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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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1 **REVIEW ARTICLE**

2 Title: Current Methods and Future Directions in Avian Diet Analysis

3 **ABSTRACT**

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

20

21 **KEY WORDS**

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

24

25 **LAY SUMMARY**

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

38

39 INTRODUCTION

40 Evaluating the composition of avian diets has been a focus of ornithological inquiry for
41 over a century (Slater 1892). Dietary studies have helped to characterize ecological interactions
42 of birds (Burin et al. 2016) and identify prey preference as a driving force behind the evolution
43 of the immense biodiversity across the Class Aves (Kissling et al. 2012, Barnagaud et al. 2014).
44 Diet has long been recognized as a defining life history trait (Eaton 1958), and characterizing the
45 dietary niche is an important step in identifying the roles of species within ecosystems (Elton,
46 1927). A baseline understanding of avian prey preferences has helped researchers to better
47 identify dietary shifts caused by natural (Jaksic 2004) and anthropogenic disturbances (Murray et
48 al. 2018, Trevelline et al. 2018a) as well as the population- (English et al. 2018) and community-
49 wide (Spiller and Dettmers 2019) consequences of these disturbances. Studies of dietary
50 composition also inform our understanding of biotic interactions, such as those stemming from
51 intraspecific competition (McMahon and Marples 2017), interspecific competition (Trevelline et
52 al. 2018b, Sherry and Kent 2021) and trophic cascade events (Mäntylä et al. 2011). Finally,
53 studies of bird diets have been used to highlight the ecological services that birds provide
54 (Whelan et al. 2008). In short, understanding the dietary niche of a species allows researchers to
55 quickly describe important life history traits (Abrahamczyk and Kessler 2015) as well as the
56 complex interactions that birds have with their environments (O'Donnell et al. 2012) and, in
57 turn, provides essential information for the management and conservation of avian species and
58 their habitats (Ontiveros et al. 2005).

59 Early investigation of avian diet relied upon direct methods such as the observation of
60 foraging (Croxall 1976) and provisioning events (Snyder and Wiley 1976) or morphological
61 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

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

87 **MORPHOLOGICAL IDENTIFICATION**

88 **History and Focus**

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

101 **Methodological Considerations**

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

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

115 When samples must be collected, diet items should be analyzed and classified soon after
116 collection to avoid issues caused by tissue degradation. However, if samples cannot be processed
117 immediately, preservation via freezing or storage in high concentration ethanol or formalin
118 enables long-term storage with minimal loss of morphological integrity (Duffy and Jackson
119 1986). For studies using both observational and laboratory-based techniques, storage methods
120 must be compatible as they may influence the chemical make-up of prey tissue (Sarakinis et al.
121 2002) or the ability to retrieve high-quality DNA (Williams et al. 1999) (Figure 1).

122 **Prey classification.** Expertise in prey systematics or the aid of detailed taxonomic keys
123 (Merritt and Cummins 1996, Williams and McEldowney 1990) increases prey classification
124 accuracy (Ralph et al. 1985, Sullins et al. 2018). However, even expert taxonomists are
125 challenged to provide complete and detailed taxonomic classifications (Ralph et al. 1985, Parrish
126 1997), especially if prey remains are difficult to detect in feces or stomach contents (Deagle et al.
127 2007, Thalinger et al. 2017). Fortunately, characteristic hard parts of prey, such as sclerotized
128 arthropod mandibles or wing fragments (Sherry et al. 2016), chitinous beaks of cephalopods
129 (Xavier et al. 2011), bones of vertebrate prey (Dirksen et al. 1995), and seeds from fruits

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

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

138 **Future Potential**

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

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

153 or absolute importance of individual prey taxa (reviewed in Duffy and Jackson 1986). Finally, as
154 morphological identification of prey is minimally destructive, researchers can glean nutritional
155 information on prey (Grémillet et al. 2004) as well as digestion-related information (Barton and
156 Houston 1993) from bolus (Boyle et al. 2014), pellet (Wallick and Barrett 1976), lavage (Cherel
157 and Ridoux 1992), or fecal samples (Varennnes et al. 2015), to assess gross energy content
158 (Karasov 1990), the caloric value of different prey sizes (Stephens and Barnard 1981) or species
159 (Guillemette et al. 1992), as well as concentrations of prey-derived macronutrients (Albano et al.
160 2011).

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

172 **DNA-BASED METHODS**

173 **History and Focus**

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

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

184 **Methodological Considerations**

185 **Sample collection, storage, and processing.** Most DNA-based avian dietary analyses
186 are performed on fecal samples (Ando et al. 2020), which can be collected directly from birds
187 (Trevelline et al. 2018b, Jarrett et al. 2020), from holding bags (paper: Trevelline et al. 2016,
188 Southwell 2018; or cloth: Karp et al. 2013), or even from the environment, although the risk of
189 sample contamination is greater (Oehm et al. 2011, Gerwing et al. 2016, McClenaghan et al.
190 2019). Similar to fecal samples, boluses are a minimally invasive source of dietary DNA. Other
191 sample types have been used for genomic diet analyses, but these techniques are more invasive
192 (i.e. lavage, induced regurgitation) or otherwise hold no obvious advantage over fecal samples
193 (cloacal or mouth swabs, Vo and Jedlicka 2014; stomach samples, Snider et al. 2021). Though
194 not frequently used, stomach samples in natural history collections hold great potential for
195 molecular diet analyses (Remsen et al. 1993). However, this approach may not always be
196 suitable because many historic samples are stored in formalin, a chemical that crosslinks DNA
197 and complicates downstream amplification and sequencing techniques. Freezing samples upon
198 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

