Diet tracing in ecology: method comparison and selection

Jens M. Nielsen[1,3], Elizabeth L. Clare[1], Brian Hayden[2], Michael T. Brett[3] & Pavel Kratina[1]

[1]School of Biological and Chemical Sciences, Queen Mary University of London, London E1 4NS, UK

[2]Canadian Rivers Institute, Biology Department, University of New Brunswick, Fredericton, New Brunswick E3B 5A3, Canada

[3]Department of Civil and Environmental Engineering, University of Washington, Seattle, Washington 98105, USA

Running Head: Diet tracing in ecology

***Corresponding Authors:** jensmunielsen@gmail.com, p.kratina@qmul.ac.uk

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Summary

1. Determining diet is a key prerequisite for understanding species interactions, food web structure and ecological dynamics. In recent years, there has been considerable development in both the methodology and application of novel and more traditional dietary tracing methods, yet there is no comprehensive synthesis that systematically and quantitatively compares among the different approaches.
2. Here we conceptualize diet tracing in ecology, provide recommendations for method selection, and illustrate the advantages of method integration. We summarize empirical evidence on how different methods quantify diet mixtures, by contrasting estimates of dietary proportions from multiple methods applied to the same consumer-resource datasets, or from experimental studies with known diet compositions.
3. Our data synthesis revealed an urgent need for more experiential comparisons among the dietary methods. The comparison of diet quantifications from field observations showed that different techniques aligned well in cases with less than six diet items, but diverged considerably when applied to more complex diet mixtures.
4. Efforts are ongoing to further advance dietary estimation, including how reliably compound specific stable isotope analyses and fatty acid profiles can quantify more prey items than bulk stable isotope analyses. Similarly, DNA analyses, which can depict trophic interactions at a higher resolution than any other methods, are generating new ways to better quantify diets and differentiate among life-stages of prey. Such efforts, combined with more empirical testing of each dietary method and establishment of open data repositories for dietary data, promise to greatly advance community and ecosystem ecology.

Introduction

For generations, biologists have posed the seemingly simple question: "what does this animal feed on?" Such diet estimates provide the foundation for understanding the interactions (Kartzinel *et al.* 2015) which structure ecological communities and regulate the flow of energy and nutrients through food webs (McCann 2007). The characterisation of a consumer's resource use also provides insights into intra- and inter-specific niche specialization (Kratina *et al.* 2012), and nutritional physiology (Martínez del Rio *et al.* 2009). When compiled across whole communities, diet records provide powerful insights into the structure and function of entire ecosystems (Estes *et al.* 2011). The advent of biomarker and molecular based approaches has enhanced the precision of diet estimates, and new approaches of data integration promise to greatly expand our understanding of ecological communities (Poelen, Simons & Mungall 2014). Yet, empirical characterization of feeding interactions remains challenging, as many animals utilize diverse diets and exhibit complex spatial and temporal foraging patterns (McMeans *et al.* 2015). Furthermore, local and global environmental perturbations continuously alter animal physiology and feeding behaviour (Tunney *et al.* 2014). Because trophic relationships are key mediators of community dynamics and ecosystem functioning (Cardinale *et al.* 2012), our understanding of natural systems relies on rigorous estimates of animal feeding. How to best apply and integrate these diverse methods however remains an open question.

Ecologists are faced with a range of techniques to assess consumer diets. These include common visual techniques, such as gut, stomach, faecal or scat content analyses, DNA identification of prey items, organic macromolecules (e.g. fatty acids), and stable isotope analyses of bulk or specific compounds. While all these methods have the overarching aim of retrieving feeding information for animals or even carnivorous plants, these approaches differ considerably in their ability to identify and quantify diet components (Traugott *et al.* 2013).

Recent reviews have focused primarily on one dietary method (Boecklen *et al.* 2011; Evans *et al.* 2016), or described use of selected methods for specific taxa (Bowen & Iverson 2013) or for specific ecosystems (Traugott *et al.* 2013). However, there is no general synthesis that systematically and quantitatively evaluates all common dietary approaches. Here, we provide a comprehensive comparison of techniques, guiding researchers to select the most appropriate dietary method for a specific research question. More specifically:

1. We conceptualize fundamental steps in diet tracing and highlight important assumptions, biases and uncertainties associated with each method.
2. We compare the ability of each method to identify, differentiate and quantify multiple diet items.
3. We summarize empirical evidence on how different methods quantify diet mixtures, by systematically contrasting estimates of dietary proportions from multiple methods applied to the same consumer-resource data, or from experimental studies where the diet proportions were known.
4. Finally, we provide recommendations for method selection, discuss the advantages and challenges of method integration, and summarize future avenues for advancing diet tracing.

A conceptual overview of diet tracing

Consumers are exposed to a variety of "potential diets", i.e. resources or prey available in the consumers' environment. However, due to preferential feeding, which can differ among individuals and life stages (Werner & Gilliam 1984), a consumer's "true diet" represents only a subset of this potential diet (Fig. 1A). Following successful attack and capture, a portion of ingested material is assimilated and some of the assimilated fraction is retained and

74 incorporated into consumer's tissue while the remainder is respired or egested (Fig. 1B). All
75 methods attempt to accurately represent a consumer's true diet, however different methods
76 measure different consumed components. Visual and DNA-based diet analyses measure the
77 ingested or egested material, whereas stable isotope and biomarker (specific compounds used
78 for dietary analyses) approaches estimate the assimilated fraction. Importantly, a specific
79 biomarker, such as e.g. a fatty acid or an elements isotope ratio, is assumed to reflect general
80 dietary contributions, yet in fact they measure the dietary contribution of only the specific
81 compound. The relative amounts of ingested and assimilated diet can vary substantially, by
82 the order of consumption or even among biochemical compounds (Mitra & Flynn 2007).
83 Certain components, such as cell cytoplasm, may be preferentially assimilated, whereas
84 others, such as plant lignocellulose, are difficult to digest and assimilate (Brett *et al.* 2017).
85 Furthermore, current dietary tracing methods cannot quantify energetically important
86 resources that are respired. Each method thus captures a different fraction of the true diet, and
87 the degree of overlap between the methods is still poorly resolved. See Table 1 for
88 explanation of key terms.

