Ancient feeding ecology inferred from stable isotopic evidence from fossil horses in South America over the past 3 Ma
José L Prado¹, Begoña Sánchez² and María T Alberdi²*

Abstract

Background: Stable isotope ratios (¹³C/¹²C and ¹⁸O/¹⁶O) in fossil teeth and bone provide key archives for understanding the ecology of extinct horses during the Plio-Pleistocene in South America; however, what happened in areas of sympatry between Equus (Amerhippus) and Hippidion is less understood.

Results: Here, we use stable carbon and oxygen isotopes preserved in 67 fossil tooth and bone samples for seven species of horses from 25 different localities to document the magnitude of the dietary shifts of horses and ancient floral change during the Plio-Pleistocene. Dietary reconstructions inferred from stable isotopes of both genera of horses present in South America document dietary separation and environmental changes in ancient ecosystems, including C₃/C₄ transitions. Stable isotope data demonstrate changes in C₄ grass consumption, interspecies dietary partitioning and variation in isotopic niche breadth of mixed feeders with latitudinal gradient.

Conclusions: The data for Hippidion indicate a preference varying from C₃ plants to mixed C₃-C₄ plants in their diet. Equus (Amerhippus) shows three different patterns of dietary partitioning Equus (A) neogeus from the province of Buenos Aires indicate a preference for C₃ plants in the diet. Equus (A) andium from Ecuador and Equus (A) insulatus from Bolivia show a preference for to a diet of mixed C₃-C₄ plants, while Equus (A) santeelenae from La Carolina (sea level of Ecuador) and Brazil are mostly C₄ feeders. These results confirm that ancient feeding ecology cannot always be inferred from dental morphology. While the carbon isotope composition of horses skeletal material decreased as latitude increased, we found evidence of boundary between a mixed C₃/C₄ diet signal and a pure C₄ signal around 32° S and a change from a mixed diet signal to an exclusively C₄ signal around 35°S. We found that the horses living at high altitudes and at low to middle latitude still have a C₄ component in their diet, except the specimens from 4000 m, which have a pure C₃ diet. The change in altitudinal vegetation gradients during the Pleistocene is one of several possibilities to explain the C₄ dietary component in horses living at high altitudes. Other alternative explanations imply that the horses fed partially at lower altitudes.

Keywords: Stable Isotopes, C₄ plants, Mammals, horses, South America

Background

In South America, horses are represented by two groups: equidiforms and hippidiforms. Hippidion are characterized by a retracted nasal notch which has been interpreted as an adaptation to the presence of a proboscis and limbs with robust metapodials. The upper teeth present an elongate-oval protocone with simple enamel plication and lower teeth have a deep ectoflexid, penetrating the isthmus (Figure 1). On the other hand, in Equus (Amerhippus) a retracted nasal notch is not present and the metapodials are slender. The upper teeth present a triangular protocone and multiple internal posfossette plications. There are features that are common to both of these groups, such as differentiation into horses of both small and large body size, which are possibly a consequence of convergence due to adaptation to similar environments. The three species of hippidiforms are included within the genus Hippidion Owen, 1869
isotopes from food into their teeth and bone with an additional fractionation of ~12 to 14‰ [23,24]. Mammals feeding on C3 plants characteristically have δ13C values between -14 and -8‰, while animals that eat C4 tropical grasses have δ13C values between +2 and -2‰. A mixed-feeder would fall somewhere in between these two extremes [25,26]. Hence, the relative proportions of C3 and C4 vegetation in the diet of an animal can be determined by analyzing the δ13C value of its teeth and bones.

A number of previous studies have used the carbon and oxygen isotopic abundance of fossils and paleosols from South America to reconstruct the diets of extinct herbivores and the paleoenvironmental parameters of ancient terrestrial communities and ecosystems [27-32]. Carbon isotope data for horses from South America have been presented in several papers [8,33-35]. In 1999, MacFadden et al. [36] presented the ancient distributions and latitudinal gradients of C3 and C4 grasses based on isotopic data from New World Pleistocene horses. In addition, some papers have investigated the application of geochemical techniques in conjunction with morphological and dental wear data to reconstruct the feeding ecology and niche characterization of individual species [34,36,37].

All equid taxa from South America were sampled for teeth (n:29) and bone (n:38) stable carbon and oxygen isotopes (table 1 and 2). Additional data of thirty samples were taken from MacFadden et al. [36]. Together the data represent five species within the subgenus Equus (Amerhippus): E. (A.) andium, E. (A.) insulatus, E. (A.) neogeus, E. (A.) santaeelenae and E. (A.) lasallei [3]. The genus Hippidion includes three species: H. principale, H. devillei and H. saldiasi [2]. Some of these valid species have a wide geographical distribution whilst others, such as E. (A.) andium and E. (A.) insulatus, are restricted to the Andes region. In contrast, E. (A.) neogeus, E. (A.) santaeelenae and E. (A.) lasallei are found in the non-Andean tropical or subtropical regions of South America such as Argentina, Uruguay, Colombia, Brazil, and the coastal area of Ecuador (Figure 2). H. saldiasi is restricted to a particular habitat in southern Patagonia [38,39]; while H. principale and H. devillei come from different localities in South America, such as Tarija in Bolivia and the Pampa region in Argentina, that cover a broad range of altitudes from 10 to 4000 m. One restriction to our study is the chronological control of the sample. Most of the samples were collected from old museum collections in Ecuador, Bolivia and Argentina. These old collections were recovered without sufficient stratigraphic control. Anyway we considered this limitation may condition the interpretations about altitudinal and latitudinal gradients for fossil samples, but do not invalidate the suggested patterns.
In order to demonstrate how dietary resources were partitioned we divided the samples into 10 different groups taking into account the genus, as well as the age and the altitude of the corresponding deposit. Data for these groups and descriptive statistics are listed in table 3: (A) all *Hippidion*; (B) all *Equus* (*Amerhippus*); (C) *Hippidion* from the Late Pleistocene; (D) *Hippidion* from the Late Pliocene to Early Pleistocene; (E) *Equus* (*Amerhippus*) from the Late Pleistocene; (F) *Equus* (*Amerhippus*) from the Middle Pleistocene; (G) *Equus* (*Amerhippus*) from the plains; (H) *Equus* (*Amerhippus*) from the mountain corridor; (I) *Hippidion* from the plains; and (J) *Hippidion* from the mountain corridor.

