Fecal sterols as a potential tool for conservation paleobiology in East Africa

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1. Introduction

Since the late Pleistocene the number and diversity of terrestrial megafauna (typically defined as herbivores and carnivores exceeding a threshold for body weight) has declined across all continents except Antarctica (e.g., Malhi et al., 2016). In many instances an initial decline was triggered by the arrival of early humans (Bartlett et al., 2016; Sandom et al., 2014), but accelerated during the Anthropocene through hunting and habitat loss. In relative terms, extinction of terrestrial megafauna in Africa was modest compared to other continents. Malhi et al. (2016) report that all eleven species of the continent's large Pleistocene carnivores remain today, as do 56 of the 74 species of large herbivores. The remaining megafauna of East Africa are vital for maintaining healthy ecosystem services and are valuable to regional economies. However, recent and projected changes in East African climate (particularly the amount and seasonality of rainfall) are anticipated to impact megafauna populations during the remainder of the 21st century and beyond. Throughout the Holocene, trends and events in East Africa's hydroclimate (including abrupt, century-scale changes; e.g., Tierney and deMenocal, 2013; Tierney et al., 2013) likely influenced megafauna populations. These changes may provide analogs to help anticipate how future climate changes will impact the iconic megafauna of East Africa.

Conservation paleobiology seeks to use proxy reconstructions of animal communities (composition, abundance, and geographic range), ecosystems, and paleoenvironments (e.g., frequency and severity of drought) to provide a context for recent and predicted changes that extends beyond the limited duration of historical observations and measurements (e.g., Dietl and Flessa, 2011; Dietl et al., 2015). These reconstructions can elucidate (for example) how species responded to natural climate variability across a range of timescales and capture conditions and trends that are not adequately represented in the short observational record, but may be analogous to future changes. In the specific case of terrestrial megafauna, a major challenge for conservation paleobiology is how to characterize past populations because these animals are relatively few in number, widely distributed and mobile when alive, and poorly preserved in the sedimentary record after death. Consequently, finding the remains of megafauna is not a

viable approach to quantitively describe and analyze community changes, particularly on short (centennial to millennial) timescales. An overarching goal for conservation paleobiology is to develop new proxies that overcome the limitations of using sub-fossil remains to infer changes in megafauna populations. One possibility is to utilize dung preserved in sedimentary archives as a proxy for megafauna community composition since it is likely to be abundant, widespread, and preserved in sediment cores unlike sub-fossil remains of the animals themselves.

Fecal stanols/sterols are a group of biomarkers (here we use the term fecal sterol for simplicity) present in dung (Bull et al., 2002). In the digestive tract of animals, sterols undergo modification by gut-dwelling microbes in anoxic conditions to produce 5β-stanols that are excreted in dung. Modification of the same sterols in oxic, exogenic environments instead produces 5α-stanols (Linseele et al., 2013). The make-up of fecal sterols in dung is controlled by dietary intake and metabolic processes (for example, cholesterol is present even in animals with low-cholesterol diets) that provide the sterol precursors and the type and effectiveness of biochemical alterations that occur in the digestive tract (incomplete alteration results in precursor sterols being present in dung). Therefore, variability in diet and digestive biochemistry among species may produce dung with a diagnostic profile of fecal sterol composition. Analysis of modern dung demonstrated that fecal sterols can objectively identify the origin of dung in terrestrial (e.g., Harrault et al., 2019; Leeming et al., 1996; Shah et al., 2007) and marine (e.g., Leeming et al., 2015) ecosystems, often to the species level. Fecal sterols have low solubility in water and bind readily to particulate matter, which makes them well-preserved in depositional environments such as wetland and lake sediments (e.g., Bartlett, 1987; D'Anjou et al., 2012; Schroeter et al., 2020; Vane et al., 2010) that capture sediment (and likely fecal material) from a wider catchment area. This preservation and catchment-scale integration opens up the possibility that the characteristic fingerprint of fecal sterols from particular species/groups of megafauna can be recognized in dated sediment cores to infer community changes through time.

Our goal is to investigate the potential utility of sediment-hosted fecal sterols to reconstruct megafauna communities in East Africa over the Holocene. Proxy development is a multi-stage process that begins with testing if a potential proxy (e.g., fecal sterols) can reliably recognize or reconstruct the variable of interest (e.g., species of megafauna) in samples of known origin (e.g., fresh dung). In the absence of existing data, we aim to (1) produce a modern training set of fecal sterols from the dung of East African megafauna that captures within- and among-species variability; and (2) investigate how reliably fecal sterols identify the origin of dung. Our results demonstrate that analytical measurements of fecal sterols are reproducible and that variability in fecal sterol content among individuals of the same species is typically less pronounced than variability among species. Using a statistical model, we estimate that fecal sterols can identify the species origin of a dung sample with ~72% accuracy. The dataset of modern fecal sterol measurements from East African megafauna may have utility in reconstructing animal populations during the Holocene using sediment-hosted fecal sterols.

2. Study Area

The Maasai Mara National Reserve and Serengeti National Park are two of the most well-recognized conservation areas in East Africa (Figure 1) and represent a transnational savanna grassland with varying degrees of woodland. These ecosystems include a high abundance and diversity of wildlife (e.g., Ogutu et al., 2011). However, there were drastic declines in wildlife populations in this region over the last ~40 years, with some species declining to less than a third of their former abundance inside the protected Maasai Mara National Reserve and in its surrounding rangelands. Wildlife declines were attributed to increasing abundance of livestock, land conversion around the Maasai Mara National Reserve from rangelands to agriculture, poaching, and increasing frequency and severity of droughts (e.g., Lamprey and Reid, 2004; Ogutu et al., 2011; Ogutu et al., 2016; Ottichilo et al., 2000). These changes are typical of those occurring in rangelands throughout Kenya. Despite these declines, the Mara region still contains ~30% of Kenya's wildlife (Ogutu et al., 2016).

Rainfall (amount and seasonal distribution) is the primary driver controlling reproduction, survival, and migration of African savanna ungulates within the region (Bartzke et al., 2018; Ogutu et al., 2008). The ecology of the Maasai Mara National Reserve and Serengeti National Park is strongly influenced by the annual migration of Serengeti wildebeest (*Connochaetes taurinus*) (Sinclair and Arcese, 1995). Every year at the end of the wet season (month; Figure 1B), ~1.4 million wildebeest along with large numbers of zebra (*Equus burchelli*) and Thomson's gazelles (*Gazella thomsoni*) migrate from the southern, short-grass plains of the southern Serengeti National Park to the wetter, tall-grass woodland and savanna habitats in the northern Serengeti National Park and Maasai Mara National Reserve in response to opposing rainfall and nutritional gradients (Holdo et al., 2009). This migration plays an important role in driving primary production, fire frequency, and predator population dynamics, among many other ecosystem processes.

