Stable isotope analysis of middle Miocene mammals from the Siwalik sub-Group of Pakistan

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Introduction

The temporal and spatial records of climate archived in thick Neogene and Quaternary continental deposits in northern Pakistan provide an exceptional opportunity to explore the patterns of evolution and adaptation of mammals over the time span of million years (Badgley et al. 2008; Morgan et al. 2009; Patnaik et al. 2019). Stable isotope (SI) analysis of carbon and oxygen of enamel from mammals, both expressed in the familiar delta (δ) notation in per mil (‰) according to V-PDB (Vienna Pee Dee Belemnite) standards, allow us to reconstruct the past diets (δ13C diets) and paleoenvironmental meteoric water (δ18O mw), which in turn reflect paleovegetation and paleoclimate during the Neogene and Quaternary (Quade et al. 1992, 1995a, b; Cerling et al. 1997a, 1997b; White et al. 2009; Sanyal et al. 2005, 2010; Uno et al. 2011; Ben-David and Flaherty 2012; Khan et al. 2020; Waseem et al. 2020a). The Siwalik sub-Group of northern Pakistan contains a high-resolution fossil record spanning the Neogene time period (Quade et al. 1989; Barry et al. 1997a, 2015; White et al. 2009; Sanyal et al. 2005, 2010; Uno et al. 2011; Ben-David and Flaherty 2012; Khan et al. 2020; Waseem et al. 2020a).
However, the major C₄ radiation in the Siwalik sub-Group has been documented to occur after ~10 Ma (Barry et al. 1982, 2002; Quade et al. 1995a, b; Morgan et al. 2009). In this study, we explore the isotopic composition of enamel from three families of large herbivores to reconstruct paleoenvironments and check for evidence of C₄ dietary intake in the middle Miocene (14.2–11.2 Ma) of the Siwalik sub-Group of Pakistan. Enamel was selected for the analysis due to its high resistance to diagenesis and large crystal size and low porosity, which makes it the best material for paleodietary reconstruction in ancient ecosystems (Quade et al. 1992; Kohn et al. 1996, 2002; Kohn and Cerling 2002).

There are three metabolic pathways through which carbon is incorporated in the plants. Trees, nearly all shrubs, forbs, ferns, sedges, and most non-tropical or high altitude grasses utilize the C₃ pathway of photosynthesis in which δ¹³C<sub>diet</sub> ranges from -34 to -22‰ (Koch 1998; Pérez-Crespo et al. 2017), and average around -28.2 ± 6‰ under the present δ¹³C<sub>CO₂</sub> = -8‰, or -6.7‰, pre-industrially (Kohn 2010). The C₄ pathway is found in most of the warm region-grasses and some sedges with a range of δ¹³C<sub>diet</sub> values from -14 to -10‰ (Cerling 1999). The abundance of C₃/C₄ vegetation in an ecosystem is governed by many factors, including the mean annual temperature (MAT), mean annual precipitation (MAP), and partial pressure of carbon dioxide in the atmosphere (pCO₂) (Kohn 2010; Waseem et al. 2020b). The comparatively cooler areas (MAT <25°C) with precipitation higher than 1–2 m/year show a dominance of C₃ vegetation while warmer areas (MAT >25°C) with drier conditions (MAP <1–2 m/year) show a dominance of C₄ vegetation.
grasses (Nelson 2007). Furthermore, the C₄ pathway can work efficiently under lower pCO₂ and lower humidity levels compared to C₃ pathway (Medrano and Flexas 2000; Bellasio et al. 2018) due to the fact that carbon is fixed twice in C₄ pathway as compared to C₃ pathway (Kohn and Cerling 2002). The δ¹³C values of plants are governed by other factors such as low light intensity, saline soil, water availability, and local microhabitat conditions (Ehleringer et al. 1987; Codron et al. 2005; Bibi 2007).

These averages and ranges in δ¹³C value for modern plants were ~1.3‰ higher during the middle Miocene in fossil C₃ and C₄ plants, due largely to the effect of fossil fuel burning in decreasing δ¹³C₀₂ of the atmosphere (see Tippel et al. 2010). Thus, we use the δ¹³C₀₂ (~5.2‰) as in Tippel et al. (2010) for pre-10 Ma time span. The third photosynthetic pathway, crassulacean acid metabolism (CAM), is found in succulent plants which are mostly present in deserts or ephemiphytic plants in closed forests (Passey et al. 2005; Andrade et al. 2007) and were probably negligible in the Siwaliks.

