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## A breath test to assign carnivore diets to browsers or grazers

Christian C. Voigt, Jörg Melzheimer, Susanne Thalwitzer & Bettina Wachter

Cheetahs *Acinonyx jubatus* are important predators of herbivores in African ecosystems, and several methods to determine their diet have been used in the past. We applied a novel method to quickly assess the diet of cheetahs with respect to grazing and browsing herbivores, i.e. we analysed the stable carbon isotope ratio ( $\delta^{13}\text{C}_{\text{V-PDB}}$ ) of cheetah breath to separate individuals feeding predominantly on browsers or grazers, respectively. Browsers and grazers are contrasting in their muscle  $\delta^{13}\text{C}_{\text{V-PDB}}$ , because of their isotopically distinct C3 or C4 plant diet, respectively. Muscle  $\delta^{13}\text{C}_{\text{V-PDB}}$  of six abundant local potential prey species of cheetahs confirmed that kudu *Tragelaphus strepsiceros* and springbok *Antidorcas marsupialis* browsed on C3 plants, whereas gemsbok *Oryx gazella*, hartebeest *Alcelaphus buselaphus*, warthog *Phacochoerus africanus* and cattle *Bos taurus* predominantly grazed on C4 plants. Breath  $\delta^{13}\text{C}_{\text{V-PDB}}$  of the cheetahs followed the bimodal frequency distribution of the prey species with six cheetahs being assigned to the C4 food web and three to the C3 food web. Breath tests may be a suitable method to delineate the trophic membership of carnivores to C3 and C4 food webs when animals are chemically immobilised for other purposes.

*Key words:* *Acinonyx jubatus*, Africa, cheetah, consumers, dietary analysis, food web, free-ranging, prey, stable carbon isotope ratios

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Information on the dietary habits of predators is useful when predator-prey relationships or dietary overlap of predators are studied, or when predators are suspected to feed selectively on particular prey species that are valuable to farmers or game managers outside protected areas (Höner et al. 2002, Marker et al. 2003, Hayward & Kerley 2008). Yet, dietary analysis is a time-consuming task that often requires the close monitoring of individual predators or the collection and analyses of indigestible prey remains in faeces of the predator (Schaller 1972, Weaver 1993, Caro 1994, Wachter et al. 2012). Such conventional approaches are important and essential when the diet has to be determined in detail. However, if the information on consumed prey

categories, such as grazers or browsers, is sufficient, and predators are captured and immobilised for other purposes anyway, quicker methods might be more appropriate.

We used a stable isotope approach for assigning individual cheetahs *Acinonyx jubatus* to a C3-plant based (browser) or a C4-plant based (grazer) food web. For this purpose, we analysed the carbon stable isotope ratio ( $\delta^{13}\text{C}_{\text{V-PDB}}$ ) of exhaled breath in nine adult cheetahs captured and immobilised on livestock and game farmland in central Namibia. It is known that carbon stable isotope ratios in predator tissues reflect the relative contribution of browsing or grazing herbivores to the diet of these predators (De Niro & Epstein 1977), and can thus serve as a

quantitative measure to assign predators to a C3 or C4 food web or to some intermediate position between the two isotopically distinct food webs (Voigt & Kelm 2006). For example, Codron et al. (2007) used stable isotopes measured in hair samples to allocate lions and leopards to a C3 or C4 food web, but stable isotope ratios have never been analysed before in the breath of free-ranging African carnivores.

Breath  $\delta^{13}\text{C}_{\text{V-PDB}}$  matches with the  $\delta^{13}\text{C}_{\text{V-PDB}}$  of the oxidised substrate, which can be of exogenous origin (food ingested) or endogenous origin (triacylglycerols from fat stores or glycogen). In animals that have just ingested food, breath  $\delta^{13}\text{C}_{\text{V-PDB}}$  matches  $\delta^{13}\text{C}_{\text{V-PDB}}$  of the last diet because food ingesta are used as the major fuel to power metabolism. In contrast, breath  $\delta^{13}\text{C}_{\text{V-PDB}}$  of fasting animals is depleted in  $^{13}\text{C}$  in relation to the recently ingested food item by about 3.2‰ (Voigt et al. 2008a), because lipogenesis causes a depletion in  $^{13}\text{C}$  in adipocytes in relation to non-fat tissue and the diet (De Niro & Epstein 1977, Voigt et al. 2010, 2012). Thus,  $\delta^{13}\text{C}_{\text{V-PDB}}$  provides a reliable approximation of the food web membership of consumers (Voigt & Speakman 2007, Voigt et al. 2008b,c, Engel et al. 2009), when the nutritional status of the consumer is known (Perkins & Speakman 2001, Voigt et al. 2008a,b).

