


REVIEW, EDITOR'S CHOICE

Feeding ecological knowledge: the underutilised power of faecal DNA approaches for carnivore diet analysis

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ABSTRACT

1. Accurate analyses of the diets of predators are key to understand trophic interactions and defining conservation strategies. Diets are commonly assessed through analysis of non-invasively collected scats, and the use of faecal DNA (fDNA) analysis can reduce the species misidentifications that could lead to biased ecological inference.
2. We review the scientific literature since publication of the first paper on amplifying fDNA, in order to assess trends in the use of genetic non-invasive sampling (gNIS) for predator species identification in scat-based diet studies of North American and European terrestrial mammalian carnivores (Carnivora). We quantify error rates in morphology-based predator species identification. We then provide an overview of how applying gNIS would improve research on trophic interactions and other areas of carnivore ecology.
3. We found that carnivore species identity was verified by using gNIS in only 8% of 400 studies of carnivore diets based on scats. The median percentage

of false positives (i.e. samples wrongly identified as belonging to the target species) in morphology-based studies was 18%, and was consistent regardless of species' body size. We did not find an increasing trend in the use of gNIS over time, despite the existing technical capability to identify almost all carnivore species.

4. New directions for fDNA studies include employing high-throughput sequencing (HTS) and DNA metabarcoding to identify the predator species, the individual predator, the entire assemblage of consumed items, and the microbiome of the predator and pathogens. We conclude that HTS protocols and metagenomic approaches hold great promise for elevating gNIS as a fundamental cornerstone for future research in ecology and conservation biology of mammals.

INTRODUCTION

Predators, such as mammalian carnivores, have inherently high conservation value and are often strong interactors in terrestrial ecosystems. Because numbers of carnivores are closely linked to prey density and biomass (Hatton *et al.* 2015), understanding ecosystem effects must begin with accurate knowledge of predators' diets. Also, competition for shared prey in coexisting carnivores has been advocated as the main driver of interspecific agonistic interactions (Donadio & Buskirk 2006, Ritchie & Johnson 2009), and determination of cause-effect mechanisms driving these interactions depends on accurate knowledge of feeding ecology. From an applied perspective, livestock depredation by carnivores and perceived competition with human hunters have driven the global decline and local extinction of carnivore species (Ceballos & Ehrlich 2002, Treves & Karanth 2003), underscoring the need for rigorous identification of carnivores' diets. In short, accurate diet analysis allows researchers to estimate biological parameters ranging from trophic niche breadth to trophic specialisation and prey selection, thereby enhancing our understanding of ecological structure and informing conservation and management actions.

The analysis of undigested remains in scats is the most commonly used method to assess the diet of mammalian carnivores (Klare *et al.* 2011), generally with the implicit assumption that a scat can be correctly attributed to a carnivore species based on morphology alone. However, the application of non-invasive molecular methods, available since the 1990s (Hoss *et al.* 1992, Kohn *et al.* 1995) showed that identification of carnivore species based on scat morphology is prone to error, potentially leading to biased ecological inferences (Martínez-Gutiérrez *et al.* 2015, Morin *et al.* 2016, Weiskopf *et al.* 2016).

More recently, the emergence of next-generation sequencing or high-throughput sequencing (HTS) has revolutionised molecular approaches (Schuster 2008) and

allowed the development of highly efficient protocols for analysing low-quantity and low-quality DNA samples (Pompanon *et al.* 2012, De Barba *et al.* 2017). The HTS metagenomic protocols take the next step of confirming not only the species leaving the scat, but also positively identifying the prey species in the scat, even when remains are small or too decomposed for morphological analyses. These approaches have been applied not only to large species such as carnivores, but also to small insectivores (Biffi *et al.* 2017) and rodents (Lopes *et al.* 2015). While still scant in the scientific literature, recent molecular-based dietary assessments reveal errors in previous morphology-based dietary assessments from scats (Mumma *et al.* 2016, Gosselin *et al.* 2017, Oja *et al.* 2017). Furthermore, the advent of new HTS protocols provide opportunities for further insights from non-invasively collected faecal samples, ranging from gut microbiome composition and disease dynamics to individual behaviour.

Here we focus on the application of genetic non-invasive sampling (gNIS) to terrestrial mammalian carnivores, based on their importance in launching faecal DNA (fDNA) analysis and their high profile as a taxonomic group at the centre of global conservation and management conflicts and challenges. We review the scientific literature, beginning with the first paper describing fDNA amplification in 1992, in order to: 1) assess trends in the use of gNIS for species identification of the predator in scat-based terrestrial carnivore diet studies; 2) quantify the error rates in carnivore species identification and illustrate the potential biases in the estimation of trophic niche breadth and niche overlap among coexisting mammalian carnivore species; 3) foster the implementation of gNIS among field biologists by providing guidelines for the key steps in this approach; 4) provide the state-of-the-art of gNIS in diet assessment of terrestrial vertebrates; and 5) explore the emerging fields of research currently expanding the use of non-invasive molecular tools for scat samples.

TRENDS IN THE USE OF gNIS FOR CARNIVORE IDENTIFICATION IN DIET STUDIES

The identification of the species that produced a scat is prone to two types of error: false positives, which occur when samples from another species are misidentified as the target species; and false negatives, which occur when samples of the target species are misidentified as something else, or omitted from sampling. Both types of error occur frequently in traditional scat-based diet studies in the order Carnivora, and have potentially far-reaching consequences in conservation biology and wildlife management (Lonsinger et al. 2015, Martínez-Gutiérrez et al. 2015, Mumma et al. 2016). False positives can be investigated simply from the putative target species' samples, whereas false negatives require collection and molecular identification of all scats (even those not recorded as from the target species).

To evaluate the trends in gNIS for predator species identification in scat-based studies, we searched Thomson Reuters' Web of Science database for articles assessing the diets of 32 mammalian carnivore species (see Appendices S1–S4) for the period 1992–2017. We targeted all native, wild mammalian mesocarnivores and large carnivores indigenous to the North American and European continents. By focusing on the continents with the longest history of gNIS-based tools to identify carnivore species, we characterise the best-case scenario of gNIS application in research on carnivores' diets. The details of the literature search and exclusion criteria are provided in Appendix S1.