The spatio-temporal pattern of precipitation in East Africa reflects interactions between seasonal reorganization of prevailing atmospheric circulation and regional topography such as the East African highlands (e.g., Camberlin, 2018; Funk et al., 2016). This complexity drives a corresponding diversity of landscapes and ecosystems. In the broadest terms, East Africa is an anomalously dry region of the humid tropics that typically receives less than 2 mm/day of precipitation on average compared to locations at similar latitudes in West Africa (~5 mm/day) and Indonesia (up to 12 mm/day; e.g., Figure 1 of Yang et al., 2015). This anomaly arises from zonal Walker circulation in which low-level convergence in the Indo-Pacific warm pool is associated with rising air, heavy precipitation and low surface pressure, while divergence in East Africa is associated with descending air, little precipitation, and high surface pressure. Shifts in atmospheric circulation during the year result in a strong seasonality of precipitation (Bartzke et al., 2018; Camberlin, 2018; Yang et al., 2015; Figure 1B). For the Maasai Mara National Reserve, the presence of the intertropical convergence zone in the southern hemisphere results in prevailing northeasterly winds during January and February. These winds move from the Arabian Sea across Somalia bringing dry, stable weather. In March to May southeasterly winds bring moist air from the

Indian Ocean (where sea surface temperatures are at their warmest of the year) into East Africa resulting in substantial precipitation (the so-called "long rains"). During March, April, and May approximately 34% (336 mm) of annual precipitation is received in the Maasai Mara National Reserve (Figure 1B; Bartzke et al., 2018). Changes to the strength and position of the Somali Jet and the presence of the intertropical convergence zone in the northern hemisphere during June to September reduce onshore moisture transport and bring relatively cool, dry air from the south assisted by sea surface temperatures that are at their coldest of the year. In October to December, weakening of the Indian Monsoon and reversal of Indian Ocean trade winds once again provides moisture to East Africa (the so-called "short rains"). During November and December approximately 19% (189 mm) of annual precipitation is received (Figure 1B; Bartzke et al., 2018).

On multi-annual timescales, Lyon and DeWitt (2012) proposed that precipitation during the long rains declined in East Africa since approximately 1999 CE because of concurrent changes in sea surface temperature impacting the transfer of moisture to the continent. On longer timescales, paleoenvironmental reconstructions indicate that drying of the East African climate during the 20th century was unusual in the context of the Common Era and that regionally-coherent phases of wetter and drier conditions lasting centuries to millennia occurred in the past ~20,000 years (Tierney and deMenocal, 2013; Tierney et al., 2015). Projections of 21st century climate change suggest that East Africa is likely to experience wetter conditions overall, but with a reduction/increase in the long/short rains in Kenya and Tanzania (Gebrechorkos et al., 2019).

3. Methods

3.1 Sample collection

On January 14–17th 2016, we collected 87 samples of dung in and around the Maasai Mara National Reserve (Figure 1). These samples represent 18 species of wild animals that are protected within the reserve and a further three domesticated species from small agricultural herds in Narok County on the

periphery of the reserve. In many cases, we observed the dung being deposited directly, but where this was not possible, its origin/age was confirmed by reference to published guides (Stuart and Stuart, 2013) and consultation in the field with experienced park rangers and trackers. Most samples were judged to be less than 24 hours old, except for some specimens from lions (*Panthera leo*), baboons (*Papio anubis*), and vervet monkeys (*Chlorocebus pygerythrusess*; less than three days) and crocodiles (*Crocodylus niloticus*; approximately six weeks old based on observed nesting times at the collection site). To capture a degree of variability among individual animals, we aimed to collect five samples from each species, although this was not possible in all instances (particularly carnivores). To ensure that the samples were from different individuals, we sought to collect them from sites located throughout the Maasai Mara National Reserve and on different days. We collected two samples of red oat grass to characterize the food source of herbivores in the study region. A further seven dung samples (one per species) were provided by Woburn Safari Park and West Midlands Safari Park in the United Kingdom in August 2015 and January 2016 respectively with permission from the British and Irish Association of Zoos and Aquariums. The purpose of collecting these samples was to investigate the potential role of diet in determining the sterol content of dung since captive and wild animals of the same species likely eat different food. The chosen species reflect the availability of captive animals with wild equivalents in Kenya, except for a captive African Wild Dog (retained without a wild counterpart to evaluate sterols in canine carnivores). Each sample was sealed in a labeled polyethylene bag and transported in an iced-cool box prior to be being frozen at approximately –18 °C on the day of collection. The frozen samples from Kenya were shipped overnight to British Geological Survey, where they remained frozen until processing.

In addition, we analyzed nine samples from an earlier study on nutrient input to aquatic systems from decomposition of hippopotamus dung (Subalusky et al., 2018). Hippopotamus dung was collected on October 4th 2012, homogenized, and partitioned into representative subsamples that were placed into litter bags. One sub-sample was used to represent fresh dung, while the remaining eight sub-samples were suspended in the Mara River at Purungat Bridge (near the Kenya-Tanzania border in the Masai Mara

National Reserve; Figure 1) and recovered on eight different days beginning with October 6th 2012 and ending on December 6th 2012. Each sample was rinsed to remove river sand that accumulated in the litter bag and then dried. Dried samples were shipped to the British Geological Survey.

3.2 Analytical preparation

In the laboratory all samples were freeze dried for 72 hours. Approximately 30 g (dry weight) of each dung sample (the entirety of each plant sample) was then ground to a coarse powder using a blender (De Longhi KG40) and then further ground into a fine (able to pass through a 63 µm brass sieve), homogenized powder using an agate ball mill (Retsch PM400). In most instances a single, representative sub-sample of the homogenized material was prepared for analysis. However, to investigate analytical reproducibility we repeated the extraction process described below on multiple sub-samples of the homogenized dung.

A 5 g aliquot of each powdered sub-sample was placed on a watch-glass and spiked with deuterated cholesterol (cholesterol–2,2,3,4,4,6–d6) standard in toluene (5 ng/µl; Sigma Chemical Co.). Thereafter, samples were extracted with methanol/dichloromethane (MeOH/DCM) (1:1 v/v) using an accelerated solvent extraction system (Dionex ASE 200) operated at a temperature of 100 °C and a pressure of 1500 psi. Activated copper powder (2 g) was added to remove elemental sulphur. The solvent was removed by evaporation using a turbovap system and the residue was reconstituted in 1 ml acetone, then transferred onto the surface of a silica gel column containing 5% H_2O deactivated silica (100–200 mesh) using a glass pipette. The silica column (1×9 cm) was first eluted with 20 ml hexane/DCM (3:1 v/v) then 40 ml DCM and then with 30 ml acetone/DCM (3:7 v/v). The latter two fractions were combined, the solvent evaporated under a gentle stream of N_2 gas and the residue dissolved in 0.5 ml acetone prior to quantitative transfer to a glass vial (1.75 ml). Acetone was removed by evaporation with N_2 gas and the sample reconstituted in 0.9 ml of pyridine to which perylene–d12 extraction efficiency standard in toluene

was added. Prior to analysis, mixtures were silylated by heating in an oven at 50 °C for 30 minutes with 50 µl of N, O bis (trimethylsilyl)trifluoroacetamide (BSTFA) with 1% TMCS (Sigma Chemical Co.).

3.3 Gas chromatography and mass spectrometry

Fecal sterols were analysed using a Varian CP3800 series gas chromatograph (GC) directly coupled with a Varian 1200L triple Quadrupole MS/MS system (GC/MS). Sample injection (1.0 µl) was in splitless mode. Compounds were separated using a Varian Factor 4. VF-5MS column (30 m length×0.25 mm i.d.×0.25 µl film thickness). The oven temperature was programmed from 60 °C (1 minute isothermal) to 250 °C at 20 °C/minute then to 310 °C at 4 °C/minute and held isothermally at 310 °C for 10 minutes. The mass spectrometer was operated at 70 eV with a mass range of m/z 30–550 (beam current 150 µA, source temperature 150 °C) with helium as carrier gas at a flow rate of 1 ml/minute. Data acquisition was carried out using a Varian MS workstation v6.5. Peak assignments were made by comparison with published mass spectra and mass spectra and retention times of authentic standard compounds (Vane et al., 2010). The limit of quantification for individual compounds ranged from 0.01 to 0.04 µg/g (dry weight.); procedural blanks as well as reagent blanks contained no discernible sterols. To test instrumental reproducibility some extractions were measured more than once and are referred to in the text as replicates.