## Material and methods

### Study animals

Between November 2006 and March 2008, we collected breath samples from nine adult free-ranging cheetahs (eight males and one female) on commercial livestock and game farmland in central Namibia. We also collected muscle samples from 10 individuals of each potential prey species of cheetahs to obtain isotope reference material. Potential browser and grazer prey species on Namibian farmland are: kudu *Tragelaphus strepsiceros*, gemsbok *Oryx gazella*, hartebeest *Alcelaphus buselaphus*, springbok *Antidorcas marsupialis*, warthog *Phacochoerus africanus* and cattle *Bos taurus* (Marker et al. 2003, Wachter et al. 2006). We acknowledge that cheetahs will most likely prey on the juveniles of these species (Marker-Kraus et al. 1996), but juvenile and adult ungulates of the same species should be relatively similar with respect to their stable carbon isotope ratios (Jenkins et al. 2001).

### Sample collection and analysis of reference material

We collected samples of game species from animals killed for their trophy and/or meat by hunters and farmers according to Namibian legislation, whereas cattle samples were collected from individuals at the slaughterhouse in Windhoek, Namibia. Muscle samples were air dried, stored in cryovials and transported at ambient temperature to the Leibniz Institute for Zoo and Wildlife Research (IZW) in Berlin, Germany. Stable carbon isotope analyses of muscle samples were performed at the stable isotope laboratory of the IZW using a Flash-elemental analyser (ThermoFinnigan, Bremen, Germany) connected in continuous mode to a Delta V-Advantage isotope ratio mass spectrometer (ThermoFinnigan, Bremen, Germany). Ratios of  $^{13}\text{C}$  to  $^{12}\text{C}$  were measured in relation to the international standard Vienna-PeeDeeBelemnite (V-PDB) and values are given in the delta notation following the equation:  $\delta^{13}\text{C}_{\text{V-PDB}} (\text{‰}) = ((^{13}\text{C}/^{12}\text{C}_{\text{sample}} - ^{13}\text{C}/^{12}\text{C}_{\text{standard}})/^{13}\text{C}/^{12}\text{C}_{\text{standard}})$ , with  $^{13}\text{C}/^{12}\text{C}_{\text{sample}}$  and  $^{13}\text{C}/^{12}\text{C}_{\text{standard}}$  representing the stable carbon isotope ratio in the sample and in the standard, respectively. Accuracy of repeated measurements of laboratory protein standards was better than 0.4‰ (one standard deviation).

### Sample collection and analysis of cheetah breath

Breath of free-ranging cheetahs was collected during routine handling after cheetahs had been captured in box traps at marking trees. Box traps were checked daily, and when a cheetah was captured, additional traps were set to capture other possible group members until the next morning when the cheetahs were examined. Captured animals consisted of five single cheetahs and two groups of two males. They were provided with water but no food, which means that the captured animals had fasted for several hours prior to immobilisation and breath collection. We chemically immobilised cheetahs for approximately 45 minutes, conducted a comprehensive health screening, fitted them with a collar (Thalwitzer et al. 2010, Wachter et al. 2011) and collected their breath. Under field conditions, the collection of exhaled  $\text{CO}_2$  might be hampered by the possible contamination of exhaled  $\text{CO}_2$  with ambient  $\text{CO}_2$ . For example, if half of the collected  $\text{CO}_2$  volume originates from ambient air (air  $\delta^{13}\text{C}_{\text{V-PDB}} = -8\text{‰}$ ) and the other half originates from the predator's breath after having fed from a browser (tissue  $\delta^{13}\text{C}_{\text{V-PDB}} = -25\text{‰}$ ), the resulting  $\delta^{13}\text{C}_{\text{V-PDB}}$  of the collected gas sample would falsely suggest that the predator has

fed from a herbivore consuming a mixture of C3 ( $\delta^{13}\text{C}_{\text{V-PDB}} = -12\text{‰}$ ) and C4 ( $\delta^{13}\text{C}_{\text{V-PDB}} = -27\text{‰}$ ) plants. To control for this contamination effect, we followed and evaluated the following two approaches.

