Variation in Stable Hydrogen Isotope Values in a Wetland-associated Songbird

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Abstract.—Stable isotope analysis is often used to identify the geographic origins of migratory bird populations. While this method can accurately predict the provenance of migratory species, stable hydrogen isotope values measured in feathers (δ²Hf) can be variable within a site and may be influenced by differences among age class (second year vs. after second year), type of feather (primary vs. rectrix), year of sampling, species, and local hydrology. In this study, sources of variation in δ²Hf values were assessed in a wetland-associated Neotropical migratory bird, the Prothonotary Warbler (Protonotaria citrea), by comparing δ²Hf values among age classes, sexes, years and feather types in individuals breeding in eastern Virginia, USA. Age and year were found to influence δ²Hf values, with individuals in their second year having more depleted δ²Hf values (-62.43 ± 9.56‰) than individuals older than 2 years (-53.73 ± 9.04‰). Differences between primaries and rectrices were within the range of sampling error (-2.98‰), and there was no effect of sex. For wetland-associated songbirds, age-related differences in the dietary proportion of aquatic vs. terrestrial prey may provide an additional mechanism for differences observed in δ²Hf values between age classes. In studies that use stable isotopes to assign geographic feather origin, researchers should attempt to account for and propagate known variation in δ²Hf values in assignment models whenever possible. Received 30 October 2017, accepted 30 December 2017.

Key words.—forested wetlands, hydrogen isotopes, migratory connectivity, migratory songbird, Prothonotary Warbler, Protonotaria citrea, stable isotope analysis.

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primary feathers in a raptor (Mechan et al. 2003). δ2H values can also vary seasonally.

Table 1. Comparison of the effect of age, sex, year, and feather type on stable hydrogen isotope variation as reported in six studies. Age is denoted as nestling, HY (hatch year, after fledging), SY (second year), or ASY (after second year). Feather type is denoted as P (primary) or R (rectrix). The direction of the effect is reported if known, otherwise it is reported as present or absent. N/A denotes comparisons that were not performed in that study.

<table>
<thead>
<tr>
<th>Species</th>
<th>Sex</th>
<th>Age</th>
<th>Year</th>
<th>Age*Year</th>
<th>Feather Type</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prothonotary Warbler</td>
<td>no</td>
<td>SY &lt; ASY</td>
<td>present</td>
<td>absent</td>
<td>P slightly &lt; R</td>
<td>This study</td>
</tr>
<tr>
<td>Ovenbird</td>
<td>N/A</td>
<td>nestling &lt; ASY</td>
<td>present</td>
<td>present</td>
<td>P slightly &lt; R</td>
<td>Haché et al. 2012</td>
</tr>
<tr>
<td>Wood Thrush</td>
<td>N/A</td>
<td>present</td>
<td>absent</td>
<td>N/A</td>
<td>absent</td>
<td>Gow et al. 2012</td>
</tr>
<tr>
<td>American Redstart</td>
<td>absent</td>
<td>nestling &lt; ASY</td>
<td>absent</td>
<td>N/A</td>
<td>P &lt; contour</td>
<td>Langin et al. 2007</td>
</tr>
<tr>
<td>Cooper’s Hawk</td>
<td>N/A</td>
<td>nestling &lt; adult</td>
<td>N/A</td>
<td>N/A</td>
<td>P ≠ P</td>
<td>Meehan et al. 2003</td>
</tr>
<tr>
<td>Mallard</td>
<td>N/A</td>
<td>HY &lt; adult</td>
<td>absent</td>
<td>N/A</td>
<td>N/A</td>
<td>Van Dijk et al. 2014</td>
</tr>
</tbody>
</table>
over the course of the breeding season (Bortolotti et al. 2013).

