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A long-term study of stable isotope ratios of fingernail keratin and amino acids in a mother–infant dyad

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Abstract

Objective: To evaluate the potential of compound-specific isotope analysis of amino acids (CSIA-AA) for investigating infant feeding practices, we conducted a long-term study that compared infant and maternal amino acid (AA) nitrogen isotope ratios.

Materials and Methods: Fingernail samples were collected from a single mother–infant dyad over 19 months postpartum. Carbon and nitrogen stable isotope ratios were measured in the bulk keratin of the fingernail samples. Selected samples were then hydrolyzed and derivatized for compound-specific nitrogen isotope analysis of keratin AAs.

Results: As in previous studies, infant bulk keratin nitrogen isotope values increased during exclusive breastfeeding and fell with the introduction of complementary foods and eventual cessation of breastfeeding. Infant trophic AAs had elevated nitrogen isotope values relative to the mother, while the source AAs were similar between the mother and infant. Proline and threonine appeared to track the presence of human milk in the infant's diet as the isotopic composition of these AAs remained offset from maternal isotope values until the cessation of breastfeeding.

Discussion: Although CSIA-AA is costly and labor intensive, it appears to hold potential for estimating the duration of breastfeeding, even after the introduction of complementary foods. Through the analysis of a full suite of AAs, it may also yield insights into infant physiology and AA synthesis.

KEYWORDS
amino acids, breastfeeding, fingernails, nitrogen, weaning

1 | INTRODUCTION

The study of infant feeding practices provides an intimate look into the day-to-day maintenance activities that sustain human life across cultures and through human evolutionary history (e.g., Britton et al., 2018; Cocozza, Fernandes, Ughi, Croß, & Alexander, 2021; Waters-Rist, Bazaliiskii, Weber, & Katzenberg, 2011; Wright & Schwarcz, 1999). Stable carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotope ratio analysis of human proteins, typically type I collagen or keratin, has underscored several pivotal inquiries that remain unresolved in the analysis of infant diets such as: what is the isotopic offset between maternal materials (e.g., tissue or milk) and diet (Herrscher et al., 2017; Reynard & Tuross, 2015)?; how does physiological stress during pregnancy or infancy affect the balance of nitrogen in the

neonate (Beaumont et al., 2015; Fuller et al., 2005; Walter et al., 2020)?; and can the consumption of human milk be distinguished from foods from high trophic levels, such as aquatic resources (Cheung et al., 2022)?

Higher resolution methods, including incremental analysis of infant tissues and compound-specific isotope analysis of amino acids (CSIA-AA), are poised to address the compelling challenges associated with past infant feeding practices. Widely employed in ecology, CSIA-AA has only recently been applied to analyses of age-related changes in the human diet (Cheung et al., 2022; Mora et al., 2022). Rather than measure entire tissues, in CSIA-AA, bulk proteins are hydrolyzed into their constituent amino acids (AAs), which are then measured individually, typically using gas chromatography combustion isotope ratio mass spectrometry for nitrogen (GC-C-IRMS). To categorize AAs, here we use a classification that is based on the nitrogen isotope fractionation; AAs can be classified as "trophic" or "source" by the degree to which they undergo nitrogen isotope fractionation due to trans- and deamination with the free AA pool (McClelland & Montoya, 2002; Popp et al., 2007). Researchers have used the spacing between trophic and source AAs, usually glutamic acid (Glu) and phenylalanine (Phe), to estimate the trophic position of a target organism (e.g., Chikaraishi et al., 2010; McClelland et al., 2003; McClelland & Montoya, 2002) while others have examined a wider range of AAs using principal components analysis and Bayesian modeling (Chen et al., 2023; Soncin et al., 2021). The nitrogen isotopic patterning in AAs is key to understanding the variability observed in infant nitrogen isotope compositions and more accurately interpreting infant life events (O'Connell, 2017). Bulk tissue $\delta^{15}N$ values are a weighted average of the isotopic composition of the individual AAs and a variety of other N-containing compounds composing the tissue; therefore, by measuring the isotopic composition of AAs, it is possible to ground-truth the assumptions developed from bulk protein isotope measurements of archaeological populations.

1.1 | Stable isotope analysis of bulk tissues

Stable nitrogen isotope values undergo a stepwise increase of approximately +3 to +6‰ between trophic levels and are useful for assessing protein sources in consumer diets (Fernandes et al., 2015; Hedges et al., 2009; Hedges & Reynard, 2007; O'Connell et al., 2012). Trophic enrichment is due to the transamination of some AAs during their incorporation into body tissues and the preferential excretion of ^{14}N in nitrogenous waste products (Minagawa & Wada, 1984; Miura & Goto, 2012). Physiological stress may also increase tissue $\delta^{15}N$ values as catabolic processes cause AAs from skeletal muscle to be released back into the free AA pool (D'Ortenzio et al., 2015; Fuller et al., 2005; Hobson et al., 1993; Mekota et al., 2006, 2009; Neuberger et al., 2013). Differences in stable carbon isotope values are largely caused by multiple photosynthetic pathways (e.g., C_3, C_4, and Crassolucean acid metabolism) among plants and between terrestrial and marine primary producers (O'Leary, 1981; Schoeninger & DeNiro, 1984). The $\delta^{13}C$ value of protein in primary producers at the base of the food chain is transferred to consumers with a comparatively small isotopic fractionation (~1‰; McCutchan et al., 2003). The $\delta^{13}C$ value of a consumer's proteins is elevated by approximately 5‰ relative to whole diet, but this offset can vary with diet composition and the isotopic composition of dietary macronutrients (Ambrose & Norr, 1993; Fernandes et al., 2012; Froehle et al., 2010). The isotopic composition of consumer proteins, such as keratin, will largely reflect that of dietary protein over other sources of carbon, such as lipids or carbohydrates (Fernandes et al., 2012; Jim et al., 2004).