89 An optimal method should provide full coverage of the potential diet, clear partitioning
90 among diet items (hereafter diet separation), and the capability to identify (and when possible
91 quantify) diet items to a suitable taxonomic resolution, e.g. species, genus or family (Table 1,
92 Fig. 1C). Identifying diet items requires *a priori* developed dietary reference material (e.g.
93 genetic reference libraries, end-members fatty acid profiles or isotope values of expected
94 diets). Even visual methods rely on taxonomic knowledge of potential prey items. Reference
95 material can be constructed using: i) general reference libraries, which cover potential diets in
96 the broadest sense without being specific to the particular consumer studied (e.g. GenBank,
97 Benson *et al.* 2013), ii) direct experimental manipulations (Galloway *et al.* 2015), or iii)
98 targeted field sampling, focussing on the particular habitat and the foraging behaviour of the

specific consumer (García-Robledo *et al.* 2013). If a library comprises only a subset of the true diet, some components will remain undetected or misattributed to the resources included in the library, resulting in erroneous diet estimates. Critical consideration of each tracing step and understanding how each diet component can be modified before, during and after capture, ingestion and assimilation is essential to retrieve accurate estimates of a consumer's true diet.

Dietary tracing techniques

Visual analyses of stomach, gut or scat content

A direct observational record of a consumer's diet is, in principle, the best characterization of the trophic relationships between species (Stuart-Smith *et al.* 2013). Visual observations of foraging strategies can be effective for large terrestrial mammals and birds (Pineda-Munoz & Alroy 2014), but this is impractical for nocturnal, rare or aquatic consumers (Hyslop 1980). Furthermore, it is often impossible to directly observe the consumers' diet over an extended period, although remote observations from e.g. cameras, can partly overcome such challenges (Tollit *et al.* 2006; Giovanni, Boal & Whitlaw 2007). For feeding habits that cannot be directly observed, visual analyses of stomach, gut, faecal, or scat material facilitate the characterization of prey items to species level. Because the stomach content or faeces typically represent consumption over the preceding 6–48 hours, such analyses can provide insights into daily to weekly variation in consumption, but are not well suited for integrating across longer time scales (Hayden, Harrod & Kahilainen 2014).

At a rudimentary level, visual analyses can detail the presence or absence of specific diet items ingested by the consumer. Moreover, when prey items show large differences in body size, these estimates do not accurately measure quantitative resource contributions. Diet can be quantified as relative abundance by number, wet or dry mass, or visually using a points

123 method, whereby the relative abundance of specific prey items is recorded on a scale of 1-10,
124 to estimate the relative contribution of the various diet items (see Hyslop 1980 for a review
125 focusing on fish). Individual metrics can also be combined into a composite index of relative
126 prey importance, though this may not be an optimal solution when sample sizes are low
127 (Baker, Buckland & Sheaves 2014). By contrasting quantitative assessments of diet with
128 quantitative assessments of resource availability, visual methods can be used to calculate the
129 dietary preferences of specific populations or individuals (Hayden *et al.* 2015).

130 Nonetheless, visual methods can be biased towards depicting indigestible resources, while it
131 is impossible to detect liquid feeding (Davidson, Cook & Snelling 2004). The taxonomic
132 impediment of identifying partially digested material can render some components unknown
133 or resolved only to a coarse taxonomic level. Despite these drawbacks and the need for expert
134 taxonomical identification of often partially digested prey, visual methods are highly
135 informative and are still applied in most monitoring programs. Finally, visual techniques are
136 the only methods that can consistently identify different life-stages of prey.

137

138 *Molecular approaches*

139 High throughput DNA sequencing of stomach content or faeces has emerged as a popular
140 option for characterizing trophic interactions. This is primarily due to molecular methods
141 ability to provide high taxonomic resolution of prey and their sensitivity to rare, soft or highly
142 degraded items and those that leave no visual trace, such as liquid feeding (Piñol *et al.* 2014).
143 Older technologies such as protein electrophoresis, immunoassays, monoclonal antibodies,
144 and DNA techniques based on cloning and targeted gene approaches (reviewed by
145 Symondson 2002) still play a role in diet tracing, but most approaches now rely on high
146 throughput DNA sequencing.

147 Most molecular approaches either (i) target a single species of interest within a mixed sample,
148 or (ii) analyse a broader diversity of taxa in a mixed sample. Both approaches are similar to
149 environmental analyses (eDNA is reviewed by Bohmann *et al.* 2014), though inhibitors (DNA
150 preservation, extraction, amplification) in the gut may present problems (Schrader *et al.*
151 2012). The objective of the broad diversity option is to identify all organisms ingested by the
152 consumer using either PCR targeting or PCR-free approaches. PCR based approaches tend to
153 rely on some common target genes such as 16s for bacteria (Huber *et al.* 2007), ITS for fungi
154 (Lindahl *et al.* 2013), COI for animals (reviewed in Pompanon *et al.* 2012), combinations of
155 ITS, rbcl, matK, trnL etc. for plants (Valentini *et al.* 2009), or a series of ribosomal genes for
156 higher phylogenetic placement when broader taxonomic targets are expected (reviewed by
157 Pompanon *et al.* 2012). No target region is universal and all suffer from primer biases,
158 amplification and sequencing artefacts (Deagle *et al.* 2013; Clare 2014). PCR-free methods
159 face challenges with enriching the target (usually mitochondrial DNA, Liu *et al.* 2016) so that
160 non-target DNA (e.g. predator, nuclear, bacterial) does not swamp all the intended dietary
161 information (the capacity of the sequencer vs. the number of samples and the costs of multiple
162 sequence runs).

163 The resolution of molecular approaches makes it possible to identify species and even strains
164 but is dependent on available reference databases, such as BOLD (Ratnasingham & Hebert
165 2007) for COI, SILVA (Pruesse *et al.* 2007) for bacterial 16s V6 identification and UNITE
166 (Abarenkov *et al.* 2010) for ITS identification, or general sequence repositories such as
167 Genbank (Benson *et al.* 2013). Though all reference databases are still incomplete these are
168 rapidly expanding, and using multiple target regions may improve both the taxonomic
169 coverage and the probability of identification (Pompanon *et al.* 2012; Clare 2014).

