Feeding ecological knowledge through noninvasive genetics: advancing towards accurate carnivore identification in diet analyses

Running title: Noninvasive genetics in carnivore identification

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Tweetable abstract (mas 120 char.)

1. 78% carnivore diet studies use scats. 8% use genetics for species identity, leading to 19% false positives with bias in estimated parameters.

Abstract

1. Accurate analyses of predator diets are key for understanding trophic interactions and defining conservation strategies. The diet of carnivores is most commonly assessed through analysis of scats. The emergence of fecal DNA analysis (fDNA) methods provided new approaches for improving the accuracy of species identification of scat samples and revealed misidentifications associated with traditional methods that could lead to biased ecological and conservation inference.

2. We review the scientific literature since the first paper amplifying fDNA, and assess the temporal trends in the use of noninvasive genetic sampling (NGS) for carnivore species identification in scat-based diet studies. We quantify error rates derived from false positive and false negative samples, and illustrate the potential biases in the estimation of biological parameters. Finally, we propose a decision tree protocol to guide wildlife biologists, ecologists and managers in implementing this approach.

3. We retrieved a total of 489 papers evaluating the diet of carnivores and 380 (78%) relied on the analysis of scats. Surprisingly, only 30 studies (8%) used NGS to verify species identity. We observed a 12-year time lag since fDNA was introduced in the scientific literature and its inclusion in carnivore diet studies, and did not find an increasing trend in the use of NGS through time. Only a modest 16% of the scat-based diet studies published in the 2004-2015 period used NGS to confirm carnivore identity.

4. The median proportion of false positives was 0.19, and this trend was consistent regardless of body size of the target species, with less than one quarter of all studies reporting a proportion of false positives < 0.05. Conversely, 17% of studies reported a proportion of false positives > 0.50. We retrieved 42 papers describing NGS-oriented markers for species identification, which allow the identification of 31 (97%) of our target species. These results

indicate that the technological capability for NGS is available for virtually all North American and European meso and large carnivores. The solution to more accurately assess carnivore diet is readily available; researchers need to acknowledge the risks of misidentification of scats in diet studies and address it by conducting genetic identification.

Key-words: Conservation Genetics, Noninvasive Genetic Sampling (NGS), Scat misidentification, Sampling protocol, Carnivore diet, Carnivore conservation, Identification accuracy, Species identification

Knowing what carnivores eat: why is it important?

Predators, such as mammalian carnivores, have the potential to impose top-down forces on trophic webs via trophic cascades, with impacts that reverberate across the entire ecosystem (Estes *et al.* 2011; Ritchie *et al.* 2012; Terborgh 2015). They can regulate the population of prey species with influence cascading to the composition and structure of vegetation and geomorphology across landscapes (Ripple & Beschta 2004; Beschta & Ripple 2012; Ripple *et al.* 2014). However, the numbers of predators are closely linked to prey density and biomass (Carbone & Gittleman 2002; Hatton *et al.* 2015), hence accurately knowing what they eat is of primary importance. For example, Carbone *et al.* (1999) identified a threshold of carnivore body mass (21.5kg) above which they cannot be sustained with small prey. This energetic model strongly relies on knowledge about carnivore diets. Also, competition for shared prey in coexisting carnivores has been advocated as the main driver for interspecific agonistic interactions, often leading to the death of one of the competitors through interspecific killing or intraguild predation (when the killed species is consumed: Palomares & Caro 1999; Donadio & Buskirk 2006; Ritchie & Johnson 2009). Proposing cause-effect mechanisms driving these interactions between carnivores depends on accurate knowledge of feeding ecology.

The importance of understanding mammalian carnivore diets expands beyond purely natural ecosystems, as they are also at the heart of many conservation conflicts worldwide (Treves & Karanth 2003; Inskip & Zimmermann 2009; Thorn *et al.* 2014). The intersection between human and carnivore activities occurs in rural and even urban areas across the world, and has been the main driver for the global decline and local extinction carnivore species (Ceballos & Ehrlich 2002; Pimm *et al.* 2014). Many stakeholders in rural communities believe that carnivores are damaging economic activities, particularly in regards to livestock depredation (Ogada *et al.* 2003; Thorn *et al.* 2014). Hence, a thorough understanding of the interactions between wild carnivores and human activities is vital for effectively managing human-carnivore conflicts (Redpath *et al.* 2013).

