

Stable Isotope Values Suggest Opossums (*Didelphis virginiana*) at the Northern Edge of Their Range Do Not Seasonally Molt

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Abstract - *Didelphis virginiana* (Virginia Opossum) continues to spread further north into temperate North America, raising questions regarding how they survive harsh winters. Very few marsupials exhibit winter adaptations such as torpor or seasonal molting. We used stable isotopes to evaluate evidence of seasonal molting in Opossums. We serially cut hairs from captive Opossums to determine if a documented diet shift early in life was reflected along the hair shaft. Isotope values along the hair mirrored a shift in captive Opossum's diets. We compared isotope values between Opossums trapped in different seasons and found no differences. The data indicate Opossums do not seasonally molt at the northern edge of their range.

Introduction

Molting strategies vary across mammals, with limited phylogenetic signal (Ling 1970). Biannual or seasonal molting is a strategy observed in mammals experiencing winter environments that challenge homeothermy enough to outweigh the energetic costs of molting, including the production of new tissue. Alternative strategies include annual molting, incomplete biannual molting (additional winter hair is grown and subsequently shed), and continuous shedding (replacement hair growth as hair is lost) (Beltran et al. 2018). Both temperature and photoperiod can influence the timing of a seasonal molt (Marchand 2014); thus, the phenology of molting changes based on local conditions—colder temperatures promote winter molt or delayed spring molt (e.g., Lynch 1973, Rust 1962).

Didelphis virginiana (Kerr) (Virginia Opossum, henceforth Opossum) is an omnivorous marsupial found across much of North America (Gardner and Sunquist 2003). The Opossum has been expanding northward for over a century in both the Great Lakes region and New England (Gardner and Sunquist 2003, Walsh and Tucker 2018). It does not hibernate or exhibit torpor, and extremities can become necrotic or lost due to frostbite (Brocke 1970, Walsh et al. 2017). Whether Opossums seasonally molt for a heavier or whiter winter coat is unknown (Hinds and Loudon 1997). Opossums at the northern edge of their range are sympatric with mammals known to exhibit complete (*Mustela* [weasels]; Bissounette and Bailey 1944) and incomplete (*Neovison vison* (Schreber) [Mink]; Martinet et al. 1992]) biannual molting. By analyzing the Opossum, a species with an evolutionary history in the Neotropics that has spread into temperate

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climates previously hypothesized to be too harsh (Walsh and Tucker 2018), we can identify ecological and evolutionary traits that could allow mammals to cope with changing climate in the years to come.

Opossums are dietary generalists, and gastrointestinal dissections suggest they consume more vertebrates and fewer plants in the winter (Brocke 1970, Stieglitz and Klimstra 1962). Their behavior varies seasonally, and successful foraging in northern populations is critical to surviving winter (Brocke 1970). As temperatures begin to cool in October, Opossums forage to bulk up for winter. From December through March, Opossum foraging is frequently hindered by harsh winter conditions. By June, the breeding season concludes for most individuals, and invertebrate prey are abundant (Brocke 1970, Gardner and Sunquist 2003).

Here, we leverage stable isotope analysis (SIA) to conduct a pilot study using captive Opossums and museum specimens to test 2 hypotheses regarding seasonal molting in Opossums: (1) if Opossums do not shed seasonally, then we expect stable isotope values along the guard hair to mirror a diet shift in captive Opossums and (2) if Opossums exhibit seasonal or incomplete seasonal molting, then stable isotope values will vary between Opossums collected in different seasons.

The stable carbon and nitrogen isotopic composition of animal tissues changes in a predictable way according to an animal's diet. Nitrogen isotope ($\delta^{15}\text{N}$) values increase by 2–5‰ from diet to consumer, allowing for inference of an animal's trophic level via $\delta^{15}\text{N}$ values (Ambrose 1991, DeNiro and Epstein 1978b). Because of differences in photosynthetic pathways, C_4 plants have a higher carbon isotope ($\delta^{13}\text{C}$) value than C_3 plants, and this difference is maintained up their respective food chains (DeNiro and Epstein 1978a, Kelly 2000). While not naturally found at high biomasses across much of North America outside of the Great Plains (Still et al. 2003), C_4 plants include corn, a common additive to livestock feed and human food in the forms of corn meal and corn syrup (Jahren and Kraft 2008).