We retrieved a total of 518 studies characterising the feeding ecology of terrestrial carnivores. Fieldwork spanned 93 states or countries, and was similarly distributed between the European and North American continents ($n_{\text{America}} = 241$ vs. $n_{\text{Europe}} = 277$; Fig. 1). Studies were focused on canids (54%), mustelids (20%), felids (19%) and ursids (15%). The three most common target species were canids that are frequently at the core of intense conservation conflicts: the grey wolf *Canis lupus* ($n = 98$), the coyote *Canis latrans* ($n = 88$) and the red fox *Vulpes vulpes* ($n = 73$). Carnivore scats were the source material in 400 studies; $77 \pm 1.8\%$ (mean \pm SE) of all studies of carnivore feeding ecology rely on scat samples. The constant rate of publication (15 ± 0.6 studies per year, Fig. 2a) and the high representativeness of scat-based studies in carnivore feeding ecology research suggest that scat analysis remains relevant and contemporary, and that it continues to be the primary method to resolve the dietary patterns of this taxonomic group. We observed a 12-year time-lag between the introduction of gNIS as a diagnostic tool for scat species and diet item identification (Hoss et al. 1992)

and the first diet studies implementing it (Fig. 2a, Appendix S3). Moreover, the rate of publication of studies that used molecular methods to identify the predator species post-2003 was 2.4 ± 0.4 per year, nearly six times lower than the rate of publication of scat-based diet studies. This indicates that most researchers continue to neglect molecular tools as a means to correct potential biases in morphology-based scat identification. Even though gNIS continues to become easier and cheaper (Waits & Paetkau 2005, Rodgers & Janečka 2012), it is not increasing in use in the carnivore diet studies.

A quarter of a century after the emergence of gNIS, the authors of only 33 studies have used this approach to assess the identification of the species that produced the scats used for analysis. This corresponds to a modest 16% of the 211 scat-based diet studies published in the 2004–2017 period. The majority of the scientific literature on carnivore feeding ecology published during the gNIS era may be biased due to false positive and false negative samples. The low use of gNIS is observed in all carnivore taxonomic families. For example, of a total of 225 cases when the diet of canids was assessed from scats, gNIS was used to confirm target species identity in fewer than 5% ($n = 12$). This scenario does not differ much in other taxonomic groups, as fewer than 15% of all published scat-based diet studies used molecular techniques to confirm carnivore species identification per taxonomic group (Fig. 3a).

We believe that the primary reasons for the surprising paucity of use of gNIS to identify the predator species include a lack of operational guidance (e.g. a perception that the cost of gNIS is excessive, or limited access to expertise or equipment), and unawareness by many field scientists about the rate of scat misidentifications. To raise awareness and hopefully stimulate an increase in the implementation of gNIS in this field of research, we describe the patterns and implications of scat misidentifications (see also the case studies in Appendix S4) and provide a protocol describing key steps in the implementation of gNIS procedures for field ecologists.

POTENTIAL CAVEATS ASSOCIATED WITH gNIS IN THE STUDY OF CARNIVORE DIETS

While we argue strongly that gNIS provides an efficient means to address field identification uncertainty (Kelly et al. 2012), non-invasive molecular methods do have limitations. First, the samples may yield DNA that is low in quantity or quality depending on environmental factors, the freshness of the sample and the storage conditions (Murphy et al. 2007, Nakamura et al. 2017). Natural contamination due to behavioural features (e.g. territorial marking by individuals of the same or different species) may also be

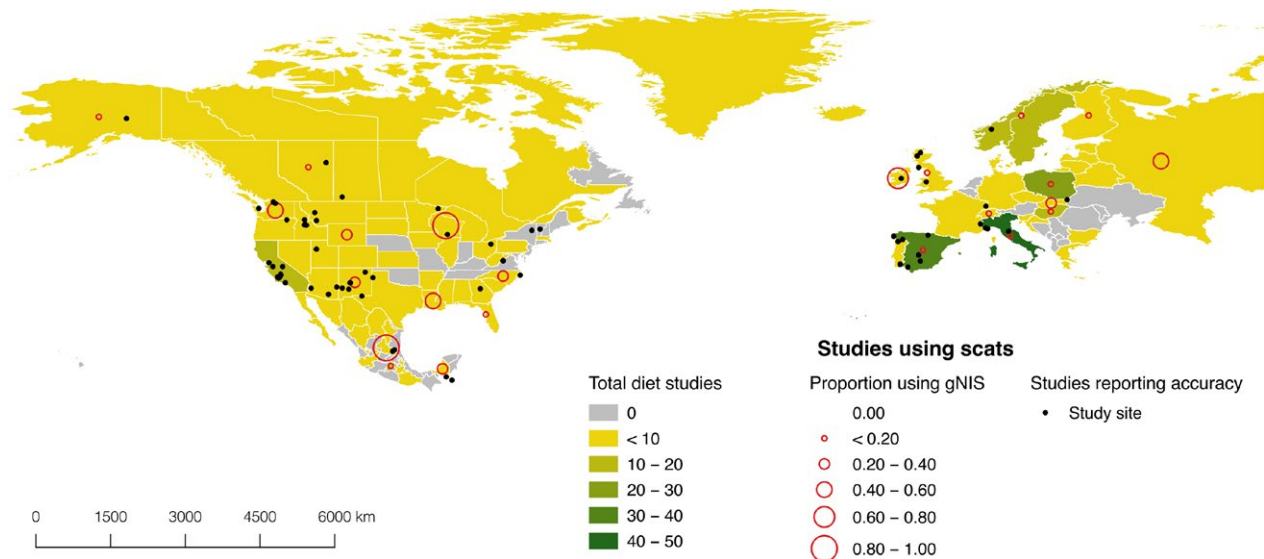


Fig. 1. Geographic distribution, by state or country, of the number of scat-based carnivore diet studies in North America and Europe, published in Science Citation Index journals during the period 1992–2017, the proportion that used genetic non-invasive sampling for carnivore species identification, and the location of study areas of scat-based studies that report field identification accuracy rates. [Colour figure can be viewed at wileyonlinelibrary.com]

an issue, but can be resolved depending on the specificity of primers and the molecular technique used to perform species identification. For example, Sanger sequencing may be affected by contamination if species-specific primers are not used, whereas methods based on DNA fragment size allow the identification of several species in a single DNA amplification (Palomares et al. 2002, De Barba et al. 2014a). Other sources of field or lab contamination due to human error or the low quantities of DNA extracted from non-invasive samples are documented elsewhere (Waits & Paetkau 2005, Beja-Pereira et al. 2009).

