24–65 scats per trial). Across the carcass trials, specific amplification of scats occurred for all collection days of each trial. Few cases of non-specific amplifications across trials were observed, which were even fewer with higher threshold for binary detection (Figure 1). The method accuracy across the six ungulate species was on average 0.92 (range 0.85–0.98), i.e. 0.91 (range 0.89–0.97), 0.92 (range 0.90–0.98), 0.93 (range 0.89–0.98), and 0.91 (range 0.85–0.98) when setting thresholds of a minimum of 4, 3, 2 and 1 markers respectively.

The DNA extracted from the tissues of 38 ungulate carcasses offered to the wolves correctly amplified with the corresponding species-specific markers, indicating that all prey carcasses were specifically identified. The tissues from one fallow deer carcass and one red deer carcass amplified non-specifically, respectively for red deer with threshold up to 4 markers, and for wild boar with threshold up to 3 markers. Only one scat from the fallow deer trial and no scats from the red deer trial followed this non-specific amplification pattern, suggesting tissue sample contamination rather than false positive as potential explanation.

3.2 Method sensitivity

The method sensitivity in detecting prey in wolf scats was affected by the set threshold, the target prey species, the feeding regime, and the number of available markers (Figures 2, 3). The effect of feeding regime on sensitivity appeared different among species and thresholds, where the model with interaction between feeding regime and species' and between feeding regime and threshold featured lower AICc scores compared to alternative models (Appendix S2). Regarding the carcass trials, a difference in sensitivity was observed among the target species, with sensitivity being significantly higher than 0.5 for moose, reindeer, roe deer and wild boar, while red deer and fallow deer sensitivity were non-significantly different from 0.5 (Figure 2, Appendix S3). Higher thresholds of number of amplifying markers required to give a positive call resulted in lower sensitivity, ranging from an average of 0.76 (range 0.53–1.00) with 1 amplifying marker as threshold, to 0.5 (range 0.26–0.8) with 4 amplifying markers (Appendix S3). When considering the feeding regime, a higher sensitivity was found when providing the wolves with a whole carcass (average 0.64, range 0.26–1.00) compared to when feeding them with only meat (average 0.24, range 0.00–0.87) (Figure 2, Appendix S3). Moreover, a different sensitivity among carcass and meat feeding regimes was detected for the different species, with a higher sensitivity of carcass feeding regime for all species, except for red deer that showed the opposite pattern (Figure 2, Appendix S3). Additionally, as shown by the interaction between feeding regime and threshold, the reduction in sensitivity observed with increasing thresholds was more pronounced for the meat feeding regime compared to the carcass feeding regime (Appendix S3). When testing the effect of a lower number of available markers on the method sensitivity, a decrease was observed when reducing the number of markers available (Figure 3, Appendix S4).

4. Discussion

Feeding experiments with captive animals are useful for estimating the performance of molecular diagnostic methods and disentangling factors that can introduce biases in species detection. Here we expanded upon a previous study on a developed molecular method to detect prey DNA in wolf scats (Di Bernardi et al., 2021). We validated the method by conducting feeding experiments with controlled diet provided to captive wolves. In terms of detection performance, the molecular method by Di Bernardi et al. (2021) evaluated and maximized specificity in the development and optimization stages using specific and non-specific reference tissue samples and empirical thresholds tailored for each species-specific molecular marker, to minimize false positives, i.e. non-target species calls. In this study, we found that the method sensitivity for scats from wolves in captivity depended on the species they consumed. A species-specific sensitivity was observed, with a variation between 0.45 and 0.95 among the six ungulates given a chosen threshold of 2 amplifying markers and a carcass feeding regime. The cause behind these differences in detection probability between target prey species is still unclear. The DNA extracted from the tissue samples of the carcasses consistently amplified with target species specific markers, thus making it unlikely that individual variation at primer annealing (e.g. due to intraspecific sequence variation at primer sites) would explain the differences in detection probability. Confounding factors, which might have differentially affected sensitivity for the target prey species, could for example be differences in prey digestibility (Deagle & Tollit, 2007) or environmental or technical factors in the trials and in the processing of samples (Alberdi et al., 2019; Oehm et al., 2011).

The effective application of species detection data to ecological and management frameworks relies on minimizing and accounting for detection errors that can otherwise generate severe biases in the ecological inferences (Yoccoz et al., 2001). Along the findings in this study, three other studies analyzing sensitivity have also found tendencies for DNA detection probability to vary among target prey species (Broadhurst et al., 2021; Schattanek et

298 al., 2021; Thuo et al., 2019). Although biased species detection may have important
299 implications for the ecological interpretation of diet analyses, it is largely overlooked and rarely
300 accounted for (Alberdi et al., 2019). A procedure that acknowledges the existence of such errors
301 would account for the level of uncertainty gained from experimental studies with true presence
302 to make reliable ecological inferences and thereby get closer to a correct description of species
303 composition (Lahoz-Monfort et al., 2016; Thomas et al., 2014, 2016; Valentini et al., 2016). In
304 some species detection studies, where empirical data on sensitivity was not available a level of
305 uncertainty has been included by using a comparative approach with other methods such as
306 camera traps (Abrams et al., 2019; Sales et al., 2020).

307 When using diagnostic molecular methods, the trade-off between false negatives and
308 false positives usually needs to be balanced depending on the research question and species
309 detection studies. Diet analyses generally prioritize specificity using conservative cut-offs that
310 may result in the loss of sensitivity, i.e. failure to detect a prey species that was actually
311 consumed (Darling & Mahon, 2011; Divoll et al., 2018). The approach used by Di Bernardi et
312 al. (2021) of utilizing multiple species-specific molecular markers is in line with previous
313 attempts to increase sensitivity through additively pooling results of multiplexing primers
314 (Alberdi et al., 2018; De Barba et al., 2014). By targeting several loci with different markers
315 for the same species, the method aims at increasing taxonomic coverage within each species
316 (Di Bernardi et al., 2021). In this study we observed how the use of several markers, instead of
317 only one per species, resulted in higher sensitivity for all target species (Figure 3). However,
318 pooling results from multiple markers can reduce the number of false negatives (Gibson et al.,
319 2014), but it may also increase the risk of introducing false positives (Alberdi et al., 2018). We
320 saw this pattern when setting too low thresholds of minimum number of markers for confirmed
321 detection (Figure 1). Through the analysis of false negatives with empirical data from feeding
322 experiments, we can therefore include sensitivity in our evaluation of the optimal threshold to

balance the trade-off between sensitivity and specificity for the detection method by Di Bernardi et al. (2021). On one side the cut-offs based on reference samples tailored for each marker maximize specificity (Di Bernardi et al., 2021). On the other side, we recommend the use of a low threshold (intended as the number of amplifying markers required to confirm detection) to concurrently maximize sensitivity, suggesting the use of 2 markers as threshold. Despite the development of markers as specific as possible and the use of tailored cut-offs for each marker maximizing specificity, occasional non-specific amplifications can occur in the developed markers (Di Bernardi et al., 2021) and we therefore caution against the use of only one marker as threshold.

The identification of non-specific detections can occur from false positives but also potentially from true positives deriving from cross-trial contamination, i.e. through the true occurrence of traces of DNA of a non-target ungulate from a previous trial that were retained in the wolf's intestine or were ingested through feeding on non-detected cashed food remains. However, we see no indication of non-specific calls from pre-fed species outnumbering those of post-fed species, which in such a case would indicate cross-trial contamination. Digestion degrades DNA and differences in digestibility among food items can produce a bias in the DNA presence and hence in its detection from scats (Dahl et al., 2022; Symondson, 2002; Thomas et al., 2014; Tollit et al., 2009). A possible explanation for the different sensitivity observed in this study for the two feeding regimes could be a higher amount of indigestible prey remains left in the scat when wolves fed on carcasses compared to only meat. Further investigation would be needed to verify this hypothesis. We however refer to the feeding regime with the whole carcass as it resembles the actual conditions of wolves feeding in the wild. We find it relevant to report the low sensitivity of the molecular method when feeding wolves with only meat as this could occur in some scats from the wild, and possibly reside among the causes of a not full sensitivity with a carcass feeding regime as well.

Our study adds to the small body of literature validating molecular methods for diet analysis with experimental feeding trials, a field that needs to receive more attention in order to accurately exploit the rapidly developing analytical tools to investigate diet from DNA (Alberdi et al., 2019; Dahl et al., 2022; Nielsen et al., 2018). A differential sensitivity for the target ungulate prey species was identified in this study, and the acknowledgement and consideration of such bias aids to correctly interpret results and draw appropriate conclusions when applying such molecular detection method into management and ecological frameworks.

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6. Data Accessibility

Compiled data generated during the feeding experiments will be publicly archived in Dryad upon acceptance.

7. Author Contribution

The study was conceived and designed by C.D.B., M. Å., C.W., H.S. with the contribution of P.C. and L.B. C.D.B. led the feeding experiments, conducted the molecular analyses, and

analysed the data. C.D.B. wrote the original draft, and all co-authors contributed to improve the manuscript and approved the final submitted version.

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Figures

Figure 1. Specific and non-specific ungulate species detection for each collection date of carcass feeding experiments with captive wolves in Sweden, 2019-2021. Detection is measured as proportion of scats giving a positive call (in percentage). The numbers noted above indicate sample size. For each target species, a minimum of 1 marker, 2 markers, 3 markers, 4 markers giving a positive call were required to confirm a final detection.

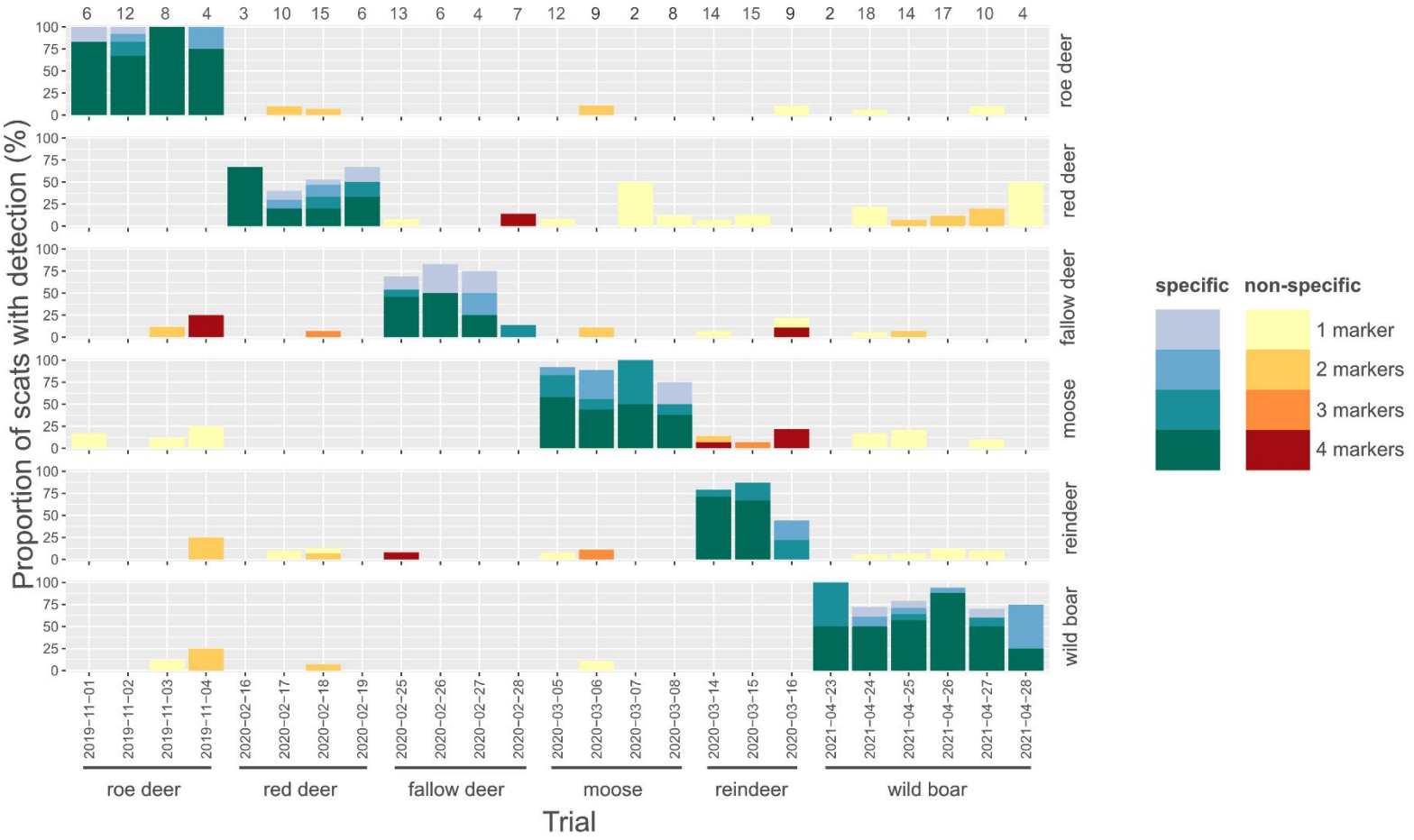


Figure 2. Sensitivity of the molecular method, tested with feeding experiments with captive wolves in Sweden during 2019-2021, estimated from the data for the trials with carcass and meat feeding regime, separated for the four thresholds (minimum of 1, 2, 3, 4 markers with a positive call to give a final call), for the six ungulate target prey species. Error bars represent 95% binomial confidence intervals.

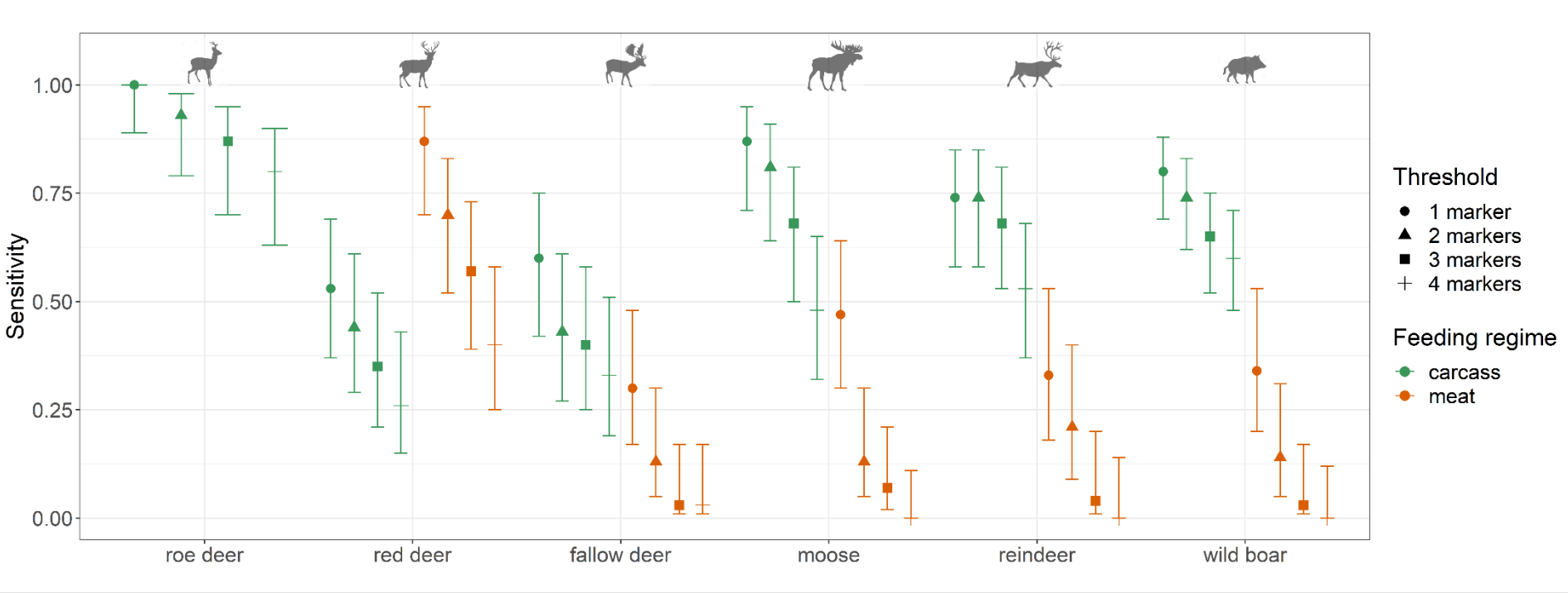
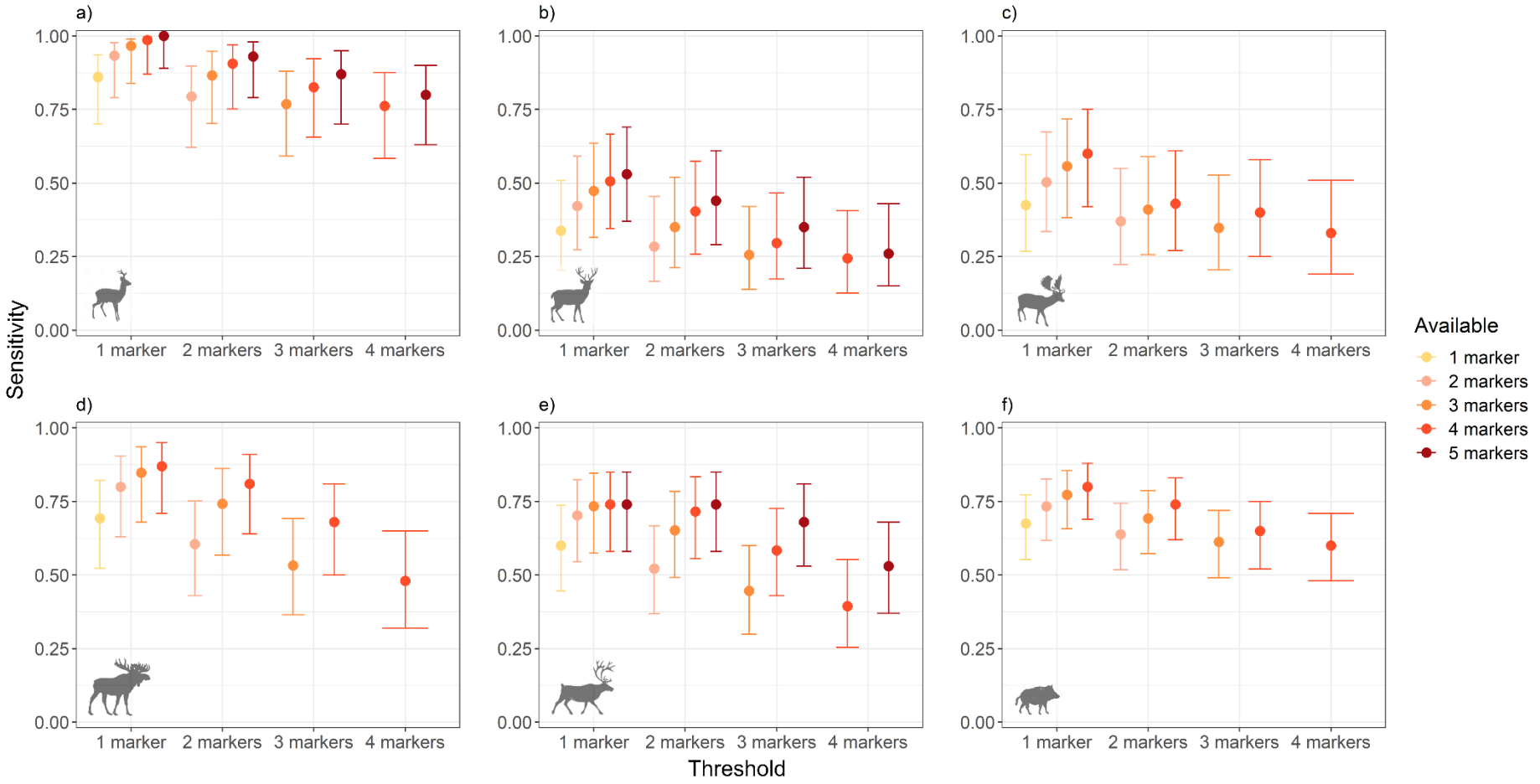


Figure 3. Sensitivity of the molecular method estimated from the data, tested with feeding experiments with captive wolves in Sweden during 2019-2021. Sensitivity values are shown for each combination of threshold (number of markers with a positive call to give a final call) and number of available markers. The different scenarios of number of available markers show a reduction in sensitivity when reducing the number of available markers. The sensitivity is presented for the carcass trials of the six target ungulate species, a) roe deer, b) red deer, c) fallow deer, d) moose, e) reindeer, f) wild boar. Error bars represent 95% binomial confidence intervals.



Supplemental Information for CHAPTER 2:

Experimental feeding validates nanofluidic array technology for DNA detection of ungulate prey in wolf scats

Di Bernardi Cecilia, Camilla Wikenros, Paolo Ciucci, Luigi Boitani, Håkan Sand, Mikael Åkesson

Corresponding author: Cecilia Di Bernardi, cecilia.dibernardi@uniroma1.it

Appendix S1. Table of feeding trials conducted with captive wolves in Sweden during 2019-2021, number of ungulate carcasses provided to the wolves, scat samples collected and analysed during the feeding trials, and final sample size after sample invalidation through the detection protocol (Di Bernardi et al., 2021). The order of trials is displayed as the temporal order of execution.

Trial	Feeding regime	Prey species	Carcasses provided	Collected scats	Analysed scats	Sample size
1	meat	reindeer	2	52	30	24
2		wild boar	5	37	30	29
3		red deer	3	49	31	30
4		fallow deer	3	65	31	30
5		moose	1	65	30	30
6	carcass	roe deer	10	93	30	30
7		red deer	1	35	35	34
8		fallow deer	3	32	30	30
9		moose	2	34	31	31
10		reindeer	3	38	38	38
11		wild boar	5	113	65	65
			38	**613**	**381**	**371**

Appendix S2. Alternative candidate generalized linear models (GLM with binomial distribution) on sensitivity of the molecular method tested with the feeding experiments conducted with captive wolves in Sweden during 2019-2021. Models are ranked by lowest AICc, number of parameters, difference in AICc scores (Delta AICc), and model weights.

Model	**n_{par}**	**ΔAICc**	**weight**
feeding regime × species + feeding regime × threshold	17	0.000	0.924
feeding regime × species + threshold	14	4.989	0.076
feeding regime + species + threshold	10	135.222	0.000
feeding regime × threshold + species	13	137.284	0.000

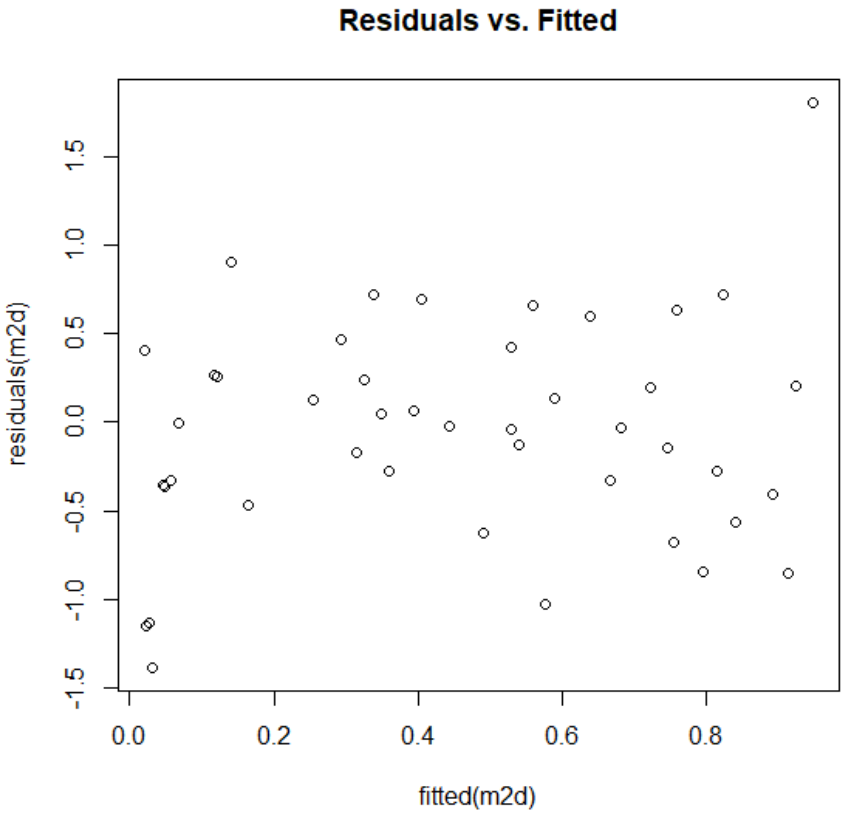
Appendix S3. Parameter estimates for generalized linear model (with binomial distribution) on sensitivity of the molecular method in relation to target ungulate species, threshold, feeding regime, and the interactions between feeding regime and species and between feeding regime and threshold using data from feeding experiments conducted with captive wolves in Sweden during 2019-2021. For target species the estimates are unique intercepts for each category, while the reference value is carcass as feeding regime, and one marker as threshold.

