The Auk
Ornithological Advances
AmericanOrnithology.org

Volume 136, 2019, pp. 1–11
DOI: 10.1093/auk/uky003

RESEARCH ARTICLE

DNA metabarcoding reveals the broad and flexible diet of a declining aerial insectivore

Beverly McClenaghan,[1,*] Erica Nol,[2] and Kevin C. R. Kerr[1,3]

[1] Environmental and Life Sciences Graduate Program, Trent University, Peterborough, Ontario, Canada
[2] Department of Biology, Trent University, Peterborough, Ontario, Canada
[3] Toronto Zoo, Toronto, Ontario, Canada
* Corresponding author: beverlymcclenaghan@trentu.ca

Submitted March 24, 2018; Accepted August 31, 2018; Published February 5, 2019

ABSTRACT

Aerial insectivores are highly mobile predators that feed on diverse prey items that have highly variable distributions. As such, investigating the diet, prey selection and prey availability of aerial insectivores can be challenging. In this study, we used an integrated DNA barcoding method to investigate the diet and food supply of Barn Swallows, an aerial insectivore whose North American population has declined over the past 40 yr. We tested the hypotheses that Barn Swallows are generalist insectivores when provisioning their young and select prey based on size. We predicted that the diets of nestlings would contain a range of insect taxa but would be biased towards large prey items and that the diet of nestlings would change as prey availability changed. We collected insects using Malaise traps at 10 breeding sites and identified specimens using standard DNA barcoding. The sequences from these insect specimens were used to create a custom reference database of prey species and their relative sizes for our study area. We identified insect prey items from nestling fecal samples by using high-throughput DNA sequencing and comparing the sequences to our custom reference database. Barn Swallows fed nestlings prey items from 130 families representing 13 orders but showed selection for larger prey items that were predominantly from 7 dipteran families. Nestling diet varied both within and between breeding seasons as well as between breeding sites. This dietary flexibility suggests that Barn Swallows are able to adjust their provisioning to changing prey availability on the breeding grounds when feeding their nestlings. Our study demonstrates the utility of custom reference databases for linking the abundance and size of insect prey in the habitat with prey consumed when employing molecular methods for dietary analysis.

Keywords: aerial insectivore, Barn Swallow, diet, DNA barcoding, *Hirundo rustica*, metabarcoding

El meta-código de barras de ADN revela la dieta amplia y flexible de un insectívoro aéreo en disminución

RESUMEN

Los insectívoros aéreos son depredadores muy móviles que se alimentan de presas muy diversas con distribuciones muy variables. Por ende, investigar la dieta, la selección de presas y la disponibilidad de presas de los insectívoros aéreos puede ser un desafío importante. En este estudio, usamos un método integrado de código de barras de ADN para investigar la dieta y el suplemento de alimento de *Hirundo rustica*, un insectívoro aéreo cuya población de América del Norte ha disminuido a lo largo de los últimos 40 años. Evaluamos la hipótesis que *H. rustica* es un ave insectívora generalista cuando se encuentra aprovisionando a sus polluelos y que selecciona sus presas en base al tamaño. Predijimos que la dieta de los polluelos contendría un rango de taxa de insectos pero que estaría sesgado hacia grandes presas y que la dieta de los polluelos cambiaría a medida que cambie la disponibilidad de presas. Colectamos insectos usando trampas Malaise en diez sitios de cría e identificamos los especímenes usando código de barras de ADN estándar. Se usaron las secuencias de estos especímenes de insectos para crear una base de datos de referencia personalizada de las especies de presas y sus tamaños relativos para nuestra área de estudio. Identificamos los insectos presa a partir de muestras de heces de los polluelos usando secuenciación de ADN de alto rendimiento y comparando las secuencias con nuestra base de datos de referencia personalizada. Los adultos de *H. rustica* alimentaron a los polluelos con presas provenientes de 130 familias pertenecientes a 13 órdenes, pero mostraron selección de presas más grandes que pertenecieron predominantemente a siete familias de dípteros. La dieta de los polluelos varió tanto dentro como entre estaciones de cría, así como entre sitios de cría. Esta flexibilidad en la dieta sugiere que *H. rustica* es capaz de ajustar su aprovisionamiento a una disponibilidad variable de presas en los sitios reproductivos cuando alimenta a sus polluelos. Nuestro estudio demuestra la utilidad de las bases de datos de referencia personalizadas para vincular la abundancia y el tamaño de los insectos presa del hábitat con las presas consumidas, cuando se emplean métodos moleculares para el análisis de las dietas.

Palabras clave: código de barras de ADN, dieta, *Hirundo rustica*, insectívoro aéreo, meta-código de barras

INTRODUCTION

Insects represent an abundant and diverse food source that is well utilized by avian predators (Morse 1971). Aerial-foraging insectivorous birds, in particular, take advantage of flying insects, a resource that cannot be used by many other predators since predation occurs on the wing (Nebel et al. 2010). Aerial insectivores are believed to typically be generalists that feed on a range of insect taxa (Morse 1971) although some prey selectivity has been observed (e.g. Brigham 1990, Csada et al. 1992). Dietary generalists are able to take advantage of fluctuating prey availability making them more resilient to changes in the food supply than specialists (McKinney 1997, Rutz and Bijlsma 2006). Knowledge of predator diets and their responses to fluctuating prey availability in changing environments is important to our understanding of food web structure as well as predator behavior and population dynamics. However, studying the predator-prey interactions between aerial insectivores and insects can be challenging since predation is hard to observe directly, and indirect observations, for example, through stomach and fecal contents, are often complicated by the variety and digestibility of prey items (Major 1990). Monitoring prey availability can also be difficult since insects are a diverse group that occupy various habitats and functional niches, and they are highly variable in their distribution across time and space (Kremen et al. 1993).

Molecular methods, notably including DNA barcoding, have been used more recently to facilitate non-invasive diet analyses for various species. DNA barcoding of animals uses the sequence from a standardized region of the mitochondrial genome—the 5' end of cytochrome *c* oxidase I (COI)—to taxonomically identify unknown specimens using a reference database of sequences from known specimens (Hebert et al. 2003). DNA barcoding can be used to identify large numbers of diverse specimens at high taxonomic resolution and requires less time and expertise than morphological identification (Hebert et al. 2003). DNA metabarcoding uses the same principles as DNA barcoding but makes use of high-throughput sequencing to sequence DNA from multiple specimens in a single sample (Pompanon et al. 2012). In the context of dietary analysis, this method can be used to sequence DNA from different prey items in a fecal sample. Sequences are identified by matching unknown prey sequences from the fecal samples to a reference database of known sequences. By sequencing prey items in the habitat and using these sequences as a custom reference database, we can link prey items in the diet to their availability in the habitat. DNA metabarcoding has been used successfully to study the diet of a variety of taxa, including insects (Ibanez et al. 2013), ungulates (Erickson et al. 2017), bats (Clare et al. 2014) and birds (Trevelline et al. 2016). For generalists that consume a variety of different prey species, this method can reveal a greater diversity of prey items than alternative methods of diet analysis (Bowser et al. 2013, Gerwing et al. 2016). Furthermore, DNA metabarcoding has higher sensitivity and taxonomic resolution compared with other methods of diet analysis (Zeale et al. 2011). This method cannot be used to differentiate life stages nor to reliably quantify prey items within a sample (Deagle et al. 2013). However, the frequency of occurrence across samples can be used as a semi-quantitative measure to compare how often predators are consuming different prey items (Bowser et al. 2013).