Carnivore species identification using gNIS has primarily relied on mitochondrial DNA (mtDNA), in large part because of the much higher DNA copy number compared with nuclear DNA. Nevertheless, mtDNA has important limitations. Nuclear copies of mitochondrial genes may be present (Triant & DeWoody 2007), and can be co-amplified with orthologous mtDNA (or even preferentially identified); this may cause error in species identification (Kim et al. 2006, Ermakov et al. 2015). The transference of mitochondrial genes from one population or species into another by mtDNA introgression has been widely described in mammals (Mallet 2005), and can compromise the correct species identification for closely related species. Finally, the use of only mtDNA is highly limiting in cases of natural or human-induced hybridisation, for example between the European mink *Mustela lutreola* and the polecat *Mustela putorius* (Lodé et al. 2005), or the grey wolf and the domestic dog (Godinho et al. 2011). In this respect, the studies by Oliveira et al. (2009) with Iberian carnivores and McVey et al. (2013) with red wolves and coyotes provide good examples of the

use of different types of nuclear DNA markers to increase species identification accuracy.

The methodological limitations of gNIS are comparatively minor, and are easier to overcome than misidentifications associated with morphology-based scat sampling. Furthermore, ongoing development of laboratory techniques and analysis inevitably improves the precision and accessibility of gNIS approaches. To quantify the continued evolution of molecular tests for carnivore species identification, we searched for studies describing markers applicable to gNIS of our target species (details provided in Appendix S1). We retrieved 42 papers matching these criteria. Diagnostic markers for gNIS have been steadily increasing, with a mean publication rate of 1.6 ± 0.3 studies per year (Fig. 2b). Diagnostic gNIS markers were available for 50% and 75% of our target species by 2002 and 2007, respectively (Fig. 2b). By 2016, molecular markers were available for 97% ($n = 31$) of North American and European mesocarnivores and large carnivores (Fig. 2b).

PATTERNS AND IMPLICATIONS OF CARNIVORE SCAT MISIDENTIFICATION

Our carnivore diet and marker development literature searches showed that 64 studies reported scat misidentification rates, representing 15 (47%) of the species considered in this review. While the overall distribution of the proportion of false positive identifications was skewed towards lower values (Fig. 3b), the median percentage of false positives (18%) suggests that approximately one-fifth of all samples in these diet studies were incorrectly identified as the target species. This was consistent, regardless of the

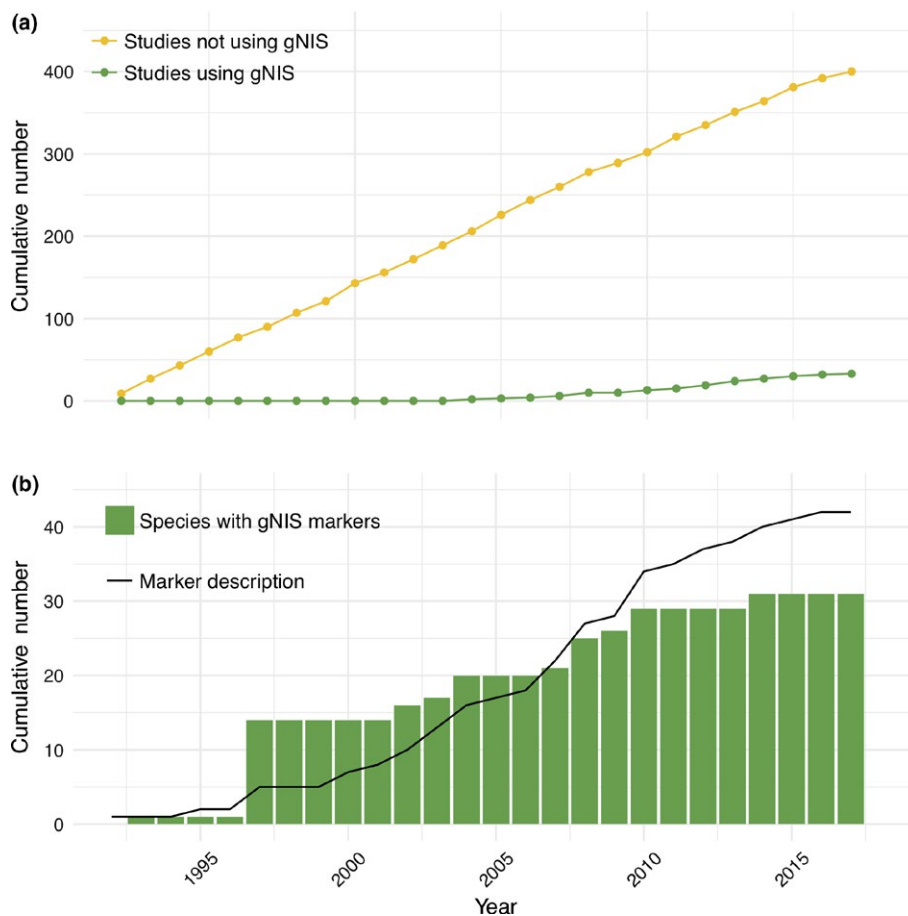


Fig. 2. Trends in the use of genetic non-invasive sampling (gNIS) for species identification in scat-based carnivore diet studies published yearly in Science Citation Index papers in 1992–2017: (a) cumulative number of scat-based studies and the number using gNIS to identify carnivore species; (b) cumulative number of papers describing molecular markers designed for identification for any of our target species with direct application to gNIS published yearly (line), and cumulative number of our target species with markers allowing for species identification (bars). [Colour figure can be viewed at wileyonlinelibrary.com]