The interpretation of the biological processes underlying ecological theories, and its application to conservation practice rely on the assumption that we have accurate knowledge about carnivore dietary patterns and prey requirements. However, recent studies have suggested that our knowledge about such basic attributes of diet might not be as solid as we previously thought, mostly due to errors associated with assigning biological samples to particular carnivore species (Davison *et al.* 2002; Janečka *et al.* 2008; Rodgers & Janečka 2013; Monterroso *et al.* 2013). The diet of terrestrial carnivores is traditionally estimated with one of three of methods (Nilsen *et al.* 2012): a) analysis of undigested contents in scats; b) analysis of partly digested contents in carnivores' digestive tract; and c) analysis of killed prey. Among these alternatives, scat analysis is the most commonly used method (Klare *et al.* 2011), with the implicit assumption that a scat can be correctly attributed to a carnivore species. However, the application of noninvasive molecular methods in the 1990s to carnivore ecology (Höss *et al.* 1992, Kohn *et al*. 1995, Foran *et al*. 1997) showed that the species that produced the undigested remains was often misidentified by traditional methods, potentially leading to biased ecological inferences drawn from inaccurate species assignment (Martínez-Gutiérrez *et al.* 2015; Weiskopf *et al.* 2016; Morin *et al.* 2016).

Accurate diet analysis underlies biological parameters such as trophic niche breadth, trophic specialization and prey selection, which enhance our understanding of the ecological structure and inform conservation and management actions (Fig. 1). In this paper, we review the scientific literature since the first paper describing DNA amplification in scats to a) assess the trends in the use of noninvasive genetic sampling (NGS) for species identification in scat-based carnivore diet studies; b) 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; and c) propose a NGS protocol oriented to field ecologists to guide the implementation of this approach in conservation biology and wildlife management.

Trends in the use of NGS for carnivore identification in diet studies

The misidentification of carnivore scats is prone to two types of errors, each with different implications for the subsequent patterns and processes of interest in diet and trophic research. False positives occur when samples from another species are misidentified as the target species. Conversely, false negatives occur when samples of the target species are misidentified as something else, or omitted from sampling. Given the subjective nature of field identification of carnivore scats, both types of errors can frequently occur in traditional scat-based diet studies in the order Carnivora, with potentially far-reaching consequences in conservation biology and wildlife management (Martínez-Gutiérrez *et al.* 2015; Lonsinger et al 2015; Mumma *et al.* 2016; Weiskopf *et al.* 2016; Morin *et al.* 2016). False positives are detected simply by analyzing the sample set collected for a target species, and are more commonly identified and quantified than false negatives, which requires collection and molecular identification of all scats, even if they are not believed to be from the target species.

We searched Thomson Reuters' Web Of Science (WOS) database for articles assessing the diets of 31 mammalian carnivore species (Supporting Information) for the period 1992-2015 to evaluate the trends of including NGS for predator species identification in scat-based studies. For the purpose of this analysis we targeted all meso and large mammalian carnivores indigenous to the North American and European continents, wild-living in their native range. We limited the geographic extent of this review to the North American and European continents because we expected the use of NGS to be the highest in these regions, as most laboratories developing and employing DNA-based methods in wildlife research and conservation biology are located Europe and North America. Therefore, we aimed at characterizing the best-case scenario in the research of the trophic dimension of carnivores' ecological niche. The details of the literature search and exclusion criteria are provided in Supporting Information.

We retrieved a total of 489 studies published during the period of 1992-2015 characterizing the feeding ecology of terrestrial carnivores. Fieldwork spanned across 92 states/countries, and was performed in fairly equivalent proportions between the European and North American continents ($n_{America}$=227 *vs.* n_{Europe}=262; Fig. 2). Most research on the feeding ecology of mammalian carnivores was focused on canids (47.4%), felids (18.7%), mustelids (15.4%) and ursids (14.8%). All three of the most common target species were canids, frequently at the core of intense conservation conflicts: the gray wolf (n=96), the coyote (n=95) and the red fox (n=71) (Supporting Information).

Carnivore scats were the source material in 380 studies, suggesting that over three quarters (77.7 ± 1.9%) of all research on carnivore feeding ecology relies on these types of samples. The yearly publication rate of studies using carnivore scats was surprisingly consistent across the considered time period (Fig. 3a), with a mean of 15.8 ± 0.6 studies being published every year. The constant rate of publication, and the high representativeness of scat-based studies in carnivore feeding ecology research, suggests that it remains relevant and contemporary, and that scat analysis comprises the primary method to resolve the dietary patterns of these species. In spite of its importance in assessing carnivore dietary patterns and the potential biases introduced by false positive and false negative samples (Davison *et al.* 2002; Witczuk *et al.* 2013; Martínez-Gutiérrez *et al.* 2015; Morin *et al.* 2016), we observed a 12 year time-lag between the introduction of NGS as a diagnostic tool for scat species identification (Höss *et al.* 1992) and the first studies implementing it in diet analysis (Fig. 3b, Supporting Information). Moreover, the rate of publication of these studies post-2003 was 2.5 ± 0.4 per year, nearly six times lower than the rate of publication of diet scat-based studies. This indicates that most researchers continue to neglect genetic tools as a means to correct potential bias in expert-based scat identification. Even though NGS is increasingly becoming an easier, cheaper and widespread tool for carnivore research (Waits & Paetkau 2005; Schwartz *et al.* 2007; Schwartz & Monfort 2008; Kelly *et* al. 2012, Rodgers & Janečka 2013; Taylor & Gemmell 2016), there is no observable increase in its inclusion in trophic ecology

research in carnivores. Surprisingly, almost a quarter century after the emergence of NGS only 30 studies used this approach to assess the identification of the species that produced the analyzed scats. This corresponds to a modest 16% of the 191 scat-based diet studies published in the 2004-2015 period. In fact, it is striking that the gross majority (72 ± 2%) of the scientific literature on carnivore feeding ecology published during the NGS era may potentially be biased due to false positive and false negative samples. Particularly, from a total of 253 cases when the diet of canids is assessed from scats, less than 5.0% (n=12) confirmed target species identity. This scenario does not differ much in other taxonomic groups, with less than 20% of all published scat-based articles diet studies using molecular techniques to confirm carnivore species identification (Fig. 4).

