

# The problem of omnivory: A synthesis on omnivory and DNA metabarcoding

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## Abstract

Dietary analysis using DNA metabarcoding is a powerful tool that is increasingly being used to further our knowledge of trophic interactions in highly complex food webs but is not without limitations. Omnivores, the most generalist of consumers, pose unique challenges when using such methods. Here, we provide the rationale to understand the problems associated with analysing the complex diets of omnivores. By reviewing existing metabarcoding studies of omnivorous diet, and constructing hypothetical scenarios arising from each, we outline that great caution is required when interpreting sequencing data in such cases. In essence, the problems of accidental consumption and secondary ingestion are significant sources of error when investigating omnivorous diets. The integration of multiple high throughput sequencing markers increases the taxonomic breadth of taxa detected but we reveal how some detections may be misleading. Disentangling which taxa have been deliberately or accidentally consumed by the focal omnivore is challenging and can falsely emphasise those that were not intentionally consumed, obscuring biologically meaningful interactions. Although we suggest ways to disentangle these issues, we urge that the results of such analyses should be interpreted with caution and all possible scenarios for the presence of biota within omnivores given due consideration.

## KEYWORDS

diet analysis, generalist, high-throughput sequencing, multiple markers, secondary predation, trophic interactions

## 1 | THE BENEFITS AND LIMITATIONS OF DIETARY ANALYSIS USING DNA METABARCODING

### 1.1 | Benefits

A key objective of ecology is to determine the structure of complex species-interaction networks and analyse the processes driving their dynamics. The consumption of one individual by another is an important biological interaction that helps shape species' distributions, behaviour, anatomy, and abundance. Environmental

DNA metabarcoding, the amplification and sequencing of short sections of DNA from samples taken from the environment (Pompanon et al., 2011; Taberlet et al., 2012), is a promising and powerful tool that, when combined with high-throughput sequencing (HTS), can simultaneously elucidate a broad range of trophic interactions in complex food webs (Littlefair et al., 2016; Pompanon et al., 2012). This method offers an improvement over morphological identification of consumed taxa from gut contents or faeces, which may fail to detect small or soft-bodied organisms and can be taxonomically imprecise (Pompanon et al., 2012; Symondson, 2002). Moreover, some predator-prey interactions

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are difficult to directly observe. This is especially true of invertebrate predators and/or prey, where microscopic identification is frequently impossible. Metabarcoding can resolve prey taxa to species level in systems where this would be otherwise impossible, e.g., in fluid-feeding invertebrates such as spiders (Cuff et al., 2020; Krehenwinkel et al., 2017), most insects (Pompanon et al., 2012), and centipedes (Eitzinger et al., 2018). Similarly, there are many prey species that do not possess morphologically identifiable structures that resist digestion, such as earthworms (Brown et al., 2012). In recent years, HTS approaches have revolutionised our understanding of predator-prey relationships in complex food webs and a diverse array of ecosystems, overcoming many of the shortfalls inherent to other methods.

## 1.2 | Limitations

Despite the clear benefits and demonstrable utility metabarcoding provides to unveil trophic interactions, there are several shortfalls with the methodology and these have been extensively reviewed in detail (Lamb et al., 2019; Pompanon et al., 2012; Symondson & Harwood, 2014; Taberlet et al., 2018). Briefly, there are inherent biases associated with several key steps in DNA metabarcoding, from amplification through to sequencing, but arguably the most critical lies in the selection of polymerase chain reaction (PCR) primers (Piñol et al., 2019). DNA used for metabarcoding-based dietary analysis is, almost by necessity, relatively low concentration and degraded by the consumer's digestive system (Symondson, 2002). Therefore, short DNA strands, amplified through PCR, must be used to identify prey species. For metabarcoding, these are usually 100–350 bp long (Elbrecht & Leese, 2017; Leray et al., 2013; Symondson, 2002; Zeale et al., 2011), with a few successful longer exceptions (Elbrecht et al., 2018; Lafage et al., 2020). These short, degraded sections of DNA are, however, often outcompeted during amplification by the fresh and abundant DNA of the focal consumer where the PCR primers used amplify both. This can, however, be circumvented by using primers that amplify a broad taxonomic range of consumed species but do not also amplify host DNA (Cuff et al., 2020; Lafage et al., 2020; Piñol et al., 2014; Vestheim & Jarman, 2008) or by using blocking probes (Vestheim & Jarman, 2008), both of which can introduce taxonomic biases to those prey detected (Murray et al., 2011; Piñol et al., 2015, 2019). For analysing omnivorous diets, using multiple HTS markers is optimal to determine the complete range of consumed prey given few options are currently available for the adequate amplification of animals and plants with a single primer pair. Such primers have been designed (Berschick, 1997), although the taxonomic resolution achieved by these and the less populated reference databases associated with the loci may reduce the utility of this approach for many studies. The direct competition of animal and plant DNA in the amplification process of these primers could, however, mitigate some of the issues discussed later in this manuscript.