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

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

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

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

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

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

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

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

290 **Quality control.** The degree of biological and technical replication necessary for
291 maximizing detectability of diet-derived sequences must be balanced with minimizing false
292 positives caused by contamination or sequencing errors (Taberlet et al 2018). The use of positive
293 and negative controls during sample collection and DNA extraction, amplification, and
294 sequencing processes can guide how reads are filtered during the sequence analysis stage
295 (reviewed in Zinger et al. 2019) (Figure 2). Additionally, technical replicates, in the form of
296 multiple PCR reactions for each DNA extract, can minimize false negatives in DNA
297 metabarcoding data, especially for diet items with low detection probabilities (Ficetola et al.
298 2015) or poor DNA amplification efficiency (Jusino et al. 2019).

299 **Data Analysis**

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

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

313 a measure of distance in niche space between two species, PCA is generally preferable to
314 NMDS, t-SNE (Maaten and Hinton 2008) or UMAP (McInnes et al. 2018) because these
315 methods do not preserve distances in multivariate space. Following data ordination, hypothesis
316 testing can be implemented. For example, criteria can be developed to identify groups in
317 multivariate space and test whether these accord with the bird species or groups in question (e.g.,
318 k-means clustering, Forgy 1965), they might derive multivariate hypervolumes (Blonder et al.
319 2014), and implement a randomization, null-model approach, or describe the qualitative
320 differences in multivariate niche space among species or other groups.

321 **Future Potential**

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

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

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

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

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

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

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

377 DNA-based dietary studies have mostly focused on the description of prey taxa and the
378 ecosystem services of avian predators (e.g. Crisol-Martinez et al. 2016); however, we can also
379 leverage DNA-based methods to examine diet overlap of sympatric species (Trevelline et al.
380 2018b), and thus address theoretical questions related to competition and resource partitioning
381 (e.g., Spence et al. 2021; Stillman et al. 2021). There is also considerable scope to examine

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

395 **STABLE ISOTOPE ANALYSIS**

396 **History and Focus**

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

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

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

421 **Methodological Considerations**

422 **Sample collection, storage, and processing.** Because stable isotopes are incorporated
423 during tissue synthesis, any tissue that can be retrieved from a bird can be used for stable isotope
424 analysis; though, selection of tissue will depend on the focus and timescale of the research
425 question (Figure 4). To assess dietary isotopic endpoints, researchers should be sure to analyze
426 the tissues of the main dietary items that birds consume, such as fruits (Vitz and Rodewald
427 2012), prey muscle tissue (Anderson et al. 2009), or even the entire body (Herrera et al. 2003) to

428 ensure that the isotopic sources are representative of the prey pool contributing to the nutrition of
429 the consumer. For all tissues, freezing is the preferred preservation method (Bond and Jones
430 2009) followed by air drying with a smokeless heat source (Bugoni et al. 2008), or storage in
431 70% ethanol (Hobson et al. 1997). Preservation media, such as formalin, genetic buffer solutions
432 (Hobson et al. 1997), or high percentage ethanol (Bugoni et al. 2008) can replace isotopes within
433 dietary or avian tissues with their own, which can be particularly problematic for carbon,
434 nitrogen, and hydrogen stable isotope analyses. For lipid-rich tissues, chemical lipid-extraction
435 may be needed prior to analysis (Bond and Jones 2009) to facilitate accurate diet reconstruction
436 (Kojadinovic et al. 2008). Similarly, diets or avian tissues rich in carbonates often require
437 acidification prior to analysis to obtain the unbiased $\delta^{13}\text{C}$ values of the organic matrix (Polito et
438 al. 2009, Mackenzie et al. 2015). However, chemical lipid-extraction and acidification have the
439 potential to bias tissue $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values (Jaschinski et al. 2008, Elliott and Elliott 2016). As
440 such, mathematical normalization for tissue lipid and/or carbonate content represents an
441 alternative method when chemical lipid-extraction or sample acidification is not feasible or
442 advisable (Post et al. 2007, Jaschinski et al. 2008, Ooppel et al. 2010).

