Received: 7 February 2023 | Revised: 4 May 2023 | Accepted: 9 May 2023

DOI: 10.1002/edn3.434

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ORIGINAL ARTICLE

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

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Funding information
Marie Claire Cronstedt's Foundation; Naturvårdsverket, Grant/Award Number: 2020-00092; University of Rome La Sapienza

Abstract

The study of carnivores' 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 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 specificity and sensitivity and, whereas priority is usually given to the former to avoid false positives, sensitivity is rarely investigated so that false negatives may be largely overlooked. We conducted feeding trials with captive wolves (*Canis lupus*) to validate a nanofluidic array technology recently developed for the detection of multiple prey species in scats. Using 371 scat samples from 12 wolves fed with a single-prey diet, the sensitivity of our nanofluidic array method varied between 0.45 and 0.95 for the six main ungulate prey species. The method sensitivity was enhanced by using multiple markers per species and by a relatively low threshold of number of amplifying markers required to confirm a detection. Yet, at least two markers should be used to avoid false positives. By acknowledging sources of bias in sensitivity to reliably interpret the 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 the occurrence and extent of biases in sensitivity.

KEYWORDS
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 tracking-collar (VHF or GPS) (Merrill et al., 2010; Sand et al., 2005). Predation can also be examined by visiting kill sites of prey equipped with a tracking collar and identifying the predator species (Mumma et al., 2014). Additionally, feces can be

collected and macroscopically or molecularly analyzed for prey content (Mech & Boitani, 2003). In particular, the investigation of carnivore food-habits through molecular detection of prey DNA from predator feces 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 over- or underestimation, respectively, 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, for example, 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). On the other hand, method sensitivity is likely to be imperfect due to the low amount and/or poor quality of DNA resulting from prey remains after digestion (Pompanon et al., 2005; Symondson, 2002). Sensitivity can be improved by a careful design of DNA primers, which are firmly dependent on the richness of available reference sequence databases (Gibson et al., 2014). Attempts to increase sensitivity have been done by pooling sequences of PCR replicates employing a multiplexing strategy with multiple universal primer sets targeting the same taxonomic group but amplifying several loci (Alberdi et al., 2018; De Barba et al., 2014), or multiple primer sets amplifying the same locus (Gibson et al., 2014). Assessing sensitivity of the molecular methods adopted in dietary studies can be dealt with concurrently analyzing samples using a complementary method, such as traditional macroscopic identification (Deagle et al., 2009; Nørgaard et al., 2021; Tollit et al., 2009). The limitation of comparative approaches, however, is that estimates of sensitivity can only be made if tested against an error-free method, a condition which is difficult to achieve in practice. An alternative approach to measure detection probability of target prey DNA from scats is to study captive animals fed with a known diet. Although this approach may be time consuming and logistically complex, it provides experimentally reliable estimates of method sensitivity and the factors affecting it (Pompanon et al., 2012; Schattanek et al., 2021).

Usually, there is a trade-off between sensitivity and specificity, as conservative approaches that seek to minimize the probability of false positives also risk to increase the probability of false negatives, thus reducing the method sensitivity (Clare et al., 2016; Darling & Mahon, 2011; Ficetola et al., 2016). The definition of a binary detection using molecular methods is often not straightforward and requires the use of cut-offs, which are generally fixed, arbitrarily defined, and conservatively chosen to avoid erroneous detections (Darling & Mahon, 2011; Divoll et al., 2018; Pompanon et al., 2012). However, the application of such fixed thresholds can have the downsides of missing rare food components and resulting in low 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 to attain detections better fitted to the actual sample and to each specific situation (Alberdi et al., 2018; De Barba et al., 2014). 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). Such approach was for instance used 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). With high genotyping success and low error rates, nanofluidic array technology is used for genotyping several large carnivore species from non-invasive samples (bears, Norman & Spong, 2015; lynx, Förster et al., 2018; mountain lions, Buchalski et al., 2022; wolves, Kraus et al., 2015; wolverines, Lansink et al., 2022; European wildcats, Nussberger et al., 2014). With the benefit of multiplexing and high-throughput analysis of small quantities of DNA, such technology has been successfully used to determine ungulate species from browsed twigs (Nichols & Spong, 2017) as well as detecting pathogen species in ticks (Michelet et al., 2014), lastly, it has also been recently used to identify prey DNA from wolf fecal samples (Di Bernardi et al., 2021). In the latter work, target detection was based on four markers per prey species, where thresholds were tailored to each marker in each run to obtain full specificity in relation to the non-target reference tissues from the run. For each species, the binary detection was determined by the cut-off minimum number of any of the 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; pinnipeds: Deagle et al., 2010; Deagle & Tollit, 2007; cheetah: Thuo et al., 2019).