3.4 Fecal biomarker nomenclature

Common compound names are used throughout this work to facilitate comparison with previous studies. The eleven fecal sterols measured were cholestane (5α-cholestane), coprostanol (5β-cholestan-3β-ol), 5β-epicoprostanol (5β-cholestan-3α-ol), cholesterol (cholest-5-en-3β-ol), 5α-cholestanol (5α-cholestan-3β-ol), coprostan-3-one (5β-cholestan-3-one), campesterol (24α-methyl-5-cholesten-3β-ol), stigmasterol (3β-hydroxy-24-ethyl-5,22-cholestadiene), fucosterol ((3β,24E)-stigmasta-5,24(28)-dien-3-ol), β-sitosterol (24-ethylcholest-5-en-3β-ol) and 5β-stigmastanol (24α-ethyl-5α-cholestan-3β-ol); chemical structures are presented in Appendix 1.

3.5 Statistical Analysis

Statistical analysis was performed after transforming sterol concentrations to percentages because of large differences among samples in absolute concentration. We used random forests to test if a dung sample could be accurately attributed to a species using the relative abundance of fecal sterols. A classification tree sequentially partitions a multi-variate dataset into subgroups. Each branch of the tree divides at a node resulting in an increasing number of subgroups and splitting continues until the subgroups cease to become more homogenous. The random forests approach builds a large number (n = 1000 in our analysis) of classification trees (the forest) in which subsets of the original variables (i.e., types of fecal sterol) and samples are excluded. For a more complete description and underlying mathematics see (Breiman, 2001; Cutler et al., 2007). A key strength of random forests is that the importance of each sterol for determining the origin of a dung sample is quantified for the dataset as a whole and for each species (incorporated as *a priori* groups). We analyzed a dataset comprised 87 observations of 11 fecal sterols. Each observation represents an individual animal (wild only and excluding the samples from a hippopotamus used to examine decomposition). Where multiple extractions and measurements were made on the same dung sample they were averaged prior to analysis using the *randomForests* package (Breiman et al., 2018) for R. Model performance is quantified using the rate of mis-classification (in permutations of out-of-bag data), and node impurity from splitting on each fecal sterol (measured by the Gini impurity index).

4. Results

4.1 Analytical and instrumental reproducibility

We selected nine species to investigate the analytical and instrumental reproducibility of fecal sterol measurements (Figure 2). The species were selected to include domestic and wild animals as well as herbivores, carnivores, birds, and primates to ensure that each sterol was adequately represented. From the homogenized dung sample of one individual per species, we extracted sterols from three

representative sub samples. In addition, we performed an extraction on six subsamples from the homogenized dung of a second elephant (sample MMF–1E). Up to nine replicate measurements (in different batches to ensure they were separated by time and other samples) were made on some of these extractions to investigate instrumental reproducibility. Since the original sample was homogenized, variability among extractions represents a test of analytical reproducibility and replicate measurements are a test of instrumental reproducibility. All measured values are tabulated in Appendix B.

The five most abundant sterols in the samples used to investigate analytical and instrumental reproducibility were cholesterol (up to 98%), coprostanol (up to 74%), 5β-stigmastanol (up to 58%), β-sitosterol (up to 53%), and stigmasterol (up to 26%; Figure 2). For these sterols the mean/maximum range of measured values across all species, extractions, and replicate measurements was 1.05/4.78%, 0.51/1.36%, 0.86/2.23%, 0.88/2.95%, and 0.52/2.83% respectively (Figure 2). There is no indication that measurements of these five fecal sterols are more/less reproducible for any species. We conclude that sub sampling of homogenized dung samples from which fecal sterols are then extracted and measured using the methods described previously (section 3) generates reproducible results for the most common fecal sterols and therefore differences among species and/or individuals are unlikely to be attributable to analytical or instrumental bias and/or error.

The remaining six measured fecal sterols had a maximum relative abundance of less than 7% across all species and samples (Figure 2), of which two had a maximum abundance of less than 1%. The mean/maximum range of measured values was 0.75/3.17% for coprostan.3.one, 0.82/3.85% for campesterol, 0.37/1.38% for 5α-cholestanol, 0.17/0.89% for 5β-epicoprostanol, 0.31/0.83% for fucosterol, and 0.03/0.08% for 5α-cholestane. Therefore, the absolute reproducibility of these measurements among sub-samples, extractions, and replicates is similar to the more common fecal sterols, but in some cases it is large compared to the underlying relative abundance. We conclude that

subtle changes in the least common fecal sterols among individuals and species may not exceed variability that arises from sample processing in a laboratory and instrumental measurement.

4.2 Variability among individuals

We collected dung samples over a period of several days at sites throughout the Masai Mara National Reserve. Therefore, each dung sample is assumed (and in many cases observed) to represent a different individual animal given the time and distance between collections. We analyzed dung samples from single individuals of six species held in UK safari parks to investigate if diet influenced the fecal sterol content of animal dung under an assumption that wild and captive individuals of the same species differ principally by diet. A dung sample from the seventh captive species (African wild dog) did not have a wild counterpart and therefore could not be used in this way. Figure 3 shows the variability in fecal sterol content of dung among individuals of each species.

The most abundant fecal sterol in carnivorous mammal dung is cholesterol (more than 85% for all individual lions, hyenas, leopards, cheetahs, and African wild dogs) and our analysis demonstrates that the abundance of this sterol in dung from captive animals (lion and cheetah) lies within the range of measured values for wild animals in Kenya (Figure 3). For example, dung from wild lions included 88.6–96.4% cholesterol, compared to 89.7% in a captive individual. The observed differences between wild and captive carnivores are small for other fecal sterols except for 5α-cholestanol, which was more common in captive animals than in their wild counterparts. For example, three wild lions produced dung with 1.1–4.0% 5α-cholestanol compared to 7.6% for a captive lion. 5α-cholestanol is a thermodynamically stable stanol produced by microbial reduction of cholesterol outside of the gut (Leeming et al., 1996). Therefore, differences among wild and captive lions/cheetahs in 5α-cholestanol are unlikely to be the influence of diet.

The other captive animals analyzed were herbivores (giraffe, zebra, wildebeest, and elephant) and differences between captive and wild individuals suggests that diet may play an important role in determining fecal sterol composition (Figure 3). Dung from a captive giraffe was relatively enriched in coprostanol (28.7% compared to a range of 5.2–7.3% in five wild individuals) and depleted in 5β-stigmastanol (28.6% compared to 36.1–58.3% in wild individuals), β-sitosterol (13.0% compared to 17.4–29.4 in wild individuals) and stigmasterol (1.2% compared to 2.5–7.2% in wild individuals). Dung from captive elephants and zebras included larger proportions of 5β-stigmastanol than wild individuals (23.4%/25.8% compared to 12.0–17.2%/14.2–18.6% respectively), but relatively less of its phytosterol precursor stigmasterol (4.4%/4.0% compared to 18.4–26.0%/5.6–10.2% respectively). Fecal sterols from a captive wildebeest also contained relatively little cholesterol (6.6% compared to 11.2–16.5%) and relatively more coprostan.3.one, campesterol, and 5β-epicoprostanol (7.4% compared to 1.9–2.6%) than five wild individuals.

4.3 Variability among species

We qualitatively identify four principle groups of animals based on measured fecal sterol (wild individuals only) content using the chord square distance metric to measure dissimilarity (Figure 4). This metric is appropriate for closed compositional data (e.g., Simpson, 2012) such as percentages of fecal sterols. Fecal sterols in dung from carnivores (lion, hyena, leopard, cheetah, and crocodile) are almost exclusively cholesterol (Figure 4). Across all species and individuals of wild carnivore the mean abundance of cholesterol was 90.5% and spanned a range of 75.6–97.3%. There was no notable difference in cholesterol abundance among species of mammalian carnivores, but crocodiles have lower cholesterol content. In comparison, non-carnivores were characterized by cholesterol content that was less than 34.5%.