443 **Isotope systems.** The most common elements used in isotopic dietary studies are those of
444 carbon ($^{13}\text{C}/^{12}\text{C}$; $\delta^{13}\text{C}$) and nitrogen ($^{15}\text{N}/^{14}\text{N}$; $\delta^{15}\text{N}$), which typically provide information on
445 source of feeding and trophic position, respectively (Figure 4). Stable isotopes of hydrogen
446 ($^2\text{H}/^1\text{H}$; $\delta^2\text{H}$) and oxygen ($^{18}\text{O}/^{16}\text{O}$; $\delta^{18}\text{O}$) are tightly linked to the hydrological cycle and ambient
447 temperature, and have also been used to identify nutrient inputs from terrestrial and aquatic
448 origins (Figure 4). Sulfur ($^{34}\text{S}/^{32}\text{S}$; $\delta^{34}\text{S}$) isotope ratios have been used to identify nutrients
449 derived from marine vs. terrestrial sources, proximity to coastlines, benthic vs. pelagic energy
450 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 ($^{87}\text{Sr}/^{86}\text{Sr}$; $\delta^{87}\text{Sr}$), 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 $\delta^{15}\text{N}$ 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 $\delta^{15}\text{N}$ values with each trophic transfer (Macko et al. 1986), and this has allowed
465 researchers to use tissue $\delta^{15}\text{N}$ values to estimate consumer trophic position (DeNiro and Epstein
466 1981, Hobson and Welch 1992). Trophic discrimination factors (TDFs) based on $\delta^{15}\text{N}$ values, or
467 the differences in $\delta^{15}\text{N}$ 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).

473 For $\delta^{13}\text{C}$ values, it is generally assumed that TDFs are relatively low with average values
474 centering around +0.4‰ (Post 2002). However, TDFs can vary by avian tissue type even when
475 synthesized under the same diet due to differences in biochemical processes and macromolecule
476 routing, which is especially apparent among lipid-rich and keratin-based tissues that may require
477 correction factors prior to analysis (Hobson and Clark 1992b, Cherel et al. 2014b). Stable sulfur
478 isotope measurements ($\delta^{34}\text{S}$) appear to also have low TDF values ($\sim 0.0\text{‰}$ to $+1\text{‰}$) and so can be
479 more readily linked to food web source inputs (Richards et al. 2003, Arneson and MacAvoy
480 2005, Florin et al. 2011). Even so, $\delta^{34}\text{S}$ TDFs can vary due to the input of endogenous sulfur
481 from the recycling of body proteins when individuals consume low-protein diets (Richards et al.
482 2003). Little is currently known about TDFs associated with $\delta^2\text{H}$ values and whether or not
483 patterns of trophic enrichment are due to isotopic discrimination or ambient exchange (reviewed
484 in Vander Zanden et al. 2016).

485 **Isotopic turnover.** The residency time of elements in animal tissues varies
486 approximately by the metabolic rate of that tissue (Figure 4). This means that metabolically
487 active tissues will assimilate isotopic information on diet over different timescales, and thus
488 present an opportunity to choose a tissue most appropriate for the dietary integration period of
489 interest (Hobson 1993, reviewed by Thomas and Crowther 2015, Carter et al. 2019a).
490 Researchers have performed stable isotope analysis on various avian tissues to understand an
491 individual bird's diet composition at scales ranging from hours (breath and plasma; Hatch et al.
492 2002; Podlesak et al. 2005; Pearson et al. 2003), days and weeks, (red blood cells; Podlesak et al.
493 2005; Hobson and Clark 1993), to months (feathers and claws; Hedd and Montevecchi 2006;
494 Bearhop et al. 2003) or even years (bone collagen; Stenhouse et al. 1979; Hobson and Clark
495 1992a; Hobson and Sealy 1991; Hedges et al. 2007). Indeed, it is possible to estimate year-round

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

504 Isotopic turnover rates can also differ due to diet composition (Hobson and Clark 1993,
505 Podlesak et al. 2005), tissue type (Vander Zanden et al. 2015), an individual’s physiological state
506 (Carleton and Martínez del Rio 2005, Cherel et al. 2005), and energy expenditure. For instance,
507 in proteinaceous tissues, structural turnover is the main driver of isotopic turnover (Carter et al.
508 2019a), but in lipids, it appears to be influenced by energy expenditure (Foglia et al. 1994, Carter
509 et al. 2018). Though there is now a greater understanding of isotopic turnover both among
510 individuals and tissue types, uncertainty remains for less-studied systems (Carter et al. 2019a). In
511 addition, drivers of tissue-specific and macromolecule-specific turnover rates as well as the
512 development of mechanistic models of isotopic turnover that can be applied across a broad
513 diversity of taxa are needed (Carter et al. 2019a, Caut et al. 2009). The derivation of allometric
514 relationships driving isotopic turnover rates will assist research on birds that differ in body mass
515 (Carleton and Martinez del Rio 2005, Carter et al. 2019a).

516 **Macromolecule routing.** While isotope-based dietary reconstruction is founded on the
517 notion that “animals are what they eat plus a few parts per mil” (DeNiro and Epstein 1976), the
518 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

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

549 **Data Analysis**

550 **Mixing models, trophic position, and isotopic niche analyses.** Isotopic values of a
551 consumer's tissue are a mixture of the isotopes derived from their prey, thus stable isotope
552 mixing models can be used to determine the relative contributions of each prey taxon (Phillips
553 2012) (Figure 5). To accurately quantify prey composition, researchers must not only know the
554 potential prey groups that birds eat, but also the isotopic values of each potential prey group,
555 ensuring that the isotopic values of each group are distinct. If unique prey sources are not
556 isotopically distinct, but belong to a shared functional group, researchers should consider
557 combining these sources in downstream analyses (Phillips et al. 2005). While all mixing models
558 work under the principle that a consumer's isotopic ratio is proportional to that of its assimilated
559 prey, earlier iterations of these models have been improved by including the elemental
560 concentrations of prey sources (Phillips and Koch 2002), considering isotopic routing (Martínez
561 del Rio and Wolf 2005), and working within a Bayesian framework to allow for better
562 propagation of uncertainty and use of informative priors (Parnell et al. 2013). Mixing models can
563 be applied to both bulk tissue stable isotope analysis and CSIA data to reconstruct avian diets
564 (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 $\delta^{15}\text{N}$, 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 $\delta^{15}\text{N}$
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 $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ 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), $\delta^{13}\text{C}$ 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.