We conducted feeding trials with captive wolves provided with a known diet to validate the molecular method developed by Di Bernardi et al. (2021) to detect prey in wolf. 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 thresholds, prey species, and feeding regime (i.e., entire carcass or only meat).

2 | MATERIALS 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 (two adults, three subadults, seven 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 prey species except roe deer, which was only offered in a carcass trial. The amount of food provided daily to the captive wolves was in line with the individual requirement of energy intake based on the zoo guidelines, that is, 2 kg of meat per individual per day, or 3 kg of carcass per individual per day.

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

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

2.2 | DNA extraction and molecular analysis for prey detection

Approximately 150 mg of fecal material was sub-sampled from each scat, following the manufacturer's instructions of the QIAamp DNA Stool Kit (Qiagen) used for DNA extraction. For tissue samples, the standard phenol/chloroform-isoamylalcohol extraction was conducted. The tissue samples included (i) fresh tissue samples from the carcasses given to the wolves during the feeding trials, and (ii) reference tissues for the 18 different target species, including moose, roe deer, red deer, fallow deer, wild boar, reindeer, sheep (*Ovis orientalis*), cattle (*Bos taurus*), European badger (*Meles meles*), beaver (*Castor fiber*), European hare (*Lepus europeus*), mountain hare (*Lepus timidus*), Western capercaillie (*Tetrao urogallus*), black grouse (*Lyrurus tetrix*), bear (*Ursus arctos*), lynx (*Lynx lynx*), wolverine (*Gulo gulo*) and red fox (*Vulpes vulpes*). All tissue samples were collected from animals that had died in Sweden and the reference tissues 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™ 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 analyzed DNA extracted from scat samples and DNA from 18 reference tissue samples as positive control, one wolf tissue, and a sample of distilled water used as negative control. All the tissue samples from the

ungulate carcasses were analyzed to verify that the animal tissues correctly amplified with the specific markers.

Following the protocol described in Di Bernardi et al. (2021), thresholds were set for each marker to get a binary detection from the DNA amplification intensity, reflected by the fluorescence signal obtained with the EP1 system upon amplification. The reference tissue samples were included in each run to set thresholds based on empirical data for each marker in each run and therefore increase the marker specificity. Scat samples with amplification intensities below the baseline were regarded as not amplifying, being the baseline determined by the intensity of 0.2 (reference value for low-amplification intensity) and by the intensity of non-specific reference tissues from the run. Finally, to get a binary detection of a target species in each scat sample, a threshold defining the minimum number of any of the species-specific markers with a positive call (indicating amplification) out of the total number of used markers was required to determine the presence of DNA from a target species in the scat sample.

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 measuring sensitivity, that is the proportion of true positives on the total sum of true positives and false negatives. For instance, a scat collected during a trial with moose that rendered moose DNA was a true positive, whereas failure to detect moose DNA in that sample corresponded to a false negative. Sensitivity was estimated separately for each threshold (minimum number of any of the species-specific markers with positive call), for the six target prey species, and separately for the meat and carcass feeding trials. The 95% binomial confidence interval for sensitivity was calculated with the R package *binom* (Dorai-Raj, 2022). We estimated the method accuracy as the sum of true positives and true negatives on the total of samples. For instance, a true positive for roe deer would be the correct detection of roe deer DNA from a scat collected in a roe deer trial, while a true negative for roe deer would be the correct non-detection of roe deer DNA from a scat collected in a moose trial.

