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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 is often 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 ($n = 37$). We determined that nitrogen concentration in tooth enamel ($\bar{x} = 5.0 \pm 1.0$ 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 record 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.

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 (Balter et al., 2012, 2019; Bourgon et al., 2020; Chu et al., 2006; Costas-Rodríguez et al., 2014; Heuser et al., 2011; Jaouen et al., 2016, 2018, 2019; Jaouen and Pons, 2017; Knudson et al., 2010; Martin et al., 2014, 2015, 2017, 2020; Sillen and Balter, 2018). 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

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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; Ambrose, 2002; Sponheimer et al., 2003a,b; 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 tissues (Schoeninger and DeNiro 1984; Bocherens and Drucker 2003; Fox-Dobbs et al. 2007; Krajcarz et al. 2018; and see Caut et al. 2009 for discussion of variability in trophic enrichment).

While nitrogen isotope analyses are widely utilized in modern systems, similar analyses of fossil material have largely been restricted to bone- and dentin-derived collagen from relatively young (typically <100,000 years), well-preserved samples in which the original nitrogen isotope composition is unaltered (Bocherens and Drucker 2003; Britton et al., 2012; Jaouen et al. 2019; but see Ostrom et al., 1990, 1993, who measured $\delta^{15}N$ values in fossils from the Late Cretaceous). The organic matter (OM) in bone is nevertheless particularly susceptible to diagenetic alteration over time because bone is porous, has a large surface-to-volume ratio, and its mineral phase is characterized by bioapatite nanocrystals of low crystallinity (e.g., Clementz 2012; Keenan, 2016). In contrast, tooth enamel is highly mineralized (95 % wt. vs. 65 % wt. for bone), and hence more resistant to diagenetic alteration and more frequently well-preserved in deep time (Koch et al. 1997; Lee-Thorp and van der Merwe 1987; Wang and Cerling, 1994; Zazzo et al., 2004; Koch 2007). Most stable isotope analyses of teeth have focused on the mineral fraction of the enamel as opposed to the organic matter. Efforts to measure the $\delta^{15}N$ values of tooth enamel organic matter using traditional combustion methods have been hampered by the low nitrogen content in enamel material (<0.01 % wt.; Savory and Brudevold 1959; Robinson et al. 1995; Robinson, 2014).

Here, we present a novel application of the oxidation-denitrification method that allows high-precision measurement of the nitrogen isotopic composition of tooth enamel ($\delta^{15}N_{enamel}$). This method involves the conversion of nitrogen in enamel-bound organic matter to nitrate (oxidation) followed by bacterial conversion of nitrate (NO_3^-) to nitrous oxide (N_2O) (denitrification). The oxidation-denitrification method was first used by Robinson et al. (2004) for the analysis of (base-soluble) opaline diatom microfossil-bound $\delta^{15}N$, later adapted for (acid-soluble) calcitic microfossils (foraminifera-bound $\delta^{15}N$) by Ren et al. (2009), and is now routinely used for determining $\delta^{15}N$ values of mineral-bound organic matter from a variety of modern and fossil marine invertebrates (e.g., foraminifera, diatoms and corals; Robinson et al. 2004; Ren et al. 2009, 2012; Straub et al. 2013; Martínez-García et al. 2014; Wang et al. 2014; Studer et al. 2015, 2018; Smart et al. 2018, 2020). The method is ≥100 times more sensitive than traditional combustion methods allowing us to reduce the amount of material required for analysis of tooth enamel to ≤5 mg.

The primary goal of this study was to determine whether $\delta^{15}N_{enamel}$ values record the isotopic composition of the diet consumed during tooth formation (i.e., amelogenesis). To test this, the ever-growing mandibular incisors of two rodent species (rat and guinea pig) from a controlled feeding study were analyzed for $\delta^{15}N_{enamel}$ values. Due to the constant, incremental growth of these incisors, this enamel is expected to record the nitrogen isotope composition of the diet continuously. Animals were fed a custom-made plant-, insect-, or meat-based pelleted diet for 54 days. Importantly, experimental diets were isonitrogenous (i.e., contain the same percentage nitrogen) but had distinctly different $\delta^{15}N$ values to simulate different trophic levels. We sought to determine (i) whether $\delta^{15}N_{enamel}$ values record differences between diet groups; (ii) how $\delta^{15}N_{enamel}$ values correlate with $\delta^{15}N_{soft\ tissue}$ data (i.e., liver, muscle, kidney) from the same individuals, (iii) if and to what extent $\delta^{15}N_{enamel}$ values vary between species, and(iv) whether $\delta^{15}N_{enamel}$ values vary according to the degree of enamel maturity.

