Research Article

Rapid Communications in Mass Spectrometry

Received: 16 January 2015 Revised: 4 March 2015 Accepted: 6 March 2015 Published online in Wiley Online Library

Rapid Commun. Mass Spectrom. **2015**, *29*, 1062–1068
(wileyonlinelibrary.com) DOI: 10.1002/rcm.7194

Discrimination factors of carbon and nitrogen stable isotopes from diet to hair and scat in captive tigers (*Panthera tigris*) and snow leopards (*Uncia uncia*)

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RATIONALE: In order to use stable isotope ratio values obtained from wild animal tissues, we must accurately calculate the differences in isotope ratios between diet and consumer ($\delta_{tissue} - \delta_{diet}$). These values, called trophic discrimination factors (TDFs, denoted with Δ), are necessary for stable isotope ecology studies and are best calculated in controlled environments.
METHODS: Scat, hair, and diet samples were collected from captive tigers (n = 8) and snow leopards (n = 10) at the Bronx Zoo. The isotope ratios of carbon and nitrogen, the two most commonly used in ecological studies, of the samples were measured by continuous-flow isotope ratio mass spectrometry. The trophic discrimination factors were calculated for both carbon ($\delta^{13}C$ values) and nitrogen ($\delta^{15}N$ values).
RESULTS: It was found that the only significant TDFs in this study were diet-hair, $\Delta^{13}C_{Hair}$, for snow leopards (5.97 ± 1.25‰) and tigers (6.45 ± 0.54‰), and diet-scat, $\Delta^{15}N_{Scat}$, in snow leopards (2.49 ± 1.30‰). The other mean isotope ratios were not significantly different from that of the premixed feline diet. The $\Delta^{15}N_{Hair}$ values for both species were unusually low, potentially due to the protein content and quality of the feline diet.
CONCLUSIONS: The discrimination factors of the stable isotopes of carbon and nitrogen calculated in this study can be applied to ecological studies of wild, non-captive terrestrial mammals. The effect of protein quality in isotope discrimination is also worthy of further investigation to better understand variation in TDFs. Carnivore scat is shown to be a valuable material for isotopic analysis. Copyright © 2015 John Wiley & Sons, Ltd.

The management of large predators in any ecosystem is crucial; top predators constitute an essential component of ecosystem stability and integrity.[1,2] A major threat to these apex predators is a deterioration of the quantity and quality of prey in their environment. It is often necessary to have a detailed understanding of the dietary ecology of top predators in order to effectively conserve threatened populations. Directly monitoring the diets of endangered vertebrates, such as large felids, can be difficult and time-consuming due to their dwindling numbers, low population densities, nocturnal hunting strategies, and behavioral avoidance of humans. Non-invasive monitoring methods have already obtained considerable popularity for examining the population genetics of felids.[3,4] Collecting found materials from the target species, such as shed hair and scat (carnivore feces), allows conservation biologists to characterize population structure and relatedness of uncommon species in an inexpensive and easy way. Fortunately, although not usually done, these same materials can also be utilized to quantify the diets of these animals by using stable isotope analysis. Vertebrate diets are typically quantified through macroscopic stomach and fecal analysis or observational studies; these methods, however, only lead to a snapshot of an animal's diet rather than a long-term perspective. Isotopic and macroscopic methods, when used in concert, can provide complementary dietary evaluations over different timescales.

The stable isotope ratios of nitrogen ($\delta^{15}N$ values) and carbon ($\delta^{13}C$ values) in the tissues of animals are directly reflective of their diets (hereinafter, the term 'tissue' will include scat material). Variations of these isotope ratios in predator tissues can provide information on trophic cascades, prey sources, and habitat use.[5] Diets of top carnivores specifically are an 'emergent property' of ecological and physiological processes of other herbivores, omnivores, and carnivores in the food web.[6] In modern isotope ratio studies, tissues such as hair, muscle, and blood are used to garner dietary and foraging information about a wide range of vertebrates in both marine and terrestrial habitats. In a terrestrial system, the $\delta^{13}C$ values in an animal tissue vary based on the types of plant metabolism (C3, C4, CAM) at the base of the food web, which have systematically variable $\delta^{13}C$ values. The $\delta^{15}N$ values of consumers are used to make

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inferences about diet due to the systematic increase of ^{15}N with trophic level, thus revealing detailed information about trophic cascades and baseline food web shifts.[7,8] Examining the isotope ratios of both these elements in consumer tissues is a powerful way to deduce dietary ecology and foraging behavior that would remain otherwise elusive.