The most common fecal sterol in primates (baboon and vervet monkey) was coprostanol (mean/range of 54.8/38.0–74.3%; Figure 4). Among non-primates, the maximum occurrence of coprostanol was 29.9% in

a dung sample from a topi. Although there is overlap in measured values, baboon dung included relatively less coprostanol (38.0–55.9%) and 5α-cholestanol (1.23–3.8%) than vervet monkey dung (51.3–74.3% and 3.0–5.6% respectively). Compared to vervet monkeys, baboon dung was enriched in coprostan-3-one (Figure 3). One baboon returned an anomalously high value of 27.1% (compared to a maximum of 7% among all individuals that were not baboons) and four of the five highest measured abundances (>6%) of coprostan-3-one were in baboon dung. The highest abundance of coprostan-3-one measured in vervet monkey dung was 3.9%.

For many species of even-toed ungulates that are herbivorous ruminants, the most common fecal sterol was 5β-stigmastanol (Figure 4). Among topi, impala, giraffe, wildebeest, buffalo and domesticated cattle, sheep and goats the mean/range of 5β-stigmastanol was 47.6/28.6–58.3%. For these species there is a gradient of 5β-stigmastanol, with impala dung having the highest abundance (mean of 55.4%) and domesticated goats having the lowest (mean of 44.1%). In comparison, the mean 5β-stigmastanol content for other species was 8.8%.

A fourth group of species is distinguished by elevated concentrations of β-sitosterol (mean/range of 34.3%/17.4–52.7%). This group is comprised of species with diverse digestive systems including elephants, warthog, zebra (hindgut fermenters), hippopotamus (often described as pseudo-ruminants since they have a three chambered stomach), Thomson's gazelle (herbivorous ruminant) as well as ostrich and Egyptian goose. For species not in this group, the mean concentration of β-sitosterol was 11.2%. Elephant and hippopotami dung combine high concentrations of β-sitosterol (minimum of 37.2% and mean of 41.5%) with high concentrations of stigmasterol (minimum of 18.4% and mean of 21.5% when analysis is limited to wild individuals only). Zebra dung is unusually enriched in 5β-epicoprostanol (Figure 3), five wild individuals included a mean of 16.2% (range of 9.1–20.6%), compared to a mean/maximum for all other species of 2.6%/12.5%. Among hindgut fermenters, warthog dung includes an elevated concentration of coprostanol (19.6% and 24.9% in the two individuals studied).

4.4 Statistical model

We used random forests to determine the likelihood that fecal sterols could reliably identify the origin of a dung sample to the species level. Input for this analysis was fecal sterols measured in dung samples from 87 wild animals (averaged across multiple subsamples, extractions, and/or measurements where available) spanning 22 species (we include domesticated livestock from Kenya as wild animals and exclude only the captive individuals from the UK). For the dataset as a whole, 72% of dung samples were correctly classified to the species level during cross validation (Figure 5). Random forests quantified the importance of each fecal sterol in classifying dung samples using the Gini impurity index and mean decrease in accuracy. For the entire dataset, the sterol with the greatest predictive power was 5β-stigmastanol (Gini Impurity = 10.7; Figure 6a), after which six fecal sterols had very similar importance as evidenced by a narrow range of Gini Impurity (8.4 for campesterol to 10.0 for coprostanol). Despite spanning the widest range of measured abundance (1.6% in a baboon dung to 97.3% in cheetah dung), cholesterol had a relatively low an importance of 6.6, likely because of the bipartite division into species with high (carnivores) or low (non-carnivores) cholesterol content in their dung. The least important variables (fucosterol with Gini Impurity = 2.7 and 5α-cholestane with Gini Impurity = 0.16) were also the least abundant. For example, 25.8/93.5% of animals across all species produced dung with no fucosterol/5α-cholestane and these fecal sterols had a maximum abundance of 1.3/0.02% (Figure 3). The relative of importance of each fecal sterol was similar when measured using the mean decrease in accuracy (Figure 6b).

There are notable differences in how frequently some species are (mis)classified compared to the overall success rate of 72% (Figure 5). Dung samples from Egyptian goose, domestic sheep, wildebeest, giraffe, Thomson's gazelle, hippopotamus, and hyena were correctly classified in all instances. The most important sterols for recognizing several of these species were 5β-stigmastanol, 5α-cholestanol, and 5β-sitosterol (Figure 7). For Thomson's gazelle, campesterol was unusually important. Conversely, our

analysis indicates that carnivores are particularly challenging to differentiate since only 40% of samples (four from ten) were correctly classified (including three samples from hyenas). Carnivore dung is characterized by high abundances of cholesterol (at least 75% of fecal sterols if crocodiles are included and at least 87.5% when crocodiles are excluded), which likely leaves little scope for differentiating among species. The correct classification of hyenas despite high cholesterol content, appears to arise from the importance of 5α-cholestanol (Figure 7), which is more abundant in dung from hyenas (4.9–7.0%; Figure 3) than other carnivores (less than 4.0%). Elevated concentrations of 5α-cholestanol in hyena dung could be attributed to aerobic microbial action cholesterol after the dung was excreted. However, two of the hyena samples were collected after known individuals were observed defecating. The third sample was visibly paler/drier and estimated by hyena researchers to be less than three days old. Despite a longer exposure to environmental conditions outside of the body, this sample yielded the least 5α-cholestanol, which suggests that the distinctiveness of hyena dung was not caused by a post-depositional alteration.

There is bi-directional misclassification of dung from ververt monkeys/baboons and buffalo/domesticated cattle (Figure 5). For example, 20% (one of five) vervet monkey samples were misclassified as baboon dung, compared to 40% (two of five) in the opposite direction. This likely occurs because dung from both species is characterized by high abundances of coprostanol. However, our analysis indicates that baboon and vervet monkey dung can be regularly distinguished from one another because of the importance of coprostan-3-one (higher in baboon dung; Figure 5) for recognizing baboon dung (Figure 7). The single most important combination of sterol and species for improving classification of dung samples was coprostanol and vervet monkeys (mean decrease in accuracy of 25.9; Figure 7). 5β-stigmastanol and stigmasterol are also important for differentiating baboon and vervet monkey dung, despites no clear differences in abundance. Rather there appears to be minimal variability of 5β-stigmastanol/stigmasterol measured in baboon/vervet monkey dung, which likely makes specific abundances of these sterols characteristic of the two species.

Impala, and topi were misclassified at a rate of 40% or greater with dung from these species most commonly being misattributed to domesticated goats/sheep/cattle, or one another. The second most important combination of sterol and species for improving classification of dung samples was 5β-epicoprostanol and zebra (mean decrease in accuracy of 24.6; Figure 7), which reflects the unusually high amount of 5β-epicoprostanol in zebra dung (Figure 3). Despite this, one of the five zebra samples was misclassified as coming from a Thomson's gazelle. Inspection of the random forests results indicates that this sample is characterized by markedly lower 5β-epicoprostanol (9.1%) than the other four zebra samples (15.4–20.6%; Figure 3).

The five species represented by dung from two or fewer individuals (ostrich, warthog, crocodile, leopard, and cheetah) were misclassified at a rate of 87.5% (seven of eight samples) which illustrates the challenge of predicting sample origin for underrepresented species during cross validation. Removal of these species in a second random forests analysis marginally increased the overall correct rate of classification.

