Nitrogen isotopes in tooth enamel record diet and trophic level enrichment: results from a controlled feeding experiment

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ABSTRACT

Nitrogen isotope ratios ($\delta^{15}N$) are a well-established tool for investigating the dietary and trophic behavior of animals in terrestrial and marine food webs. To date, $\delta^{15}N$ values in fossils have primarily been measured in collagen extracted from bone or dentin, which is susceptible to degradation and rarely preserved in deep time (>100,000 years). In contrast, tooth enamel organic matter is protected from diagenetic alteration by the mineral structure of hydroxyapatite and thus can be preserved over geological time. However, due to the low nitrogen content (<0.01 %) of enamel, the measurement of its nitrogen isotopic composition has been prevented by the analytical limitations of traditional methods. Here, we present a novel application of the oxidation-denitrification method that allows measurement of $\delta^{15}N$ values in tooth enamel ($\delta^{15}N_{enamel}$). This method involves the oxidation of nitrogen in enamel-bound organic matter to nitrate followed by bacterial conversion of nitrate to N_2O and requires ≥100 times less nitrogen than traditional approaches. To demonstrate that $\delta^{15}N_{enamel}$ values record diet and trophic behavior, we conducted a controlled feeding experiment with rats and guinea pigs (n=36). We determined that nitrogen concentration in tooth enamel ($\bar{x}$ = 5.0 ± 1.0 nmol/mg) is sufficient for $\delta^{15}N_{enamel}$ analyses with ≥5 mg untreated enamel powder. The nitrogen isotope composition of enamel reflects diet with an enrichment ($\Delta^{15}N_{enamel\text{-}diet}$) of ca. 2–4 ‰. $\delta^{15}N_{enamel}$ values differ significantly between dietary groups and clearly records a shift from pre-experimental to experimental diet. The small sample size required (≤5 mg) by this method permits analyses of sample size-limited, diagenetically robust tooth enamel, and, as such it represents a promising new dietary proxy for reconstructing food webs and investigating the trophic ecology of extant and extinct taxa.

Keywords: nitrogen isotopes, tooth enamel, paleodiet, trophic level, rodents

1. INTRODUCTION

Paleontologists have long sought a reliable geochemical proxy for inferring the trophic position of fossil organisms. Multiple approaches, including trace element ratios (e.g., Sr/Ca, Ba/Ca) and traditional (e.g., C) and non-traditional (e.g., Ca, Sr, Mg, Zn) isotopic systems have been employed with varying degrees of success (Chu et al., 2006; Knudson et al., 2010; Heuser et al., 2011; Balter et al., 2012, 2019; Costas-Rodriguez et al., 2014; Martin et al. 2014, 2015, 2017, 2020; Jaouen et al., 2016, 2017, 2018; Sillen and Balter, 2017; Bourgon et al., 2020). While some non-traditional isotopic systems hold great promise, the physiological mechanisms (i.e. element cycling in the body) which drive fractionation in these systems are not yet well understood and thus their implementation as trophic proxies are still in their infancy.

In contrast, nitrogen isotope ratios (expressed as $\delta^{15}N$ values) are a well-established tool for investigating the dietary and trophic behavior of animals in terrestrial and marine food webs (e.g., Minagawa and Wada, 1984; Schoeninger and DeNiro, 1984; Ambrose and DeNiro 1986; Sealy et al., 1987; Hilderbrand et al., 1996; Roth and Hobson, 2000; Jenkins et al., 2001; Bocherens, 2015), and $\delta^{15}N$ values have been studied in a variety of natural and experimental settings (DeNiro and Epstein, 1981; Hare et al., 1991; Hobson et al., 1996; Sponheimer et al., 2003a, 2003b; Wolf et al., 2009). These studies show that, within well-constrained ecosystems, consumer tissues are enriched in ^{15}N relative to their diet (i.e., the tissues of vertebrate herbivores typically record higher $\delta^{15}N$ values than the plants they consume, while the tissues of animals which consume other animals record higher $\delta^{15}N$ values than their prey). Thus, animals which consume meat and/or protein rich resources (carnivores, insectivores, some omnivores), usually have higher $\delta^{15}N$ values relative to those which consume only plants (herbivores). Large-scale ecological studies indicate an average trophic enrichment of 3–4 ‰ between diet and consumer

81 tissues (Schoeninger and DeNiro, 1984; Bocherens and Drucker, 2003; Fox-Dobbs et al., 2007;
82 Krajcarz et al., 2018; and see Caut et al., 2009 for discussion of variability in trophic
83 enrichment).

84 While nitrogen isotope analyses are widely utilized in modern systems, similar analyses
85 of fossil material have largely been restricted to bone and dentin derived collagen from relatively
86 young (typically <100,000 years), well-preserved samples in which the original nitrogen isotope
87 composition is unaltered (Bocherens and Drucker, 2003; Britton et al., 2012; Jaouen et al., 2019;
88 but see Ostrom et al., 1993, who measured $\delta^{15}N$ values in fossils from the Late Cretaceous). The
89 organic matter (OM) in bone is nevertheless particularly susceptible to diagenetic alteration over
90 time because bone is porous, has a large surface-to-volume ratio, and its mineral phase is
91 characterized by bioapatite nanocrystals of low crystallinity (e.g., Clementz, 2012; Keenan et al.,
92 2016). In contrast, tooth enamel is highly mineralized (95 % wt. *vs*. 65 % wt. for bone), and
93 hence more resistant to diagenetic alteration and more frequently well-preserved in deep time
94 (Koch et al., 1997; Lee-Thorp and van der Merwe, 1987; Wang and Cerling, 1994; Zazzo et al.,
95 2004; Koch, 2007). Most stable isotope analyses of teeth have focused on the mineral fraction of
96 the enamel as opposed to the organic matter. Efforts to measure the $\delta^{15}N$ values of tooth enamel
97 organic matter using traditional combustion methods have been hampered by the low nitrogen
98 content in enamel material (<0.01 % wt.; Savory and Brudevold, 1959; Robinson et al., 1995,
99 2014).

100 Here, we present a novel application of the oxidation-denitrification method that allows
101 high-precision measurement of the nitrogen isotopic composition of tooth enamel ($\delta^{15}N_{enamel}$).
102 This method involves the conversion of nitrogen in enamel-bound organic matter to nitrate
103 (oxidation) followed by bacterial conversion of nitrate (NO_3^-) to nitrous oxide (N_2O)

104 (denitrification). The oxidation-denitrification method was first used by Robinson et al. (2004)
105 for the analysis of (base-soluble) opaline diatom microfossil-bound $\delta^{15}N$, later adapted for (acid-
106 soluble) calcitic microfossils (foraminifera-bound $\delta^{15}N$) by Ren et al. (2009), and is now
107 routinely used for determining $\delta^{15}N$ values of mineral-bound organic matter from a variety of
108 modern and fossil marine invertebrates (e.g., foraminifera, diatoms and corals; Robinson et al.,
109 2004; Ren et al., 2009, 2012; Straub et al., 2013; Martínez-García et al., 2014; Wang et al., 2014;
110 Studer et al., 2015, 2018; Smart et al., 2018, 2020). The method is ≥100 times more sensitive
111 than traditional combustion methods allowing us to reduce the amount of material required for
112 analysis of tooth enamel to ≤5 mg.
113 The primary goal of this study was to determine whether $\delta^{15}N_{enamel}$ values record the
114 isotopic composition of the diet consumed during tooth formation (i.e. amelogenesis). To test
115 this, the ever-growing mandibular incisors of two rodent species (rat and guinea pig) from a
116 controlled feeding study were analyzed for $\delta^{15}N_{enamel}$ values. Due to the constant, incremental
117 growth of these incisors this enamel is expected to record the nitrogen isotope composition of the
118 diet continuously. Animals were fed a custom-made plant-, insect-, or meat-based pelleted diet
119 for 54 days. Importantly, experimental diets were isonitrogenous (i.e., contain the same
120 percentage nitrogen) but had distinctly different $\delta^{15}N$ values to simulate different trophic levels.
121 We sought to determine (i) whether $\delta^{15}N_{enamel}$ values record differences between diet groups; (ii)
122 how $\delta^{15}N_{enamel}$ values correlate with $\delta^{15}N_{soft\ tissue}$ data (i.e. liver, muscle, kidney) from the same
123 individuals, (iii) if and to what extent $\delta^{15}N_{enamel}$ values vary between species and, (iv) whether
124 $\delta^{15}N_{enamel}$ values vary according to the degree of enamel maturity.
125

126 ## 2. MATERIALS AND METHODS

2.1 Experimental design

Thirty-six rats and guinea pigs received (in groups of 6) the same plant-, insect- or meat-based pelleted diet for a duration of 54 (+ 5 acclimatization) days. Adult female WISTAR (RjHan:WI) rats (*Rattus norvegicus* forma domestica; n=18; initial body mass, 198 ± 12 g, 11–12 weeks old) and adult female Dunkin Hartley (HsdDhl:DH) guinea pigs (*Cavia porcellus*; n=18; initial body mass, 401 ± 16 g, age 4–5 weeks old) (breeder: Envigo) were housed in groups of six in indoor stables (0.58 m^2 each), each equipped with two open food dishes containing their assigned experimental diet and two nipple drinkers of local tap water. All animals received exactly the same experimental foods and were housed under the same conditions. All animals were free to engage in caecotrophy (ingestion of feces), a natural behavior in both species (Björnhag et al., 1999). The feeding experiment and euthanization were performed with ethical approval of the Swiss Cantonal Animal Care and Use Committee Zurich (animal experiment licence N° ZH135/16).

Water and food were provided for *ad libitum* consumption. The experimental diet was supplemented with the pelleted diet provided by the animal breeder for an acclimatization period of 5 days, during which the breeder food was gradually phased out. One rat that received only the breeder diet was sampled for measurement (no equivalent guinea pig individual was available) and the experimental period of 54 days began after this acclimatisation period. Each animal was fed one of three pelleted experimental diets including; (i) a plant-based (P), (ii) an insect-based (I), or (iii) a meat-based (M) pellet. Each diet consisted of a primary ingredient of lucerne (P), black soldier fly larvae insect protein meal (I), or lamb meal (a ruminant herbivore) blended with additional plant-derived ingredients and minerals/vitamins (see Table 1) according

149 to the nutritional requirements of the animals. To avoid isotopic variation resulting from
150 differences in protein content, all pelleted diets were formulated to be isonitrogenous (21.5 %).

Table 1: Composition of pelleted diets. Ingredients are reported in weight % (wet matter).

Ingredient	Plant-based Pellet in % wt.	Insect-based Pellet in % wt.	Meat-based Pellet in % wt.
Primary Ingredient	(Lucerne)	(Protix Insect Protein Meal)	(Lamb Meat)
	56	26	25
Potato Protein	13	–	–
Wheat Meal	10	18	18
Oat Meal	7	16	15
Apple Bits	5	14	15
Soy Husks	3	10	13
Straw Meal	–	10	9
Molasses	3	3	3
Vitamins and Minerals	3	3	2

151

152 Aliquots of each experimental feed were collected every two weeks (to confirm pellet
153 homogeneity) during the experiment, homogenized with a ball mill, and analyzed for $\delta^{15}N$ (see
154 Section 2.4). Breeder diets for rats and guinea pigs differed both in ingredient and isotopic
155 compositions. The guinea pig breeder pellet was higher than the rat breeder pellet in $\delta^{15}N$ value
156 (4.8 ‰ *vs*. 2.4 ‰) and thus the two taxa should have begun the experiment with different tissue
157 isotopic compositions. Experimental diets differed in $\delta^{15}N$ by +3.4 ‰ between plant- and meat-
158 based diet, with +1.1 ‰ between the plant- and insect-based diet and +2.3 ‰ between the insect-
159 vs. meat-based diet (Figure 1; Table 3). Hence, although the pelleted diets do not represent a
160 direct link in a single food chain, the $\delta^{15}N$ values of the feeds nonetheless approximate trophic
161 level enrichment: the plant pellet has a lower $\delta^{15}N$ value than the animal-product containing
162 pellets.