In order to use isotope ratio values obtained from non-invasively collected remains, the fractionation process from diet to tissue has to be better understood. The difference in isotopic composition between a tissue and the diet derives from many processes (isotopic fractionation during metabolism, isotopic routing, etc.) and is known as the discrimination factor. The discrimination factor is represented by Δ and defined as $\Delta = \delta_{tissue} - \delta_{diet}$,[9,10] with δ_{tissue} being the stable isotope ratio of the analyzed consumer tissue and δ_{diet} the stable isotope ratio of the total consumed diet. The discrimination factors for isotopes of both C and N can vary between species, tissues, diet, and age.[11–13] Usually, $\Delta^{13}C$ is between 1 and 3‰ for hair,[14] but $\Delta^{15}N$ is typically a larger value because of trophic enrichment, as first demonstrated by DeNiro and Epstein.[15] As $\Delta^{15}N$ shows experimental variation due to the factors listed above, the most commonly reported average value, 3.4‰,[16] is often used in papers where experimental determination of discrimination factor was not carried out.[10] Importantly, reliable $\Delta^{15}N$ values are needed when using stable isotope mixing models. Mixing models are able to determine what the weighted components of the assimilated diet of a consumer are using the tissue stable isotope value and the values of potential prey items.[17] Caut *et al*.[18] determined that the dietary components from mixing models were most accurate in rats when $\Delta^{15}N$ was experimentally estimated for that species.

In most cases, a controlled diet to tissue fractionation experiment cannot be implemented for every single species studied. Instead, it is better to rely on the range of $\Delta^{15}N$ values obtained experimentally from closely related species fed a variety of foods.[10] Unfortunately, discrimination factors are unavailable in the literature for many species of large terrestrial carnivores (see Table 2 in Crawford *et al*.[8]). Recently, the first ever discrimination factors for diet-hair were determined for four species of large felids.[19] The aforementioned reference does not include any TDF values for the two felids included in this study, tigers (*Panthera tigris*) and snow leopards (*Uncia uncia*) and also does not include terrestrial carnivore diet-scat TDFs. Tigers and snow leopards are both charismatic conservation targets and some of the most endangered large cats in the world with populations hovering around 3500 tigers[20] and 6000 snow leopards.[21] As previously mentioned, non-invasively collected materials from these animals, such as scats and shed hair, are frequently used in population genetics studies of these elusive creatures. Felid scats can also be used to create a snapshot of a dietary profile of the animal through visible inspection of prey remains[22] or DNA metabarcoding.[23] While scat is used for DNA-based studies for both population genetics and diet, it stands to reason that stable isotope analysis on scats can provide added value for ecological studies and help better understand potential shifts in the trophic levels of top predators. Stable isotope analysis on the feces of non-herbivores has mainly been focused on small insectivores such as bats[24] but has the untapped potential to be applied to other terrestrial omnivores and carnivores.

Tigers and snow leopards can both be considered umbrella species – meaning that while they are the specific targets for conservation, they also earn protection for other species in the same ecosystem. Their home range requirements are so large that effective conservation of these species across a landscape would provide significant area-based conservation for most other wildlife in those ecosystems. The historic range of tigers (*Panthera tigris*) once extended over an extensive portion of Eurasia from Iran and Turkmenistan through most of China, Southeast Asia, and Russia.[25] Currently, the range is limited to disjointed populations in 13 countries including India, Myanmar, Thailand, Malaysia, Russia and China. The number of people living in the range of extant tigers has doubled to over 3.4 billion in the 40 years since they were declared endangered.[26] Over the past 200 years, wild tiger populations have decreased by 98% on the Indian subcontinent, and probably by a similar amount in the rest of the remaining range.[26,27] The loss of tigers across their historical range is indicative of ecosystem erosion, caused by habitat loss and other forms of human encroachment.[26] Tigers need substantial access to large ungulate prey for survival, and understanding the food web that they are a part of has the potential to mitigate loss of resilience in these ecosystems.

Snow leopards (*Uncia uncia*) are enigmatic wild cats that live in the high altitudes of 12 countries including regions such as the Himalayas, Tibetan Plateau, Siberia, Mongolia, and China. They are well adapted to harsh conditions but remain threatened due to habitat loss, fragmentation and reduction of their natural prey.[21] Snow leopards subsist primarily on large ungulates such as argali, but will also feed on smaller prey such as hares, weasels, and rodents.[23] Unfortunately, the large natural prey of snow leopards is being reduced via illegal and unregulated hunting of mountain ungulates for trophies and traditional medicines.[21] If snow leopards are being forced to feed on smaller prey, this trophic level shift can be detected through the stable isotope analysis of their hair or scats, which is extremely valuable conservation information that cannot be gained in any other fashion.

