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TECHNICAL REVIEW

Molecular tools reveal diets of insectivorous birds from predator fecal matter

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Abstract The emerging field of molecular scatology enables critical testing of food web theory. The non-invasive application of molecular tools allows for sequencing of prey DNA from predator fecal matter, evaluating diet breadth and foraging guild. While insectivorous bats are obscure foragers compared to most insectivorous birds, more is known about which arthropod species bats consume because molecular techniques have been optimized for mammalian systems, not avian physiology. Our research objective was to use molecular tools to detect arthropod prey in the fecal matter of an insectivorous avian predator. We used Western Bluebird (*Sialia mexicana*) as a model predator due to its generalist foraging strategy. We compared two fecal DNA extraction kits: (1) Qiagen's DNA stool mini kits, used widely in dietary studies on bats and (2) Zymo's Soil/Fecal DNA MiniPrep kits, not currently cited in the molecular scatology literature. We successfully extracted DNA only with the Zymo kit, amplified mitochondrial cytochrome oxidase *c* subunit I genes, sequenced, and identified the arthropod prey. A spiked PCR experiment showed evidence of possible inhibitors remaining in the Qiagen kit extractions. Overall, arthropod prey from seven different orders and five different classes were identified. We discuss the ecological implications of these data and suggest areas of future research applying molecular techniques to avian fecal matter. Consistent methodological advancement will enable molecular scatology to identify ecosystem services provided by insectivorous birds, develop ecological theory, and inform predator conservation efforts.

Keywords Avian predator · Community ecology · Diet breadth · Insectivore · Trophic interactions · Molecular scatology

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Introduction

By incorporating molecular tools to sequence prey DNA from predator fecal matter, molecular scatology critically tests food web theory, evaluating diet breadth and foraging guild. The annual number of molecular scatology publications is increasing in number and expanding in scope (see King et al. 2008; Pompanon et al. 2011). Publications have targeted marine predators (Deagle et al. 2007; Jarman et al. 2002) and insectivorous bats (Bohmann et al. 2011; Clare et al. 2009). Although insectivorous bats forage nocturnally while insectivorous birds are diurnal predators, studies have uncovered the components of many bat diets because molecular methods were designed for mammalian (not avian) systems. For example, Qiagen QIAamp® DNA stool mini kits contain protocols for DNA extractions from human samples. Bats, like humans, are mammals and possess a digestive system for solid waste in addition to a separate urinary system (Neuweiler 2000). The pathogen detection protocol from these Qiagen kits extracts microbiome and dietary DNA and are broadly used for isolating prey DNA from bat feces (Bohmann et al. 2011; Razgour et al. 2011; Zeale et al. 2011). Birds (Aves), amphibians (Amphibia), and reptiles (Reptilia), on the other hand, have one single orifice (the cloaca) for all elimination. As a result, avian

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fecal matter is combined with uric acid, a highly acidic and concentrated substance containing more toxic nitrogen, secondary metabolites, and by products in comparison to bat feces (Howard and Heiser 2004). Our research objective was to develop a molecular scatology protocol that would successfully identify arthropod prey of an avian predator's fecal matter. If successful, such a protocol may be applicable to a broader range of predators and expedite a shift in focus to more powerful analyses for researching predator–prey dynamics and trophic interactions.

Using molecular approaches to study predator diets is less invasive and less biased than alternative methods involving gut content analysis from sacrificed birds (Beal 1915), emetics that lower avian survivorship (Carlisle and Holberton 2006), neck ligatures on nestlings (Mellott and Woods 1993), and gastric lavage (Moody 1970). Manually dissecting avian fecal samples, although non-invasive, is usually limited to taxonomically crude and biased results (Symondson 2002). Isotope analysis techniques do not allow for the identification of specific prey items (Newsome et al. 2007). Consequently molecular scatology is a powerful tool that has the potential to revolutionize ecological studies with birds.

To develop and test molecular techniques as applied to insectivorous avian fecal matter, we compared diets from a small sample of Western Bluebird (*Sialia mexicana*) nestlings. We used Western Bluebird, hereafter simply bluebird, as a model predator because as a generalist insectivore, it forages for arthropods on the ground, air, or vegetation (Guinan et al. 2008). Results from bluebird diets are compared to previous research collected from sacrificed birds approximately 100 years ago. Our research compares two different extraction kits, the popular Qiagen kits used for bat diets, and newer Zymo kits. Because only one of these kits successfully amplified PCR product showing visible bands on our gels, we conducted an additional spiked PCR experiment to test whether PCR inhibitors were coextracted from both kits. We use this case of molecular extraction techniques to model a methodology and discuss the benefits, limitations, and ecological consequences of interpreting results using such an approach.

