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RESEARCH ARTICLE

# Differentiating siliceous particulate matter in the diets of mammalian herbivores

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## Abstract

1. Silica is crucial to terrestrial plant life and geochemical cycling on Earth. It is also implicated in the evolution of mammalian teeth, but there is debate over which type of siliceous particle has exerted the strongest selective pressure on tooth morphology.
2. Debate revolves around the amorphous silica bodies (phytoliths) present in plants and the various forms of siliceous grit—that is, crystalline quartz (sand, soil, dust)—on plant surfaces. The problem is that conventional measures of silica often quantify both particle types simultaneously.
3. Here we describe a protocol that relies on heavy-liquid flotation to separate and quantify siliceous particulate matter in the diets of herbivores. The method is reproducible and well suited to detecting species- or population-level differences in silica ingestion. In addition, we detected meaningful variation within the digestive tracts of cows, an outcome that supports the premise of ruminal fluid 'washing' of siliceous grit.
4. We used bootstrap resampling to estimate the sample sizes needed to compare species, populations or individuals in space and time. We found that a minimum sample of 12 individuals is necessary if the species is a browser or as many as 55 if the species is a grazer, which are more variable. But a sample size of 20 is adequate for detecting statistical differences. We conclude by suggesting that our protocol for differentiating and quantifying silica holds promise for testing competing hypotheses on the evolution of dental traits.

KEYWORDS
functional morphology, opaline phytoliths, siliceous particulate matter, silicon biology, tooth wear

## 1 | INTRODUCTION

Silicon (Si) is the second most abundant element in soils. Ecologists use the term silica because Si is always associated with oxygen ($SiO_2$) in ecosystems, where it forms an essential component of the biogeochemical cycle (Cooke & Leishman, 2011; Vandevenne et al., 2013)—indeed, every vascular plant incorporates silica for a wide range of reasons (Epstein, 1994; Strömberg et al., 2016); e.g., stress from shifts in local abiotic conditions, such as low atmospheric $CO_2$, heat or drought (Strömberg et al., 2016), or to defend against biotic threats such as microscopic pathogens (Fauteux et al., 2005), animal herbivores (Massey et al., 2007; McNaughton et al., 1985), or both (Cooke & Leishman, 2011). Paleobiologists, in turn, have focused their attention on silica when debating the evolutionary consequences of chewing it, with a special emphasis on the dental adaptations of mammalian herbivores (Damuth & Janis, 2011; Fannin et al., 2021; Kaiser et al., 2018; Madden, 2014).

For paleobiologists, there is a crucial material difference between the endogenous silica bodies of plants (opaline phytoliths) and exogenous 'grit', a term with dual meaning. Grit can describe heterogeneous mixes of particles, but many authors use it as a synonym for crystalline quartz, a major component of soil, sand, and ash, as well as aerosolized atmospheric dust (Fannin et al., 2021; Glaccum & Prospero, 1980; Perlwitz et al., 2015). Chewing plant foods exposes teeth to phytoliths and grit (i.e., quartz), and it is this inextricable mix of siliceous particles that underlies decades of spirited debate. One or the other is usually implicated in the evolution of major dental innovations, such as the high-crowned (hypsodont) teeth of ungulates and the ever-growing (hypselodont) teeth of lagomorphs and many rodents (Damuth & Janis, 2011; Jardine et al., 2012; Tapaltsyan et al., 2015; Williams & Kay, 2001). The central problem is that most methods measure silica in toto—a new protocol is needed to differentiate and quantify phytoliths and grit in the diets of herbivores.

Numerous approaches exist to quantify silica, including gravimetry (Morikawa & Saigusa, 2004), trace element analysis (Arthur & Alldredge, 1979; Sneva et al., 1983), solubilization and colorimetry (Brizuela et al., 1986; Kolesnikov & Abaturov, 1997; Vandevenne et al., 2013), and the measurement of either faecal acid insoluble ash (AIA) or faecal acid detergent insoluble ash (ADIA) (Beyer et al., 1994; Healy & Ludwig, 1965; Hummel et al., 2011; Skipworth, 1974). The colorimetric method of Kolesnikov and Abaturov (1997) differentiated siliceous particles, but their protocol, like those that preceded it, required specialized instrumentation and/or many analytical steps involving hazardous chemicals. More recently, Katz et al. (2010) described a simplified method that relies on heavy-liquid flotation to rapidly isolate plant phytoliths from soil matrices for the purpose of identifying plant species in antiquity (review: Cabanes, 2020). If this method can be modified and repurposed to separate phytoliths (specific gravity range: 1.5–2.3; Piperno, 2006) from quartz (specific gravity range: 2.51–2.65; Götze, 2009) in biological samples, then it would hold promise for informing debate on the evolution of silica–animal ecological interactions.

