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Advancing Methods of Diet Analysis: A Case Study Using Degraded Merlin (Falco columbarius) Prey Remains

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Abstract

Prey remains have long been used as a mechanism to approach diet analyses. As understanding diet is key to comprehending ecosystem dynamics, prey remains identification requires a unique methodological approach to determine diversity within a sample. With the advancement of technology, molecular protocols designed for species-specific identification have improved to incredible accuracy and precision. Yet, the visual identification method has remained a predominant technique within diet studies. With entry-level observers, we matched visual identifications with molecular-based methods to quantify the accuracy of the visual identification method. This study determined what fraction of visually identified prey remains could be correctly identified to a high degree of certainty. Using the mitochondrial DNA of > 40-year-old Merlin (*Falco columbarius*) feather samples, we found that the correct identification of visually identified "high" certainty samples was 41.7%. Furthermore, visually identified samples with a "medium to low" certainty plummeted to 19.0%. This study reveals that correct identification of visually identified samples is significantly lower than previously considered but that certainty level has a significant role in correct identification. Similarly, visual identification can provide rapid determination of separate taxa and the number of species in a sample. It is critical to assess prey remains using multiple techniques to procure definitive identification of

individual prey items. Anecdotally, I found that the primers AWF2-R4 and AWF4-R6 targeting regions within the cytochrome c oxidase subunit-1 gene are effective for degraded (i.e. > 40 years old) feather samples of Passeriformes and Charadriiformes.

Introduction

Diet analyses reveal a plethora of information about an ecosystem, including ecological niche, species relatedness, hierarchy, and even the biotic and abiotic factors influencing a species (Romàn-Palacios et al. 2019). As prey remains have been used as a key source of data for diet analyses, proper identification of remains is critical to understanding species-specific questions and ecosystem queries (Speller et al. 2011, Sheppard & Harwood 2005). Furthermore, identifying prey items to the species level is fundamental to understanding a predator's diet as it provides a more comprehensive understanding of predator-prey dynamics and its role within the ecosystem. While using prey remains for dietary studies is not new, identification techniques have begun to incorporate new technological advancements (Redpath et al. 2001, Sheppard & Harwood 2005, Hoenig et al. 2022). Genetic technology is just one advancement that has sparked questions about the efficacy of the visual identification method (Speller et al. 2011, Monterosso et al. 2019, Hoenig et al. 2022).

Prey remains are defined as feathers, fur, pellets or bones, left after an animal has killed and consumed a prey species (Lewis et al. 2004, Monterosso et al. 2019). Occurring throughout the animal kingdom, prey remains are relatively easy to collect and store (Redpath et al. 2001, Monterosso et al. 2019). For raptors, prey remains are located in, near or around nest sites after a kill (Lewis et al. 2004). These remains can be collected, dried, and identified to more effectively understand a raptor's dietary needs and preferences. For merlins (*Falco columbarius*) specifically, these falcons have been understood to consume small passerines or shorebirds

(Laing 1985, Wilbor 1996). As passerine and shorebird prey remains look very similar in composition when consumed by a merlin, this leads to questions about visual identification accuracy, or the percentage of correctly identified prey species.

Historically, visual identification is the most accessible method for identifying prey remains (Wilbor 1996, Symondson 2002, Hoenig et al. 2022). Comparisons of visual identification accuracy with molecular methods have been observed in species-specific identification of mammals (i.e. Monterosso et al. 2019) but have not been thoroughly investigated in bird species. When identifying prey of merlins, visual identification consists of singling out primary, tail, and body feathers along with other readily identifiable components (e.g., feet, heads, etc.) to confirm species identification (Lewis et al. 2004). If identifiable components are unavailable, correct identification is difficult and time-consuming (Lewis et al. 2004, Pompanon et al. 2012). Hoenig et al. (2022) provide a comprehensive review describing procedural aspects, including strengths and limitations, of several prey remains identification methods, such as morphological-based methods (i.e. visual identification), DNA-based methods (i.e. molecular identification), stable isotope analysis, and alternative dietary biomolecule training. While Hoenig et al. (2022) is one of the most thorough reviews of avian prey remains methodologies to date, quantifying the visual identification technique for bird species remains a knowledge gap.