Barn Swallows are aerial insectivores with a worldwide distribution, but they have experienced population declines in many parts of their range, including North America (Nebel et al. 2010, Lee et al. 2011, Ambrosini et al. 2012, Vickery et al. 2014). In Canada, they have declined by more than 70% since 1980 (Environment Canada 2014). This is part of a guild-wide decline in aerial insectivores that represents the steepest decline seen in any ecological guild of birds in North America (NABCI 2012). While there have been some previous studies on Barn Swallow diet in North America (e.g. Beal 1918, Law et al. 2017), DNA metabarcoding has the potential to broaden our knowledge of their diet and improve comparisons between diet and prey availability. An understanding of the diet of Barn Swallows and how they respond to fluctuating prey availability in changing environments is important to our understanding of their population dynamics and is a necessary first step in determining whether declining prey population might be contributing to the decline in North American Barn Swallows.

Our objective was to use an integrated DNA barcoding method to investigate the diet and food supply of Barn Swallows (*Hirundo rustica*) during the breeding season in North America. Barn Swallows are altricial passerines whose young rely entirely on adult provisioning for their food, although the prey consumed by adults can differ from the prey fed to nestlings (Turner 2006). The period when Barn Swallows are feeding young in the nest is the most energetically demanding time during the breeding season, so our dietary analysis focused on prey fed to nestlings (Turner 1982). Based on previous work on the nestling diet of this species in Europe (Turner 1982, Orłowski and Karg 2013a,b), we hypothesized that Barn Swallows are generalist insectivores when provisioning their young and select prey based on size. We predicted that the diets of nestlings would contain a wide range of insect taxa but would be biased towards large prey items because these would maximize energy intake (Turner 1982), and that the occurrence of larger prey items in the diet would exceed their availability in the habitat. We also predicted that the

diet of nestlings would change as a function of time (both within and between seasons) and breeding sites to track changing insect availability across time and space.

METHODS

Study Site and Sample Collection

We studied Barn Swallow nestling diet at 10 breeding sites in Peterborough County and the City of Kawartha Lakes in Ontario, Canada (centre of study area: 44°18'N, 78°23'W). Each breeding site had between one and 25 nesting pairs (mean: 8.1 ± 1.48 SD) and consisted of a barn and associated buildings surrounded by a mixture of cropland, pasture and/or hayfields. At each site, nests were monitored every 2–3 days and active nests were identified. Barn Swallow nestlings begin defecating off the side of the nest as early as day 5 after hatching (Turner 2006) and all nestlings defecate off the side of the nest by day 12 after hatching (Brown and Brown 1999). We placed clean, plastic squares (~30 × 30 cm) below all active nests. We collected and pooled 3 fresh nestling fecal samples from each nest every 2 days from the plastic squares using flame-sterilized forceps, starting at day 8 after hatching and ending at fledging (date range: June 3–August 20, 2015; June 10–August 25, 2016). Pooled fecal samples represented multiple nestlings, but provisioning by one breeding pair. After sampling, we placed a new plastic square below each nest. Fecal samples were stored in 95% ethanol at −20°C until processing.

We used land and air Malaise traps with bottom collectors (BioQuip #2869 & #2892) to monitor insect abundance and diversity at each breeding site. At each site, we set up a trap 1 m above the ground and 15–30 m from the structure used for nesting. Barn Swallows usually forage <10 m in height and often <1 m from the ground (Brown and Brown 1999) and spend most of their time foraging within a few hundred m of the nest site (Møller 1987). Malaise traps paired with bottom collectors are effective at collecting flying insects from a range of taxa and should represent the diversity of prey available to Barn Swallows (Campos et al. 2000, Van Achterberg 2009). Collection bottles were placed on traps at dawn (sunrise ± 30 min) and removed at dusk (sunset ± 30 min). In 2015, traps were open for 2 days per wk at 9 sites and 6 days per wk at one site. We began collecting insects on May 20 at 2 sites and began collecting insects on June 1 at the remaining 8 sites. Collection continued at all sites until August 29. In 2016, traps were open for 2 days per wk at all sites and collection began on April 25 and continued through to August 29. Specimens were stored in 95% ethanol at −20°C until processing.

DNA Extraction, Amplification and Sequencing

Fecal samples were processed at the Canadian Centre for DNA Barcoding (CCDB) at the University of Guelph, Guelph, Ontario, Canada. The samples were homogenized by vortexing in a 15 mL tube containing 2 ceramic beads, followed by subsampling and extraction using one of 2 protocols. Both protocols used a manual plate-based method of DNA extraction modified from Ivanova et al. (2006). In the first protocol, the homogenate was dried to remove excess liquid and a subsample of ~100 µL was transferred to 1.5 mL tubes. To lyse samples, 350 µL of an insect lysis buffer, ProK, and 2% polyvinylpyrrolidone (PVP) mixture was added and samples were incubated at 56°C overnight. The whole lysate was mixed with 700 µL of binding mix and 850 µL of the mixture was transferred to a glass fiber plate. The plate was washed with 700 µL of protein wash buffer followed by 2 washes with 700 µL of wash buffer and then eluted in 40 µL of elution buffer. The second protocol was an updated extraction protocol by the CCDB to maximize DNA recovery, in which the entire homogenate was lysed using 5 mL of insect lysis buffer, ProK, and 2% PVP mixture and incubated at 56°C overnight. A 50 µL subsample of lysate was mixed with 100 µL of binding mix and transferred to a glass fiber plate. The plate was washed with 180 µL of protein wash buffer followed by 2 washes with 750 µL of wash buffer and then eluted in 40 µL of elution buffer. See supplementary material for a comparison of protocols.