Differences in habitat, migration distance, and foraging pattern have also been considered as sources of variation in $\delta^2$H values across species (Hobson et al. 2012; Nordell et al. 2016). For example, Hobson et al. (2012) found that migration distance and foraging guild influence $\delta^2$H values and the relationship between $\delta^2$H and $\delta^2$H. Many studies now use guild-specific transfer functions to relate $\delta^2$H and $\delta^2$H, which classify species as short-distance, long-distance, or non-migratory and whether they forage on the ground or in the canopy (Hobson et al. 2014; Pillar et al. 2015; Gonzalez-Prieto et al. 2017). While Hobson et al. (2012) did not find support for an effect of aquatic vs. upland habitat use on $\delta^2$H, hydrologic processes occurring within aquatic habitats may distort the relationship between $\delta^2$H and $\delta^2$H (Hobson et al. 2004; Clark et al. 2006; Coulton et al. 2009; but see Hebert and Wassenaar 2005). Bowen et al. (2011) found that surface water $\delta^2$H values may be between 30‰ more depleted to 20‰ more enriched relative to local precipitation, differences that likely propagate through the trophic web to influence $\delta^2$H values of individuals in these ecosystems. Some studies have excluded aquatic-associated species from analyses (Hobson et al. 2004) or justified the exclusion of outliers for this reason (Hobson et al. 2012), but few studies have explicitly investigated sources of variation in $\delta^2$H values of wetland-associated birds (but see Betini et al. 2009; Bortolotti et al. 2013).

There has recently been considerable interest in using stable isotope methods to determine migratory connectivity for the many species of long-distance migratory birds that rely on wetlands (Hobson et al. 2006; Pérez et al. 2010; Bridge et al. 2015). Thus, there is a need to explicitly assess both how much variation in $\delta^2$H values occurs in these wetland-associated species relative to terrestrial species (Table 1), and how much variation can be identified and accounted for when using stable isotope analysis to investigate dispersal or migratory movements. In this study, we measured $\delta^2$H values in a population of Prothonotary Warblers (Protonotaria citrea) breeding in eastern Virginia, USA. Our goal was to assess variation in $\delta^2$H values in Prothonotary Warbler feathers as a function of age, sex, year, and feather type.

### Methods

#### Study Area

We collected feather samples from adult male ($n = 33$) and female ($n = 63$) Prothonotary Warblers during the 2013-2016 field seasons at long-term study sites in the Lower James River Important Bird Area (Blem and Blem 1994; Bulluck et al. 2013; Table 2). The majority of samples used in this study ($n = 89$) were from Deep Bottom Park ($37^\circ 24'\ N, 77^\circ 18'\ W$), with additional samples ($n = 7$) from Presquile National Wildlife Refuge ($37^\circ 21'\ N, 77^\circ 15'\ W$), both in Henrico County, Virginia, USA. At both sites, we obtained samples from individuals attending nest boxes positioned over water near the shore of tidal freshwater creeks and the main stem of the James River.

#### Study Species

The Prothonotary Warbler is a Neotropical migratory songbird and a habitat specialist of forested wetlands such as bald cypress-tupelo swamps, riparian corridors, and flooded bottomland hardwood forests (Petit 1999). Their breeding range encompasses the southeastern United States, extending through the Atlantic Coastal Plain and the Mississippi Alluvial Valley north to Ontario, Canada, and they winter in mangroves and

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Table 2. Sample sizes of feathers in which $\delta^2$H was measured from Prothonotary Warblers from Henrico County, Virginia, USA. Age is reported as second-year (SY) or after second-year (ASY).

<table>
<thead>
<tr>
<th>Year</th>
<th>Age</th>
<th>Sex</th>
<th>Feather Type</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SY</td>
<td>ASY</td>
<td>Female</td>
</tr>
<tr>
<td>2013</td>
<td>8</td>
<td>14</td>
<td>16</td>
</tr>
<tr>
<td>2014</td>
<td>10</td>
<td>15</td>
<td>16</td>
</tr>
<tr>
<td>2015</td>
<td>6</td>
<td>12</td>
<td>13</td>
</tr>
<tr>
<td>2016</td>
<td>14</td>
<td>17</td>
<td>18</td>
</tr>
<tr>
<td>Total</td>
<td>38</td>
<td>58</td>
<td>63</td>
</tr>
</tbody>
</table>

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other forested wetlands in Central and South America (Petit 1999). As a riparian- and wetland-associated species, they consume both terrestrial and aquatic prey throughout the nesting season (Petit 1999; Dodson et al. 2016).

Data Collection

We captured males by playing conspecific songs and calls near a mist net with a decoy. We captured females using a hand held net placed over the cavity entrance hole during incubation. We determined age for each bird as after second-year (ASY, \( n = 58 \)) or second-year (SY, \( n = 38 \)) following molt criteria outlined in Pyle (1997), and we determined sex using plumage characteristics and presence of breeding condition (i.e., brood patch or cloacal protuberance). Feathers sampled from all individuals had been grown the previous summer; SY individuals would have molted their flight feathers while nestlings, and ASY individuals would have molted their flight feathers following breeding. Seventy-one percent of individuals had been banded at the study site previously. We collected one outermost rectrix (R6) in all individuals that were known to have been present on the study site previously. All individuals used in the comparison between primaries and rectrices had been encountered at the site previously. We conducted all statistical analyses in statistical program R (R Development Core Team 2016) using an alpha value of 0.05 when applicable, and we report all results as mean ± SD.

