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DEUTERIUM MEASUREMENTS OF RAPTOR FEATHERS: DOES A LACK OF REPRODUCIBILITY COMPROMISE GEOGRAPHIC ASSIGNMENT?

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Abstract.—Despite the widespread use of stable isotopes in studies of avian movement, key assumptions on which the methodology is based remain unsubstantiated, including the assumption that measurements of stable hydrogen isotopes in feathers (δD_f) are consistent across time within the same laboratory or among laboratories using the same analytical protocols and keratin standards. We tested this assumption by remeasuring δD_f from 211 raptor feathers within and between laboratories. Initial and repeat samples were prepared and analyzed using identical protocols but analyzed in distinct automated runs with laboratory staff blind to sample identity. Reproducibility of δD_f measurements varied significantly and substantially among nine independent sample groups. Feather δD measurements among sample groups exhibited average isotopic shifts from –15.6‰ to +27.5‰ (an absolute difference of 43.1‰), with standard deviations from 6.0‰ to 12.4‰. Therefore, despite existing analytical protocols to address issues of reproducibility, empirical data suggest that comparing δD_f measurements among studies or labs and pooling samples analyzed during different automated runs within a laboratory remain problematic. More importantly, poor reproducibility compromises the geographic assignment of origins based on δD_f, because the substantial differences in δD_f measurements between automated runs can result in spurious inferences regarding the origins of migratory birds. We caution against the continued use of δD_f for predicting geographic origin, and for addressing important conservation questions, until the factors affecting poor reproducibility are identified and improved reproducibility is demonstrated within and among laboratories across time and taxa. *Received 14 January 2008, accepted 15 June 2008.*

Key words: accuracy, bias, IRMS, migration, origins, precision, stable hydrogen isotopes.

Mediciones de Deuterio en Plumas de Rapaces: ¿La Falta de Reproducibilidad Compromete la Asignación Geográfica?

Resumen.—A pesar del uso difundido de los isótopos estables en los estudios de movimiento de las aves, las suposiciones clave en las que se basa esta metodología continúan sin ser verificadas, incluyendo la suposición de que las mediciones de los isótopos estables de hidrógeno en las plumas (δD_p) son consistentes en el tiempo en el mismo laboratorio o entre laboratorios usando el mismo protocolo analítico y los mismos estándares de queratina. Evaluamos esta suposición mediante la remedición de δD_p de 211 plumas de rapaces en un mismo laboratorio y entre laboratorios. Las muestras iniciales y repetidas fueron preparadas y analizadas usando protocolos idénticos pero analizadas en corridas automáticas diferenciadas, sin el conocimiento de la identidad de la muestra por parte del personal del laboratorio. La reproducibilidad de las medidas de δD_p variaron significativamente y de modo substancial entre nueve grupos de muestras independientes. Las mediciones de δD de las plumas entre los grupos de muestreo exhibieron cambios isotópicos promedio desde –15.6‰ a +27.5‰ (una diferencia absoluta de 43.1‰), con desviaciones estándar desde 6.0‰ a 12.4‰. Por tal motivo, a pesar de que existen protocolos analíticos para considerar los aspectos de reproducibilidad, los datos empíricos sugieren que la comparación de mediciones de δD_p entre estudios o entre laboratorios y combinando muestras analizadas durante diferentes corridas automáticas en un mismo laboratorio siguen siendo problemáticas. De modo más importante, la baja reproducibilidad dificulta las asignaciones geográficas de origen basadas en δD_p, debido a que las diferencias substanciales en las mediciones de δD_p entre las corridas automáticas

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pueden resultar en inferencias espurias sobre el origen de las aves migratorias. Lanzamos una advertencia en contra de continuar usando δD_p para predecir el origen geográfico y para abordar importantes preguntas sobre conservación, hasta que los factores que afectan la baja reproducibilidad sean identificados y que se demuestre una mejor reproducibilidad en y entre laboratorios a través del tiempo y de los taxones.