We conducted an analysis of isotope composition along guard-hair shafts of 2 captive Opossums with known changes in diet during captivity to evaluate the evidence that Opossums seasonally shed. If Opossums do not shed biannually or annually, the recorded shift in diet when the captive Opossums were juveniles would be reflected in isotope values along the hair shaft. In addition, we compared isotope values of wild Opossums collected in 3 seasons. If Opossums shed seasonally or grow additional hair for the winter, we predicted isotopic values from whole guard hairs will vary between seasons, especially between summer and winter, given evidence of seasonal variation in diet based on gastrointestinal dissections (Brocke 1970, Stieglitz and Klimstra 1962).

Methods

In July 2016, we obtained guard hairs from the base of the right hind leg from 1 female and 1 male held as education animals in outdoor enclosures at the Ohio Wildlife Center (OWC) (Table 1). Hair was cut and collected by wildlife staff who routinely handled the Opossums (Ohio Division of Wildlife Permit 18-60, Columbus, OH). To evaluate diet over time, we took serial samples in ~10-mm increments

along the length of a shaft of hair, from the base to the tip of the hair shaft. The tips of hair represent the oldest section of hair, whereas the base reflects the most recent hair growth and the most recent diet of the Opossums. We took 4 serial segments from the male and 5 segments from the female because she had longer hair. To obtain enough mass for stable isotope analysis, we needed ~20 guard hairs to be serially cut for each segment. We also processed entire guard hairs to compare the serial cuts to whole bulk samples. We sampled guard hairs from 42 museum specimens collected in the northern part of the Opossum's range (Illinois, Indiana, Iowa, Massachusetts, Michigan, New York, North Dakota, and Ohio) to compare $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values from Opossums collected in different seasons (specimens listed in Supplementary File 1, available online at <https://www.eaglehill.us/NENAonline/suppl-files/n28-1-N1831-Walsh-s1>, and, for BioOne subscribers, at <https://dx.doi.org/10.1656/N1831.s1>).

We treated hair samples with a 2:1 chloroform:methanol solution to remove external oils and lipids and then dried them under a fume hood. For each sample, we placed 0.5–1.0 mg of hair in a tin capsule (Costech, Valencia, CA) for SIA of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values conducted by the University of New Mexico Center for Stable Isotopes (UNM-CSI, Albuquerque, NM). Analytical error across 56 runs of the UNM-CSI protein standard (casein) was 0.11 standard deviations (SD) for $\delta^{15}\text{N}$ and 0.05 SD for $\delta^{13}\text{C}$. For museum specimens collected before 2017, we adjusted $\delta^{13}\text{C}$ values to account for the decrease in atmospheric ^{13}C through time with a spline annual adjustment of -0.005‰ per year between 1925 and 1961 and -0.022‰ per year after 1961 (Chamberlain et al. 2005). The adjusted $\delta^{13}\text{C}$ values (Seuss $\delta^{13}\text{C}$) and unadjusted $\delta^{13}\text{C}$ values (raw $\delta^{13}\text{C}$) are listed in Supplementary File 1.

Both captive individuals were orphaned and delivered to the OWC hospital between 10 and 11 wks old (aged by veterinarian staff based on development and weight) and later transferred to the OWC education center when deemed non-releasable (Table 1). Before arriving at the education center when 23 wks old, the female was trained for handling with horse meat (Table 1). If Opossums do not shed annually or biannually, one would expect to see a decrease in $\delta^{15}\text{N}$ values associated with a decrease in meat in the diet once at the education center. To statistically evaluate the female's change in $\delta^{15}\text{N}$, we used a one-sample *t*-test to compare her

Table 1. Dates in which 1 female and 1 male orphaned *Didelphis virginiana* (Virginia Opossum) were delivered to a wildlife hospital, transferred to an education center, and sampled, along with diet shifts experienced due to the transfer that would have impacted their nitrogen ($\delta^{15}\text{N}$) and carbon ($\delta^{13}\text{C}$) isotope values.

