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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 (δ^2H_f) 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 δ^2H_f values were assessed in a wetland-associated Neotropical migratory bird, the Prothonotary Warbler (*Protonotaria citrea*), by comparing δ^2H_f values among age classes, sexes, years and feather types in individuals breeding in eastern Virginia, USA. Age and year were found to influence δ^2H_f values, with individuals in their second year having more depleted δ^2H_f 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 δ^2H_f 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 δ^2H_f 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.

Migratory birds can be traced across their annual cycle using intrinsic markers such as stable isotopes, genetic markers, or trace elements (Webster *et al.* 2002; Hobson and Wassenaar 2008). Stable isotope ratios are often used for provenance studies because they exhibit predictable geographic variation, can be measured from a single capture, and are relatively inexpensive to measure (Hobson 1999). Feathers assimilate the isotope ratio of the local environment and are often retained for an entire migration cycle (Hobson and Wassenaar 2008); additionally, the location of feather growth is known for many species of migratory birds (Pyle 1997; but see Leu and Thompson 2002; Nordell *et al.* 2016). Stable isotopes, especially stable hydrogen, have been widely used to assign migratory birds captured on the nonbreeding grounds to their location of feather growth on the breeding grounds (Chamberlain *et al.* 1997; Hobson and Wassenaar 1997; Rushing *et al.* 2014; Gonzalez-Prieto *et al.* 2017) and to quantify natal dispersal movements (Studds *et al.* 2012; Haché *et al.* 2014). This allows the degree of migratory connectivity, or the degree to which breeding populations mix on the wintering grounds, to be measured (Webster *et al.* 2002), and allows demographic units to be delineated (Haché *et al.* 2014).

Stable hydrogen isotopes are useful for migration and dispersal studies because patterns of atmospheric circulation and temperature create a latitudinal gradient of stable hydrogen values in precipitation (δ^2H_p) across North America (Fry 2006; Hobson and Wassenaar 2008). Studies typically take advantage of the strong correlation between δ^2H_p and stable hydrogen values measured

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in bird feathers (δ^2H_f) by correcting an interpolated δ^2H_p map (isoscape) using the regression equation between the two values and assigning individuals of unknown origin to this surface in a spatially explicit manner (Bowen *et al.* 2005; West *et al.* 2010). However, δ^2H_f is often variable among (Powell and Hobson 2006; Oppel *et al.* 2011) and within (Tonra *et al.* 2015) individuals from a single sampling location. Thus, large-scale processes like continental precipitation patterns do not fully describe the mechanisms that result in a given individual's δ^2H_f value in a given year.

For studies using precipitation-dependent stable isotope patterns to measure migratory connectivity accurately, Wunder *et al.* (2005) suggested that δ^2H_f and δ^2H_p should have a linear relationship that does not depend on species, age, location, or time, and called for more direct tests of these assumptions. Langin *et al.* (2007) further highlighted several assumptions of such studies, including that all individuals at a given location should have similar δ^2H_f, regardless of sex, and that a given location should have a similar δ^2H_f within and between breeding seasons. However, previous studies have found that δ^2H_f can vary within a site (Table 1). Specifically, δ^2H_f can vary among individuals of different ages (Meehan *et al.* 2003; Langin *et al.* 2007; Gow *et al.* 2012; Haché *et al.* 2012), and between years at the same site (Haché *et al.* 2012; Van Dijk *et al.* 2014; Tonra *et al.* 2015). An interaction between year and other sources of variation has been found in some studies, with species-specific (Nordell *et al.* 2016) and age (Gow *et al.* 2012; Haché *et al.* 2012) effects being different among years. Most studies have not investigated differences between sexes, but those that did have been inconclusive (Langin *et al.* 2007) or found no effect (Nordell *et al.* 2016). A further assumption is that δ^2H_f values should not vary between feather types in the same individual, but small differences have been found between primary and rectrix feathers in a songbird (Haché *et al.* 2012) and large differences were seen between various primary feathers in a raptor (Meehan *et al.* 2003). δ^2H_f 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.

Species	Sex	Age	Year	Age*Year	Feather Type	Study
Prothonotary Warbler (*Protonotaria citrea*)	no	SY < ASY	present	absent	P slightly < R	This study
Ovenbird (*Seiurus aurocapillus*)	N/A	nestling < ASY	present	present	P slightly < R	Haché *et al.* 2012
Wood Thrush (*Hylocichla mustelina*)	N/A	present	absent	present	absent	Gow *et al.* 2012
American Redstart (*Setophaga ruticilla*)	absent	nestling < ASY	absent	N/A	P < contour	Langin *et al.* 2007
Cooper's Hawk (*Accipiter cooperii*)	N/A	nestling < adult	N/A	N/A	P ≠ P	Meehan *et al.* 2003
Mallard (*Anas platyrhynchos*)	N/A	HY < adult	absent	N/A	N/A	Van Dijk *et al.* 2014

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 δ^2H_f 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 δ^2H_f values and the relationship between δ^2H_f and δ^2H_p. Many studies now use guild-specific transfer functions to relate δ^2H_f and δ^2H_p, 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 δ^2H_f values, hydrologic processes occurring within aquatic habitats may distort the relationship between δ^2H_p and δ^2H_f (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 δ^2H 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 δ^2H_f 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 δ^2H_f 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 δ^2H_f 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 δ^2H_f values in a population of Prothonotary Warblers (*Protonotaria citrea*) breeding in eastern Virginia, USA. Our goal was to assess variation in δ^2H 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° 24′ N, 77° 18′ W), with additional samples ($n = 7$) from Presquile National Wildlife Refuge (37° 21′ N, 77° 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

Table 2. Sample sizes of feathers in which δ²H was measured from Prothonotary Warblers from Henrico County, Virginia, USA. Age is reported as second-year (SY) or after second-year (ASY).