EXPERIMENTAL

Hair, diet, and scat samples

The snow leopards and tigers in this study are maintained at the Bronx Zoo (Wildlife Conservation Society, New York, NY, USA) in the Himalayan Highlands and Tiger Mountain exhibits. We obtained samples from 10 snow leopards and 8 tigers. The scat and hair samples were collected opportunistically from the ground and backscratchers in the enclosures over the course of two days in June 2013. Each sample collected was from a different individual animal and labeled appropriately; one hair sample and one scat sample were analyzed per individual, except in the case of one unavailable tiger scat sample and one unavailable snow leopard hair sample. Subsamples (n = 7) of the premixed feline diet (Nebraska Brand Classic Feline Carnivore Diet; Nebraska Brand, North Plate, NE, USA) were also taken at the same time. This is a commercial blend commonly fed to zoo cats. It includes a blend of horsemeat, meat, meat by-products, fishmeal and premixed quantities of vitamins and trace minerals, and it

makes up the primary component of both the tiger and the snow leopard diets at the Bronx Zoo, with occasional treats of cow bones, chicken pieces, and fish. Because these animals are held on a steady diet of primarily feline diet on a daily basis (~90% of their diet), and only occasionally receive treat items, only feline diet samples were used to calculate discrimination factors.

Stable isotope analysis

Stable isotope analysis was conducted at the Environment and Natural Resources Institute Stable Isotope Laboratory at the University of Alaska, Anchorage (Anchorage, AK, USA). Samples were analyzed for their $\delta^{13}C$ and $\delta^{15}N$ values using a ECS 4010 elemental analyzer (Costech, Valencia, CA, USA) interfaced to a ThermoFinnigan Delta V Advantage continuous-flow isotope ratio mass spectrometer (Thermo Scientific, Bremen, Germany). The instrument was calibrated against international reference standards from the International Atomic Energy Agency (IAEA, Vienna, Austria; IAEA-N1,IAEA-CH7, IAEA-C3, and IAEA-600) and the USGS (USGS Isotope Library, Reston, VA, USA; USGS-25, USGS-40, and USGS-41). We included internal standards of purified methionine (Alfa Aesar, Heysham, UK; $\delta^{13}C$ = –34.58‰, $\delta^{15}N$ = –0.94‰), homogenized peach leaf (NIST 1547, $\delta^{13}C$ = –25.89‰, $\delta^{15}N$ = 1.89‰), and homogenized bowhead whale baleen (University of Alaska, $\delta^{13}C$ = –18.37‰, $\delta^{15}N$ = 14.44‰) with all samples as quality controls. The stable isotope ratio values are reported in standard δ notation (‰) and are referenced to Vienna Pee Dee Belemnite (VPDB) for $\delta^{13}C$ values and to air for $\delta^{15}N$ values where $\delta = (R_{sample}/R_{standard} - 1)$ and $R = {}^{13}C/{}^{12}C$ or ${}^{15}N/{}^{14}N$. Long-term records of internal standards yield an analytical precision of 0.2‰ for $\delta^{15}N$ values and 0.1‰ for $\delta^{13}C$ values.

In order to remove surficial contaminants and lipids, all hair samples were immersed in a 2:1 chloroform/methanol solution and were agitated for 24 h on a random orbit shaker table. The samples were then rinsed five times in purified water and oven dried at 50 °C for 24 h. The scat and diet samples were subsampled and analyzed both with and without lipid extraction treatment. The samples were lipid extracted using a modified Bligh and Dyer method.[28] Dried homogenized samples were immersed in a 2:1 ratio chloroform/methanol with a solvent volume ~3–5 times > sample volume. The samples were then mixed for 30 s, left undisturbed for approximately 30 min, centrifuged for 10 min at 3400 rpm, and the supernatant containing solvent and lipids was removed. This process was repeated at least three times until the supernatant was clear and colorless following centrifugation. The samples were then re-dried at 50 °C for 24 h to remove any remaining solvent.

Statistics and data analysis

Discrimination factors were calculated using individual measured isotope ratio values for tissue and average diet for both $\delta^{13}C$ and $\delta^{15}N$ values using the equation: $\Delta X_{tissue} = \delta X_{sample} - \delta X_{diet}$. The ΔX_{tissue} value is in per mil (‰) while δX_{sample} is either the $\delta^{13}C$ or $\delta^{15}N$ value of hair or scat and δX_{diet} is the average $\delta^{13}C$ or $\delta^{15}N$ value of the homogenized non-lipid-extracted carnivore diet samples. Statistical tests, specifically t-tests to compare means between species and lipid treatment groups, were performed in R[29] and plots were created in R using ggplot2.[30]

RESULTS

All statistical tests performed were parametric as the data met the conditions (normal distribution) for parametric testing. An F-test was performed and used to choose a t-test for unequal (Welch two sample t-test) or equal variance based on the results. The means are reported ± standard deviation (SD). The significance is reported for α = 0.05. A summary of these results can be found in Table 1. The full isotope dataset can be found in the Supporting Information.