Upon feeding on plants, the carbon is incorporated in the herbivores tooth enamel during the period of tooth formation. When carbon isotopes are incorporated in the enamel of herbivores, δ¹³C enamel values are fractionated by an enrichment factor (e_{enamel-diet}):

\[ e_{enamel-diet} = \frac{a_{enamel-diet} - 1}{1000} \times 1000 \]  

where:

\[ a_{enamel-diet} = \frac{d_{enamel} + 1000}{d_{diet} + 1000} \]

Thus, middle Miocene animals consuming C₃ vegetation outside of rainforests and deserts with δ¹³C_{diet} values from ~28.1 to -23.1‰ should show δ¹³C_{enamel} values ranging from about ~14 to -9‰, whereas middle Miocene animals feeding on C₄ vegetation should show δ¹³C_{enamel} values ranging from ~2 to ~2‰. The values between ~9 to ~2‰ are considered to reflect mixed feeding on C₃/C₄ vegetation (MacFadden and Cerling 1996). In this study, we assume that δ¹³C_{enamel} values ≤-16.1‰ denote browsing under closed-canopy forests, ~16 to -14‰ for sub-canopy browsing, ~14 to -12‰ for browsing in forests, and ~12 to -8‰ for browsing in woodlands (Cerling and Harris 1999; Domingo et al. 2012). The δ¹³C_{enamel} between ~8 to -3‰ will be considered as mixed feeding on C₃/C₄ vegetation while values >-2‰ can be interpreted as grazing on dominantly C₄ grasses (Cerling et al. 1997a, b, 2004; Kohn 2010). However, e_{enamel-diet} does not solely depend upon one factor. Tejada-Lara et al. (2018) argue that a single value of e_{enamel-diet} of ~14.1‰ does not account for all the mammals from monkeys to elephants by overlooking the other metabolic and physiological effect on carbon fractionation. For example, different mammals show different e_{enamel-diet} depending on body mass and digestive physiology (Tejada-Lara et al. 2018). Combining all factors, we assume an average e_{enamel-diet} of ~14.1‰ for large-bodied rhinocerotids and deinotheres and ~13.1‰ for suids according to Codron et al. (2011) and Tejada-Lara et al. (2018).

Oxygen enters an animal’s body in three main ways: (i) from drinking water, (ii) water derived from food, and (iii) inhalation, and leaves the body through sweat, exhalation, and urination (Sponheimer and Lee-Thorp 1999; Koch 2007; Sánchez 2005; Blumenthal et al. 2017, 2018). Of these sources, the majority of oxygen isotopes incorporated into enamel are from drinking water and diet, and δ¹⁸O values of local meteoric water (δ¹⁸O_{water}) are affected by environmental temperature, amount of precipitation, latitude, and altitude (Dansgaard 1964). The general trend of δ¹⁸O_{enamel} values is set by the ecology as the animals living under closed habitat and in humid conditions (forests) tend to show lower δ¹⁸O_{enamel} values compared to the animals living in open and arid (savannah or grasslands) areas (Feranec and MacFadden 2006). Levin et al. (2006) categorized the animals under two broad categories on the basis of their physiology and behavior. Evaporation sensitive (ES) taxa tend to obtain most of their water from their diet (leafy plants) and generally are ruminants (δ¹⁸O values of their enamel is correlated to the evaporation of leaf water), whereas evaporation insensitive (EI) taxa are mostly non-ruminants that depend on surface water for their body water requirements (δ¹⁸O_{enamel} is correlated to local drinking water sources). In this study, all the families sampled can be categorized as EI taxa on the basis of the physiology of the modern analogues, providing better insights into the δ¹⁸O value of palaeo-precipitation (Levin et al. 2006; Blumenthal et al. 2017; Faith 2018).

The fossil fauna of Chinji Formation includes Rhinocerotidae, Suidae, Tragulidae, Bovidae, Giraffidae, Deinotheriidae, Gomphotheriidae, and other taxa including Hominidae, Rodentia, and Carnivora (Pilgrim 1937; Khan et al. 2013; Barry et al. 2013; Flynn et al. 2016). We focus here on the paleodiet, paleoenvironments, and niche partitioning among Rhinocerotidae, Suidae, and Deinotheriidae from the middle Miocene of northern Pakistan. We selected these three EI taxa over non-EI taxa as δ¹⁸O_{enamel} value of EI taxa is correlated to their water source (Faith 2011, 2018). Furthermore, we selected one specialized browser (deinotheres), one taxon (i.e., Hyotherium pilgrimi) which disappeared from the Siwalik record during the late Miocene, and one taxon (rhinocerotid, Chilotherium intermedium) which shifted towards a C₄ diet during the late Miocene (Badgley et al. 2008; Morgan et al. 2009) to evaluate whether these taxa ate C₃ or C₄ plants during the middle Miocene time span in the Siwalik sub-Group of Pakistan.

