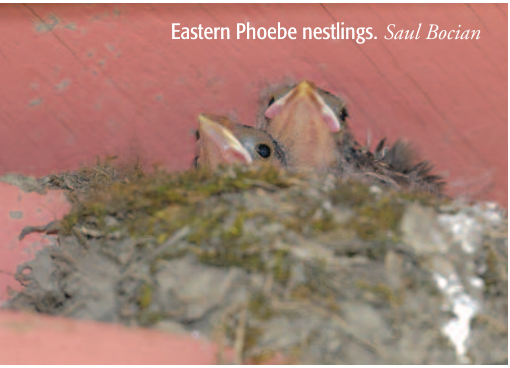
Using next-generation sequencing of DNA to determine nestling diet of two double-brooded avian insectivores

Al Sandilands and Derek Morningstar

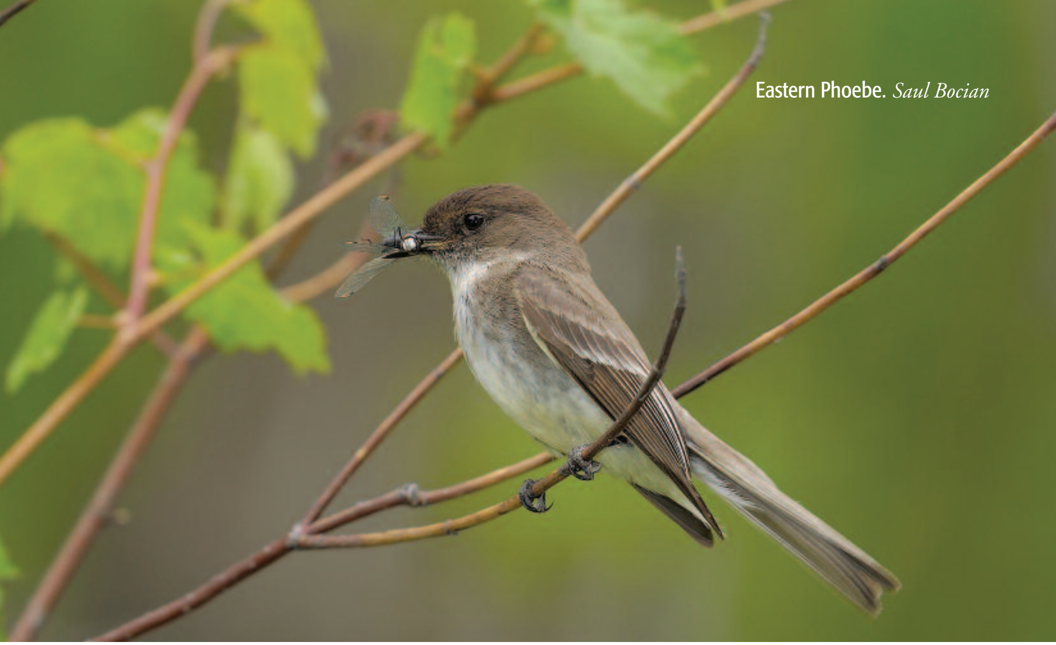


Eastern Phoebe nestlings. *Saul Bocian*

Introduction

Diet is a critical component of the ecology of birds that is poorly understood for many insectivorous birds. Many passerines subsist predominantly on insects during the breeding season, occasionally supplementing their diet with spiders and fruit. Difficulty is often experienced in determining the diet because many of the prey items are small and need microscopic examination to be identified. Samples from stomachs or fecal matter may yield biased results because of different rates of digestion of prey, with beetles and other species with chitinous body parts often being over-represented in the results.

Several techniques are commonly used to determine avian diet such as sacrificing birds to analyse stomach or gizzard contents, analyzing food items in fecal matter, or causing birds to eject their stomach contents. Turner (1980) tied temporary ligatures around the necks of nestlings so that they could not swallow the food that the adults delivered to them; food was then removed from their throats for analysis. MacDade *et al.* (2011) used stable isotopes to analyze breath samples in exhaled carbon dioxide which gave an indication of the relative contributions of terrestrial and aquatic prey to the diet. Other studies have used tissue stable isotopes to compare the diets of species (Hipfner *et al.* 2013), but these give very general results with no indication of the food items that are actually consumed. A high proportion of information on the diet of birds is anecdotal,



Eastern Phoebe. *Saul Bocian*

typically from observations of birds consuming a given prey item, and these data may not be representative of the diet.