Measurement of stable hydrogen isotope ratios (deuterium: protium; δD) in feathers, which are indicative of the location of feather growth (Chamberlain et al. 1997, Hobson and Wassenaar 1997), has shown the potential to bring previously intractable questions related to bird migration, dispersal, and migratory connectivity into the purview of researchers, particularly when used in combination with other intrinsic markers (reviewed in Rubenstein and Hobson 2004, Norris et al. 2006). Interestingly, the proliferation of δD-based applications in studies of avian movement has not been mirrored by tests of key assumptions on which the methodology is based, despite the recognized need for careful examination of assumptions in new stable-isotope applications (Gannes et al. 1997). Norris et al. (2006) recently enumerated several assumptions in need of validation pertaining to the distribution of δD in precipitation and its relation to feather δD (δD_f). Likewise, natural variation in δD_f within and among individuals remains poorly characterized (but see Smith and Dufty 2005, Wunder et al. 2005, Langin et al. 2007, Smith et al. 2008). Despite these unresolved issues, stable-isotope applications have been described as the cornerstone of a new era of bird migration research (Hobson 1999, Webster et al. 2002, Rubenstein and Hobson 2004, Hobson 2005). In our effort to capitalize on the power of this emerging methodology, we may have neglected a question fundamental to the application of δD to the study of avian migration: does inaccuracy in δD_f measurements compromise geographic assignment?

Inferences of geographic origin are reliable only when they are based on accurate measurements of δD_f. Two types of error potentially affect the accuracy of δD_f measurements: systematic (bias) and random (imprecision) error. The supposed predominant source of bias in δD_f measurements, exchangeable hydrogen, is estimated from the measurement of keratin standards and a correction is devised and applied to feather samples measured concurrently (see below; Wassenaar and Hobson 2003). Despite the correction, an unknown amount of bias persists and contributes to measurement error (Rabinovich 2005). Imprecision in studies that rely on δD_f measurements is typically reported as the standard deviation (SD) of repeated measurements of isotopically homogeneous reference materials (Jardine and Cunjak 2005). However, feather samples typically are not homogenized prior to isotopic analysis. Consequently, decreased precision is to be expected in δD_f measurements, compared with isotopic reference materials or keratin standards, for two primary reasons: (1) lack of homogenization and (2) an inability to measure repeatedly the same sample of feather. The second point suggests that precision estimates based on repeat δD_f measurements may be complicated by systematic isotopic differences between adjacent samples within a feather (e.g., Smith et al. 2008). Furthermore, the conditions of measurement influence precision estimates (International Organization for Standardization [ISO] 1995). Specifically, we distinguish between repeatability and reproducibility of δD_f measurements: "repeatability" denotes the precision of multiple measurements under the same measurement conditions (e.g., within an automated run); "reproducibility" denotes the precision of repeated measurements when one or more measurement condition has changed (e.g., between automated runs or labs; ISO 1995). Reproducibility may be the more relevant metric to quantify in association with δD_f measurement to assess comparability of measurements within and among studies.

Initially, the exchange of approximately 10–20% of the hydrogen in feathers with ambient hydrogen introduced considerable bias and imprecision into δD_f measurements, resulting in δD values reflecting ambient laboratory δD in addition to environmental δD indicative of the location of feather growth (Chamberlain et al. 1997, Wassenaar and Hobson 2000). Subsequently, Wassenaar and Hobson (2003) developed several homogenized keratin standards and a protocol for the comparative equilibration of feather samples with these standards to nullify the influence of exchangeable hydrogen on δD_f measurements. Specifically, the comparative equilibration method improves δD_f measurements by correcting measured δD values of feather samples based on a formula derived from a regression of measured δD values of keratin standards against their calibrated δD values (see Wassenaar and Hobson [2003] for details of the correction); current keratin standards range between –190‰ and –100‰ (Wassenaar and Hobson 2003). Although the comparative equilibration correction reduces bias in δD_f measurements, measurement precision is not improved by such a correction factor, nor can it be (Rabinovich 2005). The utility of the comparative equilibration method lies in the prospect of direct comparison of δD_f measurements throughout the year, as ambient δD shifts, and among labs, thus enabling comparison of results among studies (Wassenaar and Hobson 2003).