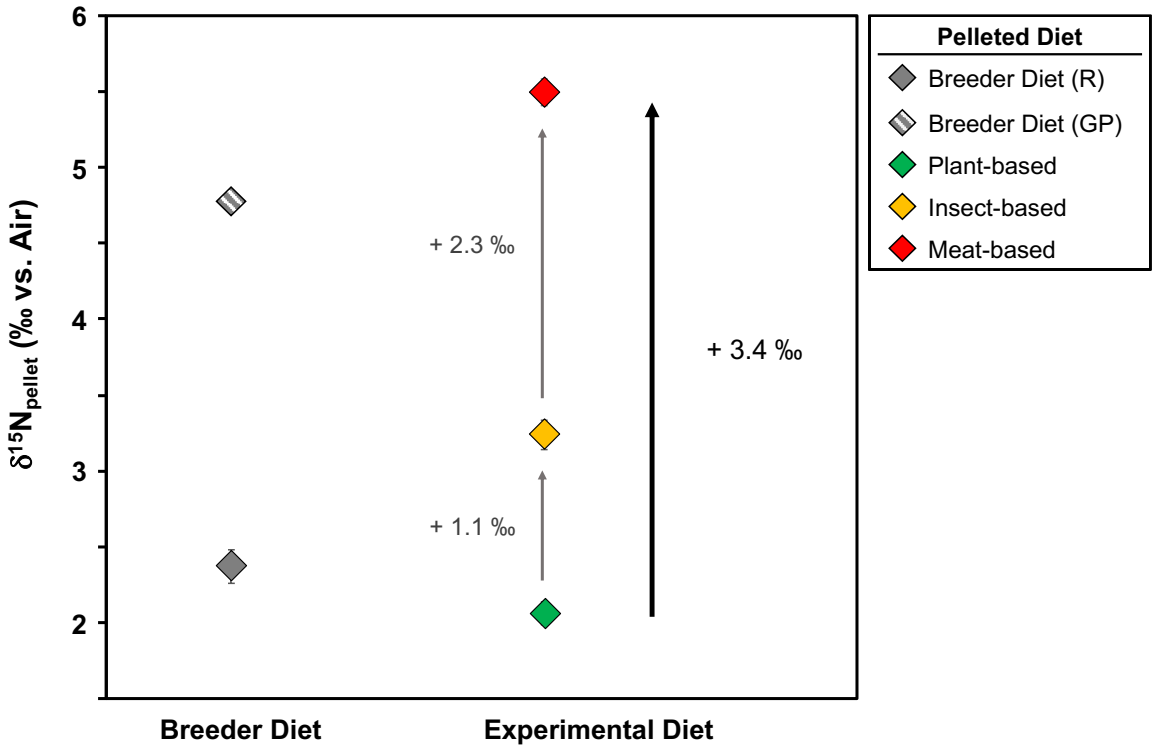


Figure 1: $\delta^{15}N$ values of breeder and experimental pelleted feeds. Differences in the $\delta^{15}N$ values of experimental feeds are given (in ‰) and indicated with arrows. The offset between the plant- *vs*. meat-based experimental diet (+3.4 ‰) approximated the average trophic offset observed between herbivores and carnivores in a natural food web (Schoeninger and DeNiro, 1984; Caut et al., 2009).

Following the conclusion of the experiment, animals were euthanized with carbon dioxide. The bodies were immediately dissected, and the soft tissues were stored at −20 °C. Enzymatic maceration of the skulls was conducted at the Center of Natural History (CeNak) of the University of Hamburg, Germany. Enamel of one mandibular incisor from each individual was prepared for $\delta^{15}N$ analysis using the novel oxidation-denitrification method.

Soft tissues, including whole liver, whole kidney, and ~1 cm^3 muscle from the upper right quadriceps, were sampled from each individual for comparison of $\delta^{15}N_{soft\ tissue}$ values with $\delta^{15}N_{enamel}$ values. Tissue samples were freeze-dried for 24 h, milled and homogenized using a

177 Retsch Cryomill, and 700 ± 0.2 µg of material was weighed into tin capsules for $\delta^{15}N_{soft\ tissue}$
178 analyses (see Section 2.4). Lipid extractions were not performed on these samples due to the
179 potential for alteration of tissue $\delta^{15}N$ values by the extraction process (Pinnegar and Polunin,
180 1999; Sotiropoulos et al., 2004; Post et al., 2007).
181
182 **2.2 Tooth enamel nitrogen isotope measurement**
183 *2.2.1 Standards and consumables*
184 $\delta^{15}N_{enamel}$ analyses were performed in the laboratories of the Organic Isotope
185 Geochemistry Group of the Department of Climate Geochemistry at the Max Planck Institute for
186 Chemistry (MPIC), Mainz, Germany. All standards used are listed in Table 2. In-house standards
187 include a crushed, homogenized sceleractinian coral *Porites* sp. (PO-1) and *Lophelia pertusa* a
188 deep-sea coral (LO-1), as well as two tooth enamel standards, a modern African elephant
189 *Loxodonta africana* (AG-Lox; Gehler et al., 2012) and a fossil (ca. 2.5 to 2.3 Ma) suid
190 *Notochoerus scotti* (Noto-1) from Zone 3A-2 of the Chiwondo Beds in Malawi (i.e. HCRP-
191 RC11-762 in Kullmer et al., 2008) developed for this study. The values of the in-house standards
192 were determined using the same oxidation-denitrification procedure.
193 Throughout the entire study, all standards, samples and other solutions were prepared
194 with ultrapure Milli-Q water (18.2 MΩ cm). Reagents used for reductive-oxidative cleaning
195 include: sodium citrate, sodium bicarbonate, sodium dithionite, potassium persulfate (four times
196 re-crystalized), and sodium hydroxide (ACS grade). Reagents used for de-mineralization of
197 enamel and oxidation of organic matter include: hydrochloric acid (Optima grade), potassium
198 persulfate for oxidation (four times re-crystalized), and sodium hydroxide (ACS grade). All
199 glassware was pre-combusted to minimize contamination with organic matter; this included all

4ml borosilicate glass vials (VWR Part No. 548-0051), 20 ml headspace vials, rinse-water beakers, and pipette tips. All centrifugation steps were performed at 3000 rpm for 5–10 min.

Table 2: Certified $\delta^{15}N$ values of international standards and $\delta^{15}N$ values of in-house standards (determined using the oxidation-denitrification method) used in this study.

International Standard	Material	$\delta^{15}N$ (‰ vs Air)
USGS40	L-glutamic acid	- 4.52 ± 0.06
USGS41	L-glutamic acid	47.57 ± 0.11
IAEA-NO-3	Potassium Nitrate	4.7 ± 0.2
USGS34	Potassium Nitrate	-1.8 ± 0.2
MPIC In-House Standard		
PO-1	Coral	6.05 ± 0.2
LO-1	Coral	10.01 ± 0.2
AG-Lox	Modern Enamel	4.2 ± 0.5
Noto-1	Fossil Enamel	13.7 ± 0.5

2.2.2 Oxidation-denitrification method overview

The oxidation-denitrification procedure is outlined briefly here and detailed descriptions of each step are given in the following sections. The method consists of four main steps. First, tooth enamel powder is subjected to cleaning to remove exogenous organic matter. Samples are subsequently demineralized, and the remaining endogenous organic matter is oxidized to nitrate. This nitrate is then quantitatively converted to N_2O via bacterial denitrification (Sigman et al., 2001; Casciotti et al., 2002; Mcilvin and Casciotti, 2011; Weigand et al., 2016). Finally, the N_2O is extracted, and its isotopic composition is measured to obtain $\delta^{15}N$ values. International and in-house standards are included in each step of every run to monitor the process and two analytical blanks are measured in each batch of samples to quantify blank size and isotopic composition during the oxidation step.

2.2.3 Preparation of enamel samples

The lower incisor was removed from the jaw of each individual after maceration in an enzymatic bath, and any remaining adherent tissue was gently buffed away using a hand-held drill with a cleaning/grinding tip. Teeth were then rinsed with Milli-Q water, dried, weighed and measured prior to enamel preparation. Using a Dremel mounted under a microscope fitted with diamond-studded drill bits (0.3–1.5 mm), dentin was carefully removed leaving only the thin enamel layer remaining. Each tooth was then cut in half (perpendicular to the growth axis of the tooth) ~ 3–5 mm above the boundary between translucent and opaque enamel (opaque boundary) to ensure separation of the highly mineralized, "mature" enamel (i.e. tip half of the tooth) from the partially mineralized, recently formed "immature" root enamel (see Figure A1; Robinson et al., 1977, 1995, 2014). Separate halves were crushed and ground to a fine powder in an agate mortar and pestle. Mature and immature enamel $\delta^{15}N$ values are compared in Section 3.2.4 and discussed in Section 4.3. The $\delta^{15}N_{enamel}$ values presented in this paper are values for *mature* enamel from the tip half of the tooth only (see Figure A1) unless otherwise indicated.

2.2.4 Removal of exogenous organic matter in tooth enamel

For the reductive-oxidative cleaning, untreated modern and fossil enamel powder was weighed (3–4 mg and 4–6 mg, respectively) into 15 ml polypropylene centrifuge tubes (Falcon®). Then, 7 ml of sodium bicarbonate-buffered dithionite citrate was added to the samples, which were shaken and placed, loosely capped to allow degassing, in an 80 °C water bath for ca. ten minutes. This step is designed to reduce oxidized contaminants, particularly metal oxide coatings, which could potentially trap exogenous nitrogen during the fossilization process (Mehra and Jackson, 1958; Ren et al., 2012). Samples were then immediately centrifuged and decanted. The remaining material was rinsed three times with 10 ml Milli-Q water (with

centrifugation between each rinse), before the powder was transferred to pre-combusted 4 ml glass vials. Residual water from the transfer process was removed by aspiration after centrifuging a final time.

Following the reductive cleaning, a 3 ml aliquot of a basic potassium persulfate solution consisting of a 1:1 ratio of sodium hydroxide to potassium persulfate per 100 ml of Milli-Q water (i.e. 2 g NaOH and 2 g $K_2S_2O_8$) was added to each sample. Samples were then autoclaved for 65 minutes at 120 °C. During this process, exogenous nitrogen in organic matter is oxidized to nitrate in solution, which is then rinsed away. After autoclaving, the oxidative solution was removed by aspiration and then rinsed four times with 4 ml Milli-Q water. Again, samples were centrifuged after oxidation and between every rinse. After the final rinse, the Milli-Q water was removed by aspiration, and the samples were loosely covered with pre-combusted aluminum foil and placed in a dedicated drying oven at 60 °C for 36 hours to dry. Once completely dry, samples were transferred into vials and precisely weighed (for the calculation of N content) in preparation for oxidation. Sample loss varied between 30–60 % for enamel powder as a result of handling and dissolution during cleaning.

2.2.5 Conversion of organic nitrogen to nitrate via persulfate oxidation

First, 2–4 mg of cleaned enamel powder was demineralized using 40 µl of 4N hydrochloric acid. Nitrogen in the freed organic matter was then oxidized to nitrate using 1 ml of basic potassium persulfate solution. To prepare this solution, 0.67–0.70 g of four times re-crystalized potassium persulfate (to ensure low nitrogen content) was added to 4 ml of 6.25 N NaOH solution in 95 ml Milli-Q water. Samples were autoclaved for 65 min at 120 °C to ensure

complete oxidation. After oxidation, the samples were centrifuged to ensure separation of supernatant and any precipitate.

To monitor and correct for the nitrogen content and $\delta^{15}N$ value of the oxidizing solution, we prepared ten 1 ml oxidation blanks consisting only of HCl and oxidizing solution. In addition, triplicates of two amino acid isotope reference standards (USGS40, USGS41; see Table 2 for values) were oxidized with the samples. These standards were prepared at a concentration of 10 nmol N to match the N concentration of the in-house standards. These amino acid references served as a means to ensure that complete oxidation of the nitrogen in the samples took place, as well as to provide a secondary control on the blank contribution of the persulfate solution.

2.2.6 Determination of sample nitrate concentration

To minimize uncertainties associated with nonlinearity in the mass spectrometer, the nitrate concentration of each sample was first determined in order to ensure a consistent final quantity of N_2O for both samples and standards (Sigman et al., 2001; Weigand et al., 2016). This measurement was performed via chemiluminescent detection on a Teledyne NOx analyzer (NOxBox) after reduction of nitrate to nitrous oxide with Vanadium(III) (Braman and Hendrix, 1989). This analysis provided an estimate of the nitrate concentration of the sample, which was then used to calculate the volume of sample to be injected for conversion by bacteria to achieve the desired 5 nmol N for measurement.