The rhinocerotid, Chilotherium intermedium, was widespread in the Siwalik sub-Group of India and Pakistan, and existed up to the late Miocene (~7 Ma) in China. This species is characterized by low-crowned cheek teeth (brachydonty) with thin enamel folding and less complexity, indicating a diet composed of soft and less-gritty vegetation, preferably leaves of plants and trees (Khan 2009). C. intermedium was comparatively smaller (height 1.5 m and weight 1–2 tons) than modern black rhinos (Diceros bicornis) (height up to 1.8 m and weight up to 2.2 tons) (Decher 1999; Deng 2005).

Deinotherium pentapotamiae and D. indicum (Deinotheriidae) (body weight up to 11 tons and height up to 5 m, Larramendi 2016) was widespread in the Siwalik Group
and contemporaneous African sites for most of the middle Miocene, and it persisted as late as ~9 Ma in the Indian sub-continental region (Sarwar 1977). Two hooked shaped lower tusks were present in the species of Deinotherium with a flat skull, unlike other proboscideans, along with low-crowned teeth, indicating a browsing diet (Sarwar 1977; Shoshani and Tassy 2005).

_Hyotherium pilgrimi_ (Suidae) was a species with bunodont (rounded cusps) and brachyodont cheek teeth which existed during the middle Miocene time span in the Siwaliks. It had a range of body weight from 150–200 kg and height up to 1.1 m (Sorkin 2008). The mesowear and morphological studies (low hypsodonty index of 1.1, simple bunodont dentition with thick enamel) show that this species browsed on leaves and fruits (Barry et al. 2002; Tariq 2010; Aslam 2018). In general, all the middle Miocene mammals of the Siwalik sub-Group of Pakistan show morphological characters indicating browsing diets in forests and woodland settings, like their Indian counterparts (Patnaik et al. 2019).

**Institutional abbreviations.**—EB, Environmental Biology Laboratory, Institute of Zoology, University of the Punjab, Lahore, Pakistan.

**Other abbreviations.**—CAM, crassulacean acid metabolism; EI, evaporation insensitive; ES, evaporation sensitive; MAP, mean annual precipitation; MAT, mean annual temperature; SI, stable isotopes; V-PDB, Vienna Pee Dee Belemnite.

**Geological setting**

The middle Miocene is represented by the Chinji Formation in the Siwalik sub-Group of Pakistan (Fig. 1), which spans 14.2–11.2 Ma (Barry et al. 2002). The thickness and near-continuous deposition of the strata have permitted the development of a robust chronostratigraphic framework for the Chinji Formation on the basis of paleomagnetic dating. The samples for this study were collected from the Chinji-Nagri and Dhok Bun Ameer Khatoon sections (Fig. 1). The Chinji-Nagri section (Fig. 1B, C) is present in Chakwal District, Punjab (32°39’N, 72°22’E), whereas the Dhok Bun Ameer Khatoon section (DBAK) (Fig. 1B, D) is located 50 km north of Chinji Village (32°47’N, 72°55’E). The boundary between Chinji and Nagri formations can be placed at 11.2 Ma (Fig. 1A), whereas Suidae, Rhinocerotidae, and Deinotheriidae returned average values of $\delta^{13}$Cenamel of -11.2 ± 1.4‰ (n = 10), -12.3 ± 0.8‰ (n = 10), and -12.5 ± 1.3‰ (n = 10), respectively (Table 1). Normality tests indicate that data were normally distributed. One-way ANOVA along with the post-hoc (Tukey’s HSD) test show no significant differences in $\delta^{13}$C values between Rhinocerotidae and Deinotheriidae (p = 0.061), whereas Suidae showed significant differences compared to Deinotheriidae (p = 0.039) and Rhinocerotidae (p = 0.041).