A 157 base pair (bp) target region of the COI gene was amplified using primers described in Zeale et al. (2011). For each 96-well plate, the samples were uniquely tagged using a combination of 12 forward multiplex identifier (MID) tags and 8 reverse MID tags (Clarke et al. 2014). The PCR mixture consisted of 6.25 µL of 10% trehalose, 2 µL of dH_2O, 1.25 µL of 10X buffer, 0.625 µL 50 nM $MgCl_2$, 0.125 µL of 10µM of each of the forward and reverse primer, 0.0625 µL of 10 µM dNTP, 0.06 µL of Platinum Taq (5U/µL) and 2 µL of DNA. PCR conditions were as follows: 2 min at 94°C, followed by 60 cycles of 30 s at 94°C, 30 s at 53°C and 30 s at 72°C, followed by a final extension of 5 min at 72°C and then held at 10°C. Amplicons were visualized using 4 µL on an E-Gel (Invitrogen G700802, Thermo Fisher Scientific, Waltham, Massachusetts, USA).

PCR products for each plate were pooled and amplicons were purified using a magnetic bead protocol outlined in Prosser and Hebert (2017) using double-size selection to purify for the target amplicon length (~284 bp). The cleaned product was quantified using a Qubit 2.0 fluorometer (Invitrogen Q32866, Thermo Fisher Scientific) and adjusted to 1 ng μL^{-1}. The sequencing library was prepared by templating and enriching with the Ion OneTouch 2 System (Ion 4474779, Thermo Fisher Scientific). The library was sequenced using a 316 v.2 chip on an Ion Torrent PGM (Ion 4462921, Thermo Fisher Scientific) following the manufacturer's instructions.

Custom Reference Library

To create a custom reference database of insect prey available to Barn Swallows, we sorted insect specimens collected in Malaise traps to morphospecies (identified by B. McClenaghan; Oliver and Beattie 1996). We measured the body length to the nearest 0.1 mm (excluding appendages such as antennae or cerci) of up to 5 randomly chosen specimens of each morphospecies if available. A representative of each morphospecies was identified using DNA barcoding. Specimens were processed at the CCDB according to their standardized extraction, amplification and unidirectional sequencing protocols (see Ivanova et al. 2006, deWaard et al. 2008). The resulting sequences were used to identify the specimens via the BOLD Identification System (http://www.boldsystems.org/; Ratnasingham and Hebert 2007) Taxonomic identifications were assigned at 98% similarity to the lowest possible taxonomic level that did not have conflicting taxonomy. These sequences were subsequently used as reference sequences to identify unknown specimens from the fecal samples. Matching individual specimens in the diet to specimens in the habitat would typically require species-level resolution. Developing a custom reference database allowed us to match sequences from prey items consumed by Barn Swallows to those from specimens collected in traps and their associated abundance and size, even when sequence identification at high taxonomic resolution was not possible. This in turn allowed us to infer the size of prey items detected in fecal samples. For any insect specimens that did not sequence successfully or did not match a reference sequence on BOLD, we used photos to identify the specimens to order and, where possible, to family, using Marshall (2006).

Taxonomic Identification

For each sequencing run, the reads were separated into 12 subsets by forward MID tag using the Ion Torrent Server and those subsets were then separated into 96 samples by reverse MID tag using Galaxy (https://usegalaxy.org/). The primer and adapter sequences were trimmed using Cutadapt (Martin 2011). Reads were filtered based on quality (minimum quality score of 20) and length (minimum length of 100 bp) using Sickle (github.com/ucdavis-bioinformatics/sickle). The filtered reads were dereplicated to remove duplicated reads using FASTX Collapser (http://hannonlab.cshl.edu/fastx_toolkit/index.html) and clustered into operational taxonomic units (OTUs) at 97% using UPARSE (Edgar 2013). OTUs with infrequent haplotypes (<10 reads) were excluded from downstream analysis. Taxonomic identification was assigned to each OTU using a BLAST search against our custom reference database. Species-level identifications were assigned at a minimum of 99.3% sequence similarity and genus-level identifications were assigned at a minimum of 95% based on Zeale et al. (2011). Any sequences that did not match a reference sequence in the custom reference database were identified by performing a BLAST search against all Canadian animal sequences in the BOLD database (downloaded December 2016). Spurious identifications, including sequences identified as non-arthropod (e.g., algae, nematodes), were omitted from subsequent analysis.

Data Analysis

Data analysis was conducted using R Studio 1.0.136. Amplicon read counts do not reliably reflect abundances of prey items in fecal samples (Deagle et al. 2013), therefore, arthropod sequences were considered as present or absent within each fecal sample. We calculated frequency of occurrence (FOO) of prey families and of inferred prey size classes in the diet based on presence/absence across our samples (e.g., if a taxon was present in 10 of 100 samples, the frequency would be 0.10). To calculate the frequency of occurrence of different prey sizes, we used only the prey items identified using our custom reference database because they were associated with voucher specimens whose length was measured. We used the package *mvabund* (Wang et al. 2017) to model the change in the family-level prey community in the diet over time and by site using multivariate generalized linear models (GLMs). Site, year and brood were included as categorical predictor variables and significant effects were determined using the *anova.manyglm* function with $\alpha = 0.05$. We ran the same models for the family-level prey community available in the habitat. Families whose frequency of occurrence in the diet changed significantly with site, year or brood were identified using univariate tests in the *anova.manyglm* function. For these families, we compared the change in frequency of occurrence in the diet to the change in availability in the habitat between sites, years and broods.

RESULTS

Custom Reference Library

We collected over 60,000 insect specimens over 2 breeding seasons. Of the 7,600 specimens submitted for sequencing, we recovered sequences from 87.4% of specimens, which were then used as our custom reference library. Of these sequences, 99.1% were identified to the level of order, 97.5% to the level of family, 71.7% to the level of genus, and 42.0% to species-level. In total, we identified over 1,000 species from 262 different families. All sequences are publicly available on BOLD under the dataset DS-AABARS.

Prey Identity

DNA was successfully extracted and amplified from 271 fecal samples (129 from 2015, 142 from 2016) out of a total

281 samples. We recovered 12,479,453 reads representing 472 unique taxa (see Supplementary Table S1 for detailed sequencing results). Overall, 88.8% of OTUs matched a reference sequence and, of these, 53.1% matched a reference sequence in our custom reference database. Of the OTUs that successfully matched a reference sequence, 100% identified to the order level, 98.5% identified to the family level, 82.8% identified to the genus-level and 14.5% identified to the species-level. Family-level identifications represented the highest level of taxonomic resolution where a high proportion of OTUs were identified so we performed subsequent analyses at the family level. We identified 130 arthropod families in the diet from 13 different orders. The order most frequently detected in nestling diet was Diptera (FOO: 1.00), the most frequently detected family was Tachinidae (FOO: 0.57), and the most frequently detected species was *Pollenia pediculata* (Calliphoridae; FOO: 0.074), a species of cluster fly that is parasitic on earthworms as a larva and whose winged adult form is a pollinator (Jewiss-Gaines et al. 2012). Many human and livestock pests were frequently fed to nestlings as prey, such as mosquitoes (Culicidae; FOO: 0.28), horseflies (Tabanidae; FOO: 0.34) and stable flies (Muscidae; FOO: 0.35). Agricultural crop pests, such as alfalfa pest *Hypera postica* (Curculionidae; FOO: 0.03) and the tarnished plant bug *Lygus lineolaris* (Miridae; FOO: 0.01) were also fed to nestlings but at a lower frequency. Out of the 472 unique prey items we identified, 35 were identified as pests (Supplementary Table S2). Additionally, Barn Swallow nestlings consumed specialized nest parasites of the nestlings themselves, *Protocalliphora* sp.