To explore this possibility, we sampled dung from wild and domesticated ungulates inhabiting the Upper Connecticut River Valley and coastal Maine, USA. The species have different dietary preferences and varying levels of tooth hypsodonty, and so represent, potentially, a broad range of silica-animal interactions in New England habitats (a mix of pastoral settings and temperate broadleaf mixed forest). In addition to this comparative sample, we collected food and digesta from ruminally cannulated Holstein dairy cows. Such animals are ideal for testing the extent to which rumen fluid 'washes' exogenous silica from food surfaces (Hatt et al., 2019, 2020, 2021). Our aim in producing these complementary datasets is twofold: first, we describe and validate a protocol for differentiating and quantifying silica in the diets of mammalian herbivores; and second, we report pilot findings to highlight the practical value and future promise of this approach.

## 2 | MATERIALS AND METHODS

### 2.1 | Sample collection

Fresh-voided dung samples (~30 g each) were collected from seven ungulate species, four of which are human domesticates with distant geographic origins: alpaca *Vicugna pacos* ($n = 1$); goat *Capra aegagrus hircus* ($n = 7$); horse *Equus ferus caballus* ($n = 7$); sheep *Ovis aries* ($n = 6$). These animals, together with plains bison *Bison bison* ($n = 5$)—a species formerly present in New England during the Late Pleistocene (Bonnichsen et al., 1985), but as a smaller variant, the woodland bison, which some authors recognize as a subspecies, *B. b. athabascae* (McDonald & Lammers, 2002)—were sampled in summer 2020, a period when these animals subsisted on natural vegetation and pasturage. Wild species include moose *Alces alces* ($n = 2$) and eastern white-tailed deer *Odocoileus virginianus* ($n = 5$). Care was taken to avoid soil contamination by collecting solely from the upper surface or center of the dung sample, prior to storage in sterile Falcon polypropylene sampling tubes.

Samples were also collected from two multiparous lactating ruminally cannulated Holstein dairy cows at the Paul R. Miller Research and Educational Center, University of Vermont. In addition to food ($n = 5$)—a continuous diet of nutritionally balanced total mixed ration (TMR)—and dung ($n = 6$), one of us (S.L.G.) sampled digesta in the dorsal sac of the rumen ($n = 6$); that is, the surface of the floating mat (raft). S.L.G. also sampled digesta ventrally at a reach of ~30 cm, hereafter 'ventral' rumen ($n = 6$). In both cases, we collected 50 ml of combined fluid and solid. Every sample was collected ~5 hr after the onset of morning feeding on a single day. Access to the rumen via the cannula is described by Honan and Greenwood (2020) and approved by the Institutional Animal Care and Use Committee of the University of Vermont (protocol no. 201900019).

### 2.2 | Sample preparation

Samples were stored frozen and thawed over a period of 12–48 hr. Thawed subsamples were put into aluminium weighing boats

(diameter: 60 mm), weighed and dehydrated at 55°C (131°F) for 12–15 hr in a commercial dehydrator (Pro-1200; Weston) or until drying was complete (review: Rothman et al., 2012). Longer durations or higher temperatures may be needed if moisture content is extremely high (Cabanes, 2020). Following dehydration, the faecal samples were weighed, ground with a handheld analytical mill (IKA), put into capped 50 ml Falcon tubes, and stored over $CaSO_4$ desiccant (Drierite; W. A. Hammond Co.) in a desiccator cabinet (Dry-Keeper; SP Bel-Art).

Samples were then dry-ashed, a step that separates plant phytoliths from any adhering organic matter (Parr et al., 2001). In other words, dehydrated samples were weighed again, put into covered aluminium weighing boats, and combusted in a muffle furnace (F-A1740; Thermolyne) at 500°C for 4 hr. The residual content (dry ash) in each boat was weighed to calculate the fraction of each sample lost on ignition and stored in 15-ml Falcon tubes.

## 2.3 | Silica extraction

Dry-ashed samples were put into pre-weighed 1.5-ml conical centrifuge tubes. We found that 10–20 mg of dry-ashed sample was sufficient for effective separation of phytoliths and quartz particles—but 10 mg was optimal for some phytolith-rich samples, such as those of goats and horses (Gur-Arieh et al., 2013). [Phytoliths are hyperabundant in fresh dung due to the selective digestion of organic matter, hence the lower range than that (20–50 mg of sediment) favoured by Katz et al., 2010]. 50 µl of 6 N HCL was pipetted into each tube to dissolve carbonate minerals and other acid-soluble inorganics, a process that took several minutes under vortexing and gentle agitation of the open tube. This initial dissolution step is functionally analogous to AIA methods (Van Keulen & Young, 1977).