Case study 1: The gray wolf, coyote and bobcat in North America

Although once widely distributed throughout the northern hemisphere, predator control efforts and habitat loss have led to extirpation of gray wolves (*Canis lupus*) across much of North America (Mech 1970). The wolf's apex predator status in trophic webs makes it a key player in shaping fundamental ecological processes (Ripple & Beschta 2004, 2012; Ripple *et al.* 2013). Successful reintroduction efforts and expanding natural populations have created a need to quickly assess potential impacts of increasing wolf populations. In other areas, endangered subspecies such as the Mexican wolf continue to struggle, and estimating required food resources and competition with other predators may elucidate the mechanisms that depress recovery efforts. In addition, coyotes (*Canis latrans*) and bobcats (*Lynx rufus*) are now dominant predators across much of North America. As the populations of all three species increase and ranges overlap with each other (Fig. 5), it is critical to understand the competition dynamics between these predators and how changes in relative abundances may influence other species or result in human-wildlife conflicts.

However, the close taxonomic relation (gray wolf - coyote) and potentially similar feeding habits across the species make scat identification difficult, and the few studies that assessed

the accuracy in the identification of these species' scats indicate that it is highly variable, even when accounting for standard scat dimensions for each species (Table 1).

Overall, the accuracy of expert-based scat classification ranged between 15% to 62% (Table 1), which if uncorrected could lead to a significant misrepresentation of these species' dietary patterns. Below we describe examples of how misidentification rates of predator scats may impede conservation and conflict management over wolves, coyotes, and bobcats in North America.

i. Estimating dietary niche is a simple way to evaluate required food resources for recovering populations. False positives, where coyote and bobcat samples are identified as wolf, would likely overestimate wolf dietary breadth, describing a larger group of possible prey and diluting the importance of a particular prey species to wolves. As a consequence, managers might mistakenly assume that a recovering population would be insulated from dramatic or sudden swings in a specific prey population. Conversely, false negatives, where wolf scats are attributed to another predator, could conceal trace dietary items that may be essential or compensate when prey switching is required.

ii. Often, we estimate dietary niche overlap to determine the potential extent of exploitative competition among predators (when abundance of all predators will be reduced because of simultaneous use of the same resources). The consequences of misidentification in this case are greatest when making comparisons among sites or over time. If dietary overlap is high, false positive and negatives may balance and niche overlap estimates may be relatively unaffected. However, if we were comparing samples collected in different years, we might miss a trend in niche shift to more specialized or exclusionary diets if we are misidentifying predator species. In the same fashion, error rates could mask differences or similarities in sympatric predator diets across a spatial gradient of sympatry, misleading our conclusions of the nature of trophic relationships.

iii. Identifying carnivore species that depredate livestock or game species is a common objective of North American predator diet studies. Despite the fact that genetic identification using swabs of wounds is now available for kill site investigations, many practitioners still assume that observer identification of scat is sufficient for attributing damage to specific predator species. Unfortunately, false positives can unfairly assign predation to the wrong predator species, and false negatives miss the true culprits. Both types of error can result in inappropriate management actions with financial costs that do not ultimately resolve conflict.

iv. Signs of anthropogenic material in predator scats, such as candy wrappers in coyote scats in southern California (Larson *et al.* 2015), or bird seed in black bear scats in western Virginia (Morin *et al.* 2016) could indicate increased habituation that might lead to greater conflict in the future. However, these early signs of potential conflict are not useful and possibly misleading if we attribute the diet items to the wrong species.

Case study 2: The European wildcat, red fox and pine marten in Europe

The red fox (*Vulpes vulpes*) and the pine marten (*Martes martes*) are widely spread across Europe (Fig. 6), with Holarctic and Palearctic distributions, respectively (Hoffmann & Sillero-Zubiri 2016; Herrero *et al.* 2016). Conversely, local extinctions and severe population declines during the last centuries resulted in a current fragmented distribution of the European wildcat (*Felis silvestris silvestris*) (Fig. 6; Yamaguchi *et al.* 2015). Despite being classified as Least Concern at global scale, this small felid is a strictly protected species in Europe through the Bern Convention and Habitats Directive (Yamaguchi *et al.* 2015).

