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Identity and characteristics of feathers used as lining in Tree Swallow (*Tachycineta bicolor*) nests in Indiana and Ohio

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ABSTRACT—Nest building represents an important part of parental investment and can significantly impact reproductive success in many bird species. Tree Swallows (*Tachycineta bicolor*) are secondary cavity nesters that readily accept nest boxes and construct basic grass nest cups lined with molted feathers from other bird species. We identified and described the characteristics of the feathers used as nesting materials by Tree Swallows in 4 different habitats in Indiana and Ohio. We monitored 41 nest boxes in 2017 and 53 nest boxes in 2018. After chicks fledged, we collected feathers from nests, counted them, and characterized them by size, color, and type. Brown, medium length (5–10 cm), and contour feathers were significantly more common than other types of feathers. We compared the prevalence and characteristics of feathers in nests across different habitat types, with results showing that nests located near lakes had significantly more feathers than those in agricultural habitats. We identified which species feathers belonged to using molecular techniques and morphological comparisons and found that they belonged to 26 species from 19 families and 11 orders: Galliformes, Anseriformes, Columbiformes, Gruiformes, Charadriiformes, Pelecaniformes, Accipitriformes, Strigiformes, Piciformes, Cathartiformes, and Passeriformes, including 20 previously unreported species within those orders. Thus, feathers used as nest lining by Tree Swallows are more diverse than previously reported. Feather identities from each habitat matched avian communities in those areas, suggesting that Tree Swallows are generalist feather collectors and that the number and characteristics of feathers used as lining depends on feather availability near the nesting site. Received 11 January 2022. Accepted 24 May 2022.

Key words: avian nesting behavior, cytochrome oxidase I (COI) gene fragment sequencing, feather availability and habitat, feather identification, Hirundinidae, nest construction, nest lining.

Identificación y características de las plumas usadas como revestimiento en los nidos de la golondrina *Tachycineta bicolor* en Indiana y Ohio

RESUMEN (Spanish)—La construcción del nido representa una parte importante de la inversión parental y puede tener un impacto significativo sobre el éxito reproductivo de muchas especies de aves. La golondrina *Tachycineta bicolor* anida en cavidades secundarias, aceptando fácilmente cajas nido dentro de las que construyen un nido básico con hierbas en forma de taza y revestido con plumas de otras especies de aves. Identificamos y describimos las características de las plumas usadas como material del nido de *T. bicolor* en 4 hábitats diferentes en Indiana y Ohio. Monitoreamos 41 cajas nido en 2017 y 53 en 2018. Después que los pichones volaron del nido, recolectamos las plumas de los nidos, las contamos, y las caracterizamos por tamaño, color y tipo. Las plumas del contorno, marrones y de longitud media (5–10 cm) fueron significativamente más comunes que cualquier otro tipo de pluma. Comparamos la prevalencia y las características de las plumas entre nidos en diferentes tipos de hábitats, y los resultados mostraron que los nidos cerca de lagos contenían significativamente más plumas que los nidos de los hábitats agrícolas. Identificamos las plumas utilizando técnicas moleculares y comparaciones morfológicas, y encontramos que éstas pertenecían a 26 especies de 19 familias y 11 órdenes: Galliformes, Anseriformes, Columbiformes, Gruiformes, Charadriiformes, Pelecaniformes, Accipitriformes, Strigiformes, Piciformes, Cathartiformes y Passeriformes; incluyendo 20 especies no reportadas previamente dentro de esos órdenes. Esto indica que las plumas utilizadas por *T. bicolor* para el revestimiento de sus nidos son más diversas que lo reportado previamente. La identidad de las plumas en cada hábitat coincide con las comunidades de aves en esas áreas, sugiriendo que las golondrinas *T. bicolor* son recolectoras de plumas generalistas, y que el número y las características de las plumas utilizadas como revestimiento del nido dependen de la disponibilidad de plumas cerca del lugar de anidación.

Palabras clave: comportamiento de anidación de aves, construcción del nido, hábitat y disponibilidad de plumas, Hirundinidae, identificación de plumas, revestimiento del nido, secuenciación del fragmento del gen del citocromo oxidasa I (COI).

Nest construction is a critical component of parental investment in most birds (Collias and Collias 1984) and can have important consequences on reproductive success and individual fitness (e.g., Collias and Collias 1984, Winkler 1993, Dawson et al. 2011, de Zwaan and Martin 2018). Tree Swallows (*Tachycineta bicolor*) are secondary cavity nesters that build cup-shaped nests inside nest boxes using dry grasses lined with other species' molted feathers collected in the nest surroundings (Cohen 1985, Winkler 1993, Winkler

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et al. 2020). Tree Swallow nesting habits are relatively well documented, and they have become important model organisms to study avian reproductive behavior (Jones 2003, Winkler et al. 2020).