Even when more detailed diet analyses are undertaken, results are often reported only to the level of Order. Nolan (1978) completed an exhaustive study on the Prairie Warbler (*Setophaga discolor*). Spiders and six Orders of insects (Coleoptera, Lepidoptera, Diptera, Hymenoptera, Hemiptera, and Homoptera) constituted 97% of the diet and these same Orders include 94% of all North American insects. Consequently, he concluded that this warbler was a generalist feeder. There is a good possibility that his conclusion was incorrect. He had no information on which species were available in the environment for possible consumption or on which species were consumed from each Order of insects. His study is representative of the level of detail of most diet studies of insectivorous species. Some studies identify insects to the level of Family but very few identify them to the level of genus or species. Even when more detailed identifications are completed, biases due to different rates of digestion may still affect results and conclusions.

Determination of the diet of nestling birds is even more challenging than for adults and relatively little is known about this facet of the life history of most birds. The predominant method of nestling diet determination that has been used is visual observation of what adult birds bring to the nest. Much of the smaller prey cannot be seen using this method and only larger specimens can be identified, usually only to the Order level. For many species, the description of the nestling diet is something vague such as small green caterpillars and small flying insects.

Two concerns have recently been raised regarding insect availability and insectivorous birds, which are thought to be declining as a group. The first concern is that insect numbers and biomass may be declining and that this may have an effect on birds that prey upon them (Michel *et al.* 2016). The other concern is that climate change may be affecting the timing of insect emergence (Savignac 2011). Birds have evolved to generally time their nesting so the nestling period coincides with the time of maximum insect abundance. It is possible that the chronology of insect emergence and bird nesting will become asynchronous which has the potential to affect reproductive success. Certain bird species may be more affected than others depending upon the species of insects that they feed their nestlings. It will be very difficult to assess these potential impacts without better knowledge of which species of insects individual bird species are consuming.

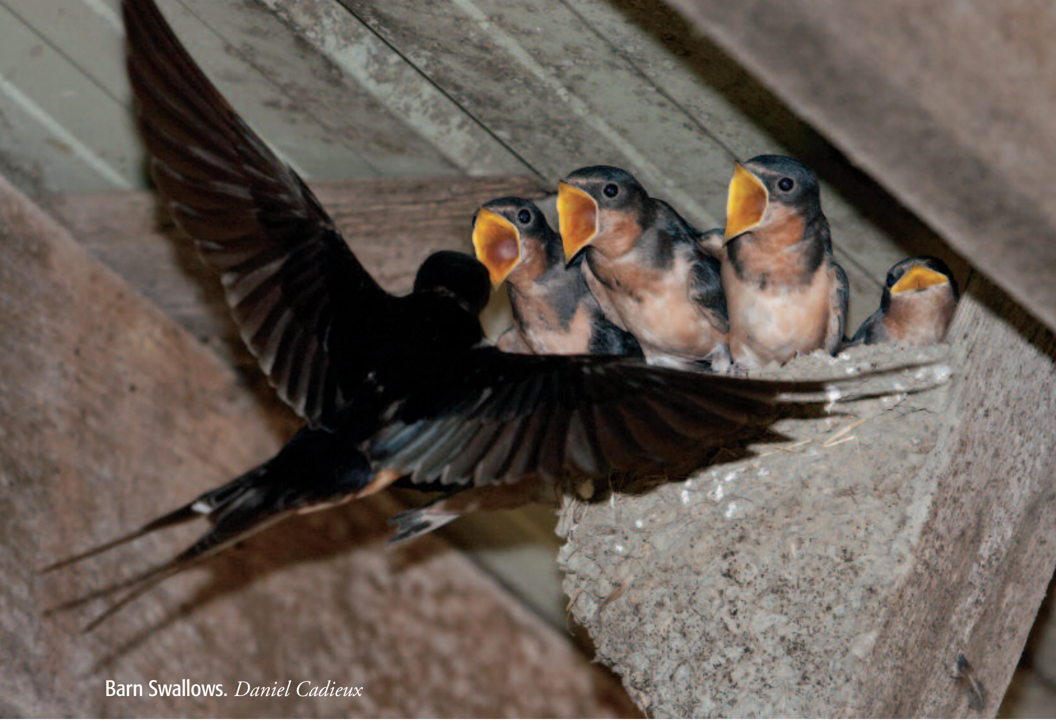
Next-generation sequencing (NGS) has become a widely used technique for identifying and classifying the DNA of species within a given sample using a standardized approach (Endrullat *et al.* 2016). NGS is a high through-put means of extraction, amplifying and sequencing the DNA. It allows for the sequencing of whole genomes or targeted gene regions called mini-barcodes. Although NGS is a general term that can include a variety of specific methods and technological platforms, our methods were based on using the Illumina MiSeq machine, as used in previous studies which can allow identification of samples to the species level (Hajibabaei *et al.* 2011).