Prey Selection

The prey families that were fed to nestlings most frequently represented a small proportion of the prey available in the habitat (Figure 1). Prey families that were most available in the habitat were also fed to nestlings but not at high frequency (Figure 1). Small aerial insects (<3 mm in length) represented most of the prey available in the habitat (76.3%) but species in these length classes occurred infrequently in nestling diets (mean FOO: 6.8%). In contrast, larger aerial insects (>3 mm in length) represented a much smaller proportion of the available prey (23.7%) but species in these length classes were detected more frequently in nestling diets (mean FOO: 16.7%; Figure 2). The 3 mm length threshold divided prey items that were consumed at a lower frequency of occurrence compared with the proportion of prey in the habitat they represented (< 3 mm in length) compared with prey items that were generally found at higher frequency of occurrence in the diet compared with the proportion of prey they represented in the habitat (>3 mm; Figure 2). The inferred size of prey items detected in the diet ranged from 1.1 to 20.3 mm (mean: 4.9 ± 3.2 mm SD) in length while prey items available in the habitat ranged from 0.1 to 38.9 mm (mean: 3.4 ± 2.6 mm SD) in length.

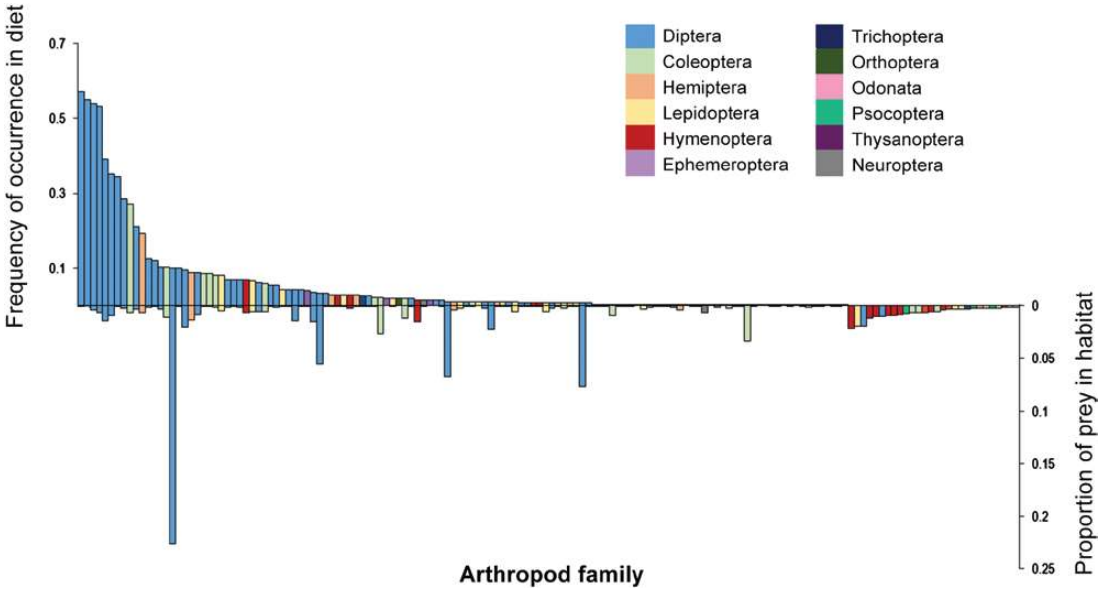

FIGURE 1. The frequency of occurrence of different arthropod families in the diet of Barn Swallow nestlings compared to the availability of these arthropod families in the habitat presented as a proportion of the total prey available in the habitat. Each bar on the x-axis represents a different family and bars are colored by order. The 5 most frequently consumed families were Tachinidae, Tipulidae, Limoniidae, Calliphoridae and Anthomyiidae. The 5 most abundant insect families in the habitat were Chironomidae, Sciaridae, Cecidomyiidae, Ceratopogonidae and Staphylinidae.

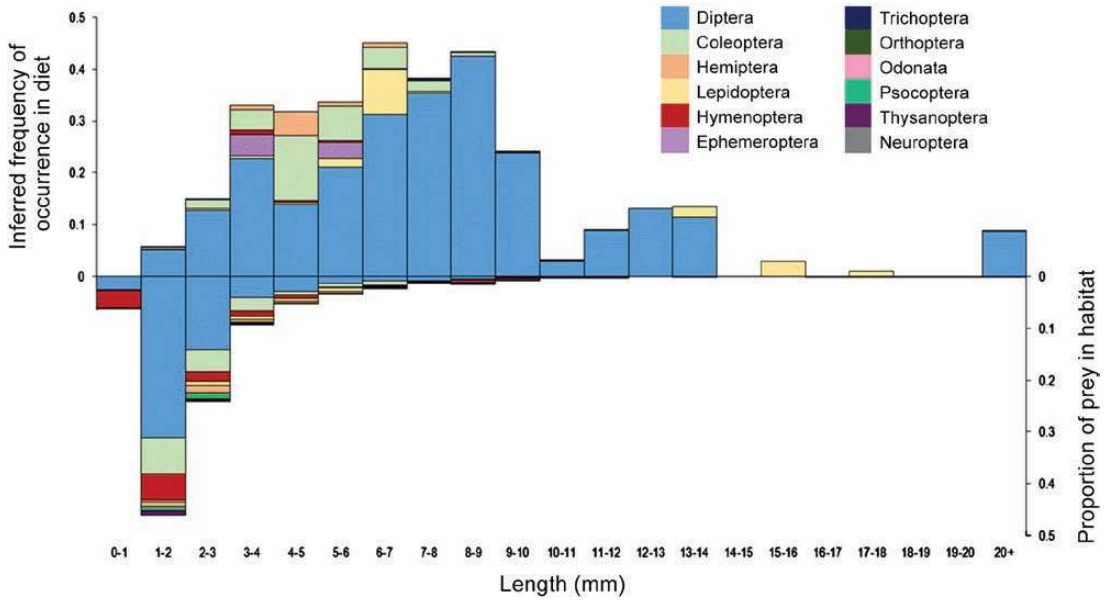

FIGURE 2. The frequency of occurrence of different sizes of arthropod in the diet of Barn Swallow nestlings compared to the availability of these sizes of arthropod prey in the habitat presented as a proportion of the total prey available in the habitat. Bars are colored to show the proportion of operational taxonomic units (OTUs) in each size class represented by each order.