Tree Swallow feather nest lining may provide a thermally stable microenvironment to mitigate the high energetic cost of incubation (Winkler 1993, Stephenson et al. 2009, Dawson et al. 2011), therefore enhancing reproductive success of swallow pairs (Winkler 1993, Lombardo et al. 1995, Stephenson et al. 2009, but see Holland and Shutler 2018). Feather lining may also reduce egg-dumping or nest parasitism by camouflaging eggs (Heinrich 2015), or act as a barrier against ectoparasites, but the evidence for these effects has been mixed (Winkler 1993, Lombardo et al. 1995, Thomas and Shutler 2001, Stephenson et al. 2009, Winkler et al. 2020).

Accounts of individuals competing vigorously for feathers (Forbush 1929, Kuerzi 1941, Cohen 1985, Winkler 1993) suggest that they can be a limiting resource in some environments, although other authors describe intraspecific interactions with feathers as play behavior (Weydemeyer 1934, Lincoln 1956). Feather availability and characteristics can vary among habitats based on composition of local avian communities and other attributes of the environment (e.g., wind, vegetation structure). Several studies have reported that Tree Swallows preferentially use certain feather types over others, although the specific types of feathers reported differ among studies. For example, Tree Swallows studied in Massachusetts and Maine were found to preferentially use large white feathers over other feathers (Forbush 1929, Heinrich 2015). The greater color contrast of white feathers against the environment was suggested to play a role in feather selection (Heinrich 2015). Barn Swallows (*Hirundo rustica*) have also been found to preferentially select white feathers over other pigmented feathers (Peralta-Sanchez et al. 2011) and nests with white feathers tended to have lower bacterial densities (Peralta-Sanchez et al. 2010) and higher hatching success (Peralta-Sanchez et al. 2011). However, research conducted in New York (Winkler 1993), Massachusetts (Austin and Low 1932), and Connecticut (Kuerzi 1941) reported that Tree Swallows use feathers of a greater diversity of colors (e.g., gray, brown, white) that belong to many different

species, suggesting that Tree Swallows might be generalists when collecting feathers for their nests.

Despite the overwhelming evidence that the number and characteristics of feathers used as lining can have strong effects on an individual's fitness (e.g., Winkler 1993, Stephenson et al. 2009, Dawson et al. 2011), there has not yet been an effort using molecular techniques to identify which species the feathers used as nest lining belong to. Moreover, there has been little research on feather use by populations breeding in the midwestern United States. Previous studies used only direct observation and comparison with museum specimens and found that Tree Swallows use feathers from species within the orders Galliformes, Anseriformes, Charadriiformes, Accipitriformes, Strigiformes, Psittaciformes, and possibly Pelecaniformes (Austin and Low 1932, Weydemeyer 1934, Kuerzi 1941, Winkler 1993, Heinrich 2015). Austin and Low (1932) visually identified feathers to species level and reported that 99% of the total feathers used in the 46 nest boxes in their Massachusetts site were Herring Gull (*Larus argentatus*) feathers, and that the remaining feathers belonged to American Black Ducks (*Anas rubripes*), scaups (*Aythya affinis*/*A. marila*), Wood Ducks (*Aix sponsa*), Canada Geese (*Branta canadensis*), Great Horned Owls (*Bubo virginianus*), Red-tailed Hawks (*Buteo jamaicensis*), and domestic fowl. Heinrich (2015) also visually identified feathers used by Tree Swallows to species level and found that non-experimental nests included feathers from Wood Duck, Canada Goose, and possibly Great Blue Heron (*Ardea herodias*), and that other Tree Swallows used experimentally offered feathers from Wild Turkey (*Meleagris gallopavo*) and Dusky Grouse (*Dendragapus obscurus*).

The goal of our study was to characterize and determine the species identity of feathers used as lining in Tree Swallow nests located in Indiana and Ohio. Our research questions were as follows: (1) How do the species identities and characteristics of molted feathers used as lining compare to findings in other geographic locations? (2) How does habitat affect the number, identity, and characteristics of feathers used? We recorded the number, size, color, and type of feathers and used a novel approach based on molecular techniques to identify feathers used as lining in nests.

Table 1. Habitats of Tree Swallow nest boxes monitored in 2017 and 2018 in Indiana and Ohio. Site coordinates for the center of each study site can be found in the methods section. The *n* nests 2017 and *n* nests 2018 columns indicate the number of nests sampled each year.

Habitat	Habitat description	Locality	<i>n</i> nests 2017	<i>n</i> nests 2018
Agricultural	Habitat predominated by agricultural fields; some forest edge habitat; small stream within a mile of nest boxes	Sites 1–2	11	6
Prairie/early successional	Habitat predominated by non-woody vegetation like grasses and forbs; some small trees but open canopy; interspersed with small ponds	Sites 3–6	10	13
Near lake	Habitat directly adjacent (within 0.40 km) to a large reservoir; some sandy and mowed grass shoreline; forbs and grasses prevalent	Sites 7–8	20	31
Wetland ^a	Habitat adjacent to large reservoir with predominantly aquatic and semi-aquatic vegetation	Site 9	0	3

^a Excluded from habitat comparison.