Alternatively, some studies accept amplification of host DNA and remove reads conspecific with the host post-bioinformatics (Piñol

et al., 2014); in such studies over 95% of the sample read depths can be lost to host DNA (Cuff et al., 2020). By limiting amplification of the host, however, a greater read depth is ensured which is increasingly likely to accurately capture the true diversity of consumed species. This choice of method can be most acute when the predator is closely related to its prey, for example, when a spider is eating other spiders (Cuff et al., 2020), requiring a fine balance between taxonomic bias and loss of sequencing depth. Few problems are encountered where the consumer is distantly related, such as an herbivore ingesting plant material. Obstacles in securing taxonomically complete coverage can theoretically be overcome by using several pairs of primers (each with their own biases, of course), but this rapidly multiplies sequencing costs. Furthermore, prospective primers should be tested for specificity against a broad range of potentially consumed organisms from the sample site.

Perhaps more fundamentally problematic, sequencing results cannot accurately predict the amount of biomass consumed by a focal species (Deagle et al., 2019; Lamb et al., 2019), nor can it quantify how much nutrition is obtained (Deagle et al., 2010, 2013; Pompanon et al., 2012). Sequencing data affords us, at best, a semi-quantitative prediction of biomass consumed from sequence reads (De Barba et al., 2014; Deagle et al., 2019; Lamb et al., 2019; Piñol et al., 2019; Pompanon et al., 2012), or can be used to infer rates of predation through knowledge of the approximate length of time for which a given length of DNA can be detected (Egeter et al., 2015). Differences in the concentration of organelles commonly scrutinised in metagenomics (e.g., mitochondria, chloroplasts, ribosomes) between tissues and species currently precludes determination of relative biomass of consumed taxa (Veltri et al., 1990). Inherent technical biases, such as those associated with sequencing and PCR, present further problems to accurately extrapolating how much of a given organism was eaten by a consumer (Deagle et al., 2013; Murray et al., 2011; Piñol et al., 2019; Thomas et al., 2016). The amount and type of tissue being consumed will partially determine the importance of a given species interaction. For example, a predator may infrequently consume large amounts of a large-bodied prey species in a single meal, perhaps even selecting specific tissues, but very frequently consume whole small-bodied prey species. Given that biomass consumed and nutritional value can only be, at best, very tentatively interpreted from read numbers in a few circumstances, analyses commonly rely on frequency of occurrence as a measure of importance, but this can conceal true biological importance to the consumer (Deagle et al., 2019). Similarly, the tissue type consumed is impossible to determine from sequencing results alone; a predator may only eat highly nutritious tissues, such as bears consuming only the brain or eggs of hyperabundant salmon (Gende et al., 2001), or lions eating the intestines of ungulate prey (removing plant matter within) before other tissues (Schaller, 1976). For plants consumed, there may be several different tissues potentially ingested, e.g., fruits, seeds, leaves, roots, pollen, nectar, or any combination of a plant's tissues. This may obscure important ecological functions, such as seed dispersal and pollination. Even when seasonality is accounted for, which may lower the number of potential tissues

consumed depending on fruiting/flowering phenology, a range of potential tissue types remain.

Finally, it is often challenging to determine if certain taxa were consumed deliberately or accidentally, and this issue is especially acute when analysing the diet of omnivores using multiple HTS markers.

