Isotopic palaeoecology of Makapansgat Limeworks Perissodactyla

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Relatively little is known about the ecology of South African Pliocene perissodactyls. Researchers usually assume that these taxa were ecologically similar to their modern counterparts, but this assumption is questionable, and can tell us nothing about the ecology of taxa without modern representatives such as chalicotheres. Here we examine the ecology of Makapansgat's Rhinocerotidae, Equidae and Chalicotheridae using stable carbon isotope analysis of tooth enamel. This allows us to test uniformitarianist assumptions, provides insight into the ecology of these taxa, and allows a glimpse into the ecological underpinnings of hominid evolution in South Africa.

The rodents,[1] suids,[2] bovids,[3-6] cercopithecids,[7] and hominids[8,9] from the 3-million-year-old Makapansgat Limeworks have been subjected to intense study. Relatively little attention, however, has been paid to the perissodactyls, which include some of the oldest specimens of the modern white (*Ceratotherium simum*) and black (*Diceros bicornis*) rhinos, as well as an extinct equid (*Hipparion lybicum*) and chalicothere (*Ancylotherium hennigi*).[10] To a large extent, our understanding of the ecology of these creatures has been based on taxonomic uniformitarianism and morphology. Specifically, it is assumed that *C. simum* and *H. lybicum* were grazers because their living relatives are, and because they had hypsodont dentition, while it is assumed that *D. bicornis* was a browser because of its low-crowned dentition and dedicated browsing habits of the modern black rhino.[10,11] As the Chalicotheridae have no living representatives, we can infer their diets from morphology only. *Ancylotherium*'s brachyodont dentition and apparent specialization for rearing up on two hindlegs suggests that it was a browser that gathered material for consumption with its forelimbs.[12] Others have argued that its unique claws were used for extracting food from underground[13]

Neither taxonomic uniformitarianism nor morphology, however, allows us to make unequivocal dietary assignments.[6,14] For example, a recent isotopic and microwear study examining the diets of North American fossil equids demonstrated that some Pliocene taxa were browsers despite hypsodont dentition and grazing survivors.[15] This necessitates tapping other sources of information to investigate the diets of fossil taxa. Here, we examine the ecology of the Makapansgat perissodactyls in the light of new stable carbon isotope data.

Stable carbon isotope analysis is useful for studying the ecology of fossil taxa because plants that use the C_3 photosynthetic pathway such as trees, bushes, and forbs have depleted ratios of $^{13}C/^{12}C^{†}$ in their tissues compared to tropical grasses and some sedges, which use the C_4 pathway.[16,17] Dietary carbon is incorporated into tooth enamel apatite of herbivores with some further fractionation.[18,19] In African savanna environments, pure grass consumers (grazers) have $\delta^{13}C$ values of about $+2‰$ to $-2‰$, while animals that eat from trees, bushes, shrubs, and forbs (browsers) have depleted $\delta^{13}C$ values of between about $-10‰$ and $-16‰$. Thus, by determining $\delta^{13}C$ of a fossil taxon's tooth enamel we can tell if it was a grazer, a browser, or a mixed feeder.

In order to perform stable carbon isotope analysis, enamel was obtained from fossil teeth using a rotary drill equipped with a diamond-tipped burr.[20] The powder was pretreated in 1.5% sodium hypochlorite solution to remove organic contaminants and in 0.1 M acetic acid to remove highly soluble diagenetic carbonates, and freeze-dried. One milligram of purified powder was weighed into individual reaction vessels in a Kiel II Autocarbonate device interfaced with a Finnigan MAT 252, for reaction with phosphoric acid at 70°C, cryogenic distillation, and measurement of the resultant CO_2. The raw data were calibrated against several international and laboratory standards before being downloaded for statistical analysis. The results for the Limeworks perissodactyls are presented in Table 1 and Fig. 1. The grazing bovid *Parmularius braini* and browsing bovid *Tragelaphus angasi* from Makapansgat are included for comparison.