610 2019b), and novel applications of $\delta^{13}\text{C}$ CSIA-FA promise to broaden our understanding of avian
611 food webs and address the limitations of previous applications (Twining et al. 2020).

612 **ALTERNATIVE DIETARY BIOMOLECULE TRACING**

613 While DNA-based and stable isotope techniques are applicable to most study systems,
614 researchers also trace other biomolecules through food webs to address more specialized
615 questions in avian dietary ecology. Useful dietary tracers include essential biomolecules that are
616 not synthesized by birds (e.g., essential lipids, amino acids, vitamins; Ruess and Müller-Navarra
617 2019), biomolecules that undergo little or no metabolic change post-consumption (e.g., long-
618 chain polyunsaturated fatty acids; Twining et al. 2016), and non-nutritional components
619 indicative of environmental contamination (e.g., lead, mercury). Because alternative dietary
620 tracers are often specific to certain environments, studies typically couple one of the previously
621 described techniques with these tracers to draw ecological inferences about the effect of diet
622 variation; though, continued development of mixture modeling approaches (e.g., quantitative
623 fatty acid analysis [QFASA]; Iverson et al. 2004) and the identification of additional dietary
624 tracers in new habitats (Hixson et al. 2015) will allow for broader application of biomolecule
625 tracing in diet reconstruction. Analytical methods for individual dietary tracers is beyond the
626 scope of this review, but has been discussed by others (Williams and Buck 2010, Nielsen et al.
627 2017, Majdi et al. 2018). Here, we focus on analyses employing multiple techniques to address
628 objectives beyond diet identification.

629 **Nutritional Components: A Healthy Diet**

630 In addition to meeting energy demands and broad macronutrient requirements, birds must
631 obtain essential biomolecules from diet to maintain optimal health and productivity (Klasing
632 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.

656 **Non-nutritional Components: A Contaminated Diet**

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

675 **Future Potential**

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

679 and $\delta^{15}\text{N}$ 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 $\delta^{34}\text{S}$ 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 $\delta^{13}\text{C}$ explain the role of a terrestrially-sourced diet on Peregrine Falcon (*Falco*
685 *peregrinus*) contaminant exposure in urban environments (Ferne 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

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

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

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

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

740 Related to the third approach, a fourth approach combines various analytical approaches
741 into a multidimensional dietary niche space (Swanson et al. 2015). Though studies frequently
742 derive values from the same type of assay (e.g., stable isotope analysis), axes can theoretically
743 include other metrics such as trace element concentrations or fatty acid concentrations. As
744 indicated above, incorporating different metrics requires that the various axes be expressed in
745 quantities that are unitless (typically expressed as mean values divided by the standard
746 deviation). Analytically, this approach has many advantages, though the main drawback is that it
747 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

