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Comparison of vitamin D metabolites in wild and captive baboons

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Abstract

Vitamin D adequacy is essential for multiple physiologic processes. With limited exposure to sunlight for vitamin D₃ synthesis, captive primates are supplemented with vitamin D₃ (cholecalciferol). Vitamin D metabolite data from wild primates living indigenously could suggest optimum levels. The purpose of this study was to: 1) to explore whether baboons, a speciose genus whose members have significant exposed skin, coat color variation and wide geographical distribution, mirrors the skin pigmentation-vitamin D relationship found in humans; 2) compare vitamin D metabolite levels in wild and captive members of the same or similar baboon species; and 3) apply a recently developed method currently used in humans for measuring multiple vitamin D metabolites as a panel to explore if/how these metabolites can inform us on vitamin D sufficiency. Serum samples from males of three baboon species in the wild: *Papio anubis* (olive baboon, dark exposed skin), *P. cynocephalus* (yellow baboon, brown exposed skin), and *P. hamadryas* (hamadryas baboon, pink exposed skin), were compared with vitamin D supplemented captive olive baboons with sun exposure. Liquid chromatography/tandem mass spectrometry

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CONFLICT OF INTEREST

There was no conflict of interest for any of the authors of this manuscript.

(LC/MS/MS) measured vitamin D and its main metabolites. Cholecalciferol, 25hydroxyvitamin D_{2&3}(25(OH)D_{2&3}), and 24,25 dihydroxyvitamin D_{2&3} (24,25(OH)₂D_{2&3}), showed significant differences by species. The levels of cholecalciferol due to supplements in the captiveolive baboons did not convert to higher 25(OH)D₃ while the wild olive baboons exhibited the lowest levels for both cholecalciferol and 25(OH)D₃. Further metabolic conversion of 25(OH)D₃ to 24,25(OH)₂D₃ indicated that all baboons had more similar conversion ratios and these were within the same range found for humans that are depicted as having adequate vitamin D levels. This study provided evidence that exposed skin color does influence vitamin D₃ levels, with lower levels in darker skinned species, but these differences are eliminated in the downstream metabolite conversion indicating strong regulatory control.

Keywords

baboons; captive; metabolic profiles; skin color; vitamin D; wild

1 | INTRODUCTION

The major source of vitamin D is cutaneous production upon sun exposure. This essential vitamin is responsible for bone health and likely for multiple other systems including immune, metabolic, cognitive functioning, and the prevention of autoimmune diseases (Kalueff, Lou, Laaksi, & Tuohimaa, 2004; Lagishetty et al., 2010; Telcian et al., 2017). Most cells in the body have the vitamin D receptor and the capacity to produce the bioactive form: 1,25-dihydroxyvitamin D (1,25(OH)₂) (Holick, 2011), which regulates multiple genes with diverse physiologic functions. As vitamin D inadequacy is common in humans, it is currently the most widely studied vitamin (Shah et al., 2017).

Vitamin D deficiency symptoms have been documented in institutions housing nonhuman primates, and the deficiency has been especially noted for nursing infants (Crissey, Pribyl, Pruett-Jones, & Meehan, 1998). Vitamin D deficiency causes inadequate bone mineralization, that is, osteomalacia in adults and rickets in infants. Vitamin D toxicity results when vitamin D levels are too high due to high consumption or supplementation and results in calcification of soft tissues, such as kidneys, lung, and heart (Klein, 1985). Determining, “normal” levels for individual non-human primate species would require measuring levels from wild, indigenous populations of each species who have evolved to maintain normal levels under their environmental conditions. Early studies based their assessment of captive nonhuman primates in comparison with the levels considered normal for humans (Crissey et al., 1998). A number of studies that have reported the measurement of the two most studied metabolites, 25 (OH)D and 1,25(OH)₂D in marmosets (*Callithrix jacchus*), rhesus (*Macaca mulatta*), and cynomolgus macaques (*Macaca fascicularis*), required separate analysis for each analyte and reported higher ranges in values than has been found for humans (see Ziegler, Kapoor, Hedman, Binkley, & Kemnitz, 2015). Whether the higher levels may be due to the different amounts of vitamin D used to supplement the diets of indoor and outdoor nonhuman primates or if nonhuman primates have higher levels than humans have not been established.

Primates have adapted to their environments by using hair, skin coloration, and hair density to control the amount of radiation absorbed from the sun (Zihlman & Cohn, 1988). Greater skin melanin increases skin darkening with sun exposure and the time required to maximize the formation of vitamin D₃, indicating that dark skin produces less vitamin D₃ than lighter skin in primates per unit of ultraviolet exposure (Holick, MacLaughlin, & Doppelt, 1981). Baboons, genus *Papio*, inhabit much of sub-Saharan Africa in varied habitats including savannas, forests, scrublands, arid semi-desert, hilly areas, alpine meadows, and mountains (DeVore & Washburn, 1963). Several papionin species have shared non-rainforest habitats in sub-Saharan Africa with hominins since their lineages emerged (Jolly, 2001). Baboons have often been used as an analogy for human evolutionary scenarios because of their similar geographic distribution and speciation patterns (Jolly, 2001). Baboon species, especially adult males, vary in the amount of pigment in their exposed skin and in hair color and distribution patterns (see Figure 1). For instance, *Papio hamadryas*, the hamadryas baboon, has the lightest skin with pink exposed skin on the face and buttocks, and silver hair, while *Papio anubis*, the olive baboon, has dark exposed skin and dense, dark hair. Both exposed skin and hair color in *Papio cynocephalus*, the yellow baboon, lies between these two species. If cholecalciferol production is related to exposed skin coloration, we would expect to see the lighter skinned baboon, the hamadryas baboon with higher levels of cholecalciferol due to higher UV absorption and production of cholecalciferol while the olive baboon would be expected to have lower absorption and production of cholecalciferol. No information exists on cholecalciferol levels in nonhuman primates to date. Captive nonhuman primates typically are limited in sun exposure and therefore, require oral cholecalciferol supplementation, but circulating levels found in their indigenous environment where their vitamin D levels reflect levels the species has been adapted to are unknown. Since the primary determinant of vitamin D levels is exposure to sunlight, we would expect the vitamin D levels in baboons living in their indigenous African environment to be adapted for optimum homeostasis, as has been suggested in studies in traditionally living human populations (Luxwolda, Kuipers, Kema, Dijck-Brouwer, & Muskiet, 2012).