	Female	Male
Hospital arrival date	5 August 2014 (10 wks old)	24 June 2015 (11 wks old)
Transfer date	3 Nov 2014 (23 wks old)	26 Aug 2015 (20 wks old)
Sampling date	July 2016 (2 yrs old)	July 2016 (1 yr old)
Diet shift related to $\delta^{15}\text{N}$	Trained w/horse meat before transfer	-
Diet shift related to $\delta^{13}\text{C}$	Increase in corn products in diet at education center	Increase in corn products in diet at education center

earliest hair $\delta^{15}\text{N}$ value (tips) to her 3 most recent hair segments (base, 10–20 mm, and 20–30 mm). We repeated this one-sample *t*-test for the male as a control.

When both Opossums arrived at the education center, the amount of corn in their diet increased based on differences in standard diets between the hospital and education center. At the hospital, Opossums were fed a mixture of approximately 32 g cat chow, 47 g vegetables, 32 g fruit, 7 g baby food, 19 g yogurt, and additional protein (boiled egg or small fish twice a week). Once in long-term captivity, to prevent or mitigate nutritional disorders including secondary hyperparathyroidism, both Opossums were fed a steady, daily diet including approximately 32 g cat chow, 20 meal worms, 1 dime-sized meatball, and a 64-g serving of a calcium-rich smoothie (2 parts yogurt, 5 parts fruit, and 2 parts corn) (McRuer and Jones 2009). Thus, corn and meal worms fed on grain meal (i.e., the C_4 plants corn and sorghum) were added to their diet at approximately 20 wks and 23 wks old, male and female respectively, when they were transferred to the education center. If Opossums do not shed annually or biannually, one would expect to see an increase in $\delta^{13}\text{C}$ values associated with the rise in C_4 plants in their diet. To statistically evaluate the change in $\delta^{13}\text{C}$ values, we used one-sample *t*-tests to compare each Opossum's earliest hair $\delta^{13}\text{C}$ value (tips) to his or her 3 most recent hair segments (base, 10–20 mm, and 20–30 mm).

Wild caught Opossums included museum specimens collected in the fall (October–November, $n = 16$), winter (December–March, $n = 16$), and summer (June–September, $n = 10$). We defined seasons based on shifts in the ecology and behavior of Opossums at the northern edge of their range (Brocke 1970, Gardner and Sunquist 2003). We evaluated differences in (Seuss adjusted) $\delta^{13}\text{C}$ values between seasons using a one-way ANOVA and differences in the non-normally distributed $\delta^{15}\text{N}$ values using a Kruskal–Wallis (K–W) test. To further evaluate seasonal variation in each stable isotope, we ran multiple regression analyses in R (version 3.6.0; R Foundation, Vienna, Austria). For carbon, we evaluated raw $\delta^{13}\text{C}$ values with the predictor variables of season and year and evaluated square root transformed $\delta^{15}\text{N}$ values with the predictor variable of season. For both analyses, we added C:N as an additional predictor variable to test for lipid contamination.

Results

The captive female Opossum that was trained for handling with horse meat until she was 23 wks old (Table 1) had a significantly higher $\delta^{15}\text{N}$ value (7.5‰) at the distal tips of the hair compared to the newer growth (mean = 7.0‰, $P = 0.017$, $t = -7.684$; Fig. 1A). The captive male did not have a significantly higher $\delta^{15}\text{N}$ value (6.4‰) at the distal tips compared to newer growth (mean = 6.2‰, $P = 0.062$, $t = -3.830$). Both captive individuals exhibited a significant increase in $\delta^{13}\text{C}$ values between the tips of hair and the more proximal hair sections (Fig. 1B). The female's tips had a mean $\delta^{13}\text{C}$ value of -18.6‰, while the first 30 mm of hair had a mean of -18.0‰ ($P = 0.041$, $t = 4.812$). The male's tips had a mean $\delta^{13}\text{C}$ value of -16.5‰ and the first 30 mm of hair had a mean of -15.8‰ ($P = 0.010$, $t = 10.017$).