170 Approaches based on the comparison of molecular operational taxonomic units are also
171 widely used in ecological analyses without taxonomy (MOTUs, Floyd *et al.* 2002). MOTU

approaches allow inclusion of both the known and unknown components of diversity but should be used cautiously as they are easily biased and may over or under estimate taxonomic proportions by orders of magnitude (Flynn *et al.* 2015; Clare *et al.* 2016). When possible, one solution is to conduct careful analyses of mock communities (mixes of known source DNA included as controls) to estimate the error rate of MOTU analysis (Flynn *et al.* 2015). Other significant analytical considerations include the choice of target (e.g. coding vs non-coding genes which face trade-offs between evolutionary rates and taxonomic resolution and ease of amplification vs. alignment issues) and the length of the amplicon (a trade-off between the capacity of a sequencer and the possible taxonomic resolution, reviewed by Clare 2014). DNA degradation rates due to digestion, assay sensitivity and the ability to quantify the outcomes are still major limitations to this approach (Deagle *et al.* 2013). DNA provides detailed information of the most recent events (e.g. 24-48 hours) but cannot provide a longer dietary perspective except by continual sampling (Thomsen *et al.* 2012).

In addition, secondary predation or cannibalism are nearly impossible to detect. Secondary predation is an issue for the study of foraging behaviour, but even if not directly hunted these species contribute to the nutritional intake and thus are also valid in dietary categorization of the consumer (Bowser, Diamond & Addison 2013). Cannibalism poses a greater challenge as the data are dismissed as predatory DNA contamination (Pompanon *et al.* 2012). Similarly, environmental contamination (e.g. DNA in the water swallowed along with DNA from a consumer's prey) cannot be differentiated from actual prey (Kelly *et al.* 2014).

Quantitative diet estimates are currently a widely debated and actively researched issue. Bacterial researchers almost always uses haplotype or OTU abundance to quantify the representation of taxa or assess abundance using qPCR (Props *et al.* 2016), however eukaryotic research almost never provides quantified estimates outside some very specifically controlled cases (Thomas *et al.* 2016). The problem is whether the number of sequencing

reads represents the biomass of the material consumed. Empirical testing (Deagle *et al.* 2013) and thought experiments (Clare 2014) suggest this is challenging particularly for partially digested multi-cellular systems of broad diversity where digestive processes and the order items are consumed and primer biases influences sequencing outcomes, and where identification is based on taxonomically biased reference databases (Clare *et al.* 2016). One suggestion is to use "correction factors" to retrieve better quantitative estimates but at present this is limited to laboratory studies of only 3-4 diet items (Thomas 2016). In most natural systems the number of potential prey combinations makes this unrealistic at the time of writing.

Despite challenges, molecular diet analyses have exploded in the last decade with hundreds of publications using these techniques for a wide range of applications. Wirta *et al.* (2014) resolved a food web using molecular techniques and found three times more feeding interactions and novel food web properties. A review of the molecular food webs (Roslin & Majaneva 2016) advocates for the adoption of molecular techniques in combination with traditional diet tracing. Even based on binary data molecular techniques have made unique applications possible. For instance, assessing aquatic habitat quality (Clare *et al.* 2014) or aiding reintroductions (Boyer *et al.* 2013). The advantage of molecular techniques is the ability to provide an unprecedented level of detail compared to other techniques.

Fatty acid and alternative biomarkers

Fatty acids are a diverse group of dietary lipids that are commonly used for diet tracing (Dalsgaard *et al.* 2003). A particular diet item often has a unique fatty acid profile, allowing depiction and differentiation among dietary sources such as bacteria, fungi, terrestrial and aquatic plants (Dalsgaard *et al.* 2003). Terrestrial plants often have high proportions of long

chain saturated fatty acids, bacteria synthesize a range of fatty acids, such as branched and odd chain fatty acids, but normally have low levels of omega-3 and omega-6, in contrast to most algae which are normally rich on these essential omega-3 and omega-6 FAs (Brett *et al.* 2009). Due to the high number of biomarkers (20-30), analyses of fatty acid profiles allow partitioning among and within specific taxonomic groups of algae (Strandberg *et al.* 2015) or terrestrial plants (Reiner *et al.* 2014), but it is rarely possible to depict all items contributing to a consumer's diet (Traugott *et al.* 2013). Commonly, only about half of the fatty acids may contribute to the diet analyses, because some are only found in low amounts, or have similar proportions in all the primary producers studied (Brett, Eisenlord & Galloway 2016).

Quantitative fatty acid signature analysis (QFASA, Iverson *et al.* 2004) can be used to quantitatively estimate consumer's diet proportions and identify trophic linkages (Tollit *et al.* 2006). An important consideration when using QFASA is that dietary fatty acid profiles are modified in consumers. Consequently, an estimate that accounts for the change in the proportion of a specific fatty acid from the diet to the consumer (Iverson *et al.* 2004 called them calibration coefficients) is required to obtain accurate dietary estimates (Happel *et al.* 2016). For example, consumers may preferentially catabolize certain fatty acids for energy (e.g., saturated fatty acids), preferentially retain others for anabolic processes (e.g., EPA, DHA and ARA), and modify other molecules to more metabolically active forms (e.g., ALA to EPA) (Taipale, Kainz & Brett 2011). Trophic discrimination can vary for different resources, especially when diet components have substantially different fatty acid profiles than their consumers (Happel *et al.* 2016), and across different environmental conditions (Wacker *et al.* 2016). Consequently, accurate dietary categorization of consumers relies on *a priori* estimates of calibration coefficients (Wang, Hollmén & Iverson 2014). One way to circumvent the uncertainties associated with trophic discrimination is a Bayesian mixing-model approach (i.e. FASTAR), where fatty acid profiles of a consumer fed various mono-

246 culture diets (Galloway *et al.* 2015) are used as the resource library. However, development of
247 consumer reference libraries is time consuming and limited to species easily cultured under
248 laboratory conditions (Galloway *et al.* 2015).

249 Alternative biomarkers, such as sterols and plant pigments can also provide valuable dietary
250 information. For instance, sterols can serve as markers for specific phytoplankton taxa
251 (Taipale *et al.* 2016a). However, many sterols have limited potential for diet partitioning, as
252 most consumers convert primary producers' phytosterols to cholesterol or oxidize them for
253 energy (Martin-Creuzburg, Oexle & Wacker 2014). Algal pigments have been used to
254 estimate the diet of zooplankton (Letelier *et al.* 1993), since pigment composition can be
255 algae-specific. However, despite a range of pigments having been identified as useful for
256 tracing resources (Kleppel 1988), pigment analyses are rarely used as dietary tracers. Most
257 pigments also degrade rapidly (< 1 hour) in consumer guts (Kleppel 1988) and thus only
258 provide a snapshot of the diet.