2.2.7 Bacterial conversion of nitrate to N_2O and measurement

Quantitative conversion of nitrate to N_2O is accomplished using a specific strain of denitrifying bacteria (*Pseudomonas chlororaphis*, grown, cultured and harvested at MPIC) which

lack N_2O reductase activity (Sigman et al., 2001; Weigand et al., 2016). Sample volumes were injected to achieve a target nitrogen content of 5 nmol per bacterial vial using the values calculated via chemiluminescent detection.

Standard protocol in the Martínez-García Laboratory is to use a nitrate free bacterial resuspension media buffered to a pH of 6.3 for the denitrifying bacteria, which is one of several recipes that has been tested for the denitrifier method (Sigman et al., 2001; Casciotti et al., 2002; McIlvin and Casciotti, 2011; Weigand et al., 2016). This relatively low-pH recipe was adopted for persulfate-denitrifier analyses at the MPIC because it was found to conveniently counter-balance the highly basic oxidized sample solutions, removing or reducing the need for pH adjustment after the oxidation step and prior to the denitrifier step.

The bacterial resuspension media consisted of Tryptic Soy Broth (TSB; 60 g), potassium phosphate monobasic (KH_2PO_4; 9.8 g), and ammonium chloride (NH_4Cl; 0.8 g). The method is optimized for 3 ml of media and the potassium persulfate recipe: 0.7 g persulfate and 1g NaOH**,** which is used to oxidize foraminifera samples. The re-suspension media, buffered at a pH of 6.3, ensures that the pH of the bacterial solution remains between 6.5 to 7.0 upon sample injection, thereby preventing bacterial death, and guaranteeing full conversion of the nitrate into N_2O.

Samples with low nitrate concentration (i.e., samples which had inherently low N content per mg cleaned powder, small amounts of available sample material, or significant sample loss during cleaning) require injection of a larger sample aliquot that could exceed the buffering capacity of the media. Thus, oxidation blanks and samples requiring >550 µl injection volume were adjusted by stepwise addition of 4 N hydrochloric acid to achieve a near-neutral pH of 5–7 to ensure full conversion during the bacterial step.

Two nitrate reference standards, (IAEA-NO-3, USGS34) were analyzed at concentrations of 1, 3, 5, and 10 nmols to calculate nitrogen concentration and calibrate the isotopic composition of samples relative to air. The $\delta^{15}N_{enamel}$ values of each sample is reported vs. air N_2, by calibration with IAEA-NO-3 and USGS34 (Weigand et al., 2016).

Oxidation blanks were prepared by combining five individual 1 ml aliquots of the oxidation solution into a single aliquot, which was then injected into a bacterial vial. This ensures accurate measurement of nitrogen content and nitrogen isotope composition for the oxidation blanks, which have extremely low nitrogen contents (typically 0.3 to 0.5 nmol/ml).

$\delta^{15}N$ values of bacterially converted N_2O was measured via gas chromatography-isotope ratio mass spectrometry (GC-IRMS) on a purpose-built system for N_2O extraction and purification online to a Thermo Scientific 253 Plus isotope ratio mass spectrometer (see Casciotti et al., 2002; McIlvin and Casciotti, 2011; Weigand et al., 2016 for detailed information regarding this setup). Several standard N_2O gas aliquots at measurement quantity (5 nmol N) were included throughout each run to monitor instrumental drift. No significant drift was observed for any of the analytical runs included in this study.

Tooth enamel samples were analyzed for $\delta^{15}N_{enamel}$ using the oxidation-denitrification method at the MPIC in seven batches for all 36 individuals included in the experiment. All samples analyzed for nitrogen isotopes were measured in duplicate or triplicate and in separate batches whenever possible (see Table A2).

2.3 Isotopic notation and blank correction

All isotopic values are reported in the standard delta notation (δ), (parts per thousand, ‰). The delta value is given by:

330 $$\delta = \left(\frac{R_{sample} - R_{standard}}{R_{standard}}\right) * 1000$$

331 where R is the ratio of the abundance of ^{15}N to ^{14}N of the sample or standard. All nitrogen
332 isotope data are reported relative to Air (atmospheric N_2).

333 Individual analyses are referenced to injections of N_2O from a pure N_2O gas cylinder and
334 then standardized using two international nitrate reference materials IAEA-NO3 (International
335 Atomic Energy Agency, Vienna, Austria) and USGS34 (The National Institute of Standards and
336 Technology, Gaithersburg, MD, USA). These standards were used to calibrate the isotopic scale
337 and allow the reporting of $\delta^{15}N$ values *vs*. Air. Additionally, the data were corrected for the
338 contribution of the blank using the nitrogen content, and $\delta^{15}N$ values of the oxidation blanks
339 prepared during oxidation. The blank correction, taking the fraction of the blank (f_{blank}) into
340 account, was calculated as follows:

341 $$\delta^{15}N_{sample} = \frac{\delta^{15}N_{measured} - (f_{blank} * \delta^{15}N_{blank})}{f_{sample}} \quad (1)$$

342 where both $\delta^{15}N_{measured}$ and $\delta^{15}N_{blank}$ values were measured directly via GC-IRMS and

343 $$f_{blank} = \frac{N\ content_{blank}}{N\ content_{measured}} \quad (2)$$

344 $$f_{sample} = 1 - f_{blank} \quad (3)$$

345 Differences in isotopic composition in ‰ were calculated using the Δ notation, where:

346 $$\Delta_{A\text{-}B} = \delta_A - \delta_B \quad (4)$$

347 The precision and accuracy of this correction for the contribution of the oxidation blank
348 was evaluated using the international reference materials USGS40 and USGS41. In addition, the
349 precision of the entire analytical procedure, including the cleaning step, was estimated by
350 replicate measurements of our in-house coral and enamel standards (see Section 3.1).
351

2.4 Measurement of $\delta^{15}N$ values in pelleted feed and soft tissues

Pelleted feeds and soft tissues were analyzed for $\delta^{15}N$ (with $\delta^{13}C$) using a Costech Elemental Analyzer (ECS 4010) in continuous flow mode coupled to a Thermo Scientific Delta V Plus isotope ratio mass spectrometer at the GeoZentrum Nordbayern, Friedrich-Alexander University Erlangen-Nürnberg, Germany and the Institute for Analytical and Applied Paleontology, Johannes Gutenberg University Mainz, Germany. The datasets were corrected for linearity and instrumental drift with laboratory standards (acetanilide, casein, and urea) which were calibrated directly against USGS40 and USGS41, and values were normalized for carbon to Vienna Pee Dee Belemnite (VPDB) and for nitrogen to atmospheric nitrogen (Air). Precision of the laboratory standards was better than 0.1 ‰ for $\delta^{15}N$ values.

2.5 Statistical Analyses

Statistical analyses were performed using JMP Version 15.1 and PAST Version 4.0 . Statistical significance was evaluated using a One-way ANOVA with a Tukey-Kramer post-hoc test, unless otherwise indicated. The significance level was set to 0.05.

3. RESULTS

All results are given in Tables A2 to A4 in the Appendix.

3.1 Blanks, reproducibility and precision

The average nitrogen content of the oxidation blank was 0.36 ± 0.07 nmol/ml (n=15) and of the modern tooth enamel samples from the feeding experiment was 20.5 ± 7.0 nmol/ml (n=60). Thus, the blank typically contributed less than 2 % of the total nitrogen content in a

given sample. Due to the low f_{blank}, the majority of the blank corrections were <0.3 ‰ but varied depending on f_{sample} in each specific instance (see Table A3 and A4). Blank corrected inter-batch $\delta^{15}N$ (± 1σ) values for the international standards used to monitor the oxidation process were -4.6 ± 0.3 (n=20) and -48.2 ± 0.4 (n=14), for USGS40 and USGS41, respectively. Inter-batch precision (± 1σ) in $\delta^{15}N$ values were between 0.3 and 0.5 ‰ for the coral and tooth enamel in-house standards across all analytical batches (see Table 4).

3.2 Controlled feeding experiment

3.2.1 Tooth enamel growth

Mean total tooth length for rats was 21.8 ± 0.7 mm while guinea pig incisors were slightly longer at 25.5 ± 1.0 mm. Mandibular incisors have been documented to grow at a rate of 0.4–0.6 mm/day in rats and 0.3 mm/day in guinea pigs (Park et al., 2017; Müller et al., 2015). The experimental duration was 54 days (after a 5-day acclimatization period during which the animals also received the experimental food). Thus, based on documented enamel growth rates for each species, the enamel in the incisors of the rats is expected to have been completely replaced (21.6–32.4 mm total growth), while that of the guinea pigs is calculated to have reached approximately 64 % (16.2 mm total growth) turnover.

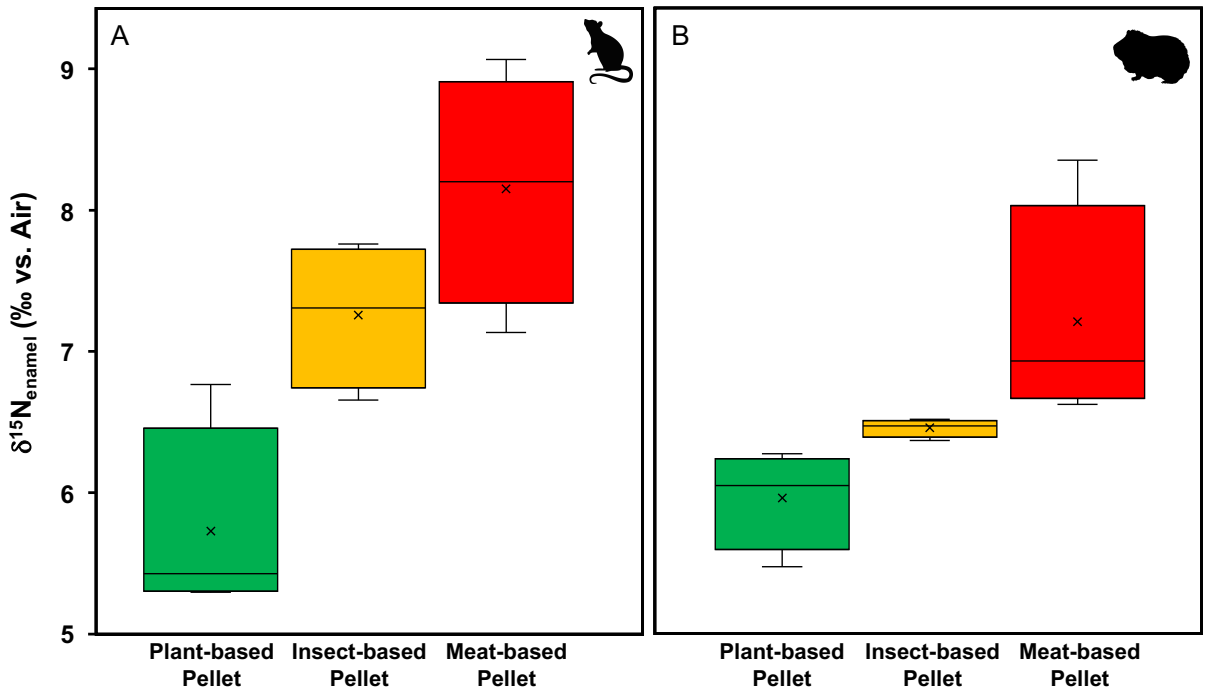
3.2.2 Variation of $\delta^{15}N_{enamel}$ values

Mature tooth enamel from experimental animals that received the plant-based pellet had the lowest $\delta^{15}N_{enamel}$ values (6.0 ± 0.5 ‰), followed by insect- (7.1 ± 0.6 ‰), and meat-based (7.8 ± 0.8 ‰) diet groups, and all diet groups differed significantly from one another. This

398 pattern of relative "trophic spacing" in $\delta^{15}N_{enamel}$ values (i.e. plant<insect<meat) was consistent

399 within species, with some variation between species $\delta^{15}N_{enamel}$ values depending on which diet

400 was consumed (Figure 2; Table 3).



401 Figure 2: $\delta^{15}N_{enamel}$ values of mature enamel for rats (A) and guinea pigs (B) by diet. Boxplots
402 show the interquartile range with the median indicated by the solid line and the mean indicated
403 by "x". Average $\delta^{15}N_{enamel}$ values for plant-, insect-, and meat-based diet groups showed the
404 same pattern of relative ^{15}N-enrichment in both species.