Hipparion lybicum was clearly a consumer of C_4 grasses like *Equus* in Africa today. Its $\delta^{13}C$ values are slightly depleted compared to the alcelaphine bovid *P. braini*, but analysis of variance shows that this difference is not statistically significant. Modern African equids tend to have slightly lower $\delta^{13}C$ values than coexisting alcelaphines.[21] While it can be argued that these small differences reflect their differing digestive physiologies, a study of the diet to enamel apatite fractionation in wild and captive animals found no differences between equids and bovids.[19] This, coupled with studies noting that modern equids occasionally browse, suggests that the lower $\delta^{13}C$ values probably reflect the inclusion of small amounts of (non-grassy) C_3 vegetation.[22] An isotopic study of equids from the late Miocene at Lothagam also suggested an early specialization on grasses within this lineage.[23] Thus, it appears likely that Pliocene *Hipparion*, and later *Equus*, filled much the same niche. Future studies of sympatric *Hipparion* and *Equus* (at Swartkrans and Koobi Fora, for example) should improve our understanding of the ecology underpinning the eventual disappearance of the former, and ascendancy of the latter.

Predictably, the $\delta^{13}C$ values of *C. simum* and *D. bicornis* are significantly different ($P < 0.0001$). The ^{13}C-depleted *D. bicornis* was clearly a browser as generally assumed, as its $\delta^{13}C$ values are nearly identical to those of the browsing bovid *T. angasi*. But although *C. simum* certainly consumed C_4 grasses, its $\delta^{13}C$ values are significantly different from those of the aforementioned grazers *P. braini* and *H. lybicum* ($P < 0.01$). Therefore, unlike the modern white rhino, the Makapansgat *Ceratotherium* was not a pure grazer. Indeed, these data indicate that browse comprised over 30% of its diet, much like the greater Indian rhinoceros today (*Rhinoceros unicornis*).[24] A hypsodonty index of 2.9 for the single unworn M_3 of Makapansgat's *Ceratotherium* falls closer to the mean hypsodonty index of modern browsing *Diceros* (HI = 2.2) than it does to modern *Ceratotherium* (HI = 3.9). This, coupled with the isotopic evidence, strongly suggests that *Ceratotherium* had neither the morphological equipment nor the behavioural propensities of the extant white rhino. The result is perplexing given that many African bovid and equid taxa had already become specialized grazers by this time.[6,14] Apparently

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[†]$^{13}C/^{12}C$ is expressed as $\delta^{13}C$ values in parts per thousand (per mil) relative to the PDB standard.

Table 1. Mean $\delta^{13}C$ values, standard deviations, and specimens sampled for the Makapansgat Limeworks perissodactyls and grazing and browsing bovid outgroups.

Species	$\delta^{13}C$ (‰)	s.d.	n
Ancylotherium hennigi	−10.3	0.3	3
Ceratotherium simum	−3.5	0.7	4
Diceros bicornis	−11.6	1.3	3
Hipparion lybicum	−0.8	0.9	5
Parmularius braini	0.7	0.7	4
Tragelaphus angasi	−11.7	1.5	4

the rhinoceros *Ceratotherium* lineage remained less-specialized until well into the middle to late Pliocene faunal turnover. Given this evidence, it is unlikely that the Makapansgat *Ceratotherium,* and the extant white rhino, are conspecific. More likely, it is a late representative of *C. praecox,* or a new South African endemic species.

The $\delta^{13}C$ values for *A. hennigi* are consistent with its being a C_3 feeder. It is slightly enriched in ^{13}C compared to the browsers *D. bicornis* and *T. angasi,* but the difference is not significant (given the small sample numbers). *Ancylotherium* was clearly a browsing herbivore, but the results allow that approximately 10% of its diet could have consisted of C_4 plants. We cannot, therefore, exclude the possibility that *Ancylotherium* dug for underground parts of C_4 plants occasionally. Moreover, $\delta^{13}C$ analysis alone does not allow us to address the possibility of their digging for the tubers of C_3 plants. We suggest that microwear studies could be used to examine this possibility.