### Table 1 Values of $\delta^{18}$O and $\delta^{13}$C of South American fossil *Hippidion*.

| Species name (age) | Specimen number | Skeletal tissue | Altitude m.asl | Locality (country) | Latitude | $\delta^{13}$C (CO$_3$) | $\delta^{18}$O (PO$_4$) | $\delta^{18}$O (CO$_3$) |
|--------------------|----------------|-----------------|----------------|--------------------|----------|------------------------|------------------------|------------------------|
| *H. devillei* (44 to 21 ka BP) | MACN 1621 | b | 1866 | Tarija (Bo) | 22S | -8.8 | 19.7 | 26.1 |
| *H. devillei* (44 to 21 ka BP) | MACN 1516 | t | 1866 | Tarija (Bo) | 22S | -10.8 | 19.3 | 28.7 |
| *H. devillei* (Late Pliocene to Early Pleistocene) | MACN 5361 | b | 4000 | Uquía (Ar) | 23S | -8.8 | 21.5 | 29.8 |
| *H. devillei* (Late Pliocene) | MACN 5361 | t | 4000 | Uquía (Ar) | 23S | -9.9 | 16.8 | 25.3 |
| *H. devillei* (Early Pleistocene) | MACN 5364 | b | 4000 | Uquía (Ar) | 23S | -10.3 | 16.0 | 25.9 |
| *H. devillei* (Late Pliocene) | MACN 2140 | t | 10 | Olivos (Ar) | 35S | -9.8 | 21.5 | 29.8 |
| *H. devillei* (Early Pleistocene) | MACN 2138 | t | 10 | Olivos (Ar) | 35S | -11.7 | 20.2 | 28.8 |
| *H. devillei* (Early Pleistocene) | MACN 2155 | b | 10 | Olivos (Ar) | 35S | -10.2 | 19.7 | 27.7 |
| *H. principale* (Late Pliocene) | MLP 6-19 | t | 10 | Paraná (Ar) | 32S | -8.4 | 19.4 | 28.9 |
| *H. principale* (Late Pliocene) | MLP 6-19 | b | 10 | Paraná (Ar) | 32S | -8.7 | 19.1 | 29.3 |
| *H. principale* (Early Pliocene) | MLP 6-441 | e | 10 | Buenos Aires (Ar) | 35S | -12.9 | 24.2 | 33.0 |
| *H. principale* (Early Pliocene) | MLP 6-435 | e | 10 | Buenos Aires (Ar) | 35S | -11.2 | 20.9 | 30.7 |
| *H. principale* (28 to 10 ka BP) | MACN 10441 | e | 10 | Luján (Ar) | 35S | -10.6 | 21.2 | 30.2 |
| *H. principale* (28 to 10 ka BP) | MACN 10441 | d | 10 | Luján (Ar) | 35S | -12.1 | 20.9 | 30.7 |
| *H. principale* (28 to 10 ka BP) | MLP 6-364 | b | 10 | Luján (Ar) | 35S | -11.3 | 19.5 | 29.6 |
| *H. principale* (28 to 10 ka BP) | MACN 6092 | b | 10 | Luján (Ar) | 35S | -11.2 | 21.7 | 30.5 |
| *H. principale* (28 to 10 ka BP) | MACN 5667 | t | 10 | Rio Salado (Ar) | 36S | -12.0 | 19.8 | 28.8 |
| *H. principale* (28 to 10 ka BP) | MACN 5667 | b | 10 | Rio Salado (Ar) | 36S | -10.1 | 19.9 | 28.3 |
| *H. principale* (28 to 10 ka BP) | MACN 5056 | b | 10 | Rio Salado (Ar) | 36S | -8.2 | 20.2 | 30.1 |
| *H. principale* (28 to 10 ka BP) | MACN 1735 | t | 50 | Arroyo Tapalqué (Ar) | 38S | -9.8 | 20.2 | 29.2 |
| *H. principale* (28 to 10 ka BP) | MACN 5265 | b | 50 | Arroyo Tapalqué (Ar) | 38S | -9.6 | 20.1 | 29.1 |
| *H. principale* (28 to 10 ka BP) | MACN 1734 | t | 50 | Arroyo Tapalqué (Ar) | 38S | -9.4 | 22.1 | 31.5 |
| *H. principale* (28 to 10 ka BP) | s/s | b | 100 | Arroyo Tapalqué (Ar) | 38S | -11.6 | 19.5 | 30.8 |
| *H. principale* (28 to 10 ka BP) | MACN 9739 | b | 100 | Rio Quequén Salado (Ar) | 38S | -8.1 | 21.2 | 29.9 |
| *H. saldiasi* (12.8 to 11.5 ka BP) | MLP 81-VII-28-S | b | 200 | U. Esperanza (Ch) | 52S | -12.2 | 17.6 | 24.9 |

Samples with the same "specimen number" correspond to the same individuals. The bones correspond to the mandibular or maxillary remains that contained the teeth that were analyzed. Plio = Pliocene; Pl = Pleistocene; e = enamel; d = dentine; b = bone; t = tooth (enamel + dentine). Ar = Argentina; Bo = Bolivia; Br = Brazil; Ch = Chile. A = Andean; PL = Plain landscape. 1 Taken from MacFadden et al. [22], 2 taken from MacFadden and Shockey [27] and 3 taken from MacFadden et al. [20].