Methods

Study area

Our study was conducted in eastern Indiana and western Ohio in 4 habitats divided across 9 study sites (Site 1: 39°34′36.444″N, 85°01′32.6634″W; Site 2: 39°30′46.6″N, 84°43′11.0″W; Site 3: 39°47′53.2314″N, 84°58′12.252″W; Site 4: 39°48′58.32″N, 84°54′50.4″W; Site 5: 39°47′49.7034″N, 84°57′57.06″W; Site 6: 39°48′26.9994″N, 84°58′09.984″W; Site 7: 39°35′16.5″N, 84°59′33.6″W; Site 8: 39°36′48.888″N, 84°57′45.4392″W; and Site 9: 39°33′47.8584″N, 85°01′21.0216″W; Table 1). All sites were within 41 km of Richmond, Indiana. In June and July 2017, we visited 41 Tree Swallow nest boxes from 3 habitats (Agricultural, Prairie/early successional, and Near lake), and during May and June 2018, we visited 53 Tree Swallow nest boxes from 4 habitats (Agricultural, Prairie/early successional, Near lake, and Wetland). Habitats were classified using the characteristics described in Table 1 and distances to large bodies of water were measured in Google Earth Pro 7.3.4.8248 (Google, Mountain View, California, USA). Only data collected in 2017 were used to characterize feathers found in nests and to compare feathers across the 3 habitats sampled, while we used feathers collected in both years and all habitats for molecular feather identification.

Feather collection

At regularly monitored nests, after all eggs had hatched but before chicks fledged, we opportunistically

collected up to 5 feathers with distinctive colors or patterns from each nest to ensure that the most diverse feathers were included in molecular identification analyses. We replaced these feathers with autoclaved domestic chicken (*Gallus gallus*) feathers of similar size and type, so as not to impact nestling development. If any feathers were later identified as belonging to a domestic chicken, we excluded them from our analysis of species identification to avoid accidental inclusion of replacement feathers. Once chicks had fledged, we collected all feathers from 41 nests in 2017 and 11 nests in 2018. At this stage we sampled additional feathers for molecular identification. The number of feathers selected for molecular identification from each nest varied, but in all cases, we included the most common feathers and added any unique feathers found.

Number and characteristics of feathers

To investigate the characteristics of feathers that were used as nest lining, we first counted all feathers from each nest. Then we categorized the size, type, and color of all feathers. Size was measured with 1 cm precision and size categories were <5 cm (small), 5–10 cm (medium), >10–15 cm (large), and >15 cm (extra-large). Categories of feather type were contour, flight, down, and semiplume. To assign colors to feathers found in nests, we compared them against Ridgway (1886) color plates and categorized them as black, brown, gray, white, or other. Although feathers were not cleaned before categorization, it was always

possible to observe their original color. We compared the number of feathers of each size, color, and morphological type in nests to determine which feathers were most used. An archived copy of our feather characteristics data is available to researchers via Dryad (<https://doi.org/10.5061/dryad.r4xgd2fj>).

Species identity of feathers

We extracted DNA from 121 feathers to be sequenced. We removed 1 mm of the calamus of each sampled feather and used the commercially available Qiagen DNeasy Blood and Tissue Kit (Qiagen, Inc., Valencia, California, USA) following the manufacturer's protocol with the following modifications: before adding ProK, we added 30 μ L of 10% 1,4-Dithiothreitol (DTT) to the sample and vortexed to increase digestion. After adding ProK, we incubated samples at 55 °C until tissue was completely dissolved (3–4 d). Each day we added 20 μ L of ProK and vortexed samples. After samples dissolved, we followed Qiagen's protocol, but with two 30 μ L solutions of AE buffer to increase the concentration in our DNA stocks.

We amplified DNA with PCR using the primers COIbird_R2 and Falco_FA from Hebert et al. (2004) and Kerr et al. (2007) to target and amplify the cytochrome oxidase I (COI, 648 bp) gene. Amplification volume totaled 25 μ L including 2 μ L of DNA template, 2.5 μ L of 10XPCR Buffer, 0.75 μ L of 50 mM MgCl₂, 0.5 μ L of 10 mM dNTP, 0.5 μ L of each primer, 18.15 μ L of nuclease-free water, and 0.1 μ L of Platinum Taq DNA Polymerase (Invitrogen Corp., Carlsbad, California, USA). Reactions were denatured at 94 °C for 2 min, followed by 6 cycles of denaturing at 94 °C for 1 min, annealing at 45 °C for 1.5 min, and extension at 72 °C for 1.5 min, after which there were 45 cycles of denaturing at 94 °C for 1 min, annealing at 55 °C for 1.5 min, and extension at 72 °C for 1.5 min. Reactions then spent 5 min at 72 °C for a final extension (Hebert et al. 2004). We visualized 7 μ L of each PCR product using a 2% agarose gel with a Promega PCR Marker G316A and 2 μ L of Blue/Orange 6X loading dye as a size marker (Promega Corp., Madison, Wisconsin, USA).

