REVIEW ARTICLE

Title: Current Methods and Future Directions in Avian Diet Analysis

Running head: Methods and Future Directions in Avian Diet Analysis

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ACKNOWLEDGMENTS

The authors wish to thank Sarah Sonsthagen for her expertise and helpful input.

Funding Statement: Not applicable.

Ethics Statement: The authors declare no conflict of interest.

Author Contributions:

Conceived the idea, design, experiment (supervised research, formulated question, or hypothesis): BDH, SCL, BAP

Performed the experiments (collected data, conducted the research): Not applicable.

Wrote the paper (or substantially edited the paper): BDH, AMS, TWS, KAH, MJP, DPLT, ETM, AJW, LLP, SLR, AMF, SCL, SST, BAP.

Developed or designed methods: Not applicable.

Analyzed the data: Not applicable.

Contributed substantial materials, resources, or funding: Not applicable.

Data Availability: Not applicable.

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ABSTRACT

Identifying the composition of avian diets is a critical step in characterizing the roles of birds within ecosystems. However, because birds are a diverse taxonomic group with equally diverse dietary habits, gaining an accurate and thorough understanding of avian diet can be difficult. In addition to overcoming the inherent difficulties of studying birds, the field is advancing rapidly, and researchers are challenged with a myriad of methods to study avian diet, a task that has only become more difficult with the introduction of laboratory techniques to dietary studies. Because methodology drives inference, it is important that researchers are aware of the capabilities and limitations of each method to ensure the results of their study are interpreted correctly. However, few reviews exist which detail each of the traditional and laboratory techniques used in dietary studies, with even fewer framing these methods through a bird-specific lens. Here, we discuss the strengths and limitations of morphological prey identification, DNA-based techniques, stable isotope analysis and the tracing of dietary biomolecules throughout food webs. We identify areas of improvement for each method, provide instances in which the combination of techniques can yield the most comprehensive findings, introduce potential avenues for combining results from each technique within a unified framework, and present recommendations for the future focus of avian dietary research.

KEY WORDS

Feeding Ecology, DNA Metabarcoding, Stable Isotope Analysis, Prey Identification, Dietary Biomolecules, Avian Diet

LAY SUMMARY

- Providing accurate assessments of diet composition is an essential step in understanding the life history of birds as well as their roles within ecosystems.
- A wide array of techniques exists to study the prey composition of birds, including recently developed laboratory-based methods, but each of these methods comes with their own strengths and weaknesses.
- This review details the benefits and drawbacks of each technique, suggests pathways to overcoming methodological limitations, and demonstrates how these techniques can be leveraged to answer cutting-edge questions in avian dietary studies.
- Finally, we discuss how the use of multiple techniques within a single study can yield a more comprehensive understanding of avian diet, present novel ways to combine data from each technique within a unified framework, and suggest areas of research to advance the field of avian dietary ecology.

INTRODUCTION

Evaluating the composition of avian diets has been a focus of ornithological inquiry for over a century (Slater 1892). Dietary studies have helped to characterize ecological interactions of birds (Burin et al. 2016) and identify prey preference as a driving force behind the evolution of the immense biodiversity across the Class Aves (Kissling et al. 2012, Barnagaud et al. 2014). Diet has long been recognized as a defining life history trait (Eaton 1958), and characterizing the dietary niche is an important step in identifying the roles of species within ecosystems (Elton, 1927). A baseline understanding of avian prey preferences has helped researchers to better identify dietary shifts caused by natural (Jaksic 2004) and anthropogenic disturbances (Murray et al. 2018, Trevelline et al. 2018a) as well as the population- (English et al. 2018) and community-wide (Spiller and Dettmers 2019) consequences of these disturbances. Studies of dietary composition also inform our understanding of biotic interactions, such as those stemming from intraspecific competition (McMahon and Marples 2017), interspecific competition (Trevelline et al. 2018b, Sherry and Kent 2021) and trophic cascade events (Mäntylä et al. 2011). Finally, studies of bird diets have been used to highlight the ecological services that birds provide (Whelan et al. 2008). In short, understanding the dietary niche of a species allows researchers to quickly describe important life history traits (Abrahamczyk and Kessler 2015) as well as the complex interactions that birds have with their environments (O'Donnell et al. 2012) and, in turn, provides essential information for the management and conservation of avian species and their habitats (Ontiveros et al. 2005).

Early investigation of avian diet relied upon direct methods such as the observation of foraging (Croxall 1976) and provisioning events (Snyder and Wiley 1976) or morphological identification of prey retrieved from gastric lavage (Moody 1970), feces (Tucker and Powell

1999), and stomach samples from sacrificed birds (Beal 1915). While these methods provide a strong foundation, they are laborious, seldom provide taxonomically-precise prey identification (Symondson 2002), and often fail to detect relatively small prey (Culicidae; Guinan et al. 2008, Jedlicka et al. 2017), rapidly digested prey (Lepidoptera; Eaton 1958, Trevelline et al. 2016), or highly fragmented prey remains (Galimberti et al. 2016). The advent of several laboratory-based methods now allows for indirect estimation of prey composition, thus permitting increased precision in prey detection and taxonomic assignment (Taberlet et al. 2012), while adding information on nutrient assimilation (Hobson and Clark 1992a) across time scales ranging from hours to years depending on the tissue sampled (Podlesak et al. 2005). However, while these laboratory-intensive techniques have revitalized studies of avian diets and trophic dynamics, they have their own drawbacks, such as an inability to accurately quantify prey counts or biomass with DNA-based methods (Piñol et al. 2015), and the variable nature of biomolecule assimilation (Galloway and Budge 2020) potentially impacting results stemming from isotopic and lipid-based methods. Because the findings of dietary studies are methodologically sensitive (Marti 1987), it is important to understand the benefits and limitations of each technique prior to use.

While valuable reviews detail the most commonly used methodologies in dietary reconstruction (Schoeninger 2010, Traugott et al. 2013, Nielsen et al. 2017, Alberdi et al. 2019), few pertain specifically to birds (Rosenberg and Cooper 1990, Barrett et al. 2007), and none discuss how these methods are currently used in avian diet research or how they can be leveraged to build on the wealth of prior research in birds, one of the best-studied taxonomic groups. Here, we review the current methods in avian dietary studies detailing the applications, limitations, and future directions of each technique. In particular, we highlight areas where additional methodological refinement is needed, the future directions for avian dietary studies, and how

data from morphological, molecular, and isotopic studies can be integrated to provide a more comprehensive understanding of avian diet.

MORPHOLOGICAL IDENTIFICATION

History and Focus

Traditional methods have informed much of our understanding of avian dietary ecology (Hyslop 1980, Rosenberg and Cooper 1990, Bent 1925), and serve as the basis for comparison with more recently developed laboratory techniques. Morphological prey identification has aided in dietary descriptions of near-threatened warblers (Deloria-Sheffield et al. 2001), helped to explain how habitat structure and search tactics are related to forest bird prey choice (Robinson and Holmes 1982), and revealed how aerial insectivores recognize differences in the quality of prey provisioned to offspring (Quinney and Ankney 1985). As these methodologies have been used for well over a century (McAtee 1912), a wealth of literature already exists that describes different approaches to the collection and identification of prey from morphological samples (Duffy and Jackson 1986, Rosenberg and Cooper 1990). Here, we briefly introduce methods for morphological prey identification to understand prey composition (versus behavioral ecology, e.g. Remsen and Robinson 1990, Ydenberg 1994).

Methodological Considerations

Sample collection, storage, and processing. Morphological prey identification techniques are diverse, and include manual identification of prey during observations of foraging (Collis et al. 2002), feeding (Fleischer et al. 2003) or provisioning events (Margalida et al. 2005) as well as monitoring nestling-provisioning attempts with nest-box cameras (Currie et al. 1996) and digital photography (Gaglio et al. 2017). Researchers have also identified prey retrieved from regurgitates collected via emetics (Prŷs- Jones et al. 1974), neck ligatures (Owen 1956), or

lavage (Brensing 1977); feces collected while handling birds (Ralph et al. 1985) or from past deposits (Waugh and Hails 1983); and samples collected directly from gizzards (McAtee 1918) or stomachs (Sherry 1984, Chapman and Rosenberg, 1991). Some types of direct prey collection can cause undue stress (Duffy and Jackson 1986), induce behavioral changes (Little et al. 2009), or have lethal outcomes (Zach and Falls 1976, Poulin et al. 1994, Carlisle and Holberton 2006), suggesting that some direct collection techniques are undesirable, particularly with at-risk species (Ralph et al. 1985).

When samples must be collected, diet items should be analyzed and classified soon after collection to avoid issues caused by tissue degradation. However, if samples cannot be processed immediately, preservation via freezing or storage in high concentration ethanol or formalin enables long-term storage with minimal loss of morphological integrity (Duffy and Jackson 1986). For studies using both observational and laboratory-based techniques, storage methods must be compatible as they may influence the chemical make-up of prey tissue (Sarakinos et al. 2002) or the ability to retrieve high-quality DNA (Williams et al. 1999) (Figure 1).

Prey classification. Expertise in prey systematics or the aid of detailed taxonomic keys (Merritt and Cummins 1996, Williams and McEldowney 1990) increases prey classification accuracy (Ralph et al. 1985, Sullins et al. 2018). However, even expert taxonomists are challenged to provide complete and detailed taxonomic classifications (Ralph et al. 1985, Parrish 1997), especially if prey remains are difficult to detect in feces or stomach contents (Deagle et al. 2007, Thalinger et al. 2017). Fortunately, characteristic hard parts of prey, such as sclerotized arthropod mandibles or wing fragments (Sherry et al. 2016), chitinous beaks of cephalopods (Xavier et al. 2011), bones of vertebrate prey (Dirksen et al. 1995), and seeds from fruits

(Gorchov et al. 1995) and grains (Desmond et al. 2008) often persist in both regurgitant and fecal samples.