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. One rat that received only the breeder diet was sampled for measurement (no equivalent guinea pig individual was available). 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 and Snipes, 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. The experimental period of 54 days began after this acclimitization 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 to the nutritional requirements of the animals. To avoid isotopic variation resulting from differences in protein content, all pelleted diets were formulated to be isonitrogenous (21.5 %).

Aliquots of each experimental feed were collected every two weeks (to confirm pellet homogeneity) during the experiment, homogenized with a ball mill, and analyzed for $\delta^{15}N$ (see Section 2.4). Breeder diets for rats and guinea pigs differed both in ingredient and isotopic

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	56 (Lucerne)	26 (Protix Insect Protein Meal)	25 (Lamb Meat)
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

compositions. The guinea pig breeder pellet had higher $\delta^{15}N$ values than the rat breeder pellet (4.8 ‰ vs. 2.4 ‰) and thus the two taxa should have begun the experiment with different tissue isotopic compositions. Experimental diets differed in $\delta^{15}N$ by +3.4 ‰ between plant- and meat-based diet, with +1.1 ‰ between the plant- and insect-based diet and +2.3 ‰ between the insect- vs. meat-based diet (Fig. 1; Table 3). Hence, although the pelleted diets do not represent a direct link in a single food chain, the $\delta^{15}N$ values of the feeds nonetheless approximate trophic level enrichment: the plant pellet has a lower $\delta^{15}N$ value than the animal-product containing pellets.

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 Retsch Cryomill, and 700 ± 0.2 μg of material was weighed into tin capsules for $\delta^{15}N_{soft\ tissue}$ analyses (see Section 2.4). Lipid extractions were not performed on these samples due to the potential for alteration of tissue $\delta^{15}N$ values by the extraction process (Pinnegar and Polunin 1999; Sotiropoulos et al. 2004; Post et al. 2007).

2.2. Tooth enamel nitrogen isotope measurement

2.2.1. Standards and consumables

$\delta^{15}N_{enamel}$ analyses were performed in the laboratories of the Organic Isotope Geochemistry Group of the Department of Climate Geochemistry at the Max Planck Institute for Chemistry (MPIC), Mainz, Germany. All standards used are listed in Table 2. In-house standards include a crushed, homogenized sceleractinian coral *Porites* sp. (PO-1) and deep-sea coral *Lophelia pertusa* (LO-1), as well as two tooth enamel standards, a modern African elephant *Loxodonta africana* (AG-Lox; Gehler et al. 2012) and a fossil (ca. 2.5 to 2.3 Ma) suid *Notochoerus scotti* (Noto-1) from Zone 3A-2 of the Chiwondo Beds in Malawi (i.e., HCRP-RC11-762 in Kullmer, 2008) developed for this study. The values of the in-house standards were determined using the same oxidation-denitrification procedure applied to the samples.

Throughout the entire study, all standards, samples and other solutions were prepared with ultrapure Milli-Q water (18.2 MΩ cm). Reagents used for reductive-oxidative cleaning include: sodium citrate, sodium bicarbonate, sodium dithionite, potassium persulfate (four times re-crystalized), and sodium hydroxide (ACS grade). Reagents used for de-mineralization of enamel and oxidation of organic matter include: hydrochloric acid (Optima grade), potassium persulfate for oxidation (four times re-crystalized), and sodium hydroxide (ACS grade). All 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.

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. The enamel from 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 Fig. A1; Robinson, 2014; Robinson et al., 1977, 1995, 1998). 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 Fig. 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.