## 2 | THE PROBLEM OF OMNIVORY

### 2.1 | Previous DNA metabarcoding of omnivorous diets using HTS

Omnivores, by their generalist nature, can elicit top-down effects across the entire breadth and depth of ecological networks. Determining the structure and dynamics of these interactions is therefore valuable in understanding the wider food-web and the biology of focal omnivores. However, these species present the greatest challenge to accurate identification of what they may have consumed (De Barba et al., 2014; Silva et al., 2019). Deciphering the complete diet of omnivores is time-consuming, taxonomically challenging and, in the case of DNA metabarcoding, quite costly (Pompanon et al., 2012; Symondson & Harwood, 2014). Thus, few studies have analysed the complete diet of omnivores using HTS. De Barba et al. (2014) used a combination of three universal primer pairs amplifying vertebrate, plant and invertebrate DNA, as well as two group-specific primers amplifying four plant families, to determine the diet of brown bears (*Ursus arctos*) from 91 faecal samples. Plants and invertebrates predominated in the diet, with Asteraceae and Formicidae most frequently occurring in these groups, respectively. The authors suggest additional precision was needed to be able to make stronger inferences relating to diet composition, such as more extensive barcoding of the plant community at sample sites or using taxon-specific markers for important taxa. A combination of study-specific factors and shortfalls inherent to HTS dietary analyses led the authors to suggest cautionary interpretation of dietary patterns using their data.

Similarly, Robeson et al. (2018) investigated the omnivorous diet of wild pigs (*Sus scrofa*) using DNA metabarcoding. This study used two primer pairs amplifying animal (COI) and plant (trnL) DNA from 48 wild pigs sampled across three US states. Analyses showed wild pigs consumed a wide variety of animal and plant resources, including species of high conservation concern and financial value. The authors suggest this information can be used to better mitigate the detrimental effects of wild pigs as an invasive species. As with the study by De Barba et al. (2014), Robeson et al. (2018) also encountered several issues, both specific to their study system and more generally when using HTS methods to determine diet, such as the inability to determine if certain plants were deliberately or accidentally consumed.

In Portugal, da Silva et al. (2019) tested the efficacy of using a multimarker metabarcoding approach to reveal the diet of black wheatears (*Oenanthe leucura*), an omnivorous passerine bird. The

study showed that using multiple markers overcame the underestimation of dietary diversity present compared to a single marker approach, as the combination of markers overcame the biases inherent in each one. Overall, results showed black wheatears to rely primarily on invertebrates (Araneae, Coleoptera, Diptera, Hemiptera, Hymenoptera: Formicidae, and Lepidoptera) and the berries of *Pistacia terebinthus*, *Solanum nigrum*, and *Vitis vinifera*. The authors point out, however, that most plants detected in the diet are probably a result of secondary consumption, concluding that only ~8.5% of plant species detected were directly eaten by the wheatears. They suggest this may be a problem inherent to researching the diet of omnivores using metabarcoding.

More recently, a study on black bears (*Ursus americanus*) in Canada by Bonin et al. (2020) used a mixed approach of HTS, morphological examination of faeces, and stable-isotope analysis to determine diet. Results revealed that black bears consumed a wide variety of vertebrate and plant species. Unfortunately, a primer pair amplifying invertebrate prey was not used, which may have obscured biologically important interactions given that insects are considered a primary food resource for black bears (Eagle & Pelton, 1983), especially social Hymenoptera (Landers et al., 1979). Furthermore, this study collected scats up to 14 days old, which the authors suggest may have led to excessive DNA degradation and false negatives. Indeed, visual examination of scats uncovered a greater dietary species diversity and frequency of occurrence in most cases. The chance of contamination of scats by species found in the immediate environment may also increase with time exposed before sample collection but obtaining samples immediately after excretion is probably challenging in free-ranging populations of black bears. Nevertheless, the use of combined methods by Bonin et al. (2020) overcame many of the issues faced by relying solely on one method of dietary determination.

Across these studies, authors highlight that some species detected in the diet may be present because of accidental consumption and secondary predation, presenting a larger problem when investigating the diet of omnivores relative to consumers with simpler diets.