The interaction between nutrient routing and lactogenesis influences the degree of isotopic offset between maternal tissues and milk. Drawing on evidence from ecological and observational studies, in humans and other species that produce milk from nutrients consumed concurrently, maternal milk has lower $\delta^{15}N$ values by 1.5 to 2‰ relative to maternal tissues (Cherel et al., 2015; Herrscher et al., 2017). The isotopic spacing of mother–infant dyads is reduced relative to the expected trophic level shift of 3–6‰; infant tissue $\delta^{15}N$ values are approximately 1–4.5‰ higher than maternal milk and only 1.2–2.5‰ higher than maternal tissues (Cherel et al., 2015; De Luca et al., 2019; Herrscher et al., 2017; Stegall et al., 2008; Stricker et al., 2015). In species that rely on stored nutritional resources to produce milk, milk proteins are enriched in ^{15}N relative to maternal tissues (Borrell et al., 2016; Habran et al., 2010; Polischuk et al., 2001).

The rapidity of isotopic change associated with maternal milk consumption and subsequent supplementation with non-milk foods also depends on the turnover rate of the sampled tissue. Human hair and, less frequently, fingernails are routinely sampled in forensic contexts as the isotopic composition of keratins is linked to that of dietary protein (Fraser et al., 2006; Lehn et al., 2011). In two longitudinal studies of healthy adult volunteers, following a dietary change, hair $\delta^{15}N$ reached isotopic equilibration after 5 months, while carbon responded more slowly (Hülsemann et al., 2009; O'Connell & Hedges, 1999). Like hair, fingernails grow incrementally and are isotopically inert once formed. However, the slower growth rate of nails has been suggested to result in greater averaging of the isotopic composition (Lehn et al., 2011). Isotopic attenuation is expected if infant proteins are sourced from multiple isotopic pools: nitrogen in human milk and nitrogen from protein breakdown and recycling (Ayliffe et al., 2004; Waterlow, 2006). Through regular sampling of the fingernails of six infants across two studies, after correcting for infant nail growth, the peak $\delta^{15}N$ value associated with exclusive breastfeeding was not achieved until ~10–30 weeks after birth (Fuller et al., 2005; Herrscher et al., 2017).

Human milk is a dynamic and bioactive substance composed of macro- and micronutrients, free AAs, urea and non-protein nitrogen, immune and growth factors, hormones, and microbiota, all of which serve critical developmental functions (Donovan & Lönnerdal, 1989), but not all of these are bioavailable or digestible in unaltered form (Fomon et al., 1988; You et al., 2023). Of these components, milk proteins are the most relevant for interpreting infant $\delta^{15}N$ data. The concentration of protein in human milk varies between 14 g/L in colostrum to 8 g/L in mature milk (Liao et al., 2017; Lönnerdal et al., 2017; Thakkar et al., 2016). Milk proteins can generally be

classified as whey proteins or caseins, and these are present in a ratio of ~90:10 in colostrum and 50:50 in later lactation (Kunz & Lönnerdal, 1992). Whey proteins include alpha-lactalbumin, lactoferrin, and secretory immunoglobulin A (IgA), while human casein is dominated by kappa-casein, beta-casein, and minor amounts of alpha-casein (Rasmussen et al., 1995). Theoretically, changes in the protein composition of milk have the potential to affect its isotopic composition, but this was not observed in the longitudinal study of human milk (Herrscher et al., 2017). The caseins have high concentrations of proline (Pro) relative to other AAs, while the whey proteins have higher concentrations of leucine (Leu) and threonine (Thr) (Brignon et al., 1985; Findlay & Brew, 1972; Greenberg et al., 1984; Metz-Boutigue et al., 1984; Mole et al., 1977; Rasmussen et al., 1995; Toraño & Putnam, 1978). Colostrum and transitional milk have lower concentrations of aromatic AAs (Phe and Tyr) as neonates have low levels of the enzymes required to metabolize them (Lawrence & Lawrence, 2011).

Archaeological interpretations assume a relatively straightforward trophic relationship between mothers and infants. If maternal milk has a higher $\delta^{15}N$ value than the maternal diet, then the bone or dentine collagen of an infant exclusively consuming human milk will have higher $\delta^{15}N$ values than the mother due to isotopic fractionation during tissue synthesis (Jenkins et al., 2001). With complementary feeding, infant $\delta^{15}N$ values will decrease, providing the foods have a lower $\delta^{15}N$ value than human milk (Fogel et al., 1989; Fuller, Fuller, et al., 2006). Otherwise, if the complementary foods are enriched in ^{15}N relative to human milk, any decline in the infant's $\delta^{15}N$ value associated with decreasing human milk consumption will be masked (Cheung et al., 2022). Among archaeological populations, infants with estimated ages below 2 years typically have collagen $\delta^{15}N$ values that are 2–3‰ higher than those of adult females from the same population (Howcroft et al., 2012; Jay, 2009; Richards et al., 2002), which is consistent with observations from modern mother–infant dyads (Fuller, Fuller, et al., 2006; Herrscher et al., 2017). A slight (~ + 1‰) increase in the $\delta^{13}C$ values of infant proteins over those of the mother may also be observed (Fuller et al., 2003; Fuller et al., 2005), but the timing and direction of change of infant $\delta^{13}C$ values are less predictable (De Luca et al., 2012; Herrscher et al., 2017).

