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Serial sampling provides chronological evidence that endogenous protein is used for primary growth in a molt-migrant goose

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ABSTRACT

This is a proof of concept paper based on chronological samples of growing feathers from geese thought to be molt-migrants. When molt-migrant birds initiate molt shortly after migrating to a new isoscape, isotope values measured along the length of their feathers should change continuously. To assess long-term changes and daily cycling in $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values, we serially sampled growing primaries of three presumed molt-migrant geese. Two showed changing $\delta^{15}\text{N}$ signatures along the length of their growing primaries, indicating they were molt-migrants, while the third, presumably a resident, showed no change. We then resampled these feathers at closer intervals for evidence of the predicted diel cycle in the use of exogenous and endogenous protein for feather growth, generated by the diel feeding cycle of these geese. As predicted, the two geese that were equilibrating to a new isoscape showed oscillations of approximately 24-hour periodicity in $\delta^{15}\text{N}$ values, measured along the length of their primaries. In contrast, the goose that was not equilibrating to a new isoscape showed no 24-hour periodicity in its $\delta^{15}\text{N}$ values. Our results demonstrate that chronological sampling along the length of individual primaries holds great potential for identifying individuals that are molt-migrants.

INTRODUCTION

Molt-migrants require time for their endogenous protein reserves to come into equilibrium with local isoscapes (Martinez del Rio and Anderson-Sprecher 2008). Thus, feathers grown during this period of equilibration should show a steady change in isotopic signature along the length of their feathers. This follows because feathers grow at a more or less constant rate throughout the 24-hour cycle (Murphy and King 1986; Schieltz and Murphy 1995, Lillie and Wang 1940); thus, when exogenous protein from local foraging is exhausted, endogenous protein reserves, which are coming into equilibrium with the new isoscape, supply the protein needed to build feathers during daily periods of fasting (Murphy and King 1990).

These facts conspire to make two predictions that help identify molt migrant birds that initiate feather growth shortly after moving to a new isoscape. First, a steady change in isotope signature along the length of flight feathers is expected for molt-migrants that begin molt before equilibrating to a new isoscape. This prediction is easily tested with coarse sampling along the length of flight feathers. Second, fine sampling along feathers, should reveal a roughly 24-hour periodicity representing the alternating use of exogenous and endogenous protein sources for feather construction. The amplitude of this cycle is likely driven by the size of the protein pool in the blood relative to the amount of protein being withdrawn for feather synthesis. If the fraction of blood protein used for feather synthesis is small, then daily cycling associated with feeding and fasting may be damped and difficult to measure, but it still should be present.

Greylag Geese (*Anser anser*) that breed in terrestrial, freshwater habitats in Sweden are known from sightings of marked individuals (Nilsson et al. 2001), to migrate in late summer to the maritime saltmarshes of the island of Saltholm (55°38'N 12°45'E) in Denmark where they graze saltmarsh plants (Fox et al. 1998) and undergo their annual molt (Fox et al. 1995). Marine environments are well known to have very different $\delta^{15}\text{N}$ signatures than freshwater environments, and they may have slightly different $\delta^{13}\text{C}$ signatures. Fox et al. (2009) confirmed that newly grown flight feathers of Greylag Geese showed stable isotope values that

were intermediate between those derived from a terrestrial plant diet (i.e. in Sweden prior to the molt-migration) and a diet of saltmarsh plants from Saltholm.

To explore expected changes in $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ along the length of primaries and to examine the potential for 24-hour cycling in the use of exogenous and endogenous protein for feather growth, we serially sampled a single primary feathers from each of three Greylag Geese from Saltholm; these feathers (and no others) were available from the study of Fox et al. (2009). Because feathers are generated from tip to base and composed of non-living keratin, primaries grown by geese that migrated to Saltholm for their molt should change chronologically from more positive (freshwater) $\delta^{15}\text{N}$ signatures at the feather tip to more negative (marine) $\delta^{15}\text{N}$ signatures at the feather base. Further, because flightless Greylag Geese forage only at night on Saltholm (Kahlert et al. 1996), a 24-h periodicity in the $\delta^{15}\text{N}$ signature should be expected in serial samples from the flight feathers of geese that were not in equilibrium with the Saltholm isoscape.