Several new advancements in measuring vitamin D have occurred. Measurement of vitamin D by LC/MS/MS, has resulted in increased sensitivity and reliability compared to the use of immunoassays or HPLC separation methods. With the development of a panel for vitamin D metabolites, now several metabolites can be measured simultaneously allowing for the first time to examine the metabolic conversions of vitamin D and how they might differ between different primates. Recently, a study reported the comparison of the circulating metabolite of vitamin D₃, 25(OH)D₃, in congeneric wild and captive nonhuman primates: captive rhesus macaques, *Macaca mulatta*, (Ziegler et al., 2015), and wild toque macaques, *Macaca sinica* (Power & Dittus, 2017). The levels in the wild toque macaques were almost a third lower than the captive, supplemented rhesus monkeys indicating that supplemental levels in captive primates may be higher than necessary. Unfortunately, the measurement was by two different methods (LC/MS/MS for the captive macaques and by immunoassay for the wild macaques). To understand the levels that nonhuman primates are optimized to function under may be important in determining how much to supplement our captive primates. We therefore planned to: 1) determine vitamin D levels in captive and wild olive baboons and 2) compare levels of vitamin D metabolites in different baboon species with differing skin

coloration and 3) compare the wild baboons to a population of olive baboons held in captivity and receiving vitamin D supplements. Additionally, the LC/MS/MS method, considered the gold standard in human studies, was used to assess vitamin D and metabolites by a panel, therefore permitting the examination of the metabolic processing for vitamin D.

In the past, Vitamin D levels have been examined in captive nonhuman primates by use of immunoassays or HPLC with ultraviolet detection. However, these methods are lacking in the sensitivity of the newer LC/MS/MS, liquid chromatography/tandem mass spectrometry, methods (Shah et al., 2017). No nonhuman primate studies have yet utilized the newly developed, highly specific LC/MS/MS methods that are preferred for human analyses for the essential vitamin D metabolites that can be measured simultaneously as a panel. Thus, no normal ranges for vitamin D metabolites have been established for non-human primates for the different metabolites that are necessary for enhanced understanding of vitamin D sufficiency (Ziegler et al., 2015). In humans, it had been accepted to use 25(OH)D levels for defining an individual's vitamin D status (Chung et al., 2009). However, recent work in humans suggests that knowledge of other vitamin D metabolites may improve the definition of vitamin D sufficiency (Luque-Córdoba & de Castro, 2016).

Cholecalciferol is produced in the skin and in humans has a rapid half-life in circulation of only 24 hr before subsequently being converted to 25(OH)D₃ in the liver. 25(OH)D₃ is the main circulating metabolite of cholecalciferol and remains in circulation for around three weeks, then is further converted to the bioactive 1,25(OH)₂D₃ or to its major metabolite, 24,25(OH)₂D₃ via an alternative enzymatic process (Figure 2). Different levels of the enzymes processing vitamin D into the different pathways may modify the concentration of the measured target metabolite. For instance, circulating 25(OH)D is converted to 24,25(OH)₂D by the 24-hydroxylase enzyme, CYP24A1. This enzyme is regulated in part by the vitamin D receptor and regulates how much bioactive vitamin D, 1,25(OH)₂D, is available for activating the vitamin D receptor (Jones, Prosser, & Kaufmann, 2014). Consequently, the concentrations of 25(OH)D and 24,25(OH)₂D are highly regulated. The ratio of 25(OH) to 24,25(OH)₂D has been advocated by some as a better predictor of vitamin D sufficiency than 25(OH)D alone (Berg et al., 2015; Fabregat-Cabello et al., 2017). The ratio of 25(OH)D₃ to 24,25(OH)₂D₃ is termed the VMR, Vitamin D Metabolic Ratio, and a ratio within the normal range established for humans indicates healthy vitamin D status. We compared the ratios of the various baboon populations, and then compared these to human ratios. This is the first study comparing a nonhuman primate with humans in the metabolic processing of vitamin D.