259

260 *Bulk stable isotopes*

261 The application of stable isotope ratios to diet tracing is based on the assumption that a
262 consumer's stable isotope ratio reflects that of its prey (Michener, Kaufman & Lajtha 2007).
263 As different primary producers often have distinct stable isotope ratios (Fogel & Cifuentes
264 1993), the relationship between the stable isotope ratio of a consumer and its putative prey
265 can be used to estimate the dietary use of the consumer. During assimilation the lighter stable
266 isotope is preferentially excreted, meaning that consumers normally become isotopically
267 enriched (heavy) relative to their prey (McCutchan *et al.* 2003), this phenomenon is known as
268 trophic discrimination. The offset between the isotope ratios of a consumer and its prey is
269 termed the trophic discrimination factor (TDF, Table 1). Commonly, for stable isotopes of

carbon (^{13}C) and sulphur (^{34}S) TDFs are minimal, typically <1 ‰, but for stable isotopes of nitrogen (^{15}N) TDF is consistently higher, typically 2 - 4‰ (McCutchan *et al.* 2003). Hence, a consumer's ^{13}C or ^{34}S isotope ratio is used to differentiate between a reliance on different primary producers, whereas ^{15}N is used to estimate the consumer's trophic position as well as diet.

Accurately estimating dietary use from a consumer's isotope ratio requires accurate estimate of TDF (Martínez del Rio *et al.* 2009). Trophic discrimination factors for a particular consumer–prey combination can be derived from laboratory studies (McCutchan *et al.* 2003), or approximated from simple trophic chains in the field (Gladyshev *et al.* 2012). However, as trophic discrimination is influenced by temperature, growth rate, isotope routing, type of excretion and resource availability, TDF values derived from experimental settings may not reflect those of the natural environment (Martínez del Rio *et al.* 2009). The advent of Bayesian mixing models facilitating the inclusion of error estimates of both the TDF and the isotope ratios of putative prey sources (Moore & Semmens 2008; Stock & Semmens 2015), has led to a proliferation of research quantifying animal diet use, primarily using carbon and nitrogen stable isotopes (Boecklen *et al.* 2011). For example, differences in $\delta^{13}C$ values can distinguish between a consumers reliance on C3 and C4 plants (Peterson & Fry 1987) or between benthic and pelagic resources (France 1995). Prey items that are enriched or depleted in ^{15}N, allow separation of for example, C3, C4 plants and phytoplankton (Hayden *et al.* 2015). Such analyses work well when putative dietary resources can be well established *a priori*, the isotope ratios of different primary producers are clearly differentiated and the TDF is well resolved. Mixing models provide good quantitative estimates of resource flow, however their diet resolution is normally limited to 3-5 diet items depending on the number of isotope tracers, and these models are rarely suitable for depicting individual diet items (Phillips *et al.* 2014). In cases where $\delta^{13}C$ and $\delta^{15}N$ of distinct prey sources overlap, additional

295 markers are required to distinguish among putative prey items. In such cases, stable isotopes
296 of sulphur (^{34}S) can partition, for example, benthic vs. pelagic or marine vs. freshwater
297 resources (Michener, Kaufman & Lajtha 2007); and hydrogen (^{2}H) can be used to partition
298 the contribution of terrestrial and aquatic resources to consumers (Doucett *et al.* 2007).
299 However, there are still knowledge gaps regarding the use of $\delta^{34}S$, $\delta^{2}H$ values, and little is
300 known about the utility of $\delta^{18}O$ ratios as a trophic marker (Vander Zanden *et al.* 2016). An
301 additional complication of using $\delta^{2}H$ and $\delta^{18}O$ isotope ratios is that their abundance in a
302 consumer is derived both from its diet and the environmental water (Vander Zanden *et al.*
303 2016).

304 An advantage of stable isotope analyses is the ability to integrate animal diet information over
305 a long time scale. Temporal integration varies depending on the element, tissue analysed,
306 temperature and organism size (Vander Zanden *et al.* 2015); by analysing a specific tissue
307 researchers can identify dietary utilization during the preceding days (blood, plasma), weeks
308 (liver), months (muscle, feathers) or years (bone, fish otoliths). Isotope labelling studies by
309 which a prey item is artificially enriched in ^{15}N, for example through the application of urea,
310 provide an additional dimension to diet estimation as this "spike" can be subsequently traced
311 through an entire food web (Galván, Fleeger & Fry 2008). This approach is however limited
312 to tracing a single diet item, or two if using dual labelling with ^{15}N and ^{13}C.

313

314 *Compound specific stable isotopes*

315 Compound specific stable isotope analyses are a relatively recent advance in ecology. This
316 method measures isotope values from individual biomolecules, yielding stable isotope ratios
317 from multiple compounds within a single bulk sample (Hayes *et al.* 1990). Such analyses
318 provide more dietary tracers than common bulk stable isotopes, allowing in principle for

partitioning among a large number or diet items. However, the taxonomic resolution of these approaches is still constrained to partitioning one more resource than the number of tracers used (McMahon *et al.* 2015).

Compound specific stable isotope analyses of amino acids is almost exclusively performed with carbon and nitrogen isotope ratios, though in principle other stable isotope values (e.g. hydrogen) can also be obtained from amino acids (Fogel, Griffin & Newsome 2016). The carbon isotope composition of amino acids, and particularly essential amino acids, which show little isotope change during trophic transfer (McMahon *et al.* 2010), can separate among, for example, fungi, bacteria, aquatic and terrestrial primary producers in natural ecosystems (Larsen *et al.* 2013). Application of $\delta^{13}C$ amino acid analysis is currently expanding and this approach has been applied to partition resources in a range of both terrestrial (O'Brien, Boggs & Fogel 2005) and aquatic ecosystems (Vokhshoori, Larsen & McCarthy 2014).

Nitrogen stable isotope analyses of amino acids are primarily used to estimate animal trophic position (Chikaraishi *et al.* 2009; Nielsen, Popp & Winder 2015). However, it can also be used to infer diets, as a subset of amino acids differ among primary resources (McCarthy, Lehman & Kudela 2013). These amino acids have been used to partition terrestrial from aquatic primary resources (Ishikawa *et al.* 2014), seagrasses from other aquatic resources (Vander Zanden *et al.* 2013), as well as to separate cyanobacteria from eukaryotic algae (McCarthy, Lehman and Kudela (2013). Interestingly, the nitrogen isotope ratios of different amino acids vary in their incorporation rates (Bradley *et al.* 2014), which can depict temporal dynamics in animal diet utilization (Madigan *et al.* 2014). Although, widely different integration times among different amino acids may complicate dietary analyses or trophic position estimation using these compounds.