### 2.2 | Accidental consumption

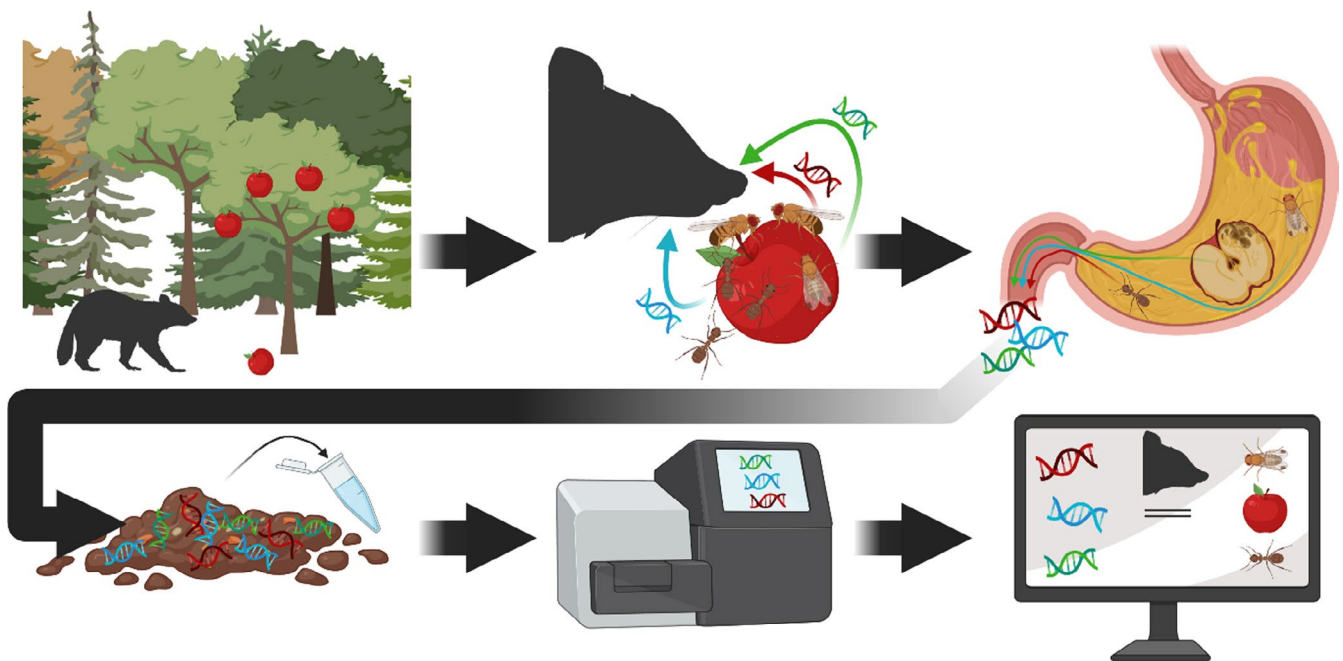
The use of multiple primer pairs is almost always necessary when investigating the diet of an omnivore using metabarcoding because it can theoretically determine the complete range of consumed species across different kingdoms, which would otherwise be difficult using a single marker (but see Berschick, 1997). A problem arises, however, when certain species are accidentally consumed. These detections may be difficult or impossible to distinguish from species eaten deliberately. For example, Robeson et al. (2018) suggest that oak may feature prominently in the diet of wild pigs (found in over 40% of pigs sampled in California) because of incidental consumption of leaves or roots whilst foraging for other resources. Forests in the California site are dominated by oak, increasing the chances a pig would consume oak tissue, such as fallen leaves or broken bark. The

authors highlight how samples were collected before the ripening of acorns, which pigs are known to consume, decreasing the likelihood of acorn consumption except in a few circumstances. Given the predominance of oak in these samples, accidental consumption therefore becomes the most parsimonious explanation. This example suggests that non-dietary plant tissues abundant in the foraging area of a study species can be frequently detected in the dietary results. These misleading positives may not be limited to fallen leaves, but any type of abundant plant tissue found on or around species deliberately consumed by an omnivore, e.g. pollen on insects and other plants, nesting material of prey, and all types of plant matter that might be common in the surroundings at the point of consumption by the consumer. A similar issue may be true if studying mycophagous omnivorous species given that fungal spores will seasonally cover most surfaces in some habitats and differentiating between consumed fungi and species naturally present in the gut could be challenging.

Accidental consumption may also confound how animal prey are consumed. For example, a hypothetical generalist vertebrate scavenger is known to feed on vertebrates, invertebrates, and plants and researchers are collecting fresh faecal samples to elucidate trophic interactions using DNA metabarcoding (Figure 1). The focal omnivore commonly feeds on fallen fruit, but these fruits are rapidly colonised by flies and ants upon reaching the ground. The omnivore indiscriminately consumes the fallen fruit, accidentally ingesting many ants and flies at the same time. When primer pairs amplifying plants and insects are used, the flies, ants, and fallen fruit are all amplified and detected in the sequencing results. Researchers are then tasked with determining if these detections are deliberate or

accidental. Given that the omnivore is known to feed on invertebrates, this may be a difficult question to answer. The ant species itself is an abundant generalist omnivore and colonises most food items before the hypothetical vertebrate, and thus appears in many faecal samples. The issue is exacerbated if little is already known about specific trophic interactions between the omnivore and its prey, making disentangling what has happened more challenging. The flies and ants in the above scenario are assumed to be highly important to the vertebrate omnivore because they frequently occur in faecal samples, but this may not be true biologically. The omnivore may not benefit from ingesting these species; indeed, the vertebrate may actually be harmed by feeding on the ants, which typically possess distasteful compounds and/or venom (Blanchard & Moreau, 2017; Hölldobler & Wilson, 1990; Schmidt, 2009).