Our previous study with LC/MS/MS analyses used separate assays for 25(OH)D_{2&3} and 1,25(OH)₂D_{2&3} to determine the levels in captive nonhuman primates who have been supplemented with vitamin D levels. The study used serum from rhesus and cynomolgus macaques, and common marmosets (Ziegler et al., 2015). The results showed that captive nonhuman primates have higher 25(OH)D_{2&3} and 1,25(OH)₂D_{2&3} levels than humans, represented by North American Caucasians, native East Africans, and highly sun exposed surfers in Hawaii (Binkley et al., 2007; Hedman et al., 2014; Luxwolda et al., 2012). Additionally, all species demonstrated high between-individual variation, indicating that supplementation with cholecalciferol or high amounts of sun exposure does not ensure a

uniform outcome. Our recently established vitamin D panel for human and nonhuman primate use measures cholecalciferol, 25(OH) D_{2&3}, and 24,25(OH)₂D_{2&3} (both the 2 and 3 forms) simultaneously by LC/MS/MS. We applied this method in the present study, making this the first nonhuman primate work to provide extensive information on these metabolites of both vitamin D₂ (ergocalciferol) and cholecalciferol in both captive and wild populations. As it has previously been advocated that highly sun-exposed humans be used to define optimal vitamin D status (Binkley et al., 2007) similarly, wild baboons might be used to define optimal levels in these species. We therefore examined vitamin D and its metabolites in serum samples from wild and captive baboon.

2 | METHODS

2.1 | Wild baboons

In the course of studies on baboon (*Papio*) population structure and natural hybridization (Phillips-Conroy & Jolly, 1986, 1988; Rogers & Kidd, 1996), we (JR, J.P.-C, CJ) collected blood samples from: olive, yellow and hamadryas baboons (*P. anubis*, *P. cynocephalus*, and *P. hamadryas*, respectively) (Figure 1). The olive and hamadryas baboons were trapped and sampled in the Awash National Park, Ethiopia (Phillips-Conroy & Jolly, 1986, 1988); the yellow baboons in Mikumi National Park, Tanzania (Rogers & Kidd, 1996) (Figure 3). Baboons in these areas are known to seek sun when it is cold and shade from direct sun when it is hot and therefore, have some control over amount of sun exposure. Samples were collected from the hamadryas and the olive baboons in the wet season in Ethiopia, and in the dry season in Tanzania for the yellow baboons. All protocols complied with institutional and governmental regulations and guidelines. Blood samples were taken in the morning from animals trapped soon after leaving their sleeping trees. Animals were trapped individually in drop-door box traps and tranquilized via a pole syringe with ketamine hydrochloride. Blood was collected into evacuated tubes with anticoagulant within 30 min of tranquilization and kept cool until the evening when the blood was centrifuged and separated and the plasma collected and stored in liquid nitrogen until shipping. The samples from the yellow baboons were centrifuged and separated for plasma, then stored in liquid nitrogen and transported to the U.S. while the olive and the hamadryas baboon blood was processed for plasma as well and stored frozen but were hand-carried on dry ice into the U.S. All African baboon samples for this project were first stored at –30 to –80 °C at New York University until shipped to the Wisconsin National Primate research Center to be analyzed.

Since increasing age is known to have a negative effect on vitamin D levels in humans (Adami, Viapiana, Gatti, Idolazzi, & Rossini, 2008), for the present study, we selected serum samples from adult, subadult or older juvenile males of each species ($N = 32$ olive, 30 yellow, and 30 hamadryas baboons). We further examined age to determine if it was a significant effect on species differences in vitamin D metabolites. Ages were estimated for each animal on the basis of tooth eruption and occlusal wear (Phillips-Conroy & Jolly, 1988). Each individual was assigned an age in months and allocated to an age class based on its dental maturation. Age class 3 comprised large juvenile males of 4–6 years; age class 4 were young adult males of 7–10 years, and age class 5 consisted of mature to old adult

males, 10–20 years old. Juveniles, age class 3, were a small fraction of the total per species except for the wild olive (captive olive 2/29; wild olive 12/32; yellow 6/30; hamadryas 4/30).

2.2 | Captive baboons from the southwest national primate research center (SNPRC)

The SNPRC at Texas Biomedical Research Institute, San Antonio, Texas is the major facility for research on captive baboons in the United States. It houses mainly olive baboons but also olive-yellow hybrids. The 29 olive baboons selected for this study lived outside in large corrals where sun exposure occurs throughout the day but can be limited by seeking shade. San Antonio has a similar amount of sun days to Awash National Park in Ethiopia (<https://www.worldweatheronline.com/san-antonio-weather-averages/texas/us.aspx>), although the low sun months are at different times of the year. Primary housing for baboons were large social enclosures of 10–20 animals encircled by a high solid fence with heated indoor areas and outdoor areas with climbing structures, perches, and swings. The baboons were fed a commercial diet that is supplemented with 6.6–7.1 IU/g of cholecalciferol (Purina 5038 or LE05, St Louis, MO) (Schlabritz-Loutsevitch et al., 2016).

The samples from the SWNPRC baboons were all collected on the same day, so there were no seasonal differences in these samples. While the wild baboons were all males, the captive baboons were female ($n = 18$) and male ($n = 11$). We therefore tested to see if cholecalciferol or 25(OH)D₃ differed significantly by sex using the nonparametric Mann Whitney U test. The means and distributions were very similar with no significant difference found for cholecalciferol ($U = 84.00$, $p = 0.51$), for 25(OH)D₃ ($U = 97$, $p = 0.95$) and for 24,25 (OH)₂D₃ ($U = 86.00$, $p = 0.57$). Therefore, the sexes were combined for evaluating the differences between captive and wild. Serum samples from six of the captive baboons were eliminated from the study since they were hybrids, *Papio anubis* × *cynocephalus*, as they could interfere with the interpretation of the results, leaving 23 samples from captive olive baboons. All sample collection from both the wild and the captive baboons adhered to the American Society of Primatologists (ASP) principles for the ethical treatment of nonhuman primates.