343 Stable isotope analyses of amino acids can include 15-20 compounds, or double that amount
344 if analysing both carbon and nitrogen. However, except for a number of well-established
345 essential amino acids (4-5 compounds, McMahon et al. 2010), further research is required to
346 determine their suitability for tracing and partitioning more complex diet mixtures.

347 The analysis of $\delta^{13}C$ ratios of specific fatty acids can distinguish between terrestrial and
348 aquatic carbon resources (Gladyshev *et al.* 2012), including partitioning between different
349 phytoplankton taxa (Taipale *et al.* 2016b). However, to date few studies have estimated
350 dietary proportions using $\delta^{13}C$ isotope ratios of fatty acid, as the TDFs for individual fatty
351 acids are unknown for most organisms. Recent work has indicated that the majority of fatty
352 acids become depleted during assimilation (Gladyshev *et al.* 2012), though this can vary (Bec
353 *et al.* 2011), and a subset of $\delta^{13}C$ fatty acid ratios show constant trophic discrimination in fish
354 (Fujibayashi, Ogino & Nishimura 2016). Given the general usefulness of fatty acid
355 biomarkers to diet tracing, gathering more information on the use of $\delta^{13}C$ ratios of fatty acids
356 for diet tracing is a priority for future research.

357

358 **Similarity comparison of multiple diet tracing methods**

359 All diet tracing methods aim to provide an accurate description of consumers' diet, yet they
360 differ substantially in their application and measure consumption of diets at various stages
361 during the feeding-assimilation process. In order to summarize current empirical evidence on
362 how different methods quantify diet mixtures, we systematically searched the *Institute for*
363 *Scientific Information Web of Knowledge* for published studies that contrasted estimates of
364 dietary proportions from multiple methods applied to the same consumer-resource data, or
365 from experimental studies where known diet proportions were fed to the consumer (Fig. 2).
366 We included studies with a minimum of three distinct diet items. Because individual studies

used different levels of taxonomic resolution in their diet items, we normalized all the data to the same level of resolution prior to the analysis. However, there were not enough studies to further contrast subsets of data with different diet resolutions. We used the Czekanowski index, ranging from 0% (no overlap) to 100% (complete overlap), to compare pairwise data accounts from field measurements (n=114) and experimental measurements (n=22), published in 40 papers. The Czekanowski index (CI) was calculated as:

$$CI = 1 - 0.5 \sum_{j=1}^{S} p_{x,i} - p_{y,i} \,,$$

where $p_{x,i}$ (estimated by method x) and $p_{y,i}$ (estimated by method y) are the proportions of the i^{th} diet item used by a consumer, and S is the number of total diet items. This index is well-suited for comparing proportional data of pairwise method comparisons, with varying numbers of diet items (Kohn & Riggs 1982). A full description of the quantitative literature synthesis is presented in the *Supplementary material X1*.

Overall there was a good agreement between methods applied to the field data with three to six dietary resources (Czekanowski index = 64±21 % to 81±11 %; mean±SD; Fig. 2). However, similarity between the methods declined with increasing number of potential resources (>6), for both comparisons of visual versus molecular methods (Czekanowski index = 44±33 %; mean±SD; Fig. 2), and especially for comparisons of visual versus isotope analyses (Czekanowski index = 27±19 %; mean±SD; Fig. 2). The high disparity between visual and stable isotope analyses applied to datasets with diverse resources likely resulted from the mixing models inability to differentiate among many more resources relative to the number of dietary tracers used (Brett 2014). This emphasises the inaccuracy of stable isotope mixing models when the assumptions of these model are violated, such as in cases where the number of diet items far exceeds the number of tracers (see Phillis *et al*., 2014 for further

details). In contrast, the low similarity between visual and molecular analyses highlights the high taxonomic resolution of molecular methods (Braley *et al.* 2010), which is generally not possible to achieve with visual methods. Nonetheless, such discrepancy with increased diet complexity among different techniques highlight the influence method choice alone can have on the ecological outcome. Strikingly few experimental studies (22 comparisons from 10 studies) have assessed how well a dietary method partitions the proportions of a known fed diet mixture, though for these studies the methods represented the known diet fairly well (Czekanowski index = 57±13 % to 91±08 %; mean±SD; Fig. 2). This surprisingly low number of experimental comparisons among methods, species and ecosystems illustrates a significant knowledge gap and a major avenue for further investigation.

Integration of multiple dietary methods

Integrating multiple methods (Chiaradia *et al.* 2014) allow researchers to describe complex food web interactions (Hambäck *et al.* 2016). Independently comparing outputs from multiple methods reduces uncertainty if the methods show similar results. This also provides additional insights about particular prey species or rare diet items (e.g. molecular analysis), prey size or life-stage (e.g. stomach content analysis), which may not be gained from other methods.

Diet tracing methods can be also combined non-independently, where diet information from one method is being integrated with another dietary technique (Kainz *et al.* 2017). Recent advances in model frameworks now allow for integration of diverse biomarkers and isotope data (Stock & Semmens 2015) and with other biological data (Swanson *et al.* 2015). For instance, using dietary estimates of visual or molecular analyses as prior information for Bayesian isotope mixing models can improve quantitative diet estimates (Chiaradia *et al.* 2014). This allows inclusion and *a priori* ranking of the most relevant resources (as identified

413 by the other dietary technique) when constructing mixing models. However, this should be
414 done with caution (Moore & Semmens 2008), especially if the dietary methods provide vastly
415 different outcomes, something that was evident in our synthesis for dietary methods
416 partitioning more than six diet items (Fig. 2). Because, constructing a Bayesian mixing model
417 with to strong priors based on one method, may influence a model so strongly that the outputs
418 from the Bayesian analysis merely reflect the prior, especially if the isotope values of the prey
419 resources are not clearly separated. Nonetheless, when correctly applied, integration of
420 diverse diet records provides new possibilities to understand complex trophic relationships in
421 natural ecosystems which are difficult to untangle when using only a single method.