Table 3: $\delta^{15}N_{pellet}$ values for pelleted feeds and $\delta^{15}N_{enamel}$ values, $\Delta^{15}N_{enamel\text{-}diet}$, and N content (nmol/mg) values for tooth enamel (mature and immature) by taxon and diet group.

	$\delta^{15}N_{pellet}$ (‰ vs. Air)	N-Content (nmol/mg)		$\delta^{15}N_{enamel}$ (‰ vs. Air)		$\Delta^{15}N_{enamel\text{-}diet}$ (‰ vs. Air)	
Rat		**Mature Enamel**	Immature Enamel	**Mature Enamel**	Immature Enamel	**Mature Enamel**	Immature Enamel
Breeder	2.4 ± 0.1	6.4 (1)	4.3 (1)	6.4 (1)	6.6 (1)	+4.0 (1)	+4.2 (1)
Plant-based	2.1 ± 0.1	5.0 (1)	4.0 ± 0.7 (4)	5.8 ± 0.6 (6)	5.3 ± 0.9 (4)	+3.7 ± 0.6 (6)	+3.2 ± 0.9 (4)
Insect-based	3.2 ± 0.1	4.6 ± 0.6 (6)	4.7 ± 1.8 (4)	7.5 ± 0.6 (6)	6.3 ± 0.4 (4)	+4.3 ± 0.6 (6)	+3.1 ± 0.4 (4)
Meat-based	5.5 ± 0.1	5.2 ± 0.5 (6)	3.9 ± 0.5 (4)	8.3 ± 0.7 (6)	7.9 ± 0.6 (4)	+2.8 ± 0.7 (6)	+2.4 ± 0.6 (4)
Guinea Pig							
Breeder	4.8 ± 0.2	-	-	-	-	-	-
Plant-based	2.1 ± 0.1	5.7 ± 2.0 (6)	4.2 ± 1.0 (4)	6.1 ± 0.4 (6)	5.2 ± 0.4 (4)	+3.8 ± 0.4 (6)	+3.1 ± 0.4 (4)
Insect-based	3.2 ± 0.1	5.1 ± 0.3 (6)	4.8 ± 0.7 (4)	6.6 ± 0.3 (6)	5.9 ± 0.3 (4)	+3.3 ± 0.3 (6)	+2.7 ± 0.3 (4)
Meat-based	5.5 ± 0.1	4.7 ± 0.5 (6)	4.0 ± 0.6 (4)	7.3 ± 0.6 (6)	7.3 ± 0.6 (4)	+1.8 ± 0.6 (6)	+1.8 ± 0.6 (4)

* Average ± 1σ (*n*)

405

406

407 Within species, differences between dietary groups were significant in several cases
408 (Table 4). In rats, $\delta^{15}N_{enamel}$ values of the plant-based diet group was significantly lower (5.8 ±
409 0.6 ‰) than both the insect- (7.5 ± 0.6 ‰) and meat-based (8.3 ± 0.7) diet groups, which did not
410 differ significantly from one another. In guinea pigs the plant-and meat- diet group differed
411 significantly from one another (6.1 ± 0.4 ‰ vs. 7.3 ± 0.6 ‰).

412 In both species, the difference in enamel $\delta^{15}N$ values between diet groups (reported as
413 $\Delta^{15}N_{\text{diet B - diet A}}$; Table 4) deviated from those measured between the pellets themselves (reported
414 as $\Delta^{15}N_{\text{pellet B - pellet A}}$). Overall, enamel $\delta^{15}N$ values of animals fed plant vs. meat-based diets (rats
415 = 2.5 ‰; guinea pigs = 1.2 ‰) did not differ as much as the pelleted diets (3.4 ‰). Differences
416 between the insect- and meat-based diet group $\delta^{15}N_{enamel}$ values were comparable for both
417 species (rats = 0.8; guinea pigs = 0.7) but again smaller than measured difference between the
418 pelleted diets (2.3 ‰). Interestingly, rats fed the insect- vs. plant-based diets differed more (1.7
419 ‰) than guinea pigs on these same diets (0.5 ‰) and exceeded the measured difference in $\delta^{15}N$
420 value between the plant- and insect-based pellets (1.1 ‰; see Table 4 and Figure 1).

Table 4: $\Delta^{15}N_{diet\ B\text{-}diet\ A}$ according to sample type and taxon. Statistical results for comparison of mean $\delta^{15}N_{enamel}$ values according to diet group and taxon. Statistical significance was determined using One-Way ANOVA for overall significance with a Tukey-Kramer post-hoc test for pairwise comparisons.

Pelleted Diet	$\Delta^{15}N_{pellet\ B\ –\ pellet\ A}$		
Plant vs. Meat	**3.4**		
Plant vs. Insect	1.1		
Insect vs. Meat	2.3		
Enamel	**$\Delta^{15}N_{diet\ B\ -\ diet\ A}$**	***p*-value**	**Significance**
All		< 0.0001	***
Plant vs. Meat	**1.8**	< 0.0001	***
Plant vs. Insect	1.1	0.0007	**
Insect vs. Meat	0.7	0.03	**
Rat		< 0.0001	***
Plant vs. Meat	**2.5**	< 0.0001	***
Plant vs. Insect	1.7	0.0008	**
Insect vs. Meat	0.8	0.10	NS
Guinea Pig		0.002	**
Plant vs. Meat	**1.2**	0.015	**
Plant vs. Insect	0.5	0.16	NS
Insect vs. Meat	0.7	0.071	NS

*** significant at $p < 0.0001$, ** significant at $p < 0.05$, NS: no significance

3.2.3 Diet-to-tissue fractionation in tooth enamel

In this study, enamel was enriched in ^{15}N relative to diet (3.3 ± 1.0 ‰; range = 1.9–4.9 ‰) (Figure 3). This enrichment differed somewhat according to both diet and species (Table 3). Comparing diet groups, $\Delta^{15}N_{enamel\text{-}diet}$ was similar for animals which received breeder, plant- and insect-based diets (+3.9 ‰) but smaller for meat-based diets (+2.3 ‰). This pattern is more pronounced in guinea pigs, in which $\Delta^{15}N_{enamel\text{-}diet}$ is low in the insect-based diet group (+3.3) compared to rats (+4.3), and markedly low (+1.8 ‰) in the meat-based diet group (Figure 4).

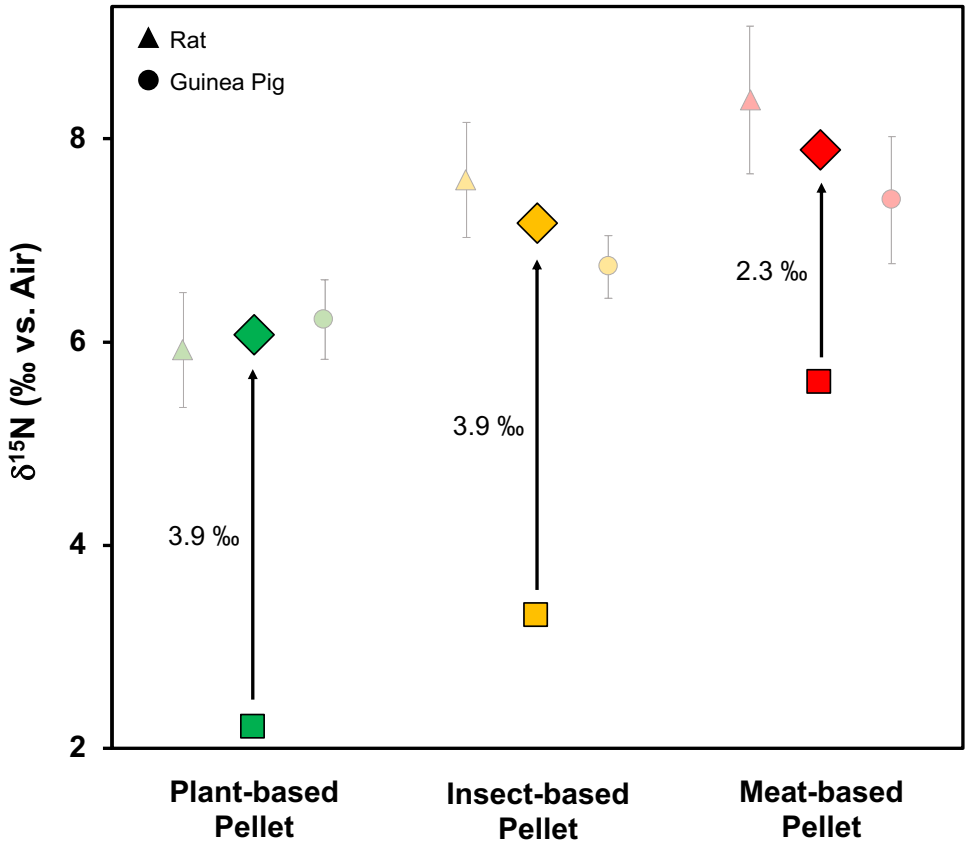


Figure 3: Mean $\delta^{15}N$ values for pelleted diets (squares) and mature tooth enamel (diamonds; both species with offset in $\delta^{15}N$ values ($\Delta^{15}N_{enamel\text{-}diet}$). Note the smaller offset for the meat-based diet. Mean ($\pm$ 1σ) $\delta^{15}N_{enamel}$ values for rats (triangles) and guinea pigs (circles) are also plotted and are depicted in more detail in Figure 4.