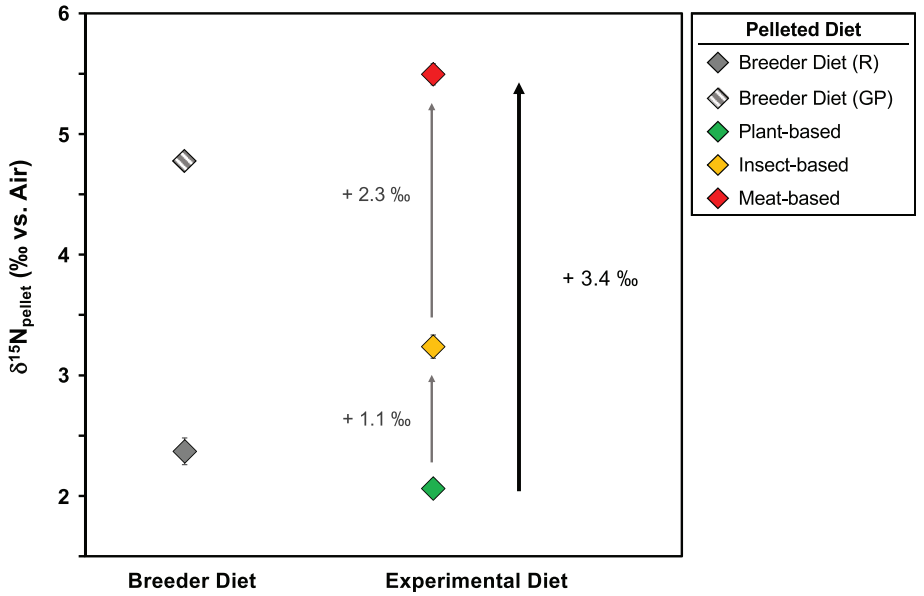

Fig. 1. $\delta^{15}N$ values of breeder and experimental pelleted feeds. Differences in the $\delta^{15}N$ values of experimental feeds are given and indicated with arrows. The offset between the plant- vs. meat-based experimental diet (+3.4 ‰) approximated the average trophic offset observed between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

Table 2
Certified $\delta^{15}N$ values of international standards and measured $\delta^{15}N$ 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	6.1 ± 0.2
LO-1	Coral	10.2 ± 0.2
AG-Lox	Modern Enamel	4.2 ± 0.5
Noto-1	Fossil Enamel	13.7 ± 0.5

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 $\delta^{15}N$ 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 1 g 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). 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:

$$\delta = \left(\frac{R_{sample} - R_{standard}}{R_{standard}}\right) * 1000 \quad (1)$$

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

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

$$\delta^{15}N_{sample} = \frac{\delta^{15}N_{measured} - (f_{blank} * \delta^{15}N_{blank})}{f_{sample}} \quad (2)$$

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

$$f_{blank} = \frac{N\ content_{blank}}{N\ content_{measured}} \quad (3)$$

$$f_{sample} = 1 - f_{blank} \quad (4)$$

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

$$\Delta_{A-B} = \delta_A - \delta_B \quad (5)$$

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

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 A1).

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 pattern of relative "trophic spacing" in $\delta^{15}N_{enamel}$ values (i.e., plant<insect<meat) was consistent within species, with some variation between species $\delta^{15}N_{enamel}$ values depending on which diet was consumed (Fig. 2; Table 3).

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

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

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 ‰) (Fig. 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 (Fig. 4).

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). N content did not differ significantly between species.

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

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.

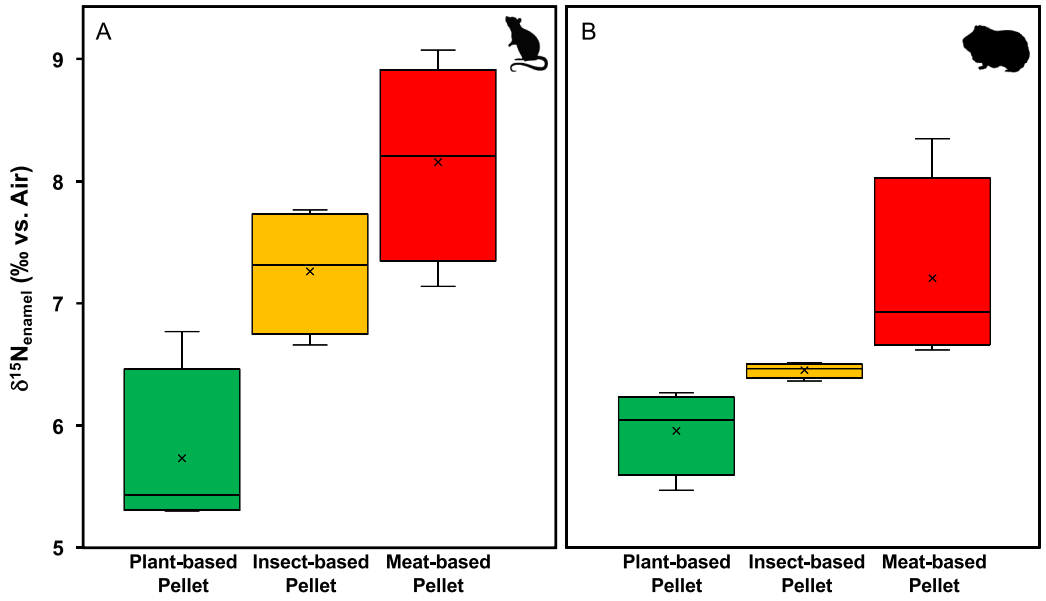

Fig. 2. $\delta^{15}N_{enamel}$ values of mature enamel for rats (A) and guinea pigs (B) by diet. Boxplots show the interquartile range with the median indicated by the solid line and the mean indicated by "x". Average $\delta^{15}N_{enamel}$ values for plant-, insect-, and meat-based diet groups showed the 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)	
		Mature Enamel	Immature Enamel	**Mature Enamel**	Immature Enamel	**Mature Enamel**	Immature Enamel
Rat							
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*).