Next, 450 µl of 2.4 g/ml sodium polytungstate, or SPT [$Na_6(H_2W_{12}O_{40})$; Sometu Ltd.], was pipetted onto the remaining solution, which contained the dilute HCL plus the acid insoluble fraction. Producing an initial concentration of 2.4 g/ml is essential for separating, via flotation, phytoliths (specific gravity range: 1.5–2.3; Piperno, 2006) from quartz (specific gravity range: 2.51–2.65; Götze, 2009). It is also advisable to prepare fresh stock solutions of SPT before benchwork as the solution crystallizes over time (see Table S1 for a volume-specific mixing table). After adding SPT, the tube was vortexed for 3 s and sonicated for 10 min (FS20D Ultrasonic Cleaner; Fisher Scientific) to disperse any clay aggregates. Samples were then vortexed (3 s) and centrifuged at 2,320 *g* (5,000 rpm) for 10 min (5415D; Eppendorf). Centrifugation separates the phytoliths, which aggregate at the top of the solution, from exogenous quartz (grit), which pellets at the bottom (Figure S1; and see Sections 2.5 and 3.1 for assessment and verification of this claim).

## 2.4 | Quantifying siliceous particulate matter

Phytoliths in suspension were withdrawn using a 1-ml pipette (recommended over smaller volumes, which can clog at the tip) and dispensed into another pre-weighed 1.5-ml conical centrifuge tube. Care was taken to remove all phytolith-containing supernatant from the primary centrifuge tube without contact between the pipette tip and centrifugate pellet. Next, deionized (Milli-Q) water was added to both tubes for two purposes: first, to dilute the SPT concentration so that phytoliths can pellet under further centrifugation; and second, to wash the SPT from each sample. Washing entailed three cycles of vortexing and centrifugation at 2,320 *g* (5,000 rpm) for 10 min, followed by withdrawal of the supernatant each time. [N.B. the preceding steps should be completed within 1 hr as SPT crystallizes rapidly after centrifugation]. Samples were dried under a heat lamp for 8–10 hr, or until all moisture had evaporated, after which each tube was re-weighed to calculate the mass of phytoliths and quartz (grit) per sample, each of which can be expressed as mg/g of either dry or fresh dung (Table 1).

**TABLE 1** Summary of study species and results

| Species and sample sizes (*n* = individuals) | Exogenous silica (mg)/g DM (±*SE*) | Endogenous silica (mg)/g DM (±*SE*) | Total silica (mg)/g DM (±*SE*) |
|---|---|---|---|
| Moose (*Alces alces*); *n* = 2 | 46.9 ± 16.3 | 5.6 ± 3.9 | 52.5 ± 20.2 |
| Bison (*Bison bison*); *n* = 5 | 87.3 ± 33.6 | 27.4 ± 5.9 | 114.6 ± 30.2 |
| Goat (*Capra hircus*); *n* = 7 | 8.8 ± 1.2 | 36.6 ± 5.0 | 45.4 ± 5.9 |
| Horse (*Equus ferus caballus*); *n* = 7 | 25.6 ± 7.3 | 27.9 ± 4.2 | 53.5 ± 8.1 |
| White-tailed deer (*Odocoileus virginianus*); *n* = 5 | 17.8 ± 7.7 | 4.6 ± 0.8 | 22.4 ± 3.9 |
| Sheep (*Ovis aries*); *n* = 6 | 5.4 ± 2.2 | 12.5 ± 2.1 | 17.9 ± 2.9 |
| Alpaca (*Vicugna pacos*); *n* = 1 | 28.2 | 36.8 | 65.0 |

## 2.5 | Validation

To validate the separation steps in Section 2.3, we withdrew the supernatant from samples of three species representing the range of variation in our study (bison, horse, deer), vortexing each for 3 s. Then we pipetted a 50 µl aliquot of supernatant onto a microscope slide and covered it with a 24 × 24 mm coverslip. [By transferring phytoliths in suspension, we assume a homogeneous distribution on the slide]. We counted particles in 16 fields at 200× magnification, following the rationale and protocol of Katz et al. (2010). As phytoliths take on the size, shape and texture of cells in or around which they were deposited, there is a limited range of distinct morphotypes; thus, we classified particles as phytoliths if they matched one of 19 morphotypes established by the International Code for Phytolith Nomenclature 2.0 (Neumann et al., 2019). Counts included fragmented morphotypes (from mastication, among other causes, but distinguishable on the basis of texture) and anatomically connected particles (multicellular structures). Given that the total amount of particulate matter on the slide represented 5% (50 of 1,000 µl) of sample mass, we calculated phytolith and grit concentrations as millions per g of dry ash. The centrifugate pellet (grit) was dried, weighed, and mounted on a microscope slide using Entellan and covered with a 24 × 24 mm coverslip. Again, we classified and counted particles in 16 fields at 200× magnification and calculated concentrations as millions per g of dry ash (here, as 100% of pellet mass).