We purified PCR products using ExoSAP-IT (Thermo Fisher Scientific, Waltham, Massachusetts, USA) with the thermocycler set to 1 cycle of

37 °C for 4 min followed by 1 min at 80 °C. Samples were sent to the Yale University DNA Analysis Facility for sequencing. We edited DNA sequences for accuracy using chromatograms and FinchTV 1.4.0 (Geospiza Research Team 2004). Sequences were entered into NCBI's Nucleotide Basic Local Alignment Search Tool (BLAST; Altschul et al. 1990) and compared to existing sequences in the GenBank database (Clark et al. 2015). For each BLAST search we optimized for highly similar results using Megablast, filtered for results with Expectation Value (E-Value) < 0.0000001, and sorted results by Percent Identity (% Ident). When the closest species match for the cytochrome oxidase I gene had a high similarity ($\geq 95\%$ Ident) and a low E-Value, we considered it a positive identification. E-Values represent a measure of statistical significance and indicate the probability that high scores between the unknown sequence and one sequence in the GenBank database are obtained by random chance (Pertsemlidis and Fondon 2001). We uploaded all sequences to GenBank (accession numbers: ON158868–ON158887; ON158889–ON15893). For 1 species (Wild Turkey) we were not able to get high-quality sequences, and thus we used microscopic identification to Order level (Dove and Koch 2011) and morphological comparisons to museum specimens and other available resources (Scott and McFarland 2010, USFWS 2020) to support species identification.

Statistical analyses

To assess how many feathers of each color, size, and type category were used per nest, we used the number of feathers as our response variable, and either feather color, feather size, feather type, or habitat as our independent variable. We conducted generalized linear models (GLMs) with negative binomial error distribution to account for the over-dispersion of our data. To assess whether an interaction existed between habitat and feather characteristics (i.e., does habitat drive the number of feathers with various characteristics used in Tree Swallow nests?), we fit our data to negative binomial models with and without an interaction and used Akaike information criterion (AIC) scores to determine the fit of the models.

We assessed differences among the independent variable categories in each model using the

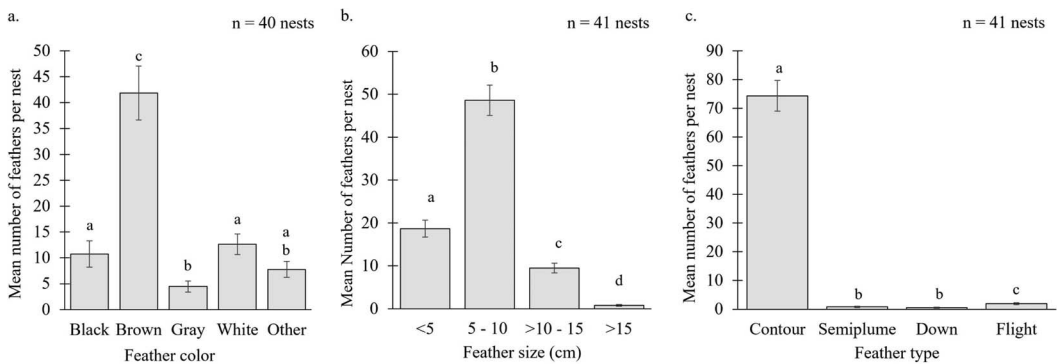


Figure 1. Mean number of feathers ($\pm$ SE) from (a) each color category, (b) each size category, and (c) each feather type category found in Tree Swallow nests from Indiana and Ohio in 2017. There were more brown, medium, and contour feathers. Non-overlapping letters represent significant differences in means.

Anova() function from the *car* package (Fox and Weisberg 2019). In 2-way analyses with an interaction specified, we ran Type III sums of squares (SS) in order to best capture the interaction; otherwise, we used Type II SS, which is a more powerful analysis when interactions are not present.

When we found significant differences, we conducted post hoc pairwise comparisons using estimated marginal means (EMMs) adjusted using the Tukey method in the *emmeans* package (Lenth 2020) and produced a compact letter display (CLD) of EMMs at $\alpha = 0.05$ using the package *multcompView* (Graves et al. 2019). All analyses were conducted in RStudio 1.2.2019 (RStudio Team 2020) using R 64.4.1.0 (R Core Team 2021).

Results

Number and types of feathers

In 2017 nests contained a mean of 77.34 ± 34.01 feathers (range 19–175; in all cases, the mean value is followed by $\pm$ SD), whereas in 2018 nests contained a mean of 43.91 ± 21.99 feathers (range 4–88). For 2017 nests ($n = 41$ nests), among a total of 3,181 feathers collected, the most common feather size was medium (62.68%), the most common type was contour (95.82%), and among the 3,098 feathers categorized by color ($n = 40$ nests), the most commonly used was brown (54.03%). The same general pattern occurred within individual nests. We found significant differences in the number of feathers of different colors in nests ($\chi^2_4 = 97.016$, $P < 0.001$,

$n = 40$ nests), with more brown feathers than all other colors (EMMs post hoc: $P < 0.001$ for all pairwise comparisons), and fewer gray than black feathers (EMMs post hoc: $P = 0.006$) and white feathers (EMMs post hoc: $P = 0.001$; Fig. 1a). We also found significant differences in the number of feathers of different sizes ($\chi^2_3 = 468.38$, $P < 0.001$, $n = 41$ nests). There were more medium feathers than other sizes, more small than large and extra-large feathers, and more large than extra-large feathers (EMMs post hoc: $P < 0.001$ for all pairwise comparisons; Fig. 1b). Moreover, we found significant differences in number of feathers of different types ($\chi^2_3 = 1152.7$, $P < 0.001$, $n = 41$ nests), with more contour feathers than any other type of feather (EMMs post hoc: $P < 0.001$ for all pairwise comparisons), as well as more flight feathers than semiplume feathers (EMMs post hoc $P = 0.003$) or down feathers (EMMs post hoc: $P < 0.001$; Fig. 1c).