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.

Measured enamel and soft tissue $\delta^{15}N$ values from the same individual were positively correlated (Fig. 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, $r = 0.76$; $p < 0.001$). Liver had higher $\delta^{15}N$ values relative to enamel, while kidney and muscle exhibited slightly lower values.

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 Fig. 6).

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

^{15}N enrichment in soft tissues between the insect- and meat-based diet groups were smaller than the measured 2.3 ‰ offset between these two pelleted diets in all tissues and in both species. In the rats, this reduced spacing (between 0.4 and 0.5 ‰) was driven by the relatively enriched $\delta^{15}N$ values of the insect-based diet group. Spacing between

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.

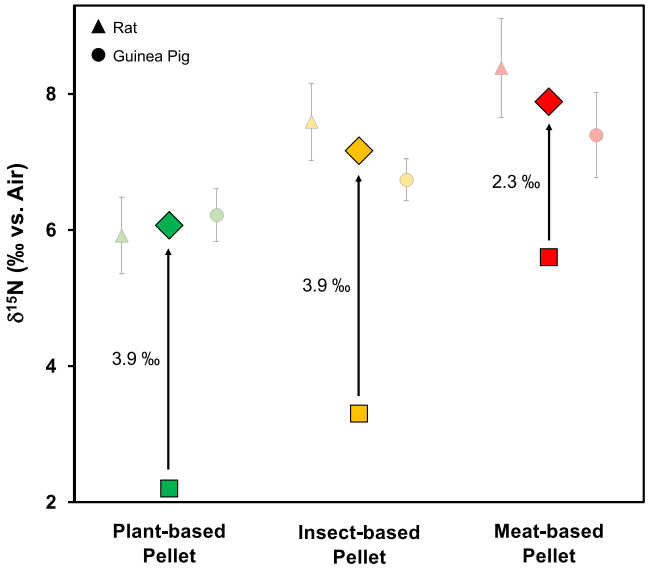

Fig. 3. Mean $\delta^{15}N$ values for pelleted diets (squares) and mature tooth enamel (diamonds; both species with offset in $\delta^{15}N$ values ($\Delta^{15}N_{enamel\text{-}diet}$). Note the smaller offset for the meat-based diet. Mean ($\pm$ 1σ) $\delta^{15}N_{enamel}$ values for rats (triangles)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

the insect- and meat-based diet groups is similar (0.8 ‰) in enamel. In contrast, the offset between these two diets in the soft tissues of guinea pigs was larger (between 1.2 and 1.8 ‰) than that observed in enamel (0.7 ‰) (see Fig. 7).

4. Discussion

In this study, we measured $\delta^{15}N_{enamel}$ values from the tooth enamel of 37 rodents that received isotopically different diets in a controlled feeding experiment. These measurements demonstrate that the average nitrogen concentration in modern tooth enamel (5.0 $\pm$ 1.0 nmol/mg) is sufficient for $\delta^{15}N_{enamel}$ analyses at sample sizes of $\geq$ 5 mg, representing a >100-fold reduction in the amount of material required for traditional combustion methods. We find that $\delta^{15}N_{enamel}$ values reflect the isotopic

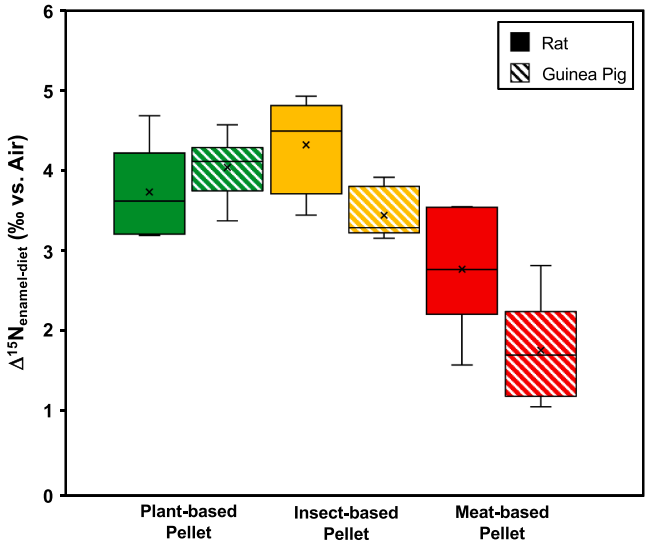

Fig. 4. $\Delta^{15}N_{enamel\text{-}diet}$ for mature enamel of rats (solid) compared to guinea pigs (striped) in ‰ according to diet. Boxplots show the interquartile range with the median indicated by the solid line and the mean indicated by "x". Diets are indicated by color. $\Delta^{15}N_{enamel\text{-}diet}$ 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 and is especially low (+1.8 ‰) in the herbivorous guinea pigs.

