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RESEARCH ARTICLE

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Stable isotope evidence (Fe, Cu) suggests that sex, but not aging is recorded in rhesus macaque (*Macaca mulatta*) bone

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Abstract

Objectives: Here, we examine (1) if the sex-related differences in iron (Fe) and copper (Cu) isotope ratios, represented as δ^{56}Fe and δ^{65}Cu values, respectively observed in humans exist in bulk occipital bone and incisors of male and female non-human primates, and (2) if the variation of Fe and Cu isotope ratios, known to vary in human blood as a factor of age are similar in non-human primate bone.

Materials and Methods: Isotope ratios were measured from the skeletal elements of 20 rhesus macaques (*Macaca mulatta*) with known life history traits. The metals were purified by column chromatography and their isotope ratios measured by MC-ICP-MS. Data were analyzed using generalized additive models (GAM).

Results: When accounting for age and sex independently, we found a significant relationship between δ^{65}Cu values and occipital bone, but not in incisors. There were no significant relationships observed between δ^{56}Fe values, occipital bone, or incisors. Similarly, there were no significant relationships observed between δ^{56}Fe values, δ^{65}Cu values, and age.

Discussion: We suggest that Cu and Fe isotope ratios have the potential to be useful supplementary tools in future research in biological anthropology, but additional studies are needed to further verify the relationship between sex, age, δ^{65}Cu, and δ^{56}Fe values in primates.

KEYWORDS
bone, copper, iron, non-traditional stable isotopes, rhesus macaques

1 | INTRODUCTION

Trace metal stable isotopes have been used to explore a variety of questions relevant to human evolution including mobility (strontium [Sr]; Ericson, 1985; Sillen, Hall, Richardson, & Armstrong, 1998; Bentley, 2006; Faure & Powell, 2012), diet (zinc [Zn]; Jaouen et al., 2013; Jaouen, Beasley, Schoeninger, Hublin, & Richards, 2016; Bourgon et al., 2020), and sex (iron (Fe), copper (Cu); Jaouen et al., 2012). In humans, Fe and Cu stable isotopes have been shown to differ significantly in females relative to males in blood (Fe: Walczyk & von Blanckenburg, 2002; Cu: Albarède, Telouk, Lamboux, Jaouen, & Balter, 2011) and bone (Jaouen et al., 2012), but not in tooth enamel (Jaouen, Herrscher, & Balter, 2017). Fe and Cu are incorporated in the bioapatite of tooth enamel and bone, having been suggested as potentially useful diagnostic tools in the absence of organic preservation in archeological samples (Jaouen et al., 2012; Jaouen, Gibert, et al., 2013; Jaouen, Szpak, & Richards, 2016; Jaouen, 2018). Our understanding of how transition metal (Fe, Cu) metabolism, in the context of human evolution, varies with sex would be furthered by expanding the reference dataset to include non-human primates.

For a given element, mass-dependent stable isotope fractionation occurs when the relative abundance of stable isotopes changes after passing through a biological barrier or phase, resulting in variable

abundances of each isotope in biological compartments. This is due to preference or discrimination in the biological barrier, where slight differences in mass lead to bond energy changes. When bonds involve high oxidation states (e.g. Fe^{3+}, Cu^{2+}), there is a preference for heavy isotopes (Albarède et al., 2011; Albarède et al., 2015; Albarède et al., 2016). During non-steady state conditions or competing reaction pathways e.g. oxidation–reduction (redox) reactions, the smaller activation energy of the lighter isotopes allows them to react faster, leading to kinetic effects, where light isotopes are preferentially bound (Albarède, 2015; Albarède et al., 2011; Albarède et al., 2016; Hotz, Krayenbuehl, & Walczyk, 2012). For the transition metals, Fe and Cu, isotope fractionation is proposed to be the result of kinetic effects during intestinal absorption and metabolic reactions (Albarède, 2015; Albarède et al., 2011; Albarède et al., 2016; Hotz et al., 2012; Jaouen et al., 2012; Jaouen & Pons, 2017).

Fe and Cu are vital components in a multitude of biological processes related to oxygen transport, electron transport, and cellular respiration in organisms (Arredondo & Núñez, 2005; Albarède, 2015; Larner, 2016). Additionally, Fe and Cu are the main components of human serum and blood, which irrigates bone, therefore Fe is incorporated, and the isotopic compositions are recorded in bone (Balter et al., 2013;Jaouen et al., 2012; Jaouen et al., 2017). Prior research has shown that in the cortical bone of male and female humans, the Fe and Cu isotopic pattern are the same as in blood, suggesting they are recorded the mineral phase (Jaouen et al., 2012; Jaouen et al., 2017). Bones are remodeled throughout life and thus, they reflect the last 10–20 years of life (Hedges, Clement, David, Thomas, & O'Connell, 2007; Jaouen et al., 2017; Pate, 1994; Stenhouse & Baxter, 1979). Therefore, in adult primates, cortical bone should reflect Fe and Cu isotopic patterns associated with sex differences.

Similarly, certain diseases have been shown to cause metabolic dysregulation, generating Fe and Cu fractionation, such as hemochromatosis (Fe: Krayenbuehl, Walczyk, Schoenberg, von Blanckenburg, & Schulthess, 2005; Stenberg et al., 2005; Walczyk & von Blanckenburg, 2005), Wilson's disease (Cu: Aramendía, Rello, Resano, & Vanhaecke, 2013), Parkinson's (Cu: Larner et al., 2013), liver disease (Cu: Lauwens, Costas-Rodríguez, Van Vlierberghe, & Vanhaecke, 2016), cancer/tumor growth (Cu, Zn: Albarède et al., 2015; Balter et al., 2015; Larner, Shousha, & Coombes, 2015; Larner et al., 2016; Télouk et al., 2015; Bondanese et al., 2016; Chamel et al., 2017), and Alzheimer's (Cu: Moynier, Creech, Dallas, et al., 2019). For the above diseases, the measurement of Fe and Cu stable isotopes in blood and serum has been helpful for diagnostic and prognostic purposes. Despite this, we still lack an understanding of what causes Fe and Cu to fractionate within the body and if there is a measurable range of variation that can be recorded in non-human primate taxa.

The δ^{56}Fe and δ^{65}Cu values have been shown to vary in biological systems, including plants (Aucour et al., 2011; Fujii, Moynier, Blichert-Toft, & Albarède, 2014; Guelke & Von Blanckenburg, 2007; Jouvin et al., 2012; Moynier et al., 2009; Viers et al., 2007; von Blanckenburg, von Wirén, Guelke, Weiss, & Bullen, 2009; Weinstein et al., 2011; Weiss et al., 2005), human red blood cells and hair (Ohno, Shinohara, Chiba, & Hirata, 2005), in the blood and bone of sheep and mice (Balter et al., 2010; Balter et al., 2013; Moynier, Fujii, Shaw, & Le Borgne, 2013, Moynier et al., 2019), and in South African mammal trophic chains (Jaouen, Pons, & Balter, 2013). The δ^{56}Fe and δ^{65}Cu values also vary between males and females in human blood (Albarède et al., 2011; Ohno, Shinohara, Kohge, Chiba, & Hirata, 2004; Walczyk & von Blanckenburg, 2002), in human bone (Jaouen et al., 2012; Jaouen, Gibert, et al., 2013; Jaouen & Balter, 2014; Jaouen et al., 2017; von Blanckenburg et al., 2014), in domestic dog blood (Chamel et al., 2017), and in mice blood (Moynier et al., 2019). Additionally, there is evidence suggesting that Fe status is implicated in human female fertility and affected by pregnancy and lactation (Miller, 2016).