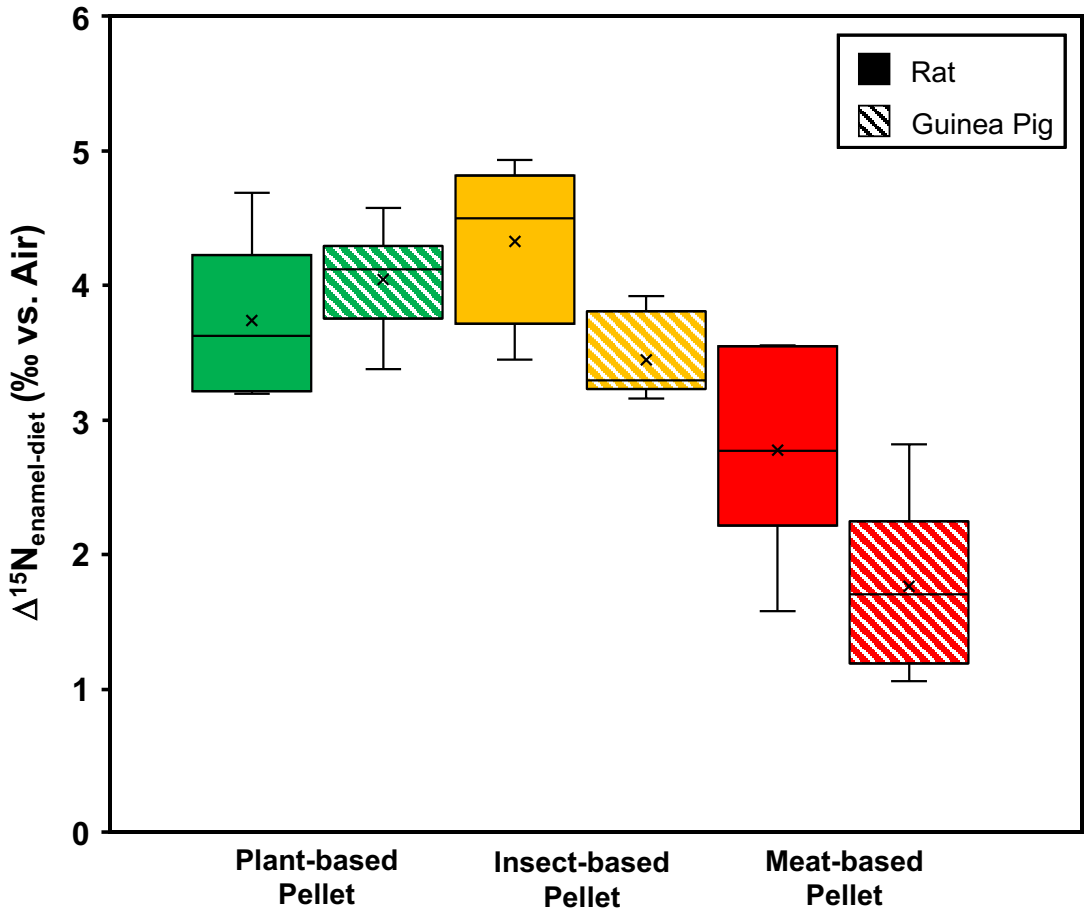
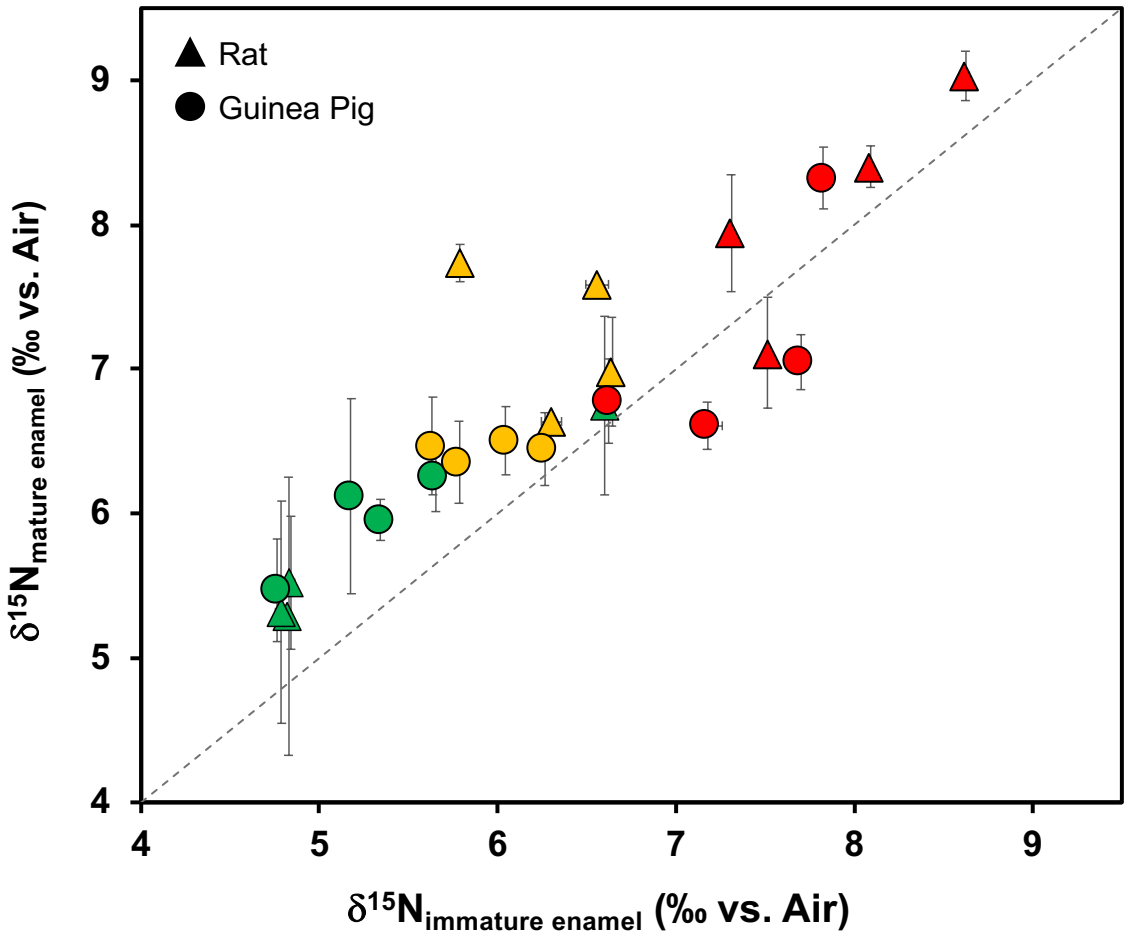


Figure 4: $\Delta^{15}N_{enamel\text{-}diet}$ for mature enamel of rats (solid) compared to guinea pigs (striped) in ‰ according to diet. Boxplots show the interquartile range with the median indicated by the solid line and the mean indicated by "x". Diets are indicated by color. $\Delta^{15}N_{enamel\text{-}diet}$ was similar in both species for the plant- and insect-based diets. $\Delta^{15}N_{enamel\text{-}diet}$ for the meat-based diet was lower than for the other diets in both species but is especially low (+1.8 ‰) in the herbivorous guinea pigs.

3.2.4 Comparison of mature vs. immature enamel

Immature enamel from the root half of the tooth was analyzed from four individuals in each diet group to evaluate the influence of enamel maturity/degree of mineralization on $\delta^{15}N_{enamel}$ values and N content. After redox-cleaning, fully mineralized (tip) enamel had a higher nitrogen content (5.0 ± 1.0 nmol/mg; n = 36) than immature (root) enamel (4.2 ± 0.9 nmol/mg; n = 24; $p = 0.006$) and N content did not differ significantly between species.

445 $\delta^{15}N$ values for immature enamel differed significantly ($p = 0.0003$; paired t-test) from,
446 but were positively correlated with, those of the fully mineralized enamel from the tip half of the
447 tooth (Pearson correlation, $r = 0.88$; $p < 0.001$; Figure 5). In general, immature enamel followed
448 the same pattern of trophic enrichment in $\delta^{15}N_{enamel}$ values observed in mature enamel (i.e. plant
449 < insect < meat), but with a smaller diet-to-tissue offset and lower average $\delta^{15}N_{enamel}$ values (see
450 Table 3).



451 Figure 5: Immature *vs*. mature $\delta^{15}N_{enamel}$ values for rats (triangles) and guinea pigs (circles) for
452 plant- (green), insect- (yellow), and meat- (red) based diets. Dashed line represents a 1:1
453 regression. Error bars represent 1σ if replicates were measured. Immature and mature enamel
454 $\delta^{15}N$ values were positively correlated ($y = 0.78x + 1.84$; $r^2 = 0.77$).
455

Differences in $\delta^{15}N_{enamel}$ values between mature and immature enamel were statistically significant for three groups, plant-pellet fed rats ($p = 0.02$), plant-pellet fed guinea pigs ($p = 0.003$), and insect-pellet fed guinea pigs ($p = 0.03$) (paired t-test). Mature and immature enamel from the same individual did not differ significantly in rats fed the insect-based diet, though there was, on average, a trend towards higher values in the mature enamel compared to immature enamel. In both species, for animals that received meat-based diets, mature and immature enamel did not differ.

3.2.5 Comparison of $\delta^{15}N_{enamel}$ and $\delta^{15}N_{soft\ tissues}$ values

Soft tissues (liver, kidney, muscle) followed the same N isotope pattern as enamel, with a $\Delta^{15}N_{tissue\text{-}diet}$ enrichment of ca. 2–6 ‰. Liver was most enriched in ^{15}N, generally followed by kidney and muscle (Table 5). As expected, soft tissues from animals that received the plant-based diet had the lowest $\delta^{15}N$ values, animals that received the meat-based diet had the highest $\delta^{15}N$ values, and animals that received the insect-based diet had intermediate values.

Table 5: Average $\delta^{15}N$ values of liver, kidney and muscle for each taxon and each diet group in ‰ vs. Air.

		Liver		Kidney		Muscle	
Taxon	Pelleted Diet	$\delta^{15}N$	n	$\delta^{15}N$	n	$\delta^{15}N$	n
Rat							
	Breeder	6.6	1	5.4	1	5.4	1
	Plant-based	6.4 ± 0.2	6	5.1 ± 0.1	6	5.2 ± 0.1	6
	Insect-based	9.0 ± 0.4	5	7.4 ± 0.1	6	6.7 ± 0.2	6
	Meat-based	9.5 ± 0.1	6	7.9 ± 0.1	6	7.1 ± 0.1	5
Guinea Pig							
	Plant-based	7.1 ± 0.6	6	4.5 ± 0.2	6	5.0 ± 0.2	6
	Insect-based	7.7 ± 0.1	6	5.7 ± 0.1	6	6.2 ± 0.2	6
	Meat-based	9.3 ± 0.1	6	7.5 ± 0.1	6	7.4 ± 0.1	6

Measured enamel and soft tissue $\delta^{15}N$ values from the same individual were positively correlated (Figure 6). Correlations between soft tissue and enamel $\delta^{15}N$ values were similar for liver (Pearson correlation, $r = 0.83$; $p < 0.001$), kidney (Pearson correlation, $r = 0.82$; $p < 0.001$), and muscle (Pearson correlation, $r = 0.76$; $p < 0.001$). Relative to enamel, liver had higher $\delta^{15}N$ values, while kidney and muscle exhibited slightly lower values.

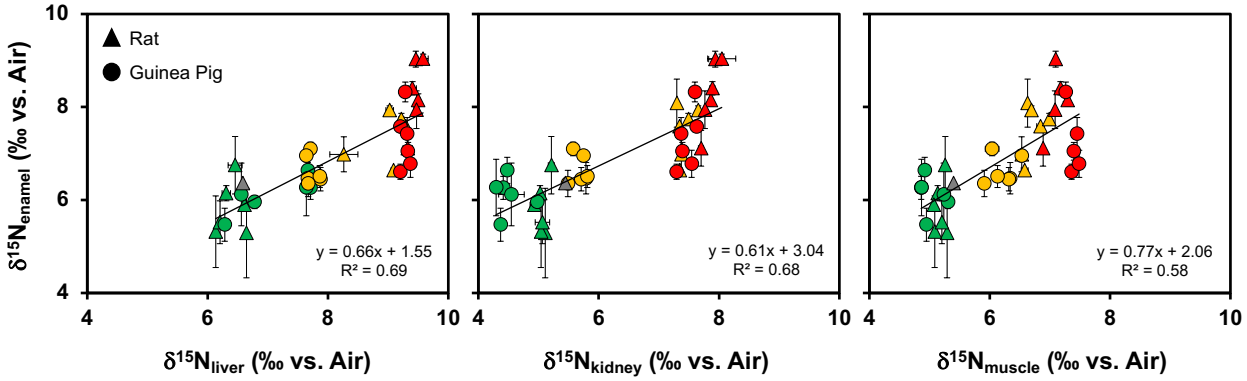


Figure 6: Soft tissue $\delta^{15}N$ *vs.* $\delta^{15}N_{enamel}$ values for rats (triangles) and guinea pigs (circles) for plant- (green), insect- (yellow), and meat- (red) based diets. Error bars represent 1σ of replicates measured. Soft tissue and enamel $\delta^{15}N$ values were positively correlated in all cases; regression shown with solid line and equation. Rat soft tissues were more enriched in ^{15}N than guinea pigs, especially in the liver and kidney.

Regardless of species, animals that received the same diet had similar $\delta^{15}N$ soft tissue values, with the exception of the insect-based diet. In this diet group, there was a marked difference between species, which was most apparent in the liver and kidney. In these tissues, rats were more enriched in ^{15}N ($\delta^{15}N_{liver} = 9.0 \pm 0.4$ ‰; $\delta^{15}N_{kidney} = 7.4 \pm 0.1$ ‰) than guinea pigs ($\delta^{15}N_{liver} = 7.7 \pm 0.1$ ‰; $\delta^{15}N_{kidney} = 5.7 \pm 0.1$ ‰) (see Figure 6).