## 2.6 | Accounting for variation

To explore the relationship between siliceous particulate matter and hypsodonty in our sample of animal species, we used regression analysis tools in JMP 15 Pro (SAS Institute). To determine the variance structure in our data and develop sampling recommendations, we focused on exogenous silica (grit) because it was more variable at the intraspecific level (Table 2). We used nonparametric bootstrap resampling with replacement for each species sample in R Studio (version 1.2.5019), increasing the theoretical sample sizes (at 1,000 iterations per bootstrap up to a maximum of $n = 100$ individuals).

# 3 | RESULTS

## 3.1 | Validation

Our protocol was effective but imperfect (Figure 1). To estimate analytical error, we counted wayward particles—that is, phytoliths in the centrifugate (grit) pellet or grit in suspension—and calculated their concentrations. The percentage of phytoliths that pelleted was 0.7 ± 0.4% for bison (0.3 ± 0.2 of 40.1 ± 3.0 millions/g dry ash), 0.4 ± 0.2% for horse (0.1 ± 0.1 of 33.1 ± 4.9 millions/g dry ash) and 0.1 ± 0.04% for deer (0.01 ± 0.003 of 10.7 ± 1.9 millions/g dry ash). Thus, the percentage of phytolith particles that failed to suspend was practically negligible (mean: 0.4 ± 0.3%). But when particles of grit are expressed as a percentage of all particles in suspension, our error was 6.0 ± 2.6% for bison (2.5 ± 1.2 of 42.3 ± 3.0 millions/g dry ash), 3.7 ± 2.1% for horse (1.2 ± 0.5 of 34.1 ± 6.3 millions/g dry ash), and 12.8 ± 2.7% for deer (1.6 ± 0.4 of 12.3 ± 2.2 millions/g dry ash). On average, grit accounted for 7.5 ± 2.5% of all particles in suspension; however, these particles were uniformly small (~5 µm; Figure 1), which suggests that the forces of surface tension can sometimes prevent the pelleting of dust-sized quartz (Fannin et al., 2021). Still, this level of analytical error was well below the variance within and between samples (Table 2).

## 3.2 | Variation and comparative analysis

Concentrations of phytoliths and grit varied across our sample (Table 1; Figure 2). Assuming the composition of dung is relatively homogeneous, we used triplicate measures to assess relative error (Table 2). The average coefficient of variation (CV) within a dung sample was 18% for exogenous silica (grit) and 31% for endogenous silica (phytoliths), a value that compares favourably to the error (30%) reported by Katz et al. (2010). In two cases (goat and sheep), the mean CV of repeat triplicates exceeded the intraspecific CV (Table 2), which we attribute to two factors: low relative amounts of grit and a murky supernatant, which affects the precision of pipetting by obscuring the pipette tip during withdrawal of

**TABLE 2** Relative variability of siliceous particulate matter in faecal samples, comparing within-sample and within-species coefficients of variation (CVs)

| | | Mean within-sample CV (range) | | | Mean within-species CV | | |
|---|---|---|---|---|---|---|---|
| **Species** | **Triplicates** | **ExSi** | **EnSi** | **Total Si** | **ExSi** | **EnSi** | **Total Si** |
| *Alces alces* | 2 | 7.5 (5.1–9.9) | 101.3 (59.5–143.1) | 11.6 (8.5–14.7) | 49.2 | 97.1 | 54.4 |
| *Bison bison* | 4 | 3.4 (1.0–4.0) | 24.5 (14.5–39.9) | 6.7 (3.7–9.9) | 86.1 | 48.2 | 58.9 |
| *Capra hircus* | 4 | 42.8 (16.3–72.7) | 19.5 (3.6–50.4) | 23.0 (12.7–44.1) | 37.3 | 36.2 | 34.7 |
| *Equus ferus caballus* | 4 | 7.4 (5.4–12.4) | 15.3 (4.3–46.2) | 6.0 (1.1–12.4) | 75.0 | 40.3 | 40.2 |
| *Odocoileus virginianus* | 2 | 14.7 (12.8–16.6) | 34.6 (26.8–42.4) | 11.8 (2.3–21.3) | 43.5 | 37.5 | 38.9 |
| *Ovis aries* | 1 | 27.7 (26.0–29.4) | 24.0 (15.5–32.4) | 16.6 (10.2–23.0) | 40.2 | 40.7 | 39.1 |