Stable Isotope Analysis

We conducted stable isotope analysis in August 2016. We cleaned all feathers in a 2:1 chloroform:methanol solution to remove oil and debris, then dried the feathers in a fume hood for 48 hr. Feather samples were equilibrated in the laboratory where analysis was conducted for ~72 hr (Wassenaar and Hobson 2003), then subsamples of feather vane from the distal end (0.3-0.4 mg) were analyzed via high-temperature combustion in an elemental analyzer (Thermo TC/EA; Thermo Scientific) and an isotope ratio mass spectrometer (Thermo Scientific Delta V Advantage). We present stable isotope values in units of per mil (‰) with non-exchangeable hydrogen reported in relation to the Vienna Standard Mean Ocean Water-Standard Light Antarctic Precipitation standard scale (Coplen 2011). We corrected measurements of stable hydrogen for exchangeable atmospheric hydrogen via the comparative equilibrium method (Wassenaar and Hobson 2003) using three in-house keratin reference standards (USGS42 [Tibetan Human Hair]: -78.5‰, CBS [Caribou Hoof Standard]: -197‰, KHS [Kudu Horn Standard]: -54.1‰). Analytical error of laboratory measurements was ±2‰.

Statistical Analysis

We used a paired t-test and linear regression to compare within-individual differences in \( \delta^2H \) between 15 paired rectrix and primary feathers sampled in 2016 from adult (10 ASY and 5 SY) female Prothonotary Warblers. We performed a two-way ANOVA to look for differences in \( \delta^2H \) between age class, year, and an interaction between age class and year. Pairwise comparisons between these categories were made using Tukey’s honestly significant difference (HSD) test. We used a generalized linear model with a Gaussian distribution to examine differences in \( \delta^2H \) between the sexes because a Levene’s test indicated unequal variances between male and female individuals (\( F_{1,11} = 4.68; P = 0.03 \)). To examine the percentage of variation explained by each mechanism of interest, we performed a multiple regression using age class, sex, year, feather type, and an interaction between age and year as predictors. We also performed each analysis using only individuals that were known to have been present on the study site previously. All individuals used in the comparison between primaries and rectrices had been encountered at the site previously. We conducted all statistical analyses in statistical program R (R Development Core Team 2016) using an alpha value of 0.05 when applicable, and we report all results as mean ± SD.

Results

The mean \( \delta^2H \) value for the study area across ages, sexes, and feather types was -57.17 ± 10.12‰ (\( n = 96 \); Range = -85.27 to -38.09‰; 95% CI = -59.2, -55.14‰). The predicted \( \delta^2H \) value for our study site based on the growing-season \( \delta^2H \) isoscape of Bowen et al. (2005) calibrated using the Hobson et al. (2012) equation for long-distance non-ground foraging migratory songbirds was -60.13‰. Primary feathers (-57.89 ± 10.91‰) had more negative \( \delta^2H \) values than rectrices (-54.914 ± 10.12‰) in paired samples (\( t_{14} = -2.66; P = 0.02 \); Fig. 1), but their values were also positively correlated (\( R^2 = 0.84; F = 69.7; P < 0.001 \); Fig. 1). Age (\( F_{3,88} = 23.6; P < 0.001 \)) and year (\( F_{3,88} = 6.39; P < 0.001 \)) had an effect on \( \delta^2H \) values, but an interaction between age and year was not found (\( F_{3,88} = 0.66; P = 0.58 \); Fig. 2). SY individuals (62.43 ± 9.56‰, \( n = 38 \)) had more negative \( \delta^2H \) values than ASY individuals (53.73 ± 9.04‰, \( n = 58 \), \( P < 0.001 \)), and feathers sampled in 2015 (-64.34 ± 8.32‰; \( n = 18 \)) had more negative \( \delta^2H \) values than feathers sampled in any other year (2013: -56.81 ± 12.01‰, \( n = 22 \); \( P = 0.02 \); 2014: -54.55 ± 7.40‰, \( n = 25 \); \( P < 0.001 \); 2016: -55.38 ± 10.11‰, \( n = 31 \); \( P = 0.001 \); Fig. 2). \( \delta^2H \) values were not different in male and female individuals (\( t_{91} = 1.77; P = 0.08 \)). When we repeated these analyses using only individuals known to have been
present on the study sites previously, the results did not change (age: \( P < 0.001 \); year: \( P = 0.002 \) [2013], \( P = 0.001 \) [2014], \( P = 0.003 \) [2016]; sex: \( t_{66} = 0.89, P = 0.377 \)). For all individuals, age explained 17.8% of the variation in \( \delta^2H_f \) values, year explained 14.2% of the variation, and all other variables (sex, feather type, and an interaction between age and year) each explained < 1.5% of the variation \( (P < 0.001; R^2 = 0.23) \). For individuals known to have been present previously at the study sites, age explained 15.3% of the variation, year explained 21.8% of the variation, and all other variables each explained < 2% of the variation \( (P < 0.001; R^2 = 0.30) \).