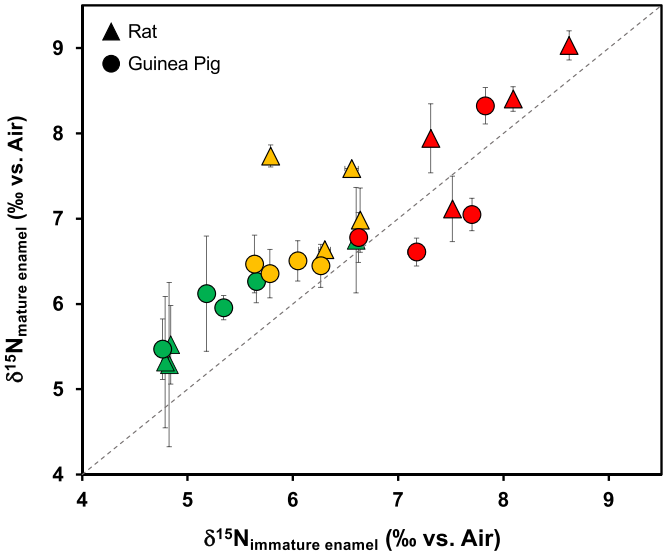

Fig. 5. Immature vs. mature $\delta^{15}N_{enamel}$ values for rats (triangles) and guinea pigs (circles) for plant- (green), insect- (yellow), and meat- (red) based diets. Dashed line represents a 1:1 regression. Error bars represent 1σ where replicates were measured. Immature and mature enamel $\delta^{15}N$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

composition and trophic spacing of the experimental diets with a diet-to-tissue enrichment of ca. 2 to 4 ‰. These $\delta^{15}N_{enamel}$ values correlated positively with $\delta^{15}N_{soft\ tissue}$ values from the same individuals, demonstrating that nitrogen isotopes in enamel reflect the isotopic composition of diet in a manner similar to other tissues. Most importantly, $\delta^{15}N_{enamel}$ values of animals that received plant- vs. meat-based diets followed a clear, expected pattern of relative ^{15}N-enrichment, and were statistically distinguishable from each other regardless of 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

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

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

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 Sections 3.2.2 and 3.2.3), and show a clear shift away from the breeder diets (see Fig. 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

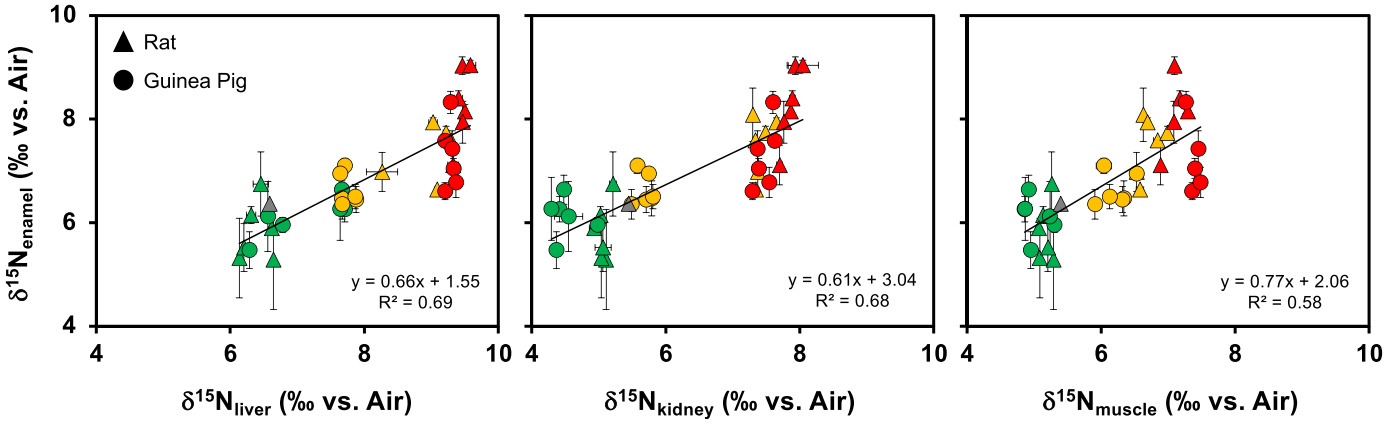

Fig. 6. Soft tissue $\delta^{15}N$ vs. $\delta^{15}N_{enamel}$ values for rats (triangles) and guinea pigs (circles) for plant- (green), insect- (yellow), and meat- (red) based diets. Error bars represent 1σ of replicates measured. Soft tissue and enamel $\delta^{15}N$ values were positively correlated 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.