Our results show that serial samples taken along the length of primary flight feathers readily show equilibration toward a new isoscape and, further, that feathers sampled at fine intervals can reveal diel shifts in exogenous and endogenous protein sources used for feather generation. We are aware of just two prior studies of hair or feathers that used chronological samples to examine changes in isotope signatures through time. Cerling et al. (2006) used Elephant hair to demonstrate the movement of individual African elephants (*Loxodonta africana*) to different foraging locations, and Church et al. (2006) used samples along the length of a growing rectrix from a California Condor (*Gymnogyps californianus*) to show a sudden deposition of lead that resulted in its death. Because our results are unavoidably based on feathers from just three geese, their importance lies in the new approaches we use to study expected changes in isotopic signatures for molt-migrant birds.

MATERIALS AND METHODS

Feathers were obtained from Greylags in active molt on Saltholm, between Copenhagen and Swedish Skania coast (Fox et al. 2009). The Danish Forest and

Nature Agency gave permission to catch and sample the geese and the landowners of Saltholm gave permission to work on the island. The geese were caught under the Copenhagen University Natural History Museum Ringing Permit A600 "DMU-Kalø Ringmærkning."

The geese included in this study were not individually tracked, so we do not know when they arrived at Saltholm, or how soon after arrival they started to molt, or even if they were all molt-migrants to Saltholm. These limitations mean that we could obtain useful information on periodicity only from birds that showed strong changes in their $\delta^{15}\text{N}$ signatures along the length of the sampled primary, indicating they were molt-migrants to Saltholm. Greylags are large geese that would require considerably more than a month to come into equilibrium with a new isoscape as different as that at Saltholm (Martinez del Rio and Anderson-Sprecher 2008). Molting geese that were already in equilibrium with the salt marsh isoscape of Saltholm should show no 24-hour cycling in isotopic signatures; these could be either local, Saltholm, geese (e.g. failed breeders) or salt marsh breeders from a similar isoscape. Although there is variation among individual feather growth rates, Greylag Geese should grow their feathers at approximately 7 mm d^{-1} , as inferred from a mean mass of 3509g (Dunning 2007) and the allometric relationship between primary growth rate and body size (Rohwer et al. 2009).

The feathers used for this study were sampled twice, once at every 5mm to plot the change in $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ over a longer period of feather time, measured as mm from the tip of the primary, and a second time at 1 or 2mm intervals, in the hope that we could detect a 24h periodicity in the protein source used for feather generation. Which primary feather was used should not affect the results of this study, as Greylag Geese, like most waterfowl, lose and replace their flight feathers simultaneously (Hohman et al. 1992).

Methods for sample preparation and analysis generally follow those in Paritte and Kelly (2009). Feathers were cleaned in dilute detergent followed by repeated rinsing. After air drying the feathers were cleaned again in 2:1 chloroform:methanol and allowed to air dry before processing. Once feathers were dried a research technician marked the rachis of each feather from its tip to the base

of the growing vane at 1, 2 or 5 mm intervals. The majority of the posterior vane was then cut away, leaving only the few mm closest to the rachis. Then, using the pen marks as a guide, an approximately 200 μ g sample of feather vane was cut immediately adjacent to the pen mark. These samples were loaded into tin capsules (3.5x5.5mm) and stored in an elisa plate until they were analyzed.

We analyzed samples in batch sequences of 49 samples and references, referred to as autoruns. Each autorun typically analyzed 39 unknown samples and 8 laboratory reference samples in positions 1, 2, 7, 13, 19, 37, 43, 49. The laboratory reference material was powdered Brown-headed Cowbird feather (*Molothrus ater*), generated as described in Kelly et al. (2009). Among sample variation in the laboratory reference material was < 0.2‰ for both $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$. In addition, to this laboratory reference we ran one sample each of two National Institute of Standards and Technologies NIST reference materials (USGS 40 in autorun position 25 and USGS 41 in autorun position 31). All stable isotope ratios are expressed in standard δ notation, where $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ = [(isotope ratio sample/isotope ratio standard) – 1] * 1000. Consequently, $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ are expressed in parts per thousand (‰) deviation from a standard, which was Vienna Pee Dee Belemnite for $\delta^{13}\text{C}$ and Air for $\delta^{15}\text{N}$. Isotope ratios were measured at the University of Oklahoma using a Thermo Finnigan Delta V isotope ratio mass spectrometer connected to a CosTech elemental analyzer.

For each autorun we corrected all measurements for instrumental drift between the first and last sample. Instrumental drift corrections were based on the slopes of best-fit lines for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values regressed against analysis time of references within each autorun. A slope was calculated for the cowbird standard in the run and this slope was used as the drift correction coefficient.