**Discussion**

In this study, we sought to explore different sources of variation in \( \delta^2H_f \) values in a wetland-associated songbird, the Prothonotary Warbler, in southeastern Virginia, USA. We found strong support for age and year effects on \( \delta^2H_f \) values, limited support for a difference between feather types, and no difference between the sexes. We found that the mean \( \delta^2H_f \) value for our study site was similar to the value predicted for this location based on growing-season \( \delta^2H_p \) values. The range of \( \delta^2H_f \) values that we observed was similar to that of other studies that have successfully measured the degree of migratory connectivity using stable isotopes (Paxton et al. 2007; Tonra et al. 2015). Of the individuals used in this study, 58% were known to have been present on the study site the previous year (i.e., the year the feather was grown) and 71% were known to have been present at the study site in at least one of the previous sampling years. However, the results did not change when we analyzed these individuals separately, which suggests that the observed variation is not a result of dispersal from other breeding areas into our study sites. Our results corroborate those found in non-wetland-associated bird species, suggesting that differences in \( \delta^2H_f \) values among age classes and years are important sources of variation regardless of the hydrological regime of a given site.

Primary feathers were found to have depleted \( \delta^2H_f \) values relative to rectrices for paired samples; however, the average difference between the two feather types (-2.98‰) was similar to the analytical error for the laboratory analysis (+2‰). Haché et al. (2012) also found that primaries were on average slightly depleted relative to rectrices (-1.9‰) in nestling Ovenbirds (Seiurus aurocapilla).

![Figure 1](https://bioone.org/journals/Waterbirds/2019/22/3/37202677.1080.2892.f1a.jpg) (A) \( \delta^2H_f \) values (%e) for primary and rectrix feathers from paired samples of 15 female Prothonotary Warblers from Henrico County, Virginia, USA. (B) Correlation of \( \delta^2H_f \) values for primary and rectrix feathers. The line of best fit (black) and a 1:1 line (gray) are shown.
In passerine birds, including Prothonotary Warblers, the innermost primary is the first primary to be molted, while the outermost rectrix is the final rectrix to be molted (Pyle 1997). A less negative $\delta^{2}H_{f}$ value in rectrices could indicate that these feathers are molted in an isotopically enriched region compared to the breeding territory (e.g., areas further south, downslope, or downriver), that a diet shift occurs during the molting period, or that the $\delta^{2}H$ value of prey items changes during this time. Because the difference in $\delta^{2}H_{f}$ values between the feather types was neither large nor systematic, it does not provide evidence that Prothonotary Warblers in our study area regularly engage in molt migration, though more study is needed to determine if and how movement during the molt period may influence $\delta^{2}H_{f}$ values for some individuals. While we suggest that future studies continue to test for differences between feather types in other species when planning to combine multiple feather types for an analysis, we conclude that primary and rectrix feathers in Prothonotary Warblers do not differ enough to preclude their simultaneous use in studies assessing migratory connectivity.