Visual identification methods are frequently criticized for their inability to classify prey items to fine taxonomic levels (Symondson 2002, Pompanon et al. 2012). However, using vouchered reference collections of locally available prey can help to alleviate these problems and can quantify prey availability in the process (Sherry et al. 2016, Kent and Sherry 2020). Additionally, species-level prey identification is not always necessary (Sherry et al. 2020), suggesting that studies will not always benefit from increased taxonomic resolution.

Future Potential

In certain cases, morphological prey identification provides greater insights than molecular or isotopic methods. For instance, the ability to distinguish caterpillars from adult moths (Barbaro and Battisti 2011) and winged from worker ants (Herrera 1983) may be important for understanding how prey are captured and for estimating the nutrient content of prey items. DNA-based methods cannot distinguish between developmental stages of prey items (Trevelline et al. 2016) while isotopic methods can only be used to do so if life stages differ in their isotopic composition (Mihuc and Toetz 1994).

Morphological techniques also provide quantitative information about prey, such as the number of distinctive prey parts and thus the number of prey individuals per sample (Sherry et al. 2016), the size of prey items (Calver and Wooller 1982), and even estimated size of partially digested prey (Hódar 1997, Rosamond et al. 2020). Furthermore, morphological techniques are unique in that they can be used to estimate prey biomass (Lalas and McConnell 2012, Ormerod and Tyler 1991), which provides critical information on energetic fluxes through food webs and can be used in conjunction with frequency of occurrence and total count to determine the relative

or absolute importance of individual prey taxa (reviewed in Duffy and Jackson 1986). Finally, as morphological identification of prey is minimally destructive, researchers can glean nutritional information on prey (Grémillet et al. 2004) as well as digestion-related information (Barton and Houston 1993) from bolus (Boyle et al. 2014), pellet (Wallick and Barrett 1976), lavage (Cherel and Ridoux 1992), or fecal samples (Varennes et al. 2015), to assess gross energy content (Karasov 1990), the caloric value of different prey sizes (Stephens and Barnard 1981) or species (Guillemette et al. 1992), as well as concentrations of prey-derived macronutrients (Albano et al. 2011).

Although researchers may turn to DNA-based methods for rapid, thorough, and precise identification of diet items or isotopic methods for information on nutrient assimilation at greater time scales, morphological prey identification will remain relevant. In addition to a list of potential prey taxa, morphological techniques can also provide the reference tissue required for laboratory-based techniques (i.e. prey DNA sequences and isotopic or lipid composition), as well as data on prey consumption, which can be used as informative priors in Bayesian stable isotope mixing models (Franco-Trecu et al. 2013). Furthermore, advances in deep learning and image processing may soon allow for computational classification and quantification of prey taxa, thus reducing the drawbacks associated with morphological identification (Høye et al. 2021) and ushering in the development of an online database of 'prey part' images, akin to the DNA barcodes found in the Barcode of Life Database (BOLD; Ratnasingham and Hebert 2007).

DNA-BASED METHODS

History and Focus

DNA-based methods have been used to study the feeding habits of birds for over 20 years (Sutherland 2000, Casement 2001) with sequence-based identification, or DNA barcoding,

evolving and improving dramatically in the last decade. The development of high-throughput sequencing used in combination with DNA barcoding across multiple taxa within a mixed sample (i.e. DNA metabarcoding), now allows for hundreds of complex samples to be processed in parallel (Pompanon et al. 2012). Although powerful, the greatest drawbacks associated with high-throughput techniques lie in the up-front costs and the computational complexity of analysis (Jo et al. 2016). However, the cost of sequencing continues to decrease—particularly the per-sample costs when highly multiplexed—and open-source software is available for the analysis of many prey types (Bolyen et al. 2019, Palmer et al. 2018).

Methodological Considerations

Sample collection, storage, and processing. Most DNA-based avian dietary analyses are performed on fecal samples (Ando et al. 2020), which can be collected directly from birds (Trevelline et al. 2018b, Jarrett et al. 2020), from holding bags (paper: Trevelline et al. 2016, Southwell 2018; or cloth: Karp et al. 2013), or even from the environment, although the risk of sample contamination is greater (Oehm et al. 2011, Gerwing et al. 2016, McClenaghan et al. 2019). Similar to fecal samples, boluses are a minimally invasive source of dietary DNA. Other sample types have been used for genomic diet analyses, but these techniques are more invasive (i.e. lavage, induced regurgitation) or otherwise hold no obvious advantage over fecal samples (cloacal or mouth swabs, Vo and Jedlicka 2014; stomach samples, Snider et al. 2021). Though not frequently used, stomach samples in natural history collections hold great potential for molecular diet analyses (Remsen et al. 1993). However, this approach may not always be suitable because many historic samples are stored in formalin, a chemical that crosslinks DNA and complicates downstream amplification and sequencing techniques. Freezing samples upon collection is ideal for most analyses (Crisol-Martínez et al. 2016, Gerwing et al. 2016, Jarrett et

199 al. 2020), and while additional preservation media are not necessary, samples can also be placed
200 in stabilizing buffer, silica, or ethanol before freezing for long-term storage (Figure 1). If
201 immediate freezing is not possible, samples stored at room temperature in ethanol are useful for
202 extended periods (Trevelline et al. 2016), although samples can degrade if ethanol concentrations
203 fall below 70% (S. Sonsthagen, USGS, Pers. Comm.).

204 Studies have tested the efficacy of different DNA extraction techniques (Oehm et al.
205 2011, Jedlicka et al. 2013), though most DNA-based studies use commercially available kits (e.g.
206 Qiagen or Zymo) with protocol modifications to optimize DNA yield and quality (Trevelline et
207 al. 2016). Phenol/chloroform extractions tend to produce inferior results at the PCR stage (Lee et
208 al. 2010), likely due to inhibitors found in fecal samples (Al-Soud and Rådström 2000). Because
209 commercial kits cannot always accommodate an entire sample, sub-sampling is common, but
210 samples should be thoroughly homogenized before sub-sampling (e.g., Forsman et al. 2021) to
211 minimize biases in prey detection (Figure 2). Increasing the number of extraction replicates
212 (Lanzén et al. 2017, Mata et al. 2019), as opposed to increased sample input amount, has been
213 shown to be more effective for capturing alpha-diversity within a sample (Brannock and
214 Halanych 2015), while chemical lysis, physical disruption (e.g., bead-beating) and
215 homogenization may minimize prey-specific DNA recovery bias.

216 **DNA barcode markers.** Identifying a suitable portion of the genome as the taxonomic
217 barcode is critical. This region must be sufficiently conserved across putative diet taxa to
218 develop generalized PCR primers, but also variable enough to distinguish prey taxa. An effective
219 barcode is one for which the divergence of species within a genus will be lower than of genera
220 within a family, and so on (Hajibabaei et al. 2006, Clare et al. 2007). Thus, only a few suitable
221 markers, such as the frequently used mitochondrial cytochrome c oxidase I (COI) gene, have

been identified and consistently used in avian diet studies (Figure 2). The specific primers and number of DNA barcoding loci used will depend on whether specific prey (Karp et al. 2014) or a wide range of taxa (Jusino et al. 2019) are targeted. However, no single primer set can perfectly amplify every species, therefore using multiple primer sets targeting different loci is advised (Corse et al. 2019, da Silva et al. 2019, Forsman et al. 2021).

Indexing. Prior to high-throughput sequencing, diet-derived DNA must be appended with oligonucleotide adapters to allow PCR amplicons to bind to the sequencing flow cell. These adapters also contain sample-specific DNA sequences (i.e. indexes) that allow for the binning of reads from each sample. Adapters can be appended directly to barcoding primers (i.e. one-step preparation) or appended to DNA barcode amplicons during a second, low-cycle PCR (i.e. two-step preparation; Zizka et al. 2019). One-step approaches are faster and reduce costs of PCR reagents, but there is evidence that PCR efficiency may be reduced compared to the two-step approach (Zizka et al. 2019). The two-step approach is often preferred because indexes can be attached to any amplicon, as long as they have a linker sequence complementary to the indexing primer. Both approaches retain information on the sample and primer set used; therefore, researchers can use the same adapters on all of the amplicons in a single sample even if multiple primers targeting various barcoding loci are used. However, if amplicon length differs greatly between the target loci, sequencing multiple barcoding regions on the same flow cell may alter the number of expected reads for each sample/primer combination due to the preferential binding of smaller sequences to the flow cell (S. Dabydeen, Illumina Inc., Pers. Comm.).

Sequence processing. Following sequencing, a number of processing steps are required before assessing diet composition (Figure 3). Reads should be trimmed and filtered to remove low-quality sequencing reads and artifacts. However, as a consensus approach has not been

reached (see Alberdi et al. 2018, O'Rourke et al. 2020), we recommend making bioinformatic pipelines open access to facilitate comparability of data across studies. Next, putative dietary taxa are delineated by clustering highly similar sequences (typically 97%) into operational taxonomic units (OTUs) and selecting a representative sequence for each cluster. Alternatively, algorithms can be used to correct sequencing errors and retain amplicon/exact sequence variants (ASVs or ESVs), which are, in effect, OTUs clustered to 100% similarity (Figure 3). Ideally, an OTU or ASV/ESV should represent a taxonomic unit corresponding to the species level (Alberdi et al. 2018).

Prey classification. Taxonomic assignment of OTUs is accomplished by comparing the representative prey-derived sequences to sequences in a reference database such as the National Center for Biotechnology Information (NCBI) nucleotide database (Benson et al. 2013) or the Barcode of Life Database (Ratnasingham and Hebert 2007) (Figure 2). Both databases tend to be biased towards areas where researchers are actively sampling biodiversity, thus representation is higher for some taxonomic groups (e.g. charismatic Lepidoptera) and for certain parts of the world (e.g. Europe, North America).