In four animals, we collected breath samples at 2-minute intervals over a period of 20 minutes ( $N = 3$ ) or 10 minutes ( $N = 1$ ). For breath collection, we placed a conic respirometry mask over the muzzle of cheetahs. We punctured the top of the mask from inside with the tip of a conventional injection needle (gauge 18) such that the tip pointed outwards. By piercing the teflon membrane of a Vacutainer™ (Labco, Buckinghamshire, U.K.) with the tip of the needle, we sucked approximately 10 ml of air from the inside of the mask into the vacutainer. Oxygen saturation of blood was constantly surveyed by an oxymeter (Nellcor N-20PA®, Nellcor Puritan Bennett Inc., Pleasanton, USA) to prevent suffocation of the animal. Since exhaled  $\text{CO}_2$  gradually accumulates under the mask and replaces the ambient  $\text{CO}_2$  in the mask, the  $\delta^{13}\text{C}_{\text{V-PDB}}$  values represent the increasing proportion of breath  $\text{CO}_2$  to ambient  $\text{CO}_2$ . Thus,  $\delta^{13}\text{C}_{\text{V-PDB}}$  of the mixture of breath and ambient air in vacutainers was more biased towards  $\delta^{13}\text{C}_{\text{V-PDB}}$  of ambient  $\text{CO}_2$  in the samples collected first than those collected afterwards.

Filled vacutainers were shipped to the stable isotope laboratory of the IZW. Before analysing the carbon stable isotope ratios, we measured the  $\text{CO}_2$  concentration (Vol%) in subsamples of the vacutainers air using conventional indirect calorimetry as outlined in Voigt et al. (2008b). Afterwards, we measured the carbon stable isotope ratios using a GasBench (ThermoFinnigan, Bremen, Germany) connected in continuous mode to a Delta V-Advantage isotope ratio mass spectrometer (ThermoFinnigan, Bremen, Germany). We used Keeling plots to determine the  $\delta^{13}\text{C}_{\text{V-PDB}}$  of exhaled breath (Keeling 1958, Carleton et al. 2004, Voigt et al. 2008b). Keeling plots are bi-variate graphs where  $\delta^{13}\text{C}_{\text{V-PDB}}$  of all breath samples of an individual are plotted against the reciprocal  $\text{CO}_2$  concentration (Vol%<sup>-1</sup>) in the vacutainer.  $\delta^{13}\text{C}_{\text{V-PDB}}$  increases with decreasing ratio of exhaled  $\text{CO}_2$  to ambient  $\text{CO}_2$ . Least squares regression models are then used to predict the intercept of the regression line with the y-axis. This intercept is identical to the  $\delta^{13}\text{C}_{\text{V-PDB}}$  of the exhaled breath and provides information on the breath  $\delta^{13}\text{C}_{\text{V-PDB}}$  of the animal without a bias of contaminating ambient  $\text{CO}_2$ .

In the other five animals, we used a refined approach by flushing the conic respirometry mask with pure  $\text{O}_2$ . The  $\text{O}_2$  replaced all ambient  $\text{CO}_2$  in the mask. We stopped the flushing of the mask after five minutes to let breath  $\text{CO}_2$  accumulate in the mask. We then collected three samples of breath in rapid succession and measured  $\delta^{13}\text{C}_{\text{V-PDB}}$  as described above. We refrained from measuring the  $\text{CO}_2$  concentration in these samples, because we assumed that no contamination with ambient  $\text{CO}_2$  took place. Thus, it was not necessary to establish Keeling plots for these animals.