Methods

Sample collection

Bluebird nestlings were sampled from nest boxes at two vineyard sites in Northern California in 2011. One site was located in Napa County, CA, USA in the city of St. Helena (38°30′N, 122°30′W) and the other in Sonoma County, CA, USA in the city of Glen Ellen (38°20′N, 122°29′W). To test each extraction kit, fresh fecal samples were collected directly from two nestlings per nest in four bluebird nest boxes at both sites (n = 16 per kit). Because each bluebird nest is fed by the same one to three adult birds (Guinan et al. 2008), nestling diets are indicative of adult bluebird foraging and prey selection for young. Upon collection, individual samples were sealed in collection tubes and placed on ice for 1–5 h during transit, then stored dry for 24–72 h at −80 °C until beginning DNA extraction.

DNA isolation and amplification

To extract prey DNA from avian feces, we used QIAamp® DNA stool mini kits (Qiagen, Hilden, Germany), following the stool pathogen detection protocol (modified for 10–100 mg starting sample used by Bohmann et al. 2011; Razgour et al. 2011; Zeale et al. 2011), and *Xpedition*™ Soil/Fecal DNA MiniPrep kits (Zymo, Irvine, California, USA) following the manufacturer's protocol with the following adjustments. Fecal matter weighing 0.10–0.25 g was added to the Zymo-provided Lysis tube, combined with Lysis/Stabilization Solution and processed in a bead beater (Precellys 24, Bertin) at 6,500 Hz per second for two cycles at 10 s each. After a 5-minute wait, the same bead beater cycle was repeated. Before proceeding to step three in the protocol, 25 µl of Proteinase K were added and the samples were vortexed and heated at 60 °C for 10–15 min.

Mitochondrial cytochrome oxidase *c* subunit I (COI) genes were amplified from bluebird fecal material, cloned, and sequenced to identify arthropod prey. Extracted DNA was PCR amplified using LCO1490 and HCO2198, a primer set originally designed to amplify a 710 bp region of the COI gene from a range of metazoan invertebrates (Folmer et al. 1994). These universal primers were used to determine whether any amplifiable DNA could be detected from the feces. DNA extractions for each sample were divided into 8 separate 20 µl PCR reactions and pooled together after amplification for DNA purification. The PCR reactions combined 0.2 µl Phusion Hot Start DNA Polymerase (ThermoFischer), 4 µl Phusion GC buffer, 1 µl DMSO, 0.4 µl dNTP, 1 µl HCO2198 [10 µM], 1 µl LCO1490 [10 µM], 7.4 µl deionized H_2O, and 5 µl DNA template. The reaction denatured at 98 °C for 2 min, then 35 cycles were preformed with denaturation at 98 °C for 8 s, primer annealing at 52 °C for 20 s, and extension at 72 °C for 30 s with one final extension of 72 °C for 7 min. DNA was purified with DNA Clean and Concentrator™ kits (Zymo, Irvine, CA) per the manufacturer's instructions.

DNA Sequencing

Only PCR products from Zymo kits were chosen for sequencing because Qiagen extraction methods yielded no visible bands (see results). Some Zymo samples showed

multiple bands, so bands corresponding to ~700 bp sequences were excised from the gel and run with Zymoclean™ Gel DNA Recovery kits (Zymo, Irvine, CA) following manufacturer's instructions. These amplicons were ligated into pCR®4-Blunt TOPO® vectors and transformed into chemically competent *Escherichia coli* cells using the Zero Blunt® TOPO PCR Cloning Kit (Invitrogen, Life Technologies, Grand Island, NY). *E. coli* were plated in LB+ Kanamycin, incubated overnight at 37 °C, and clones sent to Quintarabio (www.quintarabio.com) facilities for sequencing. Forty-five well-spaced colonies were selected per nestling sample; the M13 forward primer that flanked the insert was used for sequencing.