In this study, we conducted NGS on fecal samples of nestlings of two species of insectivorous birds: the Eastern Phoebe (*Sayornis phoebe*) and Barn Swallow (*Hirundo rustica*). The Barn Swallow is a true aerial insectivore, capturing flying insects while it is on the wing; the Eastern Phoebe captures flying insects by chasing them from a perch. They were chosen for this study because existing information on the diet of these two bird species, including nestlings, is much better than for most insectivores and it was desirable to determine if NGS would provide more detailed information than is currently available.

Additionally, these species are within a guild of birds that is generally declining in eastern North America. The Barn Swallow has been designated threatened in Canada and Ontario due to declines in breeding numbers. In contrast, the second Ontario Breeding Bird Atlas (Cadman *et al.* 2007) and the Breeding Bird Survey (Sauer *et al.* 2017) indicate that the Eastern Phoebe population is increasing or stable.

The primary purpose of the study was to demonstrate that NGS is a superior method of determining the diet of birds. This technique has the potential to identify prey items to the species level as opposed to only Order or Family level and eliminates most of the biases associated with other methods that have been traditionally used.

Our study provides some interesting insights into the diet of nestling Eastern Phoebes and Barn Swallows, but we emphasize that the results are based on a very small sample size so that none of the results can be compared statistically. With a larger sample size and more accurate sampling techniques, more definitive results could be obtained. We hope that our work will inspire others to use this method of analyzing the diet of birds.


Barn Swallows. *Daniel Cadieux*

Study Area and Methods

The study was conducted at the home of Al Sandilands, approximately 6 km south of the city of Cambridge, Ontario. Attached to the stone house are a summer kitchen and a shed, also constructed of stone. On one side, the summer kitchen is narrower than the shed, resulting in an area that is open to birds and that has a roof over it. A colony of six to seven swallows has nested in the shed for at least 32 years and a pair each of phoebes and swallows nested in the alcove formed by the summer kitchen roof in 2015.

The immediate study area consists of a rural residential lot with several mature sugar maples (*Acer saccharum*) and black walnuts (*Juglans nigra*) and windbreaks of white spruce (*Picea glauca*) and white cedar (*Thuja occidentalis*). At the broader landscape level, the house lot is surrounded by row-crop agricultural fields with three ponds within 200 m. One of the ponds is surrounded by tall willows (*Salix* spp.) whereas the others are open, and the largest is within 30 m of the bird-nesting area; there is also a grassy swale slightly over 200 m distant that holds water in springtime.

A pair each of Barn Swallow and Eastern Phoebe nested in the alcove. We hoped to obtain samples from both broods from each of these nesting pairs. Both phoebe samples were taken from the nest in the alcove, but unfortunately the first nest of the Barn Swallows failed. Consequently, the sample for the first brood of swallows was taken from a nest of a different pair within the shed and the second sample was taken from the single nest in the alcove.