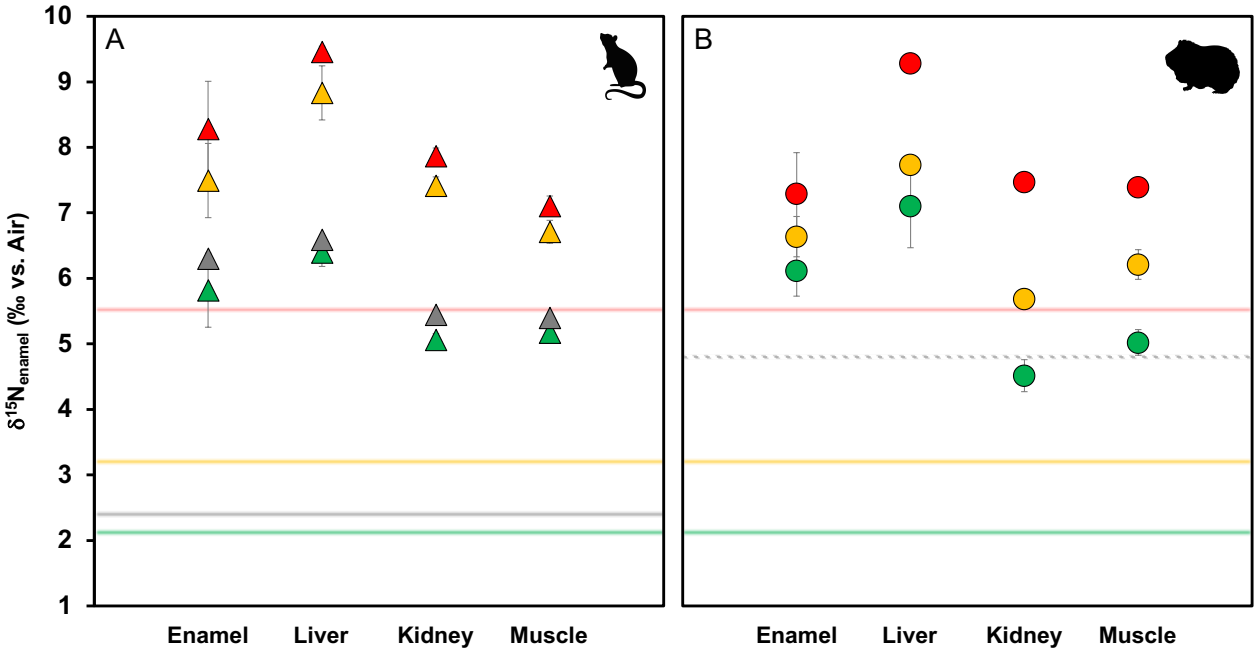


Figure 7: $\delta^{15}N$ values of pelleted diets, enamel, and soft tissues of rats and guinea pigs analyzed in this study. The measured $\delta^{15}N$ values of the pelleted diets are indicated with horizontal lines. The measured $\delta^{15}N$ values of the enamel and soft tissues for rats (A) and guinea pigs (B) are indicated with symbols. All diets are color coded (green = plant, yellow = insect, red = meat, solid gray = rat breeder, gray stripes = guinea pig breeder). Error bars represent ± 1σ for replicates. Note the similar pattern of variation in $\delta^{15}N$ values between diet groups across all tissue types.

Overall, the offset between the soft tissues of the plant- and meat-based diet group was between 2 and 3 ‰ (2.6 ± 0.7 ‰ for liver, 2.9 ± 0.2 ‰ for kidney, 2.2 ± 0.3 ‰ for muscle) for all animals. Within taxa, the offset in mean $\delta^{15}N_{enamel}$ values between these two diet groups were similar in rats (2.5 ‰) but lower (1.2 ‰) in guinea pigs compared to soft tissues (Figure 7).

In rats, the ^{15}N enrichment between the soft tissues of animals fed plant- and insect-based diets were consistently larger (between 1.5 and 2.6 ‰) than the offset measured between the pelleted diets (1.1 ‰). However, in guinea pigs the observed differences were slightly lower than, or comparable to the offset between the two pellet values (between 0.6 and 1.2 ‰). Differences in $\delta^{15}N_{enamel}$ values between plant- and insect-based diet groups (1.7 ‰ vs. 0.5 ‰ for

505 rats and guinea pigs, respectively) were therefore consistent with those observed in soft tissues
506 for each species (see Figure 7).

507 ^{15}N enrichment in soft tissues between the insect- and meat-based diet groups were
508 smaller than the measured 2.3 ‰ offset between these two pelleted diets in all tissues and in both
509 species. In the rats, this reduced spacing (between 0.4 and 0.5 ‰) was driven by the relatively
510 enriched $\delta^{15}N$ values of the insect-based diet group. Spacing between the insect- and meat-based
511 diet groups is similar (0.8 ‰) in enamel. In contrast, the offset between these two diets in the
512 soft tissues of guinea pigs was larger (between 1.2 and 1.8 ‰) than that observed in enamel (0.7
513 ‰) (see Figure 7).
514

515 ## 4. DISCUSSION

516 In this study, we measured $\delta^{15}N_{enamel}$ values from the tooth enamel of 37 rodents that
517 received isotopically different diets in a controlled feeding experiment. These measurements
518 demonstrate that the average nitrogen concentration in modern tooth enamel (5.0 ± 1.0 nmol/mg)
519 is sufficient for $\delta^{15}N_{enamel}$ analyses at sample sizes of ≥ 5 mg, representing a >100-fold reduction
520 in the amount of material required for traditional combustion methods. We find that $\delta^{15}N_{enamel}$
521 values reflect the isotopic composition and trophic spacing of the experimental diets with a diet-
522 to-tissue enrichment of ca. 2 to 4 ‰. These $\delta^{15}N_{enamel}$ values correlated positively with $\delta^{15}N_{soft\ tissue}$
523 values from the same individuals, demonstrating that nitrogen isotopes in enamel reflect the
524 isotopic composition of diet in a manner similar to other tissues. Most importantly, $\delta^{15}N_{enamel}$
525 values of animals that received plant- vs. meat-based diets followed a clear, expected pattern of
526 relative ^{15}N-enrichment, and were statistically distinguishable from each other regardless of
527 species.

Below we discuss these observations in greater detail. We first address variation in $\delta^{15}N_{enamel}$ values and $\Delta^{15}N_{enamel\text{-}diet}$ according to diet type. We then examine the differences in $\delta^{15}N_{enamel}$ values related to species (i.e. rats *vs.* guinea pigs) and degree of enamel maturation. Next, we explore the relationship between $\delta^{15}N_{enamel}$ and $\delta^{15}N_{soft\ tissue}$ values measured in the same individuals to further validate the $\delta^{15}N_{enamel}$ values as a proxy for diet. Finally, we discuss anticipated future applications for this method, particularly its use as a novel proxy to reconstruct trophic behavior in the fossil record.

4.1 Variation in $\delta^{15}N_{enamel}$ values between diet groups

The $\delta^{15}N_{enamel}$ values of mature enamel of all animals reflected diet $\delta^{15}N$ values plus a diet-to-tissue enrichment of ca. 2 to 4 ‰ in ^{15}N (see Section 3.2.2 and 3.2.3) and show a clear shift away from the breeder diets (see Figure 7). Observed inter-individual variation of $\delta^{15}N_{enamel}$ values within diet groups (0.4 to 0.7 ‰) was comparable to variation documented in other tissues by previous feeding experiments (DeNiro and Epstein, 1981; Caut et al., 2009; Webb et al., 2016) and, more specifically, was well within the range of variation (0.3 to 1.8 ‰) reported for collagen extracted from bone and dentin (Ambrose and DeNiro, 1986; Webb et al., 2016), the tissues most commonly available for nitrogen isotope analyses in the fossil record (but see Macko et al., 1999; Iacumin et al., 2006, for analyses of other tissues such as hair in the context of exceptionally preserved specimens). $\delta^{15}N_{enamel}$ values of animals that received the plant- vs. meat-based diet differed significantly in *all* cases, regardless of species, and the pattern of $\delta^{15}N_{enamel}$ values between diets followed that expected from the isotopic compositions of the pelleted feeds (see Figure 1 and Figure 2).

550 The observed enrichment in ^{15}N from diet to tissue was also in line with that documented
551 for other (non-enamel) tissues in previous feeding experiments and ecological studies (DeNiro
552 and Epstein, 1981; Vanderklift and Ponsard, 2003; McCutchan et al., 2003; Bocherens and
553 Drucker, 2003; Fox-Dobbs et al., 2007; Caut et al., 2009; Krajcarz et al., 2018). However, the
554 animals which received the meat-based diet were a notable exception; the $\Delta^{15}N_{enamel\text{-}diet}$ of this
555 group was ≥1 ‰ lower than that of animals that received plant- or insect-based diets (see Table 3
556 and Figure 3). This pattern was evident in both species, but was most pronounced in guinea pigs,
557 where $\Delta^{15}N_{enamel\text{-}diet}$ is only +1.8 ‰ (as compared to ~3–4 ‰ for the two other diets).
558 One potential explanation for this observation is that complete enamel turnover (here
559 referring to replacement of the incisor via growth and mineralization during the experimental
560 period) may not have occurred in all animals. The duration of the experiment (>8 weeks) was
561 designed such that the animal's mandibular incisors would completely (rats) or partly (guinea
562 pigs) turn over during the course of the experiment (see Section 3.2.1). However, if enamel
563 turnover was incomplete, the enamel formed during the period prior to the experiment (i.e. while
564 the animal received the breeder pellet) contributed to $\delta^{15}N_{enamel}$ values in the measured samples.
565 In guinea pigs, whose incisors grow at a slower rate than rats we can be certain that at least some
566 of the enamel from the tip half of the tooth contained material that grew before the experiment
567 began. This could have impacted the measured $\delta^{15}N_{enamel}$ values for the mature (tip) enamel (see
568 Section 4.3 for discussion of $\delta^{15}N$ values of mature *vs*. immature enamel). Interpretation of
569 $\delta^{15}N_{enamel}$ values for the guinea pigs should thus be made with this in mind.
570 In contrast, the rat's incisors should have re-grown entirely during the experimental
571 period (see Section 3.2.1). Unless the organic matrix of tooth enamel is synthesized from a pool
572 of nitrogen in the organism that is not fully equilibrated with the experimental diet, the tooth

enamel of the rats can be considered to reflect only the experimental diet. As such, the observed variation in $\Delta^{15}N_{enamel\text{-}diet}$ between rats that received the insect- and meat-based diets must be driven by additional factors beyond tissue turnover.

The observed variation in $\delta^{15}N_{enamel}$ values and $\Delta^{15}N_{enamel\text{-}diet}$ between animals that received different diets (particularly the rats which received the insect- and meat-based diets) is instead likely driven by a combination of factors including, but not limited to, (i) digestive physiology, (ii) consumed protein type, and (iii) enamel maturity. We discuss each of these factors below in relation to the inter-species and inter-diet offsets described above.

4.2 Variation in $\delta^{15}N_{enamel}$ values between species

The magnitude of the of inter-species and inter-diet differences in $\delta^{15}N_{enamel}$ values was not enough to obscure simulated trophic spacing, and thus do not overly confound the interpretation of the feeding experiment results, which are straightforward. Still, an exploration of these differences is warranted as it helps us examine the mechanisms by which isotopic differences can be imparted to the organic matter of the tooth enamel.

4.2.1 Variations in digestive physiology

The digestive physiology of a taxon is known to impact nitrogen isotope fractionation (Sealy et al., 1987; van Klinken et al., 2000; Robbins et al., 2005 DeMots et al., 2010; Cantalapiedra-Hijar et al., 2015) though the mechanisms that drive this remain elusive. Sponheimer et al. (2003), found that the $\delta^{15}N$ values of the hair of herbivores eating identical diets varied by as much as 3.6 ‰ and that rabbits had lower $\delta^{15}N$ values than the larger

595 herbivores in the study. This research suggests that interspecific variations in digestive
596 physiology can lead to large shifts in $\delta^{15}N$ values.

597 The two species of rodents used in this study have known differences in physiology that
598 may explain some of the observed inter-diet differences in $\delta^{15}N_{enamel}$ values. Guinea pigs are
599 strictly herbivorous and thus well-adapted to diets consisting entirely of plant foods. This is
600 reflected in their ever-growing, open-rooted (hypselodont, aradicular) premolars and molars,
601 whose laminate morphology makes them ideal for continuously grinding tough and/or abrasive
602 plant material throughout their lives (Ungar, 2015; Martin et al., 2019). In addition, guinea pigs
603 have a well-developed caecum that retains digesta for an extended period and aids in the
604 digestion of nutrient-poor, fibrous plant foods (Wagner and Manning, 1976; Sakaguchi, 1986;
605 Sakaguchi et al., 1987). These attributes are analogous to the adaptations of larger ruminant and
606 non-ruminant herbivores.

607 While rats also possess a well-developed caecum (Baker et al., 1979; Sakaguchi, 1986),
608 they exhibit remarkable dietary flexibility in comparison to guinea pigs, as is evident by their
609 commensality with humans. They have a generalist, bunodont dentition, and short digestive
610 tracts adapted to an omnivorous diet (Baker et al., 1979).