Although the results presented here are in accord with previous thinking for three of the four taxa analysed, the $\delta^{13}C$ values for *Ceratotherium* are unanticipated and important. Recent carbon isotope studies have shown that 25% or more Pliocene taxa had diets different from those reported (or assumed) in the literature.[6,14,25] Since many palaeoenvironmental reconstructions are founded on our reputed knowledge of the diets and habitat tolerances fossil taxa,[5,26,27] this discrepancy is highly significant. Stable carbon isotope studies can clearly improve our understanding of the ecology of these fossil species, and in turn, better our palaeoenvironmental reconstructions. Studies that combine isotopic, morphological, microwear and elemental components will improve our understanding of the processes that shaped today's biotic community.
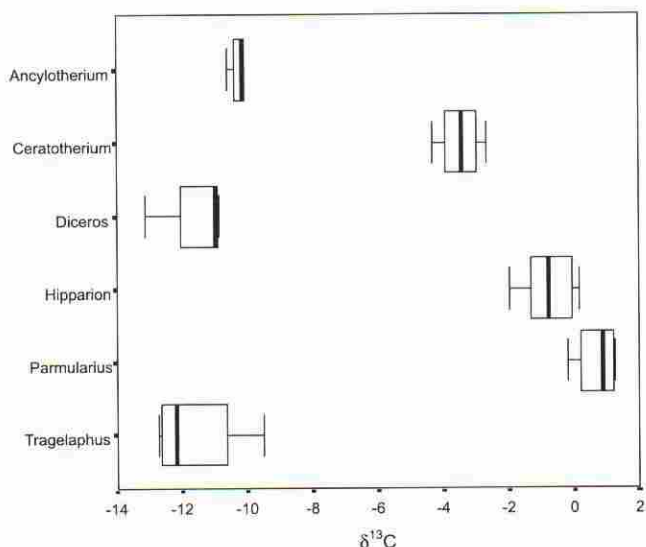
While fauna abundance-based environmental reconstructions are best made within the broad context of community ecology, these data do allow us to make some general statements about the ancient Makapansgat Valley. Some researchers believe that the ancient valley was relatively arid,[2,28–30] although recent research suggests that it contained riverine forest, bushland and edaphic grassland.[5,6,10,31] Since three of the six taxa shown in Fig. 1 were C_4 plant consumers (and grazers, fresh-grass grazers, and mixed feeders make up nearly 50% of the large-bodied herbivores),[14] it is likely that extensive grasslands or woodlands were to be found in the vicinity. Thus, while subtropical forest may have existed in patches (as it does, probably to a lesser extent, today), it would not have been the dominant habitat. On the other hand, the high percentage of browsing taxa in Fig. 1 (and about 50% of the large-bodied herbivores)[14] does suggest an abundance of woody vegetation. Thus, these results are consistent with reconstructions suggesting that the area contained riverine forest, edaphic grassland and perhaps bushland. More precise reconstructions will be possible when further analyses provide us with more information about the ecology of the entire Makapansgat fauna.

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Fig. 1. Box plot showing the interquartile range (25–75%, indicated by boxes), the means (thick vertical bars) and the range (thin horizontal bars) of the $\delta^{13}C$ values of the taxa analysed for this study.

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P-glycoprotein expression in human oesophageal squamous carcinoma cell lines

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Several tumour types show resistance to a broad spectrum of anti-cancer drugs and are referred to as expressing a multidrug-resistant phenotype. This has been associated with the over-expression of P-glycoprotein, a highly conserved transmembrane protein. Increased levels of P-glycoprotein are generated in tumour cells resistant to a range of chemotherapeutic drugs. We have examined five cultured human oesophageal squamous carcinoma cell lines and one epidermoid carcinoma cell line for the expression of P-glycoprotein using a radioimmuno assay developed for the purpose. The radioimmuno assays identified significantly different (P = 0.05) levels of P-glycoprotein among the five oesophageal carcinoma cell lines. Treating the lines with epidermal growth factor caused them to increase their P-glycoprotein expression exponentially. Exposing the lines to vinblastine identified four of the five oesophageal carcinoma cell lines as having some resistance to the drug. The SNO cell line expressed a drug-sensitive phenotype with cell death occurring at low drug concentrations. This drug sensitivity was reversed by prior treatment with epidermal growth factor. The high levels of P-glycoprotein generated by the cell lines may be linked to the poor success rate reported for chemotherapeutic treatment of oesophageal carcinoma.