After each brood had left the nest, a sample was collected from the pile of feces beneath the nest. The nesting phenology of the two species was very similar; samples from the first broods were collected on 1 July 2015 and second-brood samples were collected on 22 August 2015. Sampling consisted of simply collecting approximately equal amounts of the feces, using a new set of nitryl disposable gloves for each sample to ensure that there was no cross contamination. Samples were placed in vials and kept frozen until they were delivered to the Hajibabaei Lab at the Biodiversity Institute at the University of Guelph. The lab performed NGS on the samples using methods described by Folmer *et al.* (1994), Hajibabaei *et al.* (2011), and Gibson *et al.* (2014, 2015). The resulting DNA sequences were cross-referenced against the Barcode of Life Database (BOLD) and GenBank to determine the identity of taxa.

Only those taxa where ten or more DNA sequences were detected were reported to avoid possible identification errors. The number of sequences cannot be used as an indication of abundance of taxa so we simply identified species that were represented in each sample.

Results

In addition to having different foraging techniques, field observations indicated that the swallows and phoebes foraged in somewhat different areas. Although the observations are anecdotal, both species have been observed at the site for over 30 years and their foraging methods have been consistent. The swallows foraged over the adjacent pond and above agricultural land. They also perched on utility wires along the road and were occasionally seen foraging 300 m or further from the nesting colony. The phoebes had a much smaller home range and spent much of their time perched on lower portions of the television tower, on dead branches on trees within the yard or trees overhanging or adjacent to the closest pond, and only occasionally near the road. The different methods of foraging and different areas in which the two species concentrated would probably expose them to different species of prey. Nonetheless, swallows regularly flew through the yard while travelling to and from the colony and therefore could conceivably encounter most of the species that were accessible to the phoebes.

The results of the nestlings' diet for both broods of the swallows and phoebes are summarized in Table 1. As expected, the diet of both consisted almost entirely of insects. However, both apparently fed their young mites that they probably picked off the nestlings, with the swallows consuming both swallow mites and fowl mites and the phoebes eating only fowl mites.

Each brood of the phoebes consumed 15 different taxa and in total this species ate 24 taxa. The first brood of swallows consumed 11 taxa and the second brood consumed 13 taxa, for a total of 22 taxa.

The low number of taxa in both species' diet coupled with the fact that the number of DNA sequences cannot be used as an indicator of relative abundance of prey items means that only a qualitative comparison of the diets can be made. Of the total of 40 taxa found in all the samples, only six (15.0%) were eaten by both species indicating limited overlap in their diet. The phoebe consumed some larger and faster-flying insects that were

Table 1. Food items consumed by Eastern Phoebe and Barn Swallow nestlings.

Prey Species	Eastern Phoebe Brood 1	Eastern Phoebe Brood 2	Barn Swallow Brood 1	Barn Swallow Brood 2
ARACHNIDA (Spiders and Mites)				
Dermanyssidae (Bird Mites)				
Dermanyssus hirundinis (Swallow Mite)				+
Macronyssidae (Fowl Mites)				
Ornithonyssus sylviarum	+	+	+	
INSECTA (Insects)				
Ephemeroptera (Mayflies)				
Stenacron interpunctatum	+	+	+	+
Odonata (Dragonflies and Damselflies)				
Coenagrionidae "(Narrow-winged Damselflies)"				
Enallagma sp.	+			
Libellulidae (Skimmers)				
Leucorrhinia intacta	+			
Dermaptera (Earwigs)				
Forficulidae				
Forficula sp.	+			
Hemiptera (Bugs)				+ (ambiguous)[1]
Cicadellidae (Leafhoppers)				
Gyponana extenda		+		
Miridae (Plant Bugs)				
Lygocoris omnivagus				+
Lygus lineolaris				+
Megaloptera (Alderflies and Dobsonflies)				
Corydalidae (Dobsonflies)				
Chauliodes sp.	+	+		
Lepidoptera (Moths)				
Crambidae (Crambid Snout Moths)				
Elophila obliteralis		+		
Erebidae (Underwing, Tiger, and Tussock Moths)				
Hypena scabra		+		
Hyphantria cunea		+	+	
Spilosoma latipennis	+			