4.5 Decomposition trends

Hippopotami emerge from aquatic environments at night to feed on surrounding grasslands before returning to spend the day in pools and rivers where their dung is subsequently concentrated. This behavior results in a transfer of nutrients from the feeding grounds into aquatic environments (Dutton et al., 2020; Subalusky et al., 2015). Our analysis of two grass samples shows that the primary food source for hippopotami and other herbivores is rich in phytosterols (Figure 8). The mean composition of grass was 52.9% β-sitosterol, 31.6% stigmasterol and 9.6% campesterol.

Previous work used sub samples of homogenized hippopotamus dung suspend in litter bags in the Mara River to examine the role of decaying dung in influencing nutrient availability and cycling (Subalusky et al., 2018). We measured fecal sterols on the same samples to examine decomposition trends. Fresh hippopotamus dung collected from five individuals in the Masai Mara National Reserve in 2016 yielded

264–1206 μg/g of fecal sterols (median of 826 μg/g) and is characterized by high abundance of β-sitosterol (range of 39.4–45.8%), stigmasterol (19.3–23.6%), and cholesterol (12.6–18.6%). In contrast, the same samples contained relatively little campesterol (5.3–6.8%), although hippopotamus dung has the highest mean campesterol content (5.9%) of the species that we sampled (Figure 3). The fresh (not suspended in the Mara River) subsample of hippopotamus dung collected in 2012 yielded 270 μg/g of fecal sterols, which is within (albeit at the lower end) the range measured on fresh dung from 2016. It had a composition that was comparable to those from 2016 for most sterols (Figure 9), which suggests that our sampling captured typical variability among individuals. However, we note that the fresh dung sample from 2012 included unusually large amounts of 5β-stigmastanol (20.7%), which lies far outside of the range measured on the samples from 2016 (9.1–12.5%). It also contained a smaller amount stigmasterol (16.2%), which is a precursor to 5β-stigmastanol (Bull et al., 2002), possibly indicating a more complete digestion of grasses in the sampled individual from 2012 than those represented in our 2016 samples. This could arise from repeated ingestion of the same material since hippopotami engage in coprophagy either actively as juveniles, or passively through time spent in dung-rich hippo pools.

Over the course of 63 days suspended in the Mara River, hippopotamus dung underwent notable changes in fecal sterol content and composition (Figure 9). The concentration of fecal sterols increased through time to reach a maximum of 387 μg/g on day 63. Since this is a systematic trend it likely cannot be attributed to inadequate homogenization during sub-sampling or laboratory preparation. Rather it represents either preferential removal of material that is not fecal sterols and/or in situ creation of new sterols. The abundance of campesterol remained relatively low (<8.5%) and stable until day 16, while samples recovered at days 21–63 included elevated amounts of campesterol (range of 24.1–33.9%, mean of 29.0%). Notably, the campesterol content of the dung from day 16 onward is considerably greater than we measured in grass (mean 9.6%; Figure 7). This increase in campesterol is accompanied by decreases in β-sitosterol, sitosterol, 5β-stigmastanol, and cholesterol. Until day 16 the average β-sitosterol content of hippopotamus dung was 40.4%, but declined to an average of 32.9% for days 21–63. Similarly,

5β-stigmastanol content was relatively high and stable prior to day 16 (mean of 18.8%) and much reduced on days 21–63 (mean of 13.1%). For cholesterol, there was a rapid post submergence decline in the samples recovered on days two (5.8%) and six (10.6%), after which it remained relatively stable until day 28. The samples recovered on days 42–63 had a further decreased average cholesterol content of 5.7%.

5. Discussion

5.1 Characterization of major groups

Dung from major groups of species (carnivores, herbivorous ruminants, non-ruminant herbivores, and primates) is readily distinguished using fecal sterols (Figure 4). Carnivore dung includes very high abundances of cholesterol, primate dung includes abundant coprostanol, and two groups of (primarily) herbivores are recognized by their high abundances of 5β-stigmastanol (e.g., impala, giraffe), or β-sitosterol (e.g., elephant, hippopotamus). These patterns were recognized in other ecosystems and are attributed to well-understood combinations of diet, digestive biochemistry, and biosynthesis of endogenous sterols (Leeming et al., 1996). Comparative values discussed in the following sections should be treated with a degree of caution because of variability among studies in how samples were prepared and analyzed and which specific sterols were (not) measured (Bull et al., 2002).

There are relatively few studies that provide fecal sterol data for carnivorous terrestrial megafauna. Leeming et al. (1996) presented measurements from cats and dogs (presumably domestic and at the very least domesticated) that yielded an average cholesterol content of ~42% and 72% respectively. Similarly, Shah et al. (2007) reported average cholesterol content of ~67% for dogs. These values are considerably lower than the values that we measured in carnivore dung from East Africa (Figures 3 and 4, minimum of 75.6/87.5% including/excluding crocodiles). Although some difference in values may be attributable to the number and type of sterols measured and laboratory methods, it is likely that domestic(ated) cats and dogs do not have a fully carnivorous diet owing to their (presumed) consumption of processed pet food. Harrault et al. (2019) showed that a dog from a remote community in Siberia fed on fish and meat scraps

was classified differently to domestic dogs from Scandinavia because their different diets resulted in corresponding differences in fecal sterols. In contrast to domesticated carnivores, dung from six dingos had an average cholesterol content of ~96% (Shah et al., 2007) and we measured 98% in a sample from a captive African wild dog, which is similar to the composition of lion, cheetah, leopard, and hyena dung collected in East Africa. In marine ecosystems, the cholesterol content of seal (Weddell, elephant and leopard seals) and penguin dung from Antarctica was reported as approximately 74–99% (Leeming et al., 2015; Venkatesan and Santiago, 1989). Our measurements from East Africa further indicate that the fecal sterol content of carnivore dung is almost exclusively cholesterol despite considerable variety in the ecosystems and species studied and their diverse diets. This result is attributed primarily to a diet (meat) that is rich in cholesterol (~60-100 mg/100g for muscle of farmed animals; Chizzolini et al., 1999), coupled with the absence of a metabolic mechanism (biohydrogenation by bacteria in the anaerobic conditions of the digestive tract) for converting cholesterol into 5β-stanols such as coprostanol (as occurs for example in humans), or 5β-epicoprostanol (Leeming et al., 1996).

The consistency of cholesterol abundance among a wide variety of carnivores that consume correspondingly varied prey has two implications for reconstructing animal populations. Firstly, the diversity of prey consumed by modern carnivores can be interpreted as a proxy for how the prey consumed by a specific carnivore could alter through time in response to shifting food availability. This species-for-time substitution indicates that even pronounced shifts in diet would be unlikely to cause fecal sterols in carnivore dung to change dramatically. Differences in diet between domesticated cats/dogs and wild carnivores are likely the cause of their different fecal sterol composition, but it is unlikely that wild carnivores changed their diet to this degree through time. Similarity in fecal sterols in dung from captive and wild individuals of the same species of carnivore (lion and cheetah; Figure 3) further supports this idea. Secondly, it is unlikely that carnivores can be accurately classified to the species level using fecal sterols because of their uniformly high cholesterol content as evidenced by the high misclassification rate in the random forests model (Figure 5).