In humans, this sex difference is indicated by an on average 0.33‰ higher δ^{56}Fe values and 0.09‰ lower δ^{65}Cu values in women's bone than those of men (Jaouen et al., 2012). The first proposed reason for the difference is that during bloodletting, blood Fe becomes isotopically heavier, because Fe stored in the liver and kidneys is released to make up for the deficit (Albarède, 2015; Hotz et al., 2012; Jaouen & Pons, 2017; Krayenbuehl et al., 2005). To release Fe from hepatocytes requires Cu-hosted ceruloplasmin (Cp) to catalyze the oxidation of Fe (II, ferrous iron) to Fe (III, ferric iron), resulting in elevated Cp activity in women relative to men (Hunt, Zito, & Johnson, 2009: Jaouen & Balter, 2014). In human females, menstrual blood loss is the most significant factor affecting Fe values (Harvey et al., 2005) and due to their relatedness, most likely affecting Cu values as well (Arredondo & Núñez, 2005). The sex difference is a function of menstruation, where after menopause the difference is no longer apparent (Jaouen & Balter, 2014; Van Heghe, Deltombe, Delanghe, Depypere, & Vanhaecke, 2014), following the difference in biological turnover timelines in human blood: ~ 50 days for Cu and 8 years for Fe (Albarède, Télouk, & Balter, 2017; Jaouen & Balter, 2014; Jaouen & Pons, 2017). Therefore, post-menopausal women exhibit δ^{65}Cu similar to men and δ^{56}Fe values intermediate between men and menstruating women (Jaouen et al., 2014; Van Heghe et al., 2014). Similarly, the absence of menstruation due to consistent hormonal anti-conception treatment leads to the shifting of whole blood Fe isotope ratios to values characteristic of the male population (Van Heghe et al., 2014). Since human females experience monthly bloodletting, as a result of menstruation, the shifting of whole blood Fe and Cu isotopic compositions is in line with contemporary knowledge of female physiology. However, in most non-human primates, menstruation comparable to modern human females (blood loss around ~6–8 mL) is often not observed, but there is evidence of reproductive senescence or menopause (Alberts et al., 2013; Caro et al., 1995; de Jesús Rovirosa-Hernández, 2017; Graham, Kling, & Steiner, 1979; Gilardi, Shideler, Valverde, Roberts, & Lasley, 1997; Johnson & Kapsalis, 1998; Nichols et al., 2005; Pavelka & Fedigan, 1999; Walker & Herndon, 2008; Zuckerman & Fulton, 1934). However, recent work measuring Cu stable isotopes in the blood of mice has indicated a ~ 0.4‰ difference between wild type males and females, where the serum of females is ^{65}Cu-enriched relative to males (Moynier et al., 2019). Since the reproductive cycle of female mice does not include bleeding (Byers, Wiles, Dunn, & Taf, 2012) and

this is the opposite trend observed for humans (Jaouen, Gibert, et al., 2013), a second proposed reason for the isotope difference could be related to the speciation and relative abundance of Cu in mouse blood (Moynier et al., 2019), but this has not been tested.

We examine the variation of δ^{56}Fe and δ^{65}Cu values in the bone and incisors of rhesus macaques (*Macaca mulatta*) with documented life histories to test for sex and age-class differences characteristic of humans. Rhesus macaques (*Macaca mulatta*) were selected, because as old-world monkeys, they exhibit menstrual cycles that range from 25 to 35 days with on average 7 mL blood loss, where ~1.1% of total blood volume is lost during menstruation, which is comparable to human females (~1.2%, Brenner & Slayden, 2012; de Jesús Rovirosa-Hernández et al., 2017; Lewis & Prongay, 2015; Sharma & Sharma, 2018; Wolfensohn & Lloyd, 2008). Likewise, rhesus macaques are established non-human primate models for studying Fe deprivation and are known to assimilate dietary Fe in a manner similar to humans (Giulietti et al., 1991; Golub & Hogrefe, 2014; Rao, Prasad, & Sarathy, 1977). However, the breast milk for rhesus macaques contains more Fe (1.2–1.7 μg/mL) than modern humans (0.2–0.4 μg/mL), representing another means of iron loss other than menstruation (Miller, 2016). On average, male and female rhesus macaques weigh 7.7 kg and 5.34 kg, respectively, with an average life span of 25 years and sexual maturity is reached at 3–5 years for females and 4–6 years for males (Lewis & Prongay, 2015).

Following the first proposed reason for sex differences, we predict that since rhesus macaques have a similar hormonal cycle and menstruation (~7 mL: Brenner & Slayden, 2012) to human females (de Jesús Rovirosa-Hernández et al., 2017; Lewis & Prongay, 2015), the variation in Fe and Cu isotopic compositions in bone and teeth should reflect that of humans. We predict that based on human dental enamel and the short residence time of Cu in the body, there will be no sex differences in enamel δ^{65}Cu values (Jaouen et al., 2017). Since Fe is only present at parts per trillion in living teeth, we predict there will be no sex differences in enamel δ^{56}Fe values (Bentley, 2006; Kang et al., 2004; Kohn, Schoeninger, & Barker, 1999). Additionally, we examine if there are interspecific differences and trophic level effects between δ^{56}Fe and δ^{65}Cu values by comparing our data to published mammal data. This will help us explore the potential for a general metabolic pattern between species. By measuring the δ^{56}Fe and δ^{65}Cu values in rhesus macaques, we test if the sex differences observed in humans extend to non-human primate taxa. With a better understanding of metal metabolism in a non-human primate, we propose the extension of these measurements to other hominid taxa to explore the evolution of physiological and metabolic differences between male and female humans. By measuring δ^{56}Fe values in three age groups (juvenile, subadult, adult), we can also verify if the above phenomenon in humans occurs in rhesus macaques.

2 | MATERIALS

2.1 | Sample selection

Rhesus macaque samples were obtained from the Caribbean Primate Research Center (CPRC) in Sabana Seca, PR. The rhesus macaques were provisioned with monkey chow (Kessler & Rawlins, 2016), which provides the daily requirements of Fe, Cu, and other essential minerals. The CPRC manages the colony, collecting life history data, including date of birth, date of death, and sex. All of the individuals underwent necropsies, the majority of which have necropsy reports, including the history of health and cause of death. The individuals used were not reported to have anemia or iron-deficiency related disorders. Incisors and occipital bone were selected, because they were available for all of the selected samples and form early in life.