Prey Species	Eastern Phoebe Brood 1	Eastern Phoebe Brood 2	Barn Swallow Brood 1	Barn Swallow Brood 2
Lasiocampidae (Tent Caterpillars)				
Malacosoma americanum			+	
Lymantriidae (Tussock Moths)				
Lymantria dispar (Gypsy Moth)	+	+		+
Noctuidae (Owlet Moths)				
Orthosia hibisci	+			
Xestia dolosa		+		
Tortricidae (Leafroller Moths)				
Choristoneura fumiferana (Spruce Budworm)	+			
Choristoneura rosaceana (Oblique-banded Leafroller)	+	+		+
Tricoptera (Caddisflies)				
Hydropsychidae (Netspinning Caddisflies)				
Ceratopsyche morosa			+	
Cheumatopsyche sp.			+	
Macrostemum sp. AM1			+	
Phryganeidae (Giant Casemakers)				
Banksiola crotchi	+			
Diptera (Flies)				
Anthomyiidae (Root-Maggot Flies)				
Hydrophoria lancifer		+		
Calliphoridae (Blow Flies)				
Pollenia pediculata	+	+	+	+
Chloropidae (Frit Flies)				
Tricimba sp. BOLD:AAG1502		+		
Culicidae (Mosquitoes)			+[2]	
Limoniidae (Limoniid Crane Flies)				
Limoniidae sp. BOLD:AAO3939				+
Sarcophagidae (Flesh Flies)				
Neobellieria polistensis	+			
Sarcophaga subvicina		+		
Simuliidae (Black Flies)				
Simulium vittatum			+	
Tabanidae (Horse and Deer Flies)				
Chrysops aff. montanus morph1	+			

Prey Species	Eastern Phoebe Brood 1	Eastern Phoebe Brood 2	Barn Swallow Brood 1	Barn Swallow Brood 2
Hybomitra epistates			+	
Tachinidae (Tachinid Flies)				
Belvosia sp. WOODLEY03C				+
Leschenaultia sp. WOOD07				+
Leschenaultia sp. WOOD15				+
Coleoptera (Beetles)				
Curculionidae (Weevils)				
Rhinocyllus conicus				+
Latriidae (Minute Brown Scavenger Beetles)		+[2]		
NUMBER OF TAXA	15	15	11	13

[1] – identifiable to only the Order level   [2] – identifiable to only the Family level

Note: The codes AM, BOLD, WOOD and WOODLEY (often followed by a number) are names assigned to genetic samples that could be physically identified only to the genus level when initially entered into the Barcode of Life Database or GenBank system. The samples are considered to be unique and differentiated to the species level even if the species has never been properly described.

associated with the nearby pond. These included two species of damselflies and a dobsonfly. The phoebe ate more species of moths (nine) than did the swallow (four). One shortcoming of DNA sequencing is that one cannot determine which life stage of prey has been consumed. Because of its foraging technique, the phoebe may have captured both caterpillars and adult moths whereas the swallow may have been confined to capturing daytime-flying adult moths.

Another difference in the diet of the two species was the caddisflies that they consumed. The swallow consumed three species of netspinning caddisflies and the phoebe ate one species of giant casemaker. Larvae and pupae of the netspinners are associated with rapidly flowing water whereas the giant casemakers are associated with still water (Merritt and Cummins 1984). Because caddisfly larvae and pupae are aquatic, they are not available as prey items to Eastern Phoebes and Barn Swallows. Adult caddisflies fly nocturnally except when swarming over the breeding areas or first emerging from the water. This suggests that the swallows in our study may occasionally have travelled approximately 2 km to forage over the Grand River whereas the phoebe confined its foraging near aquatic habitat to the nearby ponds.

The composition of the diet that was provided to the young between the first and second broods changed for both species. Of the 24 taxa consumed in total by the phoebe, only six (25.0%) were eaten by both broods. Damselflies, earwigs and caddisflies were provided to the first brood only and leafhoppers and beetles were eaten by only the second brood. Moths and flies were important to both broods with five moth and three fly species being consumed by the first brood and seven moth and four fly species eaten by the second brood.