When reference libraries are incomplete, diet items may only be assignable to higher-level taxonomic ranks (e.g. Order or Family), or may be missed completely leading to false negative results (Virgilio et al. 2010). Furthermore, distinct representative prey sequences (e.g., multiple ASVs) could be assigned the same taxonomic classification, leaving open the decision whether different sequences assigned to the same taxonomic rank should be lumped or considered distinct. One approach is to aggregate diet items with the same taxonomic assignment (da Silva et al. 2020), but this can be unsatisfactory if sequencing errors cause sequences from a particular species to be assigned to the genus level instead of being aggregated with other sequences of the

same species. In this case, the prey taxon would be treated as a distinct, unidentified species within the same genus. In addition to biases stemming from incomplete and erroneous reference databases or from PCR and sequencing, prey taxa may be distinguished based on genetic divergence rather than reproductive isolation. Recently diverged species may be reproductively isolated yet genetically similar at barcoding regions unaffected by the speciation event (Wiemers and Fiedler 2007), while hybridization and introgression can cause cytonuclear disequilibrium and mask distinct species when primers target organelle DNA (Funk and Omland 2003, Toews and Brelsford 2012). Conversely, prey items with large population sizes may contain substantial genetic diversity, causing their sequences to demonstrate high intraspecific divergence (Funk and Omland 2003), though using a barcoding marker with low intraspecific variation can alleviate this issue.

Finally, DNA-based methods alone cannot determine how a diet-derived sequence became present in the sample. Probabilistic cooccurrence models (Griffith et al. 2016) have been proposed to detect accidental consumption (i.e. the consumption of prey which contains the DNA of other taxa through consumption/parasitism of another taxa), though direct observation may be necessary as these models cannot definitively indicate secondary consumption (Tercel et al. 2021) nor can they determine if an avian parasite was consumed purposefully or accidentally. Detecting cannibalism also poses a unique issue as DNA-based classification techniques rely on conspecifics sharing highly similar, if not identical, barcode sequences. However, researchers can employ barcoding markers that are conserved within the predator species but exhibit high intraspecific variation, thus allowing for the differentiation of DNA sequences stemming from an individual's diet versus its own genome.

Quality control. The degree of biological and technical replication necessary for maximizing detectability of diet-derived sequences must be balanced with minimizing false positives caused by contamination or sequencing errors (Taberlet et al 2018). The use of positive and negative controls during sample collection and DNA extraction, amplification, and sequencing processes can guide how reads are filtered during the sequence analysis stage (reviewed in Zinger et al. 2019) (Figure 2). Additionally, technical replicates, in the form of multiple PCR reactions for each DNA extract, can minimize false negatives in DNA metabarcoding data, especially for diet items with low detection probabilities (Ficetola et al. 2015) or poor DNA amplification efficiency (Jusino et al. 2019).

Data Analysis

Summary analyses. Once the taxonomic composition of the sample has been determined, data are summarized with a variety of analytical techniques (Figure 3) to create a representation of an individual's diet. Researchers frequently transform sequence data into presence-absence matrices because read abundance does not directly correlate to the biomass or frequency of corresponding prey consumed. However, this method can overestimate the importance of food consumed in small quantities (Deagle et al. 2019). Assuming the use of a presence-absence matrix of unique prey taxa or sequences, the next step is often to estimate the proportion of samples that contain a particular taxon, termed the frequency of occurrence.

Specialized analyses. More complex analytical approaches include ordination, such as principal components analysis (PCA; Crisol-Martínez et al. 2016) or non-metric multidimensional scaling (NMDS; Trevelline et al. 2018a) (Figure 3), which are statistical methods that collapse high-dimensionality data (i.e. taxonomic composition) into a smaller number of meaningful diet axes. If downstream analyses are to be implemented, such as deriving

a measure of distance in niche space between two species, PCA is generally preferable to NMDS, t-SNE (Maaten and Hinton 2008) or UMAP (McInnes et al. 2018) because these methods do not preserve distances in multivariate space. Following data ordination, hypothesis testing can be implemented. For example, criteria can be developed to identify groups in multivariate space and test whether these accord with the bird species or groups in question (e.g., k-means clustering, Forgy 1965), they might derive multivariate hypervolumes (Blonder et al. 2014), and implement a randomization, null-model approach, or describe the qualitative differences in multivariate niche space among species or other groups.

Future Potential

DNA-based methods are relatively new (Hebert et al. 2003) and are advancing rapidly to overcome current limitations. For instance, recent areas of research are exploring the use of custom positive controls, such as mock mixtures of potential prey DNA, to gauge the success of the sequencing run and the ability of primers to detect prey taxa (O'Rourke et al. 2020). The inclusion of mock mixtures may become a standard feature of DNA metabarcoding diet studies, though familiarity with potential prey taxa is essential to develop an appropriate mock mixture. Custom reference libraries may be designed for particular prey taxa within the study area to verify the accuracy of representative prey barcodes; though, such an approach necessitates the collection, identification, and sequencing of all putative prey taxa.

The inability to accurately quantify the amount of each prey type consumed, either absolutely or relative to other prey taxa, is a major weakness of DNA-based methods and may be difficult to resolve due to the variety of factors: primers are inherently more efficient at amplifying some prey (reviewed in Nilsson et al. 2019); tissue types and prey taxa may have different copy numbers of marker genes (Thomas et al. 2014, Prokopowich et al. 2003); and some prey may be

more difficult to digest, like those with exoskeletons (Clare et al. 2014). *In silico* analyses (Clarke et al. 2014) and controlled-feeding studies (Thomas et al. 2016) have shown promise in mitigating (Piñol et al. 2019), or at least accounting for quantification biases inherent to DNA-based studies (Palmer et al. 2018). However, the limited experimental work done to associate the number of reads obtained for known amounts of specific prey taxa (Deagle et al. 2010) often uses an extremely limited diversity of prey items (~2-6 taxa), suggesting that direct comparisons will be ineffective for complex dietary mixtures. Experimental designs that consider multiple consumer species, and a wider, more realistic range of diet items is necessary before its widespread application.

A semi-quantitative understanding of diet might also be possible with longer sequencing reads that are variable enough to detect and distinguish different individuals within each of the prey species present in a diet sample. However, most high-throughput sequencing methods are currently limited to short read lengths (<600bp paired-end reads) and, even if sequencing technology would allow for longer barcodes with sufficient sequence variation among conspecifics, it is possible that such long DNA fragments would not survive extended preservation or digestion (Symondson 2002), thus necessitating bioinformatic algorithms to identify unique contiguous prey sequences among highly similar barcode sequences. Finally, the use of internal standards for metabarcoding analyses may one day offer a method to compare absolute prey-derived molecule counts (Harrison et al. 2020), similar to the use of 'housekeeping' genes as internal standards for studying gene expression across samples with qPCR methodologies (reviewed in Eisenberg and Levanon 2013).

Current DNA-based approaches are also limited by their ability to identify specific prey traits, such as age or life stage, as an organism's DNA marker remains unchanged throughout its

life. However, epigenetic molecular age biomarkers (MABs; Jarman et al. 2015), such as mRNA expression levels, locus-specific DNA methylation, or telomere length, are likely to change throughout an organism's life, thus giving researchers the opportunity to glean prey life history information through the development of additional genetic tools. To date, such methods have not been implemented in dietary studies generally, let alone in avian studies. However, the development of such novel applications promises to address research questions fundamental to our understanding of avian trophic ecology.

DNA-based metabarcoding methods excel at individual prey detection and identification, and so are particularly well-suited to answer questions that require species-level data. However, given that dietary taxa can vary greatly in resource quality, an alternative approach would be to step away from taxonomic complexities and instead focus on prey characteristics (e.g., nutrient content or life history traits), as this would dramatically simplify both the analysis and, presumably, the number of samples required to reach robust conclusions. We are aware of only one avian metabarcoding study that directly assessed prey characteristics (aquatic vs. terrestrial life stages; Trevelline et al., 2018a), and while the absence of a comprehensive prey trait database currently makes such an approach challenging, we encourage future research to consider prey traits in their analyses to better illuminate the functional characteristics of avian dietary ecology.

DNA-based dietary studies have mostly focused on the description of prey taxa and the ecosystem services of avian predators (e.g. Crisol-Martinez et al. 2016); however, we can also leverage DNA-based methods to examine diet overlap of sympatric species (Trevelline et al. 2018b), and thus address theoretical questions related to competition and resource partitioning (e.g., Spence et al. 2021; Stillman et al. 2021). There is also considerable scope to examine

whether species are dietary specialists or generalists (Jesmer et al. 2020), and how prey selection is influenced by disturbance (e.g., hurricanes, fire, e.g., Stillman et al. 2021) or time of the annual cycle when nutrient requirements are high (e.g., breeding, pre-migration), thus clarifying responses to prey availability and physiological need. DNA-based methods are also well-suited for identifying the ecological services that birds offer, such as in seed dispersal (González-Varo et al. 2014) and pollination (Spence et al. 2021). From a conservation standpoint, DNA-based methods can help managers assess the foraging success of captive bred individuals reintroduced to the wild, thus lending important perspective on the potential for long term resilience (e.g. Volpe et al. 2021). Finally, there is considerable opportunity to examine how prey species communities have changed over time by taking core samples (i.e. guano at communal roosts) and extracting DNA from different layers representing different points in time. The ability to associate prey communities with climate may help to predict how climate change will affect prey availability for a range of birds.