2.4 | Statistical analyses

We fitted generalized linear models (GLM) with binomial distribution to estimate the effect on sensitivity (response variable) of the prey target species, the threshold used, and the feeding regime (meat or carcass), included as categorical variables. The interaction between the feeding regime and species was included to investigate potential differences in sensitivity of the method when providing the two distinct feeding regimes for the different target prey species. The interaction between feeding regime and threshold was included to obtain unique coefficients for the carcass feeding regime for the different species, as this is the main focus given its resemblance to the feeding conditions in the wild. We used the sample-size corrected Akaike information criterion (AICc) to compare the candidate models, correcting for small sample size (Bartón, 2013).

We tested scenarios where a lower number of markers was available per species, to assess the effect on sensitivity of the number of available tested markers. We used generalized linear 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 (five meat trials, six carcass trials), a total of 613 wolf scats were collected (32–113 scats per trial) (Appendix S1). Out of the subset of 381 samples analyzed, 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 (Di Bernardi et al., 2021). A final sample size of 371 scats was thus 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 averaged 0.92 (range 0.85–0.98), that is, 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 amplifying markers out of the total markers tested, 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. This was true except for two cases, a tissue from a fallow deer carcass and a tissue from a red deer carcass that amplified non-specifically, respectively for red deer with threshold up to four markers, and for wild boar with threshold up to three 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 cause.

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 and 3). The effect of feeding regime on sensitivity appeared different among species