body size of the target species. Less than one quarter of all studies reported a percentage of false positives below 5% (first quartile = 0.05), indicating that the inclusion of false positive samples is widespread. In fact, only eight studies (13%) did not detect false positive samples. Conversely, 17% of studies reported a percentage of false positives over 50%, and it is instructive to review these studies to understand when field identifications are most likely to be inaccurate. Four putative species-true species combinations from six study sites resulted in >75% false positives (Davison et al. 2002, Smith et al. 2006, Monterroso et al. 2013, Witczuk et al. 2013, Lonsinger et al. 2015). Each demonstrates how frequent false positives can become when the target species is rare or scarce, especially when other sympatric species are relatively abundant. Red fox samples were misidentified as the infrequently encountered European wildcat *Felis silvestris* (Monterroso et al. 2013), the European pine marten *Martes martes* (Davison et al. 2002), and the San Joaquin kit fox *Vulpes macrotis* (Smith

et al. 2006), in regions where red foxes are more widespread. Samples from the coyote were occasionally misidentified as red fox or kit fox, bobcat *Lynx rufus* and puma *Puma concolor* (Witczuk et al. 2013, Lonsinger et al. 2015). In fact, misidentified red fox (in Europe) and coyote (in North America) were the primary sources of false positives in studies of other carnivores. These misidentifications can result in high percentages of false positives when the target species is rarely or never detected, and should be scrutinised when study objectives, such as quantifying diet requirements or assessing predation pressure, are based on detecting an elusive carnivore (Weiskopf et al. 2016). Species whose scats are frequently misidentified as other carnivores are often opportunistic predators with wide trophic niche breadths and frequently reported scavenging behaviour, often consuming carrion provided by larger carnivore kills (Díaz-Ruiz et al. 2013). Other sources of high false positive percentages (>50%) include confusion between species of the same taxonomic family (Echegaray & Vilà 2010) and sympatric

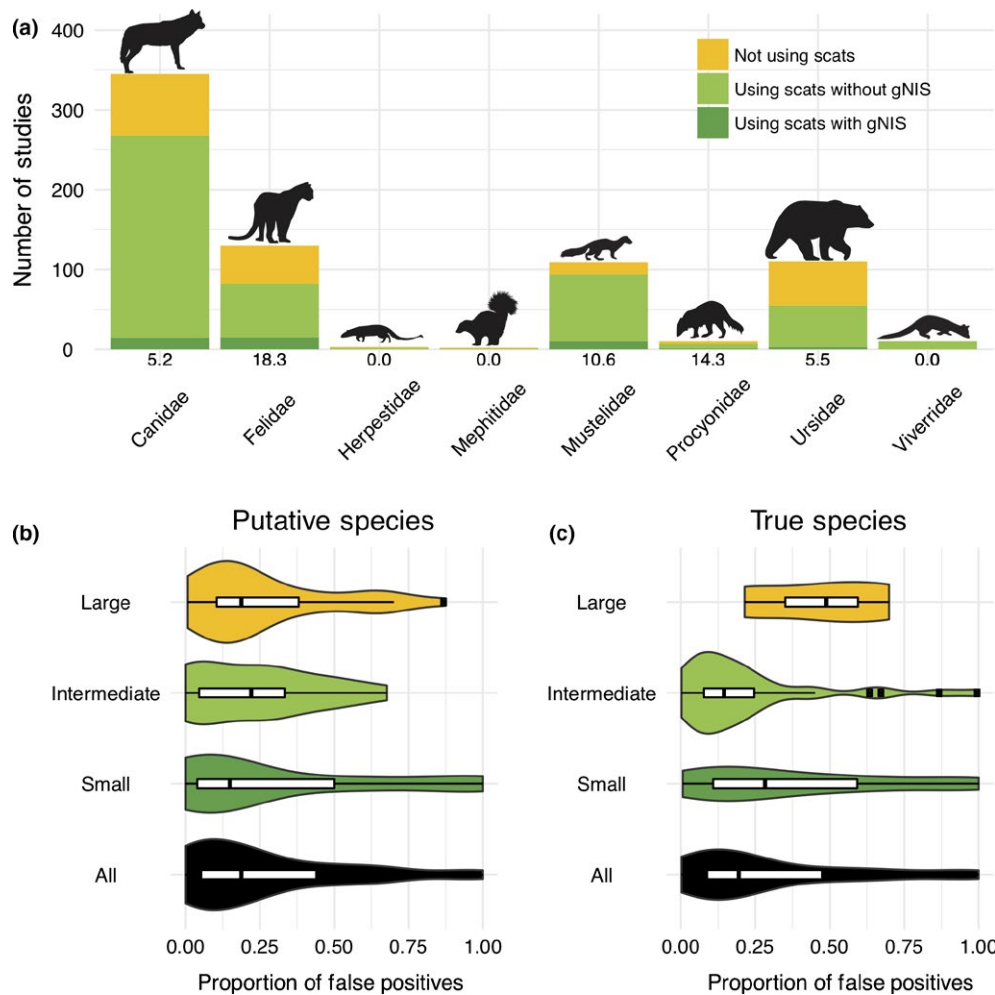


Fig. 3. (a) Total number of studies addressing carnivore diets in North America and Europe, by taxonomic family, published in Science Citation Index papers for the period 1992–2017; numbers below bars represent the percentage of studies using scats that employed genetic non-invasive sampling for carnivore species identification. Violin plots, (b) and (c), showing the skewed distribution of proportions of false positives in a data set for all putative species–true species combinations, and by body size of the putative (b) and true (c) species. Small species (1–6 kg) included foxes, martens, ringtails, and domestic cats; intermediate species (6–20 kg) included bobcats, coyotes, lynx, wolverines; and large species (20–100 kg) included wolves, pumas, jaguars, and black bears. [Colour figure can be viewed at wileyonlinelibrary.com]

species of similar sizes (Long et al. 2007, Morin et al. 2016), and it is very likely that all these factors interact and contribute to false negative rates.