Parameter	**Estimate**	**SE**	**p**
species_moose	1.5395	0.2518	<0.0001
species_roe deer	2.8826	0.3457	<0.0001
species_red deer	0.1628	0.2241	0.4677
species_fallow deer	0.3553	0.2328	0.1269
species_reindeer	1.3505	0.2307	<0.0001
species_wild boar	1.4719	0.2034	<0.0001
feeding regime_meat	-1.9291	0.3891	<0.0001
threshold_2	-0.3922	0.2224	0.0778
threshold_3	-0.7847	0.2183	0.0003
threshold_4	-1.2329	0.2175	<0.0001
species_roe deer: feeding regime_meat	NA	NA	NA
species_red deer: feeding regime_meat	4.1245	0.4578	<0.0001
species_fallow deer: feeding regime_meat	0.7946	0.4840	0.1006
species_reindeer: feeding regime_meat	0.0040	0.4896	0.9935
species_wild boar: feeding regime_meat	-0.2777	0.4671	0.5521
feeding regime_meat:threshold_2	-0.8445	0.3774	0.0252
feeding regime_meat:threshold_3	-1.4602	0.4231	0.0006
feeding regime_meat:threshold_4	-1.8009	0.4714	0.0001

Appendix S4. Parameter estimates for generalized linear mixed model (with binomial distribution) on sensitivity of the molecular method in relation to available markers and threshold, while species is included as random factor. Restricted to carcass trials of the feeding experiment conducted with captive wolves in Sweden during 2019-2021. The intercept is one marker both for threshold and available markers.

Parameter	Estimate	SE	p
Intercept	0.46832	0.34523	0.1750
threshold_2	-0.58139	0.05101	<0.0001
threshold_3	-1.11328	0.06409	<0.0001
threshold_4	-1.54551	0.10189	<0.0001
available_2	0.35398	0.08248	<0.0001
available_3	0.65346	0.08404	<0.0001
available_4	0.91542	0.09418	<0.0001
available_5	1.14679	0.14414	<0.0001

Appendix S5. Plot of residuals for the top ranked model generalized linear model (with binomial distribution) on sensitivity of the molecular method in relation to target ungulate species, threshold, feeding regime, and the interactions between feeding regime and species and between feeding regime and threshold using data from feeding experiments conducted with captive wolves in Sweden during 2019-2021.



CHAPTER 3

Predator individual traits and prey abundance affect wolf predation in a multi-ungulate system

Cecilia Di Bernardi [1, 2], Mikael Åkesson[2], Håkan Sand[2], Malin Aronsson[2], Paolo Ciucci[1], Luigi Boitani[1], Camilla Wikenros[2]

Affiliations:

[1] Department of Biology and Biotechnologies "Charles Darwin", University of Rome La Sapienza, Viale dell'Università 32, 00185, Rome, Italy

[2] Grimsö Wildlife Research Station, Department of Ecology, Swedish University of Agricultural Sciences, 739 93 Riddarhyttan, Sweden

Corresponding author: Cecilia Di Bernardi, cecilia.dibernardi@uniroma1.it

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Abstract

Among the physical, behavioural, and environmental drivers of carnivore predation patterns, those associated to individual characteristics of predators may be the most difficult to study. However, the growing field of prey DNA detection in predator scats has the potential to increase the feasibility of large-scale diet analyses and, in association with genotyping, investigate the effect of individual traits on feeding ecology.

By means of species-specific molecular markers for prey DNA detection, we analysed 2125 scats of wolves (*Canis lupus*) from Sweden to describe the use of 17 target prey and further examine the drivers of moose (*Alces alces*) and roe deer (*Capreolus capreolus*) consumption

at the landscape scale. Our results showed wolf dietary responses to changes in ungulate prey species abundances, with the use of both moose and roe deer being positively affected by their abundance and negatively affected by increasing abundance of alternative ungulates. By associating scat samples with individual wolves' genotypes, we were able to reveal that their social status affected patterns of use of both moose and roe deer, and that roe deer consumption was also affected by sex and the pedigree based inbreeding coefficient of individual wolves. The gained knowledge on prey use of an expanding wolf population contributes to our understanding of wolves' impact on wild ungulate communities and can inform the adaptive management and human harvest of these species. Finally, our results highlighted the relevance of individual predator traits when investigating predation patterns.

Key words: *Canis lupus*, diet analysis, DNA, inbreeding, pedigree, prey use, sex, social status

1. Introduction

Predation patterns by carnivores are influenced by a combination of physical, behavioural and environmental factors related to both the predator and prey species (Becker et al., 2008; Kruuk, 1986; Mech & Peterson, 2003). The prey profitability (net energy gain/handling time, Stephens & Krebs, 1986) drives carnivores' prey selection patterns and, in turn, depends on the prey abundance in the landscape, which impacts both the searching time and encounter rate (Cooper et al., 2007; Paquet & Carbyn, 2003). Prey profitability is also determined by prey vulnerability, i.e. susceptibility to predation, meaning that some prey require higher energy expenditure and are associated with higher risk of hunting failure or injury than other prey (Bergman et al., 2006). Interspecific variation in prey vulnerability can be explained by physical and behavioural characteristics such as body size and defensive behaviour, which can

50 alter the prey accessibility to predators regardless of their abundance (Garrott et al., 2007; Lind
51 & Cresswell, 2005; Tallian et al., 2017).

52 Wolves (*Canis lupus*) are generalist-opportunistic predators (Becker et al., 2008) and,
53 as most canids, foremost hunt prey by coursing and chasing vulnerable animals (Mech &
54 Peterson, 2003; Smith et al., 2004). Across their range, wolves mostly consume wild ungulates
55 and in minor part feed on smaller prey species and plants (Mysłajek et al., 2021; Nowak et al.,
56 2011). Wolves can also consume livestock or other anthropogenic foods (Vos, 2000), mostly
57 in areas characterized by high human impact and low availability of wild ungulates (Newsome
58 et al., 2016; Zlatanova et al., 2014). Across a broad range of documented multi-prey systems
59 in North America, wolves often show a preference for elk (*Cervus canadensis*), a relatively
60 profitable prey with a large body size but requiring shorter chase distances and potentially
61 lower risk of injury compared to other larger prey such as moose (*Alces alces*) (Hebblewhite
62 et al., 2003; Orning et al., 2021; Weaver, 1994). In European wolves, patterns of prey selection
63 are characterized by a latitudinal variation in relation to the relative abundance and
64 vulnerability of wild ungulates (Zlatanova et al., 2014). Large-sized ungulates such as moose,
65 and to a much less extent wild forest reindeer (*Rangifer tarandus)*, are the main wild prey in
66 northern Europe (Kojola et al., 2004; Sand et al., 2008). Red deer (*Cervus elaphus*) is the most
67 frequent prey in central-eastern Europe (Jędrzejewski et al., 2002; Okarma, 1995), while roe
68 deer (*Capreolus capreolus*) and wild boar (*Sus scrofa*) are the main wild prey species in
69 southern Europe (Newsome et al., 2016; Zlatanova et al., 2014).

70 Individual traits of predators, such as social status, sex and body condition of wolves
71 can also affect predation patterns (Imbert et al., 2016; MacNulty et al., 2012; MacNulty, Smith,
72 Vucetich, et al., 2009; Sand et al., 2006; Zimmermann et al., 2015). The social status of wolves
73 has been found to explain variation in the relative consumption of wild and domestic prey, with
74 dispersing solitary wolves showing a greater use of livestock and lower use of wild ungulates

compared to packs (Imbert et al., 2016). This may indicate that dispersing wolves were less efficient in killing wild prey and thus preferred less defensive and more vulnerable livestock (Imbert et al., 2016). Such patterns could be due to lack of local knowledge, experience, and help from partners of young dispersers compared to wolves living in packs (Imbert et al., 2016). The body size and condition of wolves may also be important for prey selection, such as lowered physical conditions of older wolves affecting predation efficacy (MacNulty, Smith, Vucetich, et al., 2009). Additionally, adult males are 25-30% larger than female wolves and may therefore be more efficient in killing larger prey (Sand et al., 2006). The age of the adult male leading the pack showed a greater positive effect on hunting success than the age of the adult female, possibly related to the greater body size (Sand et al., 2006). Another factor potentially affecting hunting performance may be inbreeding, which has shown to be associated with negative effects on body size, body condition and fitness in several wolf populations (Åkesson et al., 2016; Fredrickson & Hedrick, 2002; Keller & Waller, 2002; Liberg et al., 2005; Räikkönen et al., 2013).

In general, data on individual characteristics of predators are difficult to retrieve and even more difficult to link to individual feeding behaviour. Wolf predation studies done on GPS-collared individuals provide detailed information on feeding behaviour but are expensive and time-consuming (Sand et al., 2005). Additionally, studies on the association between hunting behaviour and intrinsic factors conducted by direct observation or cluster checks are often limited by low numbers of target individuals (but see MacNulty et al., 2009, 2012; Sand et al., 2006).

Another common and well-established methodology to characterize the diet of predators is noninvasive scat analysis (Klare et al., 2011). Through the genetic identification of wolf individuals by genotyping, diet studies based on scat analysis can aid our understanding of how individual traits are linked to predation patterns (Monterroso et al., 2019). With higher

100 taxonomic resolution and detection rate of prey, the growing field of fecal DNA-based diet
101 analysis has become a viable alternative to traditional scat analysis (Mumma et al., 2016;
102 Valentini et al., 2009). Prey DNA detection in predator scats has therefore the potential to
103 increase the feasibility of large-scale diet analyses and investigate, in association with
104 genotyping, the effect of predator-related factors on feeding ecology.

105 Since the recolonization of Scandinavia by wolves in the early 1980's, the wolf
106 distribution has mainly covered areas where moose has been the main prey, and with per capita
107 kill rates positively related to local moose density (Sand et al., 2005, 2008; Zimmermann et al.,
108 2015). Wolf expansion into areas with higher relative densities of the smaller prey roe deer
109 showed that the proportion of roe deer in wolf kills was only weakly negatively related to
110 moose density at the intra-territorial scale, while it was positively related to roe deer density
111 both at the population and intra-territorial scale (Sand et al., 2016). Thus, at low densities such
112 smaller prey might not be profitable to wolves, while with increasing roe deer densities wolves
113 were more likely to kill roe deer compared to moose. Such patterns could simply be explained
114 by the variation in encounter rate of roe deer, but also by indirect density-related variation in
115 prey vulnerability, such as the limitation of food resources for roe deer (Kjellander et al., 2004;
116 Sand et al., 2016; Vincent et al., 1995). More recently the wolf population in Scandinavia has
117 expanded further south, into areas where alternative wild ungulate species (i.e., red deer, fallow
118 deer (*Dama dama*) and wild boar) are available at higher densities in addition to moose and
119 roe deer (Rodríguez-Recio et al., 2022). With a broader community of ungulate species
120 available to wolves, the dynamics of wolves and ungulates become more complex and difficult
121 to predict, with potential implications for management and conservation of ungulate
122 populations (Rodríguez-Recio et al., 2022; Sand et al., 2016).

123 In this study we used a molecular method to detect prey DNA in wolf scats (Di Bernardi
124 et al., 2021) in concert with the wolf population genetic monitoring (Åkesson et al., 2016,

2022). This enabled us to investigate wolf feeding ecology in association with predator intrinsic factors for a high number of wolf individuals and along a spatial gradient of varying ungulate abundances. In total, we analysed 2125 wolf scats collected in Sweden using multiple species-specific molecular markers for prey DNA detection (Di Bernardi et al., 2021). Our objectives were: i) to describe prey use by Scandinavian wolves through the proportional occurrence of 17 target prey, ii) to examine if and to what extent feeding patterns on moose and roe deer were influenced (a) by the social status, sex, and inbreeding coefficient of individual wolves, and (b) by the relative abundance of moose, roe deer, and alternative wild ungulate prey species (red deer, fallow deer, wild boar). We predicted lower use of moose and higher use of roe deer for individual wolves that are expectedly less capable of killing larger prey, i.e., solitary wolves compared to wolves in pairs and packs (P1), solitary females compared to solitary larger bodied males (P2), and highly inbred individuals (P3). We also predicted that increasing abundance of moose would be related to higher use of moose and lower use of roe deer (P4), while increasing abundance of roe deer would be related to lower use of moose and higher use of roe deer (P5). Finally, we predicted that an increasing abundance of alternative wild ungulate prey species would be reflected by an overall lower use of both moose and roe deer by wolves (P6).

2. Material and methods

2.1. Study system

The wolf population in Sweden and Norway, hereafter referred to as the Scandinavian wolf population, was founded in 1983 after being declared functionally extinct in 1966 (Wabakken et al., 2001). In 1983, two wolves from the Finnish–Russian population reproduced in a cross-border territory of Sweden and Norway, and thereby founded the current Scandinavian population (Liberg et al., 2005; Wabakken et al., 2001). The founding pair bred for 3 years and from 1987 to 1990 the population was maintained by incestuous reproductions resulting in

150 inbreeding (Liberg et al., 2005). Following an immigration event in 1991, the wolf population
151 started to increase in numbers and expanded its breeding range, currently (winter 2021–2022,
152 hereafter referred to as 2021) reaching 69.5 wolf packs or pairs in Sweden, and 13.5 in Norway
153 (Wabakken et al., 2022). The inbreeding coefficient dropped after the immigration event but
154 inbreeding depression was documented for this population, with reduced litter size for more
155 inbred individuals and an increasing occurrence of congenital defects (Åkesson et al., 2016;
156 Liberg et al., 2005; Räikkönen et al., 2013). Since 1998, the monitoring of the Scandinavian
157 wolf population has been conducted each winter (October to March) with the aim to estimate
158 the number of pairs, packs, and reproduction events (Åkesson et al., 2022). Monitoring
159 techniques comprise a combination of snow tracking, identification of individual wolves from
160 DNA samples collected both non-invasively (scats, urine), invasively (hair, blood or muscle
161 tissue from captured and dead wolves), and GPS-tracking of radio collared individuals
162 (Åkesson et al., 2022). Additionally, a near complete pedigree of the population has been
163 reconstructed based on the genetic identification and parental assignment of reproductive pairs
164 in the population (Åkesson et al., 2016). This allowed us also to estimate the pedigree-based
165 inbreeding coefficient (F) for almost every wolf born in the Scandinavian wolf population
166 (Åkesson et al., 2016, 2022; Liberg et al., 2005).

167 The study included scat samples collected from wolves across Sweden (Figure 1),
168 where most wolf packs (ca. 80 %) were located (Wabakken et al., 2022). The study area was
169 mostly characterized by boreal forest including Norway spruce (*Picea abies*), Scots pine (*Pinus*
170 *sylvestris*) and some deciduous species such as birch (*Betula spp.*), aspen (*Populus tremula*)
171 and willow (*Salix spp.*). Forests were generally managed by clear-cutting regeneration resulting
172 in a mosaic of conifer stands in different age classes as well as an extensive network of forest
173 roads. The climate was continental with snow covering the ground mainly during December to
174 March (Swedish Meteorological and Hydrological Institute, www.smhi.se). Human density

was higher in urban areas in south-central Sweden (25 inhabitant/km^2) compared to rural areas (under 1 inhabitant/km^2) (2019, www.scb.se). Across the latitudinal range, winter temperatures decreased towards the north (https://www.smhi.se/data/meteorologi/kartor/), while human, road densities and proportion of agricultural land increased toward the south. In addition to large wild ungulates (see below), smaller prey species for wolves in the study area include beaver (*Castor fiber*), badger (*Meles meles*), mountain and European hares (*Lepus timidus, Lepus europeus*), capercaillie (*Tetrao urogallus*), and black grouse (*Lyrurus tetrix*) (Sand et al., 2008). Other large and medium-sized carnivores partly or entirely co-occur with wolves in the study area, including brown bear (*Ursus arctos*), Eurasian lynx (*Lynx lynx*), wolverine (*Gulo gulo*), and red fox (*Vulpes vulpes*) (Chapron et al., 2014, www.artfakta.se). Domestic species such as sheep (*Ovis aries*) and cattle (*Bos taurus*) are not free ranging and occur across Sweden with higher densities southwards (Dalerum et al., 2020; Linkowski et al., 2017). Semi-domestic reindeer are free-ranging in the reindeer husbandry area (i.e., a 230 000 km^2 area covering the northern half of Sweden, designated for indigenous Sámi reindeer-herding), where wolves are actively prevented from settling as residents (Eriksson & Dalerum, 2018).

2.2. Wild ungulate prey species distribution

Moose shows a relatively even distribution and density across the range of the Scandinavian wolf population (range 0.7-3.3 moose/km^2) (Zimmermann, 2014) and counts 265,000 individuals as total winter population in Sweden (Jensen et al., 2020). Roe deer is present in some of the northern parts of Sweden at low densities, while it increases in densities towards the south, with a total estimated population of 650,000 individuals (Linnell et al., 2020). Other wild ungulates such as red deer, fallow deer, and wild boar occur in the southern parts of Sweden where they show a steady increase in density and range (Rodríguez-Recio et al., 2022; Sand et al., 2016). After a recovery from a small remnant population in southern Sweden, red

deer counted 26,000 individuals in 2016 (Swedish Hunter's Association., 2017). The population growth of fallow deer has shown a slow positive pattern after its introduction in Sweden during the 16th century, counting 126,000 individuals in 2016 (Menichetti et al., 2019; Swedish Hunter's Association, 2017). The wild boar has been recolonizing south-eastern parts of Sweden after accidental reintroduction in the 1970s, and in 2010 the total estimated population was 150,000 individuals (Jansson et al., 2010).

2.3. Wolf scats collection and selection

The collection of scat samples was part of the wolf monitoring in Scandinavia to genetically identify individuals (Åkesson et al., 2022; Liberg et al., 2012; Wabakken et al., 2001). Scats were collected by trained field personnel during snow tracking or on bare ground (Åkesson et al., 2022). The field personnel searched for wolf tracks outside the known wolf breeding area often following wolf observation reports by the public (Åkesson et al., 2022). The majority of scat samples were collected in the wolf breeding area in south-central Sweden where most of the territories occurred, while a smaller portion of samples was collected from the most southern and northern areas with a lower wolf occurrence (Åkesson et al., 2022).

The selection of scat samples (2006-2019) was based on the following criteria: 1) all samples belonged to individually identified wolves, using methods described in Åkesson et al (2016); 2) those from southern and northern Sweden (i.e., corresponding to southern and northern large carnivore management areas, Figure 1) were prioritized given the lower occurrence of wolves and relevance in terms of multi-prey system; 3) samples were restricted to the monitoring season (October–March), a period when sample DNA quality expectedly was higher due to colder temperatures and snow on the ground; 4) samples from the same wolf had been collected at least seven days apart, considering an average kill rate for adult wolves in our study area was one prey every 4.5 days (Sand et al., 2005, 2012), in order to ensure that scats

from the same individual contained prey from different kills; and 5) samples from pups and non-dispersing yearlings were excluded as they were more likely feeding on prey killed by their parents (MacNulty et al., 2012; Zimmermann et al., 2015). We therefore excluded samples of non-territorial wolves located less than 23 km (i.e., maximum wolf territory size radius; Mattisson et al., 2013)) from the centroid of the territory where at least one parent was still present.

2.4. DNA-analysis for prey detection

DNA from scats was extracted using either the QIAamp DNA Stool Kit (Qiagen), the ISOLATE Faecal DNA Kit (Bioline, London, UK), or the PowerMaxTM Soil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, California, USA) in accordance with the manufacturer instructions, and subsequently stored in tubes in -80 °C freezers (Åkesson et al., 2016). From the extracted DNA, prey species detection was conducted using high-throughput Nanofluidic array technology with multiple species-specific markers for 18 target prey species (Di Bernardi et al., 2021). Due to hybridization among the two species, European hare and mountain hare were not always distinctly separated by the method and were therefore merged as hares, summing up to a total of 17 target prey detectable with the molecular method (Di Bernardi et al., 2021). Following the protocol to get a binary detection for a prey species in each scat sample, reference tissue samples were used as negative control in each run to set non-arbitrary cut-offs for each marker, based on empirical data. Finally, from a set of 4 species-specific markers for each species, a threshold defining the minimum number of markers with a positive call (indicating amplification) was required to determine the presence of DNA from a target prey. To prioritize the method sensitivity but simultaneously maximize specificity, a threshold of two markers was used based on an experimental method validation with feeding trials with captive wolves (Di Bernardi et al., under revision).

2.5. Covariates

2.5.1. Wolf individual traits

The *social status* (hereafter referred to as status) of individual wolves in this study was assigned based on the yearly monitoring of the Scandinavian wolf population (Åkesson et al., 2022). The monitoring prioritized the location of territorial pairs (2 individuals, a scent-marking male and female) and packs (≥3 individuals including at least one scent-marking adult), and the genetic identification of such scent-marking territory holding individuals. Observations of wolves outside known territories were investigated but not prioritized since 2014, as snow tracks with < 2 wolves were not regularly scrutinized (Åkesson et al., 2022). We assigned each scat with an individual status, including i) wolf in a *pack*, ii) wolf in a *pair*, corresponding to the status in the monitoring season when the scat was collected, and additionally iii) *solitary* wolf, including both dispersing and resident individuals, two groups that in many cases were not possible to distinguish. The *sex* of individual wolves was determined either from morphological characters of dead or captured individuals, or from DNA-analysis of scats (Seddon, 2005). The individual *inbreeding coefficient* (F; hereafter referred to as inbreeding) of wolves was calculated using CFC v1.0 (Sargolzaei et al., 2005) based on the reconstructed pedigree of the population (Åkesson et al., 2016).

2.5.2. Wild ungulate abundance index

As variation in harvest of ungulates among areas has previously been shown to be highly correlated to the population density of moose and roe deer in Scandinavia (Mattisson et al., 2013; Ueno et al., 2014), we used the yearly hunting bag records (individuals harvested/10 km^2) as an indirect index for the abundance of the five ungulate species. Data on relative abundance was generated for moose at the level of moose management unit and was available

from 2012 onward from the County Administrative Boards (www.algdata.se). For roe deer, red deer, fallow deer, and wild boar, a smaller management unit (hunting districts) was used and available from 2008 onward from The Swedish Association for Hunting and Wildlife Management (www.jagareforbundet.se). We assigned scats to the different management units using a buffer of 1 km radius on the sample and if overlapping with two units then a weighted average was measured, proportionally to the coverage of the respective units in the buffer. The extraction was conducted in R (R Core Team, 2021) and QGIS 3.16.3 (https://qgis.org/en/site/).

2.6. Data analysis

In a first descriptive section, the proportional occurrence (%) of a given target prey *i* was measured as the number of its occurrences N_i on the total number of occurrences N. For each of the 17 target prey, the proportional occurrence was calculated on the total number of occurrences instead of the total scats, as multiple species sometimes occurred in the same scat.

In a second analytical section, we examined to what extent the relative use by wolves of moose and roe deer was influenced by wolf individual characteristics and prey abundances. This was done by modelling the occurrence (presence/absence) of the target prey DNA in wolf scat samples as a Bernoulli distribution in a Bayesian framework, with the probability of occurrence (p) as the distribution parameter (i.e. $occurrence_i$ ~Bernoulli(p_i)). The probability of occurrence was the response variable in the following deterministic model:

$$\text{logit}(p_i) = a + \beta x_i + \alpha_{unit(i)} + \alpha_{year(i)} \quad (1)$$

where a is the intercept, β is a vector of regression coefficients linked to the explanatory variables, α_{unit} and α_{year} are the group level random effects for social unit ID (either as ID for the adult wolves in a pack or pair, or as ID for solitary wolves) and year. The social unit ID was included to account for multiple scats belonging to individuals in the same pair or pack or to scats belonging to the same solitary wolf, while the year was included to account for different

sample sizes between years (Appendix S1) and potential interannual variation across years. The group level effects were distributed according to a normal distribution with mean zero and standard deviations σ_{unit} and σ_{year}. The explanatory variables included were status (pack, pair, solitary), sex (female, male) as categorical, and inbreeding, moose abundance, roe deer abundance, and alternative ungulates abundance as continuous. The alternative ungulates abundance was grouped together as the sum of the abundance of red deer, fallow deer and wild boar, due to the limited number of scats collected in areas that overlapped with the distribution of each species separately. Furthermore, the following interactions were included: status × sex, status × inbreeding, status × moose abundance, and status × roe deer abundance to assess difference in probability of occurrence for wolves in pack, pair, or solitary in response to sex (P2), inbreeding (P3), moose abundance (P4) and roe deer abundance (P5). Pearson correlation coefficients were analysed to assess potential collinearity between continuous variables, and they ranged between -0.16 and 0.63, indicating none of the variables were correlated to an extent that made unfeasible to be included in the same models (Appendix S2). Scat samples collected before 2012 (n=560) were excluded from the statistical analysis because of missing data on moose abundance. Scat samples from the northern management area (n=87) were excluded from the analysis due to the lack in variation in sex and status in this particular area (53% of the scats from solitary males). Fixed effect parameters were assigned normal vague priors with mean zero and standard deviation 100,000. The standard deviation, σ_{unit} and σ_{year} for social unit and year, were given uniform priors over the interval (0, 10).