We compiled additional mammalian isotope data from the literature for within-class comparison, from the following published articles: Jaouen et al. (2012) for humans (*Homo sapiens*), Balter et al. (2013) for sheep (*Ovis aries*) and mice (*Mus musculus*), and Jaouen et al. (2013) for lion (*Panthera leo*), bushbuck (*Tragelaphus scriptus*), zebra (*Equus burchelli*), common duiker (*Sylvicapra grimmia*), sable antelopes (*Hippotragus niger*), and cape grysbok (*Raphicerus melanotis*). The skeletal elements from Jaouen et al. (2012) were archeologically obtained distal phalanxes, whereas those from Balter et al. (2013) and Jaouen et al. (2013) were a combination of fresh and fossil bones. For the published mammalian isotope data, we are proceeding under the following assumptions (1) the mechanism for integrating Fe and Cu into bone is similar in the placental mammals reported (Balter et al., 2013), (2) redox and coordination induced biological fractionation occurs (Balter et al., 2013) and (3) except in the case of mice (Moynier et al., 2019), sex differences are ignored, because the sex of the mammals reported in Balter et al. (2013) are not reported.

2.2 | Sample preparation

All rhesus macaque individuals died in Sabana Seca and were necropsied, before skeletal elements were processed by the CPRC. First, necropsied individuals underwent cold water maceration (26–30°C) in buckets for 1 week to 3 months depending on the temperature and season. The remains were then rinsed with cold water over multiple sieves to separate skeletal elements from detritus. Next, the skeletal elements underwent warm water maceration for 1 h in a solution of 4–7 L of water and 15 g of sodium carbonate depending on individual weight. After maceration, the clean bones were laid out to dry for 24–48 h and then bagged.

3 | METHODS

3.1 | Analytical methods

100–150 mg (~0.1–0.15 g) of occipital bone from the skull and enamel, dentin, and cementum of the first maxillary incisor (I^1) was used for samples. The sample size included 20 rhesus macaques [2 female juveniles (~1–2 years), 8 female adults (~6–18 years); 2 male juveniles (~1–2 years), 8 male adults (~5–27 years)]. For rhesus macaques, sex assessment was performed by the CPRC.

The occipital bone in the skull and the entire cortex of the incisor were extracted with a drill, scalpel, and pliers in the rhesus macaques.

	Parametric terms	Smooth terms		
Model	Sex	Age	Age:female	Age:male
1 (no interaction)				
$n = 20$	$T = 0.86$	$F = 1.97_{(4.33)}$		
AICc = 79.70	$p = .40$	$p = .15$		
DE = 41.7%				
$r^2_{adj} = 0.22$				
1 (interaction)				
$n = 20$	$T = 1.10$	$F = 1.36_{(3.88)}$	$F = 4.29_{(0.52)}$	$F = 1.27_{(1.08)}$
AICc = 82.47	$p = .29$	$p = .32$	$p = .16$	$p = .36$
DE = 48.4%				
$r^2_{adj} = 0.26$				
2 (no interaction)				
$n = 19$	$T = 2.94$	$F = 0.18_{(1)}$		
AICc = 62.21	$p = .01^*$	$p = .67$		
DE = 36.4%				
$r^2_{adj} = 0.29$				
2 (interaction)				
$n = 19$	$T = 0.73$	$F = 2.97_{(3.39)}$	$F = 1.89_{(2.87)}$	$F = 5.68_{(0.52)}$
AICc = 68.05	$p = .48$	$p = .06$	$p = .27$	$p = .11$
DE = 78.7%				
$r^2_{adj} = 0.65$				
3 (no interaction)				
$n = 17$	$T = -1.03$	$F = 4.21_{(1)}$		
AICc = 37.26	$p = .32$	$p = .06$		
DE = 27.4%				
$r^2_{adj} = 0.17$				
3 (interaction)				
$n = 17$	$T = -1.11$	$F = 6.15_{(0.95)}$	$F = 0.04_{(0.84)}$	$F = 1.68_{(3.2)}$
AICc = 47.00	$p = .29$	$p = .03^*$	$p = .86$	$p = .28$
DE = 56.9%				
$r^2_{adj} = 0.34$				
4 (no interaction)				
$n = 20$	$T = -1.32$	$F = 1.12_{(1.74)}$		
AICc = 49.72	$p = .21$	$p = .26$		
DE = 25.3%				
$r^2_{adj} = 0.14$				
4 (interaction)				
$n = 20$	$T = 0.46$	$F = 3.85_{(3.4)}$	$F = 3.25_{(4.3)}$	$F = 7.6_{(0.52)}$
AICc = 63.11	$p = .65$	$p = .04^*$	$p = .05$	$p = .07$
DE = 75%				
$r^2_{adj} = 0.55$				

TABLE 1 Variation between sex in the isotopic compositions of δ^{65}Cu and δ^{56}Fe values in rhesus macaques (*Macaca mulatta*) with accounting for age and sex. Deviance explained is referred to as DE. Significant values, defined as $p < .05$ are marked with a *

After bagging, individual samples were separated, double bagged, and then crushed.

The crushed material was then charred in covered aluminum boats for 24 h at 500–600°C in a muffle furnace. To remove the remaining organics, the material was switched to sealed porcelain crucibles and reduced to ashes over 24 h at 1000°C. When the samples cooled, the porcelain crucibles were lifted out with metal prongs and the samples were transferred to 2 mL eppendorf vials.

These ashed samples were dissolved in 2 mL of aqua regia (3:1 $HCl + HNO_3$). The dissolved samples were then evaporated to