1.2 | Compound-specific isotope analysis of AAs

AAs are composed of an amine (NH_2) group, a carboxyl (COOH—) group, and a unique side chain, or R-group. The trophic AAs, Pro, alanine (Ala), Leu, isoleucine (Ile), valine (Val), and aspartic acid (Asp) exchange nitrogen with glutamine (Gln) and Glu in the central body nitrogen pool (O'Connell, 2017) which produces $\delta^{15}N$ values that are 3–8‰ higher than those measured in diet (Chikaraishi et al., 2007; Chikaraishi et al., 2009; McClelland & Montoya, 2002; Popp et al., 2007). Phe, lysine (Lys), methionine (Met), and tyrosine (Tyr) do not undergo trans- or deamination during the synthesis of tissues, resulting in $\delta^{15}N$ values that approximate those in dietary proteins (Chikaraishi et al., 2007, 2009; Popp et al., 2007). Phe appears to undergo some fractionation in humans with liver cirrhosis (Fuller & Petzke, 2017; Petzke et al., 2010). Glycine (Gly) and serine (Ser) are no longer understood to be source AAs as they can undergo fractionation depending on the organism under study (McMahon & McCarthy, 2016). Thr also undergoes isotopic fractionation but becomes progressively depleted in ^{15}N with trophic level, although the exact biochemical mechanism requires further clarification (Fuller & Petzke, 2017; Wallace & Hedges, 2016).

To date, few studies have investigated infant diets using CSIA-AA. Romek et al. (2013) found no correlation between the $\delta^{15}N$ values of human milk and infant hair from mother–infant dyads. These data are challenging to interpret because, at the time of sampling, 1 month postpartum, it is unlikely that infant hair would have reached isotopic equilibrium with human milk (Hülsemann et al., 2009; O'Connell & Hedges, 1999). Further, contaminants found in cosmetic hair products can penetrate and persist in the hair shaft even after treatment with solvents and may alter the in vivo isotopic composition of hair (Santos et al., 2015). Despite previous negative results, the analysis of $\delta^{15}N$ from AAs in mother–infant dyads may be a promising means for distinguishing human milk from high trophic level complementary foods or between dietary and physiological influences on the isotopic composition of infant tissues.

Here, we track the changes in infant and maternal fingernail AA $\delta^{15}N$ values of a single mother–infant pair over 19 months, including the period of exclusive breastfeeding, the introduction of complementary foods into the infant's diet, and the cessation of breastfeeding. These data provide insight into the interactions between diet and physiology during infancy and represent a critical step in developing a framework for understanding variation observed in bulk keratin and collagen data from archaeological samples.

2 | MATERIALS AND METHODS

The female volunteer and her infant, born early term and assigned male at birth, were both residents of the United States at the time of sampling. Early term infants are those born between 37 weeks, 0 days and 38 weeks, 6 days gestation (American College of Obstetricians and Gynecologists, 2013). The infant was delivered via uncomplicated vaginal birth and did not require medical support or antibiotics after birth. The species diversity and composition of gut microbiomes are known to vary with gestational age, but this difference is much more pronounced between very preterm (<32 weeks gestation) and moderate preterm infants than between the latter and term infants (Chernikova et al., 2018).

Sampling of both maternal and infant fingernails began 109 days after birth and proceeded in irregular intervals thereafter as sampling was dictated by nail growth. The shortest sampling interval was 5 days and the longest was 48 days. Sample dates and intervals are recorded in Table S1. Before each fingernail collection took place, the hands of each individual were washed with water and soap. Since the mother did not use nail care products, sanding the top of the nail bed before sample collection was unnecessary. The samples were taken from the free edges of the nail plate that had reached beyond the fingertip on both hands. As the nails of each finger grow at

different rates (Gupta et al., 2005; Yaemsiri et al., 2010), multiple fingers from each hand were sampled to promote sample homogeneity. Fingernails of the dyad were bagged separately by sampling date. The samples were cryogenically frozen and reduced to fragments of <0.5 mm. The samples were then transferred to culture tubes and ultrasonicated in 4 mL of acetone (room temperature, 1 h), followed by sonication in 4 mL of ethanol (room temperature, 1 h) to remove exogenous carbon that may have contaminated the nail plate between formation and growth. Organic solvent residues were removed from the samples by multiple rinses with 18.2 MΩ DDH_2O, followed by sonication in DDH_2O for 10 min. An overall sample loss of about 10% was observed during the pretreatment due to difficulty visualizing the sample while pipetting. Finally, the samples were dried on a heating block (12 h, 70°C). Aliquots of clean, dry fingernails ranging from 0.6 to 0.7 mg were weighed individually into tin capsules and placed inside an auto-sampler drum for ^{13}C, ^{15}N, and elemental C/N determinations by a continuous flow stable isotope ratio mass spectrometer (Finnigan Delta-Plus CFIRMS) interfaced to a Fisons NA1500NC elemental analyzer (E.A.). The stable isotope results are measured as the ratio of the heavier isotope to the lighter isotope ($^{13}C/^{12}C$ and $^{15}N/^{14}N$) and reported as δ values in parts per 1000 or per mil (‰) units relative to internationally defined standards for carbon (Vienna Pee Dee Belemnite, VPDB) and nitrogen (Ambient Inhalable Reservoir, AIR) (Sharp, 2007). The analytical uncertainty of the measurements was calculated using the calibration and check standards from each run and was <0.2‰ for both ^{13}C and ^{15}N.

To align the outer nail clippings of the infant and mother to the period of growth, we subtracted eight and 16 weeks, respectively, from the dates that the fingernail free-edges were collected (Fogel et al., 1989; Runne & Orfanos, 1981) to match with dates associated to recorded diet changes. Infant feeding practices (e.g., exclusive breastfeeding) are defined following the WHO/UNICEF breastfeeding definitions (2008) (World Health Organization, 2008). The mother–infant dyad consumed a mixed C_3/C_4 omnivorous diet. The maternal diet comprised approximately 70% fruits, vegetables, and dairy products, 20% poultry and eggs, and 10% seafood, all expressed as a volume of food consumed. The infant exclusively consumed human milk between birth and 6 months of age, with two exceptions at 3 and 6 months when a cow's milk-based infant formula was consumed for 3 to 4 days. Complete dietary details, including the timing and types of complementary foods provided to the infant, are presented in Table 1.

TABLE 1 Infant diet by age.

Age of infant (months)	Diet
0–6	Human milk*
6	Human milk, pureed fruits, rice cereal
7	Human milk, pureed vegetables, fruits, rice cereal, poultry
12	Human milk, fruits, vegetables, poultry, cow's milk
15	Cow's milk, family diet without seafood

*Infant consumed baby formula (cow's milk base) at 3 and 6 months of age for 3–4 days each time.