Compared with false positives, false negatives are largely overlooked and their potential effects are ignored in many cases. False negatives were reported in only nine studies. Species commonly misidentified as another species included coyotes, red foxes, bobcats, pumas, and grey wolves, but there was insufficient information to evaluate the percentage or rate of false negative misidentifications for most cases.

It is evident that both false positives and false negatives occur frequently across all carnivore species and geographic areas, and their incidence may be particularly sensitive to abundances of target and sympatric species.

Bias in dietary estimates may be minimal when both target and non-target species have highly overlapping diets (Martínez-Gutiérrez et al. 2015, Morin et al. 2016). However, errors may severely alter the interpretation and inference when samples from multiple non-target or trophically divergent species are included, typically resulting in overestimation of dietary niche breadth for species with stricter requirements (Martínez-Gutiérrez et al. 2015, Weiskopf et al. 2016). Adopting a more conservative sample collection approach (i.e. restricted scat dimension parameters or confidence rankings) could mitigate the effects of false positives (Dellinger et al. 2011, Lonsinger et al. 2015); however, an unintended consequence of this approach is an increase in false negatives and incorrectly rejecting samples

from the target species. This may be especially true if a surveyors' preconceptions about the target species' ecology reduces the collection of atypical samples (Morin et al. 2016), which can lead to self-confirming results of the target species' diet. Thus, it is critical that potential errors in identification and possible outcomes relative to the objectives are considered when planning or evaluating a diet analysis based on scat collection. In Appendix S4, we present two groups of carnivore species for which we describe misidentification patterns

and their consequences for the estimation of trophic niche breadth and niche overlap.

INCREASING THE USE OF gNIS IN TROPHIC ECOLOGY RESEARCH

To contribute to the transfer of molecular advances to fundamental ecological research, we provide a step-by-step flowchart identifying five key steps in the design and implementation of scat-based studies to assess diet and

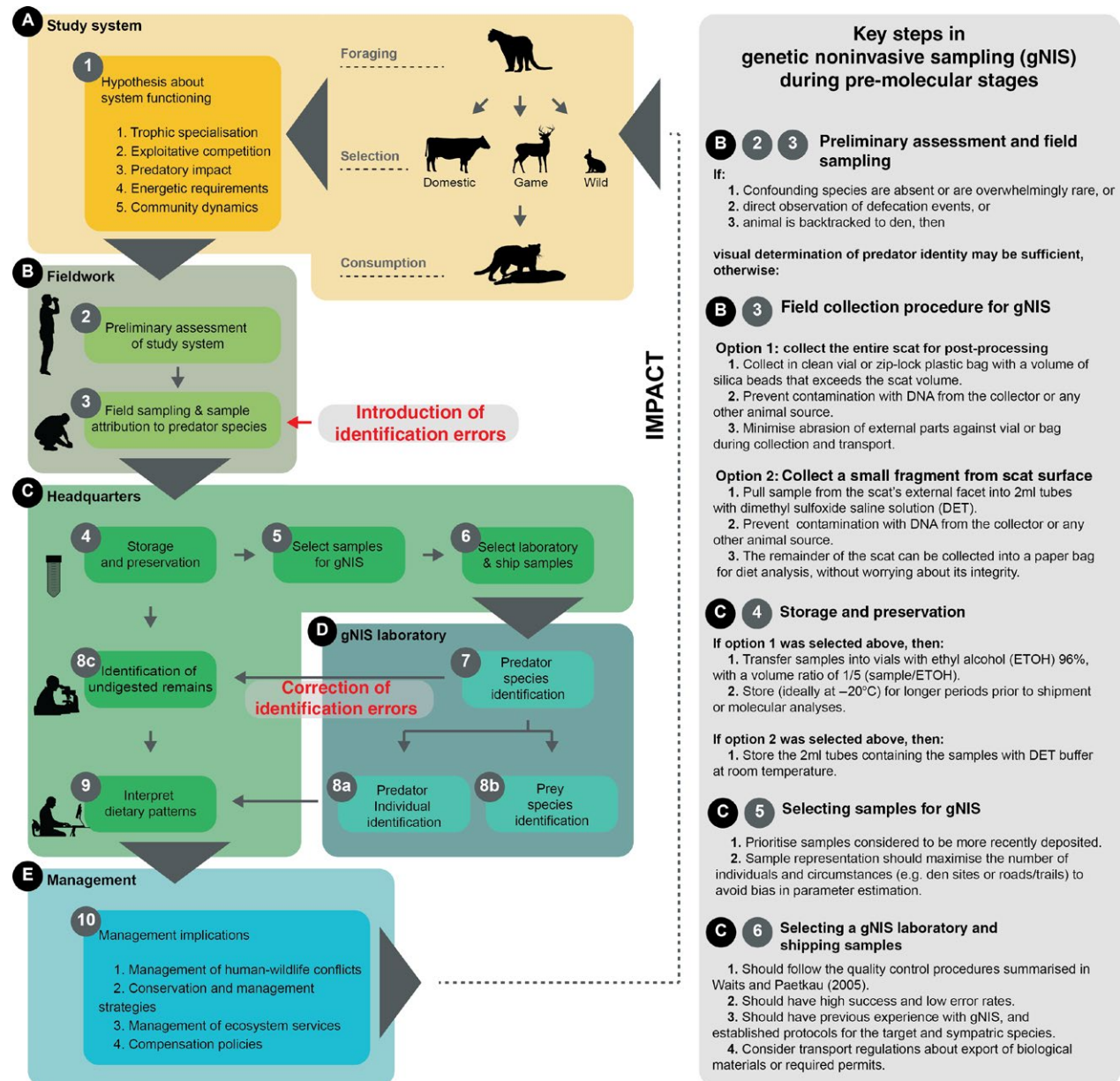


Fig. 4. Diagram depicting the step-by-step process involved in the assessment of trophic relations between predators and prey, and management implications derived from their interpretation, with emphasis on the proper procedures during the key steps of genetic non-invasive sampling during the pre-molecular stages of the study. [Colour figure can be viewed at wileyonlinelibrary.com]