Accidental consumption can cast doubt on the validity and importance of trophic interactions detected using DNA metabarcoding. It may be impossible to determine if dietary taxa have been consumed deliberately or accidentally by the study species. In these cases, the confidence that researchers may have in the importance of specific trophic interactions may be reduced, or entire data sets arising from such methods depending on the context of the research question being asked. It is important to consider this issue when investigating the diet of omnivores because of the increased taxonomic breadth of potential dietary species. Although it is being assumed that deliberately consumed species offer more meaningful nutritional benefits to a consumer than accidentally ingested species, it may be that accidentally ingested species also confer nutritional benefits to the consumer. For example, spiders may benefit nutritionally from pollen collected on their webs or on the surface of



**FIGURE 1** A hypothetical generalist vertebrate omnivore feeding on a fallen fruit colonised by insects. The apple is intentionally consumed and the insects accidentally, resulting in detection of both plant and insects in the DNA metabarcoding output, and likely equivalent representation in subsequent analyses. Figure created in Biorender.com

the prey they consume (Nyffeler et al., 2016). Unfortunately, glean-ing this information from sequencing data is not possible. Ultimately, these issues must be considered in the context of the question being asked and the impact that accidental consumption may have upon the consequent interpretation of data.

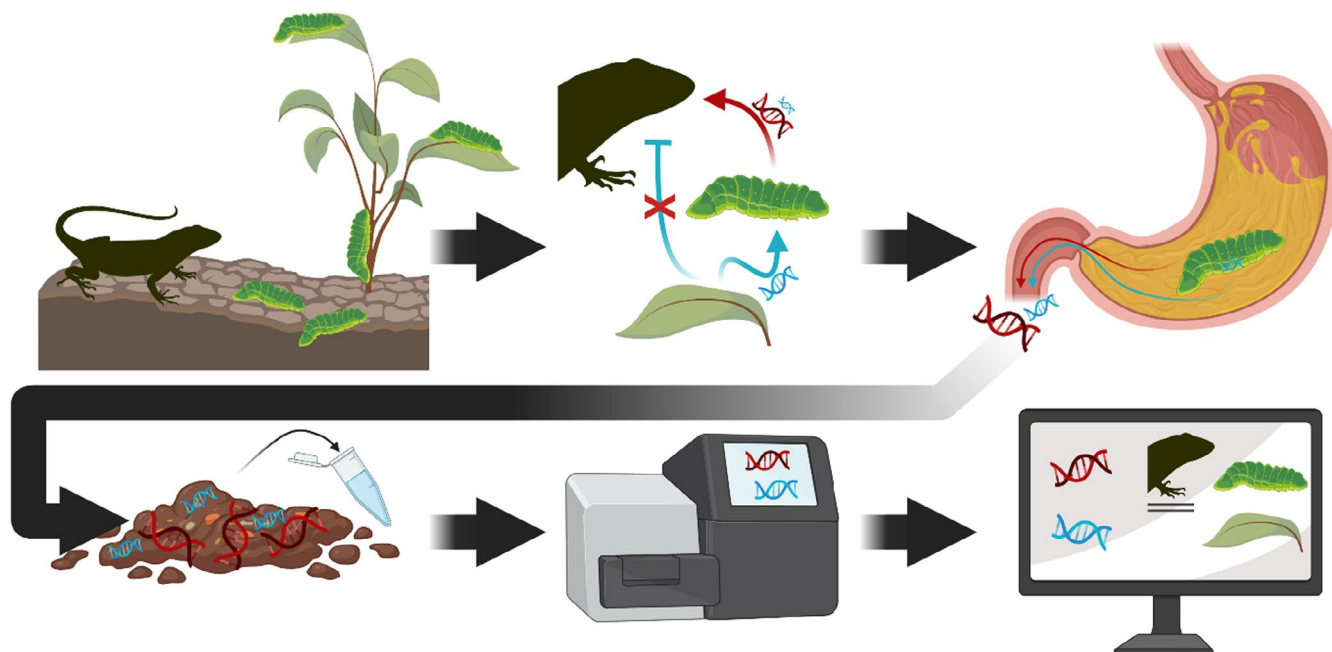
Finally, parasites and parasitoids may also be consumed by pred-ators incidentally. In some groups, such as parasitoid wasps and aphids, this may change the nutritional profile of the consumed meal for the predator given the biomass of the parasite relative to its host. Parasitism can facilitate profound morphological changes, through mummification in the example of aphids, which will undoubtedly af-fect nutritional composition. Such nutritional modulation may have positive or negative effects on predators consuming parasitised prey through altered nutritional provision (Traugott et al., 2012), although less so regarding instances of parasitism with low relative parasite biomass. Parasite DNA may nevertheless be detected frequently.