TABLE 1. Summary of multivariate generalized linear models (GLMs) of the prey community in the diet of Barn Swallow nestlings and of the available prey community in the habitat.

Predictor	Residual Degrees of Freedom	Deviance	*P*-Value
Diet			
Site	261	1054.7	0.001
Brood	260	163	0.013
Year	259	162.4	0.05
Site: Brood	250	447.2	0.001
Site: Year	242	561.8	0.001
Brood: Year	241	95.2	0.001
Site: Brood: Year	235	178.7	0.001
Habitat			
Site	231	5225	0.001
Brood	230	1447	0.001
Year	229	431	0.001
Site: Brood	220	1744	0.001
Site: Year	211	3660	0.001
Brood: Year	210	290	0.001
Site: Brood: Year	201	1244	0.001

Dietary Flexibility

Site, brood, year and their interactions were all significant predictors of variation in the diet composition of nestling Barn Swallow and prey availability in the habitat (Table 1). For both the diet and habitat models, site was the predictor that explained most of the variance. Thus, Barn Swallow nestlings were fed different prey items over time and at different sites as prey availability also changed temporally and among sites (Supplementary Tables S3 and S4). The following 8 families in the order Diptera were consistently fed to nestlings at high frequency across sites, broods and years: Tachinidae (FOO: 0.57), Tipulidae (0.55), Limoniidae (0.54), Calliphoridae (0.53), Anthomyiidae (0.39), Muscidae (0.35), Tabanidae (0.34) and Culicidae (0.28). The differences in nestling diet between sites and times were mostly driven by changes in less frequently consumed families. The change in diet we observed between sites and years reflected changes in prey availability between sites and years (Supplementary Tables S3 and S4). Most prey families were fed to nestlings more frequently when they were more abundant in the habitat. For example, Noctuidae was consumed more frequently at breeding sites where it was more abundant in the habitat and Nitidulidae was consumed more frequently in 2015 when it was more abundant. By contrast, the change in diet between the first and second broods did not mirror changes in prey availability in the habitat. Most insect families that differed in frequency of occurrence in the diet between broods were fed to nestlings more frequently in the second brood, despite greater abundance in the habitat during the first brood (Supplementary Tables S3 and S4). For example, Chironomidae was more abundant during the first brood but was fed to nestlings at higher frequency during the second brood. The most frequently provisioned prey families listed above were also more abundant in the habitat during the time that Barn Swallows were feeding their first brood. These frequently provisioned prey families decreased in abundance during the time that Barn Swallows had their second brood, when Barn Swallow nestlings were fed alternative prey at a higher frequency.

DISCUSSION

DNA metabarcoding allowed us to identify the broad taxonomic range of prey items that were fed to nestlings at higher resolution than previous studies of Barn Swallow diet. We identified most prey items to the genus-level (82.6%) and some to the species-level (14.6%). These levels of identification often cannot be reached using other methods of diet analysis, such as the visual identification of prey remains from fecal or stomach samples. Due to this high taxonomic resolution and the sensitivity of this method, we detected many prey items that were previously unreported in nestling or adult Barn Swallow diet (e.g., Fanniidae, Drosophilidae, Limoniidae, Cleridae, Ptinidae and Scirtidae; Beal 1918, Orłowski and Karg 2011, 2013b, Orłowski et al. 2014). This broad range of prey items includes species from across foraging guilds and trophic levels, from terrestrial and aquatic environments, and includes multiple pest species. Barn Swallow nestling diets also contained blowfly nest parasites, but we were unable to determine whether the parasitic larvae were being eaten by nestlings directly or whether adults were provisioning the larvae or adult flies. Blowfly parasites have been detected in the diet of other species of insectivorous birds using molecular methods and similarly life stage could not be determined (Jedlicka et al. 2017).

The presence of human, livestock and agricultural pests in the diet suggests that Barn Swallows can provide an ecosystem service by reducing the number of these pests in agricultural systems. Previous studies have also used DNA barcoding to link bird-insect interactions to pest reduction in agro-ecosystems (Crisol-Martínez et al. 2016; Jedlicka et al. 2017). This provides an economic incentive for understanding and mitigating the decline in Barn Swallows.

By simultaneously using DNA barcoding for diet analysis and to identify insects collected in the habitat, we observed evidence for a degree of prey selection. While the diet of Barn Swallow nestlings included a taxonomically broad variety of insects, they were being fed what we inferred to be larger prey items more frequently relative to their abundance in the habitat compared with smaller prey items (Figure 2). These results support our hypothesis that, as insectivores, Barn Swallows are generalists with respect to taxonomy but select prey based on size. Larger prey items are more profitable prey for Barn Swallows to capture (Turner 1982), yet we also observed evidence suggesting that Barn Swallows provisioned nestlings with taxa that had relatively small body sizes in the habitat. In Scotland, Barn Swallows ate more small prey items than would be predicted by optimal foraging theory (Turner 1982). Optimal foraging theory may not apply well to Barn Swallows since they feed on a diverse array of mobile prey; this theory applies better to systems where there is a low number of available prey types (Korpimaki 1986) and may not apply well to foragers that feed on highly mobile prey (Sih and Christensen 2001). We detected prey selection due to our ability to match prey items in the diet with specimens from the habitat. The size of prey items cannot be determined directly when relying on molecular methods for diet analysis. However, by using a comprehensive reference library that included associated specimen information, we could infer the size of prey items indirectly. This would be difficult to achieve using DNA metabarcoding without a custom reference database unless species-level taxonomic resolution was achieved for most prey items, which was not the case in this study.

We demonstrated that the diet of nestling Barn Swallows varied significantly over time and between sites as predicted (Table 1). This variation in diet suggests that Barn Swallow adults can respond to changing prey availability and take advantage of fluctuating and unpredictable prey resources. The variation in diet between sites and years appears to be opportunistic, where Barn Swallows are provisioning more of certain prey items when they are more available in the habitat, as seen in other insectivorous species (e.g., Rodenhouse and Holmes 1992, Trevelline et al. 2018). For the most part, the variation in diet matched the yearly and site-specific variation in prey availability (Supplementary Tables S3 and S4). By contrast, the change in diet between broods did not mirror the change in prey availability. Instead, the change in diet between broods could be driven by a reduced availability of preferred prey items during the second brood. Barn Swallows may have needed to include more alternative prey during the second brood as their main prey source declined. Seasonal changes in diet are observed in other insectivore species which also show a temporal change in their selectivity (Agosta et al. 2003). While swallows are flexible in their diet, prey items vary in quality in terms of the energy and other nutrients they provide (e.g., fatty acids) as well as digestibility (Bell 1990, Twining et al. 2016). Future studies should investigate the effects of food quality on the reproduction and survival of Barn Swallows throughout the breeding and post-breeding season.