STABLE ISOTOPE ANALYSIS

History and Focus

Elements may exist in forms that differ in atomic mass (i.e. isotopes) and are typically found overwhelmingly in one common form with lower abundances of rarer, usually heavier, forms. The relative abundance of rare to common isotopes can change as a result of numerous biogeochemical reactions, where abundance is expressed in delta (δ) notation relative to international standards in parts per thousand (‰, per mil; Hayes 1982). In biological systems, stable isotopes are incorporated at the base of food webs through fixation of inorganic compounds by primary producers (Kelly 2000), and their relative abundances are subsequently modified as they move through the food web via metabolic processes. For example, birds

incorporate the isotopic values of their prey into their own tissue, and the extent of subsequent isotopic change is generally dependent upon the element, dietary quality, and tissue type (Boecklen et al. 2011). Some elements (e.g., lead or strontium) with high atomic mass show little to no isotopic change with trophic position and, thus, make for useful direct tracers of basal energy pathways to consumers (DeNiro and Epstein 1978), while the lighter elements (e.g. nitrogen) show stronger isotopic changes with trophic level and can inform trophic position (Wassenaar 2019). Thus, by characterizing the stable isotope ratios of prey sources at the base of food webs and knowing how these ratios are modified between diet and consumer through isotopic discrimination, it is possible to use the stable isotope ratios in avian tissues to infer dietary source and feeding habits.

A wealth of literature discusses the details of stable isotope analyses in ecological studies (e.g., Peterson and Fry 1987, Schmidt et al. 2007, Katzenberg 2008, Hobson 2011, Boecklen et al. 2011, Layman et al. 2012, Wiley et al. 2017), and their use in the study of bird movements (Rubenstein and Hobson 2004, Hobson and Wassenaar 2019). Here, we provide a brief overview of stable isotope analysis to investigate the diets of birds by detailing the relevant applications, considerations, and future directions of this technique.

Methodological Considerations

Sample collection, storage, and processing. Because stable isotopes are incorporated during tissue synthesis, any tissue that can be retrieved from a bird can be used for stable isotope analysis; though, selection of tissue will depend on the focus and timescale of the research question (Figure 4). To assess dietary isotopic endpoints, researchers should be sure to analyze the tissues of the main dietary items that birds consume, such as fruits (Vitz and Rodewald 2012), prey muscle tissue (Anderson et al. 2009), or even the entire body (Herrera et al. 2003) to

ensure that the isotopic sources are representative of the prey pool contributing to the nutrition of the consumer. For all tissues, freezing is the preferred preservation method (Bond and Jones 2009) followed by air drying with a smokeless heat source (Bugoni et al. 2008), or storage in 70% ethanol (Hobson et al. 1997). Preservation media, such as formalin, genetic buffer solutions (Hobson et al. 1997), or high percentage ethanol (Bugoni et al. 2008) can replace isotopes within dietary or avian tissues with their own, which can be particularly problematic for carbon, nitrogen, and hydrogen stable isotope analyses. For lipid-rich tissues, chemical lipid-extraction may be needed prior to analysis (Bond and Jones 2009) to facilitate accurate diet reconstruction (Kojadinovic et al. 2008). Similarly, diets or avian tissues rich in carbonates often require acidification prior to analysis to obtain the unbiased $\delta^{13}C$ values of the organic matrix (Polito et al. 2009, Mackenzie et al. 2015). However, chemical lipid-extraction and acidification have the potential to bias tissue $\delta^{13}C$ and $\delta^{15}N$ values (Jaschinski et al. 2008, Elliott and Elliott 2016). As such, mathematical normalization for tissue lipid and/or carbonate content represents an alternative method when chemical lipid-extraction or sample acidification is not feasible or advisable (Post et al. 2007, Jaschinski et al. 2008, Oppel et al. 2010).

Isotope systems. The most common elements used in isotopic dietary studies are those of carbon ($^{13}C/^{12}C$; $\delta^{13}C$) and nitrogen ($^{15}N/^{14}N$; $\delta^{15}N$), which typically provide information on source of feeding and trophic position, respectively (Figure 4). Stable isotopes of hydrogen ($^{2}H/^{1}H$; $\delta^{2}H$) and oxygen ($^{18}O/^{16}O$; $\delta^{18}O$) are tightly linked to the hydrological cycle and ambient temperature, and have also been used to identify nutrient inputs from terrestrial and aquatic origins (Figure 4). Sulfur ($^{34}S/^{32}S$; $\delta^{34}S$) isotope ratios have been used to identify nutrients derived from marine vs. terrestrial sources, proximity to coastlines, benthic vs. pelagic energy pathways, and use of estuarine and marsh habitats (Figure 4). Analysis of "heavy" elements can

451 be useful for delineating source of feeding, especially those of strontium ($^{87}Sr/^{86}Sr$; $\delta^{87}Sr$), which
452 are associated with age of bedrock and, in North America, tend to vary along longitudinal
453 gradients (Figure 4). While the investigation of a single element's isotopic ratio within avian
454 tissues can provide details about diets and foraging habitat, using the stable isotopic values of
455 multiple elements within a single study can allow researchers to differentiate among prey sources
456 using isotopic mixing models or determining spatial origins of diets (Bowen and West 2019).
457 **Isotopic discrimination**. The change in stable isotope ratios that takes place between
458 reactants and products or as a result of kinetic processes is known as isotopic fractionation
459 (Tiwari et al. 2015). Isotopic fractionation is rarely measured in natural systems; instead the
460 isotopic discrimination that results from many individual fractionation events is measured
461 (Schoeller 1999). Isotopic discrimination patterns between diets and consumers in animal food
462 webs involving changes in $\delta^{15}N$ values are particularly useful once established. Processes of
463 amination and deamination of proteins results in step-wise and fairly predictable increases in
464 consumer tissue $\delta^{15}N$ values with each trophic transfer (Macko et al. 1986), and this has allowed
465 researchers to use tissue $\delta^{15}N$ values to estimate consumer trophic position (DeNiro and Epstein
466 1981, Hobson and Welch 1992). Trophic discrimination factors (TDFs) based on $\delta^{15}N$ values, or
467 the differences in $\delta^{15}N$ values between prey and consumer tissues, range between +2.5‰ to +5‰
468 with average values centered around +3‰ to +3.5‰ (Post 2002). A recent meta-analysis of
469 factors influencing TDFs have resulted in the development of the R-package SIDER as a tool to
470 predict TDFs when TDFs from controlled studies are not available (Healy et al. 2018) (Figure 5).
471 However, researchers are encouraged when possible to conduct controlled long-term feeding
472 trials of focal species to establish appropriate TDFs (Martínez del Rio et al. 2009).

For $\delta^{13}C$ values, it is generally assumed that TDFs are relatively low with average values centering around +0.4‰ (Post 2002). However, TDFs can vary by avian tissue type even when synthesized under the same diet due to differences in biochemical processes and macromolecule routing, which is especially apparent among lipid-rich and keratin-based tissues that may require correction factors prior to analysis (Hobson and Clark 1992b, Cherel et al. 2014b). Stable sulfur isotope measurements ($\delta^{34}S$) appear to also have low TDF values (~0.0‰ to +1‰) and so can be more readily linked to food web source inputs (Richards et al. 2003, Arneson and MacAvoy 2005, Florin et al. 2011). Even so, $\delta^{34}S$ TDFs can vary due to the input of endogenous sulfur from the recycling of body proteins when individuals consume low-protein diets (Richards et al. 2003). Little is currently known about TDFs associated with $\delta^{2}H$ values and whether or not patterns of trophic enrichment are due to isotopic discrimination or ambient exchange (reviewed in Vander Zanden et al. 2016).

Isotopic turnover. The residency time of elements in animal tissues varies approximately by the metabolic rate of that tissue (Figure 4). This means that metabolically active tissues will assimilate isotopic information on diet over different timescales, and thus present an opportunity to choose a tissue most appropriate for the dietary integration period of interest (Hobson 1993, reviewed by Thomas and Crowther 2015, Carter et al. 2019a). Researchers have performed stable isotope analysis on various avian tissues to understand an individual bird's diet composition at scales ranging from hours (breath and plasma; Hatch et al. 2002; Podlesak et al. 2005; Pearson et al. 2003), days and weeks, (red blood cells; Podlesak et al. 2005; Hobson and Clark 1993), to months (feathers and claws; Hedd and Montevecchi 2006; Bearhop et al. 2003) or even years (bone collagen; Stenhouse et al. 1979; Hobson and Clark 1992a; Hobson and Sealy 1991; Hedges et al. 2007). Indeed, it is possible to estimate year-round

dietary patterns by examining multiple tissues from the same individual (Hobson 1993, Hobson and Bond 2012, Gòmez et al. 2018). For tissues that are metabolically inactive following synthesis (e.g. claws, feathers) the tissue's isotopic information is effectively "locked in", and represents only the time window over which the tissue was grown (Hobson 2005). For birds with predictable molt cycles or those stored in museum collections, this represents an opportunity to sample feathers to infer diet at a previous time (Blight et al. 2015). Additionally, claw tissue is metabolically inert once formed but claws grow continuously, thus allowing researchers to make dietary inferences on a captured bird based on previous months (Bearhop et al. 2003).

Isotopic turnover rates can also differ due to diet composition (Hobson and Clark 1993, Podlesak et al. 2005), tissue type (Vander Zanden et al. 2015), an individual's physiological state (Carleton and Martínez del Rio 2005, Cherel et al. 2005), and energy expenditure. For instance, in proteinaceous tissues, structural turnover is the main driver of isotopic turnover (Carter et al. 2019a), but in lipids, it appears to be influenced by energy expenditure (Foglia et al. 1994, Carter et al. 2018). Though there is now a greater understanding of isotopic turnover both among individuals and tissue types, uncertainty remains for less-studied systems (Carter et al. 2019a). In addition, drivers of tissue-specific and macromolecule-specific turnover rates as well as the development of mechanistic models of isotopic turnover that can be applied across a broad diversity of taxa are needed (Carter et al. 2019a, Caut et al. 2009). The derivation of allometric relationships driving isotopic turnover rates will assist research on birds that differ in body mass (Carleton and Martinez del Rio 2005, Carter et al. 2019a).