The average lipid-extracted $\delta^{13}C$ and $\delta^{15}N$ values for the premixed zoo diet subsamples (n = 7) are –21.4 ± 2.07‰ and 6.16 ± 0.60‰, respectively. There is no significant difference between the $\delta^{13}C$ values in lipid-extracted and non-lipid-extracted diet samples (t-test, equal variance, p = 0.146, t = 1.554, df = 12). However, the $\delta^{15}N$ value of the lipid-extracted diet is significantly lower than that of the non-lipid-extracted diet (t-test, equal variance, p <0.000001, t=7.838, df=12). As has been noted from meta-analysis of discrimination factors of carbon and nitrogen from Caut *et al.*,[11] lipid extraction has no effect on the calculated TDFs, so we have opted to use non-lipid-extracted diet and scat in our TDF calculations.

The mean $\delta^{13}C$ value for the non-lipid-extracted carnivore diet samples (n = 7) is –23.61 ± 3.15‰ and the mean $\delta^{15}N$ value is 8.95 ± 0.73‰. The mean C/N ratio for the diet is 6.90 ± 0.58% and the mean N% is 7.54 ± 0.63%. The mean $\delta^{13}C$ value for snow leopard (n = 9) and tiger hair (n = 8) is –17.64 ± 1.25‰ and –17.17 ± 0.54‰, respectively. There is no significant difference between the mean $\delta^{13}C$ values of snow leopard hair and tiger

Table 1. Stable isotope results ($\delta^{13}C$ and $\delta^{15}N$ values) and statistics

	n	$\delta^{13}C$	$\delta^{15}N$	C/N	N%	C%	$\Delta^{13}C$	$\Delta^{15}N$
Snow Leopard Scat	10	−21.32±1.66	11.44±1.30	7.3±3.17	3.29±1.25	22.21±6.94	2.30±1.66	**2.49±1.30**
Tiger Scat	7	−22.36±0.62	10.52±2.04	8.49±1.66	3.89±2.06	30.23±5.80	1.25±0.62	1.57±2.04
Snow Leopard Hair	9	−17.64±1.25	9.28±0.17	2.88±0.02	-	-	**5.97±1.25**	0.33±0.17
Tiger Hair	8	−17.17±0.54	8.69±0.22	2.91±0.03	-	-	**6.45±0.54**	-0.26±0.22
Feline Diet	7	−23.61±3.15	8.95±0.73	6.90±0.58	7.54±0.63	51.6±0.49	-	-

Means are presented ± 1SD. The column heading 'n' is the number of samples analyzed for each group. Stable isotope ratios are written in delta notation (δ) and discrimination factors are noted by (Δ). The isotope values of the diet and scat presented here are non-lipid-extracted. Isotope ratios are presented in per mil (‰). Element percentages (nitrogen and carbon) are also presented. Trophic discrimination factors in bold are significantly different from zero.

hair (Welch two-sample t-test, $p = 0.325$, t = –1.03, df = 11.154). The $\delta^{15}N$ values of hair from snow leopards, averaging 9.28 ± 0.17‰, and from tigers, averaging 8.69 ± 0.22‰, are significantly different (t-test, equal variance, $p < 0.0001$, t = 6.312, df = 15). The mean $\delta^{13}C$ value for snow leopard scat (n = 10) is –21.32 ± 1.66‰ and that for tiger scat (n = 7) is –22.36 ± 0.62‰. There is no significant difference between the mean $\delta^{13}C$ values for the two species (Welch two-sample t-test, $p = 0.094$, t = 1.815, df = 12.247). The mean $\delta^{15}N$ value for snow leopard scat, 11.44 ± 1.30‰, and that for tiger scat, 10.52 ± 2.04‰, are not significantly different (t-test, equal variance, $p = 0.273$, t = 1.137, df = 15). The mean C/N ratio for snow leopard scat is 7.3 ± 3.17‰ and for tiger scat is 8.49 ± 1.66‰.