In contrast, many studies have been conducted to describe the efficacy, or the cost-benefit of method productivity, for DNA-based methods (Symondson 2002, Speller et al. 2011). These protocols can correctly identify prey items with a small sample even when degraded (Speller et al. 2011, Hoenig et al. 2022). Studies suggest that DNA-based methods have an accuracy percentage rate of molecular identification from 91.0 - 99.9% (Speller et al. 2011, Lijtmaer et al.

2012, Hacker et al. 2021). Hacker et al. (2021) found a 91.0% accuracy rate using degraded raptor pellets. Using 2-5 feather barbs with degraded samples, Speller et al. (2011) found a 98.0% accuracy. A cell lysis extraction, polymerase chain reaction (PCR) amplification, followed by DNA sequencing can produce a 98.0-100% match with certified specimen vouchers within barcoding databases such as GenBank or Geneious Prime (Symondson 2002, Ratnasingham and Herbert 2007, Speller et al. 2011, Hoenig et al. 2022). Molecular identification has proven to be an incredible tool for species-specific identification.

Using entry-level observers, my study aimed to determine how an observer's chosen level of certainty, or identification confidence, affects the accuracy rate of the visual identification method. I first recruited observers with an interest in birds and provided them with reasonable access to identification tools including field guides, photographs of feather spreads, museum specimens, and past dietary studies of merlins in Denali (Laing 1985, Wilbor 1996). Observers were given envelopes containing prey remains with the exclusion of pellets. They then marked their determined species identification, personal level of certainty (e.g., self-assessed confidence level) and relevant sample data. The visually identified contents were then split into observer designated envelopes for molecular testing. Throughout this process, I documented the significance of an observer's level of certainty in prey remains identification. In addition, this study shed light on the necessity of using multiple forms of identification techniques when attempting to identify prey remains to species.

Methods

From 1983-1987, prey remains were collected near merlin nesting sites in Denali National Park. Prey remains were initially dried, then stored in a cool location enclosed in paper envelopes. Of the ~ 800 paper envelopes available, 59 were selected randomly for this study.

Visual Identification - Observers with entry level bird identification experience examined prey remains to species using field guides, specimens from the University of Montana – Philip L. Wright Zoological Museum, past merlin diet surveys conducted by Laing (1985) and Wilbor (1996), and photographs from the U.S. Fish and Wildlife Feather Atlas database. Each observer visually observed prey remains for two hours a week for four weeks (a total of 8 hours per observer). With each envelope, observers recorded the bag ID, territory, date of collection, number of adult and juvenile merlins associated with the nest, contents of the envelope (e.g. head, feet, feathers etc.), number of individual birds within a bag (later referred to as item number), name of the identified prey species, and the degree of certainty as either low, medium, or high, defined as their self-assessment of correct identification of prey species. Low and medium certainty represented when the observer was not confident about species identification; high certainty represented when the observer was highly confident in identifying the prey item. Each observer then removed two feathers, flight feathers (i.e. primary, tail) when available, in which they identified the species and sealed the two feathers into observer-designated envelopes (ODE) used for molecular identification.

Molecular Identification - DNA was extracted and amplified at the University of Montana Conservation Genomics Laboratory. For each genetic sample, one flight feather from the individual ODE were cut above the base of the feather calamus, sliced in half and placed into a detergent-based cell lysis buffer (0.1mM NaEDTA, 0.1M Tris base, 1% SDS) with 0.2mg/ml proteinase K, and 67mM dithiothreitol. These samples were left in a shaking heater at 55°C overnight until dissolved. Protein was precipitated with Puregene Protein Precipitation Solution (Qiagen Inc.). Subsequently, DNA was cleaned with isopropyl alcohol. Dried DNA was then re-suspended in low EDTA Tris buffer. After extraction, DNA was measured for concentration and

purity using Thermo-Scientific NanoDrop Spectrophotometer. Due to the age and condition of the feather samples, primer pairs were tested from the cytochrome c oxidase subunit 1 (COI) and NADH dehydrogenase 2 (ND2) genes of mtDNA, until samples were consistently successful in sequencing (see Table 1). Species identification was determined by a 99.6% or higher rate of

Table 1. The primers utilized for PCR amplification and sequencing. Column 1 describes individual primer pairs and the gene associated with each (i.e. COI or ND2). The sequence describes the specific base pairs within each forward and reverse primer from 5' to 3'. The amplicon size describes the number of base pairs the primer can successfully sequence. The PCR column is the number of samples that underwent the PCR process using the associated primer. Success is the number of samples that produced a sequence match of 99.6% or higher to a species within the Geneious database. References show where primers in the literature. Bp = base pairs. ~ = viable primer.