A principal goal of environmental research using fecal sterols has been recognizing the characteristic fingerprint of human waste in water bodies or sediment. Commonly these studies sought to identify recent pollution from wastewater (Leeming et al., 2015; Vane et al., 2010), or to identify human settlement/behavior in the context of archaeological investigations (Bull et al., 2001; Prost et al., 2017; Schroeter et al., 2020; Zocatelli et al., 2017). Elevated concentration of coprostanol (in isolation or as part of a ratio) is taken as evidence for the presence of fecal matter from humans (Bull et al., 2002; Liebezeit and Wöstmann, 2010). Reported values for coprostanol in fresh human dung are in the range of approximately 61–71% (Leeming et al., 1996; Shah et al., 2007; Zocatelli et al., 2017). In our dataset, high concentrations of coprostanol similar to those reported for humans are found in primate (baboon and vervet monkey) dung (Figure 3). In a laboratory experiment, captive primates eating commercial, processed food produced dung with an average coprostanol content of ~38% for baboons and 32% for rhesus monkeys (Subbiah et al., 1972). This composition of human and primate dung is attributed to an omnivorous diet, coupled with microbial activity in the anaerobic digestive tract that converts cholesterol into 5β-stanols including coprostanol (Bull et al., 2002). Due to biosynthesis, cholesterol is present and available for microbial mediation in the digestive tract of humans, even if an individual eats a low cholesterol (e.g., vegetarian) diet (Leeming et al., 1996). This is also likely the case for primates in East Africa since their diet is almost exclusively (vervet monkey) or largely (baboons) vegetarian. The similarity of coprostanol among humans and primates eating highly variable diets indicates that diet plays a secondary role to gut biochemistry (endogenous production and subsequent conversion of cholesterol) in determining the sterol composition of dung. Therefore, changes in diet through time are unlikely to inhibit the characterization of dung using a modern training set. Using elevated coprostanol as a marker for pollution by human waste is underpinned by an assumption that primates are not a viable source of fecal sterols in the system being analyzed. This assumption was robust for studies in Antarctica (Leeming et al., 2015), Europe (Bull et al., 2001; Bull et al., 1999; Vane et al., 2010), Australia/New Zealand (Argiriadis et al., 2018; Leeming et al., 1996), and central Asia (Harrault et al., 2019; Schroeter et al., 2020). In the context of East Africa however, the presence of primates could lead to false positives for

pollution by human waste. Similarly, paleoecological trends inferred from time-variable inputs of coprostanol and interpreted to be shifts in primate populations could in fact be caused by human populations.

The fecal sterol composition of herbivore (plus omnivorous warthogs) dung is characterized by high abundances of 5β-stanols such as 5β-stigmastanol that are produced from precursors of biosynthesized cholesterol and phytosterols ingested from plants (Harrault et al., 2019; Leeming et al., 1996; Prost et al., 2017; Shah et al., 2007). Our analysis of grass samples confirms that the diet of many herbivorous megafauna in East Africa is likely rich in β-sitosterol, stigmasterol, and campesterol (Figure 8). Although some of the herbivores in our dataset do not feed primarily on grass, these phytosterols are common to most plants (e.g., Bot, 2019). Therefore, it is unlikely that the sterol composition of dietary input would vary significantly through time (e.g., seasonally during annual migrations, or on Holocene timescales due to wet/dry climate phases). For example, giraffe feed primarily on leaves rather than grass, but the sterol composition of the Acacia trees that they regularly browse is approximately 46–54% β-sitosterol and 14–22% stigmastenol (Nasri et al., 2012), which is similar to our measurements from grass.

The relative abundance of phytosterols in elephant and hippopotamus dung is less than in the plants they likely ingest (compare Figures 3 and 8), which indicates that there is systematic alteration during digestion. However, their dung retains relatively high proportions of β-sitosterol, stigmasterol, and campestrol compared to other herbivores, indicating a lower degree of digestion. Indeed, elephant and hippopotamus dung includes considerable quantities of identifiable un- and partially-digested plant material. The mechanism for this seemingly low conversion of phytosterols into 5β-stanols may differ between the two species. Larger herbivores typically have a longer gastrointestinal tract than smaller herbivores, which results in a longer retention time and allows them to consume lower quality forage (Clauss et al., 2003). However, forage consumed by elephants has a shorter retention time in the gut (40–50 hours) than is predicted for an animal with its body weight (65-80 hours; Illius and Gordon, 1992).

This relatively fast passage may explain the relatively high/low concentration of phytosterols/5β-stanols in elephant dung. Hippopotami are grazing, non-ruminant, foregut-fermenting herbivores that possess a very simple hindgut (Clauss et al., 2003). In contrast to grazing ruminants, hippopotami do not stratify their gut content, which makes selective particle retention unlikely and may result in a correspondingly reduced rate of phytosterol conversion to 5β-stanols since partially-digested plant material is passed more often than in ruminants.

For other herbivores the process of digestion proceeds further in the conversion of phytosterols to 5β-stigmastanol (Figure 3). As ruminants, plants consumed by these species are fermented through microbial actions and rechewing of cud likely enables more complete processing of plant material than is observed in elephants and hippopotami, resulting in depletion of phytosterols and enrichment in 5β-stigmastanol when dung is compared to dietary intake.

We note that zebra dung is unusual because it contains elevated amounts of 5β-epicoprostanol (Figure 3), which is produced by biohydrogenation of cholesterol by anaerobic bacteria (Leeming et al., 1996). This is further highlighted by the quantified importance of this fecal sterol for identifying zebra dung using random forests analysis (Figure 7). Zebras are hindgut fermenters rather than ruminants and compensate for the lower degree of digestion by consuming more and a wider variety of food and processing it quickly (Grubb, 1981). This combination of dietary intake and digestive tract appears to produce dung with a distinctive fecal sterol profile. In comparison, horses do not produce 5β-epicoprostanol to the same extent as zebras. Shah et al. (2007) reported an average 5β-epicoprostanol content of 7% for horses, compared to 8-11% in the study of Harrault et al. (2019) and ~1% in (Leeming et al., 1996; Prost et al., 2017).

We analyzed dung from two species of bird (ostrich and Egyptian goose), that yielded markedly different fecal sterol profiles. Relative to one another, ostrich dung is rich in coprostanol and 5β-stigmastanol,

while Egyptian goose dung is particularly rich in β-sitosterol (Figure 3). Reported fecal sterol measurements from bird dung in the literature crudely recognize species that produce dung rich in β-sitosterol (e.g., ducks, rosella, swans), cholesterol (e.g. sea gulls, penguins), or a combination of both (e.g., chickens, turkeys; Devane et al., 2015; Leeming et al., 1996; Shah et al., 2007; Venkatesan and Santiago, 1989). These divisions may represent a spectrum of diets from almost exclusively herbivorous to omnivorous. However, the presence of coprostanol in ostrich dung suggests that cholesterol provided by its omnivorous diet undergoes conversion to coprostanol during digestion, in a similar fashion to primates and this result appears unusual compared to other birds.

5.2 Species-level recognition of dung

The primary application of sediment-hosted fecal sterols is to distinguish dung inputs that are dominated by one group of species or another (often humans vs herbivores). In many cases this is achieved through examining ratios of sterols (or groups of sterols) and applying a threshold value for binary classification (Bull et al., 1999; Prost et al., 2017; Schroeter et al., 2020). This approach is effective for recognizing the impact of humans as recent polluters (Vane et al., 2010) and as agents of environmental change/presence in the archaeological and paleoenvironmental record (Argiriadis et al., 2018; Bull et al., 2001; Bull et al., 1999), particularly in locations lacking primates (section 5.1). Efforts to identify the origin of dung to the species level are less common because study goals are adequately met by using ratios, the limited scope of available data (number and scope of samples) to provide a modern training set or reference library, and/or the number of different fecal sterols that were measured. Directly combining results from multiple studies to create an expanded reference library remains challenging because of differences in sample preparation and measurement (Bull et al., 2002). However, some studies demonstrated the possibility that fecal sterols can recognize the origin of dung to the species level. For example, Harrault et al. (2019) showed that passive projection of samples into principal components analysis and hierarchical clustering could accurately establish the species origin of samples. They proposed that this success stemmed from the statistical technique used, coupled with increasing the number of measured sterols (11 fecal sterols), a

relatively large training set (90 samples representing ten species, although some were removed where *a priori* evidence indicated the absence of species) and limiting analysis to 5β-stanols to negate the influence of sterols with non-fecal origins.