611 Rodents also engage in caecotrophy to varying degrees. Caecotrophs selectively retain
612 nitrogen-rich material (esp. microbes) in their caecum and excrete this material as a special type
613 of feces that is immediately re-ingested from the anus (Björnhag et al., 1999). This behavior has
614 been explicitly demonstrated in rats (Sperber et al., 1983) and guinea pigs (Holtenius and
615 Björnhag, 1985; Takahashi and Sakaguchi, 2006). Indeed, evidence for microbe-related fatty
616 acids in the body fat of the experimental animals from this study suggests that the guinea pigs in
617 relied more on this mechanism than did the rats (De Cuyper et al., 2020). Unknown nitrogen

618 isotope fractionation during metabolism of nutrients by gut microbes and differential uptake of
619 the resulting microbially-derived protein, could therefore contribute to species differences in
620 diet-to-tissue fractionation. Specific studies that unravel the different contributions of
621 microbially transformed dietary nitrogen in rodent nutrition and related isotope-specific effects
622 of enzymatic protein digestion are required to resolve such issues.
623 In summary, given their distinct digestive adaptations and behaviors (e.g. dentition,
624 digestive efficiency, and caecotrophy), it is possible that rats and guinea pigs metabolized the
625 plant- and animal- based proteins in the experimental feeds differently. However, while it is
626 probable that these variables contributed to the observed inter-species inter-diet differences, the
627 specific mechanisms driving the relationship between these factors and nitrogen isotope
628 fractionation remain poorly understood, particularly for small mammals.
629
630 *4.2.2 Variation in protein type*
631 Plant proteins contain fewer essential amino acids than do proteins derived from animal
632 tissues, thus dietary amino acids derived from plants require more "metabolic processing" than
633 do proteins from animal tissues (Gaebler et al., 1966; Silfer et al., 1992). In contrast, animal
634 tissue proteins typically originate from endogenous amino acids that have been transaminated or
635 recycled from protein degradation (Waterlow, 2006). The additional metabolic processing can
636 induce greater isotopic fractionation during the digestion of plant proteins compared to animal
637 proteins (Poupin et al., 2011). Therefore, fractionation occurring as a result of plant protein
638 consumption can contribute more significantly to the trophic effect in nitrogen than fractionation
639 during animal protein consumption.

Indeed, recent studies have shown that the consumption of plant vs. animal protein impact $\Delta^{15}N_{tissue\text{-}diet}$ offsets such that nitrogen isotope discrimination increases when the efficiency of dietary protein utilization decreases. Robbins et al. (2005), found support for the hypothesis of decreasing discrimination with increasing protein quality and Poupin et al. (2011), determined that $\Delta^{15}N$ was markedly lower in the tissues of rats fed animal (milk) proteins compared to those fed plant (soy) proteins. Similarly, Webb et al. (2016) fed pigs isoproteinous diets with differing relative proportions of either plant-(soy) or animal-(fish) derived protein and found similar or smaller $\Delta^{15}N_{tissue\text{-}diet}$ offsets in most tissues (e.g. liver, muscle) with increased animal protein consumption. Interestingly, Webb et al., 2016 observed larger $\Delta^{15}N_{tissue\text{-}diet}$ offsets in bone collagen of the same animals with increased animal consumption. These studies highlight the fact that differences in the amino acid composition of consumed proteins and/or differences in how amino acids are routed through the body, influence $\Delta^{15}N$.

The pattern of fractionation observed in animals that received the meat-based pellet in this experiment (relative to those which received the plant-based pellet) (Figure 7) is consistent with both the mechanism proposed above and the results from Poupin et al. (2011). In particular, the rats, which are both omnivorous, and which should not be affected by incomplete tissue turnover, had a $\Delta^{15}N_{tissue\text{-}diet}$ offset of 2.8 ‰ on the meat diet, 1 ‰ lower than the 3.8 ‰ offset observed for rats on the plant diet.

We have thus far excluded the insect-based diet from direct comparison in evaluating these mechanisms because the comparison between plant- and meat-consumption is better constrained for nitrogen. However, the animals that received the insect diet also exhibited interesting variation in $\delta^{15}N_{enamel}$ values and may provide further insight into the importance of protein type for these rodents. In the guinea pigs from this study, for example, the $\delta^{15}N_{enamel}$

663 values for the insect- and plant-based diet groups did not differ significantly. This lack of
664 significant difference is arguably unsurprising considering (i) the probable incomplete turnover
665 of enamel in guinea pigs, (ii) the degree of individual variation *within* dietary groups (0.4–0.7
666 ‰), (iii) the current precision of the method for tooth enamel (0.5 ‰), and (iv) the relatively
667 small isotopic difference between the plant- and insect-based diets (i.e., 1.1 ‰; see Figure 1).
668 Under these conditions, this small isotopic difference is probably insufficient, *per se*, to result in
669 significant differences between these dietary groups.

670 It is therefore interesting that $\delta^{15}N_{enamel}$ values for rats in the insect-based diet group were
671 significantly higher than those in the plant-based diet group. Compellingly, the soft tissues of rats
672 that received the insect-based diet also evidence high $\delta^{15}N$ values relative to the spacing between
673 the pelleted diets and to the guinea pigs (see Figure 7). Hence, the fact that similar patterns are
674 evident in both the rats' soft tissue and enamel $\delta^{15}N$ values highlights the fact that the $\delta^{15}N_{enamel}$
675 values likely reflect a general dietary fractionation, rather than some enamel-specific effect.
676 Despite the potential confounding factor of slower tissue turnover time, one might expect that
677 this pattern would also be observed in the tissue of the guinea pigs, but no such similarity is
678 observed here. We can rule out the possibility that chitin (the primary component in the
679 exoskeletons of insects and typically very depleted in ^{15}N relative to insect soft tissues; Webb et
680 al., 1998; Schimmelmann, 2011) plays a role in these differences as chitin is removed in the
681 protein extraction process used to produce the insect meal used in the insect pellet. Perhaps the
682 inter-specific variability in $\delta^{15}N$ values between the rats and guinea pigs that received the insect-
683 based diet reflects the effects of taxon-specific digestive physiology (omnivore *vs*. herbivore)
684 and thereby protein bioavailability.

685 Nevertheless, even if these weakly-constrained processes are responsible for the inter-
686 species differences observed in this study, their confounding effects are not large enough to
687 overprint the differences induced by the feeding experiment, underlining the strength of
688 $\delta^{15}N_{enamel}$ values as a dietary proxy. The clear, consistent, and statistically significant difference
689 between plant- vs. meat-based diets in both taxa emphasize the utility of $\delta^{15}N_{enamel}$ values in
690 identifying meat consumption, even in instances where it makes up a relatively small (≤25 %)
691 proportion of the diet.

692 **4.3 Enamel turnover and effect of maturation stage on $\delta^{15}N_{enamel}$ values**

693 Enamel from the most recently formed part of the tooth (i.e. the actively growing open
694 root) might be expected to best reflect the experimental diet, particularly in the case of the guinea
695 pigs, in which enamel turnover was likely incomplete. However, enamel from the root half of
696 rodent teeth is poorly mineralized and differs in composition from fully mineralized, mature
697 enamel towards the tip (Robinson et al., 1995; Lacruz et al., 2017), which may impact $\delta^{15}N_{enamel}$
698 values. These considerations motivated our investigation of immature enamel for nitrogen
699 isotope analyses.

700 Mature tooth enamel consists of ~95 % mineral (predominantly hydroxyapatite), ~2–4 %
701 water, and ~1–2 % organic matter by weight (Lacruz et al., 2017). The crystal structure of the
702 enamel consists of many millions of nearly identical, highly ordered crystals of calcium
703 hydroxyapatite with extraneous mineral components such as carbonate and magnesium. The
704 outer enamel layer in some rodent taxa, including the rats included in this study, is rich in iron
705 which gives the teeth a characteristic orange color (Robinson et al., 1995). In contrast, immature
706 tooth enamel is comprised of a soft, partially mineralized tissue (~30 % mineral wt. at initial

707 secretion) that is predominantly organic material and water (Deakins, 1942; Robinson et al.,
708 1978, 1995; Lacruz et al., 2017).

709 The differences in mineralization between mature and immature enamel can be observed
710 in the N content of enamel from the tip *vs*. root halves of the incisors of the experimental animals
711 (see Table 3). After redox cleaning, total material loss was greater for root (30–40 %) compared
712 to tip (15–25 %) enamel, and the N content (per mg) of the root enamel was consistently lower
713 than that of the tip enamel (4.2 ± 0.9 *vs*. 5.0 ± 1.0 nmol/mg, respectively). Taken together these
714 observations suggest that the organic matter in poorly mineralized enamel is less "protected"
715 from oxidation by mature hydroxylapatite crystals and is thus more readily mobilized and
716 removed during the cleaning process.

717 Mature and immature enamel showed interesting differences in $\delta^{15}N$ values as well. $\delta^{15}N$
718 values for mature enamel were typically higher than immature enamel in both species and for
719 most diet groups (by 0.6 ± 0.4 ‰; see Table 3). (The only exceptions were the guinea pigs that
720 received the meat-based pellet and the rat that received only the breeder pellet. In both of these
721 cases mature and immature $\delta^{15}N$ values were essentially identical). The pattern of lower
722 $\delta^{15}N_{enamel}$ values in immature enamel for the rest of the diets is unexpected if the newly formed
723 enamel is approaching equilibrium with the experimental diets. We would anticipate, for
724 instance, that enamel from the root half of the tooth in both taxa, but most especially in the
725 guinea pigs (in which tip enamel contains some pre-experimental enamel), would be *higher* for
726 the animals that received the insect- and meat-based pellets, reflecting a continued shift towards
727 diet $\delta^{15}N$ values, but in fact the opposite trend is observed.

728 The difference between mature and immature enamel is likely related to differences in
729 the composition of the organic matter itself, which in turn depends on the degree of maturation

of the enamel. Immature enamel consists primarily of organic matter, specifically extracellular matrix proteins (EMPs; i.e. amelogenin, ameloblastin and enamelin) that are enamel-specific and play important roles in enamel formation (enamelogenesis) (Robinson et al., 1978). This partially mineralized enamel is rich in the amino acids proline, glutamic acid, and histidine (Robinson et al., 1995). The prevalence of these amino acids drops sharply just before the opaque boundary. As enamel matures, the organic matrix that shapes and facilitates mineralization is largely degraded and proteolytically removed (Lacruz et al., 2017). Thus, the organic matter in mature enamel is comprised of the remnants of EMPs that contribute to enamel's unique biomechanical properties (i.e. hard but resistant to fracture) as well as enamel-specific proteases (i.e. MMP20 and KLK4) (Robinson et al., 1995; Castiblanco et al., 2015; Lacruz et al., 2017; Welker et al., 2020). In mature enamel, the dominant amino acid is glycine, and aspartic acid and serine are also present (Robinson et al., 1995). We propose that the process of protein degradation may preferentially remove lighter ^{14}N, leaving the residual extracellular organic matrix enriched in ^{15}N relative to organic matter in immature enamel. This argument is supported by studies that have shown that several metabolic processes, including the deamination, transamination, and hydrolysis of proteins are associated with isotope fractionation and contribute to ^{15}N accumulation in tissues (Macko et al., 1986; Silfer et al., 1992; Martinez del Rio et al., 2009; Reitsema, 2013; Poupin et al., 2014). Similarly, the tissues of individuals are elevated in ^{15}N during fasting periods or episodes of negative nitrogen or energy balances, because tissues are catabolized to sustain metabolic function (Hobson et al., 1993; Barboza and Parker, 2006; Merkota et al., 2006), but these processes can be ruled out here as animals were fed *ad libitum*.