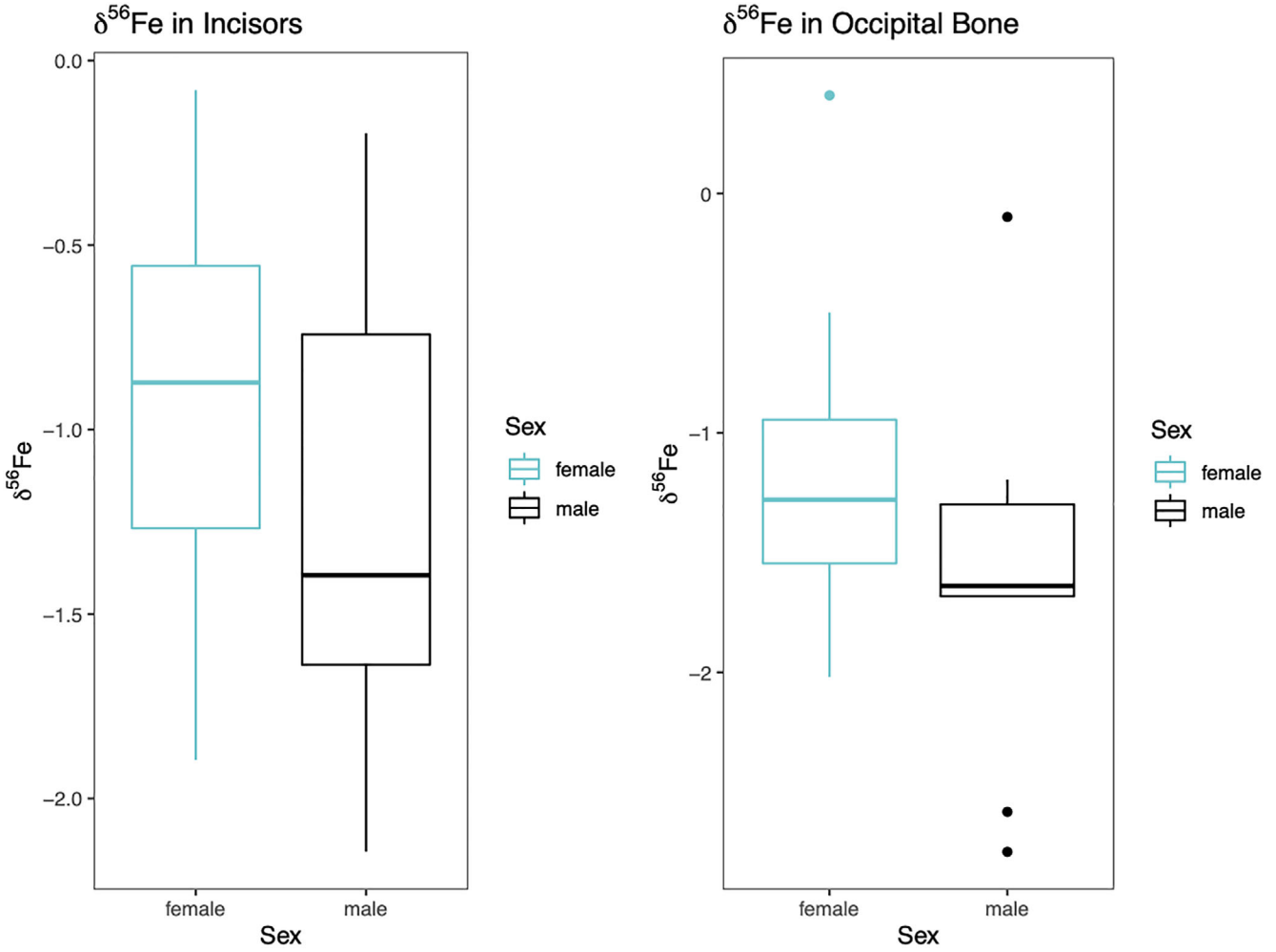

FIGURE 1 Boxplots depicting the variation of δ^{56}Fe values between male and female rhesus macaques (*Macaca mulatta*). On the *y*-axis are the δ^{56}Fe values in per mil units (‰)

dryness, saturated with hydrogen peroxide (H_2O_2) heated in closed vials, and then evaporated again. For each sample, this step was repeated until oxidation occurred, based on visual characteristics and reaction with H_2O_2. Samples were taken up in a 0.5 mL solution of 7 N HCl and 0.001% H_2O_2. The remaining solution was then processed for isotope analysis, according to the technique of Maréchal and Albarède (2002) on a 2 mm × 5 cm column filled with the AG MP-1 anion-exchange resin (100–200 mesh, Bio-Rad).

For the column chromatography, columns were cleaned 3 times by alternating cycles of 3 mL of HNO_3 and 1 mL of H_2O. Following the cleaning, the columns were pre-conditioned with two 0.5 mL rinses of 7 N HCl + 0.001% H_2O_2 before samples were loaded. After samples were loaded, the columns were rinsed three more times with 0.5 mL of 7 N HCl and 0.001% H_2O_2. Cu was eluted with a 6.5 mL solution of 7 N HCl and 0.001% H_2O_2, followed by a 0.3 mL rinse of 2 N HCl + 0.001% H_2O_2, and then Fe was eluted with a 5.5 mL solution of 2 N HCl and 0.001% H_2O_2. For some samples with a high percentage of organics, the process was repeated twice to remove interferences from calcium (Ca) and phosphorus (P). The blank contribution for the overall procedure was 0.5% Fe and 0.2% Cu. When calibrated with a tooth sample, column yields were 100%, excluding any chromatographic isotopic fractionation.

3.2 | Isotopic analyses

The δ^{56}Fe and δ^{65}Cu values were determined on a ThermoScientific Neptune Plus MC-ICP-MS. The samples were introduced by free aspiration in 2% HNO_3, with a Cetac Aridus II desolvating nebulizer. For Fe and Cu, instrumental mass fractionation was corrected using standard-sample bracketing following the technique of Albarède et al. (2004). The isotope reference standards used were NIST-976 (Cu) and IRMM-524A (Fe). During Cu analysis session, the standard utilized was ESI relative to NIST-976, where the ESI standard was 0.14 ± 0.05‰ (1SD; $n = 5$). During Fe analysis session, the standard utilized was IRMM-014 relative to IRMM-524A, where IRMM-014 was 0.01 ± 0.12‰ (1SD; $n = 5$). External reproducibility was determined by measuring ESI (Cu) and IRMM-014 (Fe) as unknowns with the reference standards and samples. The precision (external reproducibility, two standard deviations) on the isotopic ratios is ±0.07‰