Macromolecule routing. While isotope-based dietary reconstruction is founded on the notion that "animals are what they eat plus a few parts per mil" (DeNiro and Epstein 1976), the idea that the isotopes derived from prey tissues are dispersed throughout a bird's body uniformly

519 (coined the "Scrambled egg theory"; Van der Merwe 1982) is an unrealistic (Martínez del Rio et
520 al. 2009) and unsupported assumption (Ambrose and Norr 1993). Instead, stable isotopes located
521 in macromolecular pools of diets (e.g., proteins, lipids, carbohydrates) can be differentially
522 allocated to various consumer tissues through the process of isotopic routing (Schwarcz 1991),
523 an effect that may be particularly important to consider when studying omnivores (Podlesak and
524 McWilliams 2006). Thus, selection of bulk avian tissue type for stable isotope analysis is not
525 only based on the time scale of nutrient assimilation but also on the sources and destination of
526 dietary macromolecules. Dietary amino acids may be preferentially routed to more proteinaceous
527 tissues (Gannes et al. 1998, Martínez del Rio and Wolf 2005) whereas less proteinaceous tissues
528 derive the bulk of their isotopic values from dietary carbohydrates and lipids (Gannes et al.
529 1998), though some mixing of isotopic assimilation between prey sources and avian tissue is
530 expected to occur. Where possible, researchers should strive to understand the biochemical
531 processes and routing resulting in the isotopic composition of a given tissue (Voigt et al. 2008),
532 as known isotopic routing and discrimination will guide interpretation (Martínez del Rio and
533 Wolf 2005).

534 **Bulk stable isotope analysis.** Stable isotope analysis of bulk tissues (e.g., muscle, blood,
535 feather) has been the most common approach to avian dietary studies thus far. This approach has
536 been effective because sample cost is relatively low, and analyses can be performed rapidly with
537 high sample throughput. In addition, avian tissues used in non-lethal diet reconstruction studies,
538 such as feathers (Kojadinovic et al. 2008) or blood (Bond and Jones 2009), will typically require
539 little additional sample processing prior to bulk stable isotope analysis (but see Bond et al. 2010).
540 When dietary sources are well characterized and isotopically distinct, and tissue-specific TDFs
541 have been quantified, bulk stable isotope analysis can provide robust insights into the dietary

history of birds (Inger and Bearhop 2008). However, when sources and/or TDFs cannot be adequately characterized, a common challenge in the interpretation of bulk tissue stable isotope values is determining whether variation is due to changes in diet, variability in baseline food web isotope values, or some combination of these factors (Inger and Bearhop 2008). These challenges are now being overcome through more complex isotopic analyses of specific compounds (e.g., fatty acids and amino acids; Whiteman et al. 2019, Twining et al. 2020) with a method known as compound-specific isotope analysis (CSIA; Lorrain et al. 2009).

Data Analysis

Mixing models, trophic position, and isotopic niche analyses. Isotopic values of a consumer's tissue are a mixture of the isotopes derived from their prey, thus stable isotope mixing models can be used to determine the relative contributions of each prey taxon (Phillips 2012) (Figure 5). To accurately quantify prey composition, researchers must not only know the potential prey groups that birds eat, but also the isotopic values of each potential prey group, ensuring that the isotopic values of each group are distinct. If unique prey sources are not isotopically distinct, but belong to a shared functional group, researchers should consider combining these sources in downstream analyses (Phillips et al. 2005). While all mixing models work under the principle that a consumer's isotopic ratio is proportional to that of its assimilated prey, earlier iterations of these models have been improved by including the elemental concentrations of prey sources (Phillips and Koch 2002), considering isotopic routing (Martínez del Rio and Wolf 2005), and working within a Bayesian framework to allow for better propagation of uncertainty and use of informative priors (Parnell et al. 2013). Mixing models can be applied to both bulk tissue stable isotope analysis and CSIA data to reconstruct avian diets (Johnson et al. 2019), and dietary predictions can be improved through the inclusion of data from

565 morphological or laboratory-based methods (Polito et al. 2011, Chiaradia et al. 2014, Johnson et
566 al. 2019).

567 The R-package MixSIAR provides a Bayesian mixing model framework that can include
568 fixed and random effects as covariates explaining variability in mixture proportions, incorporate
569 prior data sources, and calculate relative support for multiple models via information criteria
570 (Stock et al. 2018). Another R package applying a similar Bayesian framework, tRophicPosition,
571 calculates consumer trophic positions using stable isotopes, with one or two isotopic baselines,
572 while explicitly including individual variability and propagating sampling error in the resulting
573 posterior estimates (Quezada- Romegialli et al. 2018). In addition, the SIBER (Jackson et al.
574 2011) and nicheROVER (Swanson et al. 2015) packages allow for direct comparison of isotopic
575 niche area (a proxy for trophic niches; Newsome et al. 2007) and overlap (Flaherty and Ben-
576 David 2010) across consumers and/or communities (Figure 5). While sophisticated analyses
577 continue to be published, these models are only as good as the data and study design employed,
578 and decisions about model parameterization and source grouping can influence results (Bond and
579 Diamond 2011). Phillips et al. (2014) provide a summary of the best practices for stable isotope
580 mixing models in food-web studies that are broadly applicable to avian research.

581 **Future Potential**

582 As stable isotope analysis has been used in avian diet reconstruction for nearly 40 years
583 (Schoeninger and DeNiro 1984), many of its limitations and future directions have been
584 identified—or even addressed (Post 2002, Boecklen et al. 2011, Wiley et al. 2017). However,
585 one promising new development in the field lies in CSIA or the isotopic analysis of biological
586 macromolecule groups, such as amino acids or fatty acids. Because specific compounds are
587 metabolized through unique pathways, CSIA is an improvement on bulk isotopic analysis as it

588 can quantify and account for variation in isotopic baselines over time and space, and the
589 differential routing of dietary macromolecules throughout consumer tissues (Whiteman et al.
590 2019) . For $\delta^{15}N$, some individual amino acids (e.g., glutamic acid) undergo large isotopic
591 fractionation during transamination/deamination providing greater sensitivity when estimating
592 trophic position (McMahon et al. 2015, Ohkouchi et al. 2017). In contrast, other amino acids
593 (e.g., phenylalanine) show little to no trophic fractionation between diet and consumer allowing
594 researchers to quantify isotopic baselines (McMahon et al. 2015, Ohkouchi et al. 2017). The
595 analysis of individual "trophic" and "source" amino acids can thus be used to infer trophic
596 position of avian consumers even in situations where baseline food web isotopic values are not
597 known. For example, McMahon et al. (2019) used feather glutamic acid and phenylalanine $\delta^{15}N$
598 values to calculate a nearly 100-year record of *Pygoscelis* spp. penguin trophic positions that
599 explicitly accounted for variation in food web isotopic baselines over time, while Whiteman et al
600 (2020) quantified $\delta^{13}C$ and $\delta^{15}N$ values of various amino acids to investigate nutrient allocation
601 by birds to their eggs within the context of the capital vs. income continuum.
602 Animals must acquire essential amino acids from their diet, and as these amino acids
603 undergo little to no additional isotopic change from diet to consumer (Hayes 2001, McMahon et
604 al. 2015), $\delta^{13}C$ stable isotope analysis of amino acids (CSIA-AA) can better trace energy
605 pathways from basal sources to upper-level consumers. For example, Johnson et al. (2019) found
606 that while bulk stable isotope analysis and CSIA-AA of Seaside Sparrow (*Ammospiza maritima*)
607 liver tissues predicted similar contributions of terrestrial and aquatic-derived carbon, CSIA-AA
608 did so with greater precision. CSIA of fatty acids (CSIA-FA) have also provided a glimpse into
609 the importance of fatty-acid composition in energy metabolism of migrating birds (Carter et al.

2019b), and novel applications of $\delta^{13}C$ CSIA-FA promise to broaden our understanding of avian food webs and address the limitations of previous applications (Twining et al. 2020).

ALTERNATIVE DIETARY BIOMOLECULE TRACING

While DNA-based and stable isotope techniques are applicable to most study systems, researchers also trace other biomolecules through food webs to address more specialized questions in avian dietary ecology. Useful dietary tracers include essential biomolecules that are not synthesized by birds (e.g., essential lipids, amino acids, vitamins; Ruess and Müller-Navarra 2019), biomolecules that undergo little or no metabolic change post-consumption (e.g., long-chain polyunsaturated fatty acids; Twining et al. 2016), and non-nutritional components indicative of environmental contamination (e.g., lead, mercury). Because alternative dietary tracers are often specific to certain environments, studies typically couple one of the previously described techniques with these tracers to draw ecological inferences about the effect of diet variation; though, continued development of mixture modeling approaches (e.g., quantitative fatty acid analysis [QFASA]; Iverson et al. 2004) and the identification of additional dietary tracers in new habitats (Hixson et al. 2015) will allow for broader application of biomolecule tracing in diet reconstruction. Analytical methods for individual dietary tracers is beyond the scope of this review, but has been discussed by others (Williams and Buck 2010, Nielsen et al. 2017, Majdi et al. 2018). Here, we focus on analyses employing multiple techniques to address objectives beyond diet identification.