2.3 | Sample selection and statistical analyses for vitamin d metabolites

Serum samples from the three wild baboon species were analyzed for vitamin D metabolites together with 23 captive olive baboons from the SWNPRC. Vitamin D metabolites are highly stable for variation in temperatures and time to freezing for both 25(OH)D and 24,25 (OH)₂D in serum and plasma, unlike purified vitamin D metabolites (Lissner, Mason, & Posen, 1981)

Cholecalciferol values and its metabolites were found to have a normal distribution following log transformation or using the square root (vitamin D₃, square root, 0.07, $df = 115$, $p = 0.20$; 25(OH)D₃, log, 0.04, $df = 114$, $p = 0.20$; 24,25(OH)₂D₃, 0.05, $df = 113$, $p = 0.20$). The following analyses were used to address the following questions: 1) 2-way ANOVA (fixed factors: age and species, dependent variable: vitamin D metabolite) tested whether age and species produced an interaction on the vitamin D metabolites; 2) 1-way ANOVA (fixed factor: species, dependent variable: vitamin D metabolites) with post hoc Tukey tests was used to determine if there were concentration differences by species for the

individual analytes; and 3) ANCOVA (Analyses of covariance) (fixed factor: species, dependent variable: vitamin D metabolite, covariate: vitamin D metabolite upstream of dependent variable) was used to test the differences in conversion of cholecalciferol to 25(OH)D₃, and further conversion of 25(OH)D₃ to 24,25(OH)₂D₃ by species (including captive olives as a fourth species). If the metabolic conversions are different by species, then we would expect to see differences in the regression lines that represent the ratios of conversion. For the first test, the covariate was cholecalciferol, the dependent variable was 25(OH)D₃, and the independent variable was species. For the further conversion of 25(OH)D₃ to 24,25 (OH)₂D₃, the covariate was 25(OH)D₃, the dependent variable was 24,25(OH)₂D₃, and the species was the independent variable. Post hoc pairwise comparisons were done with Tukey HDS. Outlier values (one animal each in the wild and captive olive baboon samples) were removed (Grubb's test, $p < 0.05$). Statistics were done with SPSS version 23.

2.4 | Methods for LC/MS/MS measurement of vitamin D panel metabolites

LC/MS/MS has become the preferred method for measuring vitamin D and its metabolites in humans. The National Institute of Standards and Technology (NIST) provides a quality assurance program to standardize the major laboratories in Vitamin D measurement for humans ensuring reliable and consistent values between laboratories (Dowling, Hull, Sundvall, Lamberg-Allardt, & Cashman, 2017). The vitamin D panel developed in our laboratory required less than 100 µl of serum for the analyses of five analytes and has been validated against five laboratories for accurate measurement (Dowling et al., 2017).

Powdered vitamin D metabolites and deuterated internal standards (24,25(OH)₂D₃, 24,25(OH)₂D₃-d₆, 25(OH)D₃-d₆) were purchased from Toronto Research Chemicals Inc. (Toronto, ON), cholecalciferol, 25(OH)D₃ and 25(OH)D₂ were purchased from Cerilliant (Sigma) and 24,25(OH)₂D₃ was kindly provided by Dr. Donald Wiebe (Director of Toxicology, Dept. Pathology and Laboratory Medicine, UW-Madison). The method was reported in (Dowling et al., 2017) but modified as follows. All baboon samples were aliquoted as 60–100 µl of serum (depending on volume availability) for the vitamin D metabolite panel by LC/MS/MS. To serum, 300 µl of 1% formic acid in acetonitrile was added to serum with 100 µl internal standards at 250 ng/ml of d₆-25(OH)D₃, d₆-25(OH)D₂, and 75 ng/ml of d₆-24,25(OH)₂D₃ in ethanol. The proteins were precipitated by centrifugation and the supernatant was collected and run through solid phase extraction (Hybrid SPE, Sigma, St. Louis MO) and then derivatized with 4-Phenyl-1,2,4-triazole-3,5-dione (PTAD) to increase the sensitivity of the vitamin D metabolite measurement. Liquid chromatography separated the vitamin D metabolites using a Phenomenex Kinetex column (2.6 µm C18, 100A, 100 × 2.1 mm) with the following gradient: A = 0.1% formic acid in ultrapure water, B = methanol with methylamine 0.5% program %B at 0 min, 50% up to 98% over 18 min, held until 20 min and equilibrated at 50% for 5 min on a Shimadzu Prominence (Addison, IL) integrated PLC interfaced with a SCIEX (Foster City, CA) QTRAP 5500 Quadrupole—Linear Ion Trap Mass Spectrometer equipped with an ESI source in positive mode. Coefficient of variation ranged from 7.1–17.5% for all forms of vitamin D. Serum cholecalciferol and the 2 and 3 forms of 24,25 (OH)₂D and both 2 and 3 forms of 25(OH)D were measured.