The current status and population trends of the European wildcat are still poorly known due to its cryptic behavior and the lack of large-scale surveys (Yamaguchi *et al.* 2015), but they are mainly threatened by hybridization with domestic cats (Oliveira *et al.* 2008; Hertwig *et al*. 2009; O'Brien *et al.* 2009), competition with, and disease transmission from, feral domestic

cats (*e.g.* McOrist *et al.* 1991; Germain *et al.* 2008, Sarmento *et al.* 2009), habitat fragmentation and human related mortality (road kills and persecution) (Yamaguchi *et al.* 2015). The ranges of these three species in Europe largely overlap through most of the European wildcat range: northern Iberian Peninsula, northern Britain, and central and eastern Europe. Pine marten also spatially overlaps with the red fox throughout its European range (Fig. 6). Due to the restricted geographic range of European large carnivores (Chapron *et al.* 2014), most carnivore communities are now dominated by mesocarnivore species such as foxes, martens and wildcats.

However, despite differences in body size among these species, the size and shape of their scats largely overlap. Hence, species assignment based on external features is prone to misclassification and potentially similar feeding habits across the three species make scat identification challenging. Similarly to the wolf-coyote-bobcat case study in North America (see above), the studies assessing scat identification accuracy for foxes, wildcats and pine martens in Europe indicate highly variable precision rates (Table 2). Overall, while expert-based scat classification precision averaged 88%, it ranged between 3 and 100% (Table 2). This variability in scat identification precision is likely to imply a diversity of misrepresentation patterns in the estimation of these species' trophic parameters. Below we describe examples of the potential ramifications derived from misidentification rates of these species' scats, and describe how they may hinder conservation and management plans for European wildcats, red foxes and pine martens in Europe.

i. The biogeography of feeding ecology provides a way to evaluate the relative importance of food resources across geographic gradients, informing conservation and management actions for threatened or manageable populations. Each of these species has been a target of biogeographic assessments in the consumption of feeding resources (e.g. De Marinis & Masseti 1995; Lozano *et al.* 2006; Díaz-Ruiz *et al.* 2013). False positives causing red fox scats samples to be identified as European wildcat, would likely overestimate the

wildcats' dietary breadth, describing a larger group of possible prey and diluting the importance of a particular prey species to wildcats. Given the fragmented and fragile status of many wildcat populations throughout Europe, such misrepresentation might mistakenly inform conservationists that wildcats would be insulated from dramatic or sudden swings in specific prey populations (e.g. European rabbits in Mediterranean Europe; e.g. Monterroso *et al*. 2016). Conversely, false positives where European wildcat scats samples are identified as red fox, could overemphasize the importance of game prey, supporting misguided management actions aimed at controlling red fox populations (e.g. Delibes-Mateos *et al*. 2008b).

ii. The dietary niche overlap is often used to assess the functional response of a subordinate species to the presence of a potential superior competitor. In areas with recovering apex predators, false positives in subordinate predators' scats could mask populations declines as prey switching responses, whereas false negatives could have the opposite effect, i.e. wrongly informing of a decrease in abundance in a scenario of prey switching. For example, in southern Iberian Peninsula where Iberian lynx (*Lynx pardinus*) is currently recovering, both lynx and wildcats are strictly dependent on European rabbits (Palomares 2001, Lozano *et al*. 2006). If red fox scats are misidentified as wildcats in Iberian lynx recovery areas, wildcat population declines could go unnoticed and its dietary breadth would potentially be interpreted as wider than it actually is. Likewise, red fox are negatively affected by Eurasian lynx presence (Pasanen-Mortensen *et al*. 2013). In this case, false negative fox scats (thought to be pine martens, for example) would suggest a negative numerical response, masking a potential functional response to the recovery of Eurasian lynx.

iii. Often, we estimate dietary patterns to understand predation pressure that predators exert on particular prey species. For example, predation by red foxes has been suggested to suppress the populations of several prey species (e.g. Pech *et al.* 1992; Delibes-Mateos *et al.* 2008a; Elmhagen *et al*. 2010), whereas pine martens may control populations of small

mammals (e.g. Sheehy &. Lawton 2014). Preconceptions about the study system could guide false positives and false negatives in studies on fox feeding ecology aimed at assessing predation effects on specific prey to over-estimate predation impact, and support misdirected and potentially inefficient predation management actions, such as culling or predator removal.

iv. The contact between wildlife and humans and domestic animals is widespread across Europe, and often frequent in rural and suburban areas. Opportunistic foragers such as the red fox or the pine marten (or even European wildcats) may come close to human settlements and come in contact with domestic animals potentially acting as carriers of infectious agents and pathogens to wild populations (Bradley & Altizer 2007). Therefore, false negatives could cause managers to underestimate the role these animals could play in the spread of zoonotic diseases.