Primer	Sequence	Amplicon Size (bp)	PCR	Success	Reference
COI Aves-16S-1AF Aves-16S-1AR	CATAAGACGAGAAGACCC TGTGGA TCCAAGGTCGCCCCAACC GAA	125	1	0	Dalén et al 2017
COI Aves-16S-2AF Aves-16S-2AR	CCTTGGAGAAAAACAAAN CCTCCAAA TCCCTGGGGTAGCTTGGTC CAT	120	1	0	Dalén et al 2017
COI Bird F1_COI BirdR1_COI	TTCTCCAACCACAAAGAC ATTGGCAC ACGTGGGAGATAATTCCA AATCCTG	648	9	3	Lijtmaer et al 2017
COI ~AWCintF4 AWCintR6	TCCTCAATCCTGGGAGCA ATCAACTT GGATTAGGATGTAGACTT CTGGGTG	277	5	5	Lijtmaer et al 2017
COI AWCintF1 AWCintR2	CGCYTWAACAYTCYGCCA TCTTACC ATGTTGTTTATGAGTGGGA ATGCTATG	328	3	1	Lijtmaer et al 2017
COI ~AWCintF2 AWCintR4	ATAATCGGAGGCTTCGGA AACTGA TGGGAKAGGGCTGGTGGT TTTATGTT	314	33	31	Lijtmaer et al 2017
ND2 L5215 H5578	TATCGGGCCCATACCCCG AAAAT CCTTGAAGCACTTCTGGGA ATCAGA	351	2	1	Bates et al 1999

base pairs from sequencing to a match in the Geneious database (0.4% mismatch was only accepted due to degraded DNA, not base pair differentiation). Most ODE feather samples were successfully amplified with primer pair AWF2-R4 from the COI gene of mtDNA (See Table 1). If amplification was unsuccessful with AWF2-R4, the AWF4-R6 pair was utilized. Due to low amplicon size these primer pairs were more successful as there were fewer opportunities for breakage within the DNA strand. All PCR reactions were run according to Kerr et al. (2007): thermal cycle program beginning with 5 minutes at 95°C, followed by six cycles of 30 seconds at 95°C, 90 seconds at 45°C, and 90 seconds at 72°C, then thirty-five cycles of 30 seconds at 95°C, 90 seconds at 55°C, and 90 seconds at 72°C. Amplification finished with ten minutes at 68°C. PCR fragments were purified with Ampure XP beads (Beckman-Coulter). Samples were Sanger sequenced at the University of Montana Genomics Core Laboratory on an ABI 3130xl genetic analyzer. Trimmed sequences were blasted in the GenBank database (Geneious Prime 2022). A total of 53 samples were sequenced with 33 samples successfully having a sequence match.

Data Analysis - Visual identification accuracy (A_v) of prey remains was calculated by dividing ODE sample identifications that successfully matched with molecular methods (S_m) by the total number of samples (S_t). This equation is used to quantify the accuracy of species-specific identification of avian species using the visual identification methodology with entry-level observers.

$$\boldsymbol{A_v = S_m / S_t}$$

I then tested the categorical difference between high and medium/low certainty levels with molecular/visual matches (yes or no) using an analysis of variance (ANOVA). Medium and low certainty levels were combined due to a small number ($n = 2$) of low certainty.

Results

Out of 59 prey remains envelopes, trained observers visually identified and provided ODEs for 83 individual birds. Most visual identifications were classified as Passeriformes (n = 81), with only a couple classified as Charadriiformes (n = 2, Table 2). Four samples were categorized as unidentifiable to species and excluded from further analysis. Across observers,

Table 2: The diversity of species identification within the visual identification process. Small songbirds were the most common, with a few shorebirds.