3 | RESULTS

2-way ANOVA tested whether age and species produced an interaction on the vitamin D metabolites. The model showed that species contributed the most effect on the metabolite levels, while age was significant within a species but not across species; there was no significant interaction (Table 1). *Post hoc* analyses indicated that juveniles had significantly higher circulating levels of cholecalciferol than adults ($p = 0.02$) but not for 25(OH)D₃ ($p = 1.00$) or 24,25(OH)₂D₃ ($p = 0.96$). Therefore, age did not influence the between species comparisons and could be eliminated from the model.

Table 2 lists the mean and standard deviation in the levels of cholecalciferol and metabolites for all baboons by species and for the captive versus wild baboons. We found significant differences in circulating cholecalciferol levels ($F = 23.56$, $df = 3,105$, $p = 0.0001$, $\eta^2 = 0.41$, Figure 4a). Therefore, the species of baboon contributed 41% of the effect on the amount of cholecalciferol. *Post hoc* analyses indicated that the captive olive baboons had significantly higher levels of cholecalciferol than the three wild baboon species (all, $p = 0.0001$), while the wild olive baboons had significantly lower levels than both the captive and the other wild baboon species ($p = 0.001$ – 0.0001).

For circulating 25(OH)D₃, significant differences were found between the species ($F = 13.32$, $df = 3,105$, $p = 0.0001$, $\eta^2 = 0.27$; Figure 4b) but with species having only 27% effect on the 25(OH)D₃ levels. Wild yellow baboons had significantly higher levels than other wild species (wild olive: $p = 0.0001$; wild hamadryas $p = 0.03$), while the captive olive baboons were not significantly different from any of the wild baboon species (wild olive: $p = 0.07$; wild yellow: $p = 0.10$; wild hamadryas: $p = 1.0$).

The vitamin D metabolite, 24,25(OH)₂D₃ showed significant differences by species ($F = 8.73$, $df = 3,105$, $p = 0.0001$, $\eta^2 = 0.20$; Figure 4c), however the effect size for species contributed only 20% towards the differences in 24,25(OH)₂D₃ levels between the species. The wild yellow baboon showed significantly higher levels than the other wild species (olive, $p = 0.0001$; hamadryas, $p = 0.01$) while the captive olive baboon was not significantly different from any wild species (wild olive: $p = 0.16$; yellow: $p = 0.34$; hamadryas: $p = 0.49$).

The levels of vitamin D₂ metabolites were very low (pg/ml range) compared to vitamin D₃ levels, and for the captive baboons 11 out of the 23 samples were below the level of detection for the 25(OH)D₂ assay at 0.185 ng/ml. However, levels of 25(OH)D₂ were detectable at low concentration in the three, wild species which differed significantly ($F = 18.76$, $df = 2, 90$, $p = 0.0001$; $\eta^2 = 0.36$): yellow baboons showed significantly higher levels than the other species including the captive olive (captive olive: $p = 0.001$; wild olive: $p = 0.003$; hamadryas: $p = 0.0001$; Table 2).

Metabolic conversion of cholecalciferol to 25(OH)D₃ is shown in Figure 5. ANCOVA showed there were significant differences in the slopes of the lines between the species for the conversion of cholecalciferol to 25(OH)D₃ ($F = 26.26$, $df = 4, 109$, $p = 0.0001$, $\eta^2 = 0.49$) with 49% of the variance explained by species. The pairwise comparisons by Bonferroni indicated significant differences for the slope of the line between the yellow

baboons and the captive olive baboons ($p = 0.001$), the wild olive baboons ($p = 0.001$), and the hamadryas baboons ($p = 0.001$). The yellow baboons had the highest levels of both cholecalciferol and 25(OH)D₃ while the hamadryas baboons also demonstrated higher levels of 25(OH)D₃ than either the wild or the captive olive baboon. Very little cholecalciferol was reflected in increased levels of 25(OH)D₃ in the wild olive baboons while higher levels of circulating cholecalciferol in the captive olive baboons did not increase circulating 25(OH)D₃.

The further conversion of 25(OH)D₃ to 24,25(OH)₂D₃ is shown in Figure 6 as the VMR average by species. While the slopes of the lines were significantly different ($F = 80.54$, $df = 4, 111$, $p = 0.0001$, $\text{Eta}^2 = 0.75$), only 10% of the variance was explained by species differences ($F = 4.13$, $df = 3, 111$, $p = 0.008$, $\text{Eta}^2 = 0.10$) and 66.5% was explained by levels of 25(OH)D₃ ($F = 212.62$, $df = 1, 111$, $p = 0.0001$, $\text{Eta}^2 = 0.665$). Pairwise comparisons indicated that the slope of the line for the hamadryas baboons was significantly different from that of the wild yellow baboons. However, most of the conversion differences seen with cholecalciferol to 25(OH)D₃ were eliminated in the conversion to 24,25(OH)₂D₃. The ratios of 25(OH)D₃ to 24,25(OH)₂D₃ for all of the baboons were in the range found for normal healthy humans considered to have vitamin D sufficiency (between 20 and 40 ng/ml 25(OH)D₃, Tang et al., 2017), independent of skin color.

4 | DISCUSSION

Wild baboons from three different species with different degrees of coloration of exposed skin had significantly different levels of cholecalciferol and its metabolites. These differences paralleled the patterns found between skin pigmentation and vitamin D status in humans. The low cholecalciferol levels found in olive baboons living in the wild were not seen in their captive conspecifics, which were supplemented with cholecalciferol even though they lived in sun exposed outdoor caging. Interestingly, the higher supplemented cholecalciferol did not translate to higher 25(OH)D₃ in the captive baboons. By measuring the metabolic conversions to 25(OH)D₃ and 24,25(OH)₂D₃ as ratios, the apparent differences between the species were reduced and may indicate these reflect a normal vitamin D metabolism for all the sun enriched baboon species.