Feather use across habitats

We found significant differences in the number of feathers in nests across habitats and among colors. Nests in Near lake habitats had more feathers than nests in Agricultural habitats, and brown feathers were the most prevalent color, whereas gray and other colored feathers were the least common (Habitat: $\chi^2_2 = 14.492$, $P < 0.001$; Color: $\chi^2_4 = 15.855$, $P = 0.003$, $n = 40$ nests; Fig. 2). Moreover, we found a significant interaction between habitat and color ($\chi^2_8 = 44.143$, $P < 0.001$, $n = 40$ nests; Fig. 2), with habitat types driving patterns of color frequency. There were significantly more black

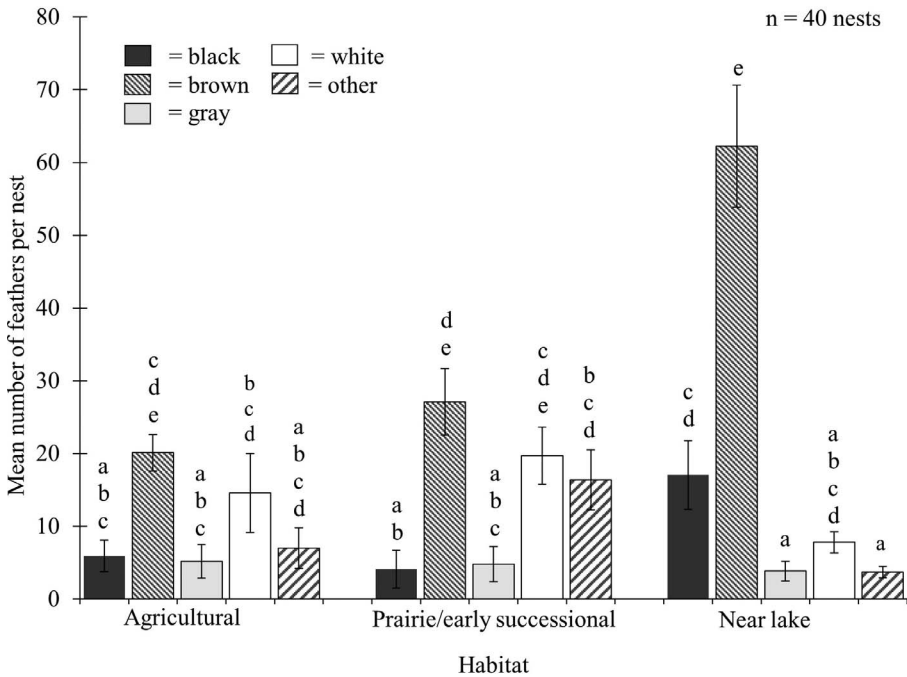


Figure 2. Impact of interaction between 5 feather color categories and 3 habitat categories on the mean number of feathers ($\pm$ SE) found in Tree Swallow nests from Indiana and Ohio in 2017. We found significantly more black feathers in Near lake habitats than Prairie/early successional habitats, and more feathers from color category “other” in Prairie/early successional habitats than Near lake habitats. Non-overlapping letters represent significant differences in means.

feathers in nests in Near lake habitats than in nests in Prairie/early successional habitats (EMMs post hoc: $P = 0.037$), and more feathers from the color category “other” in nests in Prairie/early successional habitats than in nests in Near lake habitats (EMMs post hoc: $P = 0.016$). Only nests in Near lake habitats had more black feathers than gray and other feathers (all EMMs post hoc: $P < 0.001$). There were significantly more brown feathers in nests in Near lake habitats than other colors in nests in the other habitats (EMMs post hoc: $P < 0.04$ for all pairwise comparisons), excepting white feathers in nests in Prairie/early successional habitats (EMMs post hoc: $P = 0.149$; Fig. 2). Furthermore, within the Near lake habitat, we found that nests closest to the lakeshore had significantly more feathers than nests that were farther than 75 m from the lakeshore ($\chi^2_1 = 12.275$, $P < 0.001$, $n = 20$ nests).