$\delta^{13}\text{C}$ values from hair did not significantly vary between museum specimens collected in different seasons (ANOVA: $F = 1.838$, $P = 0.173$; Table 2). Likewise, $\delta^{15}\text{N}$ values were not significantly different between museum specimens collected in different seasons (K–W: $H = 0.685$, $P = 0.710$; Table 2). The raw $\delta^{13}\text{C}$ value multiple regression analyses with and without C:N were not significant (adjusted $R^2 = 0.089$, $P = 0.114$ and $R^2 = 0.104$, $P = 0.067$, respectively). Similarly, the transformed $\delta^{15}\text{N}$ value regression analysis with and without C:N were not significant (adjusted $R^2 = -0.054$, $P = 0.824$ and $R^2 = -0.044$, $P = 0.881$, respectively).

Discussion

The SIA of the captive Opossums reveals that changes in diet at a juvenile life stage are reflected along the hair shaft, supporting our hypothesis that if Opossums do not molt, dietary shifts at a seasonal scale can be captured throughout

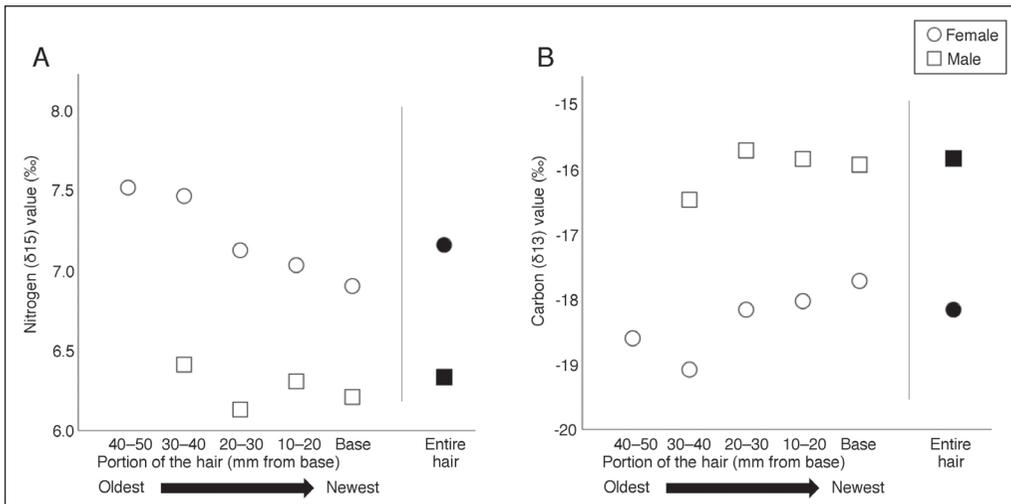


Figure 1. Trends over time of (A) nitrogen ($\delta^{15}\text{N}$) isotope values and (B) carbon ($\delta^{13}\text{C}$) isotope values from hairs of 2 captive *Didelphis virginiana* (Virginia Opossum) sampled in 10-mm increments to evaluate whether changes in diet during ontogeny are recorded in guard hair. Because the female's hair is longer, the tips of her hair are represented in the serial sample 40–50 mm.

Table 2. Mean nitrogen ($\delta^{15}\text{N}$) and carbon ($\delta^{13}\text{C}$) isotope values and standard deviations from guard hairs of *Didelphis virginiana* (Virginia Opossum) museum specimens. Museum specimens collected in the fall (October–November), winter (December–March) and summer (June–September) from Illinois, Indiana, Iowa, Massachusetts, Michigan, New York, North Dakota, and Ohio were sampled to evaluate seasonal differences.