422

423 **Recommendations for diet tracing**

424 Both molecular and visual analyses can provide high diet resolution to the species or genus
425 level, and molecular analyses even allow differentiation among strains (Pompanon *et al.*
426 2012). Fatty acids and sterols are promising for partitioning different taxa but at a courser
427 level of resolution (Taipale *et al.* 2016a), and quantification requires accurate calibration
428 factors previously determined for the focal consumers. Stable isotope analyses provide
429 quantitative estimates of resource flows integrated over a specific time scales depending on
430 the type of material analysed. However, they are normally limited to differentiating 3-5 pre-
431 defined diet groups. Selecting the best method depends on the particular focal consumer,
432 ecosystem type, environmental conditions and objective or question of the study. Nonetheless,
433 the following recommendations can improve diet tracing:

434 1) **Clearly define the research question** and the information required, as this will
435 narrow down the method selection substantially.

2) **Know the consumer's potential diet** before sampling. It is rarely possible to get a good assessment of the consumers' diet or to select the best method or reference library (see also recommendation 4), without some prior knowledge of the consumers' feeding behaviour, habitat, and the resources available in the environment.

3) **Know the limitations and advantages of each method,** such as the ability to accurately identify, partition and quantify resources (Table 2). This makes it possible to choose the most appropriate technique(s) with the required resolution to best answer a particular ecological question.

4) **Acquire adequate diet reference material** that covers all nutritionally important resources. Especially in poorly studied systems or taxa, this means initially considering a broad pool of potential resources. Obtaining additional biological information also reduces the chance of including or overestimating non-prey or rare diet items, or contrarily missing important dietary contributions.

5) **Choice of target region and length in molecular studies** is likely to result in a trade-off between broad diet coverage versus taxonomic resolution (Pompanon *et al.* 2012). Combined analyses using multiple markers targeting different DNA regions and lengths can help to retain broad coverage of food sources while enhancing resolution of particular prey-species' of interest.

6) **Evaluate the assumptions and input parameters associated with isotope mixing models**, such as the TDF values, the number of resource groups, and test the influence of the priors. Moreover, knowledge of the putative resources is needed beforehand, as inclusion of non-prey samples (i.e. potential diet items that are not actually consumed) causes errors in the diet estimation. Best practices for stable isotope mixing models are reviewed in more detail elsewhere (Phillips *et al.* 2014).

7) **Consider the temporal scale of the diet tracing method.** The different methods all provide a range of possibilities to explore feeding patterns (Table 2) as they aggregate dietary information across very different temporal scales (Davis & Pineda Munoz 2016).
8) **Account for temporal and spatial resource heterogeneity.** Consumer foraging behaviour and resource availability vary across time and space. Comparisons over time, among habitats or between migratory individuals should be accompanied with the appropriate dietary reference material, sampling and dietary method(s) scheme. This includes measuring basal resource variation (e.g. resources' isotope composition) or using repeated measures of short term diet estimates from molecular or visual analysis to better understand ecosystem variability.
9) **Combine dietary methods with other biological information.** Additional information complementing the diet data, such as nutritional composition of the diet, growth rates, animal behaviour, or inclusion of an additional dietary method that help resolve weaknesses of a particular method, is a good way to improve the accuracy of the diet estimate.

Future outlook

Our quantitative comparison of diet tracing methods identified an urgent need for more experiments where animals are fed a dietary mixture of known proportion and then analysed by a dietary estimation technique. For example, a search of "stable isotope(s)" and "mixing model(s)" in *Institute for Scientific Information Web of Knowledge Database* on 4 May 2017 yielded over 1300 studies, yet we found only 6 direct comparisons under controlled conditions of quantitative diet estimates involving stable isotope analyses. For all dietary

484 methods, only 22 direct comparisons in controlled conditions were found, and it is worrying
485 that we currently know so little about both the consistency and accuracy of the quantitative
486 estimates from most methods.

487 Each dietary method estimates different ingested or assimilated components. However, all the
488 methods aim to provide information on the consumer's true diet. The lack of empirical studies
489 points to the urgent need to test how accurately the methods quantify a consumer's true diet.
490 Future empirical tests should establish whether there are systematic differences among
491 methods, as to reduce bias due to method selection in future studies. Such tests, even includes
492 contrasting diet estimates from stomach versus scat content from the same method, e.g. visual
493 or molecular analyses. Pinpointing discrepancies among methods also assists dietary method
494 integration which has the potential to enhance comprehensive food web analyses. Such efforts
495 currently rely on the largely untested assumption, that multiple dietary outputs consistently
496 support each other in a meaningful way.

497 Molecular approaches can identify individual species' diet use. Such resolution is essential for
498 categorizing food webs (Roslin & Majaneva 2016), and this detailed information will also be
499 a vital tool for conservation management of ecosystems (Clare 2014). An important future
500 advance for molecular approaches will be to develop means whereby quantitative estimates
501 can be obtained. Promising avenues include qPCR, PCR-free methods, MOTU analyses,
502 mock communities and various forms of technical replication of extraction, PCR and
503 sequence steps to minimise random biases. However, the development of these approaches so
504 far has been somewhat haphazard for dietary tracing applications and often (in the case of
505 technical replication) prohibitively expensive. Another approach is combining field estimates
506 with laboratory data where quantities of that specific prey and the resulting number of DNA
507 sequences are known (Thomas *et al.* 2016). A further challenge is attempting to identify
508 characteristics of the prey. Was a larva or an adult being consumed? Was the organism dead

(scavenged) or alive (hunted) when consumed? There may be solutions in the use of key markers of development (life stage) or of expressed RNA (suggesting the prey was alive) (Jarman *et al.* 2015), but currently these have yet to be thoroughly tested and remain mostly speculative.

Generations of biologists have collected a vast amount of dietary assessment data and a very important future task is now to catalogue and combine such biomarker data in open repositories (Pauli *et al.* 2017). However, applications of such open source approaches in trophic ecology are still in their infancy (Hampton *et al.* 2013). Here, diet tracing can learn from molecular biologists that have developed data repositories such as GenBank for decades (Benson *et al.* 2013), efforts that now greatly benefit DNA based diet analyses (Pompanon *et al.* 2012). Data repositories also provide great opportunities to reanalyse previously collected consumer-diet data in new informative ways, such as Simons *et al.* (2013) who characterised trophic interactions in the Gulf of Mexico using the aggregation of pre-existing consumer diet datasets and Clare *et al.* (2016) who analysed the impact of bioinformatics steps on a previous set of ecological conclusions related to a molecular diet analysis. Currently, no centralised hub for dietary biomarker data exists and addressing this gap is another urgent priority (Pauli *et al.* 2017).