We have demonstrated that Barn Swallows are generalist insectivores while provisioning their young, and that their diet changes over time and space, which suggests they should be resilient to a changing food supply (McKinney 1997, Dennis et al. 2011). For generalist insectivores, total prey abundance is thought to be a more important driver of productivity than taxa-specific abundance (Durst et al. 2008). If the decline in the Barn Swallow population is related to the food supply, it would, most likely, be due to a broad-scale decline in insect prey abundance across taxa, rather than changes in the availability of specific prey

groups, for example, due to phenological shifts (Both et al. 2006).

While we were successful in using DNA barcoding for this study, there are limitations in the use of this method to identify prey items from fecal samples. DNA metabarcoding cannot be used as a quantitative method due to a variety of biological and technical factors, including the digestibility of prey items, digestion rates, and primer bias during PCR (Pompanon et al. 2012). We used frequency of occurrence as a semi-quantitative measure which applies well to the large sample sizes that we processed. Primer bias can also lead to the overrepresentation of certain taxa in sequencing results (Pompanon et al. 2012). The PCR primers that we selected for our study have since shown evidence of bias towards Diptera and Lepidoptera (Alberdi et al. 2017). Thus, primer bias may have contributed to the high FOO of Diptera in our results. However, the size selection we observed holds true within Diptera (i.e. large Diptera had higher FOO than small Diptera) suggesting that impacts of such a bias on our prey selection conclusions would be minimal.

Some insects identified from the fecal samples may not have represented prey items consumed by Barn Swallows. We collected fresh fecal samples from squares below nests, but there is a possibility that some insects came into contact with the sampling board or the fecal sample prior to sample collection. Many of the frequently consumed taxa are not taxa that are especially attracted to bird feces, so any non-prey items that we detected were likely at low FOO. One possible exception to this would be Muscidae, which includes flies known to be attracted to bird feces, which was detected at relatively high FOO (35.1%). Additionally, the detection of secondary consumption, where the predator of interest consumes another predator that contained prey in its guts, is a possibility when using DNA metabarcoding for diet analysis (Pompanon et al. 2012, Gerwing et al. 2016). Some of the taxa we detected may result from secondary consumption but the most frequently consumed prey items we detected are not predatory on insects as adults, so this bias is likely to be minimal.

DNA barcoding is very dependent on the quality of the reference database (Joly et al. 2014). A comprehensive reference database is required for unknown sequences to match a sequence in the database, and even if there is a match, the level of identification of unknown specimens can be limited by the level of taxonomic identification of the reference sequence. While we used our own custom reference database generated by DNA barcoding insect specimens, our reference library was limited by the success of DNA sequencing and the level of identification of these specimens. Approximately 83% of the insect specimens that we collected in the habitat were identified via DNA barcoding and not all of these specimens were identified to a low taxonomic level (i.e. genus or species-level). By integrating traditional and molecular taxonomic knowledge to expand reference databases, identification success and the utility of reference databases could be improved.

Most, but not all, sequences from the fecal samples were identified using our custom reference database (53.1% of OTUs were identified to at least the order level), indicating that some prey items were collected but were not successfully sequenced or that some prey items were not collected in our Malaise traps. The landscape surrounding the breeding sites was heterogeneous and prey resources may have displayed a patchy distribution with local abundances in microhabitats (e.g., hedgerows and mating swarms; Downes 1969, Lewis 1969) that were not reflected in our Malaise trap catch. Additionally, swallows typically forage within a few hundred m of the nest site and a single Malaise trap could not capture the entire diversity of prey in this area. Operating multiple Malaise traps around the breeding site could improve measures of prey availability, but logistically this was not possible for this study due to time constraints. Malaise traps can underrepresent certain taxa, such as microhymenopterans, but there is no evidence of bias against the preferred prey families Barn Swallows consumed frequently (Darling and Packer 1988).

We opted for a workflow that maximized the amount of DNA recovered and maximized the number of samples we could analyze. To achieve this, we ran our PCRs with more cycles than is typical, which increased the potential for chimeras. The bioinformatic pipeline did not control for chimeras; however, these were likely minimized by including only matches with a minimum of 95% similarity and a minimum overlap of 100 bp. With our target amplicon length of 157 bp, ~66% of the read had to match with high identity to the reference and it is unlikely that a chimeric read would match well. Secondly, we did not run multiple PCRs for each fecal sample. This has been suggested in the literature (Alberdi et al. 2017) as a way to capture more of the prey diversity in a given sample. Often, there is a tradeoff between running multiple PCRs for each sample and the number of samples that can be processed due to monetary restrictions. We opted to run more samples, giving us a larger sample size, which is better for making conclusions based on FOO.

We made use of an integrated DNA barcoding method to determine the diet of Barn Swallow nestlings and simultaneously monitor prey abundance. We found that Barn Swallows were generalist insectivores when provisioning their young but showed selection for larger prey items primarily from the order Diptera. Barn Swallow nestling diet changed in response to changing prey conditions, suggesting that Barn Swallow adults can obtain food when overall insect abundance is sufficiently high, even if their preferred prey is not available in high quantities. This information

expands our knowledge of Barn Swallow biology in North America and can be used to determine if prey availability is changing on the breeding grounds and how prey availability affects survival and reproduction. This integrated DNA barcoding method using a custom reference database of the prey available in the local area can be applied to other generalist aerial insectivores with hard to monitor prey populations.

ACKNOWLEDGMENTS

We thank the many private landowners who gave us access to their properties and allowed us to complete this project. Sean Prosser at the Canadian Centre for DNA Barcoding assisted with lab work and data analysis associated with high-throughput sequencing. We thank Joe Nocera, David Beresford, Aaron Schafer, Sabrina Taylor, Brian Trevelline and one anonymous reviewer for comments on earlier drafts of this manuscript.

Funding statement: Project funding was provided by the Species at Risk Research Fund of Ontario (Ontario Ministry of Natural Resources and Forestry), the Purple Martin Conservation Association, the Toronto Entomologists' Association, the Toronto Zoo's Endangered Species Reserve Fund and Trent University. B.M. was supported by an Industrial Postgraduate Scholarship from the Natural Sciences and Engineering Research Council of Canada in partnership with the Toronto Zoo.

Ethics statement: This study was conducted on private land for which we had permission to access. This study did not involve the direct handling of or disturbing Barn Swallows and therefore did not require animal care approval.

Author contributions: B.M. conducted data collection, performed data analysis, participated in the design of the study and drafted the manuscript; E.N. and K.C.R.K. designed the study, coordinated the study and helped draft the manuscript. All authors gave final approval for publication.