**Results**

**Stable Carbon Isotopes.**—The average $\delta^{13}$Cenamel across all the families was found to be -12.2 ± 2.2‰. The Suidae, Rhinocerotidae, and Deinotheriidae returned average values of $\delta^{13}$Cenamel of -11.2 ± 1.4‰ (n = 10), -12.3 ± 0.8‰ (n = 10), and -12.5 ± 1.3‰ (n = 10), respectively (Table 1). Normality tests indicate that data were normally distributed. One-way ANOVA along with the post-hoc (Tukey’s HSD) test show no significant differences in $\delta^{13}$C values between Rhinocerotidae and Deinotheriidae (p = 0.061), whereas Suidae showed significant differences compared to Deinotheriidae (p = 0.039) and Rhinocerotidae (p = 0.041).

**Stable Oxygen Isotopes.**—The average $\delta^{18}$Oenamel (V-PDB) across all the families was -9.9 ± 3‰, wherein Suidae, Rhinocerotidae, and Deinotheriidae yielded average values for $\delta^{18}$Oenamel of -8.2 ± 1.2‰ (n = 10), -11.4 ± 1.8‰ (n = 10), and -10.4 ± 1.7‰ (n = 10), respectively (Table 1). The data were normally distributed. One-way ANOVA along with the post-hoc test indicated that the $\delta^{18}$Oenamel of suids is significantly different from the $\delta^{18}$Oenamel of rhinos and deinotheres (p = 0.041) (Table 1, Fig. 2).
Discussion

The average $\delta^{13}C_{\text{enamel}}$ value of middle Miocene mammals (-12.2 ± 2‰) in our data overall indicates a diet composed of $C_3$ vegetation, which was likely present in the form of forests and woodlands (Kohn and Cerling 2002; Kohn 2010). Patnaik et al. (2019) report the range of $\delta^{13}C_{\text{enamel}}$ for large herbivores from -13.9 to -11.6‰ for middle Miocene Siwalik sub-Group of India, which matches our results. The lowest $\delta^{13}C_{\text{enamel}}$ value in our results (-13.9‰) was recorded at ~13 Ma interval, whereas the highest value (-9.6‰) was recorded at ~12 Ma (Fig. 2, Table 1). This shows that the lower Chinji Formation was dominated by $C_3$ vegetation, likely present in the form of forestland, whereas the upper Chinji was dominated by $C_3$ open woodlands (Kohn and Cerling 2002; Kohn 2010). Thus, vegetation and habitat appear to have changed towards more...
open ecological settings in the upper Chinji Formation. The shift to open woodland is also apparent in the suid $\delta^{13}$C enamel results, which return an estimate for suid diet of -22.7‰, assuming $e_{\text{enamel-diet}} = +13.1$‰. (Kohn 2010; Cerling et al. 2015; Table 1). Moreover, the possibility of consumption of C3 grasses cannot be ruled out as extant suids in eastern Africa also consume C3 grasses but show a low-crowned dental morphology (Harris and Cerling 2002; Souron 2017).

These findings are supported by evidence presented in Quade and Cerling (1995), who reported $\delta^{13}$C paleosol carbonate values from Kamlial and Chinji formations ranging from -12.9 to -9.5‰, indicating a forestland and woodland environment. Further afield, Quade et al. (1995b) from Pasalar, Turkey, and Uno et al. (2016) from eastern Africa report the $\delta^{13}$C values from paleosol and unguulate enamel, respectively. Both studies report a dominance of C3 vegetation with very little or no C4 vegetation during the middle Miocene time span.

The higher $\delta^{13}$C enamel values in suids compared to rhinocerotids and deinotheres indicate that this family preferred more open areas for their feeding or browsed on a leafy vegetation. Tariq (2010) reports a frugivorous component to diets in suids on the basis of mesowear analysis. The mesowear analysis coupled with the morphological features of suid teeth (bunodont teeth with thick enamel) is in agreement with the hypothesis, based upon our $\delta^{13}$C enamel data, that suids of middle Miocene utilized C3 plants in open habitats, including fruits, as their dietary resource and inhabited relatively more open areas (Tariq 2010; Aslam 2018; Waseem et al. 2020b).

On the basis of average $\delta^{13}$C enamel and $\delta^{18}$O enamel values, we may hypothesize that middle Miocene climate was sub-humid and animals drank from shaded water holes or rivers (Sanyal et al. 2010; Tada et al. 2016). If we use the equation for MAP calculation on the basis of $\delta^{13}$C diet following Kohn (2010), we can say that in middle Miocene of the Siwalik sub-Group of Pakistan MAP was lower than ~700 mm/year (see Kohn 2010: fig. 3). The average value of $\delta^{18}$O enamel (-9.9 ± 3‰) of all non-ruminant taxa is low, indicating sub-humid conditions (Fig. 2B) during the middle Miocene time span in the Siwalik sub-Group of Pakistan.