The question "what does this consumer feed on?" remains integral to studies of ecology, evolutionary biology, and research-informed ecosystem management and restoration. Elucidating feeding interactions to address such issues require using creative combinations of complimentary tracers and continuous development of more sophisticated dietary assessment methods. To this end, interdisciplinary research is critical in developing synergistic applications of diverse dietary methods to fully realize their potentials.

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Table 1. Definitions of common terms used in diet tracing.

Common terms	Definition
Assimilated diet	Diet items ingested and routed to the tissue by the organisms after ingestion. Synonym: absorption.
Biomarker	Organic compound used for dietary analyses.
Compound specific stable isotope analysis	Stable isotope analysis of specific organic molecules (e.g. fatty or amino acids).
Diet reference library	Information on the diet reference material available from literature sources, lab or field samples (e.g. isotope values of resource components or DNA sequences of diet items). A good library is essential for separating a diet mixture.
Diet resolution	The taxonomic resolution of the method. Can diet items be indentified to species, genus, family or other taxonomic level?
Diet separation	The ability of a given methods to separate individual diet items.
Estimated diet	The diet items identified by the dietary analysis method.
Ingested diet	Diet items present in a consumer's gut after capture.
Mixing model	Mathematical model based on linear algebra used to infer the proportion of diet items eaten by a consumer. Recent model are based on Bayesian frameworks, including SIAR, MixSIR, FRUITS, MixSIAR, FASTAR.
DNA diet analysis	Identification of genetic sequences of diet items in stomach, gut or faeces. Method measuring ingested components.
Molecular operational taxonomic unit (MOTU)	A grouping of sequences forming a unit which is comparable across samples and sites, but does not equate to an actual taxonomic level.
Potential diet	All diet items available to the consumer for consumption in its environment.
Stable isotope analysis	Analysis of the ratio of heavy to light isotopes of common elements in a biological tissue, denoted as e.g. $\delta^{13}C$ (‰) for carbon and $\delta^{15}N$ (‰) for nitrogen. Method measuring assimilated components.
Trophic discrimination factor (TDF)	Change in isotopic values between consumer and prey. Synonyms: trophic enrichment, trophic fractionation. Calibration coefficient is used to describe the analogue process for fatty acids.
True diet	All diet items ingested or assimilated by the consumers, which represent a fraction of the potential diet available in the environment.
Visual diet analysis	Identification of ingested diet items in stomach, gut or faeces.

Table 2. Summary of the most common methodological and ecological advantages and limitations of the different diet tracing methods. Reference numbers inside the table refer to publications provided in the *Supplementary Material X1* providing additional information concerning each method or process discussed.

	Visual	**DNA-based**	**Biomarkers**	**Stable isotopes**	**Compounds specific stable isotopes**		
Methodological attributes	Stomach, gut or feces content analyses	DNA of stomach, gut or feces content analyses	Fatty acid analyses	$\delta^{15}N$ & $\delta^{13}C$ analyses	$\delta^{15}N$ Amino acid analyses	$\delta^{13}C$ Amino acid analyses	$\delta^{13}C$ Fatty acid analyses
Measured material	Ingestion or feces	Ingestion or feces	Assimilation	Assimilation	Assimilation	Assimilation	Assimilation
New / rare diet items	Yes	Yes	No	No	No	No	No
Quantification	None to semi quantitative	None to semi quantitative/frequencies	Yes	Yes	Yes	Yes	Yes
Diet resolution	High - Species level (Family or Genus)	High - Species level (Family or Genus)	Medium nb. resource groups	Limited nb. resource groups (2-6)[1]	Assumed limited nb. resource groups	Medium nb. resource groups	Assumed medium nb. resource groups
Temporal scale	Hours to days	Hours to days	Days-weeks-months	Days-weeks-months, depends on body size and material sampled (blood, muscle, feather, otoliths)	Assumed similar to bulk but variable with specific compound[2]	Assumed similar to bulk and $\delta^{15}N$ amino acid values	Assumed similar to fatty acids and bulk isotopes
Bias due to nutritional diet composition	NA	NA	Yes[3]	Yes[4]	Yes[5]	Yes[6]	Assumed yes, not studied
Dietary reference	Taxonomic knowlegde	Custom made library or broad library reference	Field or lab resource samples, literature	Field resource samples, literature	Field or lab resource samples, literature	Field or lab resource samples, literature	Assumed similar to fatty acids
Ability to measure							
Energy flow	Limited	Medium	Limited	Good	Good	Good	Medium
Network structure	Good	Good	Limited	Limited	Limited	Limited	Limited
Prey size	Yes	No	No	No	No	No	No
Herbivory / carnivory	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Trophic discrimination	NA	NA	Variable[7]	Variable[8]	Potentially variable for both trophic and sources amino acids[9]	Variable but generally low for essential amino acids[10]	Variable[11]
Trophic position	Medium	Yes	Limited	Good	Very good	Limited	Unknown
Major strengths	Identifies life stages and size of prey	Very high diet resolution	Quantification of complex resource groups	Quantifies diverse groups, niche width, discriminates between trophic levels	Quantifies resources, trophic position	Good tracers to partition different primary producers	Unknown
Major limitations	Bias in hard vs. soft tissue, snap-shot estimate of resource use	Inability to quantify diet proportions	Discrimination factors vary	Limited to few number of resources, isotope baseline variation uncertainty	Differentiation of complex resource mixtures likely limited	Inability to discriminate among trophic levels	Variable trophic discrimination
Knowledge gaps	Few controlled studies of resource quantification	Ability to estimate life stages, differentiate dead from live prey, and diet quantification	FASTAR libraries	Few controlled studies of resource quantification, drivers of trophic enrichment largely unknown	Temporal integration time, very few tracing studies	Temporal integration time, separation of similar primary producers	Little known
Key references	Hynes 1950, Baker et al. 2014, Hyslop 1980, Rindorf & Lewy 2004	Clare 2014, Pompanon et al. 2012, King 2008, Bohmann et al. 2014, Traugott et al. 2013 Roslin & Majaneva 2016, Evans et al. 2017	Iverson 2004, Dalgaard 2003, Brett et al. 2016, Galloway 2015	Vanderklift & Ponsard 2003, McCutchan 2003, Martinez del Rio 2009, Boecklen et al. 2011, Layman et al. 2012	McCarthy 2013, Ishikawa et al. 2014, Chikaraishi et al. 2009, McMahon and McCarthy 2016	Larsen et al. 2009, 2013, McMahon et al. 2015	Gladyshew et al. 2012, 2016, Bec et al. 2011, Budge et al. 2016