### 2.3 | Secondary predation

Secondary predation/consumption involves the detection of spe-cies eaten by the prey of a focal predator (Sheppard et al., 2005), and can be considered a type of accidental consumption (Harwood et al., 2001). As well as the well-documented possibility of a primary consumer eaten by a secondary consumer being detected in the diet of a tertiary consumer (Pompanon et al., 2012; Sheppard et al., 2005; Silva et al., 2019; Symondson, 2002), omnivores present the problem that secondary consumption may disproportionately in-flate the presence of plant taxa being detected using HTS and DNA

metabarcoding (Silva et al., 2019). Plant tissues eaten by herbivores, which are then eaten by an omnivore, may be detected if appropri-ate plant primers are used. These detections can be indistinguish-able from plant tissue deliberately eaten by an omnivore, especially if the omnivore consumes one tissue type and the herbivore con-sumes another of the same plant species, e.g., fruit and leaves. This may be particularly problematic if an omnivore is feeding on insect herbivores (Guenay et al., 2020). Because of the low energy value of plant matter relative to animal tissues, some herbivorous insects, particularly larvae and nymphs, can be in a frequent state of feed-ing when active, with high volumes of fresh plant matter present in their digestive system (Chapman, 2013). Insects rely on volumetric negative feedbacks to determine when to stop feeding, coming from stretch receptors found in the alimentary canal or body wall that measure the level of distention in the gut (Chapman, 2013). These inhibit feeding when the gut is full. As plant matter moves through an insect's digestive system, the stretch receptors eventually relax and restimulate feeding behaviour, ensuring fresh plant matter once again enters the digestive system. These behaviours maximise growth in larval insects and can ensure a high volume of fresh plant matter continues to pass through an insect's digestive system.

Assuming frequent consumption, e.g., intervals between feeding in the order of 15–30 min for many caterpillars (Chapman, 2013), plant matter will follow a “degradation gradient” through the her-bivore's digestive system, from minimally degraded DNA just after consumption, to heavily degraded DNA after passing through the diges-tive system. Thus, many of these herbivores will have significant volumes of amplifiable plant DNA at some point through their diges-tive system. When such an herbivore is consumed by an omnivore, it



**FIGURE 2** A hypothetical vertebrate feeding exclusively on an invertebrate herbivore will ingest the DNA of the herbivore's recent diet. If using plant-amplifying metabarcoding primers, this DNA will be detected as a dietary component of the vertebrate and, if no other plant DNA is detected, will be represented as a large contingent of its diet, possibly larger than the consuming invertebrate itself. Figure created in Biorender.com

may be difficult to determine if plant DNA detected in the sequencing results is due to direct consumption of a plant by the omnivore in question, or by the herbivore that it has consumed. This could then confound both species level interactions as well as broader dietary patterns. For example, the proportion of animal and plant taxa consumed by an omnivore detected using metabarcoding in these scenarios might be incorrectly skewed towards increased plant consumption if such herbivorous prey are commonly consumed (Silva et al., 2019). These factors may conceal the ecology of the focal omnivore and the dynamics of the species interaction network.