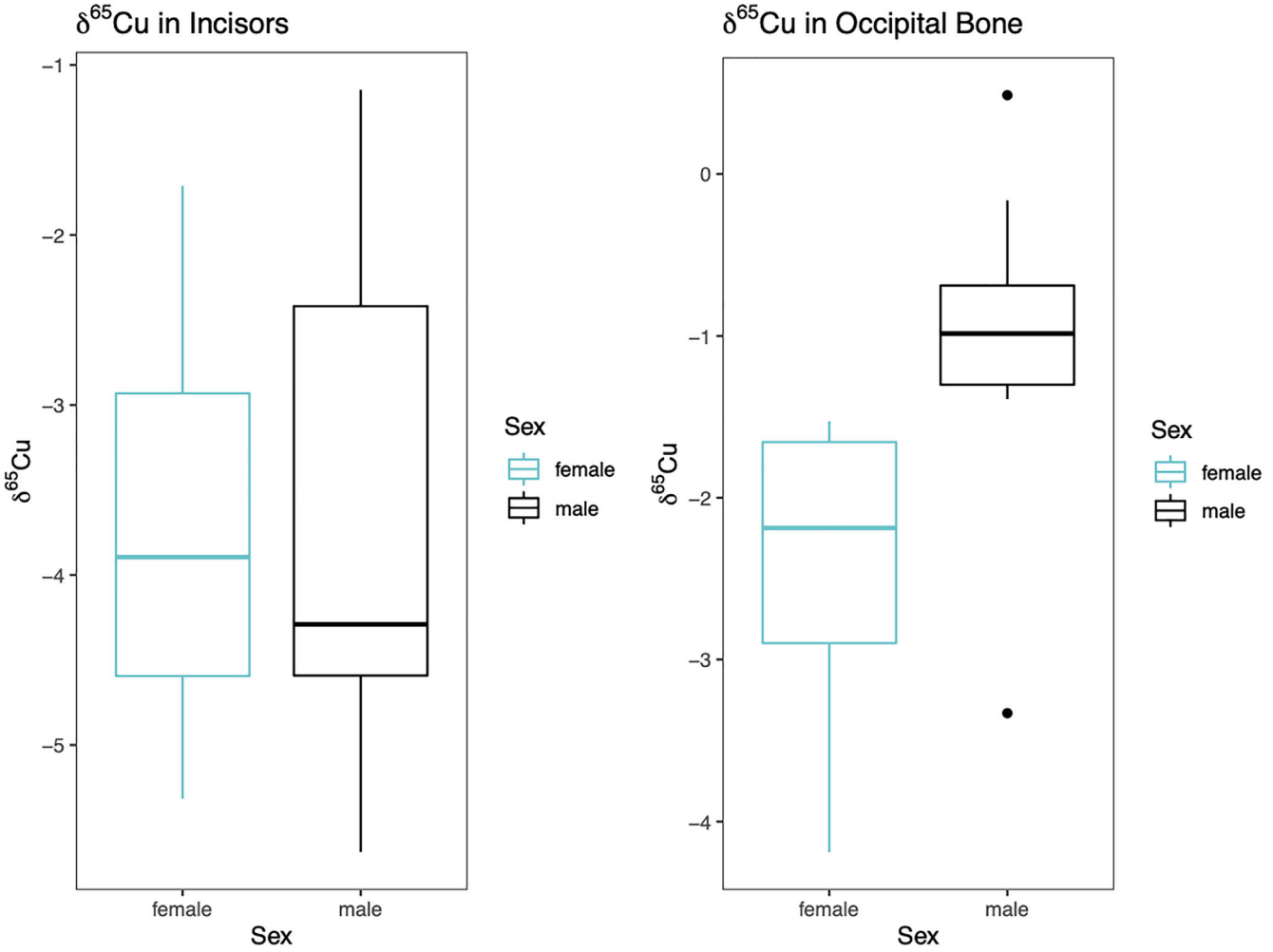

FIGURE 2 Boxplots depicting the variation of $\delta^{65}Cu$ values between male and female rhesus macaques (*Macaca mulatta*). On the *y*-axis are the $\delta^{65}Cu$ values in per mil units (‰)

for Cu and ±0.02‰ for Fe. Isotopic composition for Fe and Cu isotope values are reported on a relative scale, which is referred to as delta notation (δ) in parts per thousand or per mil (‰) units:

$$\delta^{65}Cu = \left(\frac{\left({}^{65}Cu/{}^{63}Cu \right)_{sample}}{\left({}^{65}Cu/{}^{63}Cu \right)_{NIST\ 976}} - 1 \right) \times 10^3$$

$$\delta^{56}Fe = \left(\frac{\left({}^{56}Fe/{}^{54}Fe \right)_{sample}}{\left({}^{56}Fe/{}^{54}Fe \right)_{IRMM\ 524A}} - 1 \right) \times 10^3$$

3.3 | Data analysis

To examine if there is an influence of age on sex between $\delta^{65}Cu$ and $\delta^{56}Fe$ values in rhesus macaque occipital bone and bulk incisors, generalized additive models (GAM) were run using the mgcv package in R (R Development Core Team, 2018; Wood, 2018). GAM was selected, because it is an extension of a generalized linear model (GLM) that is semiparametric and can accommodate non-linear relationships (Wood, 2017). Semiparametric methods relax the assumptions of parametric methods and the use of smoothing splines in GAM preserve our ability to detect both non-linear and linear relationships (Wood, 2017). We ran two models with the same variables, but different parameters, where one included the interaction of age and sex (interaction) and one did not (no interaction). Akaike Information Criteria corrected for small sample size (AICc) was used to select the model that best fit all of the data (Mazerolle, 2020; R Development Core Team, 2018; Wood, 2017; Wood, 2018). The best fit model was when age and sex were individually accounted for (Model 1: incisor $\delta^{65}Cu$, age, sex; Model 2: occipital bone $\delta^{65}Cu$, age, sex; Model 3: incisor $\delta^{56}Fe$, age, sex; Model 4: occipital bone $\delta^{56}Fe$, age, sex; Table S1). Only the four AICc-selected models are included in the results, but details for all eight model criteria can be found in Table 1 and Table S1. For models where there was a visual interaction, we performed a power analysis in R (Mollan et al., 2019; Shieh, Jan, & Randles, 2006; R Development Team, 2018). To analyze interspecific differences and trophic effect, Kruskal–Wallis tests were run in R (Hollander et al., 2015).