Cholecalciferol is a precursor for the active steroid-like hormone, 1,25(OH)₂D₃ that is important in many physiological, developmental, and cognitive functions. One component of regulating the production of cholecalciferol is through skin color that also limits UV absorption through skin. The UV-B rays from the sun have been the main source for cholecalciferol synthesis prior to supplementation with synthetic vitamin D₃ (Rajakumar, Greenspan, Thomas, & Holick, 2007). In humans, different geographical populations have different skin pigmentation that differs due to the melanin content in their melanocytes located in the skin's epidermal layers (Matsuoka, Wortsman, Haddad, Kolm, & Hollis, 1991). Melanin is produced from tyrosine synthesized in the melanocytes and influences differences in skin color. In humans, vitamin D₃ photoproduction is influenced by season, latitude of residence and skin color (Rajakumar et al., 2007). Light skin has a low melanin content and absorbs more UV radiation for synthesizing vitamin D while dark skin is highly melaninized for protection against UV radiation. (Zihlman & Cohn, 1988). Since melanin

absorbs UV-B radiation from the sun, it compromises cholecalciferol photoproduction in dark skin, thereby lowering the amount of cholecalciferol synthesized. Natural selection has produced variation in human skin regulation to adjust the levels of pigmentation to levels of UV radiation (Jablonski & Chaplin, 2000). Additionally, the variation in skin coloration is correlated with ambient UVB. In latitudes with low levels of UVB, there is an increase in cholecalciferol production with a decrease in melanin and skin color. Our results indicate that, as found in humans, dark exposed skin in baboons results in lower levels of circulating cholecalciferol and 25(OH)D₃. Moreover, similar to humans, the wild olive baboons have significantly lower cholecalciferol levels than the other wild species with lighter skin. Vitamin D supplementation of captive olive baboons did substantially increase the levels of 25(OH)D₃ over those found in wild olive baboons and therefore it may not be required for baboons living in captivity but with access to UV radiation year-round. While the olive baboons have overlapping habitat with the hamadryas baboons in Ethiopia and with the yellow baboons in Tanzania, their exposed skin coloration and cholecalciferol levels would argue they were selected for different climates and latitudes.

Examining 25(OH)D₃ levels provided us with an opportunity to compare captive baboons with wild, indigenous baboons who have extensive exposure to the sun. We assumed that wild-living olive baboons would have sun exposure and resultant vitamin D levels reflecting long-term physiological adaptation. Even though the olive baboons have lower levels than the other two species, it may be due to their ability to mitigate the effects of sun exposure by limiting vitamin D production as is seen in dark skinned humans and possibly maintaining higher levels of the circulating 25(OH)D₃ form by changes in the receptor binding. Using the same methodology and simultaneous assays to exclude the possibility of assay effects (Binkley et al., 2007), we found that the captive olive baboons had significantly higher levels of cholecalciferol than the wild-living species. The supplements provided for the captive baboons were more than needed to produce the levels of circulating 25(OH)D₃ comparable to those seen in the wild olive baboons, but there was no evidence that these levels were unhealthy. As captive baboons were sun exposed and receiving supplemental cholecalciferol, it is not possible to determine what proportion was sun induced rather than a result of dietary absorption. Our findings, together with similar findings of high vitamin D levels in captive versus wild macaques (Power & Dittus 2017), suggest that levels of supplementation of captive primates may exceed that needed for maintaining adequate vitamin D levels. However, due to the tight regulation of vitamin D, there do not appear to be any negative effects.

Nonhuman primates, such as the rhesus macaque, appear to tolerate higher vitamin D levels than humans (Ziegler et al., 2015). It has been proposed that the upper 25(OH)D level found in highly sun exposed humans should provide a ceiling for recommended supplements. In the humans studies with high sun exposure (Barger-Lux & Heaney, 2002 Binkley et al., 2007; Luxwolda et al., 2012), the highest 25(OH)D levels achievable by sun exposure were up to 65 ng/ml. The average levels of 25(OH)D₃ in the wild baboons were higher than the levels found in people living in the same location as the yellow baboons (Luxwolda et al., 2012). Yellow baboons had an average level of 25(OH) D₃ around 125 ng/ml which would be considered a toxic level for humans. If in our captive nonhuman primates, we are supplementing to levels well over their highest levels achievable in their native habitats, then

we have to question if these higher levels are preventative or harmful. However, once the ratios of 25(OH)D to 24,25(OH)₂D are examined, the ratios for the baboon appear to be in the range that is found for humans. It appears that vitamin D is tightly regulated as demonstrated by the levels of 24,25(OH)₂D where the differences in the species and in age have been reduced or eliminated.