2.1 | Compound-specific nitrogen isotope analysis of keratin AAs

Due to low sample mass, it was not possible to obtain $\delta^{15}N$ values for bulk keratin and keratin AAs for the same samples. For $\delta^{15}N$ values analysis of AAs, the samples were prepared and analyzed as *N*-pivaloyl-amino-acid-i-propyl esters via GC-C-IRMS following the method presented in Riekenberg et al. (2020). In brief, approximately 2 mg of cleaned, dried, and homogenized fingernail tissue was hydrolyzed in 6 M hydrochloric acid (HCl) (10–12 h, 110°C), then filtered (0.45 µm) and lipid extracted by washing with *n*-hexane/dichloromethane (v/v 6:1) after Chikaraishi et al. (2009) and Svensson et al. (2016) prior to esterification (isopropanol/acetyl chloride) and acylation (dichloromethane/pivaloyl chloride). An internal standard of norleucine was added to each sample before derivatization. During hydrolysis, asparagine (Asn) and glutamine (Gln) are deaminated to aspartic acid (Asp) and glutamine acid (Glu)and are reported as Asx (Asp + Asn) and Glx (Glu + Gln). $\delta^{15}N$ values for Ala, Asx, Glx, Gly, Leu, Lys, Ile, Phe, Pro, Ser, The, Tyr, and Val are reported. This method builds upon the method presented in Chikaraishi et al. (2007) but incorporates normalization using two standard reference mixtures, one for developing a compound-specific correction or offset and another for scale normalization, along with a reference spike of norleucine added to all samples and standards, which served as an internal reference calibration following the method from Yarnes and Herszage (2017). The data analysis, normalization methods, and known $\delta^{15}N$ values of the standards have been thoroughly documented in Riekenberg et al. (2020). A run sequence was composed of one un-derivatized alkane mix, four replicates of the offset AA mix, two replicates of the scaling AA mix, and then two sample replicates alternating with the offset AA mix until the end of the day. This analysis occurred at the Royal Netherlands Institute for Sea Research using a Trace 1310 Gas Chromatograph coupled to a Delta V Advantage isotope ratio mass spectrometer via a GC Isolink II (Thermo Fisher, Bremen). The precision of AA $\delta^{15}N$ values was <0.5‰ in the offset standards run 10× per sequence for 13 AAs throughout the analytical runs supporting this study, and sample duplicates differed by ±0.2‰. An example chromatogram of the compound-specific correction standard and one of a keratin sample are included in Figure S1. The measured mean and standard deviations for the $\delta^{15}N$ values from the offset AA standard mix are reported in Table S2.

3 | RESULTS AND DISCUSSION

3.1 | Bulk keratin $\delta^{13}C$ and $\delta^{15}N$ values

The $\delta^{13}C$ and $\delta^{15}N$ values, %C and %N, and atomic C:N of bulk keratin from infant and maternal fingernails are presented in Figure 1 and Table S1. Throughout the longitudinal study maternal fingernail $\delta^{13}C$

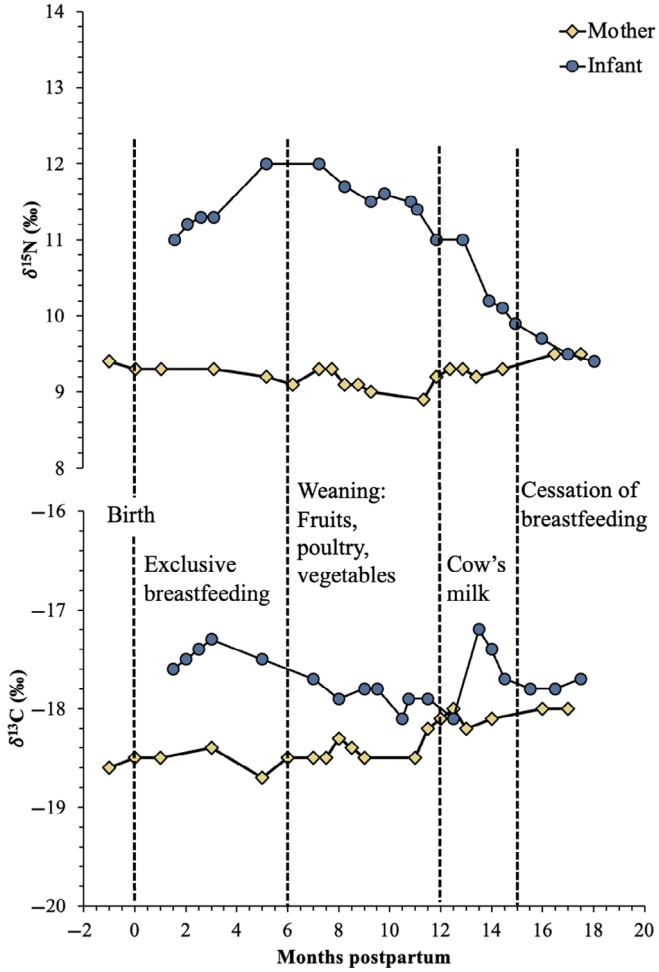

FIGURE 1 $\delta^{15}N$ and $\delta^{13}C$ values from mother and infant fingernails, adjusted for nail growth. Infant dietary events are marked between the dashed lines. The error on bulk keratin stable isotope measurements did not exceed 0.2‰ for carbon or nitrogen.

values ranged from −18.6 to −18.0‰ (mean − 18.3 ± 0.2‰, $n = 19$) and $\delta^{15}N$ values ranged from 8.9 to 9.5‰ (mean 9.3 ± 0.2‰, $n = 19$). These results are similar to fingernail samples from 496 adults from the western United States (mean $\delta^{13}C$ −18.8 ± 0.8‰; $\delta^{15}N$ 9.4 ± 0.6‰) (Nardoto et al., 2006), align with an omnivorous mixed C_3/C_4 diet and the reported range of <1‰ is to be expected with no dietary change (Fogel et al., 1989; Fuller et al., 2005; Herrscher et al., 2017). Infant $\delta^{13}C$ values ranged from −18.1 to −17.2‰ (mean − 17.7 ± 0.2‰, $n = 21$) and $\delta^{15}N$ values ranged from 9.2 to 12.0‰ (mean 10.8 ± 1.0‰, $n = 21$).