The $\delta^{13}C$ values of tiger and snow leopard hair and scat were compared with those of the diet to establish if there is a significant difference in the isotope ratios between materials. There is a statistically significant difference between both the $\delta^{13}C$ value of tiger hair and that of the diet (Welch two-sample t-test, $p = 0.002$, t = –5.352, df = 6.31), and the $\delta^{13}C$ values of snow leopard hair and the diet (Welch two-sample t-test, $p = 0.002$, t = –4.743, df = 7.47). The only other significant difference is between the mean $\delta^{15}N$ value of snow leopard scat and the $\delta^{15}N$ value of the diet (t-test, equal variance, $p = 0.0004$, t = –4.552, df = 15). The means of other pairs of materials tested were not statistically different (Table 2).

Discrimination factors were calculated by subtracting the average isotope ratio of the diet from each hair or scat sample. The average $\Delta^{13}C_{Hair}$ for snow leopards is 5.97 ± 1.25‰ and that for tigers is 6.45 ± 0.54‰. The means of $\Delta^{13}C_{Hair}$ values of snow leopards and tigers are not significantly different (Welch two-sample t-test, $p = 0.325$, t = –1.03, df = 11.15). The means of $\Delta^{15}N_{Hair}$, 0.33 ± 0.17‰ (snow leopard) and –0.26 ± 0.22‰ (tiger), are significantly different for the two species (t-test, equal variance, $p < 0.0001$, t = 6.312, df = 15). For scats, the average $\Delta^{13}C_{scat}$ is 2.30 ± 1.66‰ for snow leopards and 1.25 ± 0.62‰ for tigers; the means between species are not significantly different (Welch two-sample t-test, $p = 0.094$, t = –1.815, df = 12.247). The average $\Delta^{15}N_{scat}$ is 2.49 ± 1.30‰ for snow leopards and 1.57 ± 2.04‰ for tigers, values which are also not significantly different (t-test, equal variance, $p = 0.273$, t = 1.137, df = 15). A graphical representation of the calculated TDFs is presented in Fig. 1.

DISCUSSION

Trophic discrimination factors of snow leopards and tigers

The calculated TDFs for both tiger and snow leopard hair were similar to those measured by Parng *et al.*[19] for other species of felids. While it is usually found that ^{13}C is not enriched up to trophic levels,[18] it appears that is not the case for the felid species in this study or in the previous study. A higher than typical $\Delta^{13}C_{Hair}$ was also seen in red foxes.[13] The $\Delta^{13}C_{Hair}$ for the controlled diet experiment of the foxes was 2.6‰, which is still significantly smaller than the value obtained both by Parng *et al.*[19] (5.5 ± 0.5‰) and in this study (5.97 ± 1.25‰ and 6.45 ± 0.54‰).

The $\Delta^{13}C_{scat}$ values of both tigers and snow leopards are not significantly different, but the mean $\delta^{13}C_{diet}$ and $\delta^{13}C_{scat}$ values are also not significantly different, which indicates that the $\delta^{13}C_{scat}$ value can potentially be used as a representation of the average $\delta^{13}C_{diet}$ value in large felids, but further testing is needed. In a study of small mammals such as voles and mice, Hwang *et al.*[31] found that the carbon isotope compositions of diet and feces were not the same, which could be because these animals are herbivorous and process plant matter via hindgut fermentation. In a controlled dietary study of an insectivore (bats), there was no difference between the $\delta^{13}C_{diet}$ and $\delta^{13}C_{scat}$ values, and Salvarina *et al.*[24] suggests

Table 2. Results from t-tests (t, df, *p*-value) comparing means from materials analyzed. First, F-tests were performed to see if sample variances were equal. Depending on that result, a Welch two-sample t-test was performed on pairs of samples with unequal variance, while a standard t-test was performed for samples with equal variance. Significant differences are denoted in the last column