Season	<i>n</i>	$\delta^{15}\text{N}$	$\delta^{13}\text{C}$
Fall	16	$8.1 \pm 1.4\text{‰}$	$-22.3 \pm 0.8\text{‰}$
Winter	16	$8.3 \pm 1.8\text{‰}$	$-22.4 \pm 1.0\text{‰}$
Summer	10	$8.5 \pm 1.7\text{‰}$	$-21.7 \pm 1.0\text{‰}$
Statistical test		Kruskal–Wallis	ANOVA
Statistical significance		$P = 0.710$	$P = 0.173$

an Opossum's 1–2-year life. These results, and a wildlife veterinarian's anecdotal report that she was unaware of seasonal shedding by Opossums (M. Marks, DVM, Ohio Wildlife Center, Columbus, OH, 15 May 2016 pers. comm.), do not support a biannual or annual molt in Opossums. With an average lifespan of <2 years and an evolutionary history rooted in the Neotropics (Gardner and Sunquist 2003), it is not surprising that captive Opossums do not show signs of seasonal molting. Interestingly, the stable isotope values of entire hairs varied between the male and female. This result may partially be due to the male's strong preference for fruit and the ¹³C-rich smoothie (M. Marks, pers. comm.).

Consistent nutrition does not appear to impact molting in captive mammals (Ling 1970). Molting can be influenced by a combination of photoperiod, temperature, and precipitation (Ling 1972). While both Opossums were kept in an outdoor enclosure and would have experienced conditions similar to wild Opossums in Ohio, the definitive molting strategy of Opossums cannot be fully established from these limited results. We were fortunate to find 2 captive Opossums whose diets had been adjusted due to differences in animal husbandry practices between OWC locations, and these preliminary results can bolster additional research on captive Opossums. By examining hair samples from wild-caught Opossums from museum collections, we found no difference in stable isotope values between fall, winter, and summer guard hairs. These results suggest that the seasonal change in pelage composition is minimal, supporting continuous shedding rather than biannual molting in Opossums. Alternatively, the Opossum's diet may be consistent between seasons, but this hypothesis is not supported by gastrointestinal dissections (e.g., Brocke 1970, Stieglitz and Klimstra 1962).

North and South American marsupials are an understudied group of mammals (e.g., Hinds and Loudon 1997, Svartman 2009). No literature exists for the growth rate of Opossum guard hair, and our preliminary examination of 2 captive Opossums sought to better understand the length of time captured in guard hair. Because the captive Opossums were only sampled in the summer, we used museum specimens to evaluate the evidence of seasonal or incomplete seasonal molting. Because museums often require proof-of-concept and proof-of-method for extensive destructive sampling, we received ~25 hairs per specimen, enough to analyze duplicate samples of the entire guard hair of each specimen. We hope our preliminary results can be leveraged in the future to increase destructive sampling to at least 40 guard hairs in order to compare 10-mm increments of wild Opossums. Paired *t*-tests comparing the tips and base hairs would provide more resolving power and a direct comparison to the captive Opossums in this study. This analysis would also capture seasonal dietary shifts too subtle for our bulk analysis of entire hairs.

The Opossum's apparent lack of biannual molting may be seen as an evolutionary limitation to coping with extreme winter weather, in addition to not hibernating and having naked, fleshy paws and tail susceptible to frostbite (Gardner and Sunquist 2003). However, molting may be too energetically demanding for a mammal with a relatively low metabolic rate (Brocke 1970). Other marsupials are seemingly better adapted to low temperature extremes than the Virginia Opossum, including

multiple species of Australian *Antechinus* that exhibit seasonal molting (Leeson and Wallis 1986), as well as other didelphids, including *Gracilinanus microtarsus* (Wagner) (Brazilian Gracile Opossum) and *Thylamys elegans* (Waterhouse) (Elegant Fat-tailed Mouse Opossum) that exhibit daily torpor (Ruf and Geiser 2014).

As a species with a broad distribution encompassing both the Neotropics and Northeastern North America, the northward expansion of the range of the Virginia Opossum offers an evolutionary system to evaluate adaptations to temperate winter in a marsupial species. Future research on Virginia Opossums should include comparing isotope values in winter and summer pelage from the same individuals collected in the wild, as well as additional adaptations to winter, including diurnal foraging. This preliminary study demonstrates the useful application of stable isotope analysis in evaluating a natural history question.

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