Background

Tumours expressing a drug-resistant phenotype pose a considerable problem for chemotherapy. Several mechanisms have been implicated in the development of multidrug resistance. The mechanisms most commonly associated with the development of the multidrug-resistant phenotype include MRP (MDR-related protein), GST (glutathione S-transferase), topoisomerase II and P-glycoprotein (P-gp).[1,2] P-gp-related drug resistance in tumour cells has been linked to the expression of a 170-kDa transmembrane glycoprotein. P-gp actively expels a broad range of drugs from the cell by acting as an ATP-dependent efflux pump.[1] Elevated levels of P-gp have been detected in a number of tumour cells, either before (intrinsic) or after (acquired) chemotherapy and may cause the cells not to respond to specific drug treatments. Tumours with intrinsic expression of P-gp are usually found to express higher levels of P-gp following chemotherapy, thus complicating treatment even further.[1,2]

In addition to chemotherapeutic drugs, other factors have been argued to influence P-gp expression in tumour cell lines, including specific oncogenes (*c-ras, c-myc*), phosphorylation complexes (cAMP, protein kinase C), epidermal growth factor (EGF), and insulin-like growth factor-1 (IGF-1).[3,4] In general, these factors are shown to increase the level of P-gp expression.

Oesophageal cancer is the third most common form of cancer in southern Africa, and is the most common form of cancer in black males on the subcontinent.[5,6] Owing to the early occurrence of micrometastases by the time of diagnosis, oesophageal cancer remains, on a worldwide basis, difficult to treat using either chemo- or radiotherapeutic techniques.[7,8] Survival following surgery shows only a 15% five-year survival rate. More recently, through a combination of surgery and chemotherapy, patient survival has been extended by several months compared with surgery alone.[7,9]

This difficulty in treating oesophageal cancers chemotherapeutically may be linked to the tumour's expression of a multidrug-resistant phenotype, specifically elevated levels of P-glycoprotein. Data in this regard are scant, with P-gp expression having been demonstrated in only very few squamous carcinoma cell lines.[10,11] Recent work by Jain *et al.*[12] on oral squamous cell carcinomas, which share certain similarities with oesophageal carcinomas, showed the levels of P-gp to vary across different cell lines both before and after chemotherapy.

In this study we examined whether the oesophageal carcinoma cell lines we derived from tumours of a similar pathological grading (moderately differentiated) expressed similar levels of P-gp.[13–15] In addition, since these cell lines overexpress the EGF-receptor,[15] the levels of P-gp following EGF and vinblastine (VBL) treatment were determined. The KB epidermoid carcinoma cell line was used as a positive control, as it has been shown consistently to express the MDR phenotype and is thus considered to be an appropriate model for the study of P-gp expression.[16]

Materials and methods

Cell lines and tissue culture. The five human oesophageal squamous carcinoma cell lines studied were SNO, WHCO$_1$, WHCO$_3$, WHCO$_5$ and WHCO$_6$.[13–15] The sixth was a nasopharyngeal KB carcinoma cell line (ATCC, U.S.A.). All six cell lines were maintained in a humidified chamber at 37°C and 5% CO_2. SNO and KB cell lines were grown in Dulbecco's Modified Eagles (DME) (Highveld Biological, Midrand) growth medium containing 10% fetal calf serum (FCS) (Highveld Biological). The WHCO cell lines were grown in DME: HAMS F12 (3:1) (Highveld Biological) containing 10% FCS; 100 μg/ml streptomycin (ICN) and 100 IU/ml penicillin (ICN) was added to the growth medium of all cell lines.

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