Variable 1	Variable 2	Test	*p*-value	t	df	Significant?
$\delta^{13}C$ Hair SL	$\delta^{13}C$ Hair Tiger	t-test unequal	0.325	1.03	11.154	No
$\delta^{15}N$ Hair SL	$\delta^{15}N$ Hair Tiger	t-test equal	<0.0001	6.312	15	**Yes**
$\delta^{13}C$ Hair Tiger	$\delta^{13}C$ Diet	t-test unequal	0.001	−5.352	6.31	**Yes**
$\delta^{15}N$ Hair Tiger	$\delta^{15}N$ Diet	t-test unequal	0.398	0.9	6.924	No
$\delta^{13}C$ Hair SL	$\delta^{13}C$ Diet	t-test unequal	0.002	−4.743	7.47	**Yes**
$\delta^{15}N$ Hair SL	$\delta^{15}N$ Diet	t-test unequal	0.282	−1.172	6.49	No
$\delta^{13}C$ Scat Tiger	$\delta^{13}C$ Scat SL	t-test unequal	0.094	1.815	12.247	No
$\delta^{15}N$ Scat Tiger	$\delta^{15}N$ Scat SL	t-test equal	0.273	1.137	15	No
$\delta^{13}C$ Scat Tiger	$\delta^{13}C$ Diet	t-test unequal	0.339	1.032	6.472	No
$\delta^{15}N$ Scat Tiger	$\delta^{15}N$ Diet	t-test unequal	0.093	1.921	7.511	No
$\delta^{13}C$ Scat SL	$\delta^{13}C$ Diet	t-test equal	0.068	−1.9667	15	No
$\delta^{15}N$ Scat SL	$\delta^{15}N$ Diet	t-test equal	0.0004	−4.552	15	**Yes**
$\delta^{15}N$ Diet	$\delta^{15}N$ Diet LE	t-test equal	<0.00001	7.838	12	**Yes**
$\delta^{13}C$ Diet	$\delta^{13}C$ Diet LE	t-test equal	0.146	1.554	12	No
$\Delta^{13}C$ Hair Tiger	$\Delta^{13}C$ Hair SL	t-test unequal	0.325	−1.03	11.15	No
$\Delta^{15}N$ Hair Tiger	$\Delta^{15}N$ Hair SL	t-test equal	<0.00001	6.312	15	**Yes**
$\Delta^{13}C$ Scat Tiger	$\Delta^{13}C$ Scat SL	t-test unequal	0.094	−1.815	12.247	No
$\Delta^{15}N$ Scat Tiger	$\Delta^{15}N$ Scat SL	t-test equal	0.273	1.137	15	No

SL = snow leopard; LE = lipid extracted

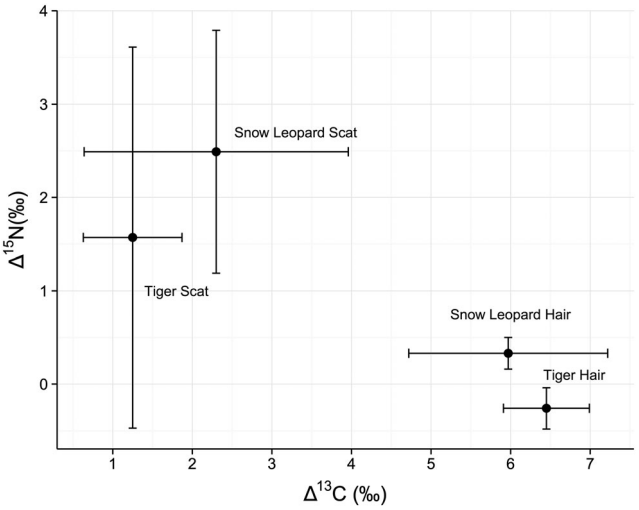

Figure 1. Bivariate plot of nitrogen and carbon isotope discrimination factors (mean ± 1SD) between diet and hair and scat (non-lipid-extracted) from captive tigers and snow leopards. TDFs presented here were calculated from non-lipid-extracted premixed feline diet.

ignoring $\Delta^{13}C_{scat}$ when elucidating bat diets from scat in the wild. It is clear more experiments are needed to determine if body size or dietary preference, among other factors, can influence the $\Delta^{13}C_{scat}$ values in mammals.

The most unexpected TDF calculated in this study was $\Delta^{15}N_{Hair}$. While the $\Delta^{13}C_{Hair}$ is higher than expected, the $\Delta^{15}N_{Hair}$ is actually lower than is typically calculated or expected for discrimination factors of nitrogen in mammals.[16] In this study, the $\Delta^{15}N_{Hair}$ for both snow leopards and tigers was very small (0.33 ± 0.17‰ and –0.26 ± 0.22‰) and the $\delta^{15}N_{diet}$ and $\delta^{15}N_{hair}$ values are not significantly different. However, in Parng *et al.*[19] the calculated $\Delta^{15}N_{Hair}$ was closer to typically expected nitrogen diet-hair discrimination factors (4.1 ± 0.1‰). While our $\Delta^{15}N_{Hair}$ values were much lower, Parng *et al.*[19] also noted that the lowest $\Delta^{15}N_{Hair}$ value calculated in their study was from lions that ate a premixed carnivore diet. It is possible that this specifically designed zoo feed for felids has high protein quality and is optimally designed for nutrition. A diet with high protein quality, meaning that dietary amino acids are supplied in the abundance that meets the animals' daily needs, could affect the $\Delta^{15}N$ values. Previous research on animals held on high protein quality diets indicates that these animals have lower $\Delta^{15}N_{Hair}$ values because of nitrogen discrimination, as the preferential retention of ^{15}N decreases with increasing protein quality.[13,32] It is notable that not just protein quality but also protein quantity may lead to some of the variation seen in the TDFs of carnivorous mammals. Feeding experiments on herbivorous mammals have shown higher $\Delta^{15}N$ values in mammals held on high protein diets.[33] The diet in our study had a nitrogen mean value of 7.54 ± 0.63%, which is similar to the 'low protein' diet treatment of Sponheimer *et al.*,[33] which had a value of 9%. It is worth noting that the other big cat TDF study of Parng *et al.*[19] also found that their lowest calculated $\Delta^{15}N_{Hair}$ value was associated with the animal with the lowest nitrogen diet (7.2%).