The analysis was conducted using JAGS (Plummer, 2003) called from R using the 'rjags' package (Plummer et al., 2016). We used two separate models, one with moose DNA occurrence as response variable and one with roe deer DNA occurrence as response variable. For each model, two MCMC chains with different initial values were used and after discarding the first 10,000 iterations we extracted parameter estimates at every 20^{th} step from a total of

100,000 accumulated samples from each chain. To promote convergence and interpretability, continuous explanatory variables were standardized subtracting the mean and dividing by the standard deviation (McCarthy, 2007; Schielzeth, 2010), and were subsequently back transformed for descriptive purposes. We assessed convergence by visual inspection of trace plots and the Gelman and Rubin diagnostic and appropriate convergence was confirmed with all estimates of the diagnostic being <1.1 (Gelman & Rubin, 1992). We evaluated model fit using posterior predictive checks, comparing the mean, variation and sums of squares of data sets simulated from the model to the observed data used to estimate model coefficients (i.e. posterior predictive checks, Hobbs & Hooten, 2015), and both the moose and roe deer model showed adequate model fit (range of Bayesian *p*-values = 0.21 – 0.51) (Appendix S3). As all covariates were included based on the biological foundations to test our hypothesis, we decided to conduct our analysis on the full model structure to make probabilistic assessments of each coefficient estimate and its importance relative to the other variables (cf. Hobbs et al., 2012). Thus, for both the probability of moose and roe deer DNA occurrence in wolf scats, our conclusions were conditional on the same full model structure. We presented posterior means with associated 95% credible intervals (CIs; the interval between the 0.025 and 0.975 quantiles of the posterior distributions) for all model coefficients and predictions, if not otherwise stated. To assess the importance of model coefficients, we examined the overlap of their posterior distributions with 0. For a regression coefficient (β), the proportion of the posterior distribution > 0 give the probability that the relationship between response and explanatory variable is positive (presented in results as $Pr(\beta > 0) = X\%$), while the proportion < 0 give the probability for a negative relationship ($Pr(\beta < 0) = X\%$). To make statement on differences between groups (sex, status) we subtracted their posterior distributions within the JAGS model structure (i.e. A-B). The proportion of the resulting probability distribution that is above zero corresponds to the probability that group A > group B (presented in results as $Pr(A > B) = X\%$). A probability

of 50% indicates the mean estimate for the difference is 0. In the results section we describe relationships with *Pr* > 90%.

3. Results

3.1. Prey use by wolves

A total of 2125 scat samples were analysed for presence of prey DNA. The scat samples came from 642 individual wolves (average 3.3 scats per wolf, range 1 to 24). Overall, results showed the following distribution of occurrence: no target prey (26%, 548 scats), 1 target prey (64%, n = 1362), 2 target prey (8%, n = 168), 3 target prey (1%, n = 23), 4 target prey (0.7%, n = 14), or >4 target prey (0.5%, n = 10). From the 1577 samples with at least one target prey detected, we obtained a total of 1926 occurrences for the 17 target prey (Figure 2). Moose and roe deer had 52.3% (1008 scats) and 23.2% (446 scats) of the occurrences, respectively (Figure 2, Figure 3). Alternative ungulates represented 6.9% (132 scats) of the occurrences. The occurrences of the following species were: 7.8% (150 scats) for small prey (beaver, badger, hares, capercaillie, black grouse), 3.5% (68 scats) for domestic species (cattle, sheep), 3.2% (62 scats) for reindeer, and 3.1% (60 scats) for carnivores (brown bear, Eurasian lynx, wolverine, red fox) (Figure 2, Figure 3). Among the carnivores, red fox was detected alone in the scats in 24% of its occurrences, while brown bear, Eurasian lynx and wolverine were never detected alone in the scats (Appendix S9). Moose, roe deer and reindeer were detected as single prey items in 84%, 72% and 75% of their occurrences, respectively. Alternative ungulates, domestic species, European badger and Eurasian beaver were detected alone in approximately half of their occurrences, while hares, Western capercaillie, and black grouse were detected in less than 30% of their occurrences (Appendix S9).

3.2. Factors related to prey use patterns

375 The analysis of probability of moose and roe deer occurrence in wolf scats (excluding 647
376 samples, see Methods) included a final sample of 1478 scats from 2012-2019 and from 434
377 wolf individuals. In the dataset analysed, the average inbreeding coefficient was 0.17 (range 0
378 to 0.40) (Appendix S8). The average abundance index of moose included in this analysis was
379 2.38 animals/10 km^2 (range 0 to 4.70), while the average abundance index of roe deer and
380 alternative ungulates were 2.69 (range 0.02 to 14.03) and 4.02 (0 to 78.57) animals/10 km^2
381 respectively (Appendix S8).
382
383 *3.2.1. Probability of moose occurrence relates to wolf social status and ungulates abundance*
384 The probability of moose DNA occurrence in scats (hereafter referred as occurrence) did not
385 differ between female and male wolves, in either packs or pairs (Figure 4A, Table 1, Appendix
386 S4). For solitary wolves instead, there was a 93% probability for a lower moose occurrence in
387 scats from females compared to males (Figure 4A, Table 1, Appendix S4). Additionally, a
388 higher occurrence of moose was observed for packs and pairs compared to solitary only for
389 females ($Pr(\text{Pack}_F > \text{Solitary}_F) = 99\%$, $Pr(\text{Pair}_F > \text{Solitary}_F) = 98\%$), while there was no such
390 effect for males (Figure 4A, Table 1, Appendix S4). For packs or pairs, there was no
391 relationship between moose occurrence in scats and the index of moose abundance in the
392 landscape (hereafter referred as abundance), whereas for solitary wolves this relationship was
393 positive with a 97% probability (Figure 5A, Table 2, Appendix S4). The occurrence of moose
394 decreased with increasing roe deer abundance for packs, pairs and solitary wolves ($Pr(\beta_{\text{roe deer}}$
395 $_{\text{abundance}} < 0) = 98\%$, 98% and 99% for pack, pair and solitary, respectively; Figure 5B). A
396 decreased occurrence of moose was also observed with increasing abundance of alternative
397 ungulates ($Pr(\beta_{\text{alternative ungulates}} < 0) = 100\%$, Figure 5C). No effect of inbreeding was observed
398 on occurrence of moose in scats for any social status of the wolf (Figure 5D, Table 2, Appendix
399 S4).

400

401 *3.2.2. Probability of roe deer occurrence relates to wolf social status, sex, inbreeding level and*
402 *ungulates abundance*

403 The probability of roe deer occurrence in scats was higher in females compared to males for
404 solitary wolves ($Pr(\text{Solitary}_F > \text{Solitary}_M) = 99\%$), but did not differ between sexes in packs or
405 pairs (Figure 4B, Table 1, Appendix S5). It was only for males that a higher occurrence of roe
406 deer was observed in packs and pairs compared to solitary wolves ($Pr(\text{Pack}_M > \text{Solitary}_M) =$
407 96%; $Pr(\text{Pair}_M > \text{Solitary}_M) = 99\%$), but there was no such an effect for females (Fig.4B, Table
408 1, Appendix S5). The occurrence of roe deer increased with increasing roe deer abundance
409 ($Pr(\beta_{\text{roe deer abundance}} > 0) = 100\%$) and this relationship did not differ according to social status
410 (Figure 6B, Table 2, Appendix S5). The relationship between roe deer occurrence and moose
411 abundance was different depending on the status; with a 99% probability for a negative
412 relationship for packs, a 95% probability for a positive relationship for solitary, and no
413 relationship for pairs (Figure 6A, Table 2, Appendix S5). As observed for moose, the
414 occurrence of roe deer decreased with increasing abundance of alternative ungulates
415 ($Pr(\beta_{\text{alternative ungulates}} < 0) = 100\%$, Figure 6C, Table 2, Appendix S5). The relationship between
416 inbreeding and roe deer occurrence showed that there was a 96% and 95% probability for
417 increasing roe deer occurrence with increasing inbreeding for packs and solitary wolves (Figure
418 6D, Table 2, Appendix S5), but no relationship for pairs.

419

420 **4. Discussion**

421 The analysis of prey use from DNA in wolf scats revealed that moose was the primary prey
422 species, followed by roe deer and, in comparable overall amounts, by alternative prey,
423 comprising other wild ungulates, smaller prey, livestock, reindeer and carnivores. We observed
424 heterogeneity in wolf diet composition at the landscape scale with both extrinsic and intrinsic

425 factors affecting prey use by wolves. Use of moose increased with its abundance but decreased
426 as the abundance of roe deer and alternative ungulates increased. The use of roe deer was
427 positively related to its abundance and negatively related to the abundance of moose and
428 alternative ungulates. In addition, by associating scat samples to individual wolves' genotypes,
429 we revealed that their social status affected the patterns of use of both moose and roe deer, and
430 that the consumption of roe deer was also affected by sex and inbreeding coefficient of
431 individual wolves.

432 We found support for our first prediction (P1), with a lower use of moose by solitary
433 wolves compared to packs and pairs, only for females. This result was in line with our
434 hypothesis that solitary wolves may be less capable of killing moose while avoiding the
435 potential increased risk of injury. Although the food reward after killing the large bodied moose
436 is high, this is also a species that can defend itself to an extent that wolves are aided by
437 cooperative hunting (Mech & Peterson, 2003; Sullivan, 1978). The fact that we found this
438 effect only for females was in line with the predicted and observed effect of sex, with a lower
439 use of moose by solitary females compared to solitary males (P2). These findings may suggest
440 that the smaller body size of females compared to males may expose them to a higher risk
441 during an attack on moose (MacNulty, Smith, Mech, et al., 2009; Sand et al., 2006). In fact, a
442 previous case study of two packs from the same wolf population showed that, after pack
443 dissolution, the lone male continued killing moose while the lone female switched to roe deer
444 (Sand et al., 2006). In contrast to our prediction, we did not find a lower use of moose by
445 solitary males compared to males in pairs and packs. This could be partly explained by that in
446 our study solitary wolves included both young dispersers and adult territorial wolves that have
447 lost their partner. These two categories of solitary wolves likely differed in their hunting
448 experience and other age-related characters such as body size (Mech & Boitani, 2003),
449 differences that may be more emphasized for males. The fact that we found no sex effect in

450 moose use for pairs and packs, in line with our prediction, may be explained by the nature of
451 cooperative hunting in wolves where the adult breeding pair share both the hunting effort and
452 the predation outcome (Sullivan, 1978; Zimmermann et al., 2015). For roe deer, our results did
453 not support our prediction of higher use for solitary wolves (P1). However, the lower use by
454 solitary males was in line with our prediction of higher use of roe deer for solitary females
455 compared to solitary males (P2). The result is however likely if ungulates other than roe deer,
456 such as red deer, fallow deer, wild boar, but also reindeer, were available and profitable for
457 solitary males. Such pattern may be partly explained by a potential geographical effect due to
458 the high dispersal rate of young males (Sanz-Pérez et al., 2018).

459 We did not find support for the effect of inbreeding on the use of moose (P3). Yet, we
460 revealed a tendency for a higher use of roe deer with increasing inbreeding coefficient, both
461 for solitary wolves and packs (P3). Although we are not aware of the inherent mechanisms
462 responsible for such relationship, inbreeding may influence the feeding pattern of individuals
463 through its negative effect on body size and condition (Fredrickson & Hedrick, 2002;
464 Räikkönen et al., 2013). Future investigations integrating such analysis with direct measures
465 of body size or condition may help clarifying the causality of the revealed pattern. Additionally,
466 the observed tendency could be an underestimation when assuming a linear relationship
467 between inbreeding and prey use because inbreeding may show non-linearity with a threshold
468 effect (Wiener et al., 1992), beyond which the body size and conditions would affect predation
469 pattern by hindering the wolf from conducting a successful hunt.

470 Our results showed wolf dietary responses to shifts in the composition of ungulate prey
471 species in the landscape. Meeting our prediction (P4), the abundance of moose played a
472 positive role in its use by solitary wolves, which is in line with kill rates found in the same and
473 other wolf populations (Vucetich et al., 2002; Zimmermann et al., 2015). The negative effect
474 of moose abundance on roe deer use by wolves in packs was in line with our prediction (P4).

Our result seems to strengthen the previously observed weak relationship between local moose density and selection of roe deer over moose in the same wolf population (Sand et al., 2016). An explanation for such stronger pattern observed in this study may be that the current study area covered a larger variation in the abundance of moose, roe deer, and other ungulates.

The importance of roe deer abundance on wolf predation patterns previously observed in Scandinavia was also confirmed in this study, in line with our prediction (P5). Our results added to an existing literature in Europe showing a high use of roe deer when available at high densities, possibly making such a small prey more profitable (Milanesi et al., 2012; Nowak et al., 2005, 2011; Sand et al., 2016; Wagner et al., 2012). Also in line with our prediction (P5), the strong relationship between increasing roe deer abundance and decreasing use of a larger but potentially more dangerous prey such as moose confirmed previous findings (Sand et al., 2016), and was additionally revealed for solitary wolves. Another novel result compared to previous research on this wolf population is the influence of alternative ungulates abundances on prey use, now possible to investigate with the recent expansion of the wolf population into the more southern multi-ungulate prey areas (Rodríguez-Recio et al., 2022). As predicted (P6), increased abundance of red deer, fallow deer, and wild boar was associated with a reduction in both moose and roe deer consumption by wolves. The observed pattern may reflect the response of an opportunistic predator to shifts in prey species composition and to a broader diversity of prey species available (Okarma, 1995). This finding is in line with the frequently observed selection for red deer by wolves in central-eastern Europe (Jędrzejewski et al., 2000; Jędrzejewski et al., 2012). However, further studies differentiating the relative abundance and use of these three ungulates are needed to reveal their relative importance for the feeding pattern of wolves in Scandinavia.

An additional potential influencing factor that is not captured by faecal diet studies is the difference in prey vulnerability, and therefore accessibility, among individuals of the same

500 species depending on sex, age and nutritional conditions (Gervasi et al., 2012; Kunkel et al.,
501 2004; Mech & Peterson, 2003). Despite its high abundance in the wolf breeding range, not all
502 segments of the moose population are similarly accessible to wolves. Indeed, moose is the main
503 prey but young-of-the-year are the most vulnerable and therefore strongly preferred by wolves
504 in Scandinavia when hunting this prey (Sand et al., 2005, 2012). As the current study is
505 conducted during winter, the observed feeding patterns on moose may not reflect the summer
506 predation pattern when calves are smaller in size and more strongly selected by wolves (Sand
507 et al., 2008).

508 Similar to any other study based on scat analysis, the investigation of food habits
509 through detection of target prey species DNA from scats does not necessarily reflect only
510 predation but can also be a result of scavenging (Cuesta et al., 1991; Symondson, 2002). The
511 possible underestimation of scavenging by wolves (but see Ciucci et al., 2020; Newsome et al.,
512 2015) would potentially argue against the use of faecal DNA-analysis to study predation
513 patterns. However, in the Scandinavian wolf population scavenging constitutes only a minor
514 proportion of the diet (6-13%) and wolves mostly rely on active killing of wild ungulates
515 (Wikenros et al., unpublished).

516 Small prey species have been widely documented as a relative minor component of
517 wolf diet (Newsome et al., 2016; Okarma, 1995; Olsson et al., 1997; Paquet & Carbyn, 2003;
518 Sand et al., 2008). The occasional feeding on small prey (e.g. small mammals, forest birds)
519 was also supported in this study by the overall low proportion of the occurrences and mostly
520 in combination with other prey in the scat. On the other hand, larger prey species were mainly
521 detected as the only prey item in the scats and constituted the bulk of wolf diet. The occurrence
522 of carnivore DNA in wolf scats may indicate different types of behaviour, including intraguild
523 predation or scavenging by wolves on dead carrions from other carnivores, although expected
524 to be rare (Martins et al., 2020). A more likely explanation could be the territorial marking

525 through urination done by carnivore species thus contaminating the wolf scat with their DNA.
526 This phenomenon has been documented for foxes as behavioural response to an apex predator
527 (Wikenros et al., 2017). For brown bear, Eurasian lynx and wolverine, this hypothesis seems
528 to be supported by the fact that these species only occurred in scats where other target prey
529 were also present. One fourth of the scats showed no occurrence of any target prey and there
530 are several potential reasons for this. Although we covered the majority of prey species
531 previously documented in the diet of wolves, some samples with no detections could
532 potentially contain other food items such as garbage, plants, or small mammals like rodents.
533 However, this hypothesis is unlikely as such food items are shown to be a minor component in
534 wolves diet (Peterson & Ciucci, 2003), and negligible for the Scandinavian wolf population
535 (Müller, 2006; Olsson et al., 1997). An alternative explanation to our failure in detecting prey
536 in some scats is degradation of prey DNA, thus hindering DNA amplification (Beja-Pereira et
537 al., 2009; Santini et al., 2007). Although the genetic identification of wolf individuals from
538 these samples may indicate an overall high quality for DNA analysis, the amplification and
539 detection rate of predator DNA could be higher than that of consumed food items
540 (Krehenwinkel et al., 2017). Despite using a method that maximized both sensitivity and
541 specificity (Di Bernardi et al., 2021), the experimental validation of the method with feeding
542 trials with captive wolves showed a false negative rate of 24% for moose and 8% for roe deer
543 (Di Bernardi et al., in revision). The findings in the validation study support the hypothesis of
544 false negatives, indicating that even freshly collected scats sometimes can result in the non-
545 detection of consumed prey.

546 In conclusion, our results revealed patterns of feeding response by wolves to shifts in
547 prey species community and the influence of intrinsic and external interacting drivers at the
548 landscape level. The gained knowledge on prey use in an expanding wolf population has the
549 potential to increase our understanding of the impact of wolves on wild ungulate communities

and inform the adaptive management of such species harvested by humans. Finally, by associating prey DNA fecal analysis to individual wolves' genotypes, our results highlighted the relevance of considering predator-related individual characteristics when investigating predation patterns.

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6. Author Contribution

MÅ, CW, HS secured funding; CDB, CW, HS, MÅ conceived and designed the study with the contribution of PC and LB; CDB conducted laboratory work for diet analysis; CDB, CW, HS, MÅ compiled data; CW, MA carried out the statistical analyses; CDB drafted the manuscript; All authors revised the manuscript and gave final approval for publication.

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Figures and Tables

Figure 1. The study area (in green) and distribution of 2125 scat samples (black dots) analysed for prey DNA detection. The samples were collected in Sweden from the Scandinavian wolf population during 14 monitoring seasons from 2006–2019. The blue lines delineate the three carnivore management regions (northern, central, southern)(Sandström et al., 2020).

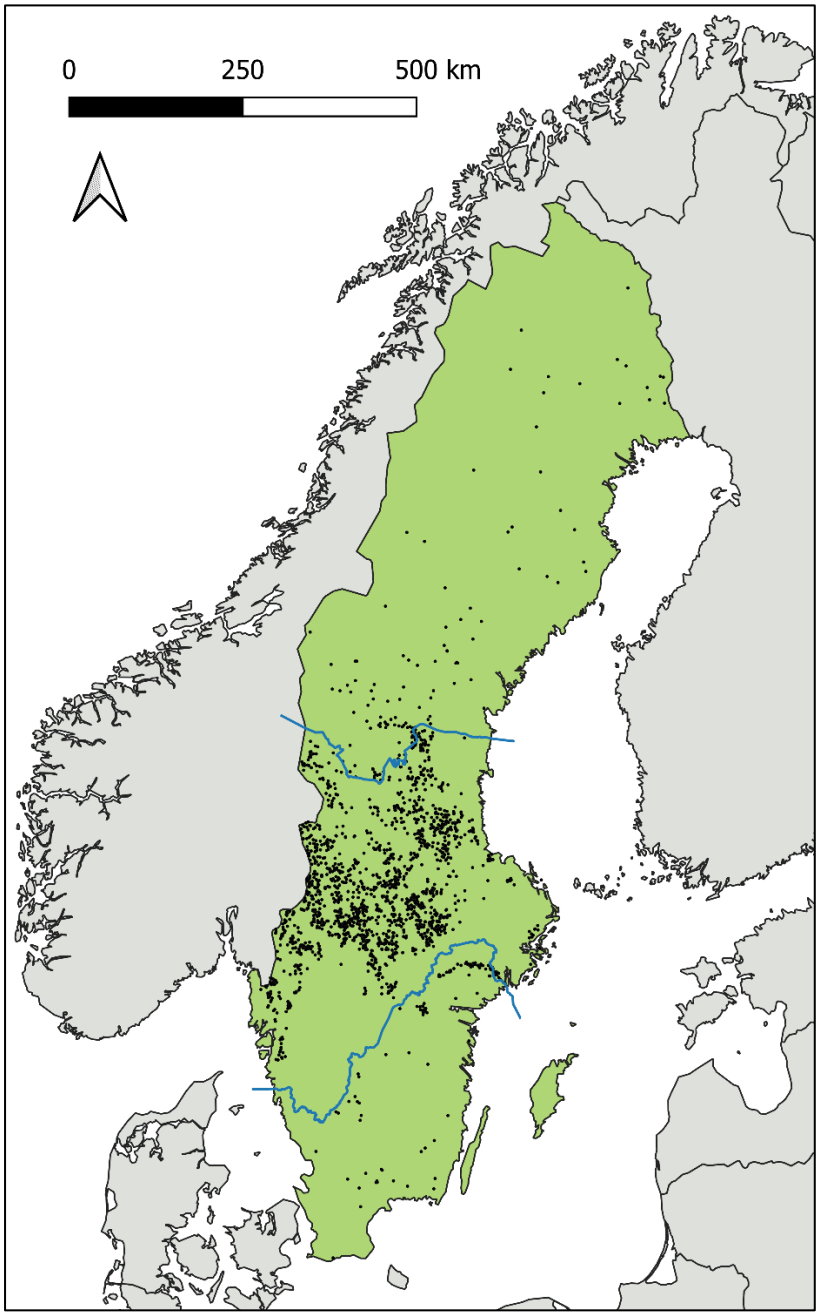


Figure 2. Proportional occurrence (%) for the 17 target prey on a total of 1926 occurrences from 1577 scats that showed detection of at least one species, out of the total samples (n = 2125) analysed for prey detection, collected during 14 monitoring seasons in 2006–2019 in Sweden. The proportional occurrence (%) of a given target prey *i* was measured as the number of its occurrences N_i on the total number of occurrences *N*.