Potential caveats associated with NGS in the study of carnivore diets

While NGS provides an efficient means to address field-identification uncertainty (Waits & Paetkau 2005, Beja-Pereira *et al.* 2009, Kelly *et* al. 2012, Rodgers & Janecka 2013), the use of noninvasive molecular methods also has some limitations. First, the samples used in NGS may yield low DNA quantity depending on environmental factors, freshness of the sample or storage conditions (Murphy et al. 2007; Vynne et al. 2012; Harms et al. 2015). DNA contamination in noninvasive DNA is also often an issue. Natural contamination of samples can happen in the field due to behavioral features like territorial marking when individuals either from the same species or different species deposit urine on scats. Such contamination may affect species identification depending on the specificity of primers used to amplify the target DNA and the molecular technique used to perform species identification. For example, Sanger sequencing will be highly affected by contamination when no species-specific primers are used while fragment size based methods allow researchers to identify several species in a single DNA amplification (e.g. Palomares et al. 2002; Livia et al. 2007; DeBarba et al 2014).

Other sources of field or lab contamination due to human error or the low quantities of DNA extracted from noninvasive samples are well discussed elsewhere (Waits & Paetkau 2005; Beja-Pereira et al. 2009).

Carnivore species identification using NGS has primarily relied on mitochondrial DNA (mtDNA), in large part because of the much higher DNA copy number in mtDNA compared to nuclear DNA. Nevertheless, mtDNA also has important limitations. The common presence of nuclear copies of mitochondrial genes (numts; Triant & DeWoody 2007) that can easily be coamplified with orthologous mtDNA or even preferentially identified may either hamper or be a main factor of error for species identification (Kim et al. 2006; Ermakov et al. 2015); heteroplasmy, the presence of different mitochondrial molecules in the same individual, has also been documented for mammalian mitochondrial genomes (Hsieh *et al.* 2001); mtDNA introgression, the translocation of mitochondrial genes from one population/species into another has also been widely described in mammals (Mallet 2005; Melo-Ferreira et al. 2012) and can compromise the correct species identification for closely related species. Finally, the use of a single locus is highly limiting in case of natural or human-induced hybridization, as might happen between the European mink and the polecat or the wolf and the dog (Lodé et al. 2005; Godinho et al. 2011). In this respect, the study by Oliveira et al. (2010) on species identification of Iberian carnivores or diet studies of red wolves and coyotes (Dellinger et al 2011, McVey et al 2013) constitute good examples of how the use of different types of molecular markers can increase species identification accuracy.

Another constraint in the molecular identification of noninvasive samples of carnivores is the absence of a universal test. In fact, most available molecular tests identify only a limited number of species (but see DeBarba et al 2014 for test of 16 species) despite the constant development of new tests since 1992. To understand the evolution of the availability of molecular tests for species identification, we performed a second review to search for studies describing or using markers and lab techniques with direct application to NGS of our target

species. The details of the literature search and exclusion criteria are provided in Supporting Information.

We retrieved a total 42 papers developing markers specifically aimed for species identification in NGS of any of our target carnivore species. Diagnostic markers for NGS have been steadily increasing in availability since its introduction in 1992 by Höss *et al.* (1992), with a mean publication rate of 1.7 ± 0.3 studies/year (Fig. 7). By 2016, molecular markers for species identification have been described for most carnivore species inhabiting North America and Europe (n=31, 97% of our target species; Fig. 7), with a mean 3.8 ± 0.6 papers per species. Moreover, in 2002 there were already NGS markers available to confirm the identity of half of North American and European meso and large carnivores, and those tools have been available for 75% of the species for nearly 10 years (Fig. 7). The only species for which we did not capture research papers describing NGS-oriented markers for species identification was the American hog-nosed skunk (*Conepatus leuconotus*). These results support that the technological capability to engage in NGS is currently available for virtually all species of North American and European meso and large carnivores.

Patterns and implications of carnivore scats misidentification

From the carnivore diet and marker development literature searches, 57 studies reported scat misidentification rates representing 18 of the species considered in this review. These studies included 87 putative species-true species combinations ie."cases" because some studies had multiple target species and multiple species putatively identified as each target species. While the overall distribution of the proportion of false positive identifications was skewed towards lower values (Fig. 8), the median proportion of false positives (0.19) indicates that on average, approximately one-fifth of all samples in a study identified as the target species are actually from another species which could easily distort results and confound understanding of ecological patterns, especially if there is correlated bias in misidentification. This trend was consistent regardless of body size of the target species, and less than one quarter of all studies

reported the proportion of false positives < 0.05 (first quartile = 0.05), indicating that the inclusion of false positive samples is widespread in scat-based studies on carnivore species. In fact, there were only seven studies (11 total cases) that did not detect false positive samples (Fig. 8). Two relied on scat detection-dogs trained to detect the target species (Smith *et al.* 2003; Shores *et al.* 2015) and one relied on snow-tracking individuals of the target species to their resting sites to collect scats (Mills *et al.* 2000). Of the remaining four, one consisted of just two bobcat samples that were correctly identified in the field (Lonsinger *et al.* 2015). However, the three other studies involved larger sample collections in study areas with sympatric carnivores (Barja *et al.* 2007; Piñeiro *et al.* 2012; Garwood *et al.* 2015), and given the pervasiveness of false positives other studies, it is surprising that they reported finding no false positives in the respective datasets.