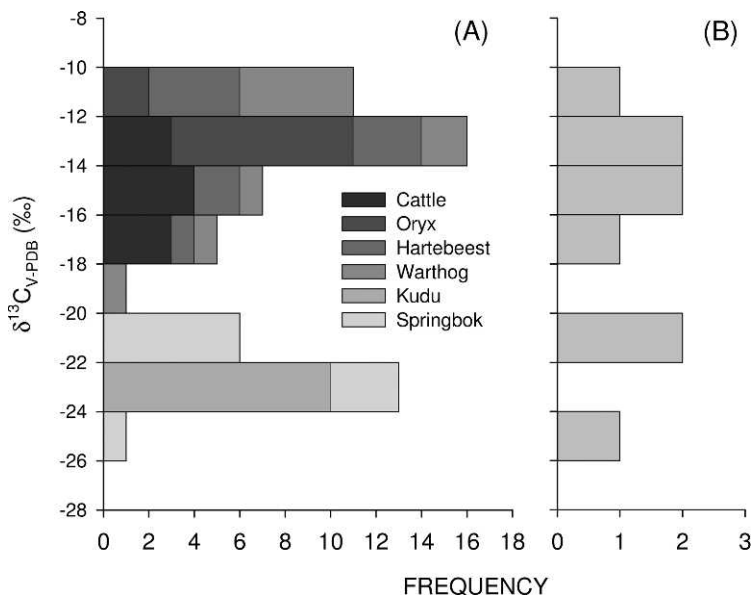
Because the cheetahs had not eaten anything since they were captured, we assumed that breath  $\delta^{13}\text{C}_{\text{V-PDB}}$  reflected mostly  $\delta^{13}\text{C}_{\text{V-PDB}}$  of endogenous oxidative fuels such as glycogen and, more importantly, triacylglycerols from adipocytes. The predominant oxidation of  $^{13}\text{C}$ -depleted triacylglycerols causes a depletion of about 3.2‰ in the breath  $\delta^{13}\text{C}_{\text{V-PDB}}$  in relation to the past diet (Perkins & Speakman 2001, Voigt et al. 2008a). We therefore added 3.2‰ to the breath  $\delta^{13}\text{C}_{\text{V-PDB}}$  value of the cheetahs to predict the  $\delta^{13}\text{C}_{\text{V-PDB}}$  of the previously ingested prey animal. For statistical tests, we assumed an alpha value of 5% and used Systat (Version 13).

## Results

### Muscle $\delta^{13}\text{C}_{\text{V-PDB}}$ of prey species

Muscle  $\delta^{13}\text{C}_{\text{V-PDB}}$  of the six potential prey species of cheetahs on Namibian farmland differed among the species (Kruskal-Wallis test:  $N = 10$  individuals for each species:  $KW = 44.6$ ,  $P < 0.0001$ ). Dunn's multiple comparisons test revealed that muscle  $\delta^{13}\text{C}_{\text{V-PDB}}$  of kudu (median =  $-23.6\text{‰}$ ) differed significantly from those of cattle ( $-15.2\text{‰}$ ;  $P < 0.05$ ), hartebeest ( $-13.3\text{‰}$ ;  $P < 0.001$ ), gemsbok ( $-12.8\text{‰}$ ;  $P < 0.001$ ) and warthog ( $-12.0\text{‰}$ ,  $P < 0.001$ ), but not from those of springbok ( $-21.7\text{‰}$ ;  $P > 0.05$ ). Muscle  $\delta^{13}\text{C}_{\text{V-PDB}}$  of springbok differed significantly from those of hartebeest ( $P < 0.01$ ), gemsbok ( $P < 0.01$ ) and warthog ( $P < 0.001$ ), but not from those of cattle ( $P > 0.05$ ). All other pairwise comparisons were not significant. The frequency distribution of muscle  $\delta^{13}\text{C}_{\text{V-PDB}}$  can be described by a bimodal distribution (Fig. 1A), suggesting two isotopically based food webs: one consisting of browsers (kudu and springbok) feeding mostly on C3 plants and one consisting of grazers (cattle,

Figure 1. Frequency distribution of 60 muscle (A) and 53 breath (B) carbon stable isotope ratios ( $\delta^{13}\text{C}_{\text{V-PDB}}$ ) of six potential prey species of cheetahs and of nine cheetahs, respectively. Breath  $\delta^{13}\text{C}_{\text{V-PDB}}$  was corrected for the oxidation of  $^{13}\text{C}$  depleted fat stores.



hartebeest, gemsbok and warthog) feeding mostly on C4 plants.