Sequences >500 bp in length were analyzed with Basic Local Alignment Search Tool (BLAST) software package optimized for somewhat similar sequences (blastn), identifying the organisms from the nucleotide collection of GenBank (Altschul et al. 1990). Those that were not identified with >96 % query coverage were manually run on BLAST using blastn and then megablast (which optimizes for highly similar sequences), and finally with distance trees to manually determine prey identification (Wilson et al. 2011).

PCR inhibition experiment

To test whether PCR inhibitors were coextracted in the DNA elutions, we ran a spiked PCR experiment of both Zymo and Qiagen extracts. Two bluebird fecal samples stored at −80 °C were divided in half and DNA was extracted with either the (1) Qiagen kit or (2) Zymo kit using the same DNA extraction protocols described above under section "DNA isolation and amplification". Initial sample weights were standardized between the kits, not exceeding 100 mg starting sample weight as recommended by Zeale et al.'s (2011) supplemental documentation. We calculated nucleic acid concentrations (ng/µl) using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, Delaware) and compared these readings to dsDNA concentrations (ng/µl) measured with dsDNA high sensitivity assay kits on a Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, California). Concentrations (ng) of dsDNA in each PCR reaction were standardized between treatments using the Qubit readings and, due to low values, a total concentration of 0.36 ng dsDNA was selected per PCR reaction. DNA extracted from a cricket femur was used as a positive control along with a negative control of double distilled water. The spiked PCR reactions combined equal concentrations of positive control DNA with all four DNA extracts, totaling 0.72 ng DNA per PCR reaction. The PCR protocol was identical to that described under the *DNA amplification* section above except that the volume of template varied due to DNA concentration as indicated above and was compensated by altering the water amounts to a total of 12.5 µl template plus water. The 10 reactions were run side by side with a 100 bp Plus ladder on a 1 % agarose gel run at 60 V for 90 min then stained with ethidium bromide.

Results

PCR products from Qiagen DNA extracts showed no visible bands whereas the Zymo DNA extracts contained visible bands for all but one sample. Arthropod DNA was successfully extracted and sequenced from 13 of the 16 nestlings using the Zymo kit. Overall prey from seven different orders and five different classes were found in bluebird fecal matter including: Arachnida, Diplopoda, and Insecta (Table 1). There were twelve clones (i.e. sequenced samples) with host amplification and several fungal

Table 1 Presence of prey DNA from fecal samples of Western Bluebird nestlings in Napa (N) and Sonoma (S) Counties

Prey Phylum	Class	Order	Query coverage (%)	N A1	N A2	N B1	N B2	N C1	N C2	N D1	N D2	S W1	S W2	S X1	S X2	S Y	Total number of clones
Arthropod	Arachnida	Araneae	96			X											1
Arthropod	Diplopoda	Julida	83			X											1
Arthropod	Insecta	Diptera	100	X			X										30
Arthropod	Insecta	Lepidoptera	82				X				X						3
Arthropod	Malacostraca	Amphipoda	70–74		X	X		X	X	X	X			X		X	81
Arthropod	Malacostraca	Isopoda	100	X	X	X	X	X	X	X	X	X	X	X	X		109
Mollusca	Bivalvia	Arcoida	74			X											2
Richness per nestling				2	2	5	3	2	2	2	3	1	1	2	1	1	

Birds with the same letters were from the same nest

contaminants that also amplified. Because BLAST is limited to comparing amplicons to known sequences in the database, query coverage for identifying prey ranged from relatively low (70 %) to high (100 %). The most common DNA sequence identified isopod prey as *Armadillidium vulgare* (Malacostracae: Isopoda) with 100 % sequence similarity. Of nestlings that were found to contain *A. vulgare*, 100 % of their siblings were also fed isopods. Only one nest contained nestlings without isopod DNA template in their feces.

Avian feces varied in prey species richness from detecting one prey item up to five different taxa per fecal sample. Clones with low frequencies of occurrence included one arachnid (Arachnida: Araneae) and one millipede (Diplopoda: Julida). Relatively more common clones were from Insecta (Lepidoptera and Diptera). Two prey items identified by BLAST with 70–74 % query coverage were a bivalve mollusk (Bivalvia: Arcoida) and Amphipoda (Malacostraca) an order of predominantly marine crustaceans. These improbable results with low confidence do not indicate that bluebirds were consuming these prey, but rather that no matches were found in the BLAST database, giving false positive hits.