Species Identification	#
American Robin (*Turdus migratorius*)	1
Black-bellied Plover (*Pluvialis squatarola*)	1
Canada Jay (*Perisoreus canadensis*)	1
Golden-crowned Sparrow (*Zonotrichia atricapilla*)	1
Least Sandpiper (*Caldris minutilla*)	1
Orange Crown Warbler (*Vermivora celeta*)	1
Say's Phoebe (*Sayornis saya*)	1
Wilson's Snipe (*Gallinago delicata*)	1
Wilson's Warbler (*Cardellina pusilla*)	1
Yellow Warbler (*Setaphagia petachia*)	1
Fox Sparrow (*Passerella illica*)	2
Horned Lark (*Eremophila alpestris*)	2
Snow Bunting (*Plectophenax nivalis*)	2
Lapland Longspur (*Calcarius lapponicus*)	4
Savannah Sparrow (*Passerculus sandwichensis*)	4
Unidentified Small Bird	4
White-Crowned Sparrow (*Zonotrichia leucophrys*)	4
Chipping Sparrow (*Spizella passerina*)	6
American Tree Sparrow (*Spizelliodes arborea*)	7
White-winged Crossbill (*Loxia leucoptera*)	7
American Pipet (*Anthus rubescens*)	9
Common Redpoll (*Acanthis flammea*)	11
Dark-Eyed Junco (*Junco hyemalis*)	15

low/medium certainty was chosen for 59.0% of samples (n = 49), whereas high certainty was chosen for 41.0% (n = 34). Extraction of DNA was attempted for each of the 83 ODE samples. However, DNA was only amplifiable in 61.0% of the ODE samples due to age and degradation (Figure 1). Sequencing was effective with primer pairs AWF2-R4 and AWF4-R6, though 4 other primers were considered (See Table 1). Using the two primers (AWF2-R4 and AWF4-R6), 33 samples were successfully amplified and sequenced (See Table 3). The sequence of each of the 33 samples was matched to specimen vouchers in the Geneious database and identified to species. I allowed a base pair match of ≥ 99.6% to account for degraded DNA. If the base pair match was < 99.6% or specific genetic identification was questioned, sequencing was performed again using the AWF4-R6 primer (n = 2). Out of the 33 successfully amplified and sequenced ODEs, 27.3% (n = 9) of samples had a match between visual identification and molecular results.

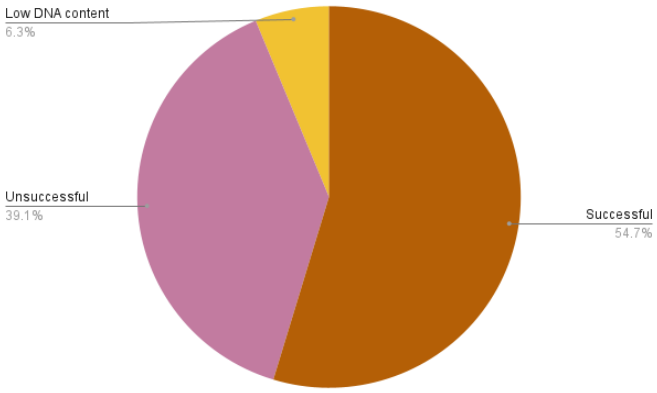

Figure 1: DNA extraction results from 83 samples. Unsuccessful DNA extraction was described as any sample with a nanodrop concentration of <15 ng/ml of DNA. Low DNA had a nanodrop concentration between 15-20 ng/ml. Successful samples were determined as any sample with a nanodrop concentration above 20 ng/ml. Most successful samples had a nanodrop concentration of 50 ng/ml or higher, with a total extraction median of 39.5 ng/ml.

When observers indicated a high level of certainty (n = 12), 41.7% (n = 5) were in agreement between the visual and molecular identification (Figure 2). When observers indicated low/medium certainty (n = 21), visual identification matched with molecular technique in 19% (n = 4) of extracted and amplified ODEs (Figure 2). As evidenced by the categorical ANOVA, in

the instance of visual identification with entry-level technicians, human certainty levels impact the accuracy of visual identification and are significant (Figure 2, p-value = 1.05 E -24).

Table 3: Represents successful sequenced samples, primers, and percent match with the genetic database, Geneious Prime. Item number describes the number of items (individual birds) within the bag. This was determined by the year, bag ID, and then item number (i.e. YYBB.01…02…03). Sequence ID is the label given for sequencing identification. The primer column describes the primer used for amplification and sequencing. Genetic identification is the identification provided via sequencing. The % match is the match between the genetic identification and Geneious vouchers. Highlighted sections are the primer AWF4-R6.