related studies in carnivores (Fig. 4). After formulating the hypotheses (Stage A1), a first decision is whether diagnostic species identification of carnivore scats is essential. As is apparent from our review, confirmation of species identity is required in most cases. Exceptions include the following situations (Fig. 4, Stages B2 and B3): 1) confounding species are either absent or overwhelmingly rare in the study area; 2) defecation is visually confirmed; or 3) animals are backtracked to their daybeds or resting sites. The first criterion is rarely met because scat misidentifications are prone to occur across a broad range of body mass values (Fig. 3b), and the probability of collecting a scat from a target species by chance depends on the percentage of scats in the population from the target species. Studies on disproportionately overabundant carnivores, as may happen with highly adaptable and opportunistic species, such as the red fox (Travaini et al. 1997), may inherently entail low-false positive rates. However, even in urban or semi-natural environments, where carnivore communities are simplified, the presence of domestic dogs can contribute to a significant number of false positive scats (Krausman et al. 2006, Echegaray & Vilà 2010). Diagnostic markers are not necessary when target animals are directly observed or scats are collected after backtracking target animals to places actively defended such as dens, daybeds, resting sites, or marking latrines. Mckelvey et al. (2006) were able to identify with 100% accuracy all scats of Canada lynx *Lynx canadensis* encountered by backtracking the animals in the snow to their daybeds. Likewise, Marucco et al. (2008) were able to only collect grey wolf scats by snow-tracking them along travel routes in the Italian Alps, and Stenglein et al. (2011) recorded a field identification accuracy of 99% when collecting grey wolf scats at rendezvous sites in Idaho, USA. However, the presence of target species' signs (e.g. tracks or scrapes) may not be sufficient to assure scat origin, as other species may also have visited and even defecated at the same site (Janečka et al. 2008, 2011). Only in those relatively few cases where at least one of the criteria is met, should ecologists proceed to identify and analyse carnivore scats without molecular diagnostic species identity confirmation (Fig. 4).

Further key steps in gNIS sampling during the pre-molecular stages, critical to ensure good quality results are (Fig. 4): field collection (Stage B3), storage and preservation (Stage C4), selection of samples for gNIS analysis (Stage C5), and selection of a gNIS laboratory and sample shipment (Stage C6). A rigorous implementation of these protocols should provide samples that yield high success rates in the extraction, amplification, and species identification using molecular methods (Piggott 2004, Waits & Paetkau 2005, Broquet et al. 2006, Beja-Pereira et al. 2009, Panasci et al. 2011).

After predator species identification for each sample using gNIS, the next step is to use these data to estimate the dietary patterns (Stage C8c, Fig. 4), adequately corrected for false positive and false negative errors. As a general rule, >100 samples of known origin are required for an adequate characterisation of dietary patterns for cross-species or spatio-temporal comparisons (Trites & Joy 2005, du Preez et al. 2017). Hence, assuming a mean scat amplification and genotyping rate of ca. 80% (Rodgers & Janečka 2012) and an expected percentage of false positives of ca. 20% (see above), ca. 160 scats should be collected from the field and sent for genetic analysis for species identification to ensure a final sample size of 100 for the target species. The approach should be even more conservative (i.e. more samples should be collected) when the target species is known to be rare. Whenever genotyping this number of samples is not possible, a subsample should be randomly selected to allow estimation of the precision (i.e. $1 - \text{proportion of false positives}$) of field identification of scats. Ideally, the dietary patterns for each target species should be assessed using only samples with confirmed target species identity (Morin et al. 2016, Weiskopf et al. 2016).

When only a fraction of all samples to be used in diet analysis are confirmed by gNIS profiling, then a more cautionary and sophisticated approach is required. In such cases, the diet reconstruction obtained from the field-identified data set should incorporate case-specific precision estimates, which will naturally increase the uncertainty in the parameters estimated (e.g. frequency of occurrence, consumed biomass or prey selection). We propose that uncertainty can be estimated using re-sampling protocols, such as bootstrapping (Manly 2006). For example, in a case where 500 samples were field-identified as belonging to coyotes, of which 50 were sent for gNIS profiling with 85% and 80% amplification and genotyping success rates, respectively, the gNIS-identified sample size would be 34. If, of those 34 samples, only 25 were actually confirmed as coyotes, then the case-specific field identification rate would be 74% ($CI_{95} = 0.55-0.84$). In this example, assuming randomness in the misidentification process, repeated random samples of $n = 367$ should be drawn from the total population of samples ($n = 500$) and the parameter of interest should be estimated at each iteration. Although assuming randomness does not allow the correction of parameter estimates for misidentification bias, it does inflate their uncertainty by incorporating the error derived from potential identification error. More accurate estimates of dietary parameters and their associated uncertainties can be obtained if false positives and false negatives are simultaneously accounted for in the re-sampling protocol. Detailed descriptions of re-sampling protocols and uncertainty analysis can be found elsewhere (Manly 2006).

TAKING DIET ANALYSIS ONE STEP FURTHER: MOLECULAR TOOLS FOR THE IDENTIFICATION OF ITEMS CONSUMED AND INDIVIDUAL CONSUMERS

The identification of consumed prey using fDNA analysis can overcome some limitations of morphology-based diet assessments. Traditional scat-based dietary assessment methods rely on macro-identification and microscopic identification of undigested items consumed by the predator; conversion factors or regressions then connect species' remains to ingested biomass (Klare *et al.* 2011, Chakrabarti *et al.* 2016). Although simple and inexpensive, this method has important shortcomings: 1) a high degree of uncertainty is associated with the species-level identification of closely related prey remains (Gosselin *et al.* 2017); and 2) dietary items composed of soft and highly digestible tissues (e.g. Gastropoda, fungi) are not detected (Nilsen *et al.* 2012), and the presence of other prey may be underestimated (Mumma *et al.* 2016, Gosselin *et al.* 2017). Using region-specific primers (for mtDNA or nuclear DNA) and Sanger sequencing technology for the identification of prey items, gNIS can minimise the effect of these potential sources of bias (King *et al.* 2008). For example, using molecular methods, Mumma *et al.* (2016) consistently found higher frequency of occurrence rates for caribou *Rangifer tarandus*, moose *Alces alces* and snowshoe hare *Lepus americanus* remains in black bear *Ursus americanus* and coyote scats than when using morphological assessments. Gosselin *et al.* (2017) obtained higher taxonomic resolution and sensitivity in the identification of leporid species from coyote scats when using molecular analysis than when using morphological methods. Also, Oja *et al.* (2017) found that the frequency of occurrence of ground-nesting birds in wild boar *Sus scrofa* scats was over four times greater when using genetic analysis than when using morphology-based estimates, revealing the role of the wild boar in the predation of the threatened capercaillie *Tetrao urogallus* in Estonia.