Estimating bias in δD_f measurements is not possible because we cannot know the true δD value of any given feather sample. We simply assume that correcting measured δD_f values on the basis of concurrent measurement of calibrated keratin standards reduces bias (but see below). Wassenaar and Hobson (2006) suggest repeatability on the order of ±3‰ as a best-case scenario for δD_f measurements, including variation strictly resulting from metabolic processes and analytical limitations; recent work agrees generally with this estimate (Paxton et al. 2007). However, comparability among laboratories and studies, as well as the validity of geographic assignment, depends fundamentally on the reproducibility of δD_f measurements. To our knowledge, a single study has reported temporally distinct repeated measurements of δD_f (i.e., reproducibility; Lott and Smith 2006); raptor feathers measured 0–14 months after an initial analysis exhibited dramatic systematic shifts in δD_f values. In the present study, we consider the reproducibility of repeated δD measurements of raptor feathers occurring in different automated runs within a laboratory and between isotope-analytical laboratories.

Methods

Feather collection, sample preparation, and stable-isotope analyses.—The raptor feathers subjected to reproducibility testing represent the work of several independent studies from throughout North

TABLE 1. Collection histories for nine sample groups used to evaluate the reproducibility of feather δD measurements in raptors. All sample groups comprise feathers from immature raptors captured during fall migration or in museum collections ("NA" sample groups; see Lott and Smith [2006] for details).

Group	*n*	Number of species	Collection location	Collection year(s)	Data source
ID	30	1	Idaho	2002–2003	K. Donahue unpubl. data
HW1	16	1	Western USA	2002–2003	HawkWatch International unpubl. data
HW2	28	7	Western USA	2002–2003	HawkWatch International unpubl. data
AR1	30	1	Florida	1998–2003	Ress 2006
HW3	12	1	Western USA	2002–2003	HawkWatch International unpubl. data
NA1	36	5	North America	1874–2003	Lott and Smith 2006
HM	20	2	Pennsylvania	2004	Hawk Mountain Sanctuary unpubl. data
AR2	19	1	Florida	1998–2003	Ress 2006
NA2	20	10	North America	1874–2003	Lott and Smith 2006

America (Table 1). However, each contributing project shared the primary objective of using measurements of δD_f to estimate the natal origins of migrating raptors (reviewed in Lott and Smith 2006). Feather samples were derived from live individuals captured during fall migration or from museum specimens, and only from individuals in juvenal plumage, given that δD measurements in both downy (Duxbury et al. 2003) and adult raptor feathers (Meehan et al. 2003, Smith and Dufty 2005) can deviate from local food-web signatures at the location of feather growth. From fall migrants and museum specimens, samples consisted of contour feathers from (1) the lower belly or breast or (2) the flanks below the folded wing, respectively. One to three contour feathers were collected from each individual.

Sample preparation prior to stable-isotope analysis occurred at various locations in North America, but the same general procedure was followed in all cases. First, feathers were cleaned of surface oils and debris using a 2:1 chloroform:methanol solution and allowed to air dry for ≥48 h. After cleaning, samples were packaged for analysis as follows: from individual feathers, portions of vane (0.35 ± 0.01 mg) cut from an area perpendicular to the rachis at the distal tip of each feather was transferred into silver capsules and stored in plastic culture trays (i.e., the initial sample). A repeat sample was taken from a location immediately proximal to the initial sample; sample preparation proceeded as described above. In some cases, samples were prepared before shipment to the lab, whereas other samples were prepared after arrival at the laboratory. We did not expect remote preparation of samples to affect δD_f measurements, because we adhered to a consistent preparation protocol and provided adequate time for samples to equilibrate with laboratory air moisture.

Once in the lab, samples air-equilibrated with ambient water vapor for ≥22 days (median = 35 days among all sample groups). The deuterium composition of the nonexchangeable component of a feather sample was measured using the online pyrolysis and CF-IRMS techniques detailed by Wassenaar and Hobson (2003, 2006). Measurement of repeat samples in one group occurred at a second stable-isotope laboratory but followed sample preparation and analysis protocols identical to those applied to other sample groups, including the use of the same keratin standards. The δD content of initial and repeat samples was measured in different automated runs, with laboratory staff blind to sample identity. However, within each sample group, initial δD_f measurements occurred within a single automated run, and the same was true of repeat δD_f measurements. Feather δD results are reported in parts per thousand (‰) deviation from the VSMOW-SLAP standard scale. Hydrogen-isotope reference material (IAEA-CH-7; –100‰ VSMOW) exhibited a measurement repeatability of better than ±2.0‰ (Wassenaar and Hobson 2006); calibrated keratin standards used for comparative equilibration have measurement repeatabilities of ±2‰ to ±5‰ (Wassenaar and Hobson 2003).