Similar to several other studies, we found that SY individuals had more depleted $\delta^{2}H_{f}$
values compared to ASY individuals (Meehan et al. 2003; Langin et al. 2007; Gow et al. 2012; Haché et al. 2012). Explanations for this variation include differences in physiology and diet between the age classes (Hobson et al. 1999; Meehan et al. 2003). Differences in δ/D values of prey items and drinking water and/or differences in the proportion of those items consumed among age classes may lead to systematic variation in δ/D values between younger and older individuals (Langin et al. 2007; Betini et al. 2009). These different sources of consumed hydrogen may also lead to individual variation within age classes because diet/resource availability may vary among individuals. For wetland-associated birds, the quantity of aquatic vs. terrestrial prey consumed may be especially important because these prey sources can have different δ/D values (Vander Zanden et al. 2016). Betini et al. (2009) suggested that the contribution of aquatic insects in the diet of box-nesting Tree Swallow (Tachycineta bicolor) nestlings from a riparian site could have led to depleted δ/D values measured in nestling blood samples. In contrast, Bortolotti et al. (2013) found that aquatic insects had slightly more enriched δ/D values compared to terrestrial insects. To date, no studies have examined δ/D values in Prothonotary Warbler food webs, but an analysis of carbon and nitrogen stable isotope ratios in Prothonotary Warbler nestling and adult breast muscle indicated that adults likely consume more terrestrial prey (L. Bulluck, unpubl. data). The majority (92%) of our feather samples came from Deep Bottom Park, where aquatic food makes up at least 68% of nestlings’ diet (Dodson et al. 2016). While it is possible that the larger proportion of aquatic food in nestlings’ diet may be responsible for the depleted δ/D values seen in SY individuals, more study is needed to understand the contributions of aquatic and terrestrial prey to adult and nestling Prothonotary Warbler diets as well as to explicitly test how those prey items differ in isotopic composition.

We examined δ/D values from 4 years of data, and found that one year (2015) had a more negative mean δ/D value compared to all other years. While the samples from 2015 were primaries, which were slightly depleted compared to rectrices, there is no evidence that differences in δ/D values between feather types is the source of between-year variation in this study, because the magnitude of the difference between years was greater than the difference between feather types. The sampling year with the most different δ/D values (2015) was the driest year of the four included in this study; eastern Virginia received 400 mm of precipitation from March through July compared with an average of 520 mm. However, 2012 was similarly dry (425 mm), and δ/D did not show a corresponding change. Other studies have also found yearly differences both in δ/D (Haché et al. 2012; Van Dijk et al. 2014) and δ/D (Van Wilgenburg et al. 2012). Yearly variation in δ/D values may be related to large-scale variation in climate processes such as the North Atlantic Oscillation or the El Nino-Southern Oscillation, which cause deviations from long-term averages in δ/D (Hobson et al. 2012). Van Wilgenburg et al. (2012) found that isoscapes based on the long-term average of δ/Dp values were accurate for predicting the mean δ/Dp across all years at their study sites, but that yearly variation in δ/Dp ranged as much as 40‰. Locally, rates of evapotranspiration may vary on a yearly or seasonal basis, which can lead to surface waters enriched in the heavy isotope of hydrogen relative to local δ/D values (Craig and Gordon 1965; Gat 2010; Bowen et al. 2011).

More study is needed to understand the mechanisms leading to variation in δ/D values beyond those addressed here. For example, we considered testing for within-season variation in δ/D values, which has been demonstrated by other studies (Bortolotti et al. 2013), but we were unable to test for this relationship because our sample sizes were too small among years. In particular, how local precipitation and surface water δ/D values interact with individual-specific differences in diet and physiology and ultimately δ/D values is poorly understood at present (Tonra et al. 2015). δ/D values measured at our study site, located along a tidal freshwater...
river, were similar to predicted values, which suggests that hydrological processes did not have an acute influence on δ²H values. However, processes such as evapotranspiration may exert a larger influence in habitats with open or standing water, such as reservoirs and swamps. Additionally, rivers fed by snowmelt may be depleted in the heavy isotope of hydrogen relative to local δ²H values (Fry 2006; Bowen et al. 2011), and large north-south flowing rivers may transport depleted δ²H values downstream. Future work should characterize the influence of both aquatic prey items and multiple hydrologic regimes on δ²H values (Bowen et al. 2011; Vander Zanden et al. 2016), which may help explain variation not accounted for by factors measured in this study. Understanding the sources of variation in δ²H values can help researchers better account for this variation, such as by limiting sampling collection to a single age class and/or year. Researchers should attempt to collect large enough sample sizes to adequately capture site-specific variation when using known-origin samples, which will allow this variation to be propagated in assignment models.

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