To determine if there was evidence of 24-hour cycling in the $\delta^{15}\text{N}$ values along the length of the primaries, we used custom Matlab code to perform an autocorrelation analysis after de-trending the data using linear regression and removing the mean. We used this method to find correlation maxima and minima that reveal periodicity in the $\delta^{15}\text{N}$ values. Using additional custom Matlab code, we then developed a bootstrap method to test for the statistical significance of having

autocorrelation minima and maxima that correspond to a periodic pattern in isotope values. To do so, we randomly permuted the data for each feather and performed autocorrelation analyses of those permuted values. Out of 10,000 permutations, we asked what fraction of the data had both a minimum less than or equal that observed in our original autocorrelation and a maximum spaced at the appropriate interval.

RESULTS

Evidence for equilibration following molt-migration

Two geese, 501 and 508, showed a clear and steady decline in their $\delta^{15}\text{N}$ signatures through time ($P < 0.0001$), while 509, showed no change ($P = 0.34$, Fig. 1). Both geese that changed did so in a way consistent with the large shift of about 8 ‰ in the $\delta^{15}\text{N}$ isoscapes suggested by the results of Fox et al. (2009). The third goose showed no change in $\delta^{15}\text{N}$ along the length of its primary and had a mean of 7.6 ‰, a value intermediate between the start and end values for the two geese that showed a steady change in $\delta^{15}\text{N}$ with time (Fig. 1), which was puzzling. If this individual had been on Saltholm long enough to be in equilibrium with the salt marsh isoscape, then its mean $\delta^{15}\text{N}$ value should have been at or below the latest values from the two geese that showed a steady change in their $\delta^{15}\text{N}$ values. However, its mean of 7.6 ‰, was considerably higher than the lowest $\delta^{15}\text{N}$ values found for those two geese (Fig. 1), suggesting that it was a resident goose that did not feed in the saltmarshes of Saltholm. Some of the hayfields used by resident Greylag Geese are less subject to marine influence than the saltmarshes where the majority of migrants feed.

The $\delta^{13}\text{C}$ signature of goose 501 increased significantly along the length of its primary ($p = 0.0003$), while $\delta^{13}\text{C}$ for goose 509 ($p = 0.09$) and goose 508 ($p = 0.46$) showed no significant change (Fig. 1). Based on the results of Fox et al. (2009), the $\delta^{13}\text{C}$ signatures of these geese should have declined if they had moved from Sweden to Saltholm for the molt. Yet goose 501 showed a significant increase in its $\delta^{13}\text{C}$ during primary growth (Fig. 1); it also showed a highly significant decline in $\delta^{15}\text{N}$, suggesting that it was indeed a molt-migrant to the island of Saltholm (Fig. 1).

Evidence for 24 hour cycling in $\delta^{15}\text{N}$

To explore the possibility of 24-hour cycling in the $\delta^{15}\text{N}$ values for these three geese, we sampled their primaries as finely as possible, at 1 or 2mm intervals along the length of the feather. Flightless Greylag Geese forage at night on Saltholm (Kahlert et al. 1996). Thus, a 24-hour periodicity in the $\delta^{15}\text{N}$ signature should be recovered from serial samples along the length of the feathers for geese 501 and 508, the two individuals that were coming into equilibrium with the Saltholm marine environment (Fig. 2). In contrast, goose 509, which showed no change in $\delta^{15}\text{N}$ along the length of its primary, was not expected to show a 24-hour periodicity in its $\delta^{15}\text{N}$ values because it apparently was in equilibrium with its Saltholm diet. The length of these fine-resolution runs was shorter than the length of feather used to generate Figure 1 because of sampling problems and a malfunction of the mass spectrophotometer.

Autocorrelation results for $\delta^{15}\text{N}$ measured in these serial samples are summarized in Figure 2. Note that, because there is significant sampling noise, the signal is not purely periodic in any of the sampled feathers. Feather 508 shows a minimum (most negative) autocorrelation value at the second autocorrelation lag. Given a sampling interval of 2 mm, this corresponds to 4 mm of feather length. Additionally, 508 showed positive autocorrelation values in the region of twice the minimum, strongly indicating periodicity in the data. The bootstrap statistics indicated that the probability of having the combination of a minimum at the lag of 2 and a max near the lag of 4 is $P = 0.032$. Feather 501 had a different sampling interval and, accordingly, showed a more expanded autocorrelation function with a minimum at lag of 3 and a maximum autocorrelation at twice that value. The bootstrap probability of having that combined maximum and minimum was $P = 0.01$. Thus, both feathers 501 and 508 show significant periodicity in their $\delta^{15}\text{N}$ values. In contrast, feather 509, which showed no sign of coming into a new equilibrium for $\delta^{15}\text{N}$, never showed a significant change in sign for the autocorrelation, suggesting, as predicted, that there was no periodic signal underlying the data for this feather.