Conversely, 17% of studies reported proportion of false positives > 0.50, and it is instructive to review these studies to understand when field identifications are most likely to be inaccurate. Four putative-true species combinations from six study sites resulted in proportions of false positive > 0.75 (Davison *et al.* 2002; Smith *et al.* 2006; Witczuk *et al.* 2013; Monterroso *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 (Bulinski & McArthur 2000; Davison *et al.* 2002; Prugh & Ritland 2005; Monterroso *et al.* 2013). Red fox samples were misidentified as the infrequently encountered European wildcat (Monterroso *et al.* 2013), European pine marten (Davison *et al.* 2002), and San Joaquin kit fox (Smith *et al.* 2006) in regions where red foxes are more widespread. However, samples from the commonly abundant coyote were occasionally misidentified as red fox or kit fox (Lonsinger *et al.* 2015), bobcat (Lonsinger et al. 2015), and puma (Witczuk *et al.* 2013, Lonsinger et al 2015). In fact, misidentified red fox (in Europe) and coyote (in North America) were the primary source of false positives in studies of other carnivores. These misidentifications can result in high proportions of false positives when the target species is rarely or never detected, and should be scrutinized 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' sample sets are often opportunistic predators with wide trophic niche breadths and frequently reported scavenging behavior, often consuming carrion provided by larger carnivore kills (Díaz-Ruiz *et al.* 2013). Other sources of proportions of high false positives (>50%) include confusion between species of the same taxonomic family (Valière *et al.* 2003; Echegaray & Vilà 2010), and sympatric species of similar sizes (Long *et al.* 2007; Morin *et al.* 2016), and it is very likely all of these factors interact, and contribute to false negative rates as well.

Compared to false positives, false negatives are largely overlooked and their potential effects completely ignored in many cases. False negatives were reported for just 23 of the putative species-true species cases analyzed (only 9 studies). Species commonly misidentified as another species included coyotes, red foxes, bobcats, pumas, and gray wolves, but there was insufficient information to evaluate the proportion or rate of false negative misidentifications for most cases.

It is evident that both false positives and false negatives can occur frequently across all carnivore species and geographic areas, and may be particularly sensitive to relative abundances of target and sympatric species. Bias in dietary estimates may be nullified when both target and non-target species have highly overlapping diets (Martínez-Gutiérrez *et al.* 2015; Morin *et al.* 2016). However, they may severely alter interpretation and inference when samples from multiple non-targets 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), potentially resulting in underestimation of the prey biomass required for stable carnivore populations to persist. Adopting a more conservative sample collection approach (i.e., restricted scat dimension parameters or confidence rankings) could mitigate the effects of false positives (Reed *et al.* 2004; Prugh & Ritland 2005; Dellinger *et al.* 2011; Lonsinger *et al.* 2015), however an

unintended consequence is an increase 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 atypical samples (Morin *et al.* 2016), which can lead to self-confirming results. Thus, it is critical that potential errors in identification and possible outcomes relative to objectives be considered when planning or evaluating a diet analysis based on scat collection.

A noninvasive genetic sampling protocol for carnivore diet studies

Reviewing past studies identifies potential flaws in past research, but does not provide a solution *per se*. We believe that a substantial reason for the surprising low use of NGS for carnivore diet studies comes from a lack of operational guidance. Twenty-five years after the publication of fecal DNA techniques, conservation biologists, ecologists and managers, particularly those not affiliated with an institution with NGS capability, still struggle to understand when such technology is required for adequate ecological inference, what is the most appropriate way to collect and store samples, how to find and choose a NGS laboratory, or how to incorporate NGS data into their ecological datasets. We propose a step-by-step protocol to guide ecologists in planning, collecting and incorporating NGS data into ecological studies relying on carnivore scats (Fig. 9).

The first decision ecologists face when engaging in field sampling for carnivore scats is whether diagnostic species identification is essential. As is apparent from our review, confirmation of species identity is required in most cases. Exceptions include the following situations: i) there are no confounding species in the study area; ii) defecation is visually confirmed; iii) animals are backtracked to their daybeds or resting sites; or iv) the target species is overwhelmingly more abundant than the remaining potential confounding species (Fig. 9). The first criterion is rarely met because scat misidentifications are prone to occur across a broad range of species body mass values (Fig. 8). Even in urban or semi-natural environments, where carnivore communities are simplified, the presence of dogs can

contribute to a significant number of false positive scats (Valière *et al.* 2003; Krausman *et al.* 2006; Echegaray & Vilà 2010). Examples that fit this criterion include studies performed in particularly inhospitable insular systems such as arctic foxes or polar bears in Svalbard, where they are the two only terrestrial carnivore species (Wilson & Mittermeier 2009).