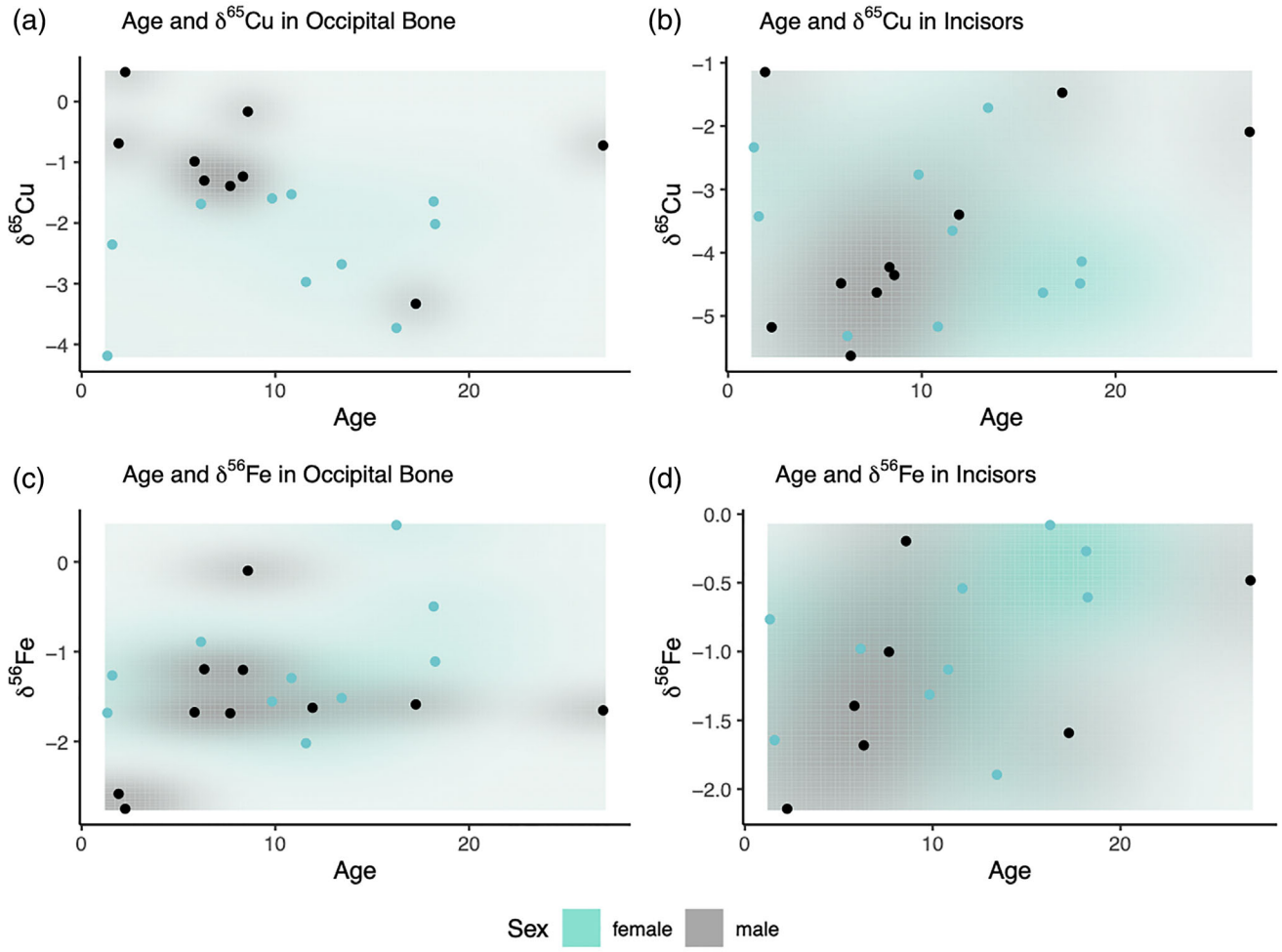

FIGURE 3 Scatterplots depicting the distribution of δ^{65}Cu and δ^{56}Fe values in per mil units (‰) throughout time. Closed, black circles represent males and open, turquoise circles represent females. Gray shading represents males, where the darker the color, the higher the density of data points at those values. Turquoise shading represents females, where the darker the color, the higher the density of data points at those values (a) age, δ^{65}Cu, occipital bone; (b) age, δ^{65}Cu, incisors; (c) age, δ^{56}Fe, occipital bone; and (d) age, δ^{56}Fe, incisors

TABLE 2 δ^{65}Cu and δ^{56}Fe values of male and female rhesus macaques (*Macaca mulatta*) and humans (*Homo sapiens*). For rhesus macaques, the occipital bone was used, and for humans, the distal phalanx was used

Sample	*n*	Mean δ^{65}Cu (‰)	2SD	*n*	Mean δ^{56}Fe (‰)	2SD
Rhesus Macaque	19			20		
Male	9	−1.04	0.09	10	−1.61	0.24
Female	10	−2.44	0.09	10	−1.22	0.24
Human[a]	30			42		
Male	18	−0.11	0.16	27	−0.45	0.84
Female	12	−0.21	0.25	15	−0.11	0.68

[a]Human data was acquired from Jaouen et al. (2012).

4 | RESULTS

4.1 | Mass-dependent isotope fractionation

The isotope data and standard error (2σ) are reported in the Supplementary Information (Table S2). We checked that isotopic fractionation was mass-dependent for all the samples, i.e., that δ^{57}Fe/3 = δ^{56}Fe/2 (Figure S3). Different slope values would have indicated instrumental mass-independent fractionation.

4.2 | Sex differences

Male rhesus macaques have δ^{56}Fe values and δ^{65}Cu values that were on average − 1.61± 0.24‰ and − 1.03± 0.09‰ for occipital bone

TABLE 3 Variation in δ^{65}Cu isotope values for mammals

Sample	Mean δ^{65}Cu (‰)	Range-min(‰)	max(‰)	2SD	*n*
Human[a]	−0.15	−0.42	0.03	0.22	30
Rhesus Macaque	−1.77	−4.19	−1.53	0.09	19
Lion[b]	0.025	−0.14	0.19	0.46	2
Bushbuck[b]	−0.14	−0.17	−0.11	0.08	2
Zebra[b]	−0.815	−0.94	−0.68	0.22	4
Common Duiker[b]	−1.16	−1.37	−0.78	0.52	4
Cape Grysbok[b]	−0.086	−0.1	−0.07	0.02	3
Sheep[c]	0.12	−0.05	0.27	0.30	4
Mice[c]	0.175	0.06	0.29	0.32	2

[a]Other data was acquired from Jaouen et al. (2012).
[b]Other data was acquired from Jaouen et al. (2013).
[c]Other data was acquired from Balter et al. (2013).

TABLE 4 Variation in δ^{56}Fe isotope values for mammals

Sample	Mean δ^{56}Fe (‰)	Range-min(‰)	max(‰)	2SD	*n*
Human[a]	**−0.33**	**−1.48**	**0.48**	**0.84**	**42**
Rhesus Macaque	−1.41	−2.75	0.399	0.24	20
Lion[b]	−0.604	−1.29	−0.25	0.94	5
Bushbuck[b]	−0.025	−0.12	0.07	0.26	2
Zebra[b]	0.005	−0.1	0.11	0.30	2
Sable Antelope[b]	−0.083	−0.61	0.44	1.04	3
Sheep[c]	−1.22	−1.37	−1.06	0.26	4
Mice[c]	−0.81	−1.03	−0.59	0.36	4

[a]Other data was acquired from Jaouen et al. (2012).
[b]Other data was acquired from Jaouen et al. (2013).
[c]Other data was acquired from Balter et al. (2013).

and −1.21± 0.24‰ and −3.66± 0.09‰ for incisors, respectively (Figures 1 and 2). Female rhesus macaques have δ^{56}Fe values and δ^{65}Cu values that were on average −1.22 ± 0.24‰ and −2.44± 0.09‰ for occipital bone and −0.92 ± 0.24‰ and −3.76± 0.09‰ for incisors, respectively (Figures 1 and 2). On average, δ^{56}Fe values and δ^{65}Cu values in occipital bone were −1.41 ± 0.24‰ and −1.77 ± 0.09‰ and in incisors were −1.04 ± 0.24‰ and −3.71 ± 0.09‰, respectively (Figures 1 and 2).

To test for sex differences, we ran four models on the responses of δ^{56}Fe values and δ^{65}Cu values in occipital bone and incisors, where age and sex were independent variables. Of the four models, the only significant relationship was found between δ^{65}Cu values and occipital bone ($T = 2.94$, $p = .01$, summarized in Table 1)

4.3 | Influence of age

To test if there is an interaction between age and sex, we ran four separate models on the responses of δ^{65}Cu and δ^{56}Fe in occipital bone and incisors with age and sex as covariates. Of the four models, there were no significant relationships between age and isotope values (Table 1 and Figure 3).