We found significant differences in the number of feathers in nests among habitats ($\chi^2_2 = 18.56$, $P < 0.001$) and feather sizes ($\chi^2_2 = 150.13$, $P < 0.001$, $n = 41$ nests; Fig. 3). All habitats had more

medium feathers than any other size category (EMMs post hoc: $P < 0.001$ for all pairwise comparisons), and more small feathers than large and extra-large feathers combined (EMMs post hoc: $P < 0.001$ for all pairwise comparisons). Furthermore, there were significantly more feathers in each size category in Near lake habitats than in the same size categories in Agricultural habitats (EMMs post hoc: $P < 0.001$ for all pairwise comparisons; Fig. 3). We found no significant interaction between habitat and feather size used in nests ($\chi^2_2 = 7.25$, $P = 0.123$, $n = 41$ nests).

Species identity of feathers

Tree Swallows in our study used feathers from 11 avian orders. Five of these (45.45%) are orders reported for the first time: Columbiformes, Gruiformes, Piciformes, Cathartiformes, and Passeriformes. Additionally, one order is reported with confidence for the first time: Pelecaniformes (Table 2). For orders previously reported in the literature, we identified feathers belonging to families and species not present in the literature. We identified

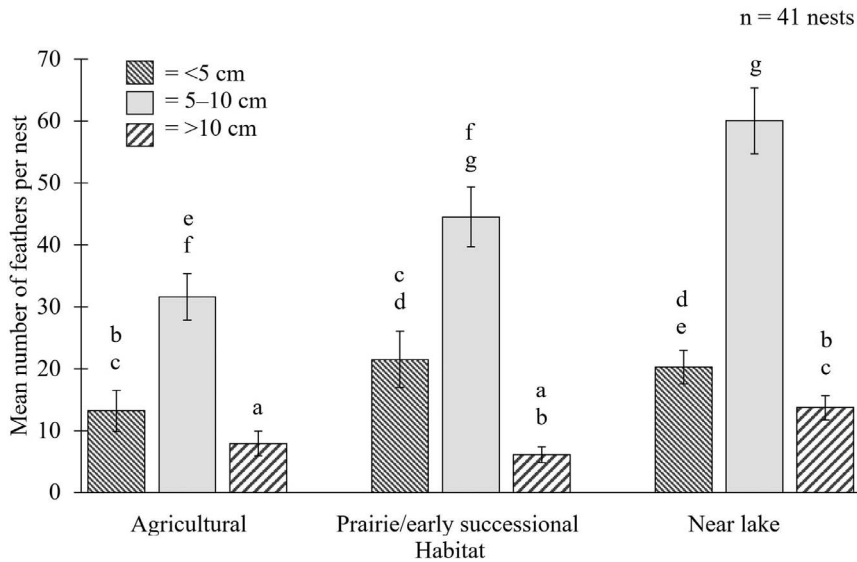


Figure 3. Mean number of feathers ($\pm$ SE) found in Tree Swallow nests in Indiana and Ohio in 2017 across 3 feather size categories and 3 habitat categories. In all habitats there were more medium feathers than small feathers, and more small feathers than large and extra-large feathers. There were more feathers of each size category in Near lake habitats than in respective sizes in Agricultural habitats. There was no significant interaction between habitat and size. Non-overlapping letters represent significant differences in means.

feathers from 19 families and 26 species (Table 2). Sequences for 25 of these species are accessioned in GenBank. Feathers from Wild Turkey were identified using morphology and microscopy due to low sequence quality (Fig. 4). Feathers from 20 (76.92%) of these species are reported for the first time. Moreover, we identified feathers from 4 nonnative species: Indian Peafowl (*Pavo cristatus*), Helmeted Guineafowl (*Numida meleagris*), European Starling (*Sturnus vulgaris*), and domestic chicken. We excluded the domestic chicken identification from our analysis, since it is possible that this was one of our replacement feathers used during data collection. The composition of feathers used as lining was different in different habitats, and Prairie/early successional habitats had feathers from the largest set of species (Table 2). It is important to note that the totals we identified represent a conservative number of species, since we only molecularly identified a subsample of feathers from each nest.

Discussion

Our study shares new insights on the use and identity of feathers as nest lining in Tree Swallow

nests in the midwestern United States. We found that the number and identity of the feathers is affected by the habitat in which the nest is located. Tree Swallows in our study sites tend to use medium (5–10 cm) contour feathers of a wide diversity of colors, with brown being the most common color used. Our data suggest that Tree Swallows might be opportunistic and willing to use feathers of different characteristics and species origins if those are the feathers available in the immediate environment. Ultimately our results show that Tree Swallows use a greater diversity of feathers than previously reported in the literature, using feathers from at least 11 avian orders and from wild and domestic species.

Our results concur with previous studies that report that feathers are important structural components of nest lining for Tree Swallow nests. All Tree Swallow nests in our study sites contained feathers as part of their lining and the mean numbers of feathers for both years were within the range reported by previous studies in the literature (Austin and Low [1932]: $72 \pm \text{NA}$, $n = 46$; Stephenson et al. [2009]: $68.3 \pm 35.8 \text{ SD}$, $n = 18$; Dawson et al. [2011]: $98.2 \pm 10.4 \text{ SD}$, $n = 10$; Heinrich [2015]: $68.4 \pm \text{NA}$, $n = 5$). Our results

Table 2. Species assignment of feathers collected from Tree Swallow nests in Indiana and Ohio identified molecularly and morphologically (i.e., Wild Turkey), and the distribution of species identifications across each of the 4 habitat types (Agricultural [A], Prairie/early successional [P], Near lake [L], and Wetland [W]). Results shown for each of the 25 species identified molecularly include the sequence with the highest Percent Identity (% Ident) and an E-value <0.0000001. “X” indicates a feather from that species was positively identified from a nest in that habitat.