PCR inhibition results

Concentrations of nucleic acid in DNA extracts from the Nanodrop were unreliable compared to the Qubit dye-based methods (Table 2). Consequently the Qubit readings were used to standardize all DNA concentrations among the PCR reactions. PCR products from Qiagen's DNA extracts produced no visible bands when run under both control and spiked conditions (Table 2). The same fecal samples extracted with Zymo's kit PCR amplified showing visible bands of our target size (~700 bp) under both control and spiked conditions.

Discussion

Although general principles for molecular scatology applications apply across predator taxa, differences in sample processing arising from distinct predator physiologies need to be addressed. For example, although Qiagen extraction kits work well for processing bat feces (Bohmann et al. 2011; Razgour et al. 2011; Zeale et al. 2011), we did not obtain amplifiable prey DNA from these kits using bluebird fecal samples, due to the coextraction of possible PCR inhibitors. Differences between levels of uric acid and secondary metabolites in bat and bird fecal matter likely contribute to the unequal performances in the kits. Oehm et al. (2011) found MoBio, Epicentre, and Qiagen stool mini kits all yielded significantly lower concentrations of prey DNA in Carrion Crow (*Corvus corone*) fecal samples than the traditional cetyltrimethyl ammonium bromide (CTAB) extraction protocol. In addition, due to low concentrations of DNA in the extracts, the Nanodrop produced unreliable readings of nucleic acid concentrations. Dye-based quantifications are necessary to accurately determine dsDNA presence and concentration in avian fecal extracts. Future research with avian predators should compare Zymo kits used successfully here with other DNA extraction options to optimize performance. Variables that could be tested include starting sample weight, time of mechanical processing, and other attempts to remove PCR inhibitors from final elutions.

Other benefits of using the Zymo protocol for DNA extraction include the ability to process samples that weigh up to 0.25 g, compared to other extraction kits that require

Table 2 Data from PCR inhibition experiment

Kit and sample number	Sample	Fecal sample weight (mg)	Nanodrop (ng/μl)	Qubit (ng/μl)	ng sample DNA in PCR	ng control DNA in PCR	Total DNA (ng)	Visible line in gel (~700 bp)
Control Qiagen 1	Bird fecal sample 1	66	1.87	0.08	0.36	0	0.36	No
Control Qiagen 2	Bird fecal sample 2	56	0.73	0.03	0.36	0	0.36	No
Control Zymo 1	Bird fecal sample 1	64	17.79	0.03	0.36	0	0.36	Yes
Control Zymo 2	Bird fecal sample 2	46	11.62	0.84	0.36	0	0.36	Yes
Positive control	Positive control cricket DNA		112.49	1.05	0	0.36	0.36	Yes
Negative control	Double distilled water		N/A	N/A	0	0	0	No
Spiked Qiagen 1	Fecal sample 1 + positive control				0.36	0.36	0.72	No
Spiked Qiagen 2	Fecal sample 2 + positive control				0.36	0.36	0.72	No
Spiked Zymo 1	Fecal sample 1 + positive control				0.36	0.36	0.72	Yes
Spiked Zymo 2	Fecal sample 2 + positive control				0.36	0.36	0.72	Yes

Each row represents one PCR reaction with controlled fecal extracts being compared to those same extracts with added amplifiable cricket DNA (spiked)

samples under 0.1 g in weight. The modification to Qiagen's pathogen detection protocol that successfully extracted prey DNA from bat feces limited the beginning sample weight to 10–100 mg (Zeale et al. 2011). Consequently if the Qiagen protocol is coextracting PCR inhibitors, larger starting samples likely have more inhibitors, affecting DNA recovery. Nestling bluebird fecal samples are visually heterogeneous and varied in weight from 0.03 to 1.16 g depending on nestling age (Jedlicka, unpublished data). If subsampling is necessary due to kit requirements, it may either (1) bias results if the remaining sample is discarded or (2) significantly increase costs by necessitating two to four extractions per fecal sample. Finally, unlike the Qiagen kit, the Zymo kit contains beads in a bead beating tube that may help extract prey DNA that is held tightly in exoskeleton fragments, increasing detection of prey items.