A power analysis showed relatively low statistical power (shieh power = 0.66, < 0.80).

4.4 | Variation of Fe and Cu metabolism in mammals

We compared our new data with the published data on δ^{65}Cu and δ^{56}Fe values in the bones of humans (*H. sapiens*) (Jaouen et al., 2012), rhesus macaques (*M. mulatta*), mice (*M. musculus*), sheep (*O. aries*) (Balter et al., 2013), lions (*P. leo*), common duikers (*S. grimmia*), zebras (*E. burchelli*), cape grysboks (*R. melanotis*), sable antelopes (*H. niger*), and bushbucks (*T. scriptus*) (Jaouen et al., 2013). The isotopic difference between species is significant in δ^{65}Cu (Kruskal–Wallis Test: $H = 47.05$, df = 8, $p < .001$) and δ^{56}Fe (Kruskal–Wallis Test: $H = 45.28$, df = 9, $p < .001$) values. However, the isotopic difference between trophic level (Carnivores, Omnivores, Carnivores, Browsers, Grazers) was not significant in δ^{65}Cu (Kruskal–Wallis Test: $H = 3.01$, df = 3, $p = .39$) or δ^{56}Fe (Kruskal–Wallis Test: $H = 5.59$, df = 3, $p = .13$) values. There is a high degree of variation between species (summarized in Tables 2–4). The highest mean δ^{65}Cu value is found in mice with 0.17 ± 0.32‰ and the lowest is for rhesus macaques with −1.77 ± 0.09‰ (Tables 2 and 3, Figure 4). The highest

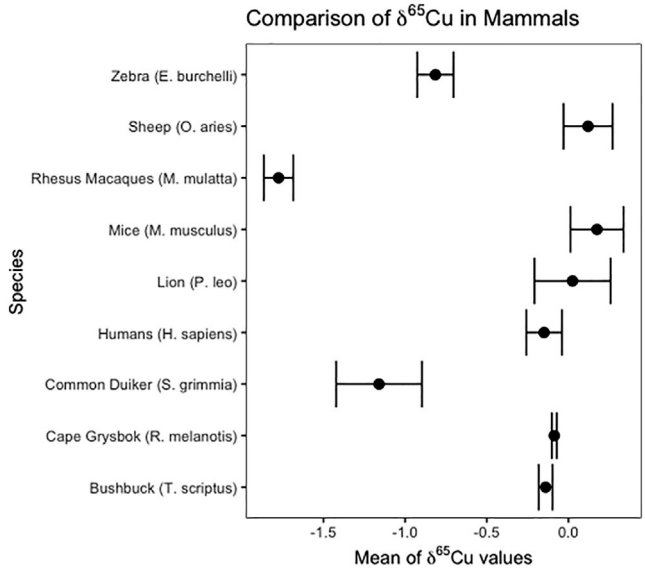

FIGURE 4 Summary of data presented in Table 2. On the *x*-axis, δ^{65}Cu in per mil units (‰), and on the *y*-axis are the species names. Each datum is depicted as a black circle and represents the mean for the relevant species with standard deviation of 2σ (95% confidence interval). There is a significant difference of δ^{65}Cu metabolism between species, with means ranging from −1.77 to 0.175 ‰ (Kruskal–Wallis test: $H = 47.05$, df = 8, $p < .001$). Other data was acquired from Jaouen et al. (2013) and Balter et al. (2013)

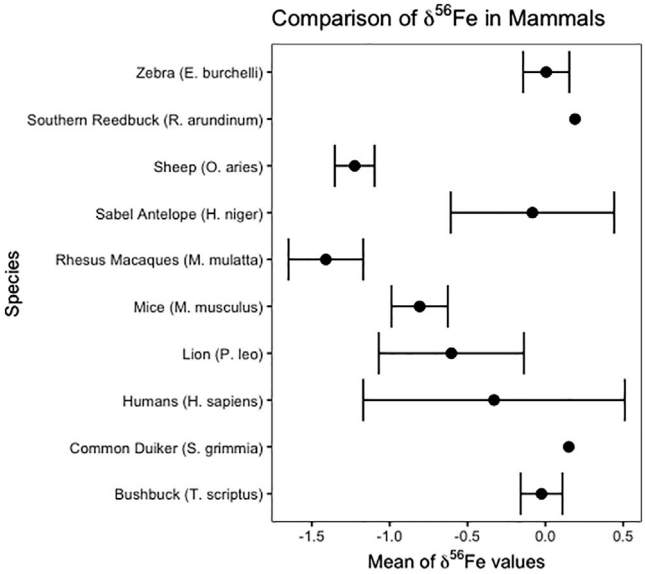

FIGURE 5 Summary of data presented in Table 3. On the *x*-axis, δ^{56}Fe in per mil units (‰), and on the *y*-axis are the species names. Each datum is depicted as a black circle and represents the mean for the relevant species with standard deviation of 2σ (95% confidence interval). There is a significant difference in δ^{56}Fe metabolism between species, with means ranging from −1.77 to 0.175 ‰ (Kruskal–Wallis test: $H = 41.637$, df = 9, $p < .001$). Other data was acquired from Jaouen et al. (2013) and Balter et al. (2013)

mean δ^{56}Fe value is in zebras at 0.005 ± 0.30‰ and the lowest is in rhesus macaques at −1.41 ± 0.24‰ (Table 4, Figure 5).

5 | DISCUSSION

Our results show that when age and sex are considered, there is no sex effect in incisors or occipital bone for δ^{56}Fe values, but there is a sex effect in occipital bone for δ^{65}Cu values, where females are ^{65}Cu-depleted relative to males. Based on previous work by Jaouen et al. (2012, 2017), this differs from modern humans and here, we suggest some potential explanations. In humans, Cu is differentially cycled in bone throughout life (Albarède et al., 2017), which is likely faster in female rhesus macaques since they are slightly smaller. Due to the low concentrations of Cu in teeth (Jaouen et al., 2017) and the faster cycling of Cu in bone, the insubstantial residence time might not record the upregulation occurring in blood from menstrual Fe losses. Additionally, in menstruating human females, it has been suggested that it could take more than 10 years for the Fe isotopic compositions of bones to reach steady state with diet (Jaouen et al., 2012). Rhesus macaques have a much shorter lifespan than modern humans: 25 years (Lewis & Prongay, 2015) vs. 74 years (Roser, Ortiz-Ospina, & Ritchie, 2019), which might explain why there are no apparent differences for δ^{56}Fe values between sex in bone. In rhesus macaques, first maxillary incisor (I^1) eruption is at 25–30 months for males and 29 months for females (Cheverud, 1981), where incisor formation time precedes this. Sexual maturity in rhesus macaques is reached at 3–5 years (36–60 months) for females and 4–6 years (48–72 months) for males (Lewis & Prongay, 2015). Adult incisors are formed before sexual maturity ensues, resulting in the absence of the attendant changes in hormonal and metabolic housekeeping that stimulate menses and the subsequent Fe loss in females. Without Fe loss in females, the subsequent enrichment of δ^{56}Fe values and concurrent depletion of δ^{65}Cu values would not occur. Fe is only present at parts per trillion in tooth enamel, therefore any flux would likely not be detectable (Bentley, 2006; Kang et al., 2004; Kohn et al., 1999). This is further supported by the short residence time of Cu in blood and bone (Jaouen et al., 2017). Subsequently, there could be an averaging effect due to the use of bulk incisors, because enamel, dentin, cementum, and the root form at differing times throughout life (Bowen & Koch, 1970; Trotter, Hixon, & MacDonald, 1977). Using one matrix of tooth tissue, such as dental enamel would help constrain the age range, if there was reason to suspect age specific fractionation before sexual maturity. However, due to the low concentrations of Fe and Cu in tooth enamel (Jaouen et al., 2017), even the use of this matrix would not likely show any significant sex effect. Overall, bulk incisors are probably not useful in indicating differences in transition metal metabolism between sexes in non-human primates and by extension, the fossil record. The absence of a sex effect for δ^{56}Fe values suggests that menstruation and menopause are not likely factors influencing the rhesus macaque transition metal metabolism used in this study, relative to humans. Since there is a sex effect observed