ID	Sequence ID	Primer	Genetic Identification	% Match:
86.13.01	TC16	AWF2-R4	American Pipet (*A. rubescens)*	100.00%
86.23.02	TC13	AWF2-R4	Savannah Sparrow (*P. sandwichensis)*	99.80%
86.26.01	TC19	AWF2-R4	Common Redpoll (*A. flammea)*	100.00%
86.57.01	TC12	AWF2-R4	American Tree Sparrow (*S. arborea)*	100.00%
86.31.01	TC22	AWF2-R4	American Tree Sparrow (*S. arborea)*	99.70%
86.29.01	TC23	AWF2-R4	American Tree Sparrow (*S. arborea)*	100%
86.46.01	TC24	AWF2-R4	Least Sandpiper (*Calidris minutilla)*	99.80%
86.53.01	TC25	AWF2-R4	American Pipet (*A. rubescens)*	100%
86.54.02	TC26	AWF2-R4	White-Crowned Sparrow (*Z. leucophrys)*	99.70%
86.18.01	TC27	AWF2-R4	American Pipet (*A. rubescens)*	100%
86.20.01	TC28	AWF2-R4	Dark-Eyed Junco (*J. hyemalis)*	100%
86.20.01	TC54	AWF4-R6	Dark-Eyed Junco (*J. hyemalis)*	100%
86.16.01	TC29	AWF2-R4	Lapland Longspur (*C. lapponicus)*	99.80%
86.36.01	TC30	AWF2-R4	White-winged crossbill (*L. leucoptera)*	100%
86.21.01	TC31	AWF2-R4	Solitary Sandpiper (*Tringa solitaria*)	99.60%
86.32.01	TC32	AWF2-R4	Common Redpoll (*A. flammea)*	100%
86.23.01	TC33	AWF2-R4	Savannah Sparrow *(P. sandwichensis)*	100%
86.14.01	TC34	AWF2-R4	Northern Wheatear (*Oenanthe oenanthe)*	99.60%
86.23.03	TC35	AWF2-R4	Tree Swallow *(Tachycineta bicolor)*	99.70%
86.23.03	TC55	AWF4-R6	Tree Swallow *(Tachycineta bicolor)*	99.70%
85.03.01	TC36	AWF2-R4	Savannah Sparrow *(P. sandwichensis)*	99.60%
85.03.02	TC37	AWF2-R4	Lapland Longspur (*C. lapponicus)*	99.60%
86.03.01	TC39	AWF2-R4	American Pipet (*A. rubescens)*	100%
86.04.01	TC40	AWF2-R4	American Pipet (*A. rubescens)*	100%
86.04.03	TC42	AWF2-R4	Horned Lark (*E. alpestris)*	100%
86.05.01	TC43	AWF2-R4	Savannah Sparrow *(P. sandwichensis)*	100%
86.05.02	TC44	AWF2-R4	Savannah Sparrow *(P. sandwichensis)*	100%
86.05.03	TC45	AWF2-R4	Baird's sandpiper (*Calidris bairdii*)	99.60%
86.07.02	TC46	AWF2-R4	White-crowned sparrow (*Z. leucophrys)*	100%
86.11.01	TC47	AWF2-R4	American Pipet (*A. rubescens)*	100%
86.34.01	TC49	AWF2-R4	Common Redpoll (*A. flammea)*	100%
86.38.01	TC50	AWF2-R4	Savannah Sparrow *(P. sandwichensis)*	100%
86.45.01	TC51	AWF2-R4	White-winged Crossbill (*L. leucoptera)*	100%
86.46.02	TC52	AWF2-R4	White-crowned sparrow (*Z. leucophrys)*	99.60%
87.01.01	TC53	AWF2-R4	Spotted Sandpiper (*Actitis macularius*)	100%