Individual identification of carnivores from their fDNA via gNIS enables individuals to be linked to their diets, and provides the means to assess intra-population and temporal variation in foraging behaviour and prey consumption patterns, hence testing previously challenging research hypotheses (Fedriani & Kohn 2001, Prugh *et al.* 2008). In pioneering work, Fedriani and Kohn (2001) combined scat genotyping and morphologic-based diet assessment to identify the diets of individual coyotes in the Santa Monica Mountains of California, USA. These authors found a significant heterogeneity in individual diet profiles, and were able to define two main groups of coyotes on the basis of their relative use of primary food sources. Furthermore, they found that the diet diversity

of individuals is only a subset of that of the entire population, and suggest that trophic diversity indices that are not based on the analysis of individuals may be downwardly biased if the samples represent a small number of individuals. More importantly, they discovered little within-individual faeces-to-faeces variation, indicating that population-level diet diversity exceeds that at the individual level. Using a similar approach, Prugh *et al.* (2008) found moderate inter-individual variability between coyotes in central Alaska, USA; trophic niche overlap was as low as 0.42 between social groups of the same population. These authors were able to test the optimal foraging theory by linking individual variability in coyote dietary breadth and consumption rates of snowshoe hares with spatio-temporal variation in the availability of this prey species. Also, using gNIS for individual identification from scats, Mesa-Cruz *et al.* (2016) divided the population of Belizean large felids into two groups according to their location, and found that diets shifted towards smaller prey outside protected areas. These advances currently allow researchers, for instance, to use non-invasive methods to test the Niche Variation Hypothesis (Van Valen 1965), which postulates that under conditions of reduced interspecific competition, a population expands its niche mostly through inter-individual variation. In recent years, evidence of individual specialisation has accumulated in many vertebrate taxa (Bolnick *et al.* 2003, Araújo *et al.* 2011), but studies are still scant for mammalian carnivores (Semmens *et al.* 2009, Robertson *et al.* 2014).

The advent of HTS technology and the continuous refinement of protocols have opened doors to innovative biological applications (Mardis 2008, Schuster 2008). The substantial expansion of sequence databases through the DNA barcoding of animals (Hebert *et al.* 2003; Barcoding of Life project - <http://www.boldsystems.org>) has unlocked the application of HTS to gNIS, by facilitating an accurate and reliable assessment of the full spectrum of prey from fDNA (Pompanon *et al.* 2012, Shehzad *et al.* 2012, De Barba *et al.* 2014b, Kartzinel & Pringle 2015, Kartzinel *et al.* 2015). Current HTS protocols even allow the identification of the individual consumer through high-throughput microsatellite genotyping (De Barba *et al.* 2017). However, determining the diet of a carnivore from HTS data still requires careful development. Two of the most commonly cited biological sources of bias in the evaluation of diet by this method are the detection of items not intentionally consumed (e.g. food of the consumed prey; Sheppard *et al.* 2005), and the contamination of faeces by environmental organisms (e.g. pollen or eggs) before sample collection (Pompanon *et al.* 2012). The differential digestibility of dietary items may also influence the amount of DNA in the faeces and its detectability in diet analysis (Deagle *et al.* 2010, Thomas *et al.* 2014). A

methodological limitation of the DNA metabarcoding method for the assessment of carnivore diets is that, while it provides an accurate assessment of consumed item assemblages, it does not allow the quantitative analysis of consumed items. This shortcoming of molecular methods could favour the use of traditional quantitative methods that allow the estimation of consumed biomass, and hence other derived parameters (e.g. kill rates, energetic requirements). However, the development of innovative approaches, such as relative correction factors (Thomas *et al.* 2016), which allow researchers to control for many of the biasing factors involved in the quantitative relationship between gene copy number and estimates of relative abundance of each prey item in scat samples, may soon facilitate quantitative assessment of consumed prey. This and other technical aspects related to low-quantity and quality of fDNA and the efficiency of metabarcoding protocols are beyond the scope of this paper, and may be found elsewhere (e.g. Pompanon *et al.* 2012, Pinol *et al.* 2014, Shehzad *et al.* 2012, Taberlet *et al.* 2012).

Consistent with the pattern found in our review of the use of gNIS for predator species identification, HTS and metabarcoding approaches have not often been applied to diet analyses, despite the falling cost and increasing power of the techniques. Nevertheless, recently published studies illustrate their applicability to carnivore research (Shehzad *et al.* 2012, Dawson *et al.* 2016, Xiong *et al.* 2017). Thus, it is currently possible to rely fully on gNIS and HTS technology to assess the carnivore species and individual, plus all consumed items, from fDNA obtained from its scats. We believe that future research should focus on individual-based hypotheses, not only revealing the answer to the question 'who is eating what?' (Pompanon *et al.* 2012), but also asking 'who is eating what, where, and why?'

CONCLUSION AND FUTURE DIRECTIONS

Carnivores are often key players affecting ecosystem dynamics (Ritchie *et al.* 2012, Ripple *et al.* 2014). Therefore, carnivore feeding ecology is a cornerstone for understanding not only predator biology, but also the effects of predators on ecosystems. We documented a high and constant rate of publication of studies using scats as a source material to evaluate carnivore dietary patterns, which highlights the importance of understanding these trophic interactions. However, it is striking that over 25 years after the emergence of gNIS applied to fDNA, only 8% of carnivore diet research utilises this more accurate technology as a diagnostic tool for carnivore identification. We demonstrate that false positive and false negative samples are frequent and widespread, and may have significantly biased our past inferences about several biological

patterns and processes. This review makes it clear that the adoption of molecular identification of the predator should be a standard and required practice in dietary analyses in most scenarios. Additionally, we provide guidance for non-geneticists engaging in carnivore diet studies to help bridge the gap between genetics and conservation practice.