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

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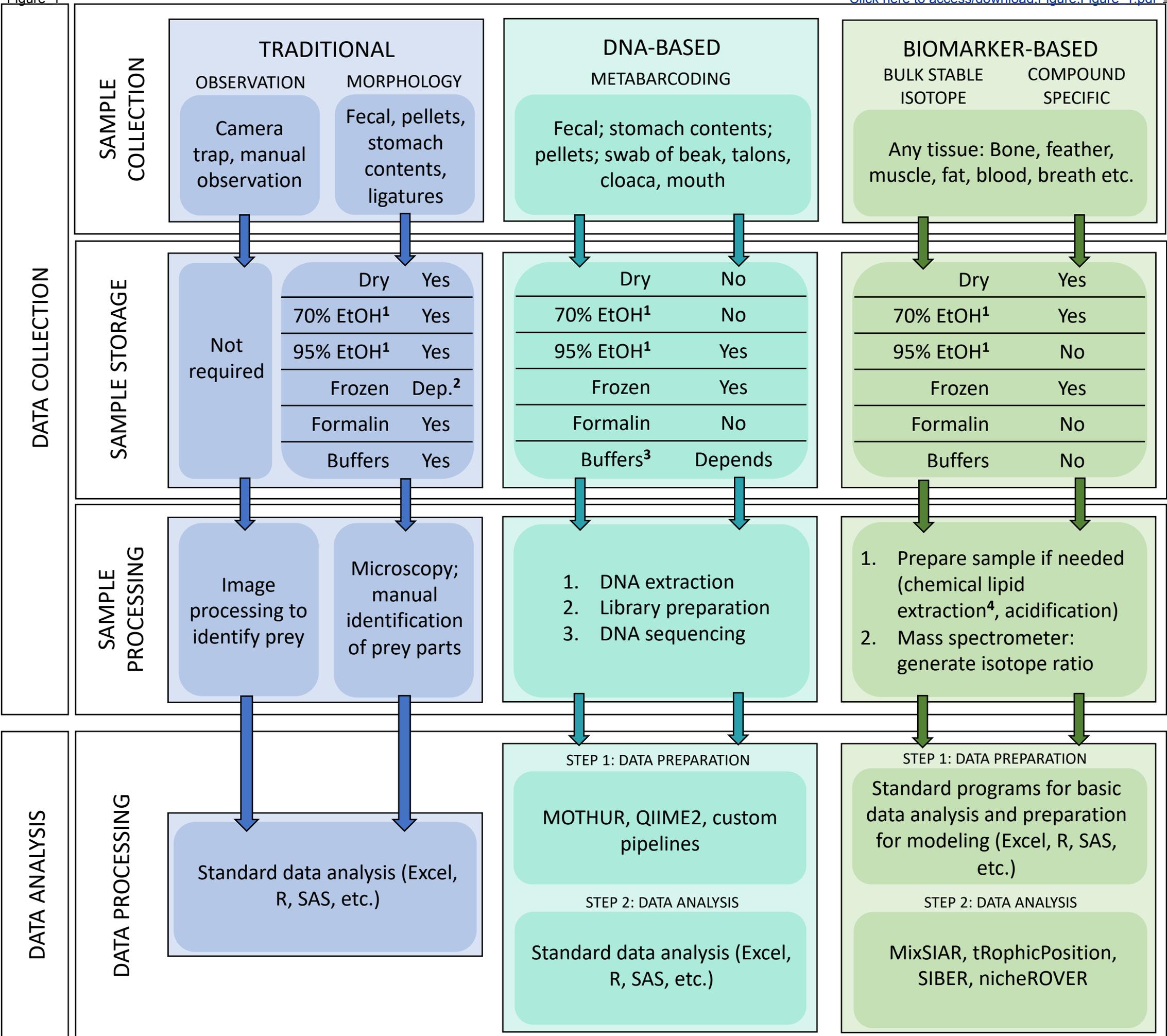


Figure 1: An outline of the general workflow from sample collection through data analysis for the most common methods used in avian diet studies.

¹EtOH 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.

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

³Longmire 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.).

⁴Lipids 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 $\delta^{13}\text{C}$ and $\delta^2\text{H}$ analyses (Soto et al. 2013).