In order to demonstrate how dietary resources were partitioned we divided the samples into 10 different groups taking into account the genus, as well as the age and the altitude of the corresponding deposit. Data for these groups and descriptive statistics are listed in table 3: (A) all *Hippidion*; (B) all *Equus* (*Amerhippus*); (C) *Hippidion* from the Late Pleistocene; (D) *Hippidion* from the Late Pliocene to Early Pleistocene; (E) *Equus* (*Amerhippus*) from the Late Pleistocene; (F) *Equus* (*Amerhippus*) from the Middle Pleistocene; (G) *Equus* (*Amerhippus*) from the plains; (H) *Equus* (*Amerhippus*) from the mountain corridor; (I) *Hippidion* from the plains; and (J) *Hippidion* from the mountain corridor.

### Methods

#### Materials

Fossil samples were collected from specimens stored at the following institutions in Argentina: Museo de La Plata, Museo Argentino de Ciencias Naturales “Bernardino Rivadavia” in Buenos Aires and Museo de Ciencias Naturales y Antropológicas “Juan Cornelio Moyano” in Mendoza. Museo de la Escuela Politécnica Nacional of Quito, in Ecuador. The museum specimen number, locality, country, age, skeletal tissue (enamel, bone and dentine) and the altitudinal and latitudinal distribution of each sample are given in Tables 1 and 2. We analyzed 26 samples of *Hippidion* and 41 carbon and oxygen.
Table 2 Values of $\delta^{18}$O and $\delta^{13}$C of South American fossil Equus (Amerhippus).

| Species name (age) | Specimen number | Skeletal number | Altitude (m. asl.) | Locality (country) | Latitude | $d^{13}$C (CO$_3$) | $d^{18}$O (PO$_4$) | $d^{18}$O (CO$_3$) |
|-------------------|-----------------|-----------------|-------------------|-------------------|---------|-----------------|------------------|------------------|
| Equus sp. (Late Pl)* | UCMP.38100 | e | 440 | La Venta (Co) | 3N | -0.7 | 29.9 |
| Equus sp. (Late Pl)* | ING 184268 | e | 440 | La Venta (Co) | 3N | -2.2 | 32.4 |
| E. insulatus (Middle Pl) | V.543 | b | 2500 | Rio Chiche (Ec) | 0 | -5.7 | 13.6 | 23.5 |
| E. insulatus (Middle Pl) | V.542 | b | 2500 | Rio Chiche (Ec) | 0 | -4.8 | 16.7 | 25.1 |
| E. insulatus (Middle Pl) | V.542 | b | 2500 | Rio Chiche (Ec) | 0 | -3.2 | 15.7 | 24.6 |
| E. insulatus (Middle Pl) | V.544 | b | 2500 | Rio Chiche (Ec) | 0 | -5.8 | 15.5 | 24.3 |
| E. andium (26 to 19 ka BP) | V.455 | b | 2500 | Rio Chiche (Ec) | 0 | -5.5 | 15.0 | 24.4 |
| E. andium (26 to 19 ka BP) | V.2495 | b | 2778 | Alangasi (Ec) | 0 | -6.9 | 13.1 | 23.3 |
| E. andium (26 to 19 ka BP) | V.2417 | b | 2778 | Alangasi (Ec) | 0 | -6.3 | 14.8 | 23.0 |
| E. santaelenae (12 to 8 ka BP) | V.3037 | d | 0 | La Carolina (Ec) | 2S | 9.2 | 22.1 | 34.9 |
| E. santaelenae (12 to 8 ka BP) | V.3037 | e | 0 | La Carolina (Ec) | 2S | -1.5 | 21.3 | 32.0 |
| E. santaelenae (12 to 8 ka BP) | V.3037 | b | 0 | La Carolina (Ec) | 2S | 1.8 | 23.2 | 32.7 |
| E. santaelenae (12 to 8 ka BP) | V.69 | b | 0 | La Carolina (Ec) | 2S | 5.4 | 33.6 |
| E. santaelenae (26 to 19 ka BP) * | FAM 131869 | e | 0 | Salinas Oil Field (Ec) | 3S | -0.8 | 32.6 |
| E. santaelenae (26 to 19 ka BP) * | FAM 131869 | e | 0 | Salinas Oil Field (Ec) | 3S | 3.0 | 25.5 |
| E. insulatus (44 to 21 ka BP) | MACN 1501A | b | 1866 | Tarija (Bo) | 22S | -5.8 | 17.4 | 25.6 |
| E. insulatus (44 to 21 ka BP) | MACN 1501B | t | 1866 | Tarija (Bo) | 22S | -5.0 | 20.9 | 26.2 |
| E. insulatus (44 to 21 ka BP) | MACN 1509 | t | 1866 | Tarija (Bo) | 22S | -8.3 | 20.0 | 28.3 |
| E. insulatus (44 to 21 ka BP) | MACN 1508 | b | 1866 | Tarija (Bo) | 22S | -4.8 | 25.2 | 25.2 |
| E. insulatus (44 to 21 ka BP) | UF.uncatalogued | e | 1866 | Tarija (Bo) | 22S | -2.9 | 24.4 | 24.4 |
| E. insulatus (44 to 21 ka BP) | UF.uncatalogued | e | 1866 | Tarija (Bo) | 22S | -2.6 | 25.8 | 25.8 |
| E. insulatus (44 to 21 ka BP) | UF.uncatalogued | e | 1866 | Tarija (Bo) | 22S | -2.3 | 26.4 | 26.4 |
| E. insulatus (44 to 21 ka BP) | UF.90750 | e | 1866 | Tarija (Bo) | 22S | -2.9 | 26.2 | 26.2 |
| E. insulatus (44 to 21 ka BP) | UF.90895 | e | 1866 | Tarija (Bo) | 22S | -3.5 | 23.5 | 23.5 |
| E. insulatus (44 to 21 ka BP) | UF.90653 | e | 1866 | Tarija (Bo) | 22S | -4.1 | 26.8 | 26.8 |
| E. insulatus (44 to 21 ka BP) | UF.90764 | e | 1866 | Tarija (Bo) | 22S | -4.1 | 24.2 | 24.2 |
| E. insulatus (44 to 21 ka BP) | UF.91972 | e | 1866 | Tarija (Bo) | 22S | -2.7 | 24.9 | 24.9 |
| E. neogeus (28 to 10 ka BP) | MCNAM-PV-83 | e | 200 | Ouroilandia (Br) | 12S | 1.1 | 29.5 |
| E. neogeus (28 to 10 ka BP) | UCMP.5219X.9-91 | e | 10 | Esperanza, Santa Fé (Ar) | 32S | -0.8 | 30.8 | 30.8 |
| E. neogeus (Middle Pl) | AMNH.11154 | e | 10 | Buenos Aires (Ar) | 3S | -10.7 | 30.8 | 30.8 |
| E. neogeus (Middle Pl) | AMNH.11154 | e | 10 | Buenos Aires (Ar) | 3S | -10.3 | 30.6 | 30.6 |
| E. neogeus (Middle Pl) | MLP.91.V1.S-1 | e | 50 | Magdalena (Ar) | 3S | -10.6 | 30.3 | 30.3 |
| E. neogeus (28 to 10 ka BP) | MACN 11636 | b | 10 | Luján (Ar) | 3S | -11.7 | 21.2 | 30.4 |
| E. neogeus (28 to 10 ka BP) | MLP 6-402 | b | 10 | Luján (Ar) | 3S | -11.2 | 20.1 | 30.6 |