Order	Family	Species	% Ident	Habitat			
				A	P	L	W
Galliformes	Phasianidae	<i>Pavo cristatus</i> ^a	100.00		X		
	Meleagridae	<i>Meleagris gallopavo</i>	NA		X		
	Numididae ^a	<i>Numida meleagris</i> ^a	99.71		X		
Anseriformes	Anatidae	<i>Aix sponsa</i>	100.00	X	X		X
		<i>Anas platyrhynchos</i> ^a	100.00	X	X	X	
		<i>Branta canadensis</i>	100.00	X	X	X	X
		<i>Cygnus olor</i> ^a	100.00	X	X	X	
Columbiformes ^a	Columbidae ^a	<i>Zenaida macroura</i> ^a	100.00	X			
Gruiformes ^a	Rallidae ^a	<i>Porzana carolina</i> ^a	100.00	X			
Charadriiformes	Charadriidae ^a	<i>Charadrius vociferus</i> ^a	100.00	X			
	Scolopacidae ^a	<i>Tringa flavipes</i> ^a	100.00	X			
Pelecaniformes ^b	Ardeidae ^b	<i>Ardea herodias</i> ^b	100.00	X	X		X
Accipitriformes	Accipitridae	<i>Buteo jamaicensis</i>	98.47	X	X		
		<i>Buteo lineatus</i> ^a	99.82		X		
		<i>Haliaeetus leucocephalus</i> ^a	100.00			X	
		<i>Pandion haliaetus</i> ^a	100.00			X	
		<i>Bubo virginianus</i>	100.00	X	X	X	
Strigiformes	Strigidae	<i>Bubo virginianus</i>	100.00	X	X	X	
Piciformes ^a	Picidae ^a	<i>Dryobates villosus</i> ^a	100.00		X		
Cathartiformes ^a	Cathartidae ^a	<i>Cathartes aura</i> ^a	100.00	X	X	X	
		<i>Coragyps atratus</i> ^a	99.83	X	X	X	
		<i>Corvus brachyrhynchos</i> ^a	100.00		X		
Passeriformes ^a	Corvidae ^a	<i>Corvus brachyrhynchos</i> ^a	100.00		X		
	Sturnidae ^a	<i>Sturnus vulgaris</i> ^a	99.50	X			
	Mimidae ^a	<i>Dumetella carolinensis</i> ^a	100.00		X		
	Turdidae ^a	<i>Turdus migratorius</i> ^a	99.37		X		
		<i>Hylocichla mustelina</i> ^a	99.37				X
	Icteridae ^a	<i>Molothrus ater</i> ^a	99.81		X		
Number of species identified per habitat				14	18	8	4

^a An order, family, or species not previously reported in the literature.

^b An identification previously recorded as an unconfirmed possibility.

also suggest that the number of feathers used as lining are affected by the nest’s environmental neighborhood (i.e., areas surrounding the nest), since nests near lakes had significantly more feathers than nests found in Prairie/early successional and Agricultural habitats (Fig. 2). Even at a smaller spatial scale (within the Near lake habitat), we found that nests by the lakeshore had significantly more feathers than nests that were farther than 75 m from the lakeshore. The relatively low average number of feathers recorded in 2018 compared to 2017 seems to be related to the fact that we focused on nests in Prairie/early successional habitats for that year, with fewer nests in Near lake habitats (Table 1). This habitat effect could also explain the different mean number of feathers reported in different geographic regions

by multiple authors (Austin and Low 1932, Stephenson et al. 2009, Dawson et al. 2011, and Heinrich 2015). Taken together, this suggests that Tree Swallows might be limited by molted feather availability, that they collect feathers mostly in the immediate nest neighborhoods, and that local nest feather availability could have important effects on the reproductive success of this species.

Several lines of evidence in our data suggest that Tree Swallows are opportunistic feather collectors, that nest lining structure and composition is influenced strongly by environmental feather availability, and that feather use is not limited to specific feather colors or sizes (e.g., white feathers), as previously suggested by some authors in the literature (e.g., Forbush 1929, Peralta-Sanchez et al. 2010, Heinrich 2015). First,



Figure 4. Wild Turkey feathers (Meleagridae: Galliformes) in Tree Swallow nests from Indiana and Ohio. Feathers (a) and (b) were identified using microscopy and morphological comparisons with museum specimens and feather guides (Scott and McFarland 2010, USFWS 2020), since we were not able to obtain high-quality DNA sequences.