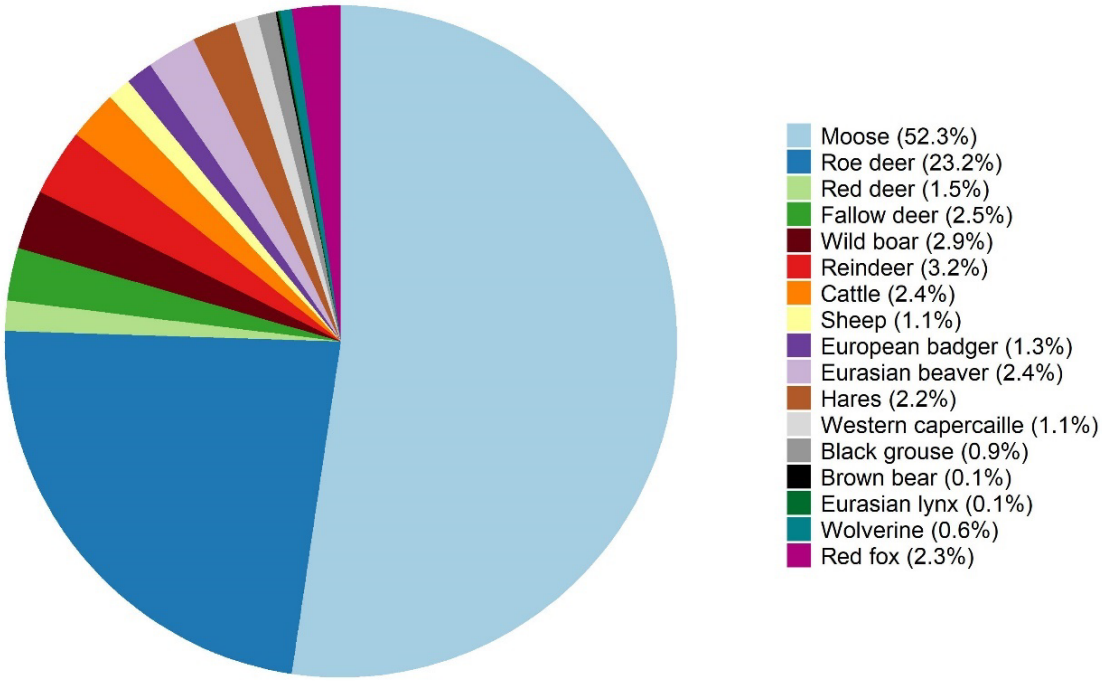


Figure 3. Prey occurrence in wolf scats from a total of 2125 samples analysed for prey detection, collected in 2006–2019 in Sweden. The number of occurrences (range 0–20) within 400 km^2 cells is displayed separately for moose, roe deer, red deer, fallow deer, wild boar, reindeer, domestic (cattle, sheep), small prey (beaver, badger, hares, capercaillie, black grouse), and carnivores (brown bear, Eurasian lynx, wolverine, red fox). In the white cells there were no scats analysed for prey detection.

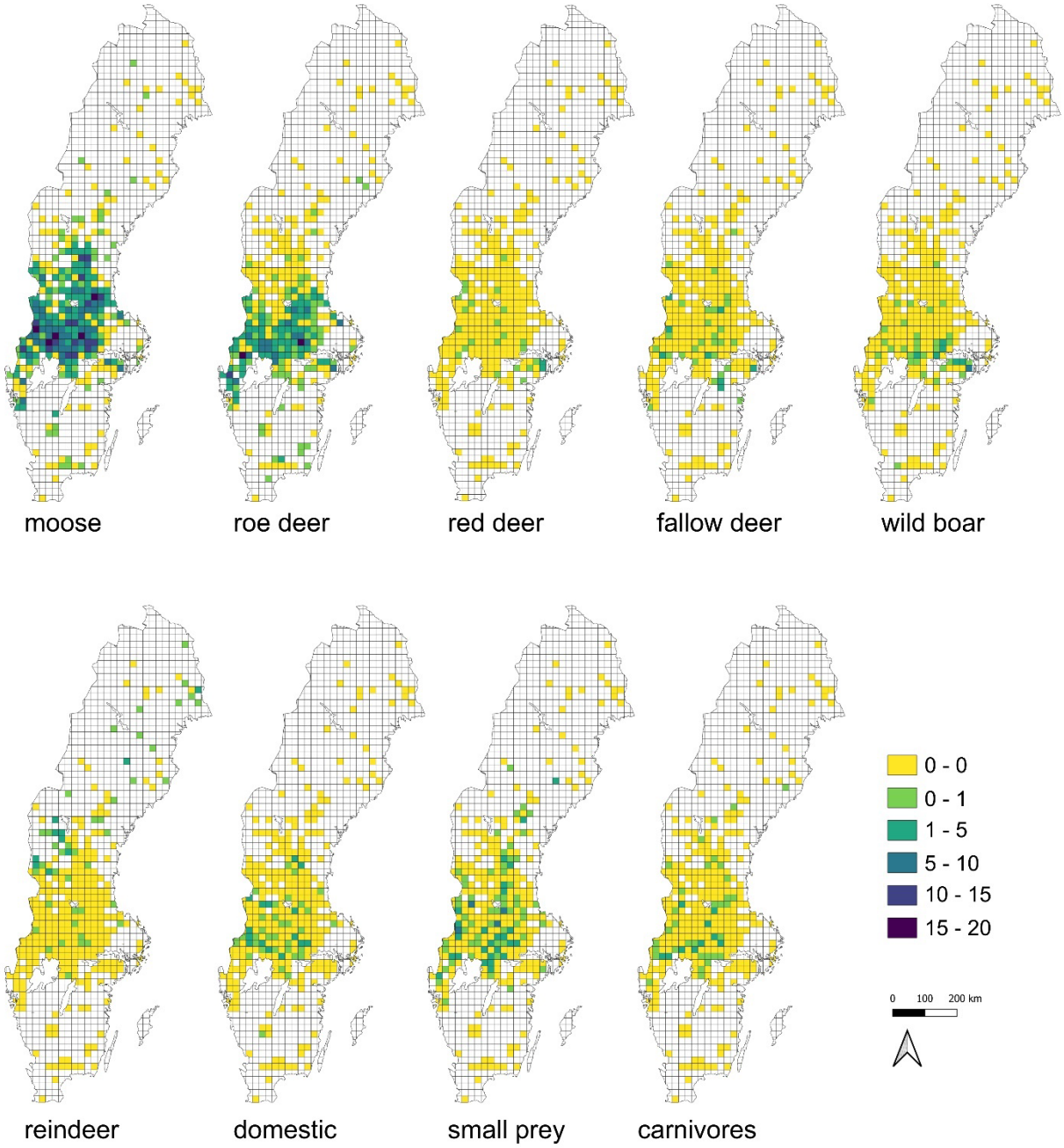


Figure 4. Mean model predictions with associated 95% CI for the probability of A) moose DNA occurrence and B) roe deer DNA occurrence in wolf scats separated by sex (Female [F], Male [M]) and social status (pack, pair, solitary) of wolves in Sweden.

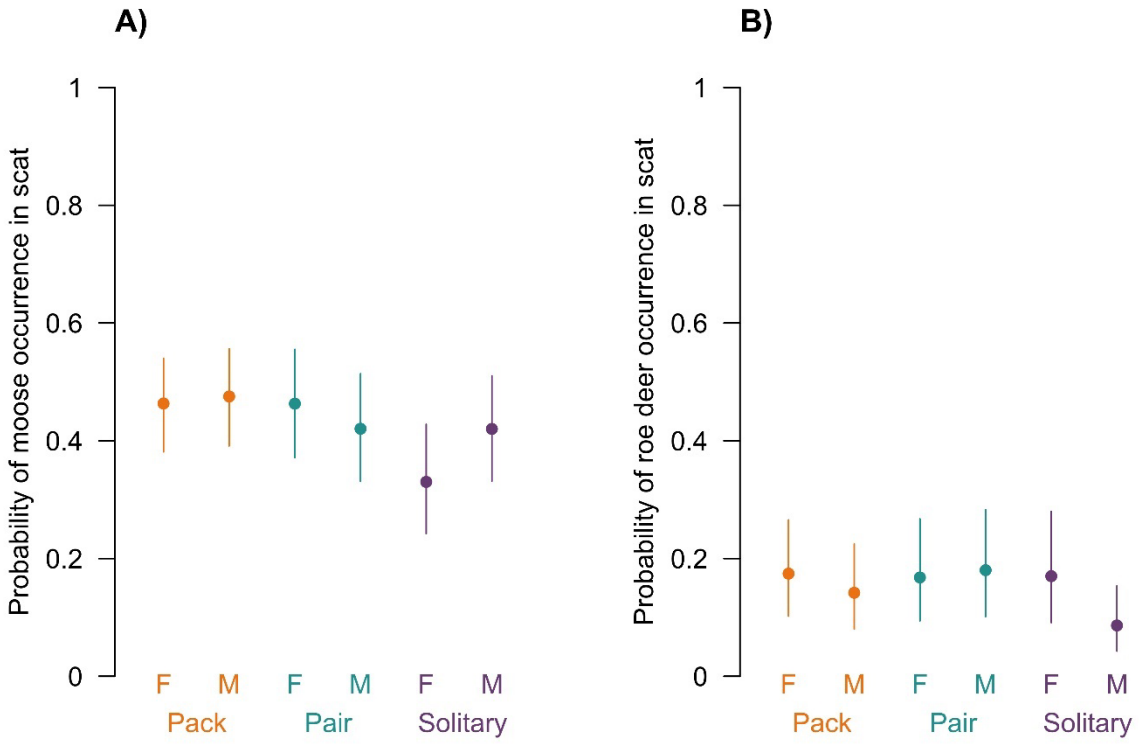


Figure 5. Model predictions with associated 95% CIs for the relationships between moose DNA occurrence in wolf scats and A) moose abundance, B) roe deer abundance, C) alternative ungulates abundance, D) inbreeding, for pack, pair, and solitary wolves in Sweden. Predictions for females are shown, as these relationships were not modelled separately for the two sexes. For figures showing the intercepts for both sexes, see Appendix S6. For coefficient estimates, see Table 2. For each graph, the other covariates are kept at their mean value.

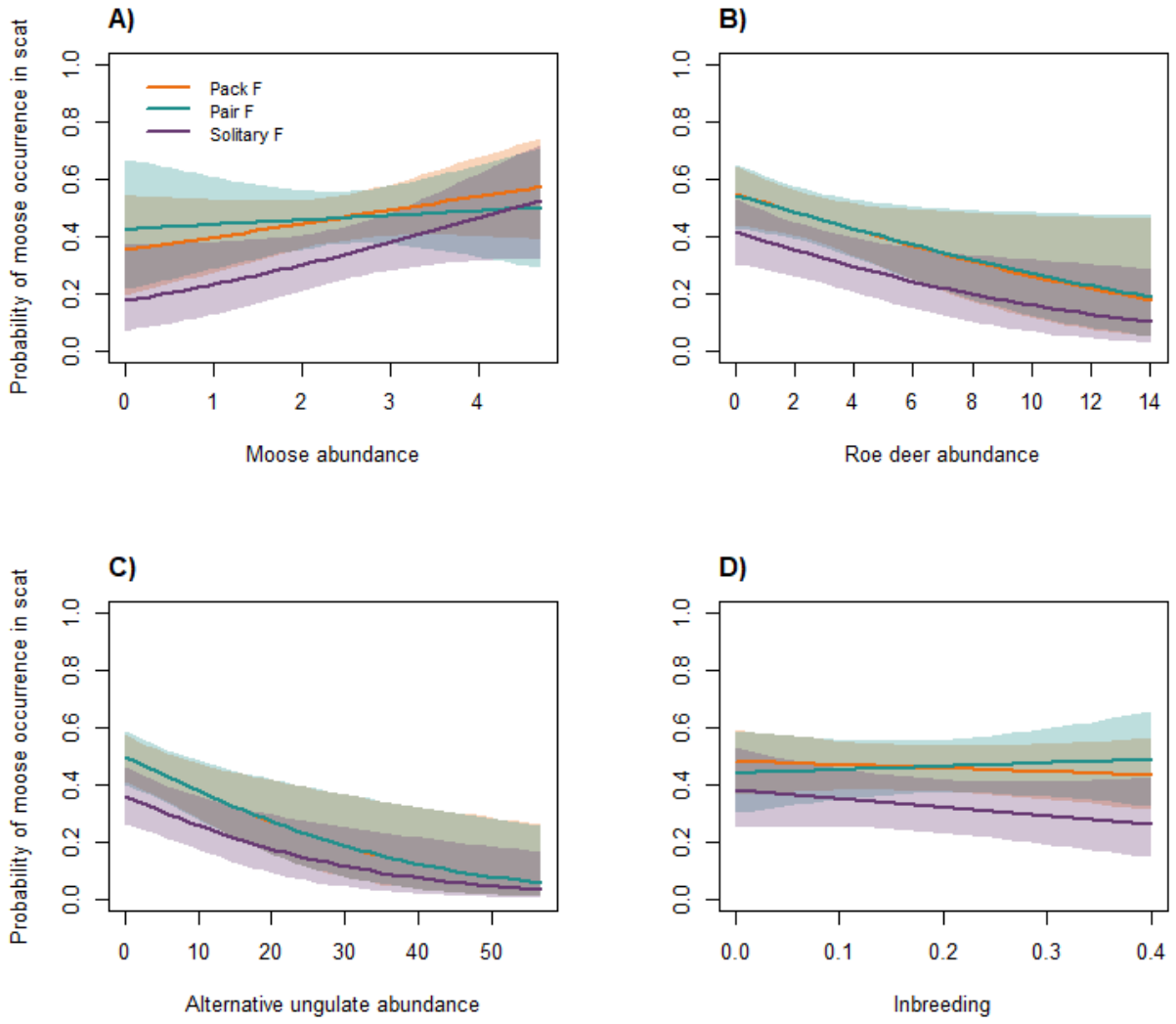


Figure 6. Model predictions with associated 95% CIs for the relationships between roe deer DNA occurrence in wolf scats and A) moose abundance, B) roe deer abundance, C) alternative ungulates abundance, D) inbreeding, for pack, pair, or solitary wolves in Sweden. Predictions for females are shown, as these relationships were not modelled separately for the two sexes. For figures showing the intercepts for both sexes, see Appendix S7. For coefficient estimates, see Table 2. For each graph, the other covariates are kept at their mean value.

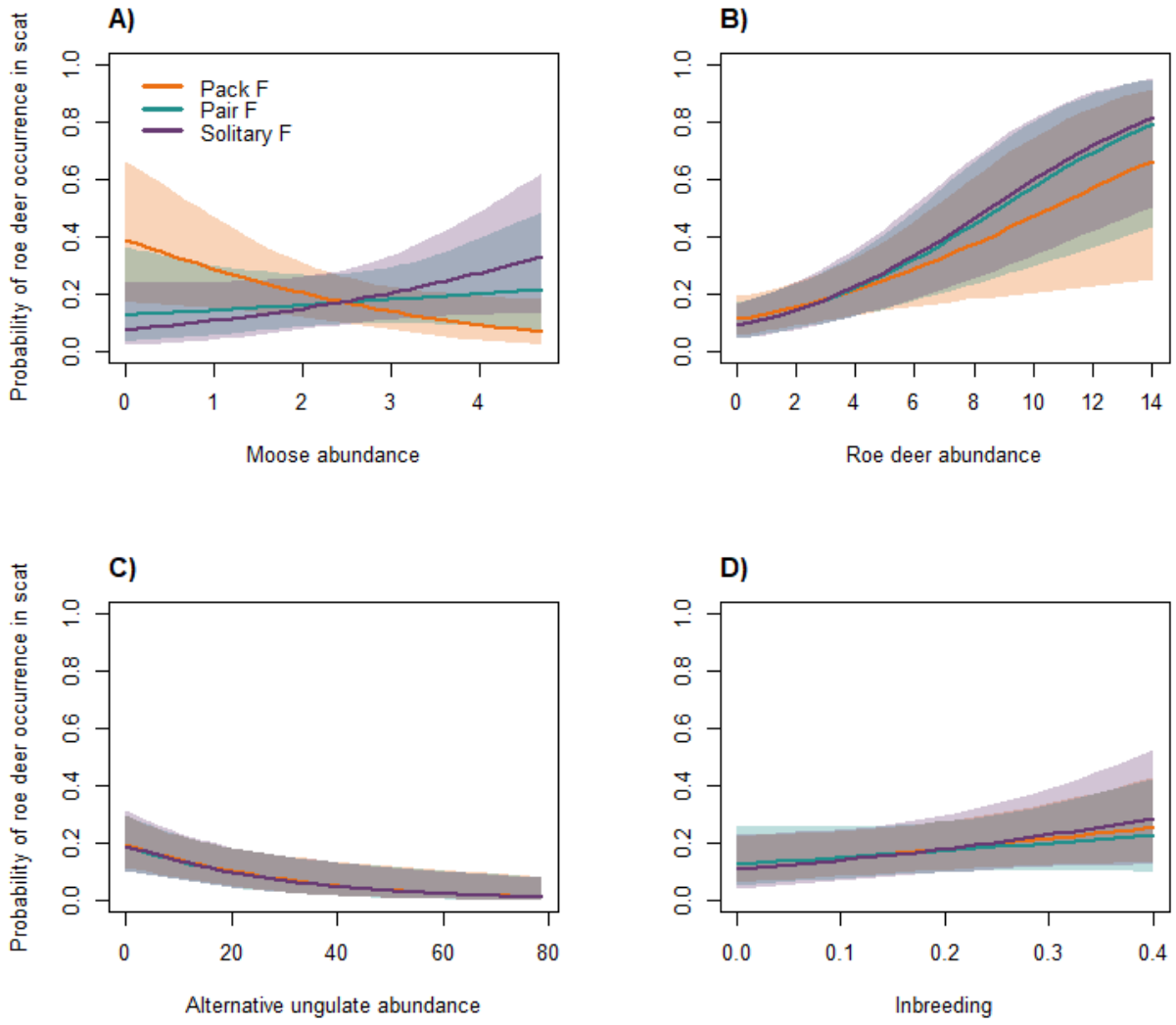


Table 1. Comparison of moose DNA occurrence and roe deer DNA occurrence in wolf scats between different sexes (Female [F], Male [M]) and statuses (Pack, Pair and Solitary). Probability (Pr) of higher occurrence in category A compared to B (*Pr*(A>B), and the estimated difference with associated 95% credible interval (CI).
Footnote: *corresponds to $Pr(Solitary_M > Solitary_F) = 93\%$

	Moose DNA occurrence		**Roe deer DNA occurrence**	
Category	**Pr**	**Mean (CI) difference**	**Pr**	**Mean (CI) difference**
$Pack_F > Pack_M$	38%	0.01 (-0.07 to 0.10)	88%	-0.03 (-0.09 to 0.02)
$Pair_F > Pair_M$	78%	0.04 (-0.06 to 0.15)	39%	0.01 (-0.65 to 0.09)
$Solitary_F > Solitary_M$	7% *	0.09 (-0.03 to 0.20)	99%	-0.08 (-0.17 to -0.01)
$Pack_F > Pair_F$	50%	0.0003 (-0.10 to 0.10)	57%	0.006 (-0.07 to 0.08)
$Pack_M > Pair_M$	85%	0.05 (-0.05 to 0.16)	16%	-0.04 (-0.12 to 0.03)
$Pack_F > Solitary_F$	99%	0.13 (0.02 to 0.23)	53%	0.003 (-0.08 to 0.09)
$Pack_M > Solitary_M$	85%	0.06 (-0.05 to 0.16)	96%	0.05 (-0.004 to 0.13)
$Pair_F > Solitary_F$	98%	0.13 (0.01 to 0.25)	48%	-0.002 (-0.09 to 0.09)
$Pair_M > Solitary_M$	50%	0.0005 (-0.11 to 0.11)	99%	0.09 (0.02 to 0.18)

Table 2. Relationship between moose DNA occurrence and roe deer DNA occurrence in wolf scats and the continuous covariates (moose abundance, roe deer abundance, alternative ungulates abundance, inbreeding coefficient) for pack, pair and solitary wolves in Sweden. Mean coefficient estimates with associated 95% credible intervals (CIs) and the probability (*Pr*) that the relationship is in the direction of the mean estimate. For example, there is a 90% probability for a positive relationship between moose DNA occurrence and moose abundance for pack and there is a 99% probability for a negative relationship between roe deer DNA occurrence and moose abundance for pack.

Footnote: * the relationship between prey DNA occurrence and alternative ungulate abundance was the same for all three statuses.

	Moose DNA occurrence		**Roe deer DNA occurrence**	
Slope	**Mean (CI)**	**Pr**	**Mean (CI)**	**Pr**
Moose abundance, pack	0.13 (-0.07 to 0.33)	90%	-0.31 (-0.60 to -0.03)	99%
Moose abundance, pair	0.04 (-0.21 to 0.29)	64%	0.091 (-0.24 to 0.43)	70%
Moose abundance, solitary	0.23 (0.00 to 0.47)	97%	0.26 (-0.06 to 0.58)	95%
Roe deer abundance, pack	-0.29 (-0.58 to -0.01)	98%	0.48 (0.14 to 0.83)	100%
Roe deer abundance, pair	-0.28 (-0.57 to 0.00)	98%	0.63 (0.31 to 0.95)	100%
Roe deer abundance, solitary	-0.32 (-0.58 to -0.07)	99%	0.66 (0.38 to 0.96)	100%
Alternative ungulates abundance *	-0.35 (-0.59 to -0.13)	100%	-0.38 (-0.65 to -0.12)	100%
Inbreeding, pack	-0.05 (-0.23 to 0.14)	70%	0.21 (-0.03 to 0.45)	96%
Inbreeding, pair	0.05 (-0.20 to 0.30)	64%	0.17 (-0.16 to 0.50)	85%
Inbreeding, solitary	-0.13 (-0.37 to 0.11)	85%	0.29 (-0.05 to 0.63)	95%

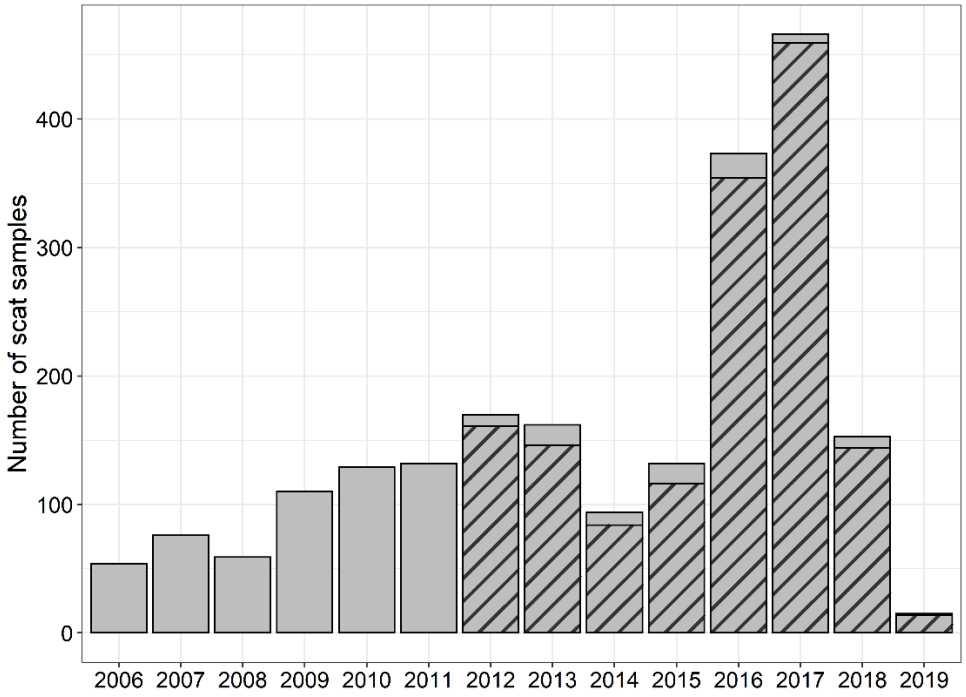
Supplemental Information for CHAPTER 3:

Predator individual traits and prey abundance affect wolf predation in a multi-ungulate system

Di Bernardi Cecilia, Mikael Åkesson, Håkan Sand, Malin Aronsson, Paolo Ciucci, Luigi Boitani, Camilla Wikenros

Corresponding author: Cecilia Di Bernardi, cecilia.dibernardi@uniroma1.it

Appendix S1. Number of scat samples analysed for prey detection (grey, n=2125), and the subset included in the modelling analyses (grey and striped, n=1478), for each year of the study period 2006–2019.