The $\Delta^{15}N_{scat}$ value for snow leopards was not significantly different from that of the tiger, although only the mean $\delta^{15}N_{diet}$ and $\delta^{15}N_{scat}$ values of the snow leopard were significantly different. While generally $\Delta^{15}N_{scat}$ calculations for non-herbivores are uncommon in the literature, the $\Delta^{15}N_{scat}$ values of both cats in this study were similar to that of bats.[24] The $\Delta^{15}N_{scat}$ value of snow leopards (2.49 ± 1.30‰), the only significant scat TDF in this study, and that of *Myotis myotis* (1.47 ± 1.51‰) calculated in Salvarina *et al.*,[24] the only significant $\Delta^{15}N_{scat}$ determined in that study, are comparable. The positive $\Delta^{15}N_{scat}$ value of snow leopards adds support to the hypothesis of fecal nitrogen loss with low protein diets previous mentioned by Sponheimer *et al.*[33] It has been shown that herbivores with low protein diets experience a significant amount of nitrogen loss through feces as opposed to urine,[34] and this is also supported in the $\Delta^{15}N_{scat}$ values of herbivores undergoing the dietary treatments in Sponheimer *et al.*[33] In that report, Sponheimer *et al.* did illustrate that herbivores on high protein diets had higher diet-tissue TDFs than the same animals on low protein diets, lending support to the idea that the amount of protein in a diet can affect the TDFs. Although in this study we did not measure the $\delta^{15}N$ values of urine so we cannot compare the fecal and urine nitrogen efflux, the snow leopards with positive fecal $\Delta^{15}N_{Scat}$ values had low $\Delta^{15}N_{Hair}$ values and this could indicate that carnivores exhibit similar TDF dynamics when fed low protein diets.

Much like Salvarina *et al.*,[24] in this study we found that the carbon and nitrogen isotope ratios displayed significant difference between diet and feces in a few cases. The $\Delta^{13}C_{Hair}$ values of both snow leopards and tigers and the $\Delta^{15}N_{scat}$ values were the only calculated TDFs significantly different from zero in this case. While it appears that scats can be used as a relatively close approximation to diet, a conclusion also found for the TDFs of bats in Salvarina *et al.*,[24] perhaps the Δ_{scat} values of a variety of carnivore species can be tested in the future to strengthen this claim and account for any variation caused in TDFs by diet. In both this study and the similar study of Parng *et al.*[19] there was variability in TDFs within and between species. There is evidence that high variability in TDFs is based on the original δ value of the diet input.[11] In a controlled experiment, rats fed a variety of diets had tissue discrimination factors ranging between –1.46‰ and 4.59‰ for $\Delta^{13}C$ and between –8.79‰ and 0.64‰ for $\Delta^{15}N$.[18] In Sponheimer *et al.*,[33] it was shown that multiple species of mammalian herbivores fed identical diets had $\Delta^{15}N_{Hair}$ values that covered a range of 3.6‰. The variation in our study confirms the caveat that variations in $\delta^{15}N$ values cannot simply be interpreted as trophic levels shifts, as is warned against in Sponheimer *et al.*[33] It has long been known that different macronutrients within bulk diets have different isotope values,[35] but it appears that both variety in diet $\delta^{15}N$ values and physiological processes may be confounding factors for overall TDF determination. In addition, it is quite possible that premixed zoo diets are not a fair representation of average non-captive diets and perhaps not ideal for drawing conclusions about what a TDF would be in the wild.

While the TDFs that we calculated are mostly the same for snow leopards and tigers, we see substantial variation in the TDFs calculated in Parng *et al.*;[19] while they had a wide range of differences between the calculated TDFs of the felids in that study, it is difficult to tell how statistically significant

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the values are since n = 1 for two of the four cat species sampled. We increased the number of individuals in this study, but it is still difficult to draw statistically significant conclusions due to the relatively small sampling pool. As most zoos and wildlife parks do not keep a large number of large felids in captivity, it is difficult to increase the number of individuals sampled in captive studies of large carnivores. While all the animals are kept in enclosures together and subjected to the same conditions, it is possible that sex, weight, or age differences could have an effect on the calculated TDFs. Perhaps in the future a larger study can be carried out that combines samples from big cats at multiple zoos and wild animal parks so that these variables can be tested with an appropriate sample size.