Nutritional Components: A Healthy Diet

In addition to meeting energy demands and broad macronutrient requirements, birds must obtain essential biomolecules from diet to maintain optimal health and productivity (Klasing 1998). Essential polyunsaturated fatty acids have been useful as tracers because vertebrates tend

633 to have a limited ability to convert these biomolecules, and controlled diet studies suggest that
634 consumer fatty acid signatures resemble the fatty acid signatures of their food (Twining et al.
635 2016). Historically, most research in avian nutrition has focused on domesticated species, but
636 there has been recent momentum in studying the nutritional response of wild populations to
637 changes in food availability resulting from anthropogenic influences and climate change (Birnie-
638 Gauvin et al. 2017). Because diet items are not all nutritionally equivalent, the impacts of
639 changes in food quality to avian health and fitness should be considered alongside prey
640 identification in shifting diets through a combination of techniques. For example, morphological
641 diet identification followed by fatty acid analysis has shown that diets containing optimal prey
642 items correlate with greater concentrations of essential polyunsaturated fatty acids as well as
643 metrics of survival and reproductive success in grassland (Zhang et al. 2020) and riparian
644 songbirds (Twining et al. 2018). Combining bulk stable isotope analysis and fatty acid analysis
645 enabled Hebert et al. (2014) to trace prey-specific fatty acids to aquatic birds foraging in benthic
646 and pelagic locations, thus explaining how shifts in bird diet were linked to disease emergence.
647 Similarly, combining fatty acid analysis and CSIA-FA showed that riparian songbirds derive
648 essential long-chain polyunsaturated fatty acids from aquatic prey, even if terrestrial prey make
649 up a greater portion of their diet (Twining et al. 2019). Furthermore, integrating morphological,
650 stable isotope and fatty acid techniques has the potential to produce a more cohesive picture of
651 avian feeding habits across short- and long-term scales, which has been influential in identifying
652 patterns of foraging plasticity (Moseley et al. 2012) and niche partitioning (Connan et al. 2014).
653 While future research will likely focus on the composition of fatty acids and amino acids, other
654 diet-derived molecules, such as carotenoids (Witmer 1996), may also enable the examination of
655 diet as well as the resulting consequences for avian populations.

Non-nutritional Components: A Contaminated Diet

In addition to nutritional components, non-nutritional chemicals and debris are also consumed directly or indirectly via contaminated prey. Anthropogenically-induced environmental contamination is a major cause of avian mortality, and also generates sublethal effects that can be tied to declining populations. For example, lead and mercury exposure can both cause immune suppression and reduce reproductive output (Whitney and Cristol 2018, Williams et al. 2018, Vallverdú-Coll et al. 2019), while brominated flame retardant exposure impacts avian courtship behavior, growth and development (Guigueno and Fernie 2017). Environmental contaminants often biomagnify at higher trophic levels, therefore, combining dietary and contaminant analyses can lead to greater insights regarding exposure risk for birds among different habitats and feeding guilds. For instance, Barn Owls (*Tyto alba*) are most heavily exposed to anticoagulant rodenticides during the fall, as estimated by diet and chemical residues in pellets (*Apodemus* spp;.Geduhn et al. 2016). Regurgitates and pellets as well as feces have also been analyzed to detect the presence of plastics ingested by wetland birds (Gil-Delgado et al. 2017, Reynolds and Ryan 2018), gulls (Lindborg et al. 2012, Furtado et al. 2016), and seabirds (Acampora et al. 2017). Although no sampling method for detecting ingested plastics is perfect (Provencher et al. 2019), tracking consumption of contaminated diet items or debris by applying morphological identification methods can support the use of avian populations as biomonitors of an increasingly polluted environment.

Future Potential

Bulk stable isotope methods have also been incorporated into studies of contaminant exposure where the effects of trophic position ($\delta^{15}N$) and dietary source ($\delta^{13}C$ and $\delta^{34}S$) influence levels of exposure. For example, positive correlations between mercury concentrations

679 and $\delta^{15}N$ values show biomagnification of lead, mercury and arsenic, resulting in higher
680 contaminant loads for aquatic and terrestrial birds feeding at higher trophic levels (Cui et al.
681 2011, Carravieri et al. 2013, Badry et al. 2019, Tasneem et al. 2020, Costantini et al. 2020).
682 Correlations between mercury concentrations and $\delta^{34}S$ have revealed a greater exposure risk for
683 gulls with a marine-sourced diet (Ramos et al. 2013), and the correlation between flame
684 retardants and $\delta^{13}C$ explain the role of a terrestrially-sourced diet on Peregrine Falcon (*Falco*
685 *peregrinus*) contaminant exposure in urban environments (Fernie et al. 2017). Stable isotope
686 reconstruction of diet over long time periods has also been useful in explaining Chimney Swift
687 (*Chaetura pelagica*) diet shifts with respect to historical use of DDT (Nocera et al. 2012) and in
688 creating an accurate mercury exposure trend for Herring Gulls (*Larus argentatus*) by
689 incorporating diet shifts (Burgess et al. 2013). These studies highlight the utility of combining
690 diet and contaminant analyses to the source, timing, and risk of exposure to avian populations.

691 **COMBINING DIETARY ANALYTICAL TECHNIQUES**

692 While the vast majority of avian dietary studies use only a single method for dietary
693 characterization, the use of multiple techniques within a single study, either independently or in
694 concert, will mitigate some of the drawbacks of each technique and yield a more accurate
695 understanding of the study system overall. There are four basic approaches to combining the
696 dietary analytical techniques we have described. All have advantages and disadvantages, and all
697 depend on assumptions related to biases inherent in any given application. First, researchers may
698 present the results of various techniques separately and consider in depth what each suggests
699 about diet (Sydeman et al. 1997, Lavoie et al. 2012, Alonso et al. 2014, Génier et al. 2021;
700 Bumelis et al. 2021). For example, researchers could apply DNA-based methodologies to
701 identify each prey taxon to the species level, morphological techniques to understand which prey

life stages and sizes are often targeted, and stable isotope analysis to quantify the assimilated nutrients that birds acquire from each prey group or life stage over a certain time period, thus gaining important information on many facets of a bird's dietary niche. Such an approach would effectively mitigate the drawbacks associated with each technique, and in many ways, would be entirely complementary as each method represents different degrees of dietary resolution and periods of assimilation. The net result of such analyses will be to provide a *weight of evidence* approach that will require a forensic reconstruction of diet similar to approaches advocated for a court of law (e.g. Ehleringer et al. 2020). This approach is appealing because all dietary evidence is presented for the reader to interpret on its own merits.

The second approach is to convert all dietary information to relative probabilities of input to a given individual or population-level diet. Once converted to probabilities, they can then be formally combined as informative priors in Bayesian mixing models (Parnell et al. 2013). For example, mixing models based on bimodal isotopic data (e.g. $\delta^{13}C$ and $\delta^{15}N$) for avian tissues and diet can be combined with morphological (Robinson et al. 2018, Johnson et al. 2020) or DNA-based data (Franco-Trecu et al. 2013, Chiaradia et al. 2014) as informative priors. In general, the formal incorporation of informative priors will improve the precision of dietary mixing models. For example, if two prey species overlap isotopically, the use of informative priors based on non-isotopic data may better resolve these inputs in the final posterior probability distributions of prey inputs. However, it is also clear that informative priors can result in misleading inferences in dietary reconstructions (Franco-Trecu et al. 2013) and considerable attention must be paid to potential biases associated with prior information. The effect of an informative prior will depend heavily on sample size and will be especially powerful with small sample sizes. As with most aspects of mixing model applications, true evaluation of the use of

priors based on controlled feeding experiments (e.g. Chiaradia et al. 2014) are rare. Currently, researchers are encouraged to present results of Bayesian mixing models with and without the use of informative priors.

A third approach is to incorporate various biomarkers directly into a multidimensional Bayesian mixing model framework (i.e. without necessarily employing informative priors). Because different biomarkers have different units of measurement, they must first be transformed to the same unitless scale by subtracting the mean and dividing by the standard deviation. The mixing model is then run in the normal fashion to discern relative dietary inputs. The approach of using stable isotope measurements and fatty acid analyses has been relatively common in marine systems (Neubauer and Jensen 2015), though O'Donovan et al. (2018) used this approach to investigate diets of wolves in northern Canada using two stable isotopes ($\delta^{13}C$ and $\delta^{15}N$) and three fatty acids of wolf and prey tissue in a five-dimensional model. While the authors found the combined approach was more powerful than using stable isotopes alone, they cautioned that adding more variables (i.e. more fatty acids) will not necessarily improve resolution.

Related to the third approach, a fourth approach combines various analytical approaches into a multidimensional dietary niche space (Swanson et al. 2015). Though studies frequently derive values from the same type of assay (e.g., stable isotope analysis), axes can theoretically include other metrics such as trace element concentrations or fatty acid concentrations. As indicated above, incorporating different metrics requires that the various axes be expressed in quantities that are unitless (typically expressed as mean values divided by the standard deviation). Analytically, this approach has many advantages, though the main drawback is that it can become difficult to interpret multidimensional niche volumes and again, multidimensional

748 niche overlap does not necessarily mean true dietary overlap. Nonetheless, if the objective is to
749 examine evidence for differences in diet among individuals or populations, the derivation of such
750 multidimensional niche hypervolumes is appealing.
751 Future dietary studies will continue to embrace ever more sophisticated forensic tools to
752 evaluate avian nutritional ecology and these approaches will benefit from vast improvements in
753 web-based analytical packages. Nonetheless, there are key knowledge gaps that should be
754 urgently addressed. First, the bulk of avian studies have been focused on describing, and often
755 re-describing, the diets of relatively few species, thus leaving gaps in our basic understanding of
756 dietary composition for many avian taxa, particularly Neotropical species (Lees et al. 2020).
757 While studies of most bird species will benefit from using any of aforementioned methods,
758 DNA-based techniques seem particularly well-suited for providing a general understanding of
759 diet for understudied species and may help build the foundation necessary for further hypothesis-
760 driven research. Similarly, most dietary studies have been biased toward the breeding season,
761 and while the importance of seasonal interactions on bird populations has been known for some
762 time (Marra et al. 1998), there has been little change in the frequency of multi-seasonal or year-
763 round avian studies (Marra et al. 2015). While evaluating diet throughout the annual cycle may
764 appear daunting, stable isotope techniques allow assays of different time periods based on a
765 single capture event (Gómez et al. 2018, Cherel et al. 2014a), with sampling of migratory birds at
766 banding stations providing such tissue samples readily (Smith et al. 2003). Finally, the
767 combination of multiple techniques together with the recent advances in temporal and spatial
768 analyses, such as Motus (Taylor et al. 2017) or GPS tags (Gyimesi et al. 2016), will provide
769 additional information on foraging areas of birds, which may ultimately lead to novel concepts,
770 such as “nutritional landscapes or seascapes”, that describe avian diets and aid in conservation

efforts (Genier et al. 2021, Bumelis et al. 2021). We are, thus, in an exciting era whereby the optimization and integration of techniques and their applications for revisiting previous studies and answering novel ornithological questions will likely lead to a stronger understanding of avian trophic ecology and a greater appreciation for the roles that birds serve in changing ecosystems around the world.