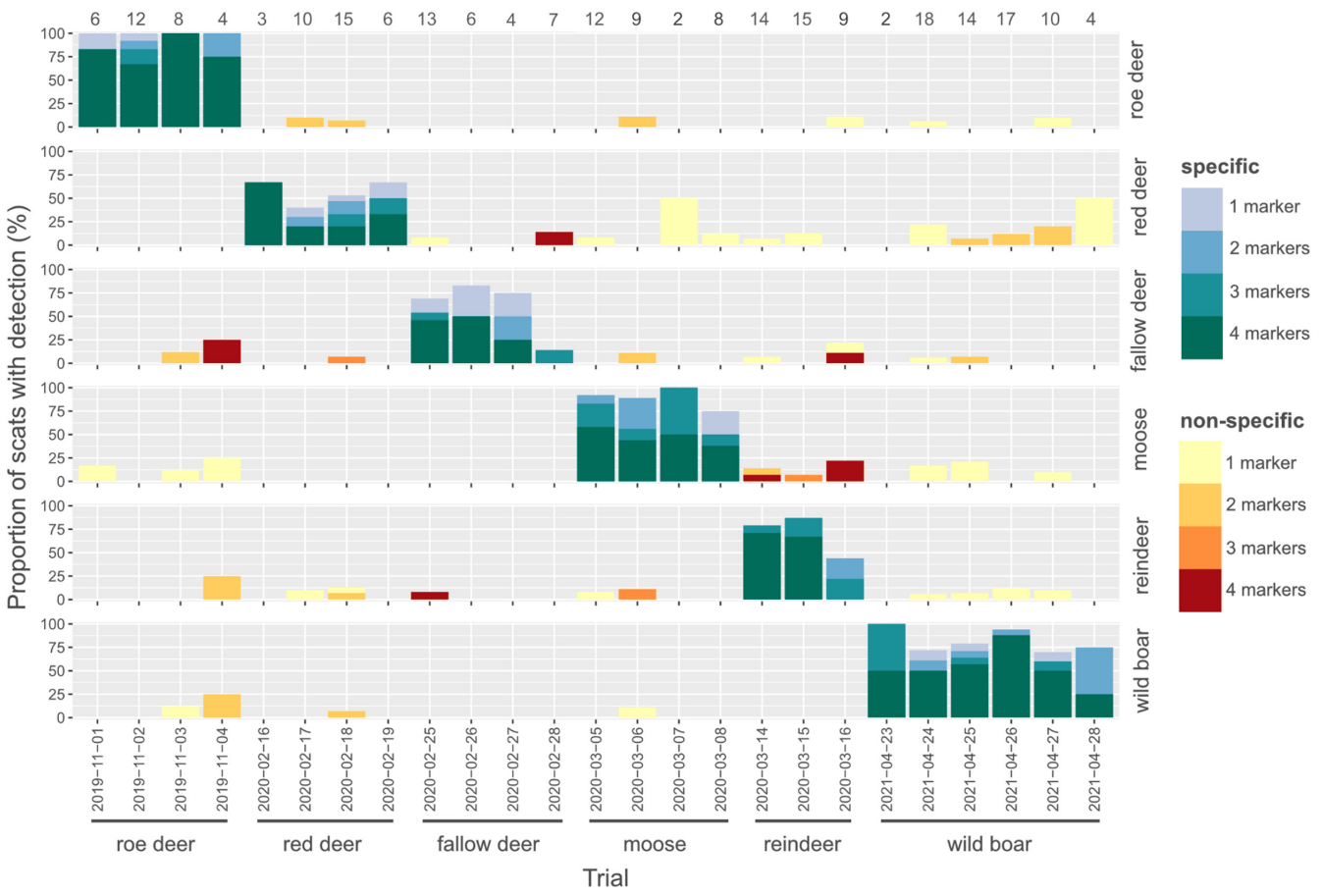

FIGURE 1 Specific and non-specific ungulate species detection for each collection date of carcass feeding experiments with captive wolves in Sweden, 2019–2021. Detection is measured as proportion of scats giving a positive call (in percentage). The numbers noted above indicate sample size. For each target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

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 one amplifying marker as threshold, to 0.5 (range 0.26–0.8) with four 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 in sensitivity 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 target and non-target reference tissue samples and empirical thresholds tailored for

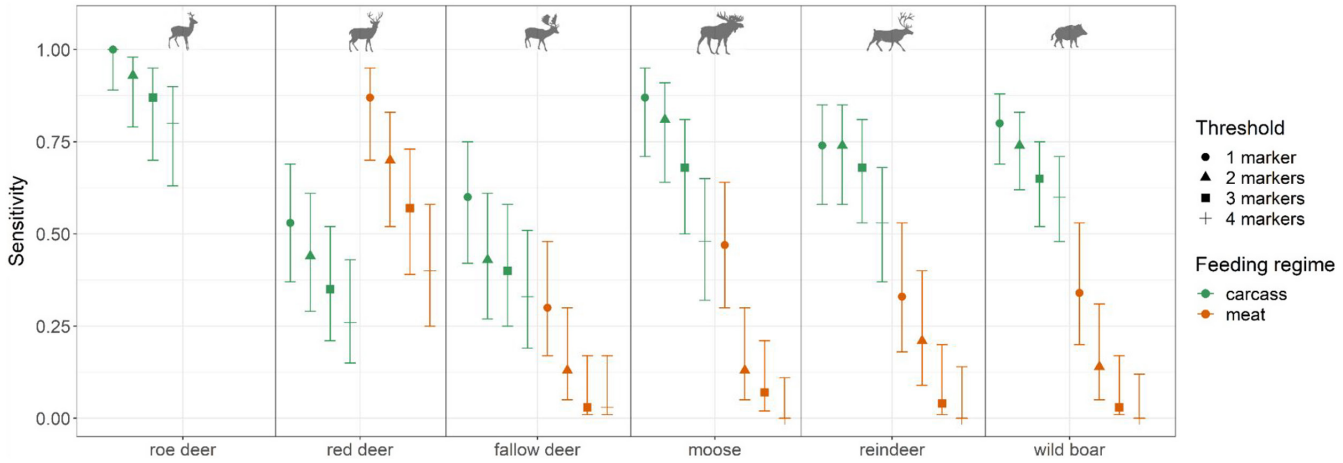

FIGURE 2 Sensitivity of the molecular method tested with feeding experiments with captive wolves in Sweden during 2019–2021. The sensitivity was estimated from the data for the trials with carcass and meat feeding regime, separated for the four thresholds (minimum of 1, 2, 3, 4 markers with a positive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