Lipid extraction

The question of whether or not it is worthwhile or even necessary to extract lipids to properly determine carbon isotope ratios is a popular one but, as previously mentioned, carbon discrimination factors calculated from both lipid- and non-lipid-extracted materials are indistinguishable from each other in both this study and other meta-analyses.[11] It is known that lipids within tissues can significantly lower the measured $\delta^{13}C$ values.[36] A more nuanced approach to examining the contributions of lipids to bulk $\delta^{13}C$ values illustrates that lipids within the tissues of dietary items can affect the $\delta^{13}C$ value of keratin, the key structural component of hair.[37] In otter populations, those otters with a higher percentage of lipids in their diet appear to have $\delta^{13}C$ values in their hair that are more lipid derived; therefore, using lipid-extracted $\delta^{13}C_{diet}$ values to calculate discrimination factors can lead to artificially low values.[37] Using the equations from Post *et al.*,[36] the C/N ratio of the feline diet from this study reveals that the diet blend is approximately 40% lipids, which is relatively high. Thus, calculating TDF with non-lipid-extracted diet values is ideal in this case.

We also used non-lipid-extracted $\delta^{15}N_{diet}$ values to calculate TDF, as the $\delta^{15}N$ values can be affected by chemical lipid extraction treatments – a small fractionation can occur when samples are treated with chloroform/methanol,[36] the treatment used this study. The isotopic fractionation that occurred within the diet samples after treatment produced changes in the $\delta^{15}N$ values that were large in magnitude and unexpected. The reason for this fractionation is unknown, as chloroform/methanol chemical extraction does not usually cause a fractionation larger than 0.25‰,[36] but in this case it was ~2.5‰. This extraction was repeated numerous times with the same results and the same issue was not seen in the treated isotopic standards. It thus appears there must be some unique fractionation occurring between the premixed carnivore diet in this study and the chloroform/methanol extraction mixture. In the future, we would like to treat these same samples with diethyl ether, another solvent used for lipid extraction, to compare lipid extraction methods; until then, we have confidence in using the non-lipid-extracted $\delta^{15}N$ values, as they have not been subjected to any outside treatments that could cause fractionation and are therefore reliable for calculating TDFs.

CONCLUSIONS

Placing this study in the context of other recent experiments, we see that scat can be considered a good proxy of diet in these carnivores. In the case of carbon isotope ratios, no discrimination factors are needed to place the isotope ratio values of scat in an ecological context. The diet-hair discrimination factors are larger than are typically seen, but are significant and fall in line with those carbon diet-hair TDFs calculated in other species of big cats by Parng *et al.*[19] It appears that the dynamics of nitrogen isotopes in both scat and hair may be slightly more complicated, as has been discussed here. The hypothesis of decreasing isotope discrimination with increasing protein quality is worthy of detailed further investigation in large carnivores specifically, as this has not been studied. In our study, the nitrogen TDFs do not seem to be significant and could potentially be ignored (except in the case of snow leopard scat), but an expanded dataset is needed to better understand the variation that is seen even between closely related species.

While we were not able to calculate a turnover rate due to the inability to alter the diets of these big cats significantly while they are in the care of the Bronx Zoo, we are confident that their tissues were in equilibrium with their diets, as their premixed feed is invariant. An ideal next step would be to perform a long-term study to track the change in TDFs along with any potential fluctuations in stable isotope ratios of the premixed diet. It is vital to study a variety of large cats (a variety of both species and individuals) in the future with a more controlled feeding experiment to determine what causes such wide variation in TDFs in large carnivores, a question that plagues many stable isotope TDF experiments. This study is meant to lay the groundwork for a rich future of further investigation into trophic discrimination factors of large terrestrial carnivores, a vast, ecologically important group in desperate need of study worldwide.

Acknowledgements

The authors thank David Powell, Pat Thomas, and the keepers at the Bronx Zoo for facilitating the collection and acquisition of all samples used in this study. Matt Rogers is also thanked for both running samples and valuable discussions relating to this manuscript while performing the stable isotope analysis at the University of Alaska, Anchorage. Thank are extended to Tom Maddox at the University of Georgia for providing additional sample preparation for stable isotope analyses. Finally, thanks go to the two anonymous reviewers whose contributions greatly enhanced the quality of this paper. Funding for this study was provided by Columbia University.

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