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isotope composition samples of *Equus (Amerhippus)*. Additional 32 samples values (Table 1 and 2) were taken from MacFadden et al. [31,33,36] and MacFadden and Shockey [34].

**Pre-treatment of the samples**

The samples were finely ground in an agate mortar. The chemical pre-treatment of the samples was carried out as described in Koch et al. [58] in order to eliminate secondary carbonate. About 40-50 mg of powdered enamel and bone samples were soaked in 2% NaOCl for three days at room temperature to oxidize organic matter. Residues were rinsed and centrifuged five times with de-ionized water, and then treated with buffered 1M acetic acid for one day to remove diagenetic carbonates. Pre-treatment of the enamel was slightly different because samples were soaked in 2% NaOCl for only one day.

**Analysis of the samples**

Carbon dioxide was obtained by reacting about 40-50 mg of the treated powder with 100% H₃PO₄ for five hours at 50°C. The carbon dioxide was then isolated cryogenically in a vacuum line. Results are reported as δ = ([R_sample / R_standard] - 1) × 1000, where R = ¹³C/¹²C or ¹⁸O/¹⁶O, and the standards are PDB for carbon and V-SMOW for oxygen. We have applied the data corrections for calcite from Koch et al. [59] to calculate the magnitude of the oxygen isotopic fractionation between apatite CO₂ and H₃PO₄ at 50°C. The analytical variation for repeated analyses was 0.1‰ for δ¹³C and 0.2‰ for δ¹⁸O. For the analysis of phosphate we followed the chemical treatment procedure described by Tudge [60], which resulted in the precipitation of the phosphate ions in the form of BiPO₄. CO₂ was obtained by reacting BiPO₄ with BrF₅ as described by Longinelli [61]. All the samples were run in duplicate and the reported results are the mean of at least two consistent results. The analytical precision for repeated analyses was 0.2‰.

We performed both parametric (t-test) and non-parametric (Wilcoxon Signed-Rank) statistical tests to evaluate δ¹³C and δ¹⁸O differences in Middle and Late Pleistocene populations. SPSS 15.0 software was used for the statistical analysis.

| Sample Details | Location | Age | Sex | Initial | Final | δ¹³C | δ¹⁸O |
|----------------|----------|-----|-----|---------|-------|------|------|
| *Equus neogeus* (Middle Pl) MLP s/s t 10 | Cant. Vial. Prov.(Ar) | 35S | -7.5 | 21.2 | 30.5 |
| *Equus neogeus* (Middle Pl) MLP s/s b 10 | Cant. Vial. Prov.(Ar) | 35S | -11.5 | 20.8 | 32.1 |

Samples with the same "specimen number" correspond to the same individuals. The bones correspond to the mandibular or maxillary remains that contained the teeth that were analyzed. Abbreviations as in Table 1. * taken from MacFadden et al. [25].

Table 2 Values of δ¹⁸O and δ¹³C of South American fossil *Equus (Amerhippus).* (Continued)
Results
Preservation state of the enamel, dentine and bone in the specimens analyzed

We checked the diagenetic alteration between the different skeletal tissues under the assumption that primary values were similar between different skeletal tissues from one individual. The phosphate oxygen isotope composition is usually considered to be more robust against diagenetic alteration than the carbonate oxygen at least if no bacteria are involved during the alteration processes. We measured both δ^{18}O_{CO3} and δ^{18}O_{PO4} values on the same specimens and obtained a regression between both, and compared those with known isotope equilibrium-relationships for biogenic apatite of modern bones and teeth [40,41]. If samples fall in the range of modern biogenic apatite this would argue for a preservation of primary δ^{18}O_{CO3} and δ^{18}O_{PO4} values, and thus likely also δ^{13}C values as they are less easily altered than δ^{18}O_{CO3} values. This was taken as an indicator that even the bone and dentine samples may be reasonably well-preserved and can be interpreted to infer feeding ecology and habitat use of these ancient horses.