For the swallows, only two of the 22 taxa (9.1%) that were consumed in total were eaten by both broods. Caddisflies were important in the diet of the first brood but were not eaten by the second brood. Flies appeared to be more important in the diet of the second than first brood, with five species consumed by the second brood compared with three species by the first brood. The locally abundant blow fly was the only species of fly that was eaten by both broods and the second brood ate three species of tachinid flies that were not consumed by the first brood.

Discussion

There was limited overlap in the diet of Eastern Phoebe and Barn Swallow with only 15.0% of the total prey items being consumed by both species. Although the nests of the two species were within 4 m of each other and their nests contained nestlings at the same time, the foraging methods and the habitats where foraging occurred differed between the two species. The phoebes foraged from a perch, mostly over the yard and often from dead branches overhanging the adjacent pond. The swallows foraged mostly while flying over agricultural fields and adjacent ponds. Additionally, based upon certain prey items that they consumed, they may occasionally have travelled approximately 2 km to feed over the Grand River. The Eastern Phoebe defends an all-purpose territory where all nesting and feeding activities are conducted whereas the Barn Swallow has a large undefended home range that allows it to forage opportunistically on seasonally and locally abundant prey. The territory size of Eastern Phoebe seldom surpasses 3 ha (Weeks 2011); in contrast, the Barn Swallow routinely forages as far away as 200 m from the nest (approximately 12.6 ha) and frequently travels much farther. In Kansas, foraging occurred as far as 800 m from the nest (Fitch 1958) and West Virginia birds routinely travelled 1.2 km from the nest (Samuel 1971). In New York, Barn Swallows travelled as far as 6 km to forage over sheltered beaver ponds (Shields 1984).

The second brood of both species consumed different prey items than the first brood. Similar results have been documented for Eastern Phoebe near Kingston, Ontario (Keast 1990). Nestlings of first nests were fed different prey than second broods due to changing availability of invertebrates; first broods were fed mostly beetles and flies whereas second broods were fed caterpillars, moths, caddisflies, and damselflies (Keast 1990). The diet of the two broods in this study differed from the Kingston study. Here, first phoebe broods ate mostly damselflies, moths, and flies and second broods focused on moths and flies. The diet of swallow broods in our study differed with caddisflies being important to first broods whereas second broods ate more flies. At the Order level, limited difference in the diet between the broods was evident for both species, but there were clear differences at the species level. Without undertaking NGS, we could not have detected differences in diet of the two species.

In Scotland, Turner (1980) determined that individual Barn Swallows may prey upon a relatively low diversity of insects; different birds consumed an average of 9.3 different taxa. She identified invertebrates mostly to the Order level and occasionally to the Family level so the diversity of insects consumed in her samples would have been greatly underestimated. Had we reported results at the same level of identification, her birds would have appeared to have a higher diversity of taxa in the diet than ours, again underscoring the superiority of the NGS method.

Brown and Brown (2011) reported 21 Families of insects in Barn Swallow nestlings' diet in Nebraska. Of the 15 most abundant Families in the Nebraska diet, only blow flies and flesh flies were documented in nestlings' diet in our study; conversely, our study identified 14 Families of invertebrates in the diet that were not documented in the Nebraska study. The Kingston study on Eastern Phoebe diet (Keast 1990) identified invertebrates to the Order level only (except for midges) but documented 11 different prey items at that level. If our study had reported at the same level of identification, only eight different prey items would have been identified rather than 24. Despite the small sample size in our study, we documented several previously unreported prey items for both species of birds.

No studies have previously reported either Eastern Phoebe or Barn Swallow consuming mites. We considered the possibility that the nestlings had not consumed the mites, but that they dropped or were dropped on the pile of feces and thus appeared in the analysis of the samples. We cannot completely rule out this possibility. We did not have the required permits to allow us to handle the nestlings or disturb them, so could not use more rigorous sampling techniques such as keeping the nestlings in a bag until they defecated or collecting feces immediately after expulsion. Use of more rigorous sampling protocols would eliminate this ambiguity.