The second situation when diagnostic markers are not necessary is when target animals are directly observed (Gese *et al.* 1996; Angerbjörn *et al.* 2013; Cassidy *et al.* 2015; Clark *et al.* 2015). A more feasible and realistic way of verifying species identification from scats consists of backtracking target animals to places actively defended such as dens, daybeds, resting sites or even marking latrines. McKelvey *et al.* (2006) were able to identify with 100% accuracy all scats of Canada lynx encountered by backtracking the animals in the snow to their daybeds. Likewise, Marucco *et al.* (2008) were able to only collect wolf scats by snow tracking them along travel routes in the Italian Alps; and Stenglein *et al.* (2011) recorded a 99.2% field identification accuracy by collecting wolf scats at rendezvous sites in Idaho. 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).

The accuracy of field identification of scats is strongly related to target and confounding species relative abundances (Bulinski & McArthur 2000; Monterroso *et al.* 2013; Lonsinger *et al.* 2015). The probability of collecting a scat from a target species by chance depends on the proportion of scats in the population from the target species. Therefore, studies on disproportionally overabundant carnivores, as may happen with highly adaptable and opportunistic species (e.g. the red fox, Travaini *et al.* 1997; Bino *et al.* 2010) will inherently produce low false positive rates.

Only in those cases where at least one of the above-described criteria is met, should ecologists be confident enough to proceed to collecting, identifying and analyzing carnivore scats without molecular diagnostic species identity confirmation. However, these consist of a small fraction of all studies being performed on carnivores.

In most studies, ecologists should engage in a noninvasive genetic sampling protocol to ascertain species identification from scats. Our proposed NGS protocol for ecologists consists of five major steps (Fig. 9): i) Field collection; ii) storage and preservation; iii) sample selection; iv) selecting a NGS laboratory; and v) using NGS data.

i) *Field collection* – Ideally, scats to be analyzed using molecular methods should be collected using sterile single-usage latex gloves into a sterile receptacle (Beja-Pereira *et al*. 2009). In the absence of latex gloves, the key is to ensure that there is no contamination of the sample with DNA from the collector or any other animal source. Therefore, the scats can simply be directed into the receptacle using a locally collected stone or stick. Ideally, the receptacle should be a closable sterile vial, with a diameter that allows the sample to be placed inside with minimum abrasion on the outside, containing target species' DNA from exfoliated intestinal epithelial cells (Waits & Paetkau 2005, Wutlsch *et al.* 2015). When such vials are not available, a paper or zip-lock plastic bag may suffice while the sample is brought from the field, after which it should be transferred into a vial with the described characteristics. However, while in the plastic or paper bag, the samples should be carried using precautions to preserve the integrity of its most external parts. Because weather conditions, moisture and exposure to sunlight (Piggott 2005; Vynne *et al.* 2011), sample age (Piggott 2005; Santini *et al.* 2007; Panasci *et al.* 2011) or cross-species contamination (Rodgers & Janecka 2013) can affect amplification success, recording such information for each scat is relevant for the post-collection procedure of selecting the samples which should be sent for analysis.

ii) *Storage and preservation* – The method of preservation and storage of fecal samples can influence DNA amplification success (Murphy *et al.* 2002; Piggott & Taylor 2003; Waits & Paetkau 2005). The most common preservation methods consist in inhibiting nucleases' activity by dehydration using drying agents such

as ethanol (ETOH, e.g. > 90%) or silica (gel or beads), through removal of cations using chelator agents (e.g. DETs buffer), or by freezing (Waits & Paetkau 2005; Beja-Pereira *et al*. 2009; Rodgers & Janecka 2013). The efficiency of each preservation technique is highly variable and is affected by target species identify, life traits, diet, environmental conditions, and even by interactions between diet and preservation methods (Panasci *et al.* 2011) and by storage and extraction methods (Franz 2003; Waits & Paetkau 2005 and Beja-Pereira *et al*. 2009). However, carrying ethanol into the field is usually not a feasible practice. Two main solutions have been utilized. Beja-Pereira *et al*. (2009) suggest that combining silica and ethanol (ETOH) protocols could provide an enhanced preservation strategy, which would also be more logistically feasible for field conditions. Using this protocol, scats can be collected into zip-lock plastic bags along with a high volume of silica beads (higher than the scat volume) under field conditions and during transport. Afterwards, namely in the lab (or office), they can be transferred into vials with ETOH 96%, using a volume ratio of 1/5 (sample/ETOH; Beja-Pereira *et al*. 2009) and stored (ideally at -20^{o}C) for longer periods prior to shipment or molecular analyses. If scats will be shipped long distances or by air,, then ETOH would not be a good solution because it is flammable and more expensive to dispatch to the NGS lab. In such situations, samples may be dried and then transferred back to a silica-containing vial for shipment purposes. Another option is to collect a small portion of fecal material from the outside of the scat for DNA analysis and store in 2ml tubes with DETs buffer at room temperature and collect the remaining scat material for diet analysis (Dellinger et al. 2011; McVey et al. 2013; Gosselin et al. 2017). Remaining scats can then be dried or frozen for storage and transport. Recent studies evaluating the amplification and genotyping accuracy of DNA extracted from scat material (Wutlsch *et al.* 2015) or surface swabs (Miles *et al.* 2015;