Statistical analyses.—Within a sample group, we considered the average difference between initial δD_f measurements and repeat δD_f measurements, and the SD of this difference, as measures of δD_f reproducibility. That is, within a sample group, a mean difference of 0 ± 3‰ (SD; i.e., best-case repeatability) would suggest perfect reproducibility. We used analysis of variance (ANOVA) and Levene's test to determine the extent to which reproducibility varied among the sample groups.

The comparative equilibration method corrects measured δD_f values of feather samples on the basis of the concurrent measurement of calibrated keratin standards. However, the calibrated values of current keratin standards span an isotopic range of only –190‰ to –100‰. Because δD_f values much higher than –100‰ were common in our samples, we considered the possibility that the reproducibility of δD_f measurements decreases above –100‰, which is outside the range of δD values used to construct regression equations used for calibration. Using all sample groups in which the initial and repeat analysis occurred in the same laboratory ($n = 191$), we constructed a linear mixed model (LMM) relating δD_f from the repeat analysis to δD_f from the initial analysis, producing a global relationship between the repeat and initial analysis. We considered sample group as a random variable to account for the lack of independence among samples analyzed together. Specifically, we allowed separate slopes and intercepts for each sample group. To determine whether reproducibility decreased at δD_f values above –100‰, we plotted the marginal residuals from the LMM model as a function of δD_f predicted for the repeat analysis. Marginal residuals were appropriate in lieu of conditional residuals, given that we were interested in the reproducibility of δD_f measurements in general, and not solely that of feathers in the present study (Schabenberger 2004).

We conducted all statistical analyses using SAS, version 8.2 (SAS Institute 1999). We corrected denominator degrees of freedom in the LMM using the Kenward and Roger (1997) method, as recommended by Schaalje et al. (2002).

RESULTS

Reproducibility of repeated δD_f measurements varied substantially among sample groups (ANOVA: $F = 52.3$, df = 8 and 202, $P < 0.001$; Levene's test: $F = 3.6$, df = 8 and 202, $P < 0.001$; Fig. 1). The average difference between initial and repeat δD_f measurements across sample groups ranged from –15.6‰ to 27.5‰, and the SD of this difference ranged from 6.0‰ to 12.4‰ (Fig. 1). Reproducibility of δD_f measurements was equally low regardless of differences in the location of initial and repeat samples preparation (Fig. 1A–B). Reproducibility of δD_f measurements assessed in separate laboratories (Fig. 1C) was within the range of that observed within a laboratory (Fig. 1A–B).

Repeated measurements of δD from the same feather related strongly to the initial measurement (LMM: $F = 1075.0$, df = 1 and 12.9, $P < 0.001$; Fig 2A), with a slope of 0.95 (95% CI: 0.88 to 1.01) and an intercept of 1.22 (95% CI: –10.39 to 12.83). These parameters agree generally with the expectation that δD measurements from adjacent locations on a raptor feather will differ slightly (i.e., because of intrafeather variation; Smith et al. 2008) but relate to one another with unit slope. However, the plot of LMM marginal residuals indicates that reproducibility of δD_f measurements decreases dramatically in feathers with δD_f values above –85‰ (Fig. 2B).