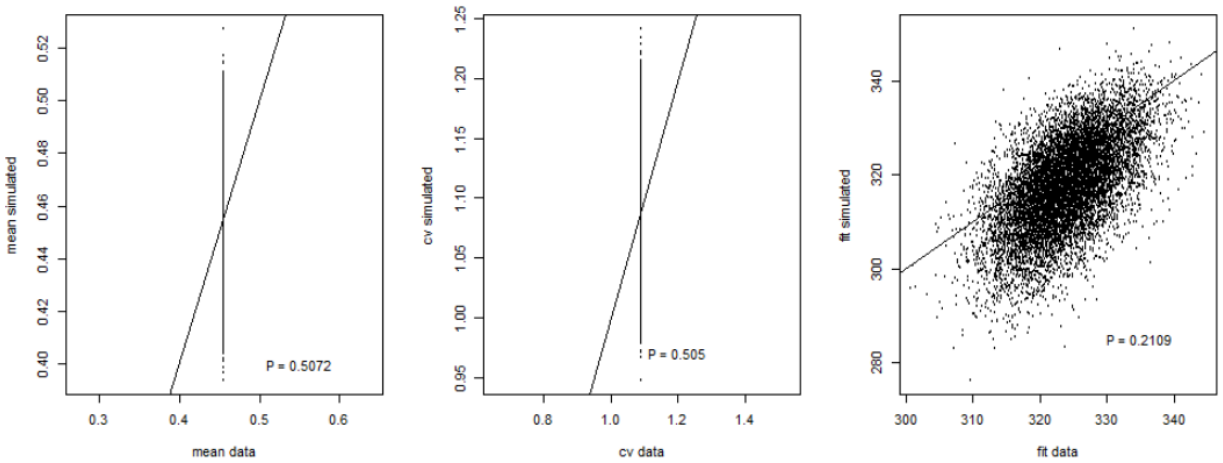


Appendix S2. Correlation index (Pearson's) among continuous explanatory variables, including inbreeding coefficient, moose abundance, roe deer abundance, and alternative ungulates abundance, used to explain variation in moose and roe deer occurrence in wolf scats.

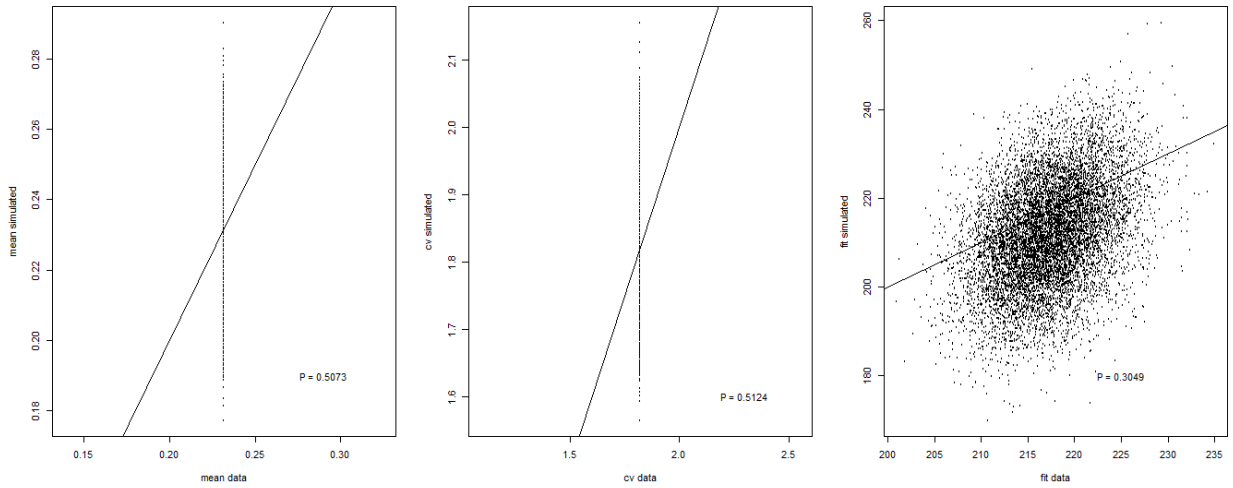
	Inbreeding coefficient	**Moose abundance**	**Roe deer abundance**	**Alternative ungulates abundance**
Inbreeding coefficient	1			
Moose abundance	-0.02	1		
Roe deer abundance	-0.11	-0.01	1	
Alternative ungulates abundance	-0.16	-0.22	0.63	1

Appendix S3. Posterior predictive checks for model fit of the A) moose model and B) roe deer model.

A)



B)



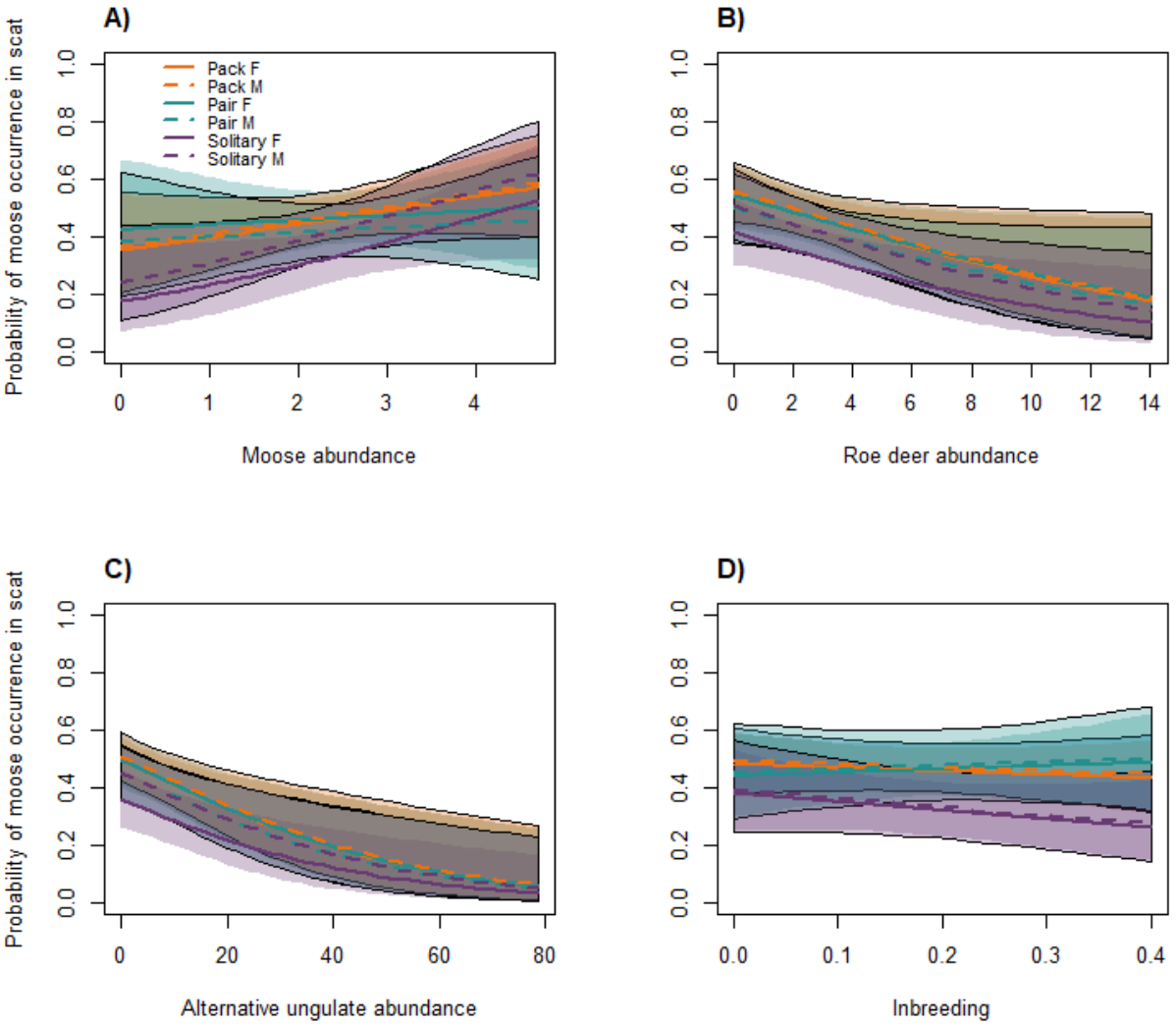
Appendix S4. Mean estimates of the parameters in the moose DNA occurrence model, with associated 95% credible intervals (CIs). The parameters are status, inbreeding, sex, moose abundance, roe deer abundance, and alternative ungulates abundance, and the interactions moose abundance × status, roe deer abundance × status, inbreeding × status, and sex × status. The reference group for the intercept is pack for status and female for sex. Pr is the probability of an association in the direction of the mean estimate of the coefficient.

Parameter	Mean	CI 95%	Pr
intercept	-0.14	(-0.46 to 0.16)	84%
pair	-0.01	(-0.40 to 0.41)	50%
solitary	-0.56	(-1.03 to -0.10)	99%
inbreeding	-0.05	(-0.23 to 0.14)	70%
male	0.05	(-0.28 to 0.39)	62%
moose abundance	0.13	(-0.07 to 0.33)	90%
roe deer abundance	-0.29	(-0.58 to -0.01)	98%
alternative ungulates abundance	-0.35	(-0.59 to -0.13)	100%
moose abundance:pair	-0.09	(-0.39 to 0.21)	72%
moose abundance:solitary	0.10	(-0.21 to 0.41)	74%
roe deer abundance:pair	0.01	(-0.36 to 0.38)	52%
roe deer abundance:solitary	-0.03	(-0.38 to 0.32)	56%
inbreeding:pair	0.09	(-0.21 to 0.39)	73%
inbreeding:solitary	-0.09	(-0.39 to 0.21)	71%
male:pair	-0.22	(-0.76 to 0.33)	79%
male:solitary	0.34	(-0.27 to 0.95)	86%

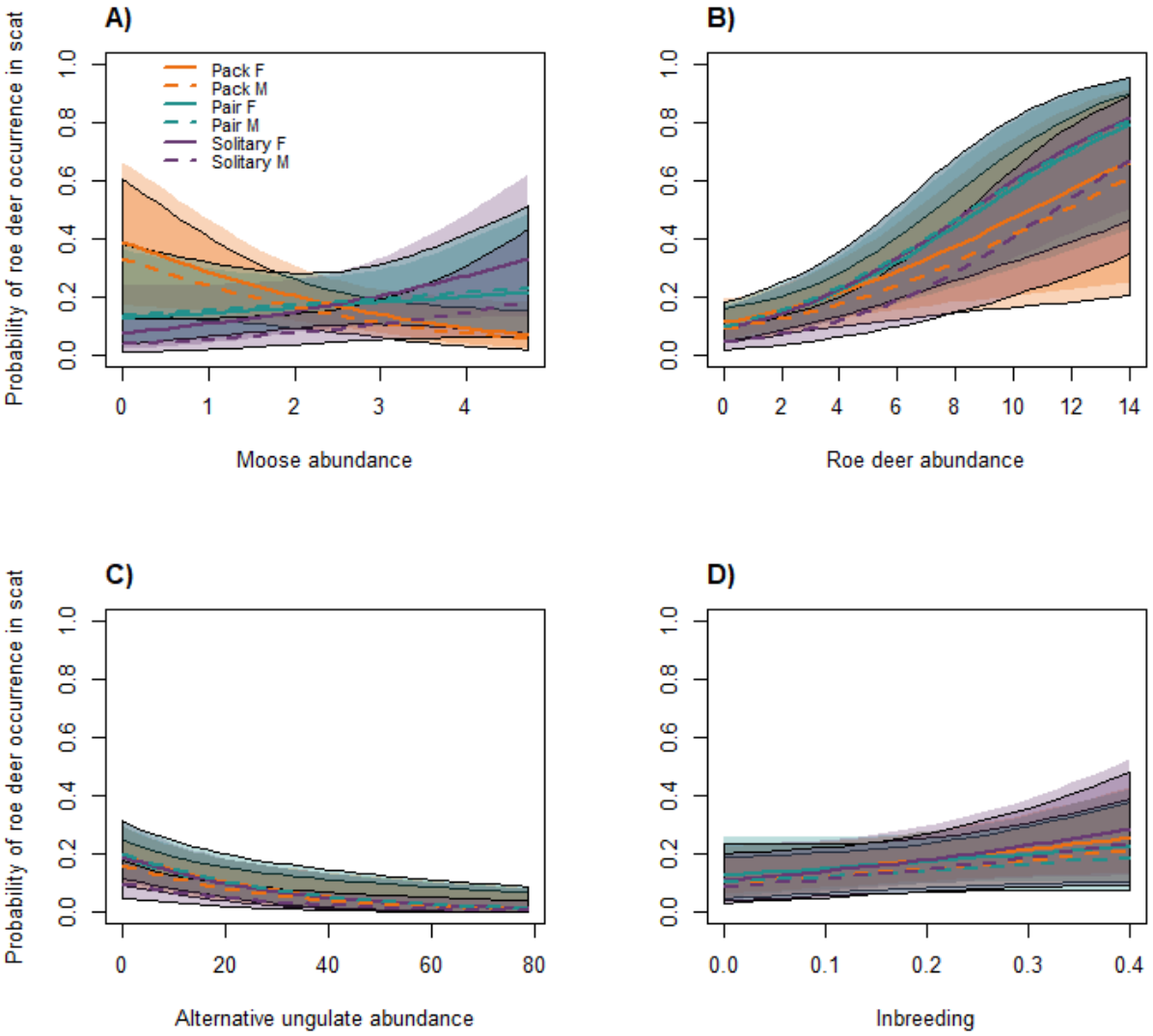
Appendix S5. Mean estimates of the parameters in the roe deer DNA occurrence model, with associated 95% credible intervals (CIs). The parameters are status, inbreeding, sex, moose abundance, roe deer abundance, and alternative ungulates abundance, and the interactions moose abundance × status, roe deer abundance × status, inbreeding × status, and sex × status. The reference group for the intercept is pack for status and female for sex. Pr is the probability of an association in the direction of the mean estimate of the coefficient.

Parameter	Mean	CI 95%	Pr
intercept	-1.56	(-2.13 to -1.04)	100%
pair	-0.04	(-0.55 to 0.47)	57%
solitary	-0.03	(-0.63 to 0.55)	53%
inbreeding	0.21	(-0.03 to 0.45)	96%
male	-0.24	(-0.65 to 0.17)	88%
moose abundance	-0.31	(-0.60 to -0.03)	99%
roe deer abundance	0.48	(0.14 to 0.83)	100%
alternative ungulates abundance	-0.38	(-0.65 to -0.12)	100%
moose abundance:pair	0.40	(0.003 to 0.81)	98%
moose abundance:solitary	0.57	(0.17 to 0.98)	100%
roe deer abundance:pair	0.15	(-0.28 to 0.57)	76%
roe deer abundance:solitary	0.18	(-0.24 to 0.61)	80%
inbreeding:pair	-0.04	(-0.43 to 0.35)	59%
inbreeding:solitary	0.08	(-0.33 to 0.50)	65%
male:pair	0.32	(-0.36 to 1.00)	83%
male:solitary	-0.54	(-1.33 to 0.25)	91%

Appendix S6. Model predictions with associated 95% CI for the relationships between moose DNA occurrence in wolf scats and A) moose abundance, B) roe deer abundance, C) alternative ungulates abundance, D) inbreeding, for pack, pair and solitary wolves. Females are represented by solid lines and males by dashed lines (95% CI for males are outlined with black lines). For each graph, the other covariates are kept at their mean value.



Appendix S7. Model predictions with associated 95% CI for the relationships between roe deer DNA occurrence in wolf scats and A) moose abundance, B) roe deer abundance, C) alternative ungulates abundance, D) inbreeding, for pack, pair and solitary wolves. Females are represented by solid lines and males by dashed lines (95% CI for males are outlined with black lines). For each graph, the other covariates are kept at their mean value.



Appendix S8. Average and standard deviation of continuous covariates included in the moose and roe deer analysis (n = 1478 scats). The abundance index for ungulates was estimated as number of harvested individuals/10km^2 (see Methods). Alternative ungulates abundance includes red deer, fallow deer, wild boar summed together.

Covariate	Average	Standard deviation	Min - Max
Inbreeding coefficient	0.17	0.09	0 - 0.40
Moose abundance	2.38	0.67	0 - 4.70
Roe deer abundance	2.69	2.38	0.02 - 14.03
Alternative ungulates abundance	4.02	10.06	0 - 78.57

Appendix S9. Number of scats with detection for each of the 17 target prey, and percentage of scats where the target species was detected as a single item in the scat, not in combination with other species.

Species	n scats with detection	% scats with species alone
Moose	1008	84%
Roe deer	446	72%
Red deer	28	50%
Fallow deer	49	22%
Wild boar	55	52%
Reindeer	62	75%
Cattle	46	46%
Sheep	22	45%
European badger	25	44%
Eurasian beaver	46	41%
Hares	41	22%
Western capercaillie	17	23%
Black grouse	21	28%
Brown bear	2	0%
Eurasian lynx	2	0%
Wolverine	11	0%
Red fox	45	24%

CHAPTER 4

Explaining the extent of scavenging by wolves in an anthropogenic landscape

Camilla Wikenros[1], Cecilia Di Bernardi[1,2], Barbara Zimmermann[3], Mikael Åkesson[1], Maike Demski[4], Øystein Flagstad[5], Jenny Mattisson[5], Aimee Tallian[5], Petter Wabakken[3], and Håkan Sand[1]

Affiliations:

[1]Grimsö Wildlife Research Station, Department of Ecology, Swedish University of Agricultural Sciences, SE-739 93 Riddarhyttan, Sweden

[2] Department of Biology and Biotechnologies "Charles Darwin", University of Rome La Sapienza, Viale dell'Università 32, 00185, Rome, Italy

[3] Faculty of Applied Ecology, Agricultural Sciences and Biotechnology, Inland Norway University of Applied Sciences, Evenstad, NO-2418, Elverum, Norway

[4] County Administrative Board of Norrbotten, SE-971 86 Luleå, Sweden

[5] Norwegian Institute for Nature Research (NINA), P.O. Box 5685, Torgarden, NO-7485, Trondheim, Norway

Corresponding author: camilla.wikenros@slu.se

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Abstract

Scavenging is an important part of food acquisition for many carnivore species that may switch between scavenging and predation. In landscapes with high anthropogenic impact humans provide food that scavenging species can utilize. We quantified the magnitude of killing versus

scavenging by gray wolves (*Canis lupus*) in Scandinavia where humans impact on the ecosystem through hunter harvest, land use practices, and infrastructure. We investigated the cause of death of different animals utilized by wolves and examined how the proportion of consumption time spent scavenging was influenced by season, social affiliation and inbreeding of the wolves, density of moose (*Alces alces*) as their main prey, density of brown bear (*Ursus arctos*) as intra-guild competitor, and human density. We used data from 39 GPS-collared wolves covering 3198 study days (2001-2019) and1362 found carcasses that was utilized by wolves. The majority of the carcasses were wolf-killed (80.5%) and a small part had died from other natural causes (1.9%). The remaining had either anthropogenic mortality causes (4.7%), or the cause of death was unknown (12.9%). The time spent scavenging was higher during winter than summer and autumn. Solitary wolves spent more time on scavenging, likely because of their poorer hunter skills compared to pack-living individuals. The scavenging time increased with the average inbreeding coefficient of the adult wolves, possible indicating that more inbred individuals resort to scavenging, which requires less body strength. There was weak evidence for competition between wolves and brown bears as well as a positive relation of human density with scavenging time. This study shows how both intrinsic and extrinsic factors relate with scavenging time for wolves but, despite a high level of inbreeding and access to carrion of anthropogenic origin, wolves mainly utilized wolf-killed ungulates.

Key words: *Canis lupus*, consumption time, intra-guild competition, human density, inbreeding, prey density, social affiliation

1. Introduction

Carnivores acquire food via predation, i.e., killing prey, and/or via scavenging, i.e., opportunistically utilizing carrion (Schaller, 1972). The level of predation versus scavenging varies between species, populations and individuals, and can change in response to intrinsic and extrinsic factors (Pereira et al., 2014). Carnivores can switch to scavenging during periods when prey are less vulnerable to predation (Pereira et al., 2014), when the density of accessible prey is low (Messier & Crete, 1985; Tallian, Smith, et al., 2017), or when anthropogenic food sources are readily available (Mattisson et al., 2016). Individual body size of carnivores can also affect levels of scavenging, as body size plays a key role in hunting success (MacNulty, Smith, Mech, et al., 2009). However, most carnivores commonly scavenge when encountering a carcass (DeVault et al., 2003; Selva et al., 2005; Wilson & Wolkovich, 2011), and scavenging is therefore an important part of food acquisition for many carnivore species.

For large carnivores, the level of scavenging versus predation can differ between human modified landscapes and protected areas. Carrion provided by humans can also be preferred, especially when the accessibility and abundance of wild prey is low (Newsome et al., 2015). For top predators like gray wolves (*Canis lupus*), the diet can be altered with the access to anthropogenic food sources like livestock (e.g., via depredation), as well as carcass dumps, and garbage sites (Newsome et al., 2015). For example, depredation was common by wolves in Portugal (Vos, 2000), the majority of scavenging done by wolves in Italy was constituted by carrion of livestock (Ciucci et al., 2020), and utilization of garbage occurred by wolves in southern Europe (Zlatanova et al., 2014).

The provision of anthropogenic food sources can show large variation in time (Wikenros et al., 2013). For example, the pulse of slaughter remains during the moose (*Alces alces*) hunting season in Scandinavia is utilized by an array of carnivore species (Gomo et al., 2017; Wikenros et al., 2013). Human activities not only result in a direct provision of food sources in terms of carrion but can also affect the access of wild prey to carnivores. Due to

74 intensive moose harvest in Scandinavia, the body condition of surviving moose is generally
75 high (Sand et al., 2012) and the rate of non-harvest mortality low (Broman et al., 2002; Ericsson
76 et al., 2001; Rönnegård et al., 2008). As a consequence, less biomass is available for scavengers
77 from moose dying of other causes than hunter harvest (Wikenros et al., 2013).

78 In this study, we explore patterns of scavenging and predation in an anthropogenic
79 landscape in Scandinavia using data from 82 study periods where we searched for carcasses
80 used by GPS-collared wolves performed between 2001 and 2019. First, we classified the cause
81 of death of different carcasses utilized by wolves, and estimated the proportion of consumption
82 time spent at scavenged carrion versus wolf-kills. Second, we examined how the proportion of
83 consumption time spent scavenging (hereafter scavenging time) by wolves was affected by a
84 set of intrinsic and extrinsic factors.

85 The Scandinavian wolf population has been subject to loss of genetic diversity and high
86 levels of inbreeding since the current population was founded in 1983 (Åkesson, Flagstad, et
87 al., 2022; Vilà et al., 2003; Viluma et al., 2022). This has caused both negative effects on
88 individual fitness (Åkesson et al., 2016; Liberg et al., 2005; Milleret et al., 2017; Wikenros et
89 al., 2021) and increasing incidence of congenital anomalies (Räikkönen et al., 2006).
90 Inbreeding has been shown to negatively affect body condition in several wolf populations
91 (Fredrickson & Hedrick, 2002; Keller & Waller, 2002; Laikre & Ryman, 1991), and highly
92 inbred wolves might therefore be less successful when hunting large ungulate prey and thus
93 more likely to resort to scavenging, which requires less body strength. We predicted that the
94 scavenging time would be greater with higher inbreeding due to associated decrease in body
95 condition. We also predicted an increased scavenging time for solitary wolves, which are
96 commonly younger and less experienced hunters, and are expected to have reduced hunting
97 efficiency compared to pack-living individuals (Sand et al., 2006; Zimmermann et al., 2015).

The season and context of the system may also affect the level of scavenging exhibited by wolves. For example, during autumn in Scandinavia there is greater availability of carrion with anthropogenic origin, i.e., hunter-killed carrion (Wikenros et al., 2013). Thus, we predicted that the scavenging time would be highest during the autumn hunting season. We also predicted that the scavenging time would increase as moose density declined, as it becomes more difficult to find vulnerable individuals in accordance with a predicted functional response (Zimmermann et al. 2015), and with an increase with brown bear (*Ursus arctos*) density. When sympatric with brown bears, wolf kill rate decreases as a result of interference competition during spring and exploitation competition during summer (Tallian, Ordiz, et al., 2017; Tallian et al., 2022). Thus, wolves living with bears may scavenge more often to make up for food lost via kleptoparasitism.

We also explored the effect of anthropogenic impact on wolf foraging pattern by testing for an effect of human density on the time spent scavenging. We predicted an increased scavenging time with higher human density as it likely results in higher presence of food sources with anthropogenic origin (Oro et al., 2013). Our study provides a detailed documentation of the feeding ecology of an inbred wolf population in a highly human-modified landscape with intensive management of ungulates and large carnivores.

2. Methods

2.1. Study area

The study was conducted in Scandinavia (Norway and Sweden) within the distribution range of the wolf population (Figure 1). The area mainly consisted of boreal forest, where most of the forest (composed of Norway spruce (*Picea abies*), Scots pine (*Pinus sylvestris*) and some deciduous tree species) was managed by clear-cutting followed by regeneration, resulting in a mosaic of conifer stands in different age classes as well as an extensive network of forest roads.