Several authors [42-44] suggest a high correlation between these two phases in some European and North American equids. In South America, the equation obtained for Equus(Amerhippus) from Argentina [45] was: δ^{18}O_{CO3} = 16.74 δ^{18}O_{PO4} + 0.64; R^2 = 0.91. More recently, Sánchez et al. [8,32] also found that a
significant relationship existed between the $\delta^{18}$O results in the carbonate and phosphate apatite phases (enamel, dentine, and bone) when they analyzed the oxygen isotopic composition of gomphotheres from several South American localities. The new data shows that $\delta^{18}$O PO$_4$ and $\delta^{18}$O CO$_3$ are highly correlated, with the latter being about 8.6‰ more positive than the former.

We analyzed the $\delta^{18}$O PO$_4$ and $\delta^{18}$O CO$_3$ of the 26 samples for Hippidion and 41 samples for Equus (Amerhippus) in this way. The results of $\delta^{18}$O PO$_4$ versus V-SMOW standard are reported in tables 1 and 2. The analytical variation for repeated analysis was 0.2‰. Each pair of $\delta^{18}$O values of teeth and bone belonged to the same individual, generally from the jaw or maxilla, the correlation being: $\delta^{18}$O PO$_4$ = $0.9452 \times \delta^{18}$O CO$_3$ - 10.456; $R^2 = 0.80$. We also calculated the correlation between pairs of $\delta^{18}$O PO$_4$ - $\delta^{18}$O CO$_3$ values for the enamel, dentine, and bone, from the same individual. The results are plotted in Figure 3. The correlations between the different PO$_4$-CO$_3$ pairs are:

- Enamel $\delta^{18}$O PO$_4$ = 2.3371 $\delta^{18}$O CO$_3$ - 19.095 $R^2 = 0.97$;
- Dentine $\delta^{18}$O PO$_4$ = 1.2999 $\delta^{18}$O CO$_3$ - 3.4034 $R^2 = 0.839$;
- and Bone $\delta^{18}$O PO$_4$ = 1.1709 $\delta^{18}$O CO$_3$ - 6.2038 $R^2 = 0.88$.

In the first case it appears that the sample might have been modified by diagenesis because the carbonate results among the three skeletal phases (enamel, dentine, and bone) are different. In fact, the range of variation is 2.6‰ in the $\delta^{18}$O PO$_4$ and 5.6‰ in the $\delta^{18}$O CO$_3$ for the samples from the same specimen. Results of $\delta^{13}$C obtained from the bone are similar to those obtained from the dentine and are, in general, equal or more negative than results obtained from the enamel. We observed the same pattern in the $\delta^{18}$O results as we did in the $\delta^{13}$C results. The range of variation between the three skeletal phases (enamel, dentine, and bone) was small and we suppose that they have not been significantly altered by diagenesis. For these reasons, we consider these $\delta^{13}$C values as representative of each group of horses.

### Dietary Partitioning

The carbon isotopic ratio of Equus (Amerhippus) and Hippidion remains indicate significant ecological differences (Figure 4). The Hippidion samples analyzed here are more homogeneous than the Equus (Amerhippus) samples, with a $\delta^{13}$C range between -12.9 and -8.0‰ (Table 1 and 2).

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**Table 3** Descriptive statistics for the eight groups of South American equids that were compared.

| Groups | n  | Mean $\delta^{13}$C (%) | SD   | Range       | n  | Mean $\delta^{18}$O (%) | SD   | Range       |
|--------|----|-------------------------|------|-------------|----|-------------------------|------|-------------|
| A      | 31 | -0.1                    | 1.39 | -12.9 to -6.6| 26 | 28.3                    | 2.20 | 24.2 to 31.5|
| B      | 68 | -5.2                    | 4.83 | -11.66 to 9.21| 68 | 28.5                    | 3.32 | 21.7 to 34.9|
| C      | 17 | -10.2                   | 1.39 | -12.22 to -8.08| 17 | 29.0                    | 2.17 | 24.2 to 31.5|
| D      | 14 | -10.0                   | 1.44 | -12.9 to -6.6| 9  | 27.0                    | 1.74 | 25.3 to 29.8|
| E      | 52 | -5.5                    | 5.43 | -11.66 to 9.21| 50 | 29.4                    | 3.17 | 21.7 to 34.9|
| F      | 16 | -4.3                    | 1.61 | -8.31 to -2.3| 16 | 25.3                    | 1.28 | 23.5 to 28.3|
| G      | 43 | -5.3                    | 5.93 | -11.66 to 9.21| 41 | 30.7                    | 1.48 | 27 to 34.9  |
| H      | 25 | -5.1                    | 1.85 | -9.86 to -2.3| 25 | 24.6                    | 1.51 | 21.7 to 28.3|
| I      | 12 | -9.8                    | 1.34 | -12.22 to -6.6| 8  | 25.8                    | 1.30 | 25.8 to 28.7|
| J      | 19 | -10.3                   | 1.42 | -8.08 to -12.90| 17 | 29.7                    | 1.00 | 29.7 to 31.5|

A: all Hippidion specimens; B: all Equus (Amerhippus) specimens; C: Hippidion from the Late Pleistocene; D: Hippidion from the Late Pliocene to Early Pleistocene; E: Equus (Amerhippus) from the Late Pleistocene; F: Equus (Amerhippus) from the Middle Pleistocene; G: Equus (Amerhippus) from the plains landscape; H: Equus (Amerhippus) from the mountain corridor; I: Hippidion from the plains landscape and J: Hippidion from the mountain corridor.

n: number of samples. SD: standard deviation.