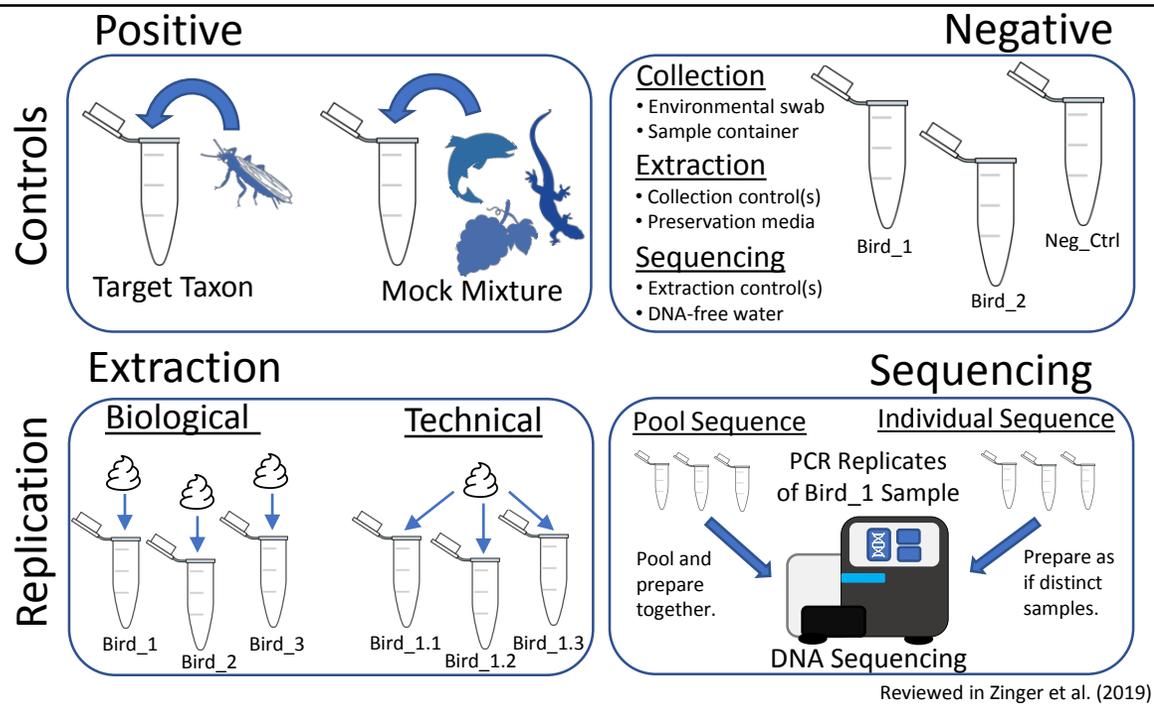
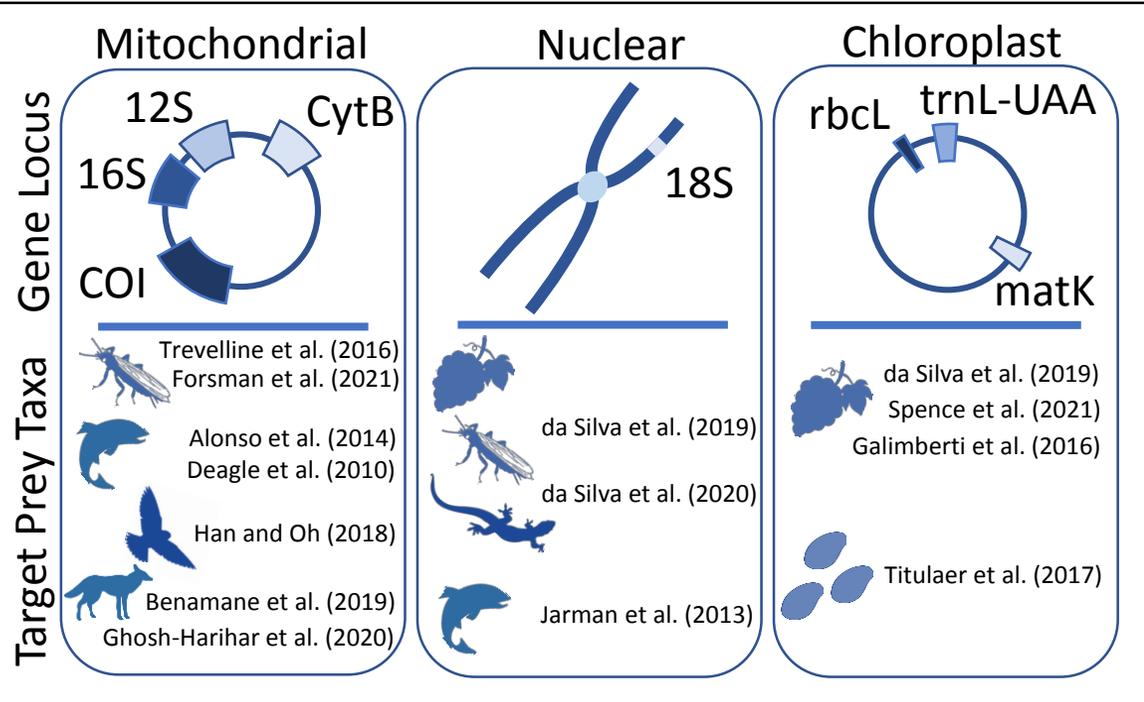
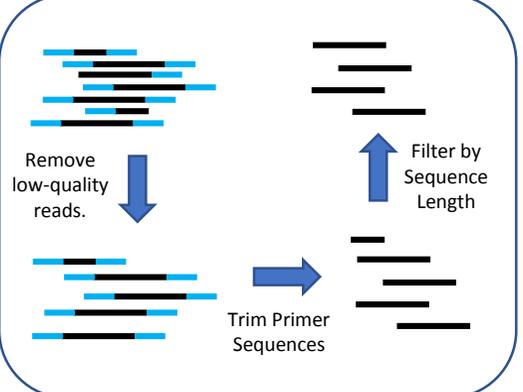


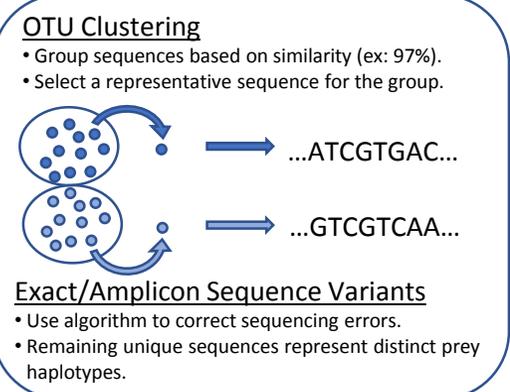
Figure 2: Considerations in DNA-based Prey Identification: Barcoding Marker Choice & Quality Control.

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.

Read Trimming



Read Collapsing



Classify Taxa

Database Choice

GenBank/BOLD	Custom
<ul style="list-style-type: none"> Highly curated Taxonomic coverage May contain errors Geographical variation 	<ul style="list-style-type: none"> Only local haplotypes Can ensure accuracy Intensive work required Difficult for generalists

Assignment Method

Score-based	Classifier-based
<ul style="list-style-type: none"> Uses query coverage & percent identity to find best match. Uses step-wise criteria (Trevelline et al. 2016). 	<ul style="list-style-type: none"> Train a taxonomic classifier using reference sequences (Forsman et al. 2021). Can adjust how classifier behaves for best assignment.