for δ^{65}Cu values, we highly suggest testing the hypotheses proposed by Moynier et al. (2019), because sex differences could be related to the speciation and relative abundance of Cu and Fe in blood. However, this does not explain why the blood of female mice is ^{65}Cu-enriched relative to males, which is the opposite of what is observed in rhesus macaque bone. If this is the mechanism for blood, then these differences would be recorded in the mineral phase (Jaouen et al., 2012; Jaouen et al., 2017).

For occipital bone and incisors, there is no relationship in isotope values (Figure 3). In the more complex models, a relationship between age was found (Figures S4 and S5), but these were excluded based on AICc. Given the relatively low statistical power (shieh power = 0.66), we cannot rule out the possibility that the lack of significant sex and age differences is the result of a Type 1 error. This is most likely due to small sample size, which is why we were conservative during model selection. While we predict a larger sample size will show the same effects for the above reasons, we cannot disregard that the lack of significant effects could be the result of low statistical power.

In addition, rhesus macaques in Sabana Seca were provisioned with monkey chow (Kessler & Rawlins, 2016) having a diet rich in Fe, Cu and other essential minerals, whereas modern humans used in Jaouen et al. (2012) were possibly suffering from inadequate diets. The individuals used in Jaouen et al. (2012) were inhabitants of the Saint-Laurent quarter during the modern period (17th-18th c. AD), where deterioration in both the economic and social statuses was observed from the modern to medieval eras (Colardelle, 2008; Jaouen et al., 2012). Similarly, paleopathological data from Herrscher, Colardelle, and Valentin (2006) reveal a significant increase in infectious and dental lesions for both adults and subadults during this epoch, suggesting that these individuals were suffering from poorer environments and diets, relative to the rhesus macaques in the present study. However, since the rhesus macaques in Sabana Seca are kept in their observed social groups with dominance hierarchies (Bercovitch & Clarke, 1995), we cannot discount the possibility that there was rank-mediated access to foods. Unfortunately, information regarding rank was not reported in the necropsy reports.

Kruskal-Wallis tests showed that δ^{65}Cu and δ^{56}Fe values were significantly different between species. When comparing dietary proclivities for all included species, where the omnivores are rhesus macaques, humans, and mice (*M. musculus*); the carnivores are the lions (*P. leo*); the grazers are zebras (*E. burchelli*) and sheep (*O. aries*); the browsers are common duikers (*S. grimmia*), cape grysbosk (*R. melanotis*), and bushbucks (*T. scriptus*), there was no significant difference contrary to what was observed in Jaouen, Pons, and Balter (2013). This could be because the data utilized in this study came from various trophic systems. Additionally, we suggest that the interspecific variation in δ^{65}Cu and δ^{56}Fe values are not likely the result of dietary intake of Cu and Fe, but probably differences in the intestinal absorption and metabolism of Cu and Fe between species (Jaouen, Pons, & Balter, 2013). Rhesus macaques have on average much lower δ^{65}Cu and δ^{56}Fe values relative to other mammal taxa. This could be a result of consuming the highly processed monkey chow, but it was not analyzed in this study for δ^{65}Cu and δ^{56}Fe values. Comparatively, humans appear to have more overlap with other species in δ^{65}Cu and δ^{56}Fe values. Zebras and the common duiker have on average, more individualistic δ^{65}Cu values (Figure 4). For δ^{56}Fe values, these differences are less clear due to large variation within species, but overall, rhesus macaques and sheep (*O. aries*) have more individualistic δ^{56}Fe values. For zebras, we suggest that this could be due increased Cu uptake in the intestinal tract, leading to ^{63}Cu-enriched blood and then bone (Jaouen, Pons, & Balter, 2013; Van Heghe, Engström, Rodushkin, Cloquet, & Vanhaecke, 2012). For common duikers, which are known to consume a wide variety of foods, including leaves, shoots, fruits, flowers, tubers, bulbs, roots, insects, and have been seen stalking and consuming lizards, frogs, rodents and birds (Estes, 2012; Kingdon, 1988), we suggest that the variable Cu sources could be a contributing factor.

Here, we have shown that a sex effect is present in rhesus macaque occipital bone for δ^{65}Cu values, but not for δ^{56}Fe values. Recent work from Moynier et al. (2019) and the lack of a sex effect for δ^{56}Fe values suggest that this is likely not due to menstruation. We propose that these sex differences could be related to the speciation and relative abundance of Cu in blood (Moynier et al., 2019). However, we cannot discount that the absence of a significant sex effect in δ^{56}Fe values could be related to a Type 1 error and low statistical power due to small sample size. Furthermore, interspecific variation of δ^{65}Cu and δ^{56}Fe values might be indicative of taxa-specific metabolic ranges. With a larger sample size of mammals, we propose δ^{65}Cu and δ^{56}Fe values could be useful supplementary identification tools for unidentified bone fragments in archaeology, paleontology, and primatology. Future research should expand the data set on Cu and Fe stables isotopes to examine other factors, such as phylogeny, metabolism, and chemical speciation that may contribute to variation among species and between sexes.

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CONFLICT OF INTEREST

There are no conflicts of interest.

AUTHOR CONTRIBUTIONS

Renee Boucher: Conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; project administration. **Shauhin Alavi:** Data curation. **Hylke de Jong:** Supervision; writing-original draft; writing-review & editing. **Linda Godfrey:** Formal analysis; methodology; supervision. **Erin Vogel:** Data curation; project administration; supervision; writing-original draft; writing-review & editing.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in Zenodo at https://zenodo.org/record/4244877#.X6LzeFNKgbl, 10.5281/zenodo.4244877.

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