Ramin-Laca *et al.* 2015) obtained under field conditions and preserved using ETOH or DET buffers, provided high success rates.

iii) *Selecting scats for genetic analysis* – Two main limitations for using NGS are collecting a large enough sample size from the target species and obtaining the necessary funding for molecular analyses. According to Trites & Joy (2005) a minimum number of 96 samples is required to detect statistically significant differences between two dietary patterns from scats with 80% power, a α level of 0.05 and an effect size of 0.30, when at least six prey species are consumed; and a minimum of 59 samples to ensure that prey items with a frequency of occurrence >5% are detected. Following a similar approach, Preez *et al.* (2017) found that no significant additional prey species were detected with sample sizes above 55 (for leopards) to 65 (for lions) scats. Using a conservative approach, a sample size of >100 samples of known origin would be required for an adequate characterization of dietary patterns for cross-species or spatio-temporal comparisons. Scat amplification and genotyping rates are variable and changing, however it is they are frequently ≥ 80% for mtDNA (Rodgers & Janecka 2013). Therefore, we recommend that a minimum of *c.* 125 samples should be collected and sent for NGS analysis to ensure a minimum effective sample size for carnivore diet assessment (i.e. n=100). However, given the mean expected proportion of false positives is *c*. 20% (see section "Patterns and implications of carnivore scats misidentification"), a total of ~160 scats should be collected from the field and sent for genetic analysis for species identification to ensure a final sample size of 100 samples. The approach should be more conservative (i.e. more samples should be collected) when the target species is known to be rare in the study site (Bulinski & McArthur 2000; Monterroso *et al.* 2013). However, it is not always possible to genotype all samples collected from the field, mostly due to financial constraints. In such situations, a subsample of the scats should be selected to

allow estimating the precision (i.e. 1 – proportion of false positives) of field identification of scats. The samples selected for NGS should be as fresh as possible to maximize amplification success (Piggott 2005; Santini *et al.* 2007; Panasci *et al.* 2011 and references therein) and utilizing a field-based classification of sample age and quality can be useful (Wulsch et al 2015). However, it is also important that sampling procedures maximize the number of individuals sampled as well as circumstances (i.e. latrines, den/rest sites, roads/trails) to avoid bias in diet estimation patterns (Steenweg *et al.* 2015).

iv) *Selecting a noninvasive genetics laboratory* – After selecting the samples to process, it is important to identify a laboratory to complete the genetic analyses. There are many factors to consider in selecting a laboratory including quality control procedures, success rates and error rates, experience with genetic identification of the species of interest, data sharing agreements, regulations for transport, and costs. To ensure accurate results and avoid contamination, many quality control procedures should be followed as summarized in Waits & Paetkau (2005). Researchers should also inquire about success rates and error rates for species ID since these can vary considerably across studies (Waits & Paetkau 2005; Broquet *et al.* 2007; Beja-Pereira *et al.* 2009), and it is best to select a laboratory with high success rates and low error rates. It can also be beneficial to select a laboratory that has previous experience and established protocols conducting species identification for the target species and sympatric non-target species. When selecting a laboratory, it is also important to consider transport regulations because some countries do not allow export of biological materials or require extensive permitting that may require or favor a local laboratory for analysis. Before initiating transfer of samples, it is important to establish data sharing agreements for use of data and publication as well as storage and use of samples after analyses are completed. Finally, cost is an important consideration

and can vary from $10 - $150 per sample depending on the type of analysis (mtDNA sanger sequencing, fragment analysis, nDNA analyses, high throughput sequencing) and labor (technician vs graduate student).

v) *Incorporating and reporting NGS data* – Whenever all scats were sent for NGS species identification, the dietary patterns for each target species should be reconstructed using only the samples with confirmed target species identity. Scats where the NGS procedure failed should be excluded from the diet estimation process to preclude biasing the parameters to be estimated (Morin *et al.* 2016; Weiskopf *et al.* 2016). However, when only a subsample was sent for NGS profiling to obtain estimates field identification precision, then a more cautionary and sophisticated approach is required. In such cases the diet reconstruction obtained from the field-identified dataset should incorporate that 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 the uncertainty can be estimated using re-sampling protocols (e.g. bootstrapping; Manly 2006), whereby random subsamples of size equaling the expected proportion of true positives are repeatedly obtained from the total population of scats used for diet analysis. For example, in a case where 500 scats were collected and field-identified as belonging to coyotes, of which 50 were sent for NGS profiling with a 85% and 80% amplification and genotyping success rates, respectively, the NGS-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 73.5% [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 estimated at each iteration, allowing the estimation of its uncertainty. More accurate estimates of dietary parameters and their