Data deposits: The sequencing results from Malaise-trapped insects can be found on BOLD in the dataset DS-AABARS (doi: 10.5883/DS-AABARS) and the sequences from high-throughput sequencing can be found on Dryad (doi: 10.5061/dryad.4r50gb0). Additional datasets supporting this article are included in the supplementary material.

LITERATURE CITED

Agosta, S. J., D. Morton, and K. M. Kuhn (2003). Feeding ecology of the bat *Eptesicus fuscus*: "Preferred" prey abundance as one factor influencing prey selection and diet breadth. Journal of Zoology 260:169–177.

Alberdi, A., O. Aizpurua, M. T. P. Gilbert, and K. Bohmann (2017). Scrutinizing key steps for reliable metabarcoding of environmental samples. Methods in Ecology and Evolution 9:134–147.

Ambrosini, R., D. Rubolini, P. Trovò, G. Liberini, M. Bandini, A. Romano, B. Sicurella, C. Scandolara, M. Romano, and N. Saino (2012). Maintenance of livestock farming may buffer population decline of the Barn Swallow *Hirundo rustica*. Bird Conservation International 22:411–428.

Beal, F. E. L. (1918). Food habits of the swallows: A family of valuable native birds. United States Department of Agriculture Bulletin 619.

Bell, G. P. (1990). Birds and mammals on an insect diet: A primer on diet composition analysis in relation to ecological energetics. In Avian Foraging: Theory, Methodology, and Applications (M. L. Morrison, C. J. Ralph, J. Verner, J. R. Jehl, Editors). Studies in Avian Biology 13:416–422.

Both, C., S. Bouwhuis, C. M. Lessells, and M. E. Visser (2006). Climate change and population declines in a long-distance migratory bird. Nature 444:81–96.

Bowser, A. K., A. W. Diamond, and J. A. Addison (2013). From puffins to plankton: A DNA-based analysis of a seabird food chain in the Northern Gulf of Maine. PLoS ONE 8:1–16.

Brigham, R. M. (1990). Prey selection by big brown bats (*Eptesicus fuscus*) and Common Nighthawks (*Chordeiles minor*). American Midland Naturalist 124:73–80.

Brown, C. R., and M. B. Brown (1999). Barn Swallow (*Hirundo rustica*). In The Birds of North America (A. F. Poole and F. B. Gill, Editors). Cornell Lab of Ornithology, Ithaca, NY.

Campos, W. G., D. B. S. Pereira, and J. H. Schoereder (2000). Comparison of the efficiency of flight-interception trap models for sampling Hymenoptera and other insects. Anais de Sociedade Entomologica do Brasil 29:381–389.

Clare, E. L., W. O. C. Symondson, and M. B. Fenton (2014). An inordinate fondness for beetles? Variation in seasonal dietary preferences of night-roosting big brown bats (*Eptesicus fuscus*). Molecular Ecology 23:3633–3647.

Clarke, L. J., P. Czechowski, J. Soubrier, M. I. Stevens, and A. Cooper (2014). Modular tagging of amplicons using a single PCR for high-throughput sequencing. Molecular Ecology Resources 14:117–121.

Crisol-Martínez, E., L. T. Moreno-Moyano, K. R. Wormington, P. H. Brown, and D. Stanley (2016). Using next-generation sequencing to contrast the diet and explore pest-reduction services of sympatric bird species in macademia orchards in Australia. PLoS ONE 11:e0150159.

Csada, R. D., R. M. Brigham, and B. R. Pittendrigh (1992). Prey selection in relation to insect availability by the Common Poorwill (*Phalaenoptilus nuttallii*). Canadian Journal of Zoology 70:1299–1303.

Darling, D. C., and L. Packer (1988). Effectiveness of Malaise traps in collecting Hymenoptera: The influence of trap design, mesh size, and location. Canadian Entomologist 120:787–796.

Deagle, B. E., A. C. Thomas, A. K. Shaffer, A. W. Trites, and S. N. Jarman (2013). Quantifying sequence proportions in a DNA-based diet study using Ion Torrent amplicon sequencing: Which counts count? Molecular Ecology Resources 13:620–633.

Dennis, R. L. H., L. Dapporto, S. Fattorini, and L. M. Cook (2011). The generalism-specialism debate: The role of generalists in the life and death of species. Biological Journal of the Linnean Society 104:725–737.

deWaard, J. R., N. V. Ivanova, M. Hajibabaei, and P. D. N. Hebert (2008). Assembling DNA barcodes: Analytical protocols. In Environmental Genomics (C. Martin, Editor). Humana Press, Totawa, pp. 275–293.

Downes, J. A. (1969). The swarming and mating flight of Diptera. Annual Review of Entomology 14:271–298.

Durst, S. L., T. C. Theimer, E. H. Paxton, and M. K. Sogge (2008). Age, habitat, and yearly variation in the diet of a generalist insectivore, the Southwestern Willow Flycatcher. The Condor 110:514–525.

Edgar, R. C. (2013). UPARSE: Highly accurate OTU sequences from microbial amplicon reads. Nature Methods 10:996–998.

Environment Canada (2014). North American Breeding Bird Survey - Canadian Trends Web Site, Data-Version 2012. http://www.ec.gc.ca/ron-bbs/.

Erickson, D. L., E. Reed, P. Ramachandran, N. A. Bourg, W. J. McShea, and A. Ottesen (2017). Reconstructing a herbivore's diet using a novel rbcL DNA mini-barcode for plants. AoB Plants 9:plx015.

Gerwing, T. G., J.-H. Kim, D. J. Hamilton, M. A. Barbeau, and J. A. Addison (2016). Diet reconstruction using next-generation sequencing increases the known ecosystem usage by a shorebird. The Auk: Ornithological Advances 133:168–177.

Hebert, P. D. N., A. Cywinska, S. L. Ball, and J. R. DeWaard (2003). Biological identifications through DNA barcodes. Proceedings of the Royal Society: Biological Sciences 270:313–321.

Ibanez, S., O. Manneville, C. Miquel, P. Taberlet, A. Valentini, S. Aubert, E. Coissac, M.-P. Colace, Q. Duparc, S. Lavorel, and M. Moretti (2013). Plant functional traits reveal the relative contribution of habitat and food preferences to the diet of grasshoppers. Oecologia 173:1459–1470.

Ivanova, N. V., J. R. deWaard, and P. D. N. Hebert (2006). An inexpensive, automation-friendly protocol for recovering high-quality DNA. Molecular Ecology Notes 6:998–1002.