Examining the metabolic pathway of vitamin D provides us with a means to understand vitamin D sufficiency (Berg et al., 2015). Since the vitamin D metabolite 24,25(OH)₂D is the major catabolic product of 25(OH)D and is partially regulated by the vitamin D receptor activity, the concentration of 24,25(OH)₂D may be a better indicator of vitamin D sufficiency than 25(OH)D (Berg et al., 2015). African Americans who have low 25(OH)D₃ concentrations but are not functionally deficient also have lower 24,25(OH)₂D₃ and their ratios are still comparable to those of light-skinned Americans. If lower 25(OH)D levels are associated with increased bioavailable 25(OH)D, then 24,25(OH)₂D would be lower as well. In our wild olive baboons both 25(OH)D₃ and 24,25(OH)₂D₃ are significantly lower than the mean values for both the wild yellow and hamadryas baboons. This would indicate that wild olive baboons are vitamin D sufficient even with lower levels than the other baboons. When we examine the ratio of 25(OH)D₃ to 24,25(OH)₂D₃, the best conversion is seen in the hamadryas baboons with pink exposed skin, which would allow a greater degree of vitamin D conversion to its metabolites. In examining the vitamin D metabolic ratio, VMR, there are differences in the mean ratio of conversion but all are within the same range that is seen in humans (Tang et al., 2017). Baboons mainly ranged in ratios between 5 and 20, which is in the same range found in normal healthy humans. This appears to indicate that while circulating vitamin D may be higher in baboons in their native habitat, their conversion to the important metabolites is similar to humans and therefore the regulatory processes are likely the same in all primates. While lower, the wild and captive olive baboons have adequate conversion and do not appear to have deficiency. Like black skinned and white skinned people living in North America, olive baboons show lower levels of the vitamin D metabolites than the lighter skinned baboons but have an adequate conversion of vitamin D₃ into its metabolites in normal healthy individuals.

Determining the correct levels of vitamin D and its metabolites for our captive nonhuman primate species may be important to consider in future studies of disease models. Recently, vitamin D levels have been associated with many disease states such as with cardiovascular, kidney disease, obesity, and brain development (Eyles, Burne, & McGrath, 2013; Kim & Kim, 2014; Norman & Powell, 2014; Vanlint, 2013). These studies mainly have investigated the effect of low levels of vitamin D, and over supplementing has been less-studied. In humans, vitamin D toxicity has increased due to consuming high levels of supplemented vitamin D (Kaur, Mishra, & Mithal, 2015). While we are just beginning to understand the levels that are optimum for humans, determining what levels are optimum for our captive nonhuman primates may be extremely important for their role as models for human research.

The nonhuman primate studies that have used LC/MS/MS technology to measure vitamin D metabolites such as 25(OH)D have indicated wide within-species variation for captive nonhuman primates (Ziegler et al., 2015). In this study, we found high variation in vitamin D metabolites for the populations of the wild olive, yellow, and hamadryas baboons. Age was

found to be a factor in the variation within the wild baboons, with higher vitamin D levels in juveniles. Additionally, it is likely that genetic factors may attribute to differences in vitamin D within a population. For instance, in vervet monkeys (*Chlorocebus aethiops*) 66 percent of their 25(OH)D level differences are due to inheritability (Chittoor et al., 2016). In human research, a genetic loci has been identified as associated with differences in 25 (OH)D₃ levels and has an heritability of 7.5% (Jiang et al., 2018). 25 (OH)D₃ has been reported to be highly inheritable, and research from classical twin studies estimates a heritability rate as high as 50–80% (Karoohl et al., 2010, Orton et al., 2008). Other factors include a high requirement for vitamin D in pregnancy as identified in pregnant female baboons and humans as it is involved in placental function and fetal growth (Bodnar, Catov, Roberts, & Simhan, 2007; Schlambritz-Loutsevitch et al., 2016). Obesity and other metabolic or kidney diseases are known to cause vitamin D deficiency (Schlambritz-Loutsevitch et al., 2016). Behavior would definitely play a role in vitamin D levels as limiting sun exposure limits the levels of vitamin D.

Vitamin D₂ is derived solely from food sources, mainly mushrooms and other fungus (Jasinghe & Perera, 2005). A notable finding was that yellow baboons in the wild had the highest level of 25(OH)D₂, significantly higher than that seen in olive and hamadryas baboons, while captive olive baboons had the lowest levels. Yellow baboons at Mikumi eat subterranean food sources most frequently of the three wild populations investigated in this study, and this would be the likely source of the vitamin D₂ (Hill & Dunbar, 2002; Norton, Rhine, Wynn, & Wynn, 1987). Vitamin D₂ is a natural source that occurs in some plant and fungi foods in response to UV radiation. Mushrooms and other fungi are one the major producers of vitamin D₂. The levels found in both humans and nonhuman primates are in the low pg/ml range and there is some evidence that the vitamin may not be as biologically active as vitamin D₃ (Horst, Koszewski, & Reinhardt, 1990). However, in the yellow baboon these food sources may also contribute to their overall intake/production.

With the use of a metabolic panel, we were able to see how different baboon species with different skin pigmentation metabolized vitamin D compared to captive olive baboons with Vitamin D₃ supplements. Vitamin D takes multiple metabolic pathways where both forms of vitamin D (D₂ and D₃) are involved (Luque-Córdoba & de Castro, 2016). Monitoring the metabolites allows the deduction of the enzymatic activity and metabolic pathway. The ratio of 25(OH)D₃ to 24,25(OH)₂D₃ is a better indicator than 25(OH)D₃ of vitamin D deficiency (Berg et al., 2015). The enzyme, CYP24A1 that converts 24,25(OH)₂D₃ from 25(OH)D₃ is partially regulated by vitamin D receptor activity. Thus, it may be helpful to establish healthy ratios at the species level to determine when individuals are experiencing a deficiency (Tang et al., 2017).