### Table 1. $\delta^{13}$C enamel and $\delta^{18}$O enamel values for thirty enamel samples of fossil mammals from the middle Miocene Siwaliks of Pakistan. The ages of the samples have been calibrated according to Gradstein et al. (2012). Abbreviations: l, left; M/m, upper/lower molar; PM, upper premolar; r, right; V-PDB, Vienna Pee Dee Belemnite.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Family</th>
<th>Species</th>
<th>Horizon</th>
<th>Type of tooth</th>
<th>Age (Ma)</th>
<th>$\delta^{13}$C enamel V-PDB (‰)</th>
<th>$\delta^{18}$O enamel V-PDB (‰)</th>
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</table>
which is consistent with Indian contemporaneous sites (Patnaik et al. 2019). The low δ¹⁸OENAMEL values also probably indicate that in the middle Miocene, animals drank from relatively unevaporated water holes or rivers.

The δ¹⁸OENAMEL and δ¹³CENAMEL values increase gradually from ~14 Ma to ~11 Ma (Fig. 2A, B), culminating in more open environments supporting a woodland or perhaps minor C₄ vegetation (0–18% cover). However, the Hysterotherium pilgrimi samples show higher δ¹³CENAMEL values in our data which may also imply that this response may be species specific. Even if we consider the values as species specific, the open ecology towards the end of middle Miocene is still supported as we consider that the studied suid species occupied more open areas similar to the late Miocene suids. However, suids in the late Miocene showed feeding in both open and closed environments (Nelson 2005, 2007).

Niche partitioning among middle Miocene mammals

The studied middle Miocene mammals show some degree of niche partitioning (Fig. 3). The deinotheres and rhinocerotids from middle Miocene of Indian Siwaliks show an average δ¹³CENAMEL values of -12.5‰ and -12.8‰, respectively, very close to the ranges in our results for these taxa (Patnaik et al. 2019), pointing to overall similar ecological and dietary preferences. The suids (average δ¹³CENAMEL = -11.2 ± 1.4‰) inhabited a woodland/forestland setting where they fed on C₃ vegetation consisting of plants, including fruits. They may have drunk from less shaded water holes as represented by their elevated δ¹⁸OENAMEL values (-8.1‰) compared to the other middle Miocene taxa (Fig. 2B, C). The rhinocerotids and deinotheres show similarly low (p >0.05) δ¹³C values (-12.2‰ and -12.4‰, respectively), indicating that both families inhabited a forestland setting and browsed from understory vegetation (Fig. 2A, C), as reported by Patnaik et al. (2019) for contemporaneous Indian sites. However, the δ¹⁸OENAMEL values significantly differ between these two families (-11.3 ± 1.8‰ and -10.4 ± 1.7‰; Fig. 2B). Heissig (1972, 2003) reports that middle Miocene rhinocerotids of Pakistan preferred a habitat close to water bodies and Biasatti et al. (2018) report the same behavior for middle Miocene rhinocerotids of China. Thus, we may assume that middle Miocene rhinocerotids lived in habitats close to water, resulting in the lowest δ¹⁸OENAMEL values among all the middle Miocene taxa analyzed in this study. Deinotheres preferred a forested habitat where they had access to water but they did not live...
very close to the water bodies like rhinocerotids as indicated by the δ¹⁸O enamel values (Fig. 3).

**Conclusions**

We analyzed thirty specimens belonging to three families of middle Miocene Siwalik sub-Group of Pakistan. On the basis of our results, we conclude that these middle Miocene species inhabited dense forests and woodlands where they fed on the C₃ vegetation and drank from shaded water resources like ponds or rivers which were frequently refilled by precipitation (Fig. 3). We did not find clear evidence of significant C₄ vegetation in their diet. The δ¹⁸O enamel results reveal that the environment was more humid at ~14 Ma and became less humid towards the upper Chinji Formation (12–11.2 Ma). In general, the middle Miocene Siwaliks of Pakistan were dominated by forests and woodlands with precipitation supporting a more evergreen ecology as in the middle Miocene of eastern Africa, India, and Nepal and present-day vegetation of Myanmar (Quade et al. 1995a, b; Uno et al. 2016).

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