The technological advances in wildlife monitoring techniques have been increasing at an unprecedented rate, providing new opportunities to improve conservation science and practice, and molecular methods play a crucial role (Shafer *et al.* 2015, Taylor & Gemmill 2016). Advances in HTS unlocked a new era for animal diet studies, allowing the identification of the predator species, the individual predator, and the prey species (Pompanon *et al.* 2012, Srivathsan *et al.* 2016); further, scat samples can be analysed much faster and more accurately than they can by using traditional molecular tools (e.g. Sanger sequencing or fragment size-based runs) or morphological identification. Moreover, metagenomic approaches applied to gNIS currently allow researchers to explore new research topics, including the identification of microorganisms and infectious agents expelled through target species' scats that provide valuable information on the health and potential adaptive mechanisms to environmental changes (Amato 2013, Bahrndorff *et al.* 2016). Currently, microbiome studies of wild terrestrial carnivores are limited. Recent articles in model and non-model systems have demonstrated that the gut microbiome can influence metabolism, nutrition, immune response, adaptation and tolerance to environmental perturbation, behaviour, anxiety levels, and, ultimately, fitness (Ezenwa *et al.* 2012, Hooper *et al.* 2012, Amato 2013, Bahrndorff *et al.* 2016, Fung *et al.* 2017). Moreover, studies of wild populations have demonstrated that phylogeny, physiology, diet, habitat quality, spatial location, sex, social system and status, reproductive status, and age all impact the microbiome composition of species and individuals (Ley *et al.* 2008, Muegge *et al.* 2011, Degnan *et al.* 2012, Phillips *et al.* 2012, Gomez *et al.* 2015, Maurice *et al.* 2015, Sommer *et al.* 2016, Wasimuddin *et al.* 2017, Wu *et al.* 2017). These studies and a growing body of literature on the role of microbiomes in influencing individual fitness clearly demonstrate the importance and value of faecal samples to characterise and understand the gut microbiome in populations of wild animals.

Infectious diseases may also have profound impacts on animal and human populations, and could be included in surveillance programs (Scott 1988, Pedersen *et al.* 2007). Faeces are known carriers of different pathogens, including micro- and macroparasites, which may cause disease to the individual, to other wild or domestic animals or, ultimately, to humans. The advent of HTS brought a breakthrough in epidemiologic surveillance, and a new paradigm

that supports the understanding of the whole pathogenic community as baseline information. A few studies have used HTS technologies to screen the fDNA of mammalian carnivores for viruses (e.g. Li *et al.* 2010, Bodewes *et al.* 2014, Conceição-Neto *et al.* 2017). Identification of macro-parasites in fDNA remains underexplored through HTS, but we foresee that HTS tools will soon become essential for research in this field. There is great potential for the use of these tools in gNIS, to move beyond purely descriptive studies of microbiomes, diet and disease to investigations that evaluate the functional links between individual behaviour, consumption patterns, genetic composition, gut microbiome and disease dynamics in the near future.

The recent scaling-up of non-invasive genetics to non-invasive genomics currently provides the setting to engage in genome-wide approaches from gNIS (Perry *et al.* 2010, Carroll *et al.* 2018), further widening our perspectives and allowing researchers to explore the full extent of inter-individual variability. Genomics may also provide more accurate and precise estimates of population structure and demographic parameters, as well as adaptive genetic variation (Perry *et al.* 2010, Russello *et al.* 2015, Shafer *et al.* 2015). Although DNA in non-invasive samples is low-quality and available in limited quantity, reducing the power of genome scans for adaptive traits, the available library preparation and sequencing technologies should, in theory, allow genomic coverage to be extended (Chiou & Bergey 2018). Likewise, functional genomics could be soon tangible through gNIS techniques (Carroll *et al.* 2018). This opens the exciting new possibility of non-invasively studying the genetic basis of specific traits, such as factors that influence dietary choices by individuals (e.g. physical condition, aggressive behaviour, boldness). Currently, a large number of species have a reference genome assembly, and many more will have in a near future (e.g. Genome 10K community of scientists 2009). Therefore, if one considers the dietary profile as a phenotype that results from specific traits reflecting either specific behaviours or physical capabilities, it is reasonable to envision genome-wide association studies aimed at identifying regions of the genome implicated in the individual manifestation of such traits. The same rationale can be used for any other traits that can be inferred from gNIS on scat samples, such as susceptibility or resistance to diseases, and adaptation to environmental gradients. To our knowledge, these approaches have never been attempted using non-invasive sampling, though they hold great promise for taking gNIS one step further to answer questions of general interest in the field of evolutionary biology – such as questions about local adaptation and host-parasite co-evolution – as well as providing a strong contribution to the conservation management of natural populations.

These exciting new research avenues still require significant technological investment, bioinformatic expertise, and establishment of standardised protocols, and will certainly entail significant research costs. Therefore, many of the new conservation genomic applications described above are not yet optimised for general use by ecologists. However, the application of rapidly evolving HTS protocols to gNIS, together with the overall reduction in laboratory costs and the increasing knowledge transfer between genetics and conservation biology, will help decrease the gap between conservation genetics and conservation practice (Shafer *et al.* 2015, Taylor & Gemmill 2016). The science of trophic interactions would benefit greatly from increased accuracy and resolution in carnivore and prey identification, to assess fine-scale patterns of resource use, and to contribute towards a better understanding of the mechanisms underlying the coexistence of species.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Appendix S1. Literature search and exclusion criteria search used in the literature reviews for carnivore diet studies and non-invasive marker description.

Appendix S2. Identification of the target species, mean body mass (kg), with respective total number of studies

addressing dietary patterns and subsets that used scats and non-invasive genetic sampling.

Appendix S3. List of research articles retrieved in the dietary patterns and marker development literature searches.

Appendix S4. Case studies identifying misidentification patterns in 1) grey wolf, coyote, and bobcat in North America; and 2) red fox, European wildcat and pine marten in Europe, illustrating the consequences of biases in the estimation of trophic niche breadth and niche overlap.