DISCUSSION

The value of sampling feathers serially

As far as we are aware, the data for $\delta^{15}\text{N}$ in Figure 1 constitute the first direct test of a gradual change in the isotopic composition of feathers being grown while a molt-migrant is coming into equilibrium with a new isoscape. Fox et al. (2009) inferred this process by sampling food plants used by Greylag Geese on their breeding grounds in southern Sweden, and on their saltwater molting grounds on the island of Saltholm in Denmark. This inference was based on the assumption that fractionation values for the conversion of $\delta^{15}\text{N}$ values in food plant to $\delta^{15}\text{N}$ values in goose feather were accurately represented by the results of an experimental study of Japanese Quail (*Coturnix japonica*) raised on a plant based diet (Hobson and Clark 1992a; Hobson and Clark 1992b). How well those values represent similar processes in Greylag Geese is an unknown, as are the confidence intervals associated with these transformations. Further, Fox et al. (2009) used only two food plants from each locality to infer the expected changes in $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values for feathers, yet Greylag Geese probably use a larger diversity of plants at each of these localities, as is known to be the case on Saltholm as the molt progresses (Fox et al. 1998). For these reasons, the direct measure of change along the length of the feather that we have established for two of the three Greylag primaries in our sample offers a powerful confirmation of the result obtained by Fox et al. (2009) for $\delta^{15}\text{N}$. Their mean value for $\delta^{15}\text{N}$ of 8.4 ‰ for feathers from 12 molting geese is reasonably close to the mean of 7.6 ‰ for the two geese in our sample that were equilibrating with the Saltholm environment. The mean $\delta^{15}\text{N}$ for the goose that showed no evidence of equilibrating was also 7.6‰ (509), considerably higher than the latest (most proximal) $\delta^{15}\text{N}$ values for the geese that showed strong declines in $\delta^{15}\text{N}$ (Fig. 1). The relatively high mean $\delta^{15}\text{N}$ for goose 509, together with the lack of change in $\delta^{15}\text{N}$ along its feather, suggests that this goose had not arrived early and delayed the start of its molt until reaching equilibrium with the Saltholm isoscape. Possibly it was a Saltholm resident with a different diet.

Fox et al. (2009) suggested that $\delta^{13}\text{C}$ also changed in a way that suggested the use of endogenous C in the generation of primary feathers during the molt.

However, the absolute difference in the expected values for $\delta^{13}\text{C}$ (again, generated by sampling two food plants from the Swedish breeding grounds and two food plants from the Saltholm molting grounds) was less than 2 ‰. While differences as small as 2 ‰ can reliably be measured, predicting differences this small by applying fraction values to the $\delta^{13}\text{C}$ values measured to samples of two food plants consumed by geese at their breeding and molting sites seems hazardous. With their sample of 12 geese, Fox et al. (2009) did find the feather values to be intermediate between the food values for Sweden and Saltholm, using the conversion figures for Japanese Quail (Hobson and Clark 1992b). Our mean $\delta^{13}\text{C}$ value of 26.2 ‰ for the three feathers we analyzed is close to their mean of 26.5 ‰ based on 12 geese (Fox et al. 2009). However, the difference in the inferred values for feather tissue generated from Swedish reserves and from Saltholm plant material suggests that $\delta^{13}\text{C}$ values should decline during primary growth. Yet, we found no evidence for such a decline in the three feathers we examined: two geese showed no change, while the third showed a significant increase in $\delta^{13}\text{C}$ along the length of its primary (Fig. 1). Further, goose 501 that showed an increase in $\delta^{13}\text{C}$ values showed a strong decline in $\delta^{15}\text{N}$ values along the length of its growing primary, so we know that this individual was not yet in equilibrium with the Saltholm $\delta^{15}\text{N}$ isoscape. The positive slope for $\delta^{13}\text{C}$ in this goose further suggests that the expected difference in feather $\delta^{13}\text{C}$, estimated from food plants sampled in Sweden and Saltholm (Fox et al. 2009), was not reliable.