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FIGURE 1.

Exposed skin coloration varies in the three species of baboons. Left is the olive baboon, *Papio anubis*, (Image: Bernard [Castelein/naturepl.com](https://www.naturepl.com)); middle is the yellow baboon, *Papio cynocephalus*, (Image: Thomas M. Butynski and Yvonne A. de Jong); right is the hamadryas baboon, *Papio hamadryas* (Image: Zoological Center, Tel-Aviv-Ramat-Gan, Israel)

FIGURE 2.

Metabolic pathways for vitamin D. UV radiation converts cholesterol in the skin to produce circulating 25 hydroxyvitamin D in the liver. In the kidney, enzymatic conversions produce either 1,25 dihydroxyvitamin D (1,25(OH)₂D) or 24,25 dihydroxyvitamin D (24,25(OH)₂D). Regulatory systems ensure 1,25(OH)₂D maintains consistent levels. CYP indicates regulatory enzyme. PTH, parathyroid hormone

FIGURE 3.

Map of the distribution of *Papio* species in Africa. The genus *Papio* consists of six different species living in sub-Saharan Africa. Three species were used for this study: *P. hamadryas*, *P. Anubis* (both from Awash National Park, Ethiopia), and *P. cynocephalus* (from Kikumi National Park, Tanzania). Map derived from Kenneth Chiou (Dissertation)

FIGURE 4.

Comparison of circulating (a) cholecalciferol, (b) 25 hydroxyvitamin D₃ (25(OH)D₃) ng/ml, and (c) 24,25(OH)₂D₃ for captive olive baboons and three species of wild baboons. (a) Captive olive baboons, (a1), had significantly higher mean levels of cholecalciferol than the wild baboons (a2), all comparisons, $p = 0.0001$) and the wild olive baboon, (a3), had significant lower levels than the other baboons (a4), yellow: $p = 0.0001$; hamadryas: $p = 0.001$; captive olive: $p = 0.0001$). (b) Wild yellow baboons had higher mean 25(OH)D₃ levels than the other wild species (wild olive: $p = 0.0001$; wild hamadryas $p = 0.03$), while

the captive olive baboons were not significantly different from any of the wild baboon species. (c) The wild yellow baboon showed significantly higher levels than the other wild species (olive, $p = 0.0001$; yellow, $p = 0.001$) while the captive olive baboon was not significantly different from any wild species ($p = 0.34$)

FIGURE 5.

The metabolism of cholecalciferol is represented by its conversion of cholecalciferol to 25(OH)D₃. The slopes of the lines were all different from each other indicating that the conversions varied by species in this intermediary step in the metabolism of vitamin D. The yellow baboons showed the highest conversion of Vitamin D₃ to 25(OH)D₃ shown as low levels of cholecalciferol are metabolized to higher amounts of 25(OH)₂D₃ whereas higher cholecalciferol did not relate to higher 25(OH)₂D₃ for the captive olive baboons who were supplemented

FIGURE 6.

The ratio of serum 25(OH)D₃ to 24,25 (OH)₂D₃ concentration is known as the Vitamin D Metabolic Ratio (VMR) serves as an indicator of vitamin D metabolic status. The red lines indicate the upper and lower median levels of ratios for humans considered to be vitamin D sufficient (Tang et al., 2017). The median range for the baboons is very similar to that of normal healthy humans independent of skin color. The hamadryas baboons, a, had significantly higher VMR than the wild olive and yellow baboons (Tukey; wild olive: $p = 0.02$; yellow: $p = 0.03$)

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Table 1

Age and species effect on cholecalciferol and its metabolites, 25(OH)D₃, 24,25(OH)₂D₃

Vitamin D metabolite	Species	Age class	Interaction
Cholecalciferol	$F = 7.56$ $p = 0.0001$ $\eta^2 = 0.53$	$F = 4.77$ $p = 0.001$ $\eta^2 = 0.16$	$F = 1.66$ $p = 0.12$ $\eta^2 = 0.12$
25(OH)D ₃	$F = 4.14$ $p = 0.0001$ $\eta^2 = 0.39$	$F = 3.90$ $p = 0.006$ $\eta^2 = 0.14$	$F = 0.86$ $p = 0.55$ $\eta^2 = 0.07$
24,25(OH) ₂ D ₃	$F = 3.13$ $p = 0.0001$ $\eta^2 = 0.33$	$F = 2.26$ $p = 0.07$ $\eta^2 = 0.09$	$F = 0.42$ $p = 0.91$ $\eta^2 = 0.03$

Table 2

Mean and S.E. for vitamin D and metabolites for captive olive, wild olive, yellow and hamadryas baboons

Vitamin D metabolites ng/ml	Captive <i>P. anubis</i>	Olive (<i>P. anubis</i>)	Yellow (<i>P. cynocephalus</i>)	Hamadryas (<i>Phamadryas</i>)
Cholecalciferol	6.84 ± 4.43	0.923 ± 9.1	3.25 ± 2.23	3.11 ± 1.85
25(OH)D ₃	88.11 ± 4.86	59.88 ± 4.18	126.4 ± 9.85	87.82 ± 6.90
24,25(OH) ₂ D ₃	10.37 ± 1.11	5.94 ± 0.55	15.03 ± 1.56	7.50 ± 0.66
25(OH)D ₂	0.21 ± 0.04	1.09 ± 0.17	3.10 ± 0.49	0.63 ± 0.13