Our data suggest that a combination of incomplete growth of enamel during the experimental period as well as effects related to the maturity of the tissue, likely impacted the

753 $\delta^{15}N_{enamel}$ values in this study. While additional sample sets would be helpful to confirm whether
754 the observed ^{15}N enrichment in mature compared to immature enamel is consistent, our data
755 again indicate that these trends do not overprint the original dietary signal. $\delta^{15}N_{enamel}$ values of
756 immature enamel follow the same pattern of "trophic" enrichment (plant < insect < meat) as
757 mature enamel, and animals that consumed plant- vs. meat-based diets are clearly distinguishable
758 (see Figure 5). Moreover, we anticipate that most future applications of the oxidation-
759 denitrification method to tooth enamel will focus on mature enamel, in which the issue of
760 potential variation in $\delta^{15}N$ values due to differences in enamel maturity does not arise.
761

762 **4.4 Comparison of $\delta^{15}N_{enamel}$ to $\delta^{15}N_{soft\ tissue}$ values**

763 The comparison of enamel to soft tissue $\delta^{15}N$ values confirms that $\delta^{15}N_{enamel}$ values
764 record the isotopic composition of diet in a manner similar to that of more commonly measured
765 types of tissues analyzed with traditional combustion methods. Overall, our measured $\Delta^{15}N_{soft}$
766 $_{tissue\text{-}diet}$ enrichment of 1.1–6.1 ‰ agrees well with published data from other controlled feeding
767 experiments which document fractionations between 2 to 6 ‰ (mean = 3.5 ‰; Caut et al., 2009
768 and references therein). Fractionation for liver (4.5 ± 0.7 ‰) is larger compared to the kidney
769 (2.7 ± 0.8 ‰) and muscle (2.7 ± 0.7 ‰) and these enrichments are in line with available data for
770 these tissues in rodents (DeNiro and Epstein, 1981; Arneson, 2005; MacAvoy et al., 2005). Thus,
771 the observed $\Delta^{15}N_{enamel\text{-}diet}$ enrichment of 2 to 4 ‰ falls within the range documented for other
772 tissues, suggesting similar diet-to-tissue fractionation in enamel.
773 In both rats and guinea pigs, we found a positive correlation (Pearson's correlation
774 coefficients 0.76–0.83) in $\delta^{15}N$ values between enamel and all measured soft tissue types (see
775 Figure 6). These correlations indicate that the organic matter preserved in enamel reflects diet-

776 related $\delta^{15}N$ values in a manner similar to soft tissues from the same individual and is therefore
777 capable of differentiating trophic level. It is not unexpected that the regressions do not reflect a
778 perfect 1:1 relationship, because fractionation is not anticipated to be identical across different
779 tissues. Indeed, tissue-specific nitrogen isotope fractionation is well-documented even within the
780 same population (Kelly, 2000; Pearson et al., 2003; Van der Zanden et al., 2015). The tissues
781 measured in this study are comprised of different proteins, that turn over at different rates, and
782 fulfill different physiological and structural roles in the body (MacAvoy et al., 2006; Phillips and
783 Eldridge, 2006; Waterlow et al., 2006; Boecklen et al., 2011). Of particular relevance to this
784 study, Lueders-Dumont et al. (2018), observed a similar correlation between the $\delta^{15}N$ values of
785 fish otoliths - measured using a version of the oxidation-denitrification method employed here -
786 and muscle from the same individual.

787 Promisingly, patterns observed in $\delta^{15}N_{enamel}$ values are also present in soft tissue $\delta^{15}N$
788 values. This is particularly apparent in the case of the rats that received the insect-based diet,
789 where the relative spacing of the tissues differs from that observed in the pelleted diets. This is
790 further evidence that the same factors (e.g., digestive physiology and protein type; see Section
791 4.2) that control soft tissue $\delta^{15}N$ values also affect $\delta^{15}N_{enamel}$ values and that the organic matter
792 preserved in tooth enamel reflects the nitrogen that is bio-available to the organism. Crucially,
793 the separation between plant- and meat-based diets is both seen in the tissues and is preserved at
794 a significant level in the enamel-bound organic matter. Given that the $\delta^{15}N_{enamel}$ values clearly
795 evidences a pattern similar to that observed in the soft tissues of our experimental animals, we
796 have confidence that $\delta^{15}N_{enamel}$ values in the mature tooth enamel records an interpretable diet-
797 related signal that is consistent with what is known about the fractionation behavior of nitrogen
798 isotopes in well-constrained trophic systems.

799

800 **4.5 Future applications of $\delta^{15}N_{enamel}$ analysis**

801 The results of this feeding experiment clearly demonstrate that the nitrogen isotope
802 composition of organic matter bound in tooth enamel records dietary information and can be
803 used as a trophic proxy under controlled experimental conditions. Large-scale ecological studies
804 of nitrogen isotopes of animal tissues document a typical enrichment of 3-4 ‰ between trophic
805 levels in natural food webs (Minagawa and Wada, 1984; Schoeninger and DeNiro, 1984;
806 Schoeninger, 1985; Sealy et al., 1987; Fry, 1988; Caut et al., 2009); the results reported here
807 suggest that this information is also recorded in tooth enamel (i.e. recorded in the organic matter
808 of tooth enamel; $\delta^{15}N_{enamel}$ values).

809 However, ecological studies have also shown that the $\delta^{15}N$ values of animal tissues vary
810 not only as a result of diet composition, but also according to aridity, altitude, and soil
811 composition. These variables play important roles in the nitrogen cycle and can impact the $\delta^{15}N$
812 values of animal's tissues such that trophic signals are potentially obscured (Heaton et al., 1986;
813 Sealy et al., 1987; Ambrose, 1991; Amundson et al., 2003; Männel et al., 2007; Hartman, 2011;
814 Loudon et al., 2016). It is therefore necessary to determine whether a trophic/dietary signal is
815 preserved $\delta^{15}N_{enamel}$ values in complex natural ecosystems that are additionally impacted by
816 ecological variables. To test this, we have analyzed tooth enamel from wild animals belonging to
817 different trophic levels (e.g. herbivores, omnivores, and carnivores) for which we have
818 complementary bone collagen $\delta^{15}N$ data using the method outlined in this paper. The results of
819 these analyses indicate that $\delta^{15}N_{enamel}$ values in natural ecosystems also preserve a trophic signal
820 and are the focus of a future publication.

The logical next step is to employ the method in the analyses of fossil tooth enamel. Validating and interpreting the results of such analyses necessarily depend on whether or not the original organic matter present in the enamel matrix during tooth formation is sufficiently preserved in fossil tooth enamel to record an interpretable nitrogen isotope signal. In this study, preliminary results of $\delta^{15}N_{enamel}$ analyses of three Plio-Pleistocene teeth (see Appendix A1) subjected to reductive-oxidative cleaning yielded N contents and $\delta^{15}N$ values consistent with the removal of exogenous N. These first results are comparable to those obtained in studies of marine fossils including shark tooth enameloid (Kast et al., 2016), fish otoliths (Lueders-Dumont et al., 2018), corals (Wang et al., 2014), and suggest that endogenous nitrogen may be similarly preserved in fossil enamel. While these results are encouraging, rigorous testing is required to determine if fossil $\delta^{15}N_{enamel}$ values preserve an interpretable trophic signal. This would best be done using material from a relatively young, well-characterized fossil assemblage for which the $\delta^{15}N$ baseline values and the trophic dynamics of the system have already been established.

Promisingly, recent efforts to recover ancient proteins in fossil tooth enamel have verified the preservation of endogenous, enamel-specific proteins and proteases, similar to those found in modern tooth enamel, in the teeth of fauna - including hominin species - as old as 1.77 Ma (Casciotti et al., 2014; Welker et al., 2020). Additionally, Welker et al. (2020), confirm that the dental enamel proteome preserves better than that of bone and dentin over geological timescales. This finding is significant, as degradation of the organic matter in bone and dentin and the low organic content of tooth enamel have long prevented nitrogen isotope analysis of fossil material older than 120,000 years (Britton et al., 2012; and see Ostrom et al., 1990, 1993). The presented method could also be of great importance even for relatively young archeological/paleontological sites situated within non-favorable climatic zones, where

844 weathering often results in the degradation of bone and dentin (and a concomitant loss of
845 collagen), but enamel remains well-preserved (e.g. Bourgon et al., 2020).
846 Another important aspect of the oxidation-denitrification method is that it requires only 5
847 mg of enamel. This analytical improvement makes feasible the measurement of $\delta^{15}N$ values in
848 precious material for which sample material is limited and can complement existing $\delta^{18}O$ and
849 $\delta^{13}C$ datasets. Thus, if the resilience of endogenous enamel-bound nitrogen to diagenetic
850 processes can be shown, this method has the potential to significantly improve our understanding
851 of the dynamics of extant and ancient food webs, especially in taxa whose dietary behavior is not
852 immediately apparent from tooth morphology. For example, we know that our early human
853 ancestors began incorporating meat into their diet at some point during the Plio-Pleistocene
854 (Blumenshine and Pobiner, 2006; Bunn, 2006), but this is difficult to infer from the evolution of
855 their tooth morphology alone (Zink and Lieberman, 2016). While stable carbon isotope analysis
856 of the bioapatite in tooth enamel have significantly advanced our understanding of the vegetation
857 consumed by (or consumed by the prey of) early hominins (Lee-Thorp et al., 2010; Ungar and
858 Sponheimer, 2011; Cerling et al., 2013; Sponheimer et al., 2013; Lüdecke et al., 2018), we
859 cannot glean much information about their trophic behavior from this proxy alone. Instead, we
860 must infer meat consumption from the appearance of stone tools and butchered animal remains
861 in the archeological record (Braun et al., 2010; McPherron et al., 2010; Thompson et al., 2015).
862 Provided that endogenous organic matter is preserved in fossil enamel, as observed by Welker
863 (2020) in two hominin teeth from different localities and of different ages, $\delta^{15}N_{enamel}$
864 measurements of early hominin teeth can provide an independent constraint on the intensification
865 of animal resource consumption across the geological record.
866

5. CONCLUSION

In this study, we present the results of a novel application of the oxidation-denitrification method to tooth enamel. We demonstrated that we require ≤5 mg of enamel in order to measure the $\delta^{15}N$ values of the organic matter bound in tooth enamel ($\delta^{15}N_{enamel}$) with a precision of 0.5 ‰. This represents more than a hundred-fold increase in sensitivity compared to traditional combustion methods.

The results of our controlled feeding experiment confirm that the nitrogen isotope composition of enamel reflects diet (with a diet-to-tissue enrichment of ca. 2 to 4 ‰), and that differences in diet are preserved across different taxa. We found that the $\delta^{15}N_{enamel}$ values of animals that received a plant-based diet were consistently lower than those of animals that received a meat-based diet. Significantly, $\delta^{15}N_{enamel}$ values were positively correlated with $\delta^{15}N_{soft\ tissue}$ (liver, kidney, and muscle) values from the same individuals, indicating that fractionation processes that impact the organic matter of tooth enamel are similar to those of other, well-studied and commonly-measured soft tissues. As such, our results demonstrate that $\delta^{15}N_{enamel}$ values have important applications as a dietary and trophic proxy in modern contexts, and also that they have the potential to significantly improve upon currently available methods for the measurement of nitrogen isotopes in the fossil record (i.e. $\delta^{15}N$ values of well-preserved collagen from dentin or bone).

This method opens new avenues of research for reconstructing (paleo)food webs and investigating the trophic ecology of extant and extinct taxa. Immediate experiments should focus on rigorously showing the preservation of trophic signals in ancient ecosystems. Providing this can be done, we may be able to more precisely characterize the dietary behavior of taxa which lived in ancient trophic systems (e.g., synapsids, dinosaurs) or which lacked highly derived

dental morphology (e.g., animals with homodont dentition). This new proxy also has the potential to help delineate major transitions in the dietary behavior of particular lineages (e.g. the transition to meat-eating in early hominins). Identifying the timing and scope of the latter event would represent a milestone in our understanding of early human evolution. In combination with recent advances in our understanding of paleoclimate and paleodiet during the Plio-Pleistocene, $\delta^{15}N_{enamel}$ analysis may offer a complementary, independent line of evidence for investigating dietary behavior in the past.

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AUTHOR CONTRIBUTIONS

Study conception and design: JL, TL, TT and AMG

Feeding Experiment Design: TT and MC

Feeding experiment execution: DW and MC

Experimental design for the analysis of enamel N isotopes: JL, TL and AMG

Measurement of $\delta^{15}N_{enamel}$: JL and TL with support from ND and AMG

Manuscript: JL and TL with significant input from AF, DS, TT and AMG

All authors contributed to the interpretation of the data at different stages of the project and provided input to the final manuscript.

SUPPLEMENTARY MATERIAL

A1: Pre-treatment evaluation for modern and fossil tooth enamel

TABLE A1: N content and $\delta^{15}N_{enamel}$ values for all in-house standards and enamel samples for each pre-treatment type.

TABLE A2: Summary statistics for $\delta^{15}N_{enamel}$ and $\delta^{15}N_{soft\ tissue}$ values for each individual from the feeding experiment

TABLE A3: $\delta^{15}N_{enamel}$ values for all measured tooth enamel samples from the feeding experiment

TABLE A4: $\delta^{15}N_{enamel}$ values for all measured samples for the cleaning test

FIG. A1: N content and $\delta^{15}N_{enamel}$ values for all in-house standards and enamel samples for each pre-treatment type.

FIG. A2: Illustration of rodent incisor sampled from the controlled feeding study

FIG. A3: $\delta^{15}N_{immature\ enamel}$ vs. $\delta^{15}N_{soft\ tissue}$ values

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