Stored reserves and 24-hour cycling

As predicted the two geese that showed strong declines in $\delta^{15}\text{N}$ along the length of their growing primaries also showed 24-hour cycling in their $\delta^{15}\text{N}$ values. Further, the goose that showed no change in $\delta^{15}\text{N}$ along the length of its primary showed no evidence of 24-hour cycling, a result we predicted because this goose was not coming into equilibrium with the Saltholm $\delta^{15}\text{N}$ environment. These results support the use of endogenous reserves for feather growth during parts of the 24-hour cycle when geese do not forage and feathers continue to grow (Murphy and King 1990). The periodicity of this cycling can also be estimated from the autocorrelation

analysis and that periodicity should match feather growth rates. Our autocorrelation analyses revealed periodicities corresponding to growth rates of roughly 8 and 6 mm d⁻¹ for feathers 509 and 501, respectively. These periodicity values accord well with the estimated primary growth rate of about 7 mm d⁻¹ for a bird the size of a Greylag Goose (Rohwer et al. 2009). Finer sampling than could be achieved by our equipment would presumably eliminate the noise in our autocorrelation results resulting from 1 or 2 mm sampling intervals, leaving only noise associated with day-to-day differences in food intake and feeding times (Moran et al. 2011).

General

Bridge et al. (2011) assessed the possible use of endogenous protein reserves for molting by studying changes in δD and $\delta^{13}C$ in the primaries of Painted Buntings (*Passerina ciris*). Like many other migrant song birds that breed in the central and southern regions of western North America, Painted bunting from the Midwestern breeding population migrate to northwest Mexico for their annual post-breeding molt (Thompson 1991; Rohwer et al. 2005; Rohwer 2013). Here they exploit a food flush generated by the late summer monsoon, which delivers most of the annual precipitation to this region of northwest Mexico in July – September (Adams and Comrie 1997; Comrie and Glenn 1998). Primary replacement in Painted Buntings in Sinaloa is so rapid that it requires an average of only 30 and 34 days in adult females and adult males, respectively (Rohwer 2013).

Bridge et al. (2011) showed that both δD and $\delta^{13}C$ values changed from primary 1 to 9 in some Painted Buntings sampled in Sinaloa. They suggest that birds that showed differences between primaries 1 and 9 should be individuals that had initiated molt shortly after arriving in Sinaloa, before their endogenous protein reserves reached equilibrium with the Sinaloa isoscape. Individuals that failed to show strong differences between these primaries either may have delayed molt until their endogenous protein reserves were in equilibrium with the Sinaloa isoscape, or the food they consumed before migrating may have matched what they were consuming on their Sinaloa molting grounds. Direct evidence of continuous

change in δD and $\delta^{13}C$ is needed to test the suggestion by Bridge et al. (2011) that buntings showing strong differences in δD and $\delta^{13}C$ signatures between primaries 1 and 9 were coming into equilibrium with a new isoscape while molting. This could now be accomplished by sampling across different primaries on the feather-time axis developed by Rohwer and Broms (2012) spanning the replacement of all primaries.

In general, measuring isotopic changes in serial samples taken at equal time intervals from flight feathers offers a powerful tool for studying molt-migration. It provides strong data for individual birds while avoiding the assumptions involved with food sampling and using fraction estimates to compute expected tissue values for isotopes. Samples from the primaries that represent equal time intervals can be generated in two ways. For large birds, finely spaced serial samples taken along the length of a primary can give such good data that the evidence of general changes can even be confirmed by evidence of 24-hour periodicity in isotope measurements, as we have shown here for Greylag Geese. For small birds, serially sampling single flight feathers generates only a limited temporal series of samples and the flight feathers of small birds grow too slowly (Rohwer et al. 2009) for traditional sampling methods to achieve a sample density sufficient to detect 24-hour cycling. However, samples representing approximately equal time intervals across the full primary molt can be taken from different primaries (Rohwer and Broms 2012), thus greatly extending the sampling period to the time required to replace all 9 or 10 primaries. Such sampling, coupled with information on how soon after arrival molt-migrants initiate primary replacement, could give excellent empirical data on the time required to reach equilibrium following changes in isoscapes by small wild birds.

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Figure 1. Values for $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ measured in serial samples along the length of the primary. Feather vein was sampled at 5mm intervals near the rachis of the growing primary for its full length, starting at the tip of the feather.