In the event that the nestlings did consume the parasitic mites, this appears to be a relatively rare phenomenon, or at least a rarely reported activity. We examined the *Birds of North America* species accounts and other literature for the 144 species of passerines that have been confirmed breeding in Ontario. Only 10 species have been documented eating mites during the breeding season: House Wren (*Troglodytes aedon*), Winter Wren (*Troglodytes hiemalis*), Marsh Wren (*Cistothorus palustris*), American Pipit (*Anthus rubescens*), American Tree Sparrow (*Spizelloides arborea*), Harris's Sparrow (*Zonotrichia querula*), Orchard Oriole (*Icterus spurius*), Yellow Warbler (*Setophaga petechia*), Black-throated Blue Warbler (*Setophaga caerulescens*), and Yellow-headed Blackbird (*Xanthocephalus xanthocephalus*). In addition to these species, the American Redstart (*Setophaga ruticilla*) has been reported eating mites during migration, but not during the breeding season. With the exceptions of the Yellow-headed Blackbird and Orchard Oriole, the Order of mite that was consumed was not identified; only adult birds were documented consuming mites. The Yellow-headed Blackbird feeds its young aquatic mites (Hydrocarina) but has not been documented consuming bird mites (Twedt and Crawford 1995). The Orchard Oriole is the only species that appears to have previously been reported consuming bird mites. Scharf and Kren (2010) assumed that they were ingested accidentally or to eliminate them from the nest. The Yellow-rumped Warbler (*Setophaga coronata*) has been observed picking lice (Philopteridae) off its feet and feeding them to nestlings (Hunt and Flaspohler 1998). Therefore, it is plausible that adults may also pick bird mites from themselves or their young and feed them to nestlings. These would provide a readily available source of protein. Another consideration is that researchers may not report mites in dietary studies. They may be included in general categories such as miscellaneous invertebrates. Most studies do not report food items that constitute a minor proportion of the diet.

It is surprising how little information is available on the diet of these two very common and widespread species. There appears to be only a single study on the nestling diet of each of these species in North America, and prey were identified only to the Family level in the more rigorous of the studies. NGS has the capacity of identifying prey to the species level. This is particularly useful in studies of birds that are in decline; their diet may be a limiting factor so it is critical to understand what they eat.

Two other diet studies have been conducted on bird species using NGS and in both studies the results were unexpected and contrary to what was previously known for the species. In the Bay of Fundy, the Semipalmated Sandpiper (*Calidris pusillus*) was thought to have a restricted diet, feeding preferentially on an amphipod (*Corophium volutator*). NGS revealed that it fed on a wide variety of prey including marine, freshwater and terrestrial invertebrates and was not specialized in its diet (Gerwing *et al.* 2016). NGS of the diet of the Louisiana Waterthrush (*Parkesia motacilla*) revealed that it was much less dependent upon aquatic prey than previously thought, with mayflies being the only aquatic invertebrates that were prevalent in the diet (Trevelline *et al.* 2016).

The tendency to place species in foraging guilds is convenient, but has limited application, and more importantly has the potential to mask significant differences among species within the group. As an example, it is typically concluded that aerial insectivores are declining. Michel *et al.* (2016) used Breeding Bird Survey data to analyze the trends of five aerial insectivores over broad geographic areas. They found that there was little concordance among the trends of the species and that only one of them exhibited significant population declines in over half of its population trajectories. To avoid overlooking significant facets in the ecology of birds, especially species at risk, they should be treated and studied individually and not as guilds. In the case of aerial insectivores, diet may be a significant component of their life history that warrants additional study. Many of them use different methods of capturing prey and forage at different altitudes over different habitats. It is especially important to know what these species are consuming if low or changing prey abundance is considered to be one of the factors limiting their populations. NGS may be a valuable technique for determining the diet of these and other species. By identifying prey items to the species level, researchers will have more detailed information to assess the ecology of birds and factors that may be limiting to them.

Our study was based on a very small sample size, yet demonstrated dietary differences between the two insectivorous birds and their two broods and identified previously unknown prey items. More detailed studies would provide a better summary of these nestlings' diet and probably demonstrate greater differences in the diets of the two species. NGS has some limitations: it is not possible to determine the abundance of individual prey items, the life stage of prey cannot be determined and prey consumed by prey may be detected in the analysis. For example, if a bird eats a dragonfly, the food that the dragonfly has eaten may be indicated in the results. These limitations are far outweighed by the superior identification capabilities that NGS provides.

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