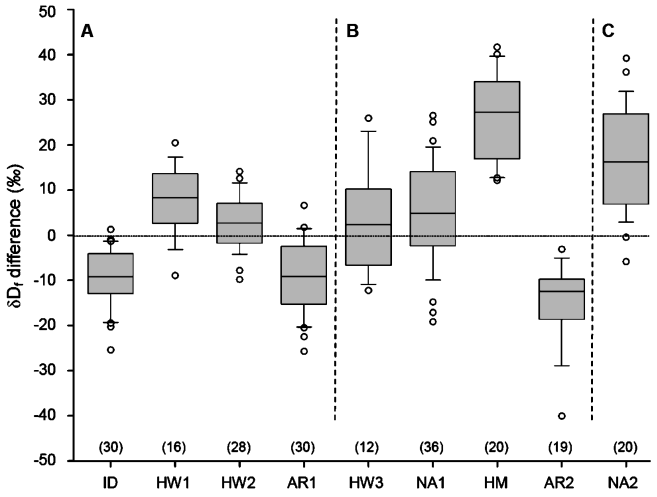

FIG. 1. Reproducibility of δD_f measurements from a single raptor contour feather measured in different automated runs for nine independent sample groups; the difference was calculated as (initial analysis – repeat analysis). Sample groups are distinguished as (A) initial and repeat samples prepared in different locations; (B) initial and repeat samples prepared in the same location; and (C) samples analyzed initially at one stable-isotope laboratory and subsequently, using identical protocols, at a second stable-isotope laboratory. Box plots indicate the mean and the 25th and 75th percentiles of the difference; whiskers indicate the 10th and 90th percentiles. Circles indicate outliers. Sample sizes are indicated in parentheses. The broken horizontal line at 0‰ indicates no isotopic difference between the initial and repeat analysis. The abbreviations along the abscissa correspond to those given in Table 1.

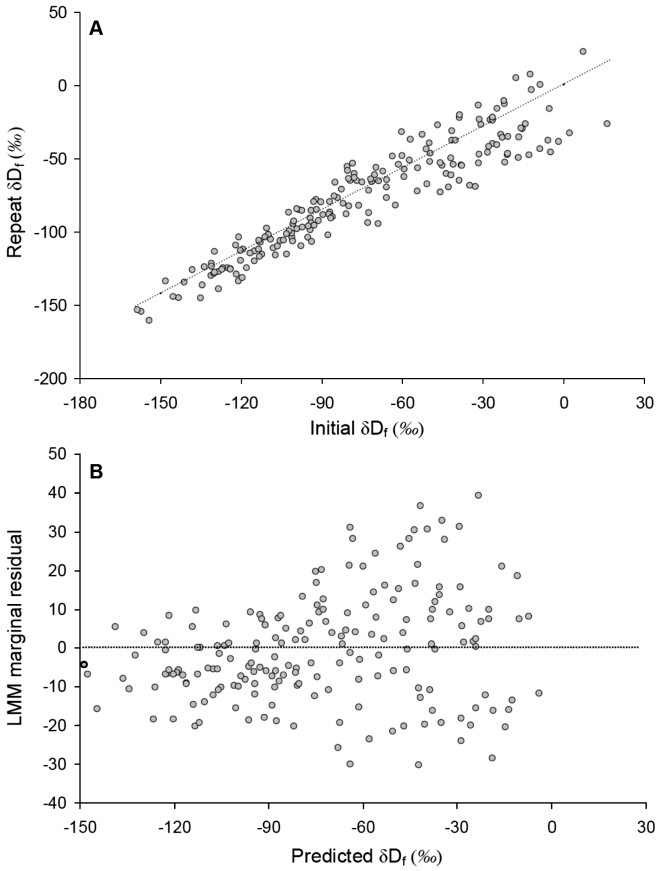

FIG. 2. (A) Raw data and (B) marginal residuals from a linear mixed model (LMM) relating a repeat measurement of δD_f to an initial measurement of δD_f from the same feather as a function of δD_f predicted for the repeat measurement. The broken line in A indicates the relationship between the repeat and initial measurements of δD_f estimated by the LMM, after accounting for the lack of independence among samples analyzed together (see text). The broken horizontal line in B indicates a marginal residual value of zero.

DISCUSSION

After the development of standard protocols for comparative equilibration of feather samples with calibrated keratin standards (Wassenaar and Hobson 2003), we expected reasonable reproducibility in measurement of δD_f. Consequently, we were surprised by the often substantial lack of reproducibility in δD_f measurements (Fig. 1). Reproducibility was inconsistent among sample groups, as indicated by both positive and negative shifts in δD_f values between initial and repeat measurements and considerable variability around these shifts (Fig. 1). As mentioned above, the inability to repeatedly measure the same sample of feather can complicate precision estimates if isotopic differences exist between adjacent sample locations within a feather. However, although the

enrichment of deuterium from distal to proximal feather vane observed in raptor feathers (about 3–11‰; Smith et al. 2008) improves reproducibility in some sample groups (i.e., those with negative average δD_f differences; Fig. 1), it also implies that reproducibility in other groups is worse than indicated (i.e., those with positive average δD_f differences; Fig. 1). We detected similarly poor reproducibility when the initial and repeat δD_f measurements occurred in different laboratories.