Our analysis using random forests was able to correctly classify the origin of a dung sample to the species level in ~72% of cases (62 out of 87 individuals) under cross validation. If carnivore dung (other than hyenas) is excluded from these results the successful classification rate is 76% and exclusion of species represented by two or fewer individuals in the current dataset also improved model performance slightly. This suggests two pathways to further refine future efforts. Firstly, it may be necessary to accept that some groups of species (e.g. carnivores, primates, buffalo/domestic cattle) cannot be adequately distinguished. Random forests (and other methods) does not require that all species be grouped or kept separate for analysis, it is statistically robust to include some taxa as species and to combine others into groups. Secondly, expansion of the reference library to include additional replicates from new individuals may improve model performance. In some cases (e.g., warthog and ostrich) this would be a trivial task, but it is more challenging for others whose population densities are lower, produce dung infrequently (crocodiles), and/or are rarely sighted (leopards). Our current reference library does not include all of the species that are encountered regularly in the Masai Mara National Reserve today. Future work may also expand the number of species as well as individuals of each species. This expansion may improve or decrease model performance.

Our results indicate that some sterols are more useful than others for establishing the species origin of dung samples (Figures 6 and 7) and these results could be used to focus analytical efforts on analyzing more samples and/or different sterols. However, one challenge with interpreting the importance of individual sterols is the influence of correlations with other sterols. If two sterols are strongly correlated in the dataset, it is possible that the importance of those sterols is underestimated. For example, in our dataset there are strong, positive correlations between stigmasterol, β-sitosterol, and campesterol since

these phytosterols comprise the principal dietary inputs for herbivores (Figure 10). During cross-validation of the random forests model, exclusion of one phytosterol may not have as large an effect as anticipated since other, strongly-correlated sterols remain in the training set.

5.3 Post-depositional decomposition and alteration

The defining characteristic of carnivore dung is high abundances of cholesterol, which allows fresh dung from these species to be readily distinguished from herbivores and primates for example. However, under aerobic conditions cholesterol is likely to undergo alteration and be converted to 5α-cholestanol (Leeming et al., 2015). Since East African carnivore dung is almost certain to experience sub-aerial exposure in terrestrial environments between the time of deposition and burial, it is possible that a modern training set of fresh (pre-alteration) dung would not be appropriate for recognizing carnivore dung in the sedimentary record. For example, we only detected 5α-cholestanol in five dung samples with a maximum abundance of 0.07%. For some of these samples, sub-sampling and replicate measurements did not detect the measurable presence of 5α-cholestanol. We estimated that the two samples of crocodile dung were approximately six weeks old at the time of collection based on observed nesting at the site. Although crocodile dung has a lower cholesterol content than other carnivores, it did not yield a detectable presence of 5α-cholestanol (Figure 3). This result suggests that post-depositional alteration of cholesterol may not be pronounced under field conditions on timescales of weeks. However, the impact of post-depositional alteration should be assessed through a future experiment in which dung (from multiple carnivore species) is left in sub-aerial conditions and repeatedly sampled over the course of a time interval that extends beyond weeks. Since 5α-cholestanol is thermodynamically stable (Leeming et al., 1996) and produced by soil microbes (Bull et al., 2002; Prost et al., 2017), it is possible that terrestrial sediments that accumulate in wetlands and lakes could include non-fecal sources of this sterol.

The difference in sterol composition between plants (Figure 8) and hippopotamus dung (Figure 3) indicates that a dietary input rich in β-sitosterol, stigmasterol, and campesterol undergoes hydrogenation

and reduction under anerobic gut conditions to produce 5β-stigmastanol (Prost et al., 2017; Schroeter et al., 2020), although the conversion appears less complete in hippopotami than other herbivores as evidenced by the relatively high amounts of phytosterols (but still less in all cases than plants) and visible, un/partially-digested grass that remains in fresh hippopotamus dung. Dutton et al. () proposed that the biochemistry of pools frequently occupied by large numbers of hippopotami with little flushing of water can become increasingly similar to hippopotamus gut conditions. Decomposition of dung drives the pool (particularly bottom water where material accumulates and is flushing is less frequent) toward anoxia which increases the likelihood that excreted gut bacteria can survive and thrive outside of the hippopotamus. They termed this modified biochemical environment the *meta gut* and demonstrated that some pools in the Masai Mara National Reserve are more similar to conditions in a hippopotamus gut than they are to other, nearby pools. For example, the gut microbiome of ten individual hippotami was dominated by *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, and *Tenericutes*. These phyla were also characteristic of water in pools with high input of dung and infrequent flushing, but distinct from water bodies upstream of hippo pools where dung input is low and flushing is more frequent.

Dung suspended in the Mara River (Figure 9) underwent modification in an aquatic environment that mimicked changes occurring within the digestive tract of hippopotami with decreases through time in phytosterols (stigmasterol and β-sitosterol in particular, but also cholesterol), which supports the concept of a *meta gut*. However, rather than producing 5β-stigmastanol as occurs in the gut of a hippopotamus, the dung in aquatic environments became enriched in campesterol. Measured values of campestrol reached a maximum of 33.9%, which is considerably greater than the maximum that we measured in grasses (9.6%; Figure 8), or fresh dung (6.8%). This trend in campesterol is accompanied by an increase in total sterol abundance (per unit mass of dung) through time. It is unclear what specific pathways result in the coincident trends of increasing relative abundance of campesterol and absolute concentration of fecal sterols. Subalusky et al. (2018) measured the mass of dung remaining in the same samples and showed that it decreased through time at an approximately log-linear rate, with a predicted loss of all

mass after ~80 days. The mass lost from litter bags is likely small particles and preferential loss of dung constituents that are not fecal sterols (e.g., cellulose) could cause the growing absolute concentration of fecal sterols. Alternatively, *in situ* production of campesterol may occur, although it is unclear through what mechanism this would be achieved since it is a phytosterol and its abundance is typically reduced through microbial action. The reduction in campesterol abundance between dietary intake (plants) and dung observed in hippopotami may suggest that pathways in the *meta gut* are distinct from those in the digestive tract despite a shared microbial community, although the site where litter bags were deployed was flushed more regularly than some the pools studied by Dutton et al. (). The elevated concentration of fecal sterols after more than 60 days suspended in the water column is an indication of their preservation potential in aquatic depositional environments (Nishimura and Koyama, 1977). Importantly, hippopotami are the only species in our dataset that spend a significant proportion of their time in aquatic environments and the dung from other species is deposited almost exclusively in terrestrial environments. Therefore, decomposition trends observed for hippopotami dung are unlikely to be representative of other species in our reference library.

5.4 Proxy development

Our goal is to develop a sediment-hosted proxy for the distribution and composition of megafaunal populations in East Africa during the Holocene. Proxy development is a multi-stage process that begins with evaluating if fecal sterols (quantified by the relative abundance of specific sterol types) can be used to objectively and accurately recognize dung samples of known origin. Cross validation performed by the random forests model indicates that we could successfully identify the origin of a dung samples at a rate of ~72%. This rate of correct classification could likely be improved further by grouping some species that are particularly difficult to distinguish from one another because of their similar sterol composition, namely vervet monkey and baboon (likely all primates), carnivores, and buffalo/domestic cattle. Expansion of the modern training set to improve representation of some under-sampled species may also be beneficial. Although we consider our approach to have passed the first step of proxy development

there are several challenges to consider before sediment-hosted fecal sterols and be reliably employed to reconstruct animal populations.