During the period of exclusive breastfeeding, the maximum offset between infant and maternal $\delta^{15}N$ values was +2.7‰, which is consistent with that reported by Fuller et al. (2006) ($n = 6$; $\delta^{15}N = 2.7$‰) and Fogel et al. (1989) ($n = 4$; $\delta^{15}N = 2.4$‰). This peak of 12.0‰ was achieved at 5 months postpartum, and a decline of 0.3‰, following the introduction of complementary foods, is recorded in samples taken 3 months later. The $\delta^{15}N$ values continued to decline slowly by 0.7‰ over the next 5 months, followed by a faster drop of 1.5‰ in the following 3 months that coincided with the introduction of cow's milk to the infant's diet. Infant and maternal $\delta^{15}N$ values reached parity (~9.5‰) at the complete cessation of breastfeeding at 15 months postpartum.

We did observe the so-called "carnivore effect" (Fuller et al., 2003), or an increase in $\delta^{13}C$ values associated with the consumption of human milk as during exclusive breastfeeding, infant and maternal $\delta^{13}C$ values were offset by +1.1‰. The timing of the decline in $\delta^{13}C$ values requires consideration. Infant $\delta^{13}C$ values began to decline by 0.4‰ from −17.3‰ just after 3 months of age and 3 months before a downward trend in $\delta^{15}N$ values was recorded. An earlier decline in $\delta^{13}C$ values relative to $\delta^{15}N$ values is thought to indicate that infant $\delta^{13}C$ values are more sensitive to the introduction of complementary foods (Fuller, Fuller, et al., 2006; Fuller, Molleson, Harris, Gilmour, & Hedges, 2006; Jay et al., 2008). However, studies of isotope turnover in human keratins show that $\delta^{15}N$ values will equilibrate with a dietary change more quickly than $\delta^{13}C$ values (Hülsemann et al., 2009; O'Connell & Hedges, 1999). In this case, the decrease predates the known date of introduction of complementary foods, suggesting another factor is at play. While the infant did receive cow's milk formula twice (once at 3 months, once at 6 months), the duration of 3–4 days may have been too short to impact the infant's isotopic composition, which reflects a longer-term average consumption of predominantly dietary protein (Fernandes et al., 2012). The timing of the decrease does correspond to a similar decrease in maternal $\delta^{13}C$ values which may simply show that a change in maternal diet is the cause. Interestingly, Fuller, Fuller, et al. (2006) also documented that four out of five exclusively breastfed infants showed a subtle decline (~0.4‰) in $\delta^{13}C$ values that predated the introduction of complementary foods; therefore, an unknown metabolic factor may also require further consideration. Infant $\delta^{13}C$ values increase again after 1 year postpartum. This may be due to the consumption of cow's milk by the infant, which would likely be higher in ^{13}C due to cattle foddering with dried ground corn and corn silage (Ishler et al., 2023; Metges et al., 1990).

3.2 | Amino acid $\delta^{15}N$ values

Mother and infant $\delta^{15}N_{AA}$ values are reported in Figure S1. The highest $\delta^{15}N$ values were measured in both mother and infant's trophic AAs Glx and Val and the lowest in Thr (Figures 2 and 3). A similar pattern of gradual increase was observed across the maternal $\delta^{15}N$ values of Ala, Asx, Glx, and Pro in the year following the infant's birth. However, except for Pro, this increase did not exceed 2‰ and did not correspond to a noticeable change in bulk keratin $\delta^{15}N$ values. Between 11 and 13 months postpartum, the $\delta^{15}N$ values of Ala, Asx, Glx, Ser, Pro, Gly, Leu, and Ile dropped by 1.3 to 5.5‰, while that of Val increased by 2‰. Maternal bulk keratin values showed only a minor increase of 0.4‰.

Infant $\delta^{15}N$ values of Asx, Glx, Ser, Leu, Gly, and Tyr all increased by at least 1‰ above maternal values between 3 and 6 months postpartum and, following the introduction of complementary foods, declined through to the last increment. Pro and Val showed a similar