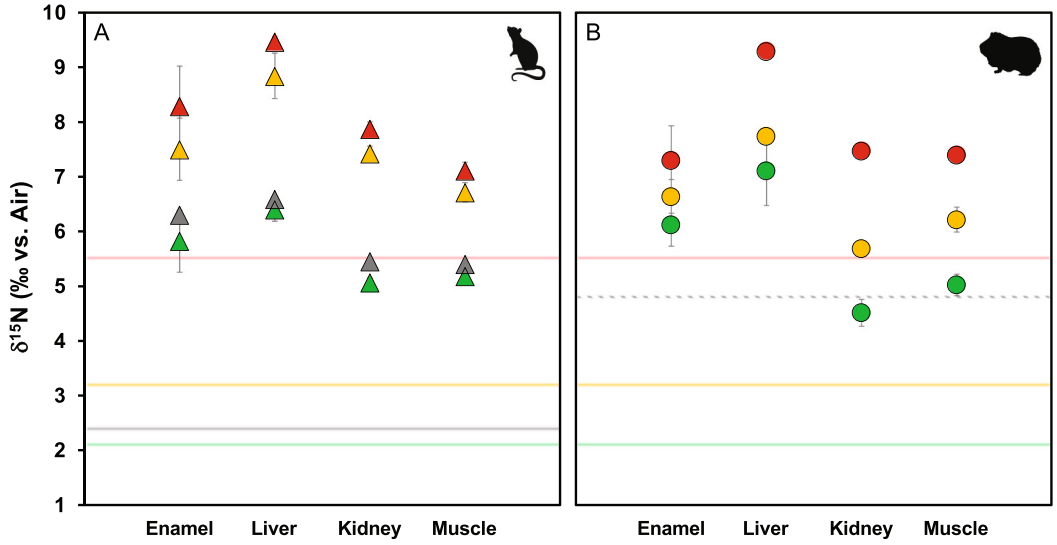

Fig. 7. $\delta^{15}N$ values of pelleted diets, enamel, and soft tissues of rats and guinea pigs analyzed in this study. The measured $\delta^{15}N$ values of the pelleted diets are indicated with horizontal lines. The measured $\delta^{15}N$ values of the enamel and soft tissues for rats (A) and guinea 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.

followed that expected from the isotopic compositions of the pelleted feeds (see Figs. 1 and 2).

The observed enrichment in ^{15}N from diet to tissue was also in line with that documented for other (non-enamel) tissues in previous feeding experiments and ecological studies (DeNiro and Epstein, 1981; Vanderklift and Ponsard, 2003; McCutchan et al. 2003; Bocherens and Drucker 2003; Fox-Dobbs et al. 2007; Caut et al. 2009; Krajcarz et al. 2018). However, the animals which received the meat-based diet were a notable exception; the $\Delta^{15}N_{enamel\text{-}diet}$ of this group was $\geq$1 ‰ lower than that of animals that received plant- or insect-based diets (see Table 3 and Fig. 3). This pattern was evident in both species, but was most pronounced in guinea pigs, where $\Delta^{15}N_{enamel\text{-}diet}$ is only +1.8 ‰ (as compared to ~3–4 ‰ for the two other diets).

One potential explanation for this observation is that complete enamel turnover (here referring to replacement of the incisor via growth and mineralization during the experimental period) may not have occurred in all animals. The total duration of the experiment (i.e., 8 weeks) was designed such that the animal's mandibular incisors would completely (rats) or partly (guinea pigs) turn over during the course of the experiment (see Section 3.2.1). However, if enamel turnover was incomplete, the enamel formed during the period prior to the experiment (i.e., while the animal received the breeder pellet) contributed to $\delta^{15}N_{enamel}$ values in the measured samples. In guinea pigs, whose incisors grow at a slower rate than rats, we can be certain that at least some of the enamel from the tip half of the tooth contained material that grew before the experiment began. This could have impacted the measured $\delta^{15}N_{enamel}$ values for the mature (tip) enamel (see Section 4.3 for discussion of $\delta^{15}N$ values of mature vs. immature enamel). Interpretation of $\delta^{15}N_{enamel}$ values for the guinea pigs should thus be made with this in mind.

In contrast, the rat's incisors should have re-grown entirely during the experimental period (see Section 3.2.1). Unless the organic matrix of tooth enamel is synthesized from a pool 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 does 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 (Cantalapiedra-Hijar et al., 2015; DeMots et al., 2010; Robbins et al., 2005; Van Klinken et al., 2002; Sealy et al., 1987) though the mechanisms that drive this remain elusive. Sponheimer et al. (2003a) 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 herbivores in the study. This research suggests that interspecific variations in digestive physiology can lead to large shifts in $\delta^{15}N$ values.