Figure 1

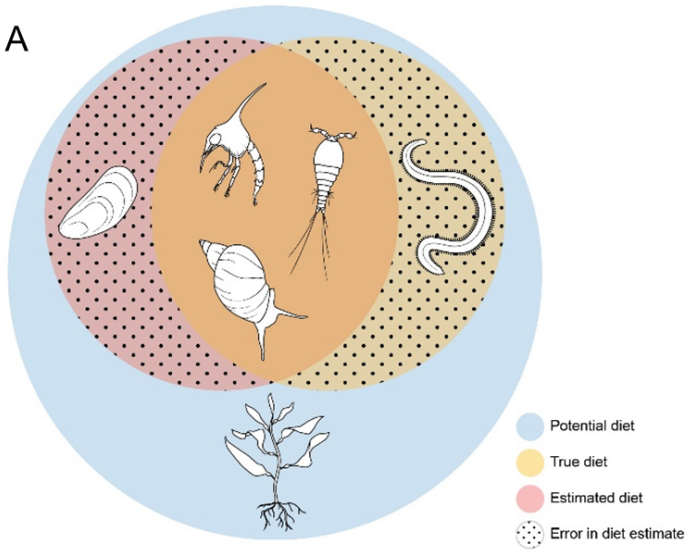
A
Potential diet
True diet
Estimated diet
Error in diet estimate
B
Respired
N
Retained
P
C
Ingested
Assimilated
Egested
(Feces)
C
Diet mixture
Proportions
Estimated diet

Figure 1. Conceptualisation of diet tracing. **(A) Diet in the environment:** A consumer's true diet (yellow shaded area) represents some fraction of all potential diet items (blue shaded area) available in the ecosystem, while the estimated diet (red shaded area) represents diet identified by the specific diet analysis method. Mismatch between yellow and red shaded areas represents error in the diet estimation (black dotted area), thus the aim of diet estimation is to maximise this overlap of the true and estimated diet (orange shaded colour). Errors can arise from: (i) true diet not detected by the selected method (right black arrow) and (ii) potential diet items in the environment not ingested by the consumer but detected by the method (left black arrow). **(B) Diet in the consumer:** The stage at which the consumed diet is measured is an important consideration. Ingested and digested diet is detected in stomach, gut or faeces content (e.g. visual or molecular diet analyses). Some fraction of ingested material is then assimilated (e.g. stable isotope or biomolecule analyses), and a subset of the assimilated material is retained in the consumer's tissue, whereas non-assimilated material is respired or excreted. It is important to note that different dietary methods measure the diet at various stages during ingestion and assimilation. **(C) Dietary analysis:** The selected method should be able to separate the different consumed items (diet mixture shown as three colours that represent individual prey taxa), and to quantify the proportions of the individual diet items.

Figure 2

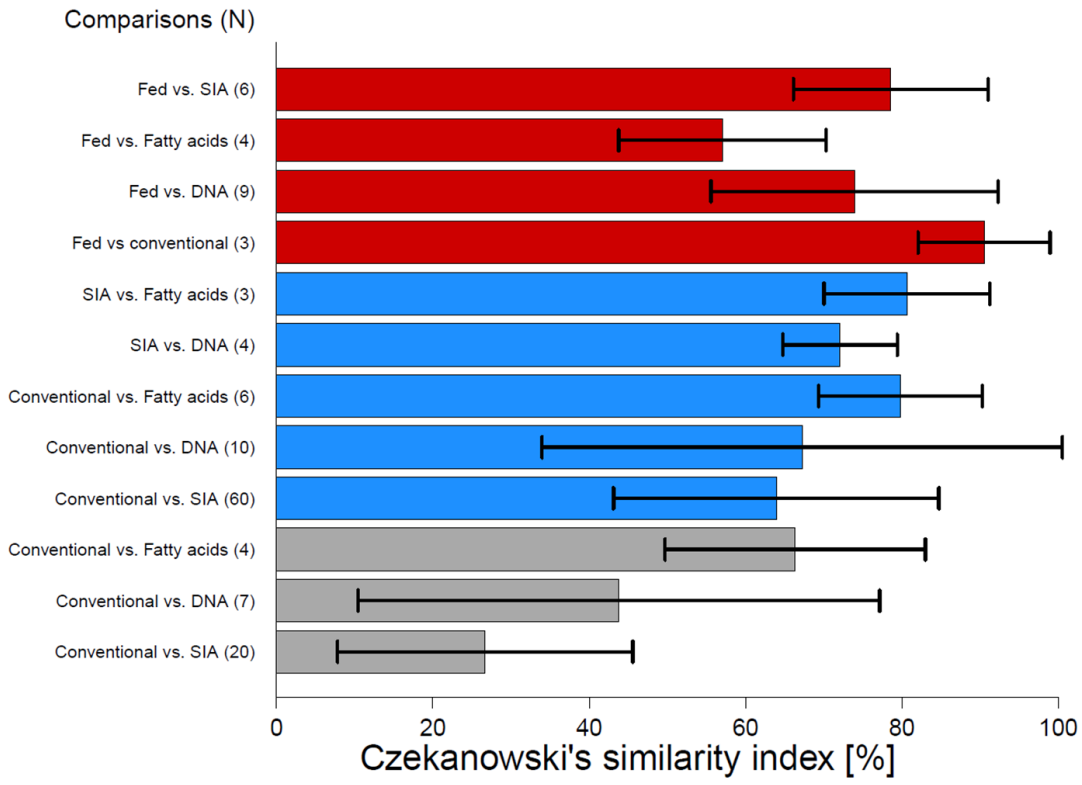

Figure 2. Pairwise comparisons of quantitative estimates from different diet tracing methods applied dietary data from laboratory (red) and field studies with 3-6 resources (blue), and >6 resources (grey). Czekanowski's similarity index of 100% denotes complete overlap between the methods, while 0% denotes no overlap. Available combinations of known diet (fed), stable isotope analyses (SIA), visual or molecular analysis of gut, stomach or faeces (DNA) were compared. Data are means ± standard deviation; values in parentheses denote the number of comparisons. The method details and the data sources are provided in the Supplementary Material X1.