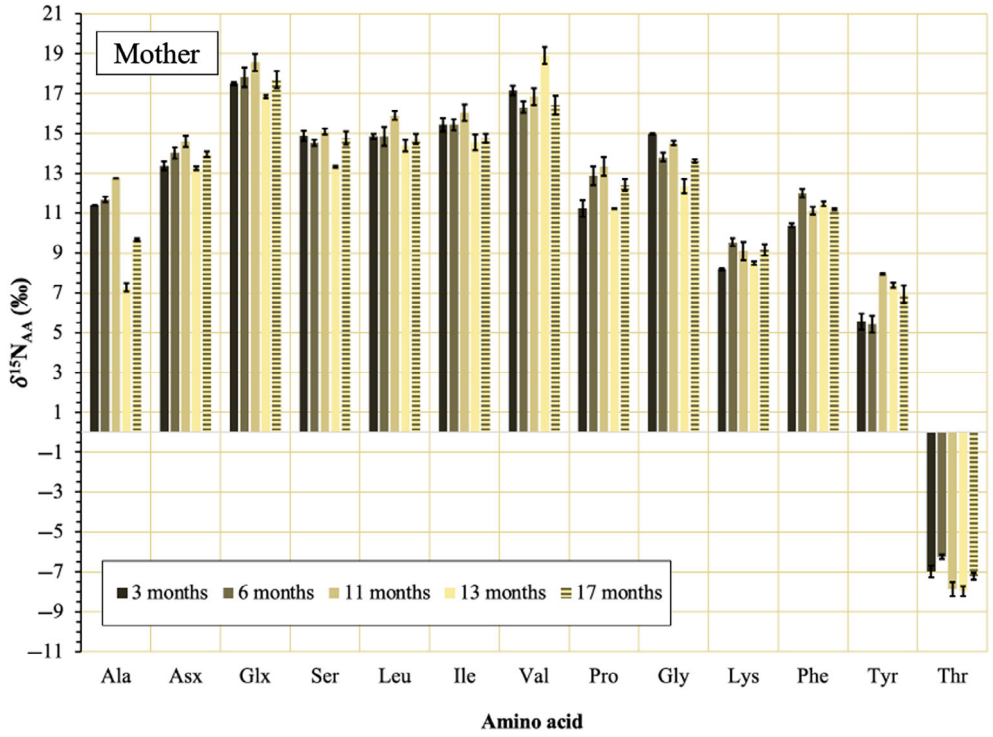

FIGURE 2 $\delta^{15}N$ values of maternal fingernail AAs. Error bars represent one standard deviation from analytical replicates. Ala, alanine, Asx, aspartic acid, Gly, glycine; Ile, isoleucine; Leu, leucine; Lys, lysine; Phe, phenylalanine; Pro, proline; Ser, serine; Thr, threonine; Tyr, tyrosine; Val, valine.

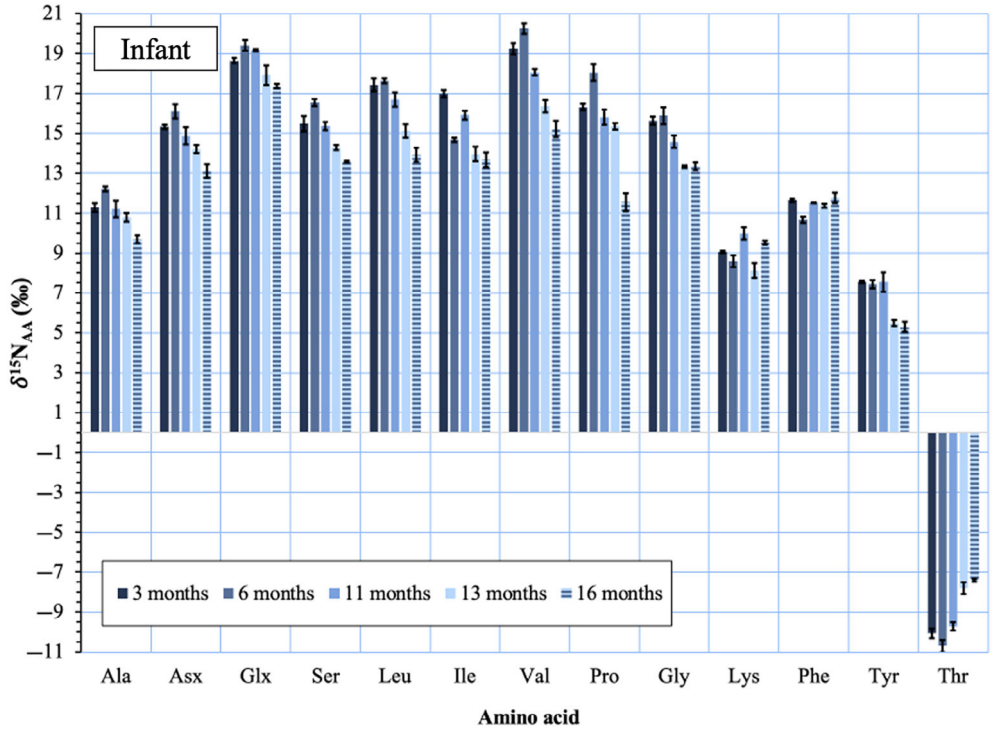

FIGURE 3 $\delta^{15}N$ values of infant fingernail AAs. Error bars represent one standard deviation from analytical replicates. Ala, alanine, Asx, aspartic acid, Gly, glycine; Ile, isoleucine; Leu, leucine; Lys, lysine; Phe, phenylalanine; Pro, proline; Ser, serine; Thr, threonine; Tyr, tyrosine; Val, valine.

pattern, but while the other trophic AAs showed a total decrease of between ~2 to 3‰ between the peak $\delta^{15}N$ values associated with exclusive breastfeeding and cessation of breastfeeding, Pro and Val decreased by 6.5 and 5.2‰, respectively. These AAs also remained elevated relative to maternal AA values until at least 13 months postpartum, whereas the others fell to within 1‰ of maternal values by 11 months postpartum.

Maternal $\delta^{15}N_{Lys}$ and $\delta^{15}N_{Phe}$ values range between 8.2 to 9.5‰ and 10.4to 12.0‰, respectively. Maternal $\delta^{15}N_{Lys}$ and $\delta^{15}N_{Phe}$ values for source AAs were highest during exclusive breastfeeding and decreased after 6 months postpartum. To our knowledge, no other study has followed a human participant or animal subject for over 1 year. As the composition of the mother's diet remained consistent throughout the study, the range of variation may be expected for an

individual consuming a mixed diet with some seasonal influence on the foddering of animals and the availability of different foods. However, it is difficult to know for certain, considering the current lack of comparative data and the potential confounding influence of lactogenesis and metabolic changes that may occur in the postpartum period. Infant $\delta^{15}N_{Lys}$ and $\delta^{15}N_{Phe}$ values ranged between 8.1 to 10‰ and from 10.7 to 11.8‰, respectively. $\delta^{15}N_{Lys}$ and $\delta^{15}N_{Phe}$ values were highest after the infant was 10 months of age, and lower values were measured in the fingernail samples corresponding to the period of exclusive breastfeeding. Lys and Phe are sourced almost entirely from dietary protein and undergo minor isotopic change, on the order of 0.5 to 1‰, between diet and consumer (relative to other AAs) (Chikaraishi et al., 2007; Fuller & Petzke, 2017; Hare et al., 1991; McClelland & Montoya, 2002; Metges, 2000; Popp et al., 2007; Steffan et al., 2013). It is impossible to explain these changes with certainty, but further studies may clarify this phenomenon.