Year	Age		Sex		Feather Type		Total
	SY	ASY	Female	Male	Primary	Rectrix	
2013	8	14	16	6	0	22	22
2014	10	15	16	9	0	25	25
2015	6	12	13	5	18	0	18
2016	14	17	18	13	30	16	46
Total	38	58	63	33	48	63	111

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 were 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 years, and in 2015 and 2016, we also collected one innermost primary (P1). As a result, we used a mixture of primary and rectrix feathers for comparisons between age classes and sexes, and all samples used in that analysis from 2013 and 2014 were rectrices, while those from 2015 and 2016 were primaries. We also compared $\delta^2 H_f$ between primary and rectrices collected in 2016.

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 $\delta^2 H_f$ 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 $\delta^2 H_f$ 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 $\delta^2 H_f$ between the sexes because a Levene's test indicated unequal variances between male and female individuals ($F_{94, 1}$ = 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 $\delta^2 H_f$ value for the study area across ages, sexes, years, and feather types was -57.17 ± 10.12‰ (*n* = 96; Range = -85.27 to -38.09‰; 95% CI = -59.2; -55.14‰). The predicted $\delta^2 H_f$ value for our study site based on the growing-season $\delta^2 H_p$ 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 $\delta^2 H_f$ 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 ($F_{1,88}$ = 23.6; *P* < 0.001) and year ($F_{3,88}$ = 6.39; *P* < 0.001) had an effect on $\delta^2 H_f$ 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 $\delta^2 H_f$ 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 $\delta^2 H_f$ 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). $\delta^2 H_f$ values were not different in male and female individuals (t_{94} = 1.77; *P* = 0.08). When we repeated these analyses using only individuals known to have been
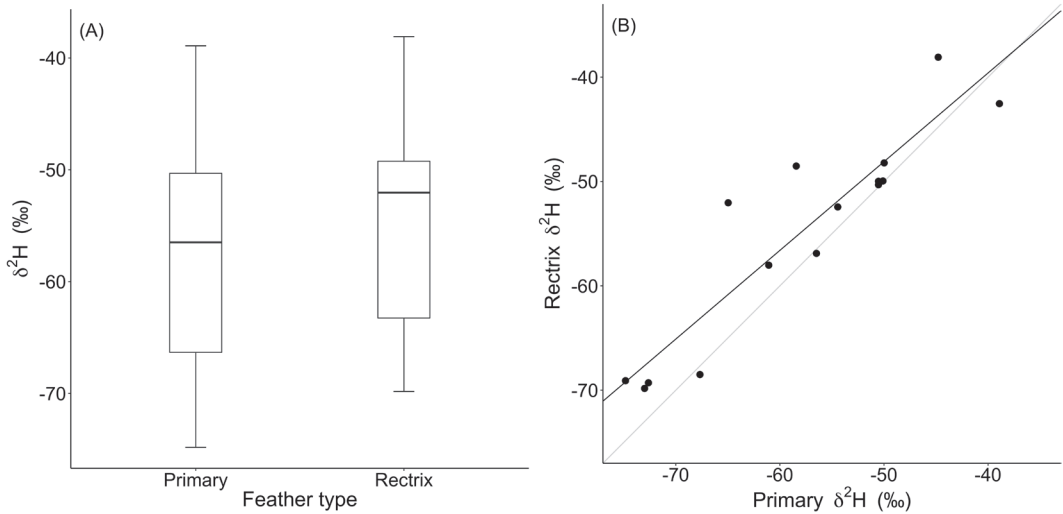
Figure 1. (A) δ^2H_f values (‰) for primary and rectrix feathers from paired samples of 15 female Prothonotary Warblers from Henrico County, Virginia, USA. (B) Correlation of δ^2H_f values for primary and rectrix feathers. The line of best fit (black) and a 1:1 line (gray) are shown.