We have no reason to believe that poor reproducibility is a problem unique to raptor feathers, and we strongly encourage similar work with other taxa (e.g., songbirds). Likewise, we encourage further work into isotopic variation within and among feathers of individuals grown simultaneously (e.g., juvenal flight feathers) and sequentially (e.g., formative and basic flight feathers).

We suggest that reduced reproducibility associated with δD_f measurements above –85‰ results from a broadening prediction interval in the inverse regression model associated with the comparative equilibration method. Specifically, the prediction interval likely broadens considerably outside the range of δD values on which the model is based (i.e., δD values less than –190‰ and greater than –100‰). Following the general recommendation of Jardine and Cunjak (2005) to use standards spanning the range of expected isotopic values in samples, we advocate the development of additional keratin standards with δD values up to 50‰ to improve the correction of higher δD_f values (i.e., greater than –100‰) using comparative equilibration. Furthermore, we recommend further distribution of all keratin standards for interlaboratory "ring-test" comparisons to generate accepted values for the standards (Jardine and Cunjak 2005).

Implications of poor reproducibility in feather δ*D.*—We cannot conclude whether the lack of reproducibility in δD_f measurements stems from some step (or steps) in the sample collection, handling, preparation, and analysis process or is an intrinsic property of unhomogenized feathers in general. However, it is clear that despite methodological advances in stable-hydrogen-isotope analysis of feathers and the distribution of calibrated keratin standards devised specifically to address the issue of comparability, δD_f measurements can vary markedly among automated runs within a single laboratory as well as among laboratories. Thus, comparing δD_f measurements among studies or labs and pooling samples analyzed during different automated runs within a laboratory remain problematic.

Moreover, poor reproducibility in δD_f measurements has a larger implication: the geographic assignment of origins based on δD_f is compromised. Take, for example, a researcher collecting feathers from hawks migrating along the Kittatinny Ridge in eastern Pennsylvania with the intent of estimating their geographic origin. Given the reproducibility observed in the present study, measurement of δD_f from nearly identical samples of feather within each individual could result in sample δD_f averages of –90‰ and –60‰. Which value best represents the average source area of the sample of hawks? Choosing one value over the other drastically influences the inference of geographic origin, whether that origin is estimated using continuous-response or discrete-response predictions (e.g., Wunder et al. 2005). In our simple example, the source area of the hawk sample may reasonably occur anywhere between northern Ontario and western New York (a difference of >1,000 km). The geographic implications of a 30‰ difference in δD_f can be visualized for all of North America using figure 4 in Lott and Smith (2006).

Identifying the source (or sources) of irreproducibility in δD_f measurements will require thoughtful experiments to determine the relative influence on reproducibility of intrinsic feather properties or sample collection, handling, preparation, and analysis methods. In the interim, as a precautionary measure, we suggest that researchers handle all feather samples as similarly as possible, including having the same person prepare every sample in the same lab, despite the apparent lack of detrimental sample-preparation effects in the present study. More importantly, given the problems we observed with reproducibility among automated runs, we suggest that researchers make all δD_f measurements for the same application in as few sequential automated runs as possible, even when feather sample collection spans multiple years. When samples from a single study are analyzed in multiple automated runs, the reproducibility of measurements should be assessed through analyses of several feathers in all automated runs (Jardine and Cunjak 2005) to ensure that results can justifiably be pooled.

Certainly, if proved reliable, the measurement of stable-hydrogen isotopes in feathers will facilitate novel insights into bird migration, dispersal, and migratory connectivity. Nonetheless, it is counterproductive to move forward without first establishing full confidence in the technique that underlies such insights and conservation recommendations. Clearly, the factors affecting reproducibility need to be identified, and improved reproducibility demonstrated within and among laboratories across multiple taxa, lest resources and time be spent generating data that are inadequate for their intended purpose of assigning geographic origins and ascertaining migratory connectivity in birds.

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