Reviewed in Alberdi et al. (2018) & O'Rourke et al. (2020)

Quantitative & Qualitative

Presence/Absence

1 = Presence 0 = Absence

Sample	Prey_1	Prey_2	Prey_3
Bird_1	1	0	1
Bird_2	0	0	1
Bird_3	1	1	1

- Common in diet studies
- Conservative estimate
- Less affected by DNA recovery bias
- Overestimates small/rare taxa
- Sensitive to contaminant DNA

Read Abundance

Sample	Prey_1	Prey_2	Prey_3
Bird_1	289	0	445
Bird_2	0	0	800
Bird_3	9001	2789	27

- Can reflect prey biomass
- Promising when used with mock mixtures
- Only tested in simple diet samples (~2-6 prey taxa)
- Sensitive to DNA recovery and amplification bias

Frequency of Occurrence

- Number of diets in which a taxon is present divided by the total number of diet samples.

The diagram shows three types of prey: a bird, a fly, and a fish. For each, the number of samples it was found in is shown as a fraction of the total samples (3). The bird was found in 2 out of 3 samples (67%), the fly in all 3 (100%), and the fish in 1 out of 3 (33%).

Specialized Analyses

NMDS1
Ordination to assess dietary dissimilarity among avian groups.

No. of Aquatic Taxa
Prey trait-based analyses as opposed to taxonomic-based.

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.

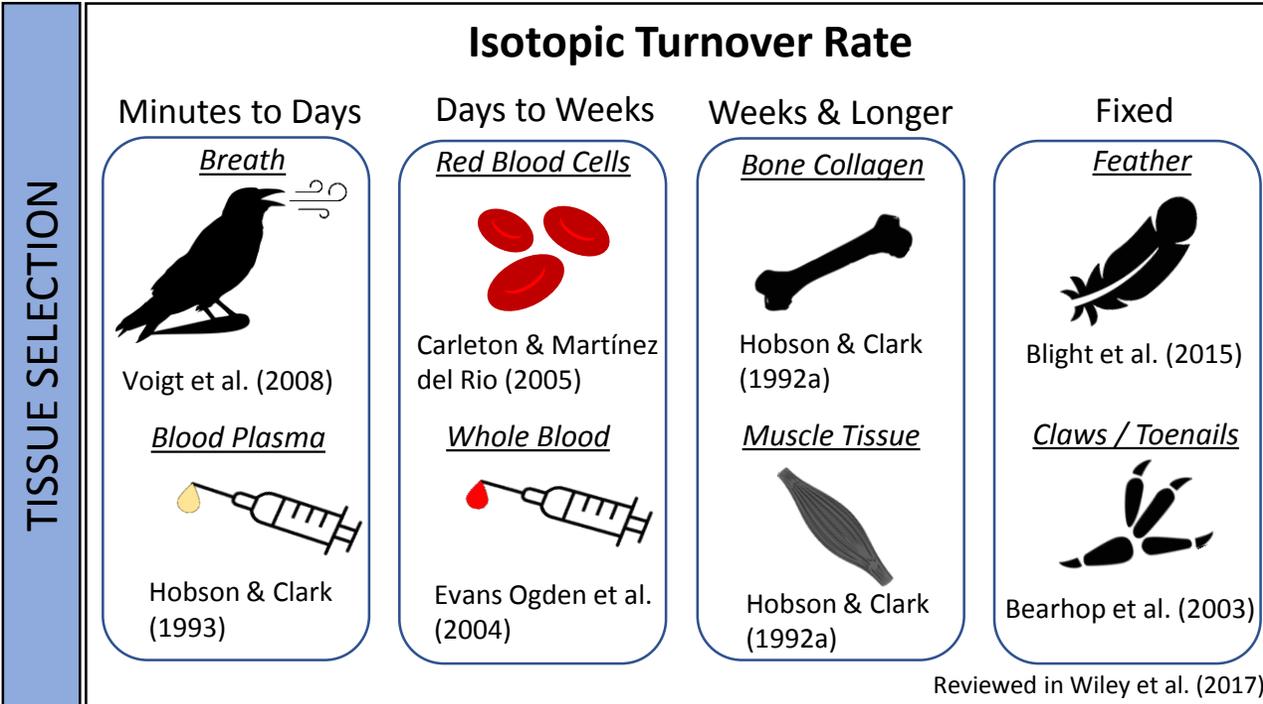
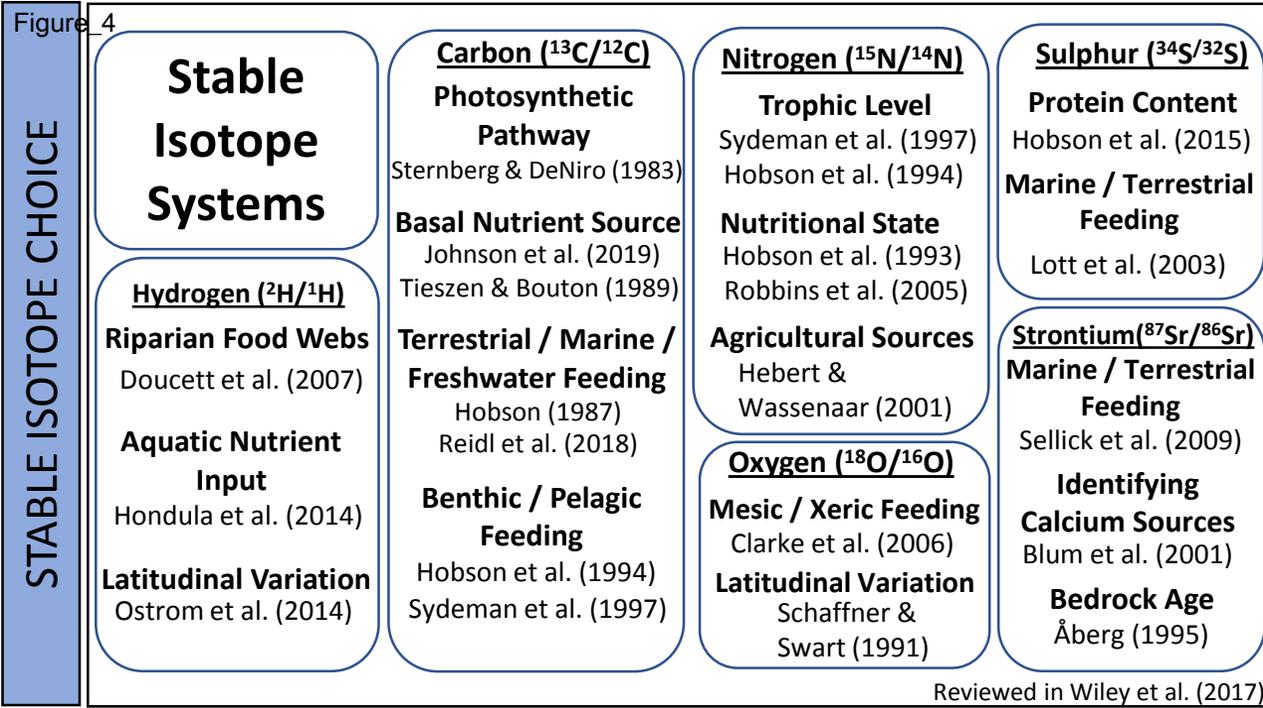