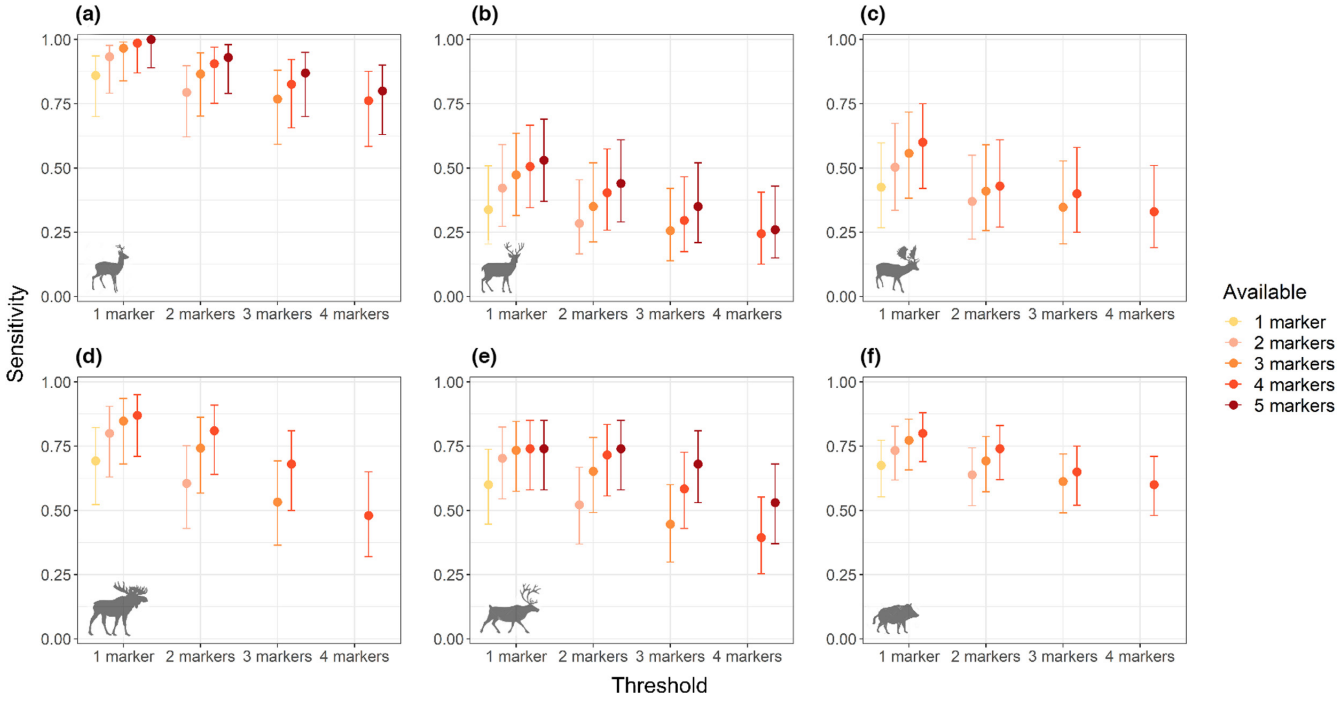

FIGURE 3 Sensitivity of the molecular method estimated from the data, tested with feeding experiments with captive wolves in Sweden during 2019–2021. Sensitivity values are shown for each combination of threshold (minimum number of any of the markers with a positive call to give a final call) and number 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. A total of 5 markers were available for roe deer, red deer, and reindeer, while a total of 4 markers were available to fallow deer, moose, and wild boar. Error bars represent 95% binomial confidence intervals.