### Breath tests in cheetahs

In cheetahs, breath  $\delta^{13}\text{C}_{\text{V-PDB}}$  ranged between  $-14.7\text{‰}$  and  $-27.8\text{‰}$  (median:  $18.3\text{‰}$ ; Table 1), suggesting that cheetahs fed on a variety of isotopically distinct prey species. Corrected breath  $\delta^{13}\text{C}_{\text{V-PDB}}$  of cheetahs showed also a bimodal distribution that almost matched the bimodal distribution of prey  $\delta^{13}\text{C}_{\text{V-PDB}}$  (see Fig. 1B). Most cheetahs ( $N = 6$ ) consumed grazing herbivores and the others ( $N = 3$ ) browsing herbivores. The two males in each of the two groups (individuals E and F and individuals G and H) showed very different breath  $\delta^{13}\text{C}_{\text{V-PDB}}$ , suggesting that they had fed on different prey species before they were captured.

### Discussion

We used a stable isotope approach to assign individual free-ranging cheetahs living on Namibian livestock and game farmland to the C3 or C4 food web of their habitat. This was possible because herbivores differ with respect to carbon stable isotopes ( $\delta^{13}\text{C}_{\text{V-PDB}}$ ) according to their browse or grass diet (Codron et al. 2007). Accordingly, predators feeding on herbivores have either a distinct C3 or C4 food web signature if they feed exclusively on browsers or grazers, respectively, or intermediate values in case they feed on a mixture of browsers and grazers. Instead of using fur or muscle tissue, we tested if breath samples are appropriate for describing the C3 or C4 origin of the cheetah diet. Breath  $\delta^{13}\text{C}_{\text{V-PDB}}$  matches with  $\delta^{13}\text{C}_{\text{V-PDB}}$  of the oxidative fuel, which is either of

Table 1. Stable carbon isotope ratio (mean  $\delta^{13}\text{C}_{\text{V-PDB}} \pm$  one standard error) in exhaled breath of nine free-ranging cheetahs. The regression equations for keeling plots with intercepts ( $\delta^{13}\text{C}_{\text{V-PDB}}$  (‰)) and slopes were calculated for the individuals marked with an asterisk (A-D); for the others regression parameters are not available (na) and mean  $\delta^{13}\text{C}_{\text{V-PDB}}$  was derived directly from the sample values.

Individual	Sex	No. of breath samples	$\delta^{13}\text{C}_{\text{V-PDB}}$ (‰)	Slope	$r^2$
A*	male	10	$-15.8 \pm 0.3$	$0.12 \pm 0.22$	0.04
B*	female	10	$-19.2 \pm 0.04$	$0.17 \pm 0.05$	0.63
C*	male	5	$-16.1 \pm 0.8$	$-1.51 \pm 0.74$	0.41
D*	male	10	$-14.7 \pm 0.6$	$-0.39 \pm 0.35$	0.14
E	male	3	$-27.8 \pm 0.1$	na	na
F	male	3	$-18.3 \pm 0.01$	na	na
G	male	3	$-17.3 \pm 0.1$	na	na
H	male	3	$-24.1 \pm 0.2$	na	na
I	male	3	$-24.0 \pm 0.2$	na	na



endogenous origin (mostly adipocyte triacylglycerols) in fasting animals or of exogenous origin in fed animals (Perkins & Speakman 2001, Voigt et al. 2008a). The stable isotope analysis of potential prey showed that herbivores can be assigned to one of two groups: either the grazer group feeding mostly on C4 grass (cattle, hartebeest, gemsbok and warthog) or the browser group feeding mostly on C3 plants (kudu and springbok). Posthoc testing suggested a single non significant difference between consumers of these two food webs, i.e. between cattle and springbok. However, this may be an artefact that has possibly resulted from the small sample size and our correction for multiple testing. A Mann-Whitney U test performed only for data of these two prey species revealed a highly significant difference in their stable carbon isotope ratios ( $U = 0.0$ ,  $P < 0.001$ ), supporting our notion of a bimodal distribution in consumer stable carbon isotope ratios. In general, our findings with respect to stable carbon isotope ratios in potential prey are consistent with other stable isotope studies performed in southern Africa on large predators (e.g. Codron et al. 2007).