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 δ^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 δ^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 δ^2H_f values, limited support for a difference between feather types, and no difference between the sexes. We found that the mean δ^2H_f value for our study site was similar to the value predicted for this location based on growing-season δ^2H_p values. The range

of δ^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 δ^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 δ^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*).
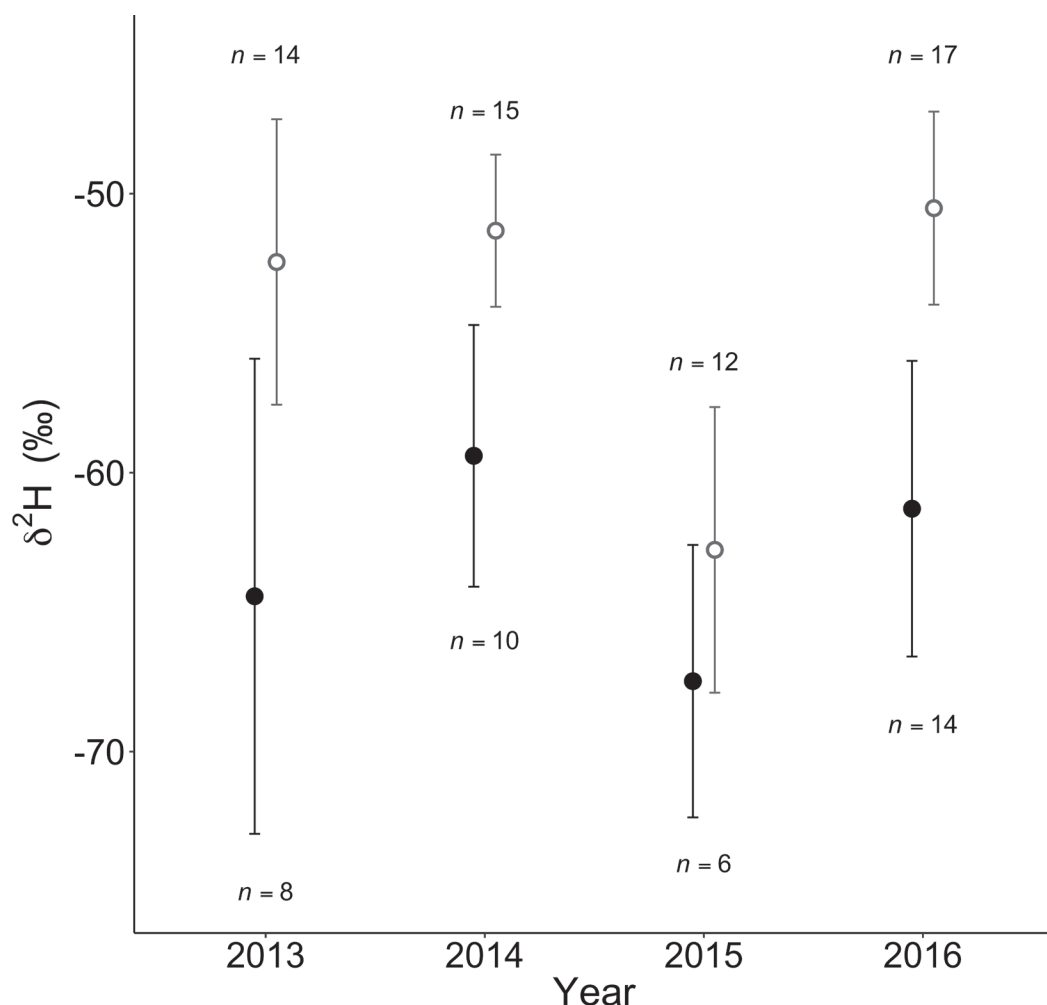
Figure 2. $\delta^2 H_f$ values (‰) across year and age classes for 96 feather samples from Prothonotary Warblers collected in Henrico County, Virginia, USA. Individuals in their first year of breeding are shown with open circles and individuals in their second year of breeding or older are shown in closed circles. Error bars show 95% confidence intervals.

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^2H 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^2H_f 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^2H 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^2H values measured in nestling blood samples. In contrast, Bortolotti *et al.* (2013) found that aquatic insects had slightly more enriched δ^2H values compared to terrestrial insects. To date, no studies have examined δ^2H 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^2H_f 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^2H_f values from 4 years of data, and found that one year (2015) had a more negative mean δ^2H_f 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^2H_f 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^2H_f 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^2H_f did not show a corresponding change. Other studies have also found yearly differences both in δ^2H_f (Haché *et al.* 2012; Van Dijk *et al.* 2014) and δ^2H_p (Van Wilgenburg *et al.* 2012). Yearly variation in δ^2H_f 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^2H_p (Hobson *et al.* 2012). Van Wilgenburg *et al.* (2012) found that isoscapes based on the long-term average of δ^2H_p values were accurate for predicting the mean δ^2H_p across all years at their study sites, but that yearly variation in δ^2H_p 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^2H_p values (Craig and Gordon 1965; Gat 2010; Bowen *et al.* 2011).

More study is needed to understand the mechanisms leading to variation in δ^2H_f values beyond those addressed here. For example, we considered testing for within-season variation in δ^2H_f 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^2H values interact with individual-specific differences in diet and physiology and ultimately δ^2H_f values is poorly understood at present (Tonra *et al.* 2015). δ^2H_f 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 δ^2H_f 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 δ^2H_p values (Fry 2006; Bowen *et al.* 2011), and large north-south flowing rivers may transport depleted δ^2H values downstream. Future work should characterize the influence of both aquatic prey items and multiple hydrologic regimes on δ^2H_f 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 δ^2H_f 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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