Jedlicka, J. A., A.-T. E. Vo, and R. P. P. Almeida (2017). Molecular scatology and high-throughput sequencing reveal predominately herbivorous insects in the diets of adult and nestling Western Bluebirds (*Sialia mexicana*) in California vineyards. The Auk: Ornithological Advances 134:116–127.

Jewiss-Gaines, A., S. A. Marshall, and T. L. Whitworth (2012). Cluster flies (Calliphoridae: Polleniinae: *Pollenia*) of North America. Canadian Journal of Arthropod Identification 19:1–19.

Joly, S., T. J. Davies, A. Archambault, A. Bruneau, A. Derry, S. W. Kembel, P. Peres-Neto, J. Vamosi, and T. A. Wheeler (2014). Ecology in the age of DNA barcoding: The resource, the promise and the challenges ahead. Molecular Ecology Resources 14:221–232.

Korpimaki, E. (1986). Diet variation, hunting habitat and reproductive output of the kestrel *Falco tinnunculus* in the light of the optimal diet theory. Ornis Fennica 63:84–90.

Kremen, C., R. K. Colwell, T. L. Erwin, D. D. Murphy, R. F. Noss, and M. A. Sanjayan (1993). Arthropod assemblages: Their use in conservation planning. Conservation Biology 7:796–808.

Law, A. A., M. E. Threlfall, B. A. Tijman, E. M. Anderson, S. McCann, G. Searing, and D. Bradbeer (2017). Diet and prey selection of Barn Swallows (*Hirundo rustica*) at Vancouver International Airport. Canadian Field Naturalist 131:26–31.

Lee, S.-D., E. R. Ellwood, S. Park, and R. B. Primack (2011). Late-arriving Barn Swallows linked to population declines. Biological Conservation 144:2182–2187.

Lewis, T. (1969). The distribution of flying insects near a low hedgerow. Journal of Applied Ecology 6:443–452.

Major, R. E. (1990). Stomach flushing of an insectivorous bird: An assessment of differential digestibility of prey and the risk to birds. Australian Wildlife Research 17:647–657.

Marshall, S. A. (2006). Insects: Their Natural History and Diversity. Firefly Books, Buffalo, NY, USA.

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet Journal 17:10–12.

McKinney, M. L. (1997). Extinction vulnerability and selectivity : Combining ecological and paleontological views. Annual Review of Ecology and Systematics 28:495–516.

Møller, A. P. (1987). Advantages and disadvantages of coloniality in the swallow, *Hirundo rustica*. Animal Behaviour 35:819–832.

Morse, D. H. (1971). The insectivorous bird as an adaptive strategy. Annual Review of Ecology and Systematics 2:177–200.

NABCI (2012). The state of Canada's birds.

Nebel, S., A. Mills, J. D. McCracken, and P. D. Taylor (2010). Declines of aerial insectivores in North America follow a geographic gradient. Avian Conservation and Ecology 5:1.

Oliver, I., and A. J. Beattie (1996). Invertebrate morphospecies as surrogates for species: A case study. Conservation Biology 10:99–109.

Orłowski, G., and J. Karg (2011). Diet of nestling Barn Swallows *Hirundo rustica* in rural areas of Poland. Central European. Journal of Biology 6:1023–1035.

Orłowski, G., and J. Karg (2013a). Partitioning the effects of livestock farming on the diet of an aerial insectivorous passerine, the Barn Swallow *Hirundo rustica*. Bird Study 60:111–123.

Orłowski, G., and J. Karg (2013b). Diet breadth and overlap in three sympatric aerial insectivorous birds at the same location. Bird Study 60:475–483.

Orłowski, G., J. Karg, and G. Karg (2014). Functional invertebrate prey groups reflect dietary responses to phenology and farming activity and pest control services in three sympatric species of aerially foraging insectivorous birds. PLOS One 9:e114906.

Pompanon, F., B. E. Deagle, W. O. C. Symondson, D. S. Brown, S. N. Jarman, and P. Taberlet (2012). Who is eating what: Diet assessment using next generation sequencing. Molecular Ecology 21:1931–50.

Prosser, S. W. J., and P. D. N. Hebert (2017). Rapid identification of the botanical and entomological sources of honey using DNA metabarcoding. Food Chemistry 214:183–191.

Ratnasingham, S., and P. D. N. Hebert (2007). BOLD : The Barcode of Life Data System (www.barcodinglife.org). Molecular Ecology Notes 7:355–364.

Rodenhouse, N. L., and R. T. Holmes (1992). Results of experimental and natural food reductions for breeding Black-throated Blue Warblers. Ecology 73:357–372.

Rutz, C., and R. G. Bijlsma (2006). Food-limitation in a generalist predator. Proceedings of the Royal Society B: Biological Sciences 273:2069–2076.

Sih, A., and B. Christensen (2001). Optimal diet theory: When does it work, and when and why does it fail? Animal Behaviour 61:379–390.

Trevelline, B. K., T. Nuttle, B. A. Porter, N. L. Brouwer, B. D. Hoenig, Z. D. Steffensmeier, and S. C. Latta (2018). Stream acidification and reduced aquatic prey availability are associated with dietary shifts in an obligate riparian Neotropical migratory songbird. PeerJ 6:e5141.

Trevelline, B. K., S. C. Latta, L. C. Marshall, T. Nuttle, and B. A. Porter (2016). Molecular analysis of nestling diet in a long-distance Neotropical migrant, the Louisiana Waterthrush (*Parkesia motacilla*). The Auk: Ornithological Advances 133:415–428.

Turner, A. (2006). The Barn Swallow. T & A D Poyser, London, UK.

Turner, A. K. (1982). Optimal foraging by the swallow (*Hirundo rustica*, L): Prey size selection. Animal Behaviour 30:862–872.

Twining, C. W., J. T. Brenna, P. Lawrence, J. R. Shipley, T. N. Tollefson, and D. W. Winkler (2016). Omega-3 long-chain polyunsaturated fatty acids support aerial insectivore performance more than food quantity. Proceedings of the National Academy of Sciences USA 113:10920–10925.

Van Achterberg, K. (2009). Can Townes type Malaise traps be improved? Some recent developments. Entomologische Berichten 69:129–135.

Vickery, J. A., S. Ewing, K. W. Smith, D. J. Pain, F. Bairlein, J. Škorpilová, and R. D. Gregory (2014). The decline of Afro-Palearctic migrants and an assessment of potential causes. Ibis 156:1–22.

Wang, Y., U. Naumann, S. Wright, D. Eddelbuettel, and D. Warton (2017). mvabund: Statistical methods for analysing multivariate abundance data. https://rdrr.io/cran/mvabund/man/mvabund-package.html

Zeale, M. R. K., R. K. Butlin, G. Barker, D. C. Lees, and G. Jones (2011). Taxon-specific PCR for DNA barcoding arthropod prey in bat faeces. Molecular Ecology Resources 11:236–244.