Fecal sterols preserved in sedimentary environments are from mixed sources since lakes and pools capture sediment and fecal sterols from a catchment area that contains many individual animals and species. Measurement of sediment-hosted fecal sterols will inherently reflect the net outcome of this source mixing. Fortunately, similar challenges are encountered when using other proxies such as isotopes in bulk sediment and efforts to produce probabilistic models to disentangle mixed inputs have proven successful and provide a framework for evaluating mixed fecal sterols (Parnell et al., 2013; Phillips et al., 2014). Mixing of fecal sterols may also reflect several ecological behaviors and physiological traits that should be evaluated. There are considerable differences among species in the amount of dung that is produced which reflects the size of individuals, their frequency of defecation, and population size. Some species display behavior during defecation that may cause their dung to be more/less likely to enter depositional environments. For example, some species deposit dung repeatedly in the same location, to mark territory (e.g., rhinoceros), or to conceal it (e.g.), while others defecate across a wide area or may actively spread dung.

6. Conclusions

Conservation paleobiology seeks to leverage paleoenvironmental reconstructions to better anticipate future changes. In East Africa, predicted shifts in hydroclimate are likely to impact the region's iconic remaining megafauna and while proxies exist for reconstructing trends and events in precipitation regime, they are currently lacking for reconstructing megaufauna populations because sub-fossil remains are ill-suited to quantitative analysis. We propose that sediment-hosted biomarkers are a potential proxy to quantitatively reconstruct megafauna populations because species (or groups of species) produce dung with a characteristic profiles of fecal sterol relative abundance and fecal sterols are well preserved in

aquatic environments after they are delivered from the surrounding catchment to integrate inputs from a geographic area.

As a first step in proxy development we collected fresh dung from 87 individuals that represent 22 species of wild and domesticated animals in and around the Masai Mara National Reserve, Kenya. Repeated sub-sampling, laboratory extractions, and instrumental measurements demonstrate that fecal sterols can be reliably quantified. We show that fecal sterol profiles in carnivore dung are dominated by cholesterol, while those from primates are (like humans) characterized by high abundances of coprostanol. Herbivores are readily divided into two groups representing (to the first order) ruminants that more effectively modify phytosterols consumed by eating plants into 5β-sterols, and non-ruminants (particularly elephant and hippopotamus) whose dung contains relatively more/less phytosterols/5β-sterols because of less effective digestion. These results are consistent with analysis of fresh dung from a diverse range of species living in different ecosystems, including the marine realm.

To evaluate the possibility that fecal sterols could be used to identify the origin of a dung sample to the species level, we applied a random forests model to the modern training set. Under cross validation, 72% of samples were correctly classified. Misclassification was pronounced for carnivores (except hyena) because all species of carnivore produce dung that is almost exclusively comprised cholesterol. Species represented by two or fewer individuals were frequently misclassified and there was bi-directional misclassification among primates (vervet monkey and baboon) and buffalo/domestic cattle. Further sampling and combining some species (e.g., carnivores) may improve future model performance. Although proxy development is a multi-stage process, our analysis indicates that fecal sterols are a promising proxy for reconstructing megafauna populations in East Africa.

A time series of fecal sterol changes in a sample of hippopotamus dung suspend in the Mara River for ~60 days showed systematic modification in aquatic conditions. Notably, conversion of phytosterols in

5β-sterols continued, which mimics trends in the digestive tract of hippopotami and supports the proposition that accumulation of dung in poorly-flushed hippo pools can result in a *meta gut* because gut microbiome are released into a reducing environment where they may survive outside of the body. However, the pathway for some changes through time are unclear, particularly a large increase in campesterol abundance which resulted in dung having up to threes time more of this phytosterol than grasses that are common in the study area. An increase in the absolute concentration of fecal sterols through time likely reflects preferential loss of non-sterol mass such as cellulose.

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Hyena ladies?

Permits?

Figure Captions

Figure 1: (A) Location of the Maasai Mara National Reserve in Kenya. **(B)** Average monthly precipitation in the Maasai Mara National Reserve (1965–2015, compiled from 15 rain gauges) and nearby Narok Town (1913–2015). Data from Bartzke et al. (2018). Mean is represented by symbols and solid line; shaded envelopes represent ± 1 standard deviation. The approximate timing of the "long rains" and "short rains" are shown for reference.

Figure 2: Analytical and instrumental reproducibility of fecal sterol measurements performed on animal dung collected in the Maasai Mara National Reserve, Kenya. The abundance of each sterol (individual panels) is expressed as a percentage of total measured sterols; note that (log) scale varies among panels. Species are organized approximately by type (herbivores, carnivores, primates, birds). For each species, one dung sample from a single individual was homogenized and extractions were performed on three sub samples (symbol fill). Up to three replicate measurements were made on each extraction. The exception to this sampling regime is elephant, where two individuals (A and E) were analyzed. From the homogenized dung sample of the individual elephant E there were six extractions and up to eight replicate measurements.

Figure 3: Sterol composition of animal dung collected from 93 individual animals, representing 23 species including wild examples from the Masai Mara National Reserve in Kenya and captive individuals in two UK safari parks (differentiated by symbol shape). The abundance of each sterol (individual panels) is expressed as a percentage of total measured sterols; note that (log) scale varies among panels. Symbol color denotes biological grouping of species. Each individual is represented by a single data point which (where available) is an average from all sub samples of a single dung and replicate measurements. Color of panel title bars indicates origin/type of fecal sterol.

Figure 4: Sterol composition of animal dung collected from 93 individual animals (bars), representing 23 species including wild examples from the Masai Mara National Reserve in Kenya and captive individuals in two UK safari parks. For individuals where multiple sub samples were processed and replicate measurements made, the presented value is an average across all measurements. Samples are ordered by their measured dissimilarity (chord distance) to an arbitrary individual (elephant A). Sample color represents *a priori* classification of species.

Figure 5: Classification of dung samples using random forests. Each of the 87 dung samples was assigned to a species based on its fecal sterol composition. Since the number of individuals varies among species, values are expressed a percentage by samples from each species (shading). Labels within cells display actual number of samples. Values equal to 0% are not shaded or labeled.

Figure 6: Predictive power of fecal sterols measured using the Gini Impurity index to quantify importance for the entire modern training set used in random forests analysis.

Figure 7: Importance of fecal sterols for recognizing dung from each species as measured by the mean decrease in accuracy (cell shading) during the random forests analysis. Sterols are ordered from the greatest (left; most important) to lowest (right; least important) mean decrease in accuracy for the dataset as a whole. Unshaded cells have no importance value, either because the sterol was not measured in samples from that species and/or because a species was represented by only one individual (cheetah, leopard) and no value of importance was generated.

Figure 8: Sterol composition of red oat grass measured on two samples (colored bars) collected in the Masai Mara National Reserve.

Figure 9: Decomposition of hippopotamus dung in an aquatic environment. Time series are for sub samples of homogenized hippopotamus dung placed into litter bags, suspended in the Mara River, and removed sequentially over a period of approximately two months (October to December 2012; blue circles). Dung at time zero was not suspended in the river and is therefore directly comparable to fresh hippopotamus dung collected in 2016 (yellow squares, representing average across all measurements made on all wild hippopotami with bars connecting minimum and maximum values). Only the five most abundant fecal sterols and concentration of total fecal sterols (μg of fecal sterol per g of dried dung) in hippopotamus dung are shown for clarity of presentation.

Figure 10: Linear correlation of fecal sterol pairs measured in the dung of wild animals from the Masai Mara National Reserve. Reported values and cell shading indicate the direction and strength of correlation.

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