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![Figure 3](http://www.biomedcentral.com/1472-6785/11/15)
All the species of *Hippidion* were almost exclusively C₃ feeders but some individuals from Bolivia and Argentina fall at the lower end of the mixed C₃/C₄ range. For instance, *H. principale* from the Eastern corridor (at sea level) and *H. devillei* from the Andes corridor yield similar δ¹³C values suggesting that they ate mainly C₃ plants. The same pattern of dietary partitioning was obtained when comparisons were made between the same taxa at different latitudes (between 22°S and 52°S). From the upper Pliocene (*H. devillei* from Uquía locality) to the lower Pleistocene (*H. principale*, from the province of Buenos Aires and *H. devillei* from the Tarija locality) the dietary partitioning remains similar. The same pattern in dietary partitioning is observed throughout the Middle to Late Pleistocene (Figure 5) showing a predominance of C₃ plants. Also, we did not find differences between *H. salдиasi* from the Ultima Esperanza in southern Patagonia and the other *Hippidion* species present at different localities across South America.

*Equus* species have predominantly been grazers, and as such, carbon isotopic values provide evidence of the C₃ and C₄ grasses. The carbon isotope data indicates that *Equus* (*Amerhippus*) shows three different patterns of dietary partitioning. Samples of *E. (A.) neogenus* from the province of Buenos Aires indicate a preference for C₃ plants in the diet. The samples from Ecuador and Bolivia [*E. (A.) andium* and *E. (A.) insulatus*] show a preference for a diet of mixed C₃-C₄ plants, while those from La Carolina (sea level of Ecuador), Bolivia, and Brazil are mostly C₄ feeders.

As mentioned before, a few outliers (e.g. δ¹³C values of 9.2; 6.1 and 5.4‰ from La Carolina) cannot be easily explained. These extremely high δ¹³C values (above 3‰) cannot be explained by consumption of C₄ vegetation, which should impart an upper limit of about 3‰. These outliers could be the result from one of several possibilities, such as individuals living in costal peninsula areas of Ecuador during the time in which C₄ grasses were abundant and may have produced δ¹³C values not observed in the modern ecosystem, or the sample presents taphonomic alteration. Specimen number V.3037 has a high variation between dentine, enamel, and bone (9.2; 1.5 and 6.1‰ respectively). MacFadden et al [36] obtained a δ¹³C value of 1.8‰ for the enamel of the same specimen but obtained 5.4‰ for the enamel of
specimen number v.68 from the same locality. A more definitive explanation for these outliers must await the analysis of additional samples from these regions.

The δ18O CO3 results of the horse remains differ according to altitudinal and latitudinal distribution. There is a clear difference in the δ18O values obtained from the populations from the lower plains and those from high elevations. The lowest δ18O CO3 values are found in high elevation species from Ecuador and Bolivia [E. (A.) insulatus and E. (A.) andium] and range from 21.3 to 28.3‰ with an average of around 25‰. The H. saldiasi samples from southern Patagonia are also included in this group, their low δ18O values being caused by the effects of altitude and latitude. The second group is mostly represented by E. (A.) neogeus, H. principale and H. devillei. These species come from the province of Buenos Aires, Brazil, and Bolivia. The distribution values range from 27.7 to 32.1‰ with an average of around 30‰. The highest values come from the Carolina Peninsula in Ecuador, with δ18O CO3 maximum of 34.9‰.

Discussion
Areas of sympathy
As might be expected from ecological theory, finer-scale preliminary results from this study suggest feeding and niche differentiation within coexisting horses. Hippidion and Equus (Amerhippus) are sympatric in the same stratigraphic level in two localities: Tarija (22 °S) in Bolivia and the Pampas in Argentina (38 °S). Data from both localities suggest some difference in dietary partitioning. In a previous study, MacFadden and Shockey [34] presented carbon isotopic results from Tarija herbivores that, based on dental morphology, span the spectrum from presumed browsers (e.g., tapirs) to presumed grazers (e.g., horses) coexisting in the same localities. They suggested that the horses clearly occupied different dietary niches and can be separated using the carbon isotopes and hypsodonty index. Hippidion is the least hypsodont taxon and has the most negative mean δ13C value, whereas the relatively hypsodont Equus has the most positive mean δ13C value. As has been shown in another article [36], Equus is usually among the most grazing adapted mammalian herbivore in Pleistocene terrestrial ecosystems. This position in the herbivore community is similar at Tarija, whereas Hippidion are more adapted to the browsing end of the spectrum and Equus are primarily C4 grazers based on their carbon isotopic signature.

Our data suggests that the range of δ13C values for H. devillei and H. principale from Tarija falls in the low end of the mixed C3/C4 range (-10.8 to -6.6‰), whereas E. (A.) insulatus falls in the higher end (-8.3 to -2.3‰). There is some overlap with at least the two values of -8.3 and -6.6‰ indicating some differences in isotopic niche, with H. devillei consuming less grass than E. (A.) insulatus at this place and time [36].

In the data from Arroyo Tapalque (38 °S), one of the localities in the Pampas, the range of δ13C values for H. principale (-11.6 to -9.3‰) and E. (A.) neogeus (-8.5 to -7.9‰) do not overlap, but both are close that they suggest the same pattern of dietary partitioning as in Tarija.

If we look at the morphology, these taxa are very different. Several previous papers based on cranial and limb morphology associate these differences with browsing and grazing diet preference [e.g. [46]]. In general, the teeth of Equus (Amerhippus) are more hypsodont than Hippidion, and the enamel patterns are more complicated in Equus (Amerhippus).