Maternal $\delta^{15}N_{Thr}$ values ranged from −8.0 to −6.2‰; infant $\delta^{15}N_{Thr}$ ranged from −10.7 to −7.4‰. Infant $\delta^{15}N_{Thr}$ were lowest in the samples corresponding to the period of exclusive breastfeeding and increased following the introduction of cow's milk and decreasing consumption of human milk, ultimately reaching parity with maternal Thr values at 13 months postpartum. In this particular context, $\delta^{15}N_{Thr}$ values may be tracking the consumption of human milk more closely than other AAs. Although the mechanism behind Thr nitrogen isotopic fractionation requires clarification, researchers have observed in rodent models that consumer Thr is lower in $\delta^{15}N$ relative to dietary Thr by 3 to 7‰, depending on the protein content of the diet (Fuller & Petzke, 2017). The $\delta^{15}N_{Thr}$ in human milk may be lower relative to maternal keratin, and further fractionation may occur as Thr is incorporated into infant tissues. Thr is only present in mature human milk in moderate abundance (mean 560 ± 17 mg/L) and may have low digestibility (Darragh & Moughan, 1998) with potential implications for how it is used in the body and isotopic fractionation. Additional experiments that measure $\delta^{15}N_{Thr}$ in human milk and infant tissues and compare these data with infant nitrogen balance are warranted. Further comparison of breastfed infants with formula-fed infants would be interesting as infant formulas tend to contain higher concentrations of Thr and protein than human milk, which has implications for Thr flux in formula-fed infants (Darling et al., 1999).

Overall, infant AA $\delta^{15}N$ values follow a similar pattern as bulk keratin values, but further information may be obtained through a comparison of maternal and infant AA values across the sampling period (Figure 4). Gln/Glu is frequently used as the canonical trophic AA in food web and archaeological paleodietary studies (e.g., Cheung et al., 2022; Chikaraishi et al., 2010; Itahashi et al., 2019), leading to the expectation that infant $\delta^{15}N_{Glx}$ values would be enriched in ^{15}N relative to maternal values. We found that infant $\delta^{15}N_{Glx}$ values were 2‰ higher than maternal values during exclusive breastfeeding. However, the greatest offset occurred in infant and maternal $\delta^{15}N_{Pro}$ values during exclusive breastfeeding (5.2‰), followed by Thr (−4.5‰) and Val (4.0‰). In the case of Ala, a drop in maternal isotope values prompted an offset of 3.5‰ following the introduction of complementary foods. These offsets are affected most by high infant $\delta^{15}N$ values in the case of Pro and Val and low infant $\delta^{15}N$ values in the case of Thr. Transamination, the process by which the 'trophic' AAs exchange nitrogen with the free AA pool, is suggested to result in greater averaging of AA $\delta^{15}N$ values, and this could explain the smaller offsets in Glx and Asx (Lübcker, et al., 2020; O'Connell, 2017). Thr does not participate in transamination reactions and can only lose nitrogen via catabolism (O'Connell, 2017). Pro has been suggested as a candidate for trophic estimations as it is less influenced by the quantity of dietary protein consumed (Fuller & Petzke, 2017; Whiteman et al., 2021) but still shows a substantial trophic shift that is usually on par with Glx, as does Val and the other branched chain AAs, due to transamination. Our data suggest that while Val and Pro may still be exchanging N with the body pool, the larger offsets reported between

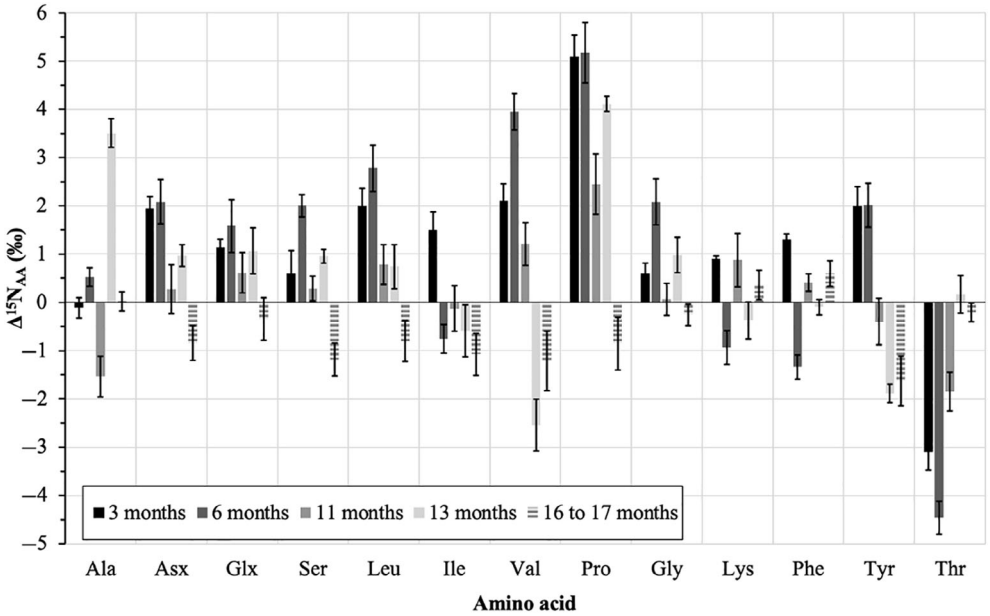

FIGURE 4 The difference between infant and maternal $\delta^{15}N$ values of fingernail AAs from 3 to 17 months postpartum. Error bars represent the propagated analytical error. AA, amino acid; Ala, alanine; Asx, aspartic acid; Gly, glycine; Ile, isoleucine; Leu, leucine; Lys, lysine; Phe, phenylalanine; Pro, proline; Ser, serine; Thr, threonine; Tyr, tyrosine; Val, valine.