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

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

Rodents also engage in caecotrophy to varying degrees. Caecotrophs selectively retain nitrogen-rich material (esp. microbes) in their caecum and excrete this material as a special type of feces that is immediately re-ingested from the anus (Björnhag and Snipes, 1999). This behavior has been explicitly demonstrated in rats (Sperber et al. 1983) and guinea pigs (Holtenius and Björnhag 1985; Takahashi and Sakaguchi 2006). Indeed, evidence for microbe-related fatty acids in the body fat of the experimental animals from this study suggests that the guinea pigs relied more on this mechanism than did the rats (De Cuyper et al. 2020). Unknown nitrogen isotope fractionation during metabolism of nutrients by gut microbes and differential uptake of the resulting microbially-derived protein could therefore contribute to species differences in diet-to-tissue fractionation. Specific studies that unravel the different contributions of microbially transformed dietary nitrogen in rodent nutrition and related isotope-specific effects of enzymatic protein digestion are required to resolve such issues.

In summary, given their distinct digestive adaptations and behaviors (e.g., dentition, digestive efficiency, and caecotrophy), it is possible that rats and guinea pigs metabolized the plant- and animal- based proteins in the experimental feeds differently. However, while it is probable that these variables contributed to the observed inter-species inter-diet differences, the specific mechanisms driving the relationship between these factors and nitrogen isotope fractionation remain poorly understood, particularly for small mammals.

4.2.2. Variation in protein type

Plant proteins contain fewer essential amino acids than do proteins derived from animal tissues, and thus dietary amino acids derived from plants require more "metabolic processing" than do proteins from animal tissues (Gaebler et al. 1966; Silfer et al. 1992). In contrast, animal tissue proteins typically originate from endogenous amino acids that have been transaminated or recycled from protein degradation (Waterlow 2006). The additional metabolic processing can induce greater isotopic fractionation during the digestion of plant proteins compared to animal proteins (Poupin et al. 2011). Therefore, fractionation occurring as a result of plant protein consumption can contribute more significantly to the trophic effect in nitrogen than fractionation 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) (Fig. 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 $\delta^{15}N_{enamel}$ values for the insect- and plant-based diet groups did not differ significantly. This lack of significant difference is arguably unsurprising considering (i) the probable incomplete turnover of enamel in guinea pigs, (ii) the degree of individual variation *within* dietary groups (0.4–0.7 ‰), (iii) the current precision of the method for tooth enamel (0.5 ‰), and (iv) the relatively small isotopic difference between the plant- and insect-based diets (i.e., 1.1 ‰; see Fig. 1). Under these conditions, this small isotopic difference is probably insufficient, *per se*, to result in significant differences between these dietary groups.

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

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

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

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

Mature tooth enamel consists of ~95 % mineral (predominantly hydroxyapatite), ~2–4 % water, and ~1–2 % organic matter by weight (Lacruz et al. 2017). The crystal structure of the enamel consists of many millions of nearly identical, highly ordered crystals of calcium hydroxyapatite with extraneous mineral components such as carbonate and magnesium. The outer enamel layer in some rodent taxa, including the rats in this study, is rich in iron, which gives the teeth a characteristic orange color (Robinson et al. 1995). In contrast, immature tooth enamel is comprised of a soft, partially mineralized tissue (~30 % mineral wt. at initial secretion) that is predominantly organic material and water (Deakins 1942; Robinson et al., 1977, 1995; Lacruz et al. 2017).

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

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

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

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

The comparison of enamel to soft tissue $\delta^{15}N$ values confirms that $\delta^{15}N_{enamel}$ values record the isotopic composition of diet in a manner similar to that of more commonly measured types of tissues analyzed with traditional combustion methods. Overall, our measured $\Delta^{15}N_{soft\ tissue\text{-}diet}$ enrichment of 1.1–6.1 ‰ agrees well with published data from other controlled feeding experiments which document fractionations between 2 to 6 ‰ (mean = 3.5 ‰; Caut et al. 2009 and references therein). Fractionation for liver (4.5 ± 0.7 ‰) is larger compared to the kidney (2.7 ± 0.8 ‰) and muscle (2.7 ± 0.7 ‰) and these enrichments are in line with available data for these tissues in rodents (Arneson and MacAvoy, 2005; DeNiro and Epstein, 1981; MacAvoy et al., 2005). Thus, the observed $\Delta^{15}N_{enamel\text{-}diet}$ enrichment of 2 to 4 ‰ falls within the range documented for other tissues, suggesting similar diet-to-tissue fractionation in enamel.