Another difference between sympatric species is body sizes. For instance, H. principale and E. (A.) neogeus are sympatric in the Pampas in Argentina. Both have a large body size, adapted to open habitat, but they differ in body mass (460 kg and 378 kg, respectively) [47]. The skulls of E. (A.) neogeus are big and show an enlarged preorbital and nasal region. The limb bones are large and robust, but more slender than in the other South American Equus species [3,4]. On the other hand, H. principale has a retracted nasal notch might signify some sort of proboscis. The skeleton is large and bulky, and the extremities are robust, mainly the metapodials and phalanges. It is the largest and strongest of the South American hippidiforms. These characteristics are classically associated with dietary and habitat preferences. The morphology indicated that H. principale may have been a browser but was able to live in open grasslands and that E. (A.) neogeus is the most hypsodont horse, and has the relatively straight muzzle characteristic of grazing horses [46].

The effect of latitude
Some of the most fundamental patterns in global biogeography are those that are structured by the latitudinal gradients that extend from pole to equator (Figure 6). The proportion of C3 to C4 grasses in most modern ecosystems rises with increasing latitude. C3 grasses are predominant (>90%) in high-latitude steppes and prairies, whereas C4 grasses are predominant (>70-90%) in most low-elevation, and equatorial grasslands. The transition between C3 and C4 dominance in grasslands occurs at about 40-45° latitude in the Northern Hemisphere [48-50]. Exceptions to this general rule include grasses at high-elevations or in climates with cool-growing seasons (e.g. the Mediterranean basin), where the grasses are predominantly C3 regardless of latitude [50-52], and the occasional C4 grass species that are found in Arctic regions [53].
MacFadden et al. [36] used the Pleistocene distribution of *Equus* (*Amerhippus*) in the Americas to present a general $\delta^{13}C$ gradient that seems to be symmetrical on either side of the Equator. They found that the isotopic transition between a full C4 signal and full C3 signal is observed at about 45°N in the northern hemisphere. Although there are considerably fewer data points in the southern hemisphere they [36] have found a signal of exclusively C3 feeders around 35°S (province of Buenos Aires). A similar pattern in the Southern Hemisphere was found by Sánchez et al. [32] in the distribution of gomphotheres in South America. Samples of gomphotheres from the province of Buenos Aires, at around 39°S latitude show a mean $\delta^{13}C$ value of -10.8‰, while samples from Chile (from several localities around 35° to 41°S) show a mean $\delta^{13}C$ value of -12.3‰. This fact would confirm the existence of a latitudinal gradient for the Southern Hemisphere, and places the transition between a full C3 signal and mixed C3/C4 signal around 35° to 41°S.

The new data for the genus *Equus* (*Amerhippus*) analyzed in this paper are in agreement with the $\delta^{13}C$ latitudinal pattern postulated by MacFadden et al. [36], while data for *Hippidion* also seem to follow a clear pattern. Samples of *Hippidion* show a boundary between an exclusively C3 to a mixed C3/C4 diet at Tarija (22°S), but in this case there is an effect of altitude combined with latitude. At the highest latitude specimens from Patagonia (52°S), the mean value is -12.2‰. The middle latitude samples, from the province of Buenos Aires (34°S), show $\delta^{13}C$ values that range between -12.9 and -8.1‰, while the lowest latitude specimens, from Tarija (22°S), show $\delta^{13}C$ values that range between -6.6 to -0.8‰.

### Altitudinal gradient

There are also significant differences between the $\delta^{18}O_{CO_3}$ values for the two genera, even when the range of $\delta^{18}O$ values for high altitude and equatorial samples are taken into account (Table 4). This demonstrates the effects of an altitudinal gradient. The *Hippidion* specimens from the Andes corridor (Bolivia and northern Argentina) and the *Equus* (*Amerhippus*) specimens from Ecuador and Bolivia show the lowest values (between 21.7 and 28.3‰). On the other hand, samples from around 500 m of altitude (Bolivia, Ecuador, Brazil, Peru, and Argentina) present values that correspond with more temperate conditions (27.7 to 32.1‰). The effects of low altitude and latitude on $\delta^{18}O_{CO_3}$ may also explain the higher value obtained for the samples from La Carolina, Ecuador (sea level, latitude 2°S), where samples showed $\delta^{18}O$ values ranging from 32 to 34.9‰.

We calculated a regression of $\delta^{18}O_{PO_4}$ values with altitude to quantify the effects of altitude. Bryant et al. [44] suggest a high correlation between $\delta^{18}O_{PO_4}$ and $\delta^{18}O_{CO_3}$ in Miocene North American equids. We found a good correlation between $\delta^{18}O_{PO_4}$ values and altitude. The equation obtained was: $\delta^{18}O_{PO_4} = -0.0016 \text{ altitude} + 20.47 \ (R^2 = 0.63)$. We obtained an altitudinal gradient of -0.16 $\delta$ unit/100 meters.