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Figure 1

Click here to access/download:Figure:Figure_1.pdf

DATA COLLECTION

SAMPLE COLLECTION

TRADITIONAL

OBSERVATION

Camera trap, manual observation

MORPHOLOGY

Fecal, pellets, stomach contents, ligatures

DNA-BASED

METABARCODING

Fecal; stomach contents; pellets; swab of beak, talons, cloaca, mouth

BIOMARKER-BASED

BULK STABLE ISOTOPE

COMPOUND SPECIFIC

Any tissue: Bone, feather, muscle, fat, blood, breath etc.

SAMPLE STORAGE

Not required

Dry	Yes
70% EtOH[1]	Yes
95% EtOH[1]	Yes
Frozen	Dep.[2]
Formalin	Yes
Buffers	Yes

Dry	No
70% EtOH[1]	No
95% EtOH[1]	Yes
Frozen	Yes
Formalin	No
Buffers[3]	Depends

Dry	Yes
70% EtOH[1]	Yes
95% EtOH[1]	No
Frozen	Yes
Formalin	No
Buffers	No

SAMPLE PROCESSING

Image processing to identify prey

Microscopy; manual identification of prey parts

1. DNA extraction
2. Library preparation
3. DNA sequencing

1. Prepare sample if needed (chemical lipid extraction[4], acidification)
2. Mass spectrometer: generate isotope ratio

DATA ANALYSIS

DATA PROCESSING

Standard data analysis (Excel, R, SAS, etc.)

STEP 1: DATA PREPARATION

MOTHUR, QIIME2, custom pipelines

STEP 2: DATA ANALYSIS

Standard data analysis (Excel, R, SAS, etc.)

STEP 1: DATA PREPARATION

Standard programs for basic data analysis and preparation for modeling (Excel, R, SAS, etc.)

STEP 2: DATA ANALYSIS

MixSIAR, tRophicPosition, SIBER, nicheROVER

Figure 1: An outline of the general workflow from sample collection through data analysis for the most common methods used in avian diet studies.

[1]EtOH is flammable and requires special packaging and labeling for shipping. EtOH is prone to leaking and can remove ink labels, although pens exist that are designed to withstand it. EtOH contains PCR inhibitors, which must be removed prior to or during the extraction process.

[2]Freezing samples may alter prey tissue making identification difficult, particularly for soft-bodied or small prey (e.g. arthropods).

[3]Longmire buffer may be preferred for fecal samples as DNA in Queen's lysis buffer tends to degrade after a few months at room temperature (S. Sonsthagen Pers. Comm.).

[4]Lipids commonly have lower stable isotope values relative to proteins within a consumer (DeNiro and Epstein 1977) and are typically routed via different metabolic pathways, a consideration especially significant for δ13C and δ2H analyses (Soto et al. 2013).

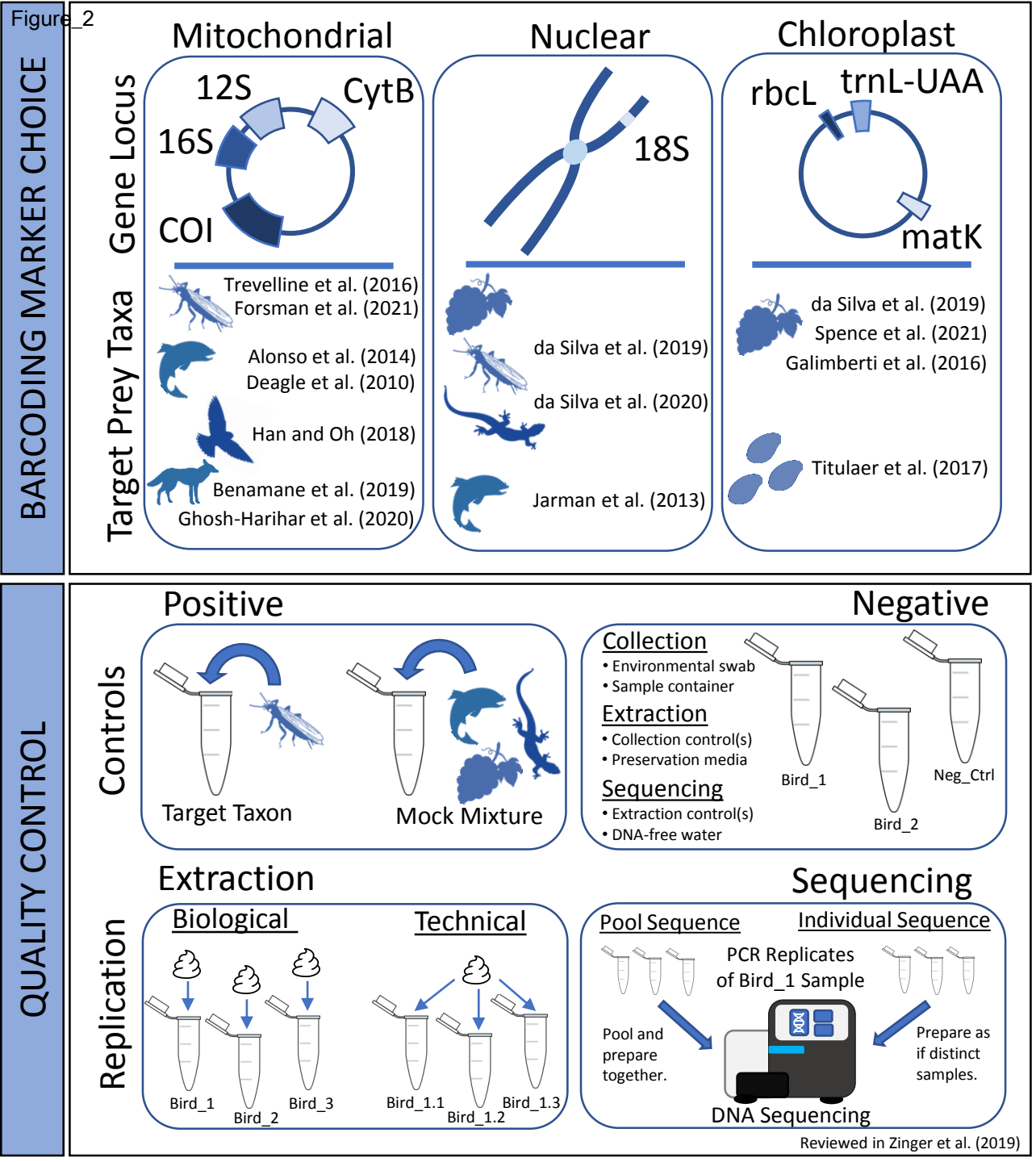

Figure 2: Considerations in DNA-based Prey Identification: Barcoding Marker Choice & Quality Control.
A diagram of common considerations when characterizing prey with DNA-based methods, including barcoding marker choice and quality control. While no consensus method exists for DNA-based dietary characterizations, articles further detailing each step are included.