123 The climate was continental, and snow covered the ground mainly during December to March.
124 Human density averaged 25 humans per km^2 in Sweden and 15 per km^2 in Norway in 2020
125 (https://www.fn.no), with a mean of 9 per km^2 (range 1-79) within the wolf territories included
126 in this study.

127 Wolves were extirpated from most of Scandinavia, including our study area, by the end
128 of the 19th century and were functionally extinct by the 1960s. They returned to the study area
129 in the late 1970s and early 1980s through natural re-colonization from the Finnish/Russian wolf
130 population and the first reproduction occurred in 1983 (Åkesson, Flagstad, et al., 2022). By the
131 winter of 2019/2020, the population consisted of 71 territories, including 26 non-reproducing
132 and scent-marking pairs and 45 family groups (≥ 3 wolves of which ≥ 1 was a scent-marking
133 adult wolf), with the majority (78%) of the territories located in Sweden (Wabakken et al.,
134 2020). Mean family group size was 5.6 individuals (95% CI = 4.6-6.7 (Chapron et al., 2016))
135 and the largest documented family group during winter was 12 individuals (Svensson et al.,
136 2021).

137 Moose is the main prey of wolves in Scandinavia (Sand et al., 2008; Zimmermann et
138 al., 2015). The Scandinavian moose population has been one of the most heavily harvested
139 ungulate populations in the world (Lavsund et al., 2003). The moose harvest season in Norway
140 starts on September 25 and lasts until December 23. The harvest season in Sweden is allowed
141 during three weeks in September and/or from the second Monday in October until the last day
142 of January or February. Mean winter moose density was 1.3/km^2 inside wolf territories
143 (Zimmermann et al., 2015). Roe deer (*Capreolus capreolus*) density, an alternative prey for
144 wolves, was mainly below 0.5/km^2 within wolf territories located in the central and northern
145 part of the wolf breeding range, but reached up to 4.5/km^2 in more southern wolf territories
146 (Sand et al., 2016).

Other large and medium-sized carnivores in the study area included brown bear, Eurasian lynx (*Lynx lynx*), and wolverine (*Gulo gulo*). The most common scavenging species included red fox (*Vulpes vulpes*), common raven (*Corvus corax*), Eurasian jay (*Garrulus glandarius*), European pine marten (*Martes martes*), golden eagle (*Aquila chrysaetos*), and hooded crow (*Corvus cornix*) (Wikenros et al., 2013).

2.2. Wolf individual traits

Wolf social affiliation was classified as either solitary or pack (a scent-marking pair or family group), based on the Scandinavian wolf monitoring system. Monitoring is conducted annually from October 1 to March 31 using snow tracking combined with DNA analysis of scats and urine (Åkesson, Svensson, et al., 2022).

Based on a reconstructed pedigree of the Scandinavian wolf population (Åkesson et al., 2016; Liberg et al., 2005), the inbreeding coefficient (F) of adult females and males in packs (solitary wolves were excluded from the analyses including the effect of inbreeding) was calculated using CFC v. 1.0 (Sargolzaei et al., 2005). The adult wolves within a pack usually move together and are primarily responsible for the hunting of ungulates among pack members (Zimmermann et al., 2015). We therefore tested the average inbreeding coefficients of the adult female and male in a pack ($F_{average}$) in the analyses, as well as the inbreeding coefficient of the adult male (F_{male}) given the greater body size of adult males compared to adult females which may imply a greater contribution to the hunting success (MacNulty, Smith, Mech, et al., 2009; Sand et al., 2006).

2.3. Intensive studies of predation

To identify carcasses utilized by wolves, we used GPS-data collected from collared wolves (GPS-Simplex or Tellus TVP Positioning/Followit, Lindesberg, Sweden and GPS-Plus

172 Vectronic Aerospace, Berlin, Germany), stored in a Wireless Remote Animal Monitoring
173 database system for data validation and management (Dettki et al., 2014). All procedures
174 including capture, handling, and collaring of wolves (Sand et al., 2006) fulfilled ethical
175 requirements and were approved by the Swedish Animal Welfare Agency and the Norwegian
176 Experimental Animal Ethics Committee.

177 A total of 82 intensive studies were conducted between 2001-2019 on 39 wolf
178 individuals; 34 in summer (15 May to 14 September, 26 collared wolves), 8 in autumn (15
179 September to 14 December, 6 collared wolves), and 40 in winter (15 December to 14 May, 28
180 collared wolves). Seven studies overlapped two seasons by 1-8 days (median 2 days). These
181 were assigned to the season including the majority of the study period. Eleven of the studies
182 were on solitary wolves (n = 7 individuals) while 71 were on packs (n = 32 individuals). We
183 used data from one of the pack-living adult wolves (n_{males} = 61, $n_{females}$ = 10). Solitary wolves
184 were either individuals captured and collared in their natal territory during their first winter as
185 pups and where intensive studies were conducted during the dispersal phase (n = 2) or after
186 established in a new territory (n = 3), or they were captured and collared as solitary, territorial
187 wolves (n = 2). The study periods included a total of 3198 study days (mean = 39, range = 8-
188 84).

189 During the intensive studies, GPS-collars were programmed to take a location every
190 half hour (n = 30) or every hour (n = 52). For equal comparison across studies, we subsampled
191 all datasets to hourly locations. Wolves were assumed to spend time at, or in the vicinity of
192 carcasses, in order to handle, consume, and digest the food. Field crew searched for carcasses
193 within a 100 m radius of clustered GPS-locations from the entire study period (Sand et al.,
194 2005). All locations within 200 meters from one another were visited in the field and searched
195 for carcasses, with the aid of dogs during summer. In addition, single locations were
196 occasionally visited and searched for carcasses.

For all carcasses found, field crew identified the species and classified the cause of death as either wolf predation or dead by other cause (Sand et al., 2005, 2008). In this study, wolf-killed carcasses included 1) “fresh wolf-killed ungulates”, that were classified as killed by wolves based on signs of hunting tracks and/or heavy bleeding/fresh blood at carcass site and if the estimated time of death of the animal coincided with the time of the first GPS-location of the collared wolf, 2) “old wolf-killed ungulates”, based on previously mentioned signs of wolf-kills when time of death of the carcass was estimated before the study period, 3) “small prey species”, when a non-ungulate prey species was utilized by wolves, 4) “carnivore prey”, including wolf-killed wolves, red foxes and domestic dogs, and 5) “livestock”, including domestic ungulates killed by wolves. Scavenged carrion included 1) “other cause of death”, including ungulates that had died from starvation, drowning, disease, or had been killed by another species than wolf, and 2) “anthropogenic origin”, when the cause of death was either linked to human activity, including vehicle collisions, carrion left after hunter harvest, or illegal dumping of livestock carrion. Finally, carcasses that were not possible to classify as either wolf-killed, other cause of death, or anthropogenic origin were classified as “unknown cause of death”. As this category could be either killed or scavenged by the wolf, we calculated both a minimum scavenging estimate by assuming the unknown to be killed by wolves, and a maximum scavenging estimate by assuming the unknown died from other reason and were scavenged by the wolves. In the unknown category, 54% of the carcasses were estimated to have died before the start of the study periods.

2.4. Consumption time

We defined wolf consumption time per carcass as the number of locations within a space-time cluster, which is a set of locations where each location was ≤ 200 meters from the next sequential location, and where ≥ 1 location within the cluster was within 200 meters of a carcass

(Carricondo-Sanchez et al., 2020; Tallian et al., 2022). Clusters were generated in R (R Core Team 2020). For each study period, we calculated total consumption time as the number of locations within space-time clusters associated with carcasses, and classified each feeding location as either predation or scavenging. When a space-time cluster overlapped several carcass sites and at least one of the carcasses was classified as a wolf kill, the cluster was assigned as predation (assuming wolves were there due to their own kill). If the wolves' first visit to the different carcass sites was done at different occasions (n = 91), then the cluster was assigned to the carcass that wolves visited most recently in time (assuming wolves were there due to the freshest carcass).

2.5. Moose density

The relative density of moose (per km^2) was estimated in the areas utilized by wolves during the winter studies, using faecal pellet group counts made during spring, after snow melt. Circular sample plots with an area of 100 m^2 were evenly distributed in a 1 km^2 grid system with 40 plots per square, 10 on each side of the square (Zimmermann et al., 2015). We counted all pellet groups deposited between leaf fall and time of spring count. Pellet counts were converted into moose winter densities by accounting for moose defecation rate (14 pellet groups per day (Rönnegård et al., 2008)) and time span between leaf fall and date of count (Sand et al., 2016). Average moose density per square was interpolated using inverse distance weighting in ArcGIS by including the 12 closest squares to any raster cell of 100 m cell size in the wolf territory. Mean moose density was extracted within the wolf territory (based on GPS-locations from wolves during the winter intensive studies) for each intensive study, as a mean of all cells falling into the wolf territory.

2.6. Brown bear density

A relative index of brown bear density was calculated using official statistics on the annual number and spatial locations of harvested brown bears (https://www.rovbase.no). Density (per km^2) was estimated using kernel density estimation in QGIS 3.16.16 with a search radius of 100 km. For each summer study for packs, the mean brown bear density was estimated within an 18 km radius buffer around the centroid of the wolf territory representing an average wolf territory size (Mattisson et al., 2013). The centroid was located during the annual monitoring of wolves (Åkesson, Svensson, et al., 2022). We used the centroid from the following monitoring season if the same adult wolf pair were still present in the territory, thus accounting for a possible change in area use due to changes in pack composition after reproduction in spring and dispersal of older pups. The centroid from the preceding monitoring season was used if the pair where not present in the territory the following season.

2.7. Human density

The number of inhabitants (per km^2) was calculated based on human population size on municipality level from Sweden (https://www.scb.se/) and Norway (https://www.ssb.no/), and estimated for each intensive study as mean human density within an 18 km radius buffer around the centroid of the wolf territory. The mean human density between the present and the following year of the study was used. This was done to better coincide with the timing of the monitoring period that overlaps two calendar years. When the buffer overlapped with several municipalities, the mean was calculated. Because solitary wolves were not monitored, we lacked official locations of centroids of their territories and instead we used centroids that were extracted from the solitary wolf locations during the study.

2.8. Statistical analyses

To analyse variation in the proportion of time spent scavenging versus consuming wolf-killed prey, we fitted generalized linear mixed models (GLMMs) with a binominal distribution using the R-package glmmTMB (Brooks et al., 2017). The dependent variable "proportion of time spent scavenging" was defined as the number of locations within space-time clusters assigned to scavenged food divided by total consumption time (i.e., the total number of feeding locations within space-time clusters) per intensive study. The total consumption time was also included as a weight to account for unequal sample size across studies. We tested with both the maximum and the minimum estimates of scavenging time as dependent variables (results for models using the minimum estimate are shown in the Supplemental Information). Wolf ID (either as a pair ID for the adult wolves in a territory or as an individual ID for solitary wolves) was included as a random factor to account for repeated observations from the same wolves. Human density was log-transformed, and all explanatory continuous variables were centred and standardized, using the *scale* command in R, to improve interpretability of regression coefficients (Schielzeth, 2010).

We first analysed the full dataset using season (summer, autumn, winter), social affiliation of wolves (solitary, pack), and human density (range: 0.82-79.02/km^2) as explanatory variables. To be able to include seasonal explicit variables, we conducted separate analyses for winter and summer for packs only; the sample size from autumn was too small and seasonal explicit variables were not available for solitary wolves. In the seasonal models, we included the variables inbreeding coefficient (range: $F_{average}$ 0.13-0.31, F_{male} 0-0.36), brown bear density in summer (as they hibernate in winter, range 0-0.0043/km^2), and moose density in winter (range 0.25-3.29/km^2), in addition to human density both in summer and winter.

We used AIC model selection, corrected for small sample sizes (AICc), to compare the performance of $F_{average}$ and F_{male} and retained the one with lowest AICc, when comparing the univariate models, for further analyses. We performed AICc model selection on all

296 combinations of explanatory variables, including an intercept only model (Table 1). We used
297 Nakagawa´s R2 (Lüdecke et al., 2021) to calculate the variance explained by the explanatory
298 variables (marginal) as well as for explanatory variables and the random factor (conditional).
299 We considered models within $\Delta AICc \leq 2$ (referred to as top models) to be equally important
300 (Burnham & Anderson, 2002), and conducted the statistical analyses in R (R Core Team 2020).
301

302 **3. Results**

303 A total of 69,616 GPS-locations (representing all wolf time) were taken during the intensive
304 studies, of which 14,205 locations were within space-time clusters (defined as consumption
305 time). The space-time clusters consisted of 12,137 locations at wolf kills, 823 locations at
306 scavenging sites, and 1245 locations at carcasses with unknown cause of death.

307 The intensive studies (n = 82) included 1362 observations of wolves utilizing carcasses
308 (in total 1426 of which 64 double or multiple carcasses). The majority of the carcasses were
309 wolf-killed (80.5%) and a small part had died from other natural causes (1.9%). The remaining
310 had either anthropogenic mortality causes (4.7%), or the cause of death was unknown (12.9%).
311 Ungulates were the most commonly found carcasses (in total 85.9% of which 69.9% was wolf-
312 killed, Figure 2a, Table S1). The major part of the remaining carcasses consisted of small prey
313 species (8.0%), depredation events (2.1%), scavenging on livestock (1.8%), and unknown
314 species (1.7%). Intra-guild predation and intraspecific killing was rare (0.5%) with four out of
315 the seven carcasses almost entirely consumed by wolves.

316 The major part of wolf consumption time was spent on fresh wolf-killed ungulates
317 (72.3%, Figure 2b). Wolves spent between 6% (mean, 95% CI: 3-9) of their consumption time
318 scavenging considering the minimum estimate per intensive study and 13% (95% CI: 9-18)
319 considering the maximum estimate.
320

3.1. Effects of season, social affiliation, and human density

When using the full dataset and the maximum estimate of scavenging time four models had a ΔAICc ≤ 2. The highest ranked model included season and social affiliation. Season was retained in all four top models, while social affiliation was only included in two of the four (Table 1). The maximum scavenging time was higher during winter compared to summer and autumn, while there was no difference between summer and autumn. The scavenging time was also higher for solitary wolves than for packs (Figure 3, Table 2). Human density was included in two of the top models (Table 1) with a negative correlation with the maximum estimate of scavenging time, but the confidence interval of one of the estimates included zero, indicating only weak evidence for a negative relation (Table 2). The top models with minimum and maximum estimates of scavenging time showed similar results (Figure S1) and the two highest ranked models had the same sets of explanatory variables for minimum (Table S2) and maximum estimates of scavenging time (Table 2). Human density was not included among the top models when using the minimum estimate of scavenging time (Table S2).

3.2. Effects of inbreeding, moose density, and human density during winter

When using the winter data and the maximum estimate of scavenging time three models had a ΔAICc ≤ 2. The highest ranked model included inbreeding and moose density with inbreeding retained in two of the three top models while moose density was retained in all (Table 1). The scavenging time increased with both moose density and the inbreeding coefficient $F_{average}$ ($F_{average}$ performed better (ΔAICc = 1.8) in the AICc model set than F_{male}, Figure 4, Table 2). Human density was additionally included in the second highest ranked model, with a positive correlation with the maximum estimate of scavenging time (Table 2). However, when using minimum estimate of scavenging time, the moose density was not included among the top models, resulting in inconsistent effects of moose density. The three top models using the

minimum estimate of scavenging time included inbreeding ($F_{average}$) and human density, as well as the intercept only model (Table S2). Both inbreeding and human density were positively correlated with the scavenging time (Table S3, Figure S2), but showed only weak evidence as the intercept only model was included among the top three models.

3.3. Effects of inbreeding, brown bear density, and human density during summer

When using the summer data and the maximum estimate of scavenging time four models had a $\Delta AICc \leq 2$. The highest ranked model for the summer dataset using maximum estimate of scavenging time was the intercept only model, and models including brown bear density and human density were each retained in two of the four top models (Table 1). The scavenging time increased with brown bear density and human density (Table 2, Figure 5), but showed weak evidence as the intercept only model was included among the top models.

The highest ranked model using the minimum estimate of scavenging time included the inbreeding coefficient $F_{average}$ only ($F_{average}$ performed better ($\Delta AICc = 2.8$) in the AICc model set than F_{male}), and the model including inbreeding and brown bear density was the second highest ranked model (Table S2) among the two top models. The scavenging time increased with both inbreeding and brown bear density (Table S3, Figure S3).

4. Discussion

We predicted that individual characteristics may affect the capability to hunt that in turn may affect the feeding behaviour of wolves. We found that solitary and inbred wolves devoted more time to scavenging. Also extrinsic factor such as densities of main prey species, intra-guild competitors, and human density affected the proneness to scavenge. However, despite the extreme inbreeding levels among Scandinavian wolves (Åkesson et al. 2016), and humans seasonally providing large amounts of biomass from hunter harvest remains (Wikenros et al.

371 2013), wolves in Scandinavia mainly consumed wolf-killed ungulates. The weak evidence of
372 several of the explanatory variables is likely due to that the scavenging time overall was low
373 for wolves in Scandinavia.

374 Despite a more than ten times higher availability of biomass from moose carrion during
375 autumn, mainly consisting of remains from hunter harvested moose (Wikenros et al., 2013),
376 the scavenging time was not higher during autumn. Instead, contrary to our prediction, wolves
377 utilised remains from moose harvest to a higher degree during winter (69%), than during
378 autumn (17%) and summer (14%). Remains during hunting included both internal organs and
379 rumen left in the forest after a moose was shot and dumps of slaughter remains (mainly bones).
380 In a previous study conducted during autumn, wolves did not turn up on camera-monitored
381 hunter harvest remains inside wolf territories (Wikenros et al., 2013). It is likely that wolves
382 avoid scavenging on remains from hunter harvest in autumn due to the pulse in human hunting
383 activity during autumn. Such avoidance could also be expected when considering that the
384 mortality of wolves is largely due to anthropogenic factors (0.15 by legal culling, verified and
385 cryptic poaching, and vehicle collisions) and to a lesser degree to natural causes of death
386 (Liberg et al., 2020). The majority of moose are harvested during October, and the harvest
387 continues at a lower intensity until latest end of February (Wikenros et al., 2013). The increased
388 scavenging time during winter may be due to less activity by hunters in the forest compared to
389 autumn. Wolves in Scandinavia are also known to avoid human settlements and main roads
390 (Carricondo-Sanchez et al., 2020), further supporting the idea that wolves may avoid areas with
391 high human activity.

392 Biomass from vehicle collisions and other causes of death (starvation etc.) constitute a
393 smaller part (7% and 10% respectively) of available carcass biomass within wolf territories as
394 compared to remains from hunter harvest of moose (57%) (Wikenros et al., 2013). However,
395 biomass from vehicle collisions is higher in winter than summer and with less variation in

availability throughout the year compared to remains from hunter harvest (Wikenros et al., 2013). Carrion due to starvation may also have contributed to the higher scavenging time during late winter when the body condition of moose are known to be at its lowest (Cederlund et al., 1991; Sand et al., 2012). When including carcasses with an unknown cause of death as scavenged carrion, i.e., maximum estimate of scavenging time, we may have overestimated the scavenging time, e.g., if some of these carcasses was caused by wolf predation prior to the start of our intensive studies. This behaviour is likely to be more common during winter when cold temperatures keep carcasses fresh and available to scavenge during longer time compared to the warmer summer period and may partly explain the higher scavenging time during winter. However, we also found that the scavenging time by wolves was higher during winter when using the minimum estimate, which did not include carcasses with unknown causes of death.

Our prediction that solitary wolves scavenge more than packs was confirmed. We were not able to separate the different age classes of solitary wolves in the analyses given the small sample size of this category. This, and the large variation in scavenging time among solitary wolves, makes it difficult to draw conclusions. However, the observed variation may reflect the diversity among solitary wolves that recently left their natal territory and dispersed through an unknown landscape, as compared to older and more experienced solitary wolves that may suffer from reduced efficiency compared to pack hunting (Sand et al., 2006; Zimmermann et al., 2015). There is evidence that larger and more experienced wolves in Scandinavia have greater hunting success (Sand et al., 2006), which is in line with findings from other systems showing an effect of sex, age, and body size on hunting success (MacNulty, Smith, Mech, et al., 2009; MacNulty, Smith, Vucetich, et al., 2009). Packs led by older males were more successful at hunting moose than packs led by younger males, and the hunting success of packs was more dependent on male age than on female age, with males being 25-30% larger than females (Sand et al., 2006).

421 The inbreeding of wolves affected individual foraging behaviour, especially during
422 winter. Inbreeding is expected to negatively affect body condition that in turn may affect
423 hunting success, leading to increased consumption time of more easily accessed carrion.
424 Scavenging time increased in areas with high moose density and highly inbred wolves.
425 Unfortunately, sample sizes were too small to test for an interaction between moose density
426 and inbreeding. As moose kill rates increase with moose density in Scandinavia (Zimmermann
427 et al., 2015), fewer moose are killed at lower densities, and therefore less biomass is available.
428 We predicted that wolves in low moose density areas would increase scavenging time as they
429 would have to devote more of their time for finding vulnerable prey. In contrast, our results
430 showed the opposite pattern. This may have been caused by an increased availability of remains
431 from hunter harvest at high moose densities that wolves, maybe especially inbred ones, could
432 utilize, but this needs to be further investigated.
433 Consumption of other carnivores within the same guild is usually rare. However,
434 Martins et al. (2020) documented a higher carnivore-carnivore consumption in human-
435 dominated landscapes with higher densities of mesopredators and lower availability of wild
436 and domestic prey species. The reason for the killing of other carnivore species is unknown,
437 and different hypotheses have been suggested, i.e., food purpose, competition, aggressive
438 behaviour (Martins et al., 2020). The high moose densities in Scandinavia make it unlikely that
439 food purpose was the reason behind the occasional intra-guild predation events despite that the
440 carcasses were partly consumed by wolves. Intraspecific killing in the Scandinavian wolf
441 population was low with only two wolf carcasses found during the study period, assuming that
442 the remains of killed wolves would be found with the same methodology used for finding other
443 carcasses. Infrequent intraspecific aggression has been reported also in other wolf populations
444 (Mech & Boitani, 2003).

We found weak evidence that the scavenging time increased with brown bear density during summer, as would be expected due to exploitation competition (Tallian et al., 2022). Both brown bears and wolves prey heavily on neonate moose during summer in Scandinavia (Ordiz et al., 2020) and brown bear predation on neonates is generally expected to be additive to wolf predation (Griffin et al., 2011). Furthermore, wolves in Scandinavia prey primarily on newly born moose calves during this time, only occasionally hunting the less vulnerable adult and subadult age classes. Together, wolves and brown bears deplete the supply of shared neonate prey on the landscape, decreasing the overall seasonal density of their main prey. Thus, there are fewer vulnerable prey on the landscape in areas where brown bear density is high, which may facilitate at least a mild shift toward wolf scavenging (Tallian et al., 2022).

The scavenging time during summer and winter increased with human density in line with our prediction, although only with weak evidence. This likely reflects that scavenging behaviour of wolves, and/or availability of human-provided carrion, may be influenced not only by human density itself but also by human activities in the landscape. Human density had on the other hand, the opposite effect on the scavenging time when assessing the full dataset. This may partly be due to a colinear effect of social affiliation and human density. Solitary wolves scavenge more and were the only wolves present in northern Sweden, since packs were not allowed to establish in the reindeer husbandry area in northern Sweden. This is also the area with the lowest human densities included in this study (less than 5.5 inhabitants/km^2 for most solitary wolves). Unfortunately, sample sizes were too small to test for an interaction between human density and social affiliation. In addition, human density may not always be a straightforward index of human activity. Most moose hunting occurs in remote areas, resulting in seasonally high availability of biomass to scavenge in low human density areas, while other types of anthropogenic food sources are likely more temporal predictable and available in areas with high human density.

The low utilization by wolf packs of human-provided carrion in combination with a low incidence of livestock depredations contrasts with other anthropogenic landscapes where depredation by wolves is high (Vos, 2000), or where humans provide carcass dumps and garbage sites that are heavily utilized by wolves (Ciucci et al., 2020; Newsome et al., 2015). High levels of depredation and use of anthropogenic food sources can increase conflicts over carnivores and their possible impacts on human livelihoods (Newsome et al., 2015). The foraging pattern dominated by predation in Scandinavia may in this respect contribute to lower levels of conflict. However, in Scandinavia, and elsewhere in Europe, humans control densities of ungulates to a large extent through hunter harvest (Jensen et al., 2020; Linnell et al., 2020). In addition, humans contribute to a high hunting success by wolves on predator-naïve moose caused by high hunter harvest pressure on the moose population (Sand et al. 2006). When wolves primarily hunt, rather than scavenge, wolf predation on important game species can have a large impact of the possible harvest yield (Wikenros et al., 2015; Wikenros et al., 2020), representing another source of conflict in highly managed landscapes where humans, and not large carnivores, are the main mortality factor in ungulate populations. The high anthropogenic impact in Scandinavia likely affects wolf feeding behaviour due to avoidance of human activities (Carricondo-Sanchez et al., 2020) as a result of humans being the main mortality factor in the wolf population (Liberg et al., 2020).