An important point concerns altitudinal gradients. It seems that the horses living at high altitudes, 1866 up to 2780 m above sea level, and at low to middle latitude

![Figure 6 Latitudinal distribution of $\delta^{13}C$ values for the two species of South American equids.](image)

**Table 4 Results of parametric (t-test) and non-parametric (Wilcoxon Signed-Rank) tests.**

| Variable | Comparison groups | t-test | Nonparametric |
|----------|------------------|-------|---------------|
| $\delta^{13}C$ | A vs. B | 3.6E-10 | 1.17E-06 |
| $\delta^{13}C$ | C vs. D | 0.97 | 0.73 |
| $\delta^{13}C$ | E vs. F | 0.12 | 0.33 |
| $\delta^{13}C$ | G vs. H | 0.15 | 0.15 |
| $\delta^{13}C$ | C vs. E | 2.2E-05 | 2.9E-04 |
| $\delta^{13}C$ | D vs. F | 3.1E-07 | 9.8E-04 |
| $\delta^{18}O$ | I vs. J | 0.17 | 0.04 |
| $\delta^{18}O$ | A vs. B | 0.85 | 0.93 |
| $\delta^{18}O$ | C vs. D | 0.01 | 0.02 |
| $\delta^{18}O$ | E vs. F | 0.08 | 0.18 |
| $\delta^{18}O$ | G vs. H | 1.4E-12 | 1.2E-05 |
| $\delta^{18}O$ | C vs. E | 0.52 | 0.46 |
| $\delta^{18}O$ | D vs. F | 0.08 | 0.09 |
| $\delta^{18}O$ | I vs. J | 0.01 | 0.03 |

Results of tests performed on twelve possible paired comparisons between two genera of South American equids. The definition of groups is the same as for table 3. *p*: significance level, *p* < 0.05 in bold.
(2° to 22°S) still have a C₄ component in their diet. This is not surprising at all given the distribution of modern C₄ plants in the central Andes. At present, three C₄ Amaranthaceae species occur at high elevations (>4000 m) where C₄ plants are rarely observed and the altitude record reported for any confirmed C₄ species worldwide is 4800 m for the grass *Muhlenbergia peruviana* [54]. However, this is not true for our specimens from 4000 m, which have a pure C₃ diet. In this central Andean region, a clear altitudinal vegetation gradient is present. Subparamo (2000-3000 m) presents mosaics where shrubs and small trees which alternate with grasslands; and Paramo proper (3000 - 4100 m), is dominated by grasslands and shows patches of woody species which occur only in sheltered locations and along water streams. This altitudinal vegetation gradient changed during the Pleistocene. The treeless vegetation above the upper forest line was most widespread during glacial times, whereas it was limited to small areas on mountain tops during interglacial times [55]. The fossil pollen records show that such oscillations in patterns of plant distribution were repeated many times during the Pleistocene Ice Ages [56]. During the Last Glacial Maximum, when atmospheric pCO₂ was reduced by some 50%, C₄ plants dominated the Paramo vegetation, while only the highest mountain tops were covered by C₃ grasses because of the low temperatures. Such small patches of C₄-rich vegetation are probably relics from the last ice age during which paramo vegetation was mainly composed of small tussocks and tufts of C₄ grasses [57].

The change in altitudinal vegetation gradients during the Pleistocene is one of several possibilities to explain the C₄ dietary component in horses living at high altitudes. Another alternative explanation might be that the horses partially fed at lower altitudes.

**Conclusions**

Based on modern analogues, Pleistocene horses are inferred to be grazers but none of the grazing horses were interpreted as consumers of only C₄ grasses. Our data shows that *Equus (Amerhippus)* had three different patterns of dietary partitioning. *E. (A.) neogeus* from the province Buenos Aires indicates a preference for C₃ plants. *E. (A.) andium* from Ecuador and *E. (A.) insulatus* from Bolivia show a preference in a mixed diet of C₃-C₄ plants, while *E. (A.) santaeleanae* from La Carolina (sea level of Ecuador) and Brazil are mostly C₄ feeders. These results confirm that ancient feeding ecology cannot always be inferred from dental morphology.

The record from South America suggests that *Hippidion* is in general a higher latitude taxon than *Equus (Amerhippus)*. The highest latitude occurrence of *Hippidion* appears to be in southern Bolivia, in contrary to *Equus (Amerhippus)* where the occurrences are further north, and alone goes a long way in explaining the isotopic differences.

The data for *Hippidion* indicates a preference for C₃ plants and mixed C₃-C₄ plants, but most of this data came from high altitude or latitude specimens. One possible, but unconfirmed explanation is that *Hippidion* were living in a “C₄ World” and were browsers as indicated by their morphology, but more southern individuals were living at latitudes high enough to support C₃ and C₄ grasses.

The current study demonstrates the utility of using wide-ranging fossil mammals to explore latitudinal gradients and patterns of C₃/C₄ grass distribution and continental palaeotemperature during the Pleistocene. The carbon isotope composition of horses decreased as latitude increased. In *Equus (Amerhippus)* we found a change in signal between a mixed C₃/C₄ diet and a pure C₄ diet around 32°S and a boundary between mixed diet and exclusively C₃ signals at 35°S.

We also found that the horses living at high altitudes and at low to middle latitudes still have a C₄ component in their diet, except for those specimens living at 4000 m, which have a pure C₃ diet. The change in altitudinal vegetation gradients during the Pleistocene is one of several possibilities to explain the C₄ dietary component in horses that lived at high altitudes. Another alternative explanation implies that the horses fed partially at lower altitudes.

**Abbreviations**

(MLP): Museo de La Plata; (MACN): Museo Argentino de Ciencias Naturales “Bernardino Rivadavia”, Buenos Aires; and (INCNAM): Museo de Ciencias Naturales y Antropológicas “Juan Cornelio Moyano”, Mendoza, in Argentina; and (MEPN): Museo de la Escuela Politécnica Nacional de Quito, in Ecuador.

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**Author details**

1INCUAPA, Universidad Nacional del Centro. Del Valle 5737. 87400JIWI Olavarria, Argentina. 2Museo Nacional de Ciencias Naturales, CSIC. José Gutiérrez Abascal, 2. 28006-Madrid, Spain.

**Authors’ contributions**

Conceived and designed the experiments: JLP MTA. Performed the experiments: BS. Analyzed the data: BS. Wrote the paper: JLP BS. Intellectual support and editorial input: JLP MTA. All authors read and approved the final manuscript.
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