In both rats and guinea pigs, we found a positive correlation (Pearson's correlation coefficients 0.76–0.83) in $\delta^{15}N$ values between enamel and all measured soft tissue types (see Fig. 6). These correlations indicate that the organic matter preserved in enamel reflects diet-related $\delta^{15}N$ values in a manner similar to soft tissues from the same individual and is therefore capable of differentiating trophic level. It is not unexpected that the regressions do not reflect a perfect 1:1 relationship, because fractionation is not anticipated to be identical across different tissues. Indeed, tissue-specific nitrogen isotope fractionation is well-documented even within the same population (Kelly, 2000; Pearson et al., 2003; Vander Zanden et al., 2015). The tissues measured in this study are comprised of different proteins, that turn over at different rates, and fulfill different physiological and structural roles in the body (MacAvoy et al. 2006; Phillips and Eldridge 2006; Waterlow, 2006; Boecklen et al., 2011; Caut et al., 2008). Of particular relevance to this study, Lueders-Dumont et al. (2018), observed a similar correlation between the $\delta^{15}N$ values of fish otoliths - measured using a version of the oxidation-denitrification method employed here - and muscle from the same individual.

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

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

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

However, ecological studies have also shown that the $\delta^{15}N$ values of animal tissues vary not only as a result of diet composition, but also according to aridity, altitude, and soil composition. These variables play important roles in the nitrogen cycle and can impact the $\delta^{15}N$ values of animal's tissues such that trophic signals are potentially obscured (Heaton et al. 1986; Sealy et al., 1987; Ambrose 1991; Amundson et al., 2003; Männel et al. 2007; Hartman 2011; Loudon et al. 2016). It is therefore necessary to determine whether a trophic/dietary signal is preserved $\delta^{15}N_{enamel}$ values in complex natural ecosystems that are additionally impacted by ecological variables. To test this, we have analyzed tooth enamel from wild animals belonging to different trophic levels (e.g., herbivores, omnivores, and carnivores) for which we have complementary bone collagen $\delta^{15}N$ data using the method outlined in this paper. The results of these analyses indicate that $\delta^{15}N_{enamel}$ values in natural ecosystems also preserve a trophic signal 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), and 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 (Cappellini et al., 2019; 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 Ostrom et al., 1993, 1994). The presented method could also be of great importance even for relatively young archeological/paleontological sites situated within non-favorable climatic zones, where weathering often results in the degradation of bone and dentin

(and a concomitant loss of collagen), but enamel remains well-preserved (e.g., Bourgon et al. 2020).

Another important aspect of the oxidation-denitrification method is that it requires only 5 mg of enamel. This analytical improvement makes feasible the measurement of $\delta^{15}N$ values in precious material for which sample material is limited and can complement existing $\delta^{18}O$ and $\delta^{13}C$ datasets. Thus, if the resilience of endogenous enamel-bound nitrogen to diagenetic processes can be shown, this method has the potential to significantly improve our understanding of the dynamics of extant and ancient food webs, especially in taxa whose dietary behavior is not immediately apparent from tooth morphology. For example, we know that our early human ancestors began incorporating meat into their diet at some point during the Plio-Pleistocene (Blumenschine and Pobiner, 2007; Bunn, 2006), but this is difficult to infer from the evolution of their tooth morphology alone (Zink and Lieberman 2016). While stable carbon isotope analysis of the bioapatite in tooth enamel have significantly advanced our understanding of the vegetation consumed by (or consumed by the prey of) early hominins (Lee-Thorp et al. 2010; Ungar et al., 2011; Cerling et al. 2013; Sponheimer et al. 2013; Lüdecke et al. 2018), we cannot glean much information about their trophic behavior from this proxy alone. Instead, we must infer meat consumption from the appearance of stone tools and butchered animal remains in the archeological record (Braun et al. 2010; McPherron et al. 2010; Thompson et al. 2015). Provided that endogenous organic matter is preserved in fossil enamel, as observed by Welker et al. (2020) in two hominin teeth from different localities and of different ages, $\delta^{15}N_{enamel}$ measurements of early hominin teeth can provide an independent constraint on the intensification of animal resource consumption across the geological record.

5. Conclusion

In this study, we present the results of a novel application of the oxidation-denitrification method for tooth enamel. We demonstrated that we require $\leq$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 demonstrating 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

Conceptualization: JL, TL, MC, TT and AMG.

Methedology: Feeding Experiment DW, TT and MC; Experimental design for the analysis of enamel N isotopes: JL, TL, ND and AMG.

Formal analysis: JL and TL with support from AF, ND and AMG.

Writing - original draft: JL and TL. Writing - review & editing: JL, TL, AF, MC, 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.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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