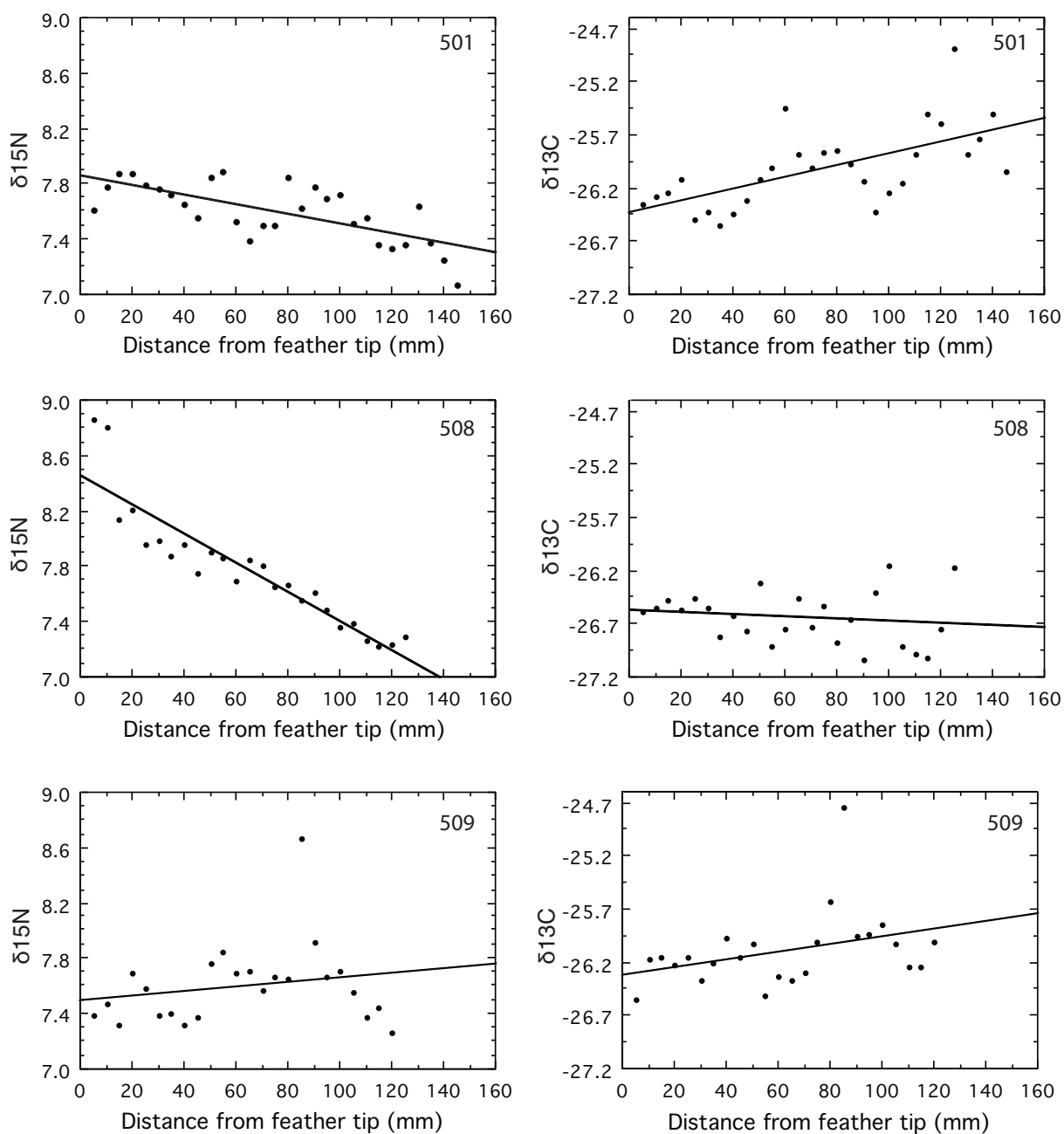


Figure 2. Autocorrelation and regression results for $\delta^{15}\text{N}$ measured at 1 or 2 mm intervals from the tips of growing primaries. Greylag Geese 501 and 508 showed decreasing $\delta^{15}\text{N}$ values, indicating they were equilibrating with the Saltholm isoscape as their primaries were growing, and both showed significant autocorrelations ($p = 0.01$ and 0.03 , respectively). Goose 509 showed no change in its $\delta^{15}\text{N}$ values and no autocorrelation ($p > 0.25$).