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 wolf scats 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 two 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 that might have differentially affected sensitivity for the target prey species could for example be differences in prey digestibility, which can be related to variations due to age, condition, and season, as well as the constituent composition of the fed prey (Deagle & Tollit, 2007; Thomas et al., 2014). Environmental or technical factors in the trials and in the processing of samples may as well affect sensitivity (Alberdi et al., 2019; Oehm et al., 2011; Thuo et al., 2019), such as variation in the amount of prey daily consumed by the target animals (Schattanek et al., 2021; Thuo et al., 2019).

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 al., 2021; Thuo et al., 2019). Although biased species detection may have important implications for the ecological interpretation of diet analyses, it is largely overlooked and rarely accounted for (Alberdi et al., 2019). A procedure that acknowledges the existence of such errors would account for the level of uncertainty gained from experimental studies with true presence to make reliable ecological inferences and thereby get closer to a correct description of species composition (Thomas et al., 2014, 2016; Valentini et al., 2016). In this respect, as the current method by Di Bernardi et al. (2021) is part of a broader array of approaches to conduct DNA-based diet analysis of predators, the comparison of these different methodologies, ideally using an experimental set-up, can aid a relative assessment of pros and cons of the available techniques and enhance the comparability of results. Sometimes, when empirical data on sensitivity was not available through controlled conditions, statistical approaches were used to account for false-positive and false-negative errors (Chambert et al., 2015; Lahoz-Monfort et al., 2016). Detection probability of DNA methods has been indirectly estimated with capture-mark-recapture or occupancy modeling for species detection from eDNA samples (Abrams et al., 2019; Broadhurst et al., 2021; Sales et al., 2020; Smith & Goldberg, 2020), as well as for prey detection in predator scats (Morin et al., 2019).

When using diagnostic molecular methods, the trade-off between false negatives and false positives usually needs to be balanced depending on the scope and research question. Diet analyses generally prioritize specificity using conservative cut-offs that may result in the loss of sensitivity, that is, failure to detect a prey species that was actually consumed (Darling & Mahon, 2011; Divoll et al., 2018). The approach used by Di Bernardi et al. (2021) of utilizing multiple species-specific molecular markers is 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, the method aims at increasing taxonomic coverage within each species (Di Bernardi et al., 2021). In this study, we observed how the use of several markers, instead of only one per species, improved sensitivity for all target species (Figure 3). However, although pooling results from multiple markers can reduce the number of false negatives (Gibson et al., 2014), it may also increase the risk of introducing false positives (Alberdi et al., 2018). We observed this pattern when setting too low thresholds of minimum number of amplifying markers (Figure 1). Through the analysis of false negatives with empirical data from feeding 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 two 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, that is, 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 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.

AUTHOR CONTRIBUTIONS

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

ACKNOWLEDGMENTS

This work was funded by the Marie Claire Cronstedt's Foundation, the University of Rome La Sapienza and the Swedish Environmental Protection Agency (grant number 2020-00092). We are grateful to the Järvzoo zookeepers A. S. Stenberg, I. Fernberg, K. Karlsson, and S. Mooij, and to L. Jelk and J. Larsson from Järvzoo for enabling the feeding trials. The genetic laboratory work for fecal DNA extraction was partly conducted by J. Märtz (SLU). We are grateful to H. Andrén for valuable statistical advice, and to R. Godinho, T. Skrbinšek, E. Hedmark, and G. Jansson for comments on earlier drafts of this paper.

FUNDING INFORMATION

Naturvårdsverket, grant number: 2020-00092; Marie Claire Cronstedt's Foundation, University of Rome La Sapienza.

CONFLICT OF INTEREST STATEMENT

No conflict of interest to declare.

DATA AVAILABILITY STATEMENT

Compiled data generated during the feeding experiments is publicly archived in the Dryad. Digital Repository at https://doi.org/10.5061/dryad.t1g1jwt70.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Di Bernardi, C., Wikenros, C., Ciucci, P., Boitani, L., Sand, H., & Åkesson, M. (2023). Experimental feeding validates nanofluidic array technology for DNA detection of ungulate prey in wolf scats. *Environmental DNA*, *5*, 723–732. https://doi.org/10.1002/edn3.434