mother and infant $\delta^{15}N_{Pro}$ and $\delta^{15}N_{Val}$ values suggest increased isotopic fractionation in the synthesis of these AA relative to other trophic AAs. One potential explanation may be due to the role of Pro in infant arginine synthesis. Arg is present in low concentrations in human milk because Arg is catabolized via arginase to produce Pro in the mammary gland (Wu et al., 2011). The requirement for Arg during infant growth, which functions as a precursor for nitric oxide and polyamines, exceeds the amount of dietary Arg available in milk, making in vivo synthesis of Arg critical for postnatal development (Tomlinson et al., 2011; Wu, 1997). In neonatal pigs and healthy preterm human infants, in vivo synthesis of Arg occurs via multiple enzymatic steps in the intestine from a Pro precursor (Tomlinson et al., 2011; Wu, 1997). It is possible that the synthesis of Arg from Pro would discriminate against ^{15}N, leaving the remaining Pro with higher $\delta^{15}N$ values (Fuller & Petzke, 2017; Macko et al., 1987; Miura & Goto, 2012).

These data may alter the way that researchers approach infant feeding in the past. There are several unknowns and sources of uncertainty that must be addressed when analyzing archaeological samples. For example, it can be difficult to differentiate the introduction of complementary foods from changes in maternal diet that are subsequently reflected in the isotopic composition of human milk as both may lower infant $\delta^{15}N$ values. A high trophic level complementary food may cause infant $\delta^{15}N$ values to remain elevated, mimicking the exclusive breastfeeding isotopic signal (Cheung et al., 2022). It is also difficult to estimate the contribution of human milk to infant diets after the introduction of complementary foods and the duration of the weaning period using bulk collagen or keratin $\delta^{15}N$ values. Based on the data presented in this paper, the $\delta^{15}N$ values of Pro, Val, and Thr may serve as markers of human milk consumption. If an infant's Pro and Val values remain high, and Thr values remain low when all other AAs have reached values similar to those in adults from the same archaeological assemblage, then this may suggest continued breastfeeding alongside complementary foods. The duration of the weaning period receives less attention in biological anthropology than types of weaning foods, or whether or not an infant was exclusively breastfed or artificially fed. However, breastfeeding duration has important implications for understanding family dynamics, women's socioeconomic roles, and cultural beliefs regarding infant feeding, social age categories, and the female body (Britton et al., 2018; Palmer, 2009; Treckel, 1989).

Further investigation into the isotopic patterns presented here using modern and archaeological samples is warranted. Our study did not collect anthropometric data from either the mother or infant, but pregnancy and postpartum changes in maternal body composition and metabolism could be relevant to the AA composition of human milk (De Luca et al., 2016). Our understanding of maternal–infant trophic dynamics and metabolism would also be enriched by $\delta^{15}N$ measurements of the AAs in human milk. For example, $\delta^{15}N$ values from the Pro, Thr, and Glx in maternal diet and keratin, milk proteins, and infant keratin would be valuable for calculating the isotopic fractionation that occurs from diet to maternal proteins and milk, and then from milk to infant proteins. The relationship among Pro and Thr and continued human milk consumption also requires further assessment. In our case study, the infant was weaned onto lower trophic level foods (fruits, cereal, later chicken, and milk). Would the same pattern of slowly declining Pro and rising Thr occur if the infant had been fed higher trophic level foods, such as fish? Alternatively, would high trophic level weaning foods be identifiable by the isotopic effects on other AAs such as Glx, Gly, and Phe? This question would be best answered by further analyses of modern mother–infant dyads, but in western societies, it is less common to provide infants with seafood/fish (Carstairs et al., 2017), therefore archaeological samples from marine-adapted populations could fill this gap. Finally, further experimentation is required to understand isotopic fractionation of Thr nitrogen. What is the relationship of protein quantity and quality, growth, pregnancy and lactation to Thr $\delta^{15}N$ values? Controlled dietary studies using animal models will be useful for addressing these questions and interpreting data from modern mother–infant dyads.

4 | CONCLUSION

Stable isotope studies are useful in the study of infant feeding practices, but various sources of uncertainty confound the analysis of bulk proteins. After obtaining bulk keratin carbon and nitrogen isotope values that were characteristic of mother–infant trophic dynamics, we investigated the change in the nitrogen isotopic composition of AAs with infant dietary events over 18 months of life using CSIA-AA. Infant AA $\delta^{15}N$ values were highest just before the introduction of complementary foods and declined throughout the weaning process. The $\delta^{15}N$ values of Lys and Phe were broadly similar between mother and infant, while those of Pro and Thr differed before the cessation of breastfeeding. Thr values remained low for the duration of breastfeeding despite the introduction of complementary foods, while Pro values remained high. This suite of AAs shows promise for identifying the isotopic effects of extended breastfeeding on infant $\delta^{15}N$ values as Pro may reveal isotopic fractionation associated with maternal milk consumption and infant AA synthesis. Future analyses may investigate these observed relationships by examining the influence of aquatic foods on Thr and Pro values, controlled animal feeding studies, and further research with modern mother–infant dyads.

AUTHOR CONTRIBUTIONS

Alison J. T. Harris: Formal analysis (lead); investigation (lead); visualization (lead); writing – original draft (lead). **Guaciara M. Santos:** Investigation (equal); methodology (equal); writing – original draft (supporting). **Kaelyn O. Malone:** Investigation (supporting). **Marcel T. J. Van Der Meer:** Methodology (supporting); writing – original draft (supporting). **Philip Riekenberg:** Conceptualization (equal); investigation (equal); methodology (equal); writing – original draft (supporting). **Ricardo Fernandes:** Conceptualization (equal); project administration (equal); supervision (lead); writing – original draft (supporting).

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the supplementary material of this article.

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