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496

497 6. Author Contribution

498 CW, BZ, PW, HS secured funding; CW, CDB, BZ, MD, JM, AT, HS conceived and designed
499 the study; CW, CDB, BZ, MD, PW, HS compiled data; MÅ, ØF was involved in the laboratory
500 work; CW, CDB, BZ, JM carried out the statistical analyses; CW drafted the manuscript; All
501 authors revised the manuscript and gave final approval for publication.

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Figures and Tables

Figure 1. Sites with carcasses (n = 1362) found during intensive studies of predation (shown in different colours) of solitary wolves (n = 11), and adult wolves in packs (≥ 2 wolves, n = 71) in Scandinavia, 2001-2019. The coloured dots represent the carcasses found per study period.

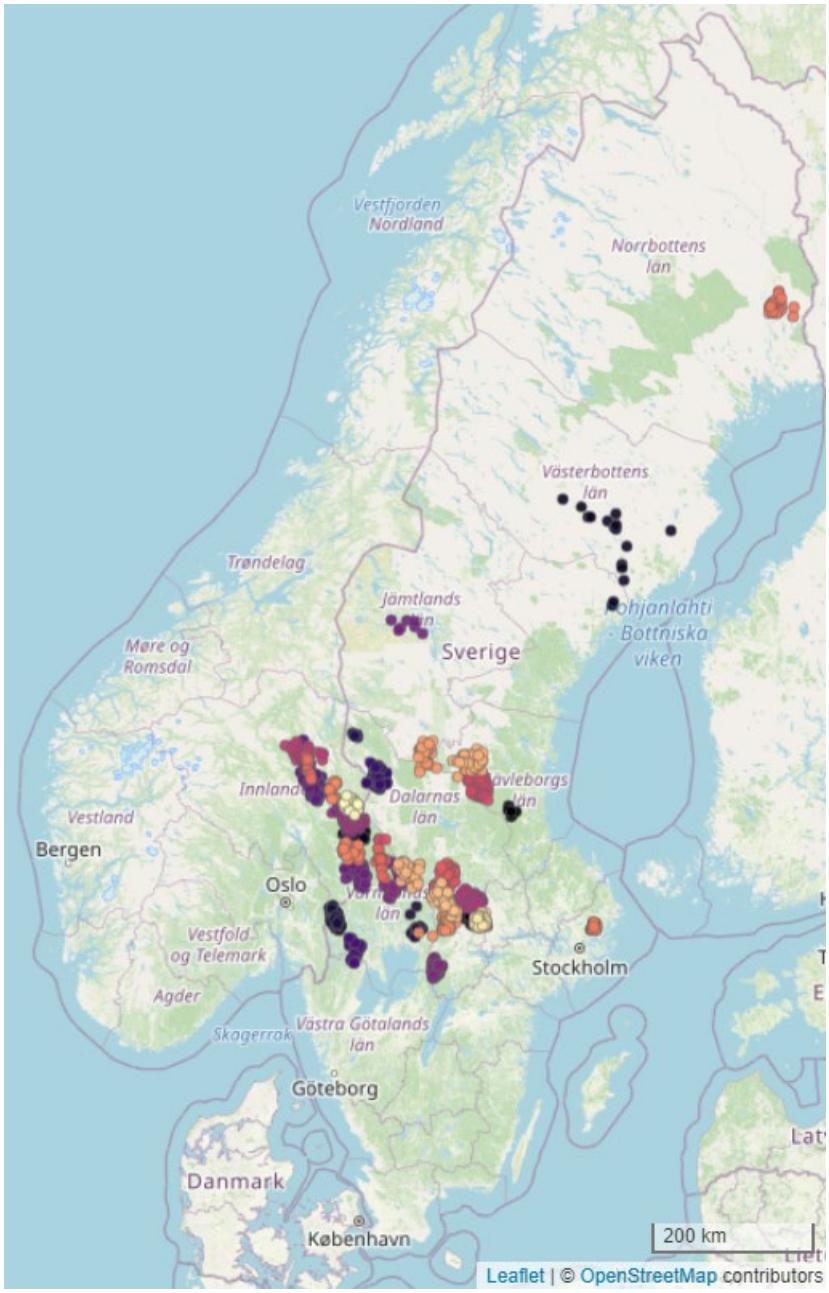

Figure 2. Relative distribution of a) number of food sources visited by wolves (n = 1362) and b) consumption time (n =14,205 GPS-locations), during intensive studies of predation (n = 82) conducted using GPS-locations from 39 wolves either solitary (n = 11) or in packs (($\geq$ 2 individuals, n = 71) in Scandinavia, 2001-2019. The inner circle shows the cause of death (wolf-killed, other cause of death, anthropogenic origin, or unknown cause of death) and the outer circle shows wolf-kills grouped as wild ungulates killed within (fresh) or before the study period (old), livestock, carnivores, or small prey species, while scavenged food sources were grouped as either wild ungulates, livestock, or unknown species.

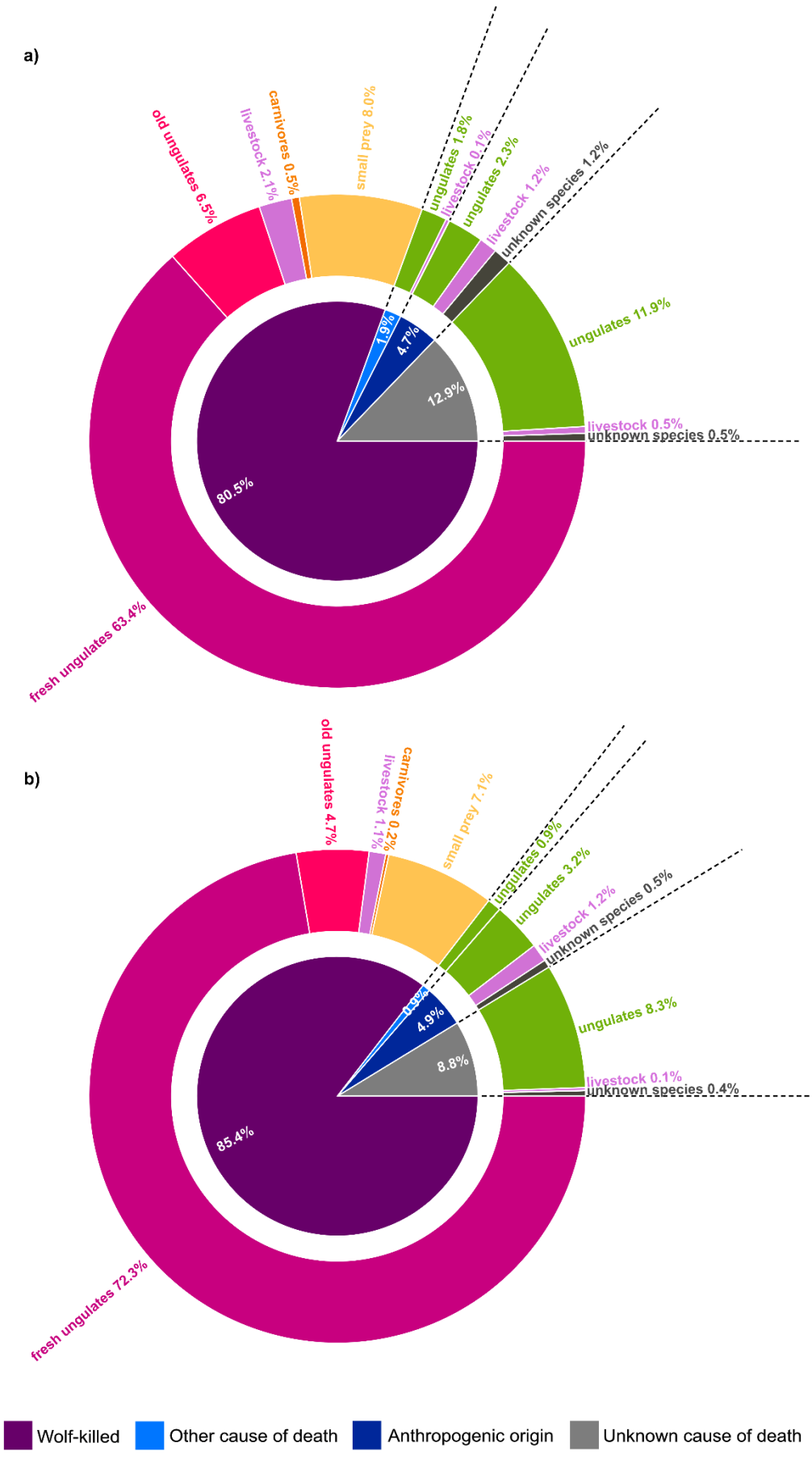
a)
old ungulates 6.5%
livestock 2.1%
carnivores 0.5%
small prey 8.0%
ungulates 1.8%
livestock 0.1%
ungulates 2.3%
livestock 1.2%
unknown species 1.2%
ungulates 11.9%
livestock 0.5%
unknown species 0.5%
fresh ungulates 63.4%
80.5%
12.9%
4.7%
1.9%
b)
old ungulates 4.7%
livestock 1.1%
carnivores 0.2%
small prey 7.1%
ungulates 0.3%
ungulates 3.2%
livestock 1.2%
unknown species 0.5%
ungulates 8.3%
livestock 0.1%
unknown species 0.4%
fresh ungulates 72.3%
85.4%
8.8%
4.9%
0.5%
Wolf-killed
Other cause of death
Anthropogenic origin
Unknown cause of death

Figure 3. Predicted proportion of maximum estimate of consumption time spent scavenging (± 95% CI) in relation to season (summer, autumn, winter) and social affiliation of wolves (solitary (grey), pack (black, ≥ 2 wolves)) from the highest ranked model based on GPS-locations of 82 intensive studies of predation in Scandinavia, 2001-2019.

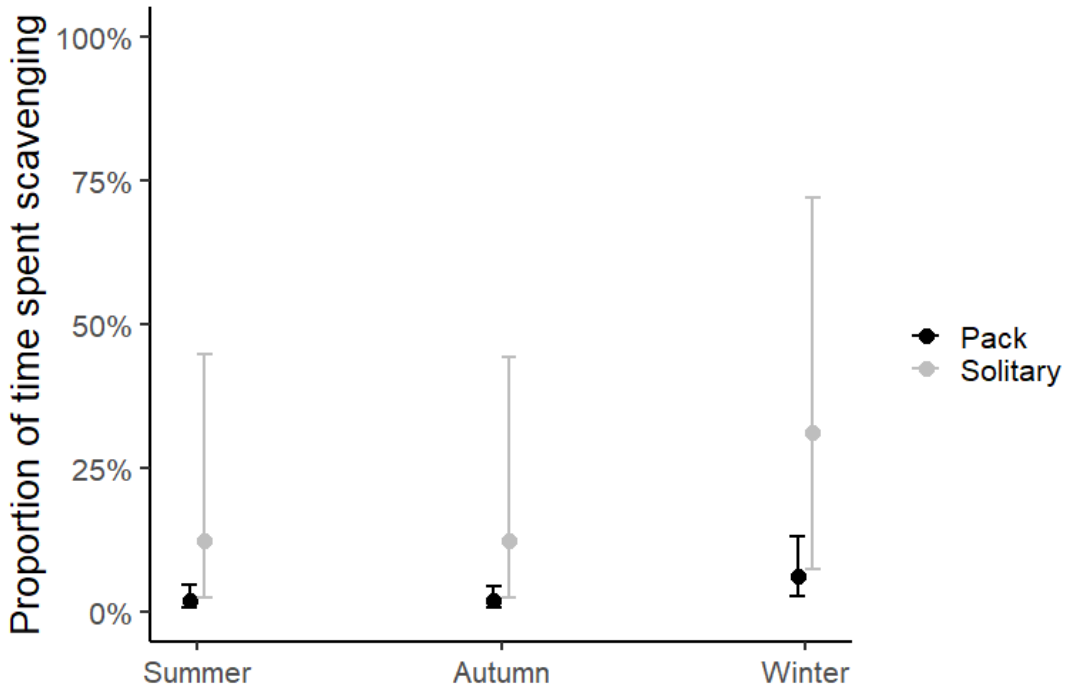

Figure 4. Predicted proportion of maximum estimate of consumption time spent scavenging during winter (± 95% CI) in relation to the average inbreeding coefficients of the adult female and male ($F_{average}$) and moose density (held constant at three different densities) for the highest ranked model. Axis are presented with original values (unscaled) and dots represent the observed values. Data was collected during intensive studies of predation (15 December to 14 May, n = 35) for wolves in packs (≥ 2 wolves) using GPS-locations from collared wolves (n = 23) in Scandinavia, 2001-2019.

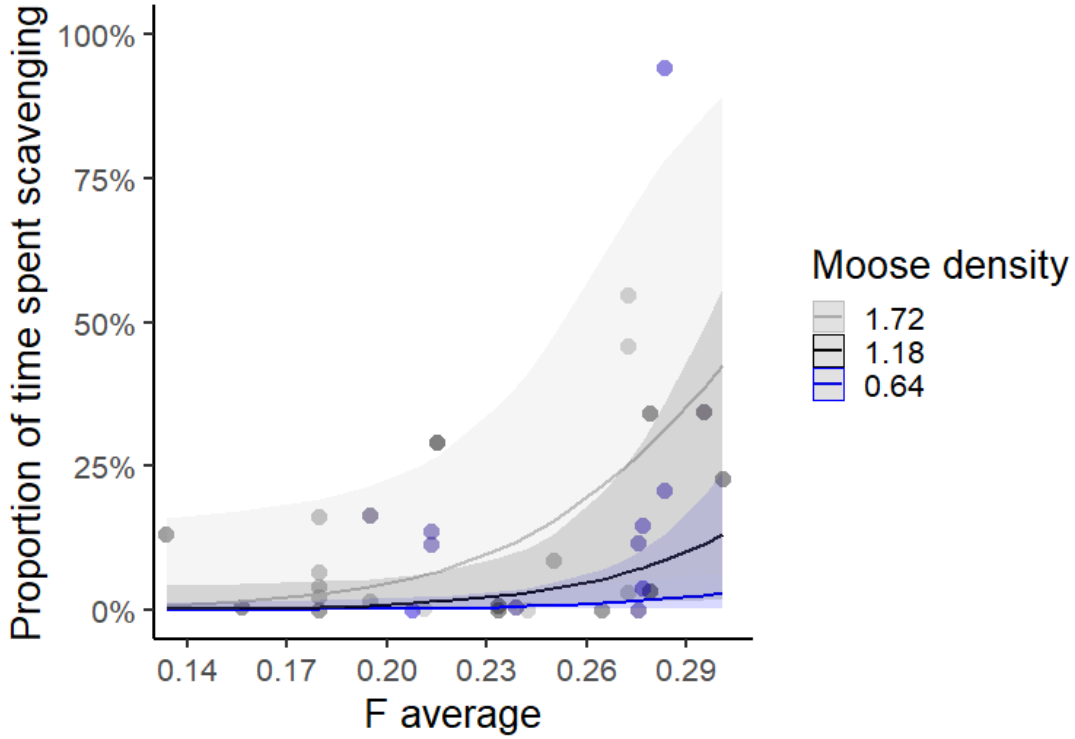

Figure 5. Predicted proportion of maximum estimate of consumption time spent scavenging during summer (± 95% CI, unscaled data) in relation to human density (log-transformed) and brown bear density (held constant at three different densities) for the third ranked model (ΔAICc = 1.2). Dots represent the observed values. Data was collected during intensive studies of predation (15 May to 14 September, n = 27) for wolves in packs (≥ 2 wolves) using GPS-locations from collared wolves (n = 21) in Scandinavia, 2001-2019.

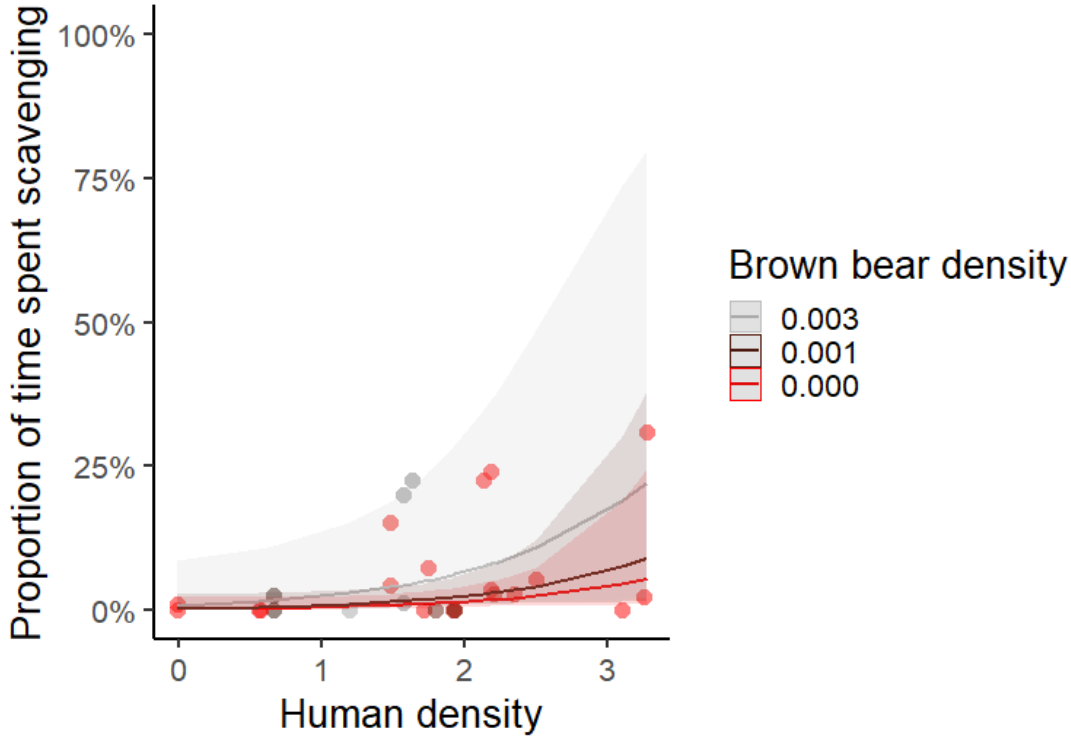

Table 1. Generalized linear mixed models to assess the effect of season (summer, autumn, winter), social affiliation (solitary, pack (≥ 2 wolves)), human density, average inbreeding coefficient of the adult female and male ($F_{average}$), and brown bear density, on the proportion of consumption time spent scavenging by wolves in Scandinavia during 2001-2019. Analyses were conducted using maximum estimates of the proportion of consumption time spent scavenging (for minimum estimates see Table S2). For all tested models, degrees of freedom (df), and difference in AICc relative to the highest-ranked model (ΔAICc) are shown. For models within ΔAICc ≤ 2, conditional ($R2_c$) and marginal ($R2_m$) Nakagawa´s R2 are also shown.

Dataset	No.	Intercept	Season	Social	Human	$F_{average}$	Moose	Bear	df	ΔAICc	$R2_c$	$R2_m$
Annual	1	X	X	X		-	-	-	5	0	0.627	0.073
n = 82	2	X	X			-	-	-	4	1.5	0.639	0.038
	3	X	X		X	-	-	-	5	2.0	0.658	0.057
	4	X	X	X	X	-	-	-	6	2.0	0.636	0.073
		X			X	-	-	-	3	188.3		
		X		X	X	-	-	-	4	189.9		
		X		X		-	-	-	3	190.8		
		X				-	-	-	2	191.6		
Winter	1	X	-	-		X	X	-	4	0	0.791	0.201
n = 35	2	X	-	-	X	X	X	-	5	0.3	0.789	0.277
	3	X	-	-			X	-	3	1.0	0.810	0.148
		X	-	-	X		X	-	4	2.4		
		X	-	-		X		-	3	11.5		
		X	-	-				-	2	11.8		
		X	-	-	X	X		-	4	13.8		
		X	-	-	X			-	3	14.2		
Summer	1	X	-	-			-		2	0	0.585	-
n = 27	2	X	-	-	X		-		3	0.6	0.577	0.072
	3	X	-	-	X		-	X	4	1.2	0.570	0.156
	4	X	-	-			-	X	3	1.7	0.577	0.028
		X	-	-		X	-		3	2.2		
		X	-	-	X	X	-		4	3.4		
		X	-	-	X	X	-	X	5	4.0		
		X	-	-		X	-	X	4	4.4		

Table 2. Conditional model parameter estimates (ß) with standard error (SE) and 95% CI (explanatory variables shown in bold when not overlapping zero) for each explanatory variable retained in the models with ΔAICc ≤ 2 (Table 1). The reference in the analyses is "autumn" for season, and "pack" for social affiliation. Analyses were conducted using maximum estimates of the proportion of consumption time spent scavenging for annual, winter and summer intensive studies of wolf predation in Scandinavia, 2001-2019 (for minimum estimate see Table S3).

Dataset	Model no.	Explanatory variable	ß	SE	95% CI
Annual	1	Intercept	-3.860	0.429	-4.701 – -3.019
n = 82		Season: summer	-0.014	0.141	-0.29 – 0.262
		Season: winter	1.159	0.131	0.902 – 1.416
		Social affiliation: solitary	1.916	0.971	0.013 – 3.819
	2	Intercept	-3.546	0.412	-4.354 – -2.738
		Season: summer	-0.014	0.141	-0.29 – 0.262
		Season: winter	1.157	0.131	0.9 – 1.414
	3	Intercept	-3.526	0.421	-4.351 – -2.701
		Season: summer	-0.024	0.142	-0.302 – 0.254
		Season: winter	1.143	0.132	0.884 – 1.402
		Human density	-0.462	0.366	-1.179 – 0.255
	4	Intercept	-3.815	0.444	-4.685 – -2.945
		Season: summer	0.019	0.141	-0.257 – 0.295
		Season: winter	1.153	0.132	0.894 – 1.412
		Social affiliation: solitary	1.695	1.072	-0.406 – 3.796
		Human density	-0.201	0.377	-0.94 – 0.538
Winter	1	Intercept	-3.594	0.703	-4.972 – -2.216
n = 35		**Moose density**	1.579	0.519	0.562 – 2.596
		$F_{average}$	1.267	0.659	-0.025 – 2.559
	2	Intercept	-3.831	0.696	-5.195 – -2.467
		Moose density	1.729	0.556	0.639 – 2.819
		$F_{average}$	1.423	0.631	0.186 – 2.66
		Human density	1.050	0.665	-0.253 – 2.3534
	3	Intercept	-3.627	0.778	-5.152 – -2.102
		Moose density	1.602	0.539	0.546 – 2.658
Summer	1	Intercept	-4.138	0.579	-5.273 – -3.003
n = 27	2	Intercept	-4.235	0.557	-5.327 – -3.143
		Human density	0.748	0.526	-0.283 – 1.779
	3	Intercept	-4.182	0.509	-5.18 – -3.184
		Bear density	0.702	0.458	-0.196 – 1.6
		Human density	0.950	0.505	-0.04 – 1.94
	4	Intercept	-4.090	0.559	-5.186 – -2.994
		Bear density	0.469	0.505	-0.521 – 1.459

Supplemental Information for CHAPTER 4:

Explaining the extent of scavenging by wolves in an anthropogenic landscape

Camilla Wikenros, Cecilia Di Bernardi, Barbara Zimmermann, Mikael Åkesson, Maike Demski, Øystein Flagstad, Jenny Mattisson, Aimee Tallian, Petter Wabakken, and Håkan Sand

Corresponding author: camilla.wikenros@slu.se

Figure S1. Model output using maximum estimate of the proportion of consumption time spent scavenging (Maximum, ± 95% CI) compared to minimum estimate (Minimum) for the highest ranked model including season (summer, autumn, winter) and social affiliation of wolves (solitary, pack (≥ 2 wolves)). The reference values are "autumn" for season and "pack" for social affiliation. Data collected during intensive studies of predation (n = 82) conducted using GPS-locations from collared wolves (n = 39) in Scandinavia, 2001-2019.