Equally, if studying a hypothetical species for which the diet is largely unknown, but is presumed to be omnivorous, this secondary consumption problem could falsely support the hypothesis of omnivory or wrongly inflate the importance of plants in its diet (Figure 2). For example, if a species were to exclusively consume herbivores, with no plant matter directly consumed, this secondary plant DNA would still be detected and, due to the representation of many metabarcoding data as presence/absence, could be considered equivalent in importance to the DNA of the herbivore. This problem will always result in the overestimation of plant consumption by omnivores unless a great quantity of plant DNA recently directly consumed by the omnivore outcompetes the secondary DNA present in its recent prey. Moreover, the above possibilities suggest it is essential to view the complete diet of an omnivore. Examining solely the plants detected in the faeces or gut contents of an omnivore may be misleading without the accompanying animals consumed to facilitate disentanglement of these relationships.

Finally, related to secondary consumption, are coprophagous species. These purposefully consume the faeces of other organisms and may present similar challenges when interpreting which taxa are being interacted with frequently from the sequencing output. Results may suggest that a specific species is being directly relied upon by an omnivore instead of the consumer of that species, which could have important ramifications in applied settings such as conservation.

### 3 | CONCLUDING REMARKS

Discerning the diet of omnivores is challenging, but DNA metabarcoding has demonstrably facilitated the most comprehensive dietary assessments to date for a number of trophic generalists (Bonin et al., 2020; De Barba et al., 2014; Robeson et al., 2018; Silva et al., 2019), revolutionising the field of trophic ecology. Nevertheless, the problems of secondary predation and accidental consumption are exacerbated when examining the diet of omnivores. There may be some tools to help researchers disentangle detections arising from accidental/secondary consumption. For example, using a post hoc probabilistic co-occurrence analysis to show which taxa are simultaneously present in the same faecal samples. This can be done using R package `COOCCUR` (Griffith et al., 2016) as per other studies using metabarcoding data (Brandl et al., 2020; Harper et al., 2020; Holmes et al., 2019); the analysis statistically measures whether species

co-occur in diet samples nonrandomly. Conducting a co-occurrence analysis can, in some circumstances, highlight whether an insect herbivore and its host plant significantly co-occur in faecal samples of the study species. Results could suggest detection of the plant species is because of secondary predation instead of direct consumption if most species detections co-occur in faecal samples with one another (da Silva et al., 2020). However, co-occurrence analyses are exploratory and co-occurrence of species is not confirmation of ecological interaction (Blanchet et al., 2020). Probabilistic co-occurrence analyses should therefore be interpreted carefully and in many cases may not facilitate interpretation e.g., because of highly polyphagous herbivores or environmentally ubiquitous plant tissue, such as pollen. Similarly, direct observation of feeding behaviours can help to tease apart how an omnivore may have consumed certain dietary items, but this is not feasible for a range of taxa or study systems (e.g., most invertebrates, nocturnal and secretive species, subterranean and fossorial consumers). It may be that many omnivores go undetected because of the assumption that they are herbivores or carnivores without sufficient research to confirm these hypotheses. For many species, direct observation studies may already exist that authors can use to inform their study design, molecular methodology and sequencing output interpretation. Metabarcoding may, however, be insufficient to detect these deviations from expected diet for cannibalistic species that are otherwise herbivorous (Booth et al., 2017); this is an important consideration for studies attempting to comprehensively characterise nutritional intake, but an equally problematic and widely-known issue for any metabarcoding study of carnivorous opportunistic cannibals.

Dietary metabarcoding has the potential to reveal these omnivores and their trophic interactions, but data will need to be carefully interpreted to accurately elucidate their trophic ecology. More generally, metabarcoding has revolutionised diet assessment and can be used in tandem with other methods (e.g., stable isotope analysis [Hambäck et al., 2016], morphological examination of faeces or gut contents [Brassea-Pérez et al., 2019] or both [Bonin et al., 2020]) to provide the most comprehensive dietary analyses to date. These methods can therefore help to identify complete ecological networks in a range of environments and with myriad applications.

Examining the dietary dynamics of omnivores is highly valuable to the field of ecology but there does not currently appear to be a panacea for the complex issues in doing so using DNA metabarcoding. Therefore, the results of such analyses should be interpreted with these issues accounted for and, ideally, paired with auxiliary behavioural observations and post hoc probabilistic co-occurrence.

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## AUTHOR CONTRIBUTIONS

Maximillian P. T. G. Tercel designed the study with substantial contributions from Jordan P. Cuff. Maximillian P. T. G. Tercel led the writing with substantial contributions from Jordan P. Cuff, whilst all authors improved the manuscript during the editing process. Jordan P. Cuff designed and produced the illustrations. William O. C. Symondson strengthened the concepts and edited the manuscript.

## DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no data sets were generated or analysed during the current study.

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