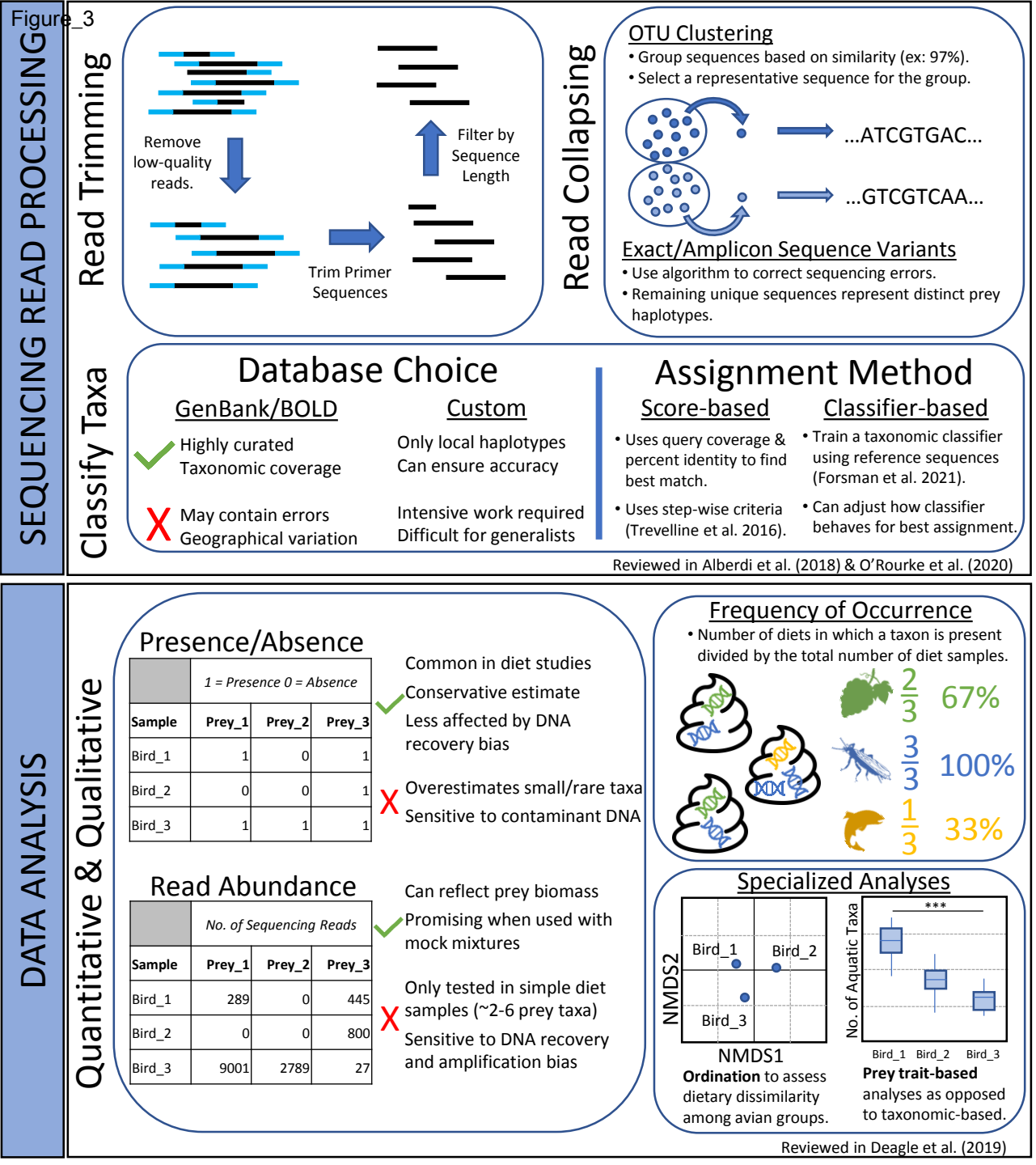

Figure 3: Considerations in DNA-based Prey Identification: Sequencing Read Processing & Data Analysis.
A diagram of the common considerations when characterizing prey with DNA-based methods, which includes sequencing read processing and data analysis. While no consensus method exists for DNA-based dietary characterizations, articles further detailing each step are included.

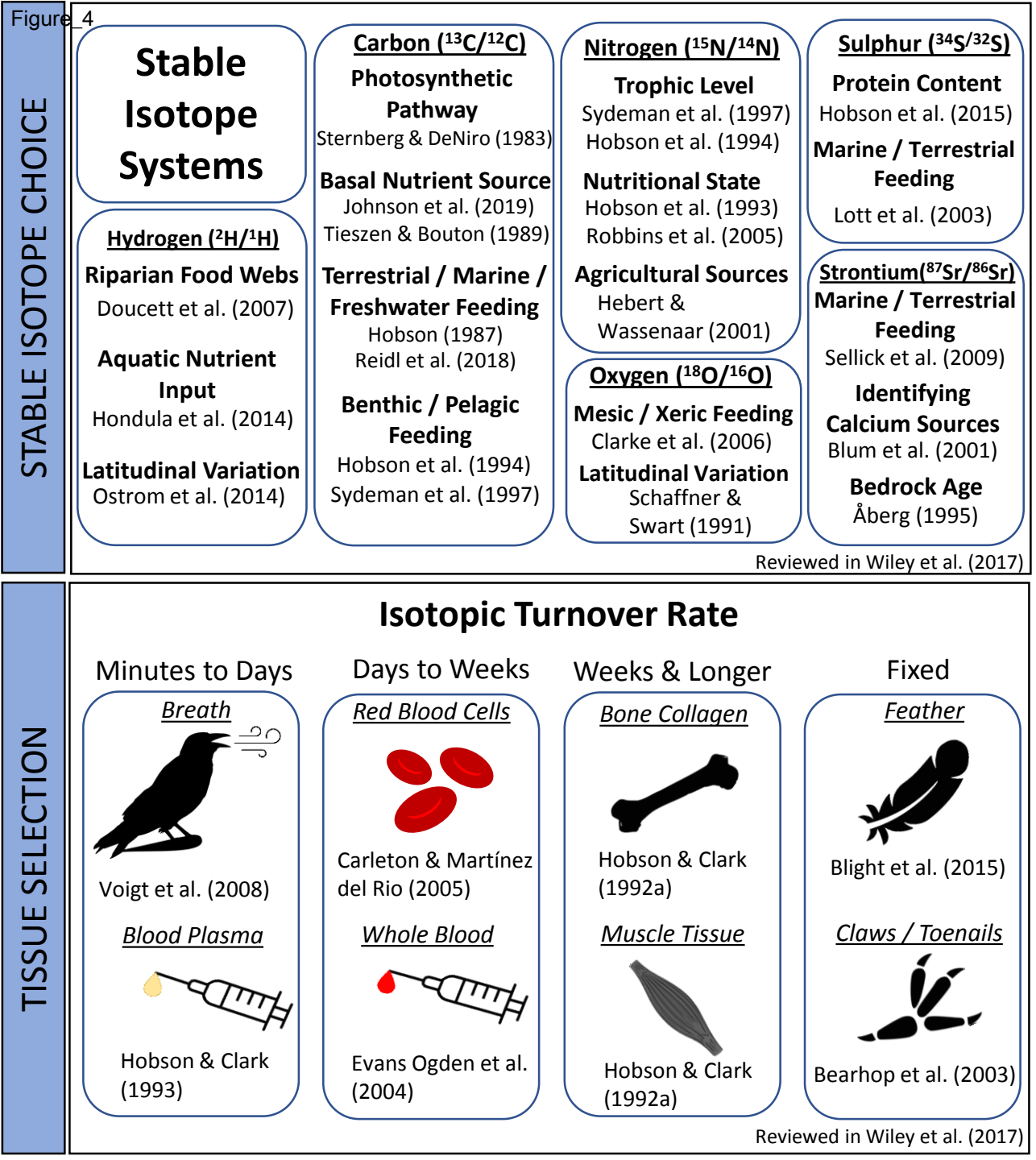

Figure 4: Considerations in Dietary Stable Isotope Analysis: Stable Isotope Choice & Tissue Selection.
A diagram of the common considerations when characterizing prey with isotopic methods, which includes stable isotope choice and tissue selection. Citations are included to provide example studies and to highlight review articles that detail each methodological consideration.

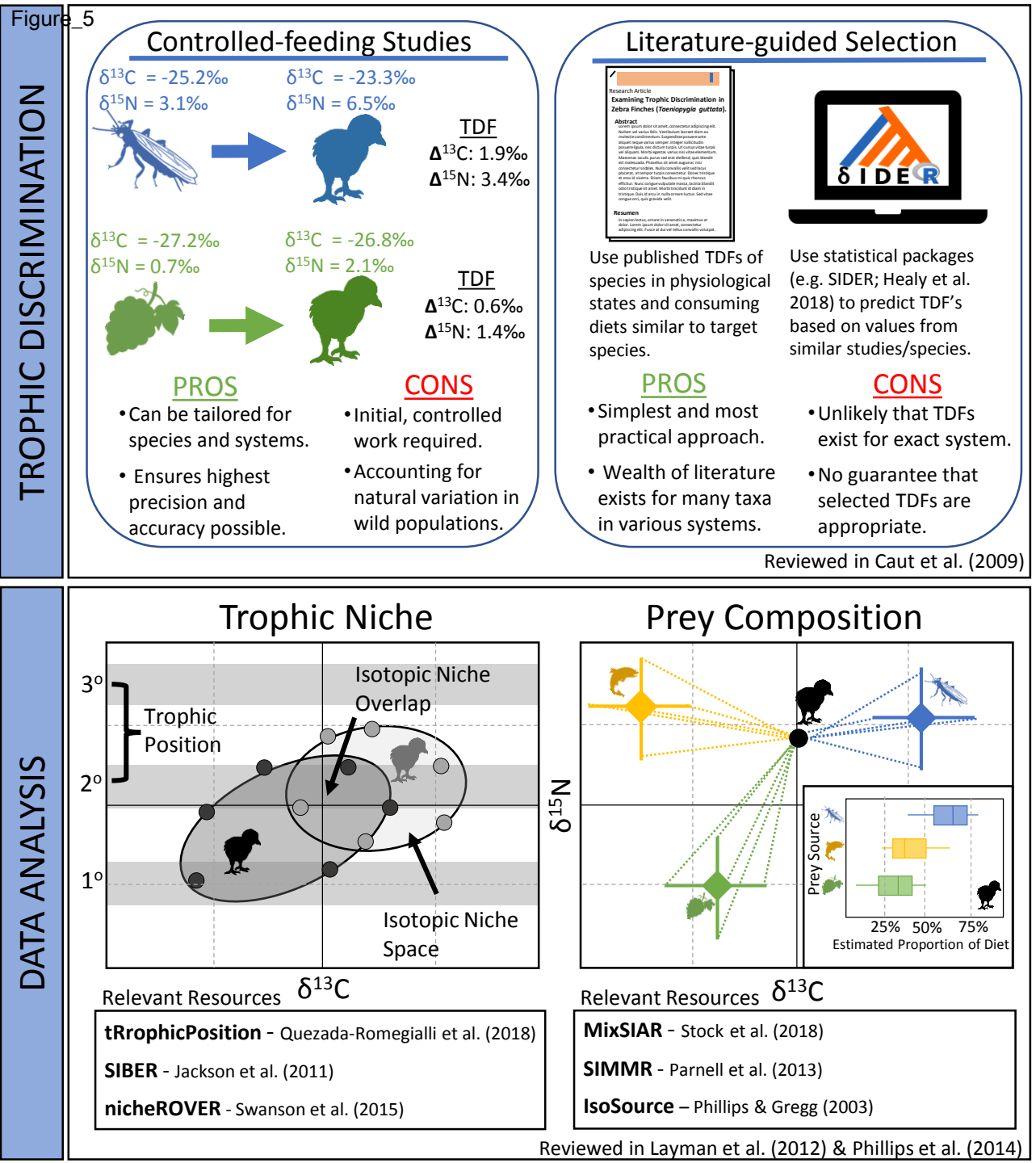

Figure 5: Considerations in Dietary Stable Isotope Analysis: Trophic Discrimination & Data Analysis.

A diagram of the common considerations when characterizing prey with isotopic methods, which includes trophic discrimination and data analysis. Citations are included to provide example studies and to highlight review articles that detail each methodological consideration.