we found that Tree Swallows in our study sites do not predominantly use white feathers as previously reported. On the contrary, brown feathers were the most common feathers used, followed by black and white feathers. This pattern is strongest in Near lake habitat, and white feathers were not consistently used more often in any of the habitats (Fig. 2). Second, a comparison of our results with other published data reveals a difference in feather size use among populations and geographic regions. The most common feather size category used as lining in our study sites was 5–10 cm in length, followed by <5 cm (Fig. 1). Contrary to this finding, Forbush (1929) reported that in Massachusetts Tree Swallows prefer “large feathers” (measurement not specified). In addition, Heinrich (2015) found that nests in Maine predominantly included “large” feathers and that breeding Tree Swallows were quick to collect feathers of 10–15 cm in length. This suggests that Tree Swallows are willing to use feathers of different sizes as lining. We argue that this might be due to different feather size availability in

different study sites. Third, Tree Swallows were found to use molted feathers from a great diversity of species. Feather identities matched the avian communities where the nests were located, rather than suggesting Tree Swallow preferences for a particular species or group of species. For example, we found that nests in the Near lake habitat predominantly used feathers from waterfowl (e.g., Canada Geese, Mute Swans [*Cygnus olor*], and Mallards [*Anas platyrhynchos*]), while nests in Prairie/early successional and Agricultural habitats used molted feathers from species associated with those environments (e.g., Killdeer [*Charadrius vociferus*], Mourning Dove [*Zenaidura macroura*], Red-shouldered Hawk [*Buteo lineatus*], Hairy Woodpecker [*Dryobates villosus*], Wood Thrush [*Hylocichla mustelina*], American Robin [*Turdus migratorius*], Wood Duck, and Lesser Yellowlegs [*Tringa flavipes*]). Moreover, feathers from common local species, for which molted feather availability would be expected to be high, such as vultures (i.e., Black Vulture [*Coragyps atratus*] and Turkey Vulture [*Cathartes*

aura]], some waterfowl (e.g., Canada Goose, Mallard), and one species of owl (i.e., Great Horned Owl), were found to be present in the lining of nests in most habitats (Table 2).

The reliance on feathers of common species was also revealed within specific habitats. For example, the intersections in habitat preference, and breeding activities of Canada Geese and Tree Swallows, suggests a high availability of Canada Goose feathers for Tree Swallows in Near lake portions of our study area. Our results match this observation, since Canada Goose feathers were among the most prevalent feathers identified in nest linings in Near lake nests. Finally, the orders and species of molted feathers in our study sites match some but not all the species reported as predominant structural components of lining in previous studies conducted in different geographic regions. This suggests that there are not specific molted feathers that Tree Swallows rely on as lining for their nests, but rather that feathers used vary by availability in different regions and habitats. Moreover, our results match previous studies that report Tree Swallows using novel feathers from domestic species if they are readily available in the environment (Weydemeyer 1934, Kuerzi 1941).

We acknowledge that our results do not allow us to make any conclusions about specific feather preferences for this species. This is because we did not collect data about environmental feather availability, and we did not conduct successful controlled preference trial experiments that would allow us to know if Tree Swallows prefer a specific feather type over another when options are available. Regardless, even if a preference for a particular feather trait does exist, our results clearly show that Tree Swallows are willing to use feathers with different characteristics as part of their nest lining and that they are more opportunistic feather collectors than previously believed.

Our results highlight the power of molecular tools to provide more resolution and confidence on molted feather identification as structural components of nest lining, particularly at the species level. In the past, researchers have identified molted feathers morphologically (Austin and Low 1932), using comparisons with museum specimens (Harwood 2011), and using microscopy (Dove 1997, Harwood 2011). Visual and microscopic identification of feathers (with or without museum specimens for comparison) can be

inaccurate due to phenotypic variability and the high similarity of feather characteristics among closely related species. Molecular techniques such as mitochondrial DNA sequencing can be readily used with feathers (Smith et al. 2003) and tools such as GenBank and BLAST can allow comparisons of DNA sequences with a library database for species identification (Sayers et al. 2019) as well as confidence thresholds for taxonomic identification assignments. Our study confirms previously reported observations such as the use of feathers from 6 taxonomic orders, 6 families, and 6 species, as well as the use of feathers from wild and domestic species. Our results show that Tree Swallows in our study site used feathers from an additional 5 orders, 13 families, and 20 species, suggesting Tree Swallows use a wider diversity of feathers than previously described.

Taken together, our results suggest that Tree Swallows readily collect a diverse set of feathers and rely on abundant populations of other bird species to provide nesting materials. Future studies on nest construction (e.g., timing and methodology of feather gathering), feather preferences (number, color, size, species origin), and the effect of environmental feather availability in various locations where swallows breed would augment our understanding of Tree Swallow nesting behavior and feather use. Additional controlled experiments on the fitness impacts of feather linings in different habitats and geographic areas would elucidate the importance and variation in feather use across Tree Swallows' breeding range, which is especially important given changes in species composition and phenology due to habitat destruction and climate change (Dunn and Winkler 1999, Winkler et al. 2002, Carey 2009, Bourret et al. 2015, Langham et al. 2015, Bateman et al. 2020).

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