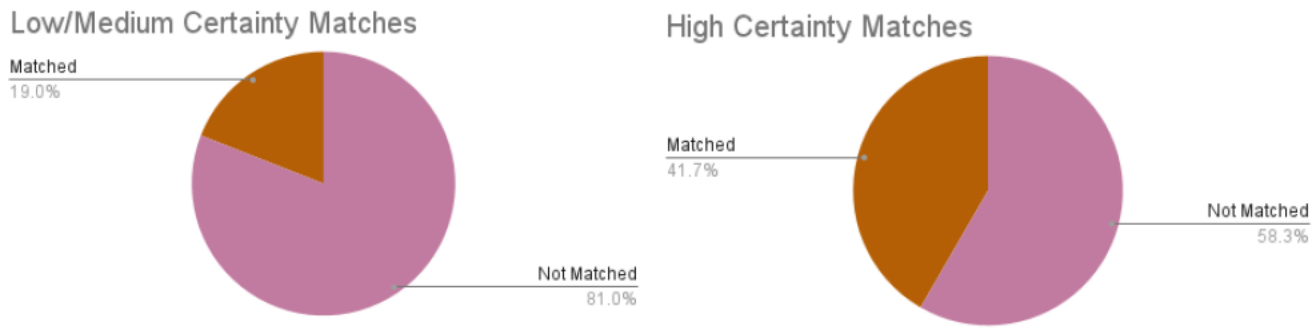

Figure 2: Pie charts for low/medium and high certainty of extracted and sequenced ODE's demonstrate the significant difference in which certainty level impacts a genetic/visual identification match. Low/medium certainty effectively matches 19% (n = 4) of the time whereas high certainty matches 41.7% (n = 5). Total low/medium certainty (n = 21) for extracted and sequenced ODE's. Total high certainty extracted and sequenced ODE samples (n = 12).

Discussion

My study suggests that observers' certainty-level influences the accuracy of identifying prey items. While the accuracy rate is still shockingly low (< 50.0%) for entry-level observers with high certainty, the significant difference between low/medium to high, demonstrates that visual identification is more effective when an observer is confident in their sample identification. Anecdotally, I noted that when observers stated they had high certainty, a larger amount of evidence (e.g., more feathers, feet, head) was often present within the overall prey remains sample collected. However, large amounts of prey remains are not always present (Lewis et al. 2004). Thus, the number of additional 'clues' within a prey remains sample impacts observer certainty levels as it is much more difficult to identify species with a smaller collection of remains. To this day, the use of visual identification to identify species-specific items in prey remains is one of the most commonly used methods for dietary studies. Yet, with all samples, observers are limited to the quality and quantity of what was collected.

While the limitations of visual identification have been a theme throughout this study, it's important to note that DNA-based methods are also limited when dealing with degraded or damaged samples. After the trimming of sequences, I was only able to successfully sequence up to around 200 definitive base pairs per identification due to the quality of DNA left in each genetic sample. This can have implications for species within sister clades, as the mtDNA for certain regions, like the COI gene used for this study, can have the same base pair composition. Within the genetic analyses for this study, I identified a 100% match to both a dark-eyed junco (DEJU) and yellow-eyed junco (YEJU). Knowledge of the geographic location within the study site of Alaska allowed the separation of these closely-related species (YEJUs in Mexico and DEJUs in the United States). For samples more intact, these problems can generally be avoided by using primers that target longer DNA sequences or different gene regions, but this is not always possible with degraded DNA. For this reason, I recommend testing multiple primer pairs in order to successfully sequence the maximum amount of base pairs possible. Additionally, it is essential to acknowledge that the GenBank and Geneious Prime databases are still acquiring specimen vouchers. Though vouchers were available for all bird species in Denali National Park, every bird is not currently in the database.

In addition, the genetic identification of species varied from similar studies conducted by Laing (1985) and Wilbor (1996) in Denali. Laing (1985) and Wilbor (1996) both utilized visual identification as their primary methodology for identification, yet, my results suggest an additional four species of sandpiper: solitary (*Actitis macularius)*, Baird's *(Calidris bairdii)*, spotted (*Actitis macularius*) and least (*Calidris minutilla)*, as well as the white-winged crossbill (*Loxia leucoptera).* White-winged crossbills are considered uncommon within Denali National Park; thus, this finding should be further investigated.

Even with the limitations of DNA-based methods, they provide a more accurate assessment of species-specific identification within prey remains. When visual observers had a *high* level of certainty, only 41.7% of the visual identifications matched the molecular technique; most observers designated their certainty level as low/medium. At its core, visual identification is up to the skill of the technician and the contents of the samples (Symondson 2002, Pompanon et al. 2017). My study suggests visual identification is ineffective when utilizing entry-level technicians for species-specific inquiries. While visual identification can still be used to differentiate between separate taxa (i.e. bird vs mammal), molecular-based techniques provide more accurate and comprehensive results for diet analyses.

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