Isopod prey were found in all nestling diets with only one exception. This was the only organism identified to species (*Armadillidium vulgare*) with 100 % sequence similarity. Previous research identifying the stomach contents of sacrificed bluebirds also found isopods, but in the analysis of 217 adult stomachs, isopods comprised only 0.11 % of the animal diet (Beal 1915). Moreover, isopod prey were absent from nestling stomachs ($n \geq 10$), which instead were comprised 90.0–97.5 % with orthopteran prey. The frequency of *A. vulgare* here may indicate that adults are responding to local prey availability in vineyard habitats, finding isopods more readily available than orthopterans. Future research quantifying prey availability in the field coupled with molecular results from predator diets will enable determination of prey selection and preferential foraging by predators.

We found that the overall species richness of prey in avian fecal samples ranged from one to five different taxa per bird. This is comparable to previous studies on bats that identified one to seven different prey taxa per guano pellet (Clare et al. 2009). Such a low diversity of prey per sample should be put in the context of predator physiology, as influenced by the gut passage time. A recent study by Oehm et al. (2011) measured gut transition time of cockchafer, *Melolontha melolontha* (Coleoptera: Scarabaeidae) meals in Carrion Crows and found there was a 30 min to 4 h window after ingestion where prey DNA could be detected in predator fecal matter. Previous studies on gut transition times for American Robins (*Turdus migratorius*) consuming fruit were estimated to range from 16 to 145 min (Levey et al. 2008; Levey and Karasov 1992). Adult bluebirds have been reported to feed nestlings approximately 4.3 times/nestling/h (Leonard et al. 1994). Consequently, increasing the frequency of fecal collection and sample size is necessary to better approximate the full diet breadth of these predators.

The total number of clones for each prey item (Table 1) is influenced by many factors and should be interpreted with caution (see Pompanon et al. 2011). High frequency of detection could signal that isopods were consumed in greater numbers than other prey or that an isopod was the most recently consumed item and clones with lower frequency were from earlier meals that already began to leave the bird's digestive system. Alternative explanations involve primer binding efficiency, biomass of the prey organism, or digestibility (e.g. how long the predator takes to fully digest the prey item). None of these factors are mutually exclusive and could be occurring in unison, so care must be taken in the ecological interpretation of these results.

We assume that entries in GenBank were correctly identified. In sequences with low confidence due to a paucity of data in the database, there are additional problems with interpreting results. Assignment to order is not always clear with BLAST results so manually checking phylogenetic tree data can help resolve identity disputes (Wilson et al. 2011). However, if the database lacks records for comparison, phylogenetic trees may be inconclusive. For example, BLAST identified two prey items with taxonomic ambiguity in our study as a bivalve mollusk and Amphipoda. While these identities are unlikely, the clones themselves were common among the fecal samples (81 clones for Amphipoda) and are undoubtedly prey DNA. Previous research has shown snails (Mollusca) to be components of bluebird diets (Beal 1915). With further efforts to expand barcoding databases and by pairing research in molecular scatology with sampling of the prey community, this identity problem can be ameliorated in the future.

Molecular methods need to be further developed to improve the efficiency and specificity of detecting prey in fecal remains. Primers that specifically target the class Arthropoda would help identify prey DNA over fungal and bacterial contamination or host amplification. Recently, Zeale et al. (2011) developed a taxon-specific primer set for bats that did not amplify host DNA or putative contaminants. Moreover their research targeted shorter mtDNA fragments that are retained in higher proportions in fecal samples, increasing detection power (Deagle et al. 2006). Such technological advancements are expanding the possibilities for ecologists to research trophic interactions, improving the soundness and efficiency of predation studies.

Next generation sequencing technology can be used to expand our knowledge of predator–prey relationships. Studies with Australian fur seals (Deagle et al. 2009), little penguins (Deagle et al. 2010; Murray et al. 2011) and bats (Bohmann et al. 2011) have proven that such technology can successfully be applied to predator fecal matter for dietary analysis. Besides sequencing methodology, recent studies have expanded upon prey identification techniques

to compare predator diet breadth and composition over time and across different habitats (Clare et al. 2011; Razgour et al. 2011). Further applications include revealing the prey of locally threatened species (Murray et al. 2011), identifying natural predators of an economically important pest (Smith et al. 2011), characterizing the microbial communities within the predators themselves (Scupham et al. 2007), and quantifying prey consumption via real-time PCR techniques (Bowles et al. 2011). Besides arthropod prey, molecular techniques were used to tease apart plant diets of herbivores via fecal samples enabling the separation of foraging niches (Corse et al. 2010; Valentini et al. 2009). Molecular scatology proves to be a rapidly developing and widely applicable tool to address vital ecological interactions between predators and their prey base.

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