Figure 4: Considerations in Dietary Stable Isotope Analysis: Stable Isotope Choice & Tissue Selection.

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.

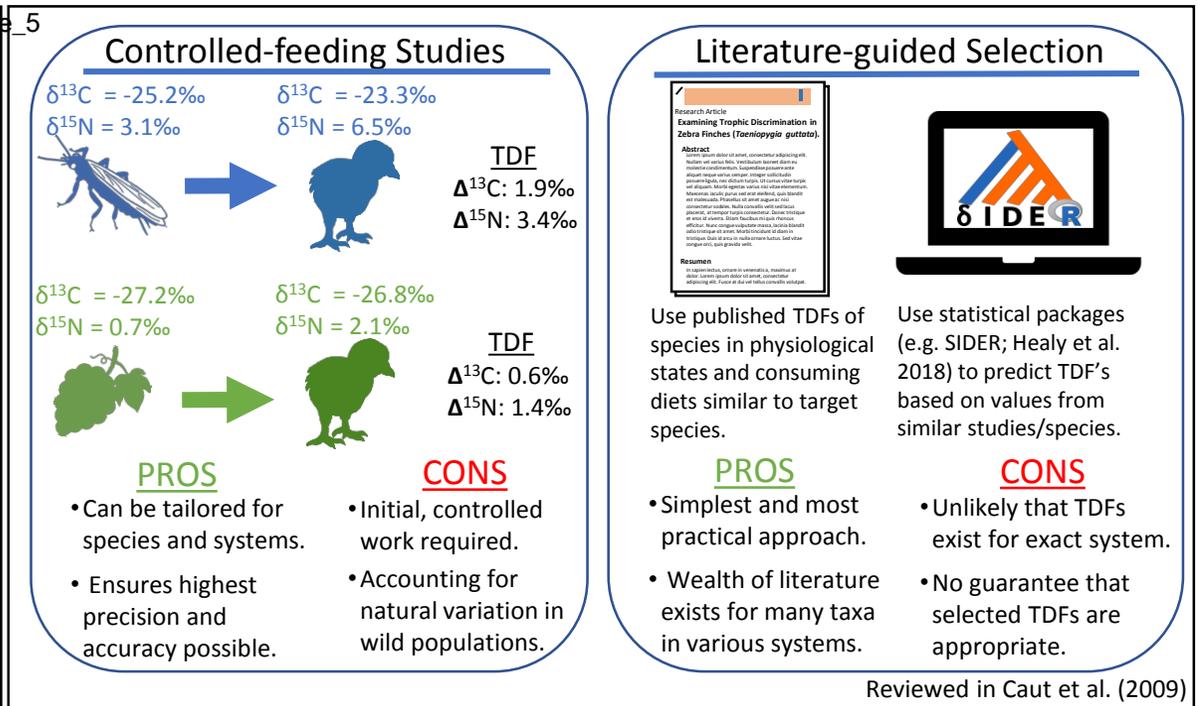


Figure 5: Considerations in Dietary Stable Isotope Analysis: Trophic Discrimination & Data Analysis.
 A diagram of the common considerations when characterizing prey with isotopic methods, which includes trophic discrimination and data analysis. Citations are included to provide example studies and to highlight review articles that detail each methodological consideration.