The analysis of cheetah breath with respect to the relative enrichment of  $^{13}\text{C}$  and  $^{12}\text{C}$  in  $\text{CO}_2$  revealed that  $\frac{2}{3}$  of the cheetahs belonged to a C3-plant based food web and  $\frac{1}{3}$  to a C4-plant based food web; at least with respect to their most recent diet absorbed in their endogenous fuels. Our data interpretation is based on the assumption that cheetahs were fasting, and that therefore, breath  $\delta^{13}\text{C}_{\text{V-PDB}}$  matched those of endogenous fuels. If this assumption is wrong, we would have expected a less pronounced bimodal distribution of breath  $\delta^{13}\text{C}_{\text{V-PDB}}$  values because varying levels of fasting in cheetahs would have introduced additional variance to the data set. Yet, a bimodal distribution of breath  $\delta^{13}\text{C}_{\text{V-PDB}}$  was clearly visible.

It is important to keep in mind that our stable isotope approach does not differentiate between prey species within a food web but depends on the pooling of prey with similar stable isotope ratios. Therefore, dietary assessments that are based on consumer breath are rough estimates of an animal's diet because they are based on isotopes of one element, i.e. carbon, only. Using conventional samples such as fur or muscular tissue would have enabled us to analyse two (e.g. carbon and nitrogen) or more isotopes and to calculate mixing models (Phillips 2012, Codron et al. 2007). Also, studying multiple organs with varying isotopic retention times may shed light on the

dietary habits over different time period. This could potentially allow a finer-scaled analysis of diet.

Using keeling plots, we were able to control for the potential contamination of breath samples with ambient  $\text{CO}_2$ . The keeling-plot approach in assigning source  $\delta^{13}\text{C}_{\text{V-PDB}}$  to a mixture of two gases has been used in environmental studies for a long time (Keeling 1958). Recently, researchers have modified this technique for use in animal ecology (Carleton et al. 2004). In our study, regression models provided good estimates for the breath  $\delta^{13}\text{C}_{\text{V-PDB}}$ . In two animals, we noted negative slopes of the regression line (see Table 1), which should be impossible when ambient  $\text{CO}_2$  with high  $\delta^{13}\text{C}_{\text{V-PDB}}$  values mix with breath  $\text{CO}_2$  depleted in  $^{13}\text{C}$  in relation to ambient  $\text{CO}_2$ . However, breath  $\delta^{13}\text{C}_{\text{V-PDB}}$  values were relatively high, and therefore, the difference between ambient and breath  $\delta^{13}\text{C}_{\text{V-PDB}}$  may have been too small to yield a statistically significant regression slope. Overall, we recommend using our second approach in collecting breath samples, i.e. flushing a respirometry mask with pure oxygen, to eliminate any potential contamination.

An advantage of this technique is that sample collection can be easily done provided that carnivores are captured and chemically immobilised anyway. Also, the export and import of breath samples does not require CITES permits as is the case with tissue samples. Therefore, shipping of breath samples is done with less administrative effort and requires less time than shipment of tissue samples. If a study is interested in whether a predator species is specialised on a particular prey category such as grazers or browsers, or needs a crude information on diet overlap of several predator species, analyses of breath  $\delta^{13}\text{C}_{\text{V-PDB}}$  of the predator species might be useful. For reliable results, reference muscle  $\delta^{13}\text{C}_{\text{V-PDB}}$  of the local prey species should also be collected and measured. These reference values can also be used for future breath samples and thus have to be collected only once.

In summary, breath  $\delta^{13}\text{C}_{\text{V-PDB}}$  of free-ranging cheetahs ranged over  $> 10\%$  and matched the bimodal distribution of muscle  $\delta^{13}\text{C}_{\text{V-PDB}}$  of the potential prey species. These findings are consistent with previous studies that analysed the diet of cheetahs based on faecal analyses (Marker et al. 2003, Wachter et al. 2006), indicating that the analysis of breath  $\delta^{13}\text{C}_{\text{V-PDB}}$  of a predator is a simple and straightforward method to reveal reliable information on the general diet of a predator.

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