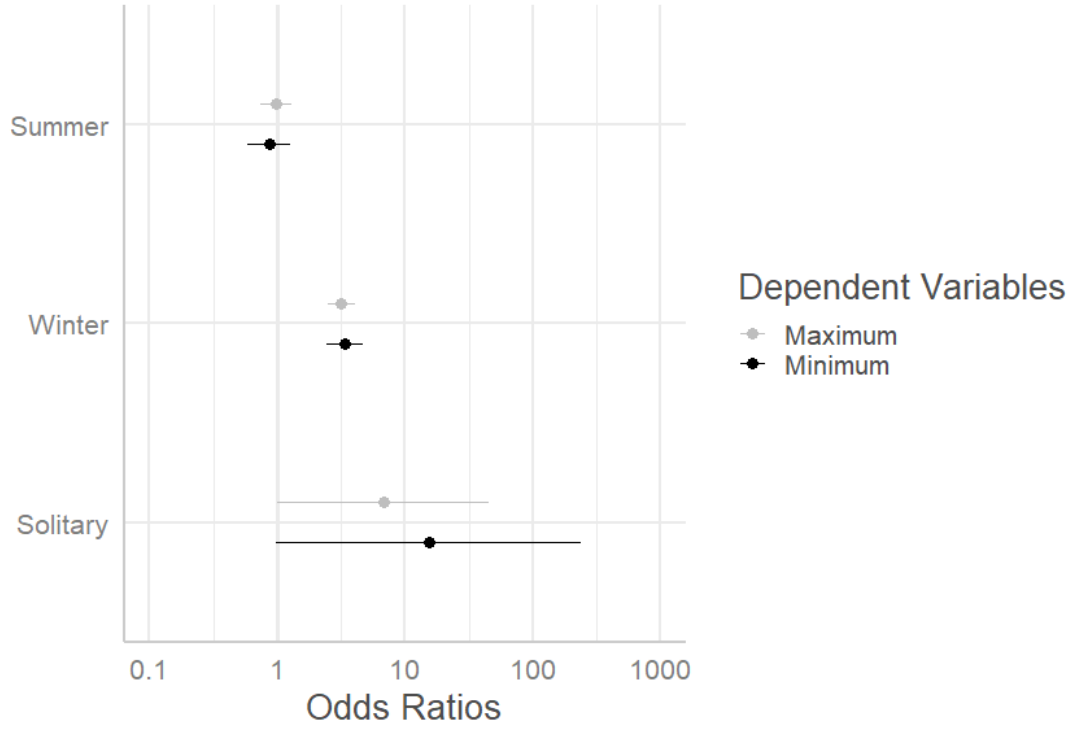

Figure S2. Predicted proportion of minimum estimate of consumption time spent scavenging during winter (± 95% CI, unscaled data) in relation to the average inbreeding coefficients of the adult female and male ($F_{average}$) and human density (log-transformed, held constant at three different values) for the second ranked model (ΔAICc = 0.9). Dots represent the observed values. Data collected during intensive studies of predation (15 December to 14 May, n = 35) for wolves in packs (≥ 2 wolves) using GPS-locations from collared wolves (n = 23) in Scandinavia, 2001-2019.

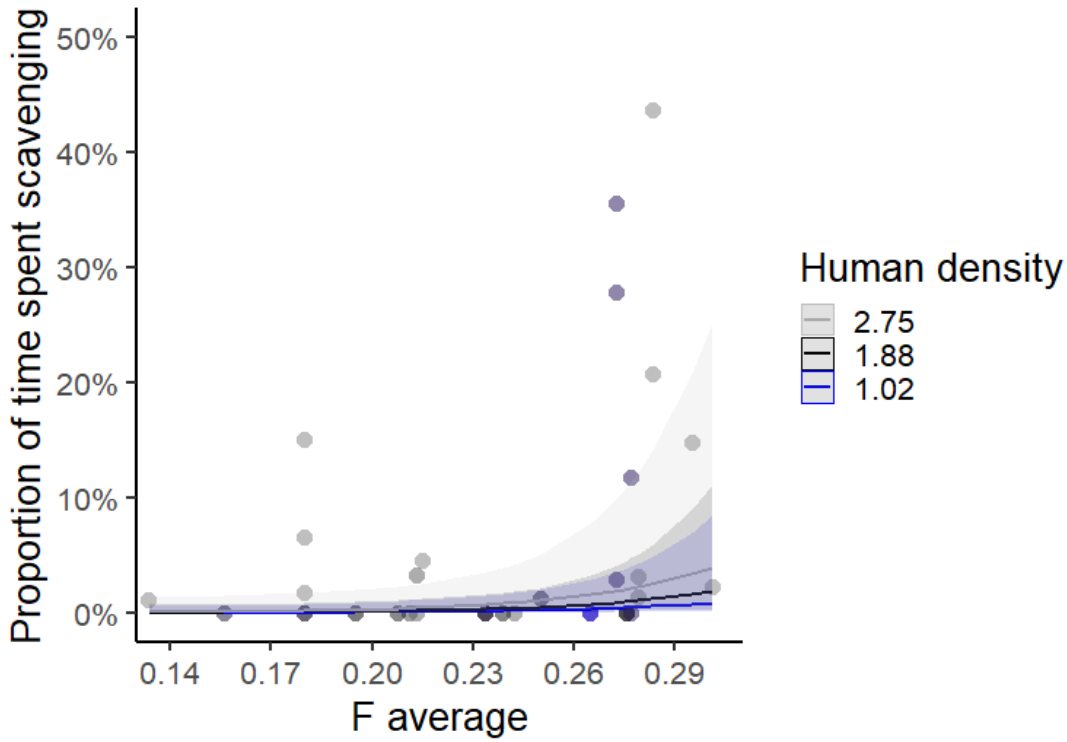

Figure S3. Predicted proportion of minimum estimate of consumption time spent scavenging during summer (± 95% CI), unscaled data in relation to the average inbreeding coefficients of the adult female and male ($F_{average}$) and brown bear density (held constant at three different densities) for the second ranked model (ΔAICc = 1.4). Dots represent the observed values. Data collected during intensive studies of predation (15 May to 14 September, n = 27) for wolves in packs (≥ 2 wolves) using GPS-locations from collared wolves (n = 21) in Scandinavia, 2001-2019.

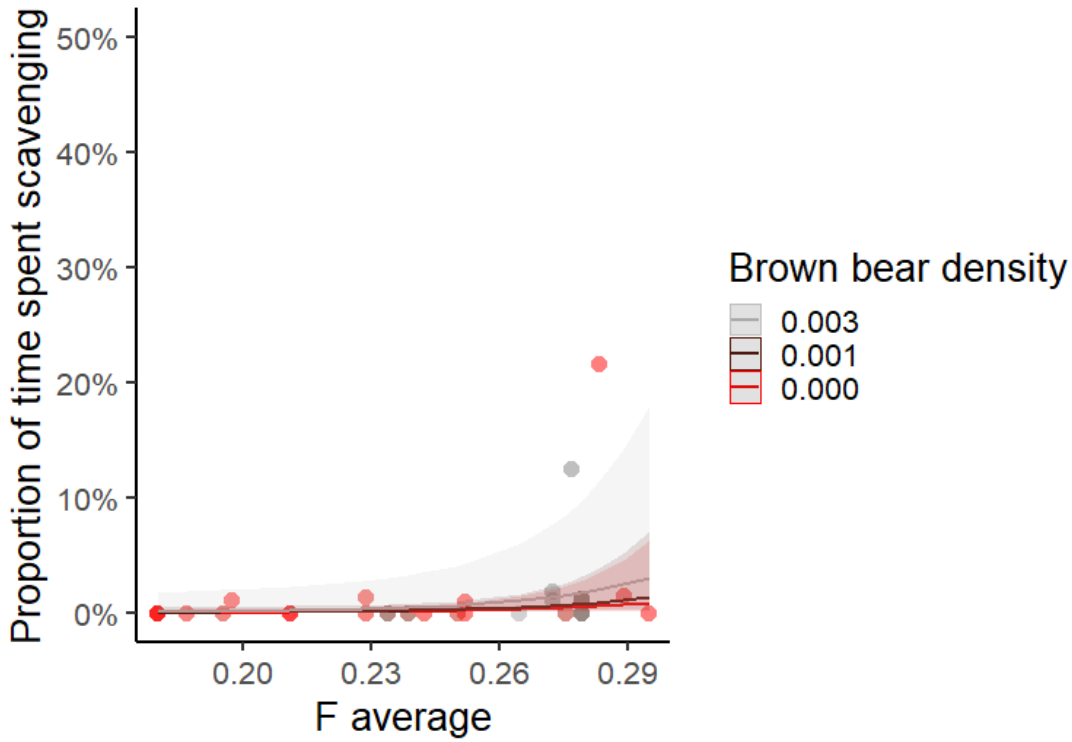

Table S1. Carcasses (n = 1362) utilized by wolves during 82 intensive studies of predation in Scandinavia during 2001-2019. Species are specified in alphabetical order according to common name, scientific name, and are grouped as wolf-killed, other cause of death, anthropogenic origin, or unknown cause of death.

Common name	Scientific name	Wolf-killed*	Other cause of death	Anthropogenic origin	Unknown cause of death
Badger	*Meles meles*	18			
Beaver	*Castor fiber*	18			
Black grouse	*Tetrao tetrix*	25			
Capercaillie	*Tetrao urogallus*	11			
Cattle	*Bos taurus*	2		8	
Hooded crow	*Corvus cornix*	1			
Dog	*Canis familiaris*	1			
Hare	*Lepus spp.*	12			
Hazel grouse	*Tetrastes bonasia*	2			
Magpie	*Pica pica*	1			
Moose	*Alces alces*	818	15	28	122
Pig	*Sus scrofa domesticus*			4	
Red deer	*Cervus elaphus*	6			1
Red fox	*Vulpes vulpes*	4			
Reindeer	*Rangifer tarandus*	22	1		5
Roe deer	*Capreolus capreolus*	124	10	3	39
Sheep	*Ovis aries*	6		5	1
Siberian jay	*Perisoreus infaustus*	1			
Squirrel	*Sciurus vulgaris*	1			
Unknown bird species	*NA*	14			
Unknown species	*NA*	2		16	7
Vole	*Cricetidae spp.*	3			
Wild boar	*Sus scrofa*	3			
Wolf	*Canis lupus*	2			

Table S2. Generalized linear mixed models to assess the effect of season (summer, autumn, winter), social affiliation (solitary, pack (≥ 2 wolves)), human density, average inbreeding coefficient of the adult female and male ($F_{average}$), and brown bear density on the proportion of consumption time spent scavenging of wolves in Scandinavia during 2001-2019. Analyses were conducted using minimum estimates of the proportion of consumption time spent scavenging. For all tested models, degree of freedom (df), and difference in AICc relative to the highest-ranked model (ΔAICc) are shown. For models within ΔAICc ≤ 2, conditional ($R2_c$) and marginal ($R2_m$) Nakagawa´s R2 are also shown.

Dataset	No.	Intercept	Season	Social	Human	$F_{average}$	Moose	Bear	df	ΔAICc	$R2_c$	$R2_m$
Annual	1	X	X	X		-	-	-	5	0	0.763	0.080
n = 82	2	X	X			-	-	-	4	1.3	0.776	0.031
		X	X	X	X	-	-	-	6	2.3		
		X	X		X	-	-	-	5	2.8		
		X		X		-	-	-	3	124.1		
		X				-	-	-	2	125.0		
		X			X	-	-	-	3	125.3		
		X		X	X	-	-	-	4	125.8		
Winter	1	X	-	-		X		-	3	0	0.713	0.106
n = 35	2	X	-	-	X	X		-	4	0.9	0.720	0.176
	3	X	-	-				-	2	0.9	0.721	-
		X	-	-		X	X	-	4	2.1		
		X	-	-	X			-	3	2.4		
		X	-	-	X	X	X	-	5	2.8		
		X	-	-			X	-	3	3.2		
		X	-	-	X		X	-	4	4.8		
Summer	1	X	-	-		X	-		3	0	0.627	0.270
n = 27	2	X	-	-		X	-	X	4	1.4	0.628	0.305
		X	-	-	X	X	-		4	2.8		
		X	-	-			-	X	3	2.8		
		X	-	-			-		2	3.2		
		X	-	-	X		-	X	4	3.8		
		X	-	-	X	X	-	X	5	3.9		
		X	-	-	X		-		3	5.3		

Table S3. Conditional model parameter estimates (ß) with standard error (SE) and 95% CI (explanatory variables shown in bold when not overlapping zero) for each explanatory variable retained in the models within ΔAICc ≤ 2 shown in Table S2. The reference in the analyses is "autumn" for season, and "pack" for social affiliation. Analyses were conducted using minimum estimates of the proportion of consumption time spent scavenging for annual, winter and summer intensive studies of wolves in Scandinavia, 2001-2019.

Dataset	Model no.	Explanatory variable	ß	SE	95% CI
Annual	1	Intercept	-6.439	0.704	-7.819 – -5.059
n = 82		Season: summer	-0.014	0.197	-0.4 – 0.372
		Season: winter	1.221	0.167	0.894 – 1.548
		Social affiliation: solitary	2.730	1.404	-0.022 – 5.482
	2	Intercept	-6.033	0.679	-7.364 – -4.702
		Season: summer	-0.140	0.197	-0.526 – 0.246
		Season: winter	1.219	0.166	0.894 – 1.544
Winter	1	Intercept	-5.423	0.769	-6.93 – -3.916
n = 35		$F_{average}$	1.104	0.608	-0.088 – 2.296
	2	Intercept	-5.581	0.777	-7.104 – -4.058
		$F_{average}$	1.190	0.593	0.028 – 2.352
		Human density	0.765	0.604	-0.419 – 1.949
	3	Intercept	-5.469	0.839	-7.113 – -3.825
Summer	1	Intercept	-6.256	0.736	-7.699 – -4.813
n = 27		**$F_{average}$**	1.543	0.641	0.287 – 2.799
	2	Intercept	-6.220	0.706	-7.604 – -4.836
		$F_{average}$	0.555	0.480	-0.386 – 1.496
		Bear density	1.349	0.649	0.077 – 2.621

8. GENERAL DISCUSSION

In this thesis, a new molecular method to detect prey DNA in wolf scats has been developed and validated through controlled feeding experiments. Such investigations highlighted the value of using multiple molecular markers for each target prey species and the relevance of including sensitivity in the validation of molecular methods. Following the initial methodological stages, this approach was applied to investigate the feeding behaviour of wolves across the anthropized landscape of Sweden, where wolves thrive on multiple wild ungulate species whose relative abundance varies across the landscape. In relation to changes in abundance of the main and alternative ungulate prey species across the landscape, marked differences were observed in wolves' feeding patterns. GPS-data showed how other extrinsic factors, i.e. season and density of brown bear and humans affected scavenging in the Scandinavian wolf population. In combination with these extrinsic factors, differential feeding patterns were related to individual traits of wolves such as sex, social status, and level of inbreeding when using both methods.

8.1. DNA-method validation for reliable implementation into ecological frameworks

The analysis of DNA for binary prey detection in predator scats is receiving increasing attention in the field of feeding ecology and trophic interactions (Traugott et al., 2021). The developed diagnostic method with species-specific markers (chapter 1) added to the growing number of studies developing DNA-methods to examine carnivore diet (e.g. Hacker et al., 2021; Quéméré et al., 2021; Roffler et al., 2021). However, the rapid development of this field largely lacks validations, and the prioritization of specificity using conservative cut-offs for binary detection risks to result in loss of sensitivity (Divoll et al., 2018). We followed recent guidelines highlighting the relevance of adjusting the detection procedure by basing thresholds

on empirical data in order to attain detections better fitted to the actual sample and to each specific situation (Alberdi et al., 2018).

In the method development and optimization stages described in the first chapter, specificity was evaluated and maximized using specific and non-specific reference tissue samples and empirical thresholds tailored for each molecular marker. Additionally, we utilized multiple species-specific molecular markers for each species in line with previous attempts to increase sensitivity through additively pooling results of multiplexing primers (Alberdi et al., 2018; De Barba et al., 2014). By targeting several loci with different markers for the same species, we aimed to increase taxonomic coverage within each species and the overall method sensitivity. In the second chapter, we experimentally examined the method sensitivity and assessed the optimal threshold to balance the occurrence of false negatives and false positives. We observed how the use of several markers, instead of only one per species, resulted in higher sensitivity for all target species. Pooling results from multiple markers can reduce the number of false negatives (Gibson et al., 2014), but it may also increase the risk of introducing false positives (Alberdi et al., 2018). This pattern was observed when setting low thresholds of minimum number of markers for confirmed detection. Through the analysis of false negatives with empirical data from feeding experiments, we could therefore include sensitivity in the evaluation of the optimal threshold. Even if specificity was maximized in chapter 1 by using cut-offs based on reference samples tailored for each marker, we recommended the use of a low threshold (intended as the number of amplifying markers required to confirm detection) to concurrently maximize sensitivity, suggesting the use of two markers as threshold. Despite the development of markers as specific as possible, occasional non-specific amplifications can occur in the developed markers and we therefore cautioned against the use of only one marker as threshold. Supporting previous findings, the results highlighted the relevance of setting cut-

offs that are systematically and empirically validated to optimize detection in order to maximize the rate of true positives.

Additionally, the method sensitivity for scats from wolves in captivity depended on the species they consumed. Potential alternative explanations were raised for the differences in detection probability observed between target prey species but the cause behind such patterns is still not known. Acknowledging detection biases represents a critical step to correctly interpret results when applying such molecular method into ecological frameworks. Our study (chapter 2) added to the small body of literature validating molecular methods for diet analysis with experimental feeding trials, a field that needs more attention in order to accurately exploit the rapidly developing analytical tools to investigate diet from DNA (Alberdi et al., 2019; Dahl et al., 2022).

8.2. Traits of individual wolves and abundance of co-occurring species as drivers of feeding ecology

Taking advantage of the genetic monitoring of the Scandinavian wolf population, we were able to associate wolf individual traits to wolves' prey consumption using the faecal DNA analysis (chapter 3) and to the extent of scavenging using GPS-data (chapter 4). Overall, the results supported our predictions of more scavenging and of higher use of roe deer while lower use of moose for individual wolves that were expectedly less skilled hunters. Specifically, solitary wolves were characterized by a higher level of scavenging, and solitary females showed a lower consumption of moose compared to territorial females living in pairs or packs. Such patterns may be explained by solitary individuals being less capable to hunt successfully compared to pack-living individuals, and possibly avoiding the potential increased risk of injury. Although the food reward after killing the large bodied moose is high, this is also a species that can defend itself to an extent that that wolves are facilitated by cooperative hunting (Mech &

Peterson, 2003). An effect of sex was observed for solitary wolves. The higher use of roe deer and tendency for lower use of moose for females likely suggests that their smaller body size compared to males may expose them to higher risks when handling moose (MacNulty et al., 2009; Sand et al., 2006). The fact that we found no differences between sexes in moose and roe deer use for pairs and packs may be explained by cooperative hunting where the two territorial members of the pair share both the hunting effort and the predation outcome (Sullivan, 1978; Zimmermann et al., 2015).

The Scandinavian wolf population is highly inbred and it is shown that inbred wolves in the population suffer from lower fitness (Åkesson et al., 2016; Liberg et al., 2005). We revealed a tendency for higher use of roe deer the higher the inbreeding coefficient, both for solitary wolves and pack members. Additionally, the proportion of consumption time spent scavenging increased with the average inbreeding coefficient of the adult female and male. Although we are not aware of the inherent mechanics of such relationships, the observed patterns may suggest reduced hunting success resulting from decreased body size and conditions, previously associated to higher levels of inbreeding (Fredrickson & Hedrick, 2002; Räikkönen et al., 2013). Future investigations integrating such analysis with direct measures of body size or conditions may help clarifying the causality of the revealed pattern. Anyhow, the relation of feeding patterns with social status and inbreeding coefficient, observed in both studies, may suggest circumstantial support for the higher hunting success among stronger and more experienced wolves in Scandinavia.

The consumption patterns observed from the scats analysed in the third chapter were affected by the relative abundance of wild ungulates at the landscape level. Positive relationships were observed between use and abundance of the two main prey of wolves, moose and roe deer. In particular, our study confirmed the role of roe deer abundance on wolf predation patterns previously observed in Scandinavia, and added to an existing literature in

Europe showing a high consumption of roe deer when available in high densities, possibly making such a small prey more profitable (Milanesi et al., 2012; Nowak et al., 2005, 2011). The strong relationship between increasing roe deer abundance and decreasing use of a larger but potentially more dangerous prey, moose, confirmed previous findings as well (Sand et al., 2016). Novel compared to previous research on this wolf population is the influence of alternative ungulates abundances, given the recent expansion south into multi-ungulate prey areas (Rodríguez-Recio et al., 2022). The observed lower use of the main prey (moose and roe deer) with increasing abundance of alternative ungulates may reflect the response of an opportunistic predator to shifts in prey species composition and to a broader diversity of prey species available (Okarma, 1995).

In the last chapter, scavenging constituted only a marginal part of the consumption time of wolves in Scandinavia (6-15%). In fact, despite access to carrion with anthropogenic origin or killed by natural causes, wolves utilized those to a minor extent compared to wolf-killed wild ungulates. The increased proportion of consumption time spent scavenging during winter may be in line with the observed avoidance of human settlement and main roads by wolves in Scandinavia (Carricondo-Sanchez et al., 2020). Indeed, even though the majority of moose is harvested in autumn (peak in October), the harvest continues until February (Wikenros et al., 2013) and the higher scavenging during winter may indicate the use of hunting remains in the end of the season when activity by hunters in the forest is reduced. In line with our prediction, the increased scavenging with higher brown bear density during summer was likely explained by competition between wolves and brown bears (Tallian et al., 2022). Both carnivores predate heavily on neonate moose in Scandinavia (Ordiz et al., 2020). When brown bear densities are high, there are fewer vulnerable prey in the landscape and this may cause a partial shift toward scavenging by wolves (Tallian et al., 2022). Despite we got a moderate support for our prediction of positive effect of human density on the extent of scavenging during summer and

winter, this result was not consistent across the analyses. Indeed, on the whole dataset we found support for the opposite pattern, and the higher scavenging at lower human densities may reflect anthropogenic food sources related to specific human activities occurring in remote areas, such as hunting. We solicit future research to further investigate how the scavenging behaviour of wolves may be influenced not only by human density itself but also by human activities in the landscape.

8.3. Concluding remarks

Based on the developed method to detect prey DNA in wolf scats, our studies highlighted the relevance of assessing method sensitivity and including it in the evaluation of optimal thresholds for binary detection. Such sensitivity validations are unfortunately largely overlooked in the growing field detecting prey DNA in predator scats, potentially leading to inaccurate answers to ecological questions. Our method is part of a broader body of multiple approaches developed to conduct DNA-based diet analysis of predators, such as metabarcoding with next-generation sequencing (Traugott et al., 2021). The comparison of these different molecular methodologies, ideally using an experimental set-up, can help identifying the pitfalls and knowledge gaps to be addressed by future lines of research in this rapidly developing field. Additionally, comparing the performance of different approaches can help increasing the comparability of the provided results.

Our findings underline the opportunistic and flexible nature of wolves' behaviour and show support for variation at the individual level in relation to intrinsic traits. Adding to a small body of literature, our study advocates a line of research looking into the range of biological and behavioural traits related to the individual condition and experience, which can play a role in determining feeding behaviour. For instance, as wolves are a group-living species, hunting experience during the early stages of life may influence their feeding behaviour once

establishing their own territory. If this learning experience improves the wolves' capability in finding and killing certain prey, it may also be expected that wolves would favour the selection of natal-like prey types later in life, influenced by the experience gained early in life. Such knowledge on potential experience-based prey preference can increase our understanding of the feeding ecology of expanding wolf populations, which move over broad areas characterized by different livestock husbandry techniques and wild prey communities.

As the developed molecular method may be adapted to different ecological settings and customized to fit local research needs with different prey species, it has the potential to be developed and applied to other areas and other large carnivores. The application of our relatively fast and cost-efficient approach could be expanded to different areas across Europe where wolves are recolonizing their former ranges, returning into areas where humans for hundreds of years have relied on hunting large game species and having livestock without the competition from large carnivores. Along gradients of variable prey densities and composition, the molecular method has the potential to be used as a tool to inform the management of ungulates and wolves by revealing the prey composition in wolves' diet. With better knowledge on wolf diet in response to presence of multiple wild ungulate prey and different livestock in anthropized landscapes it will be possible to make more informed and precise management actions to maintain the co-existence of humans and large carnivores.

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