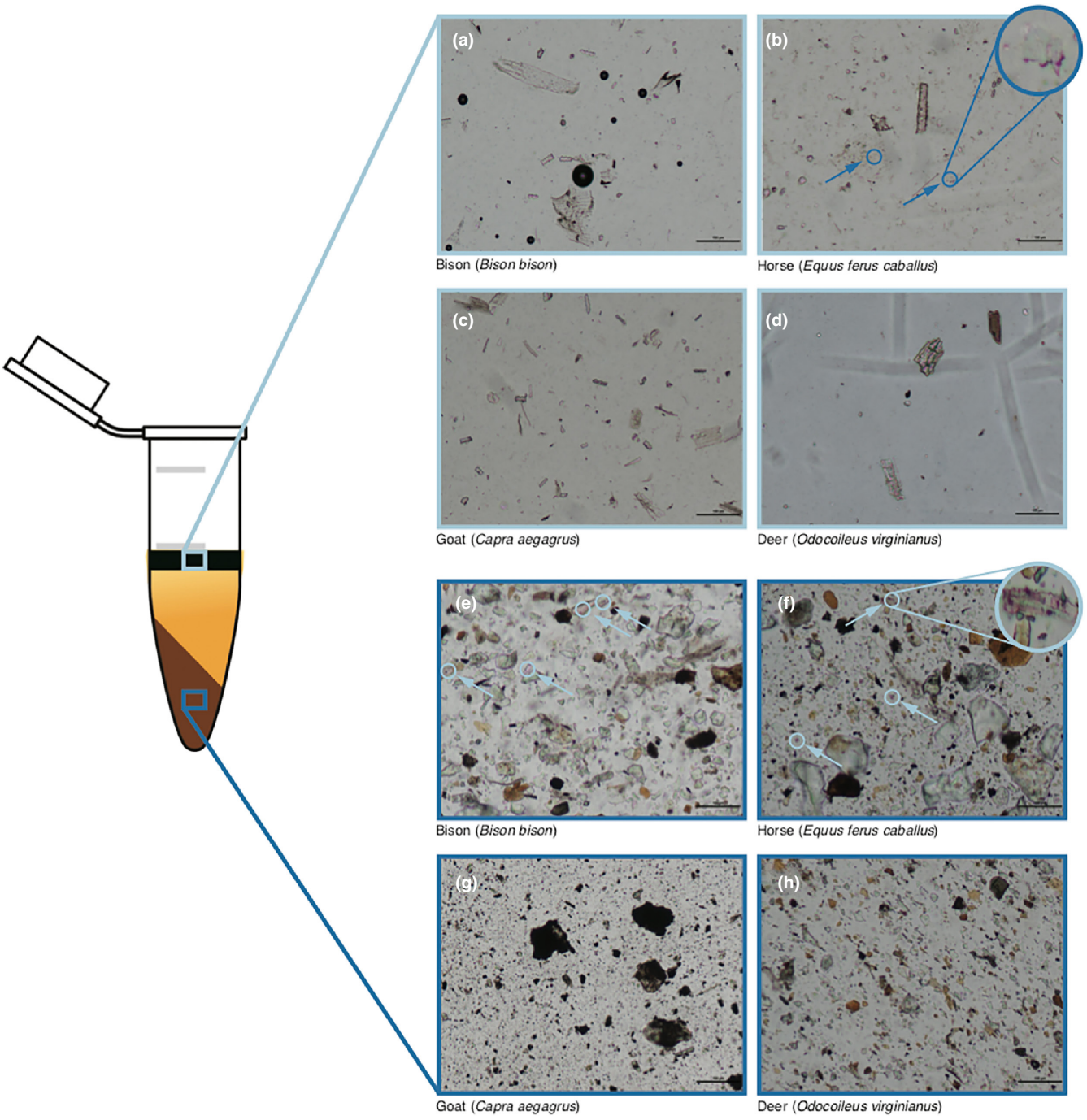


**FIGURE 1** Micrographs validate the effective separation of siliceous particulate matter during heavy-liquid flotation. Representative images from the phytolith-laden supernatant: bison (a), horse (b), goat (c), white-tailed deer (d). Dark-blue arrows signify contaminating crystalline quartz, or grit; a selected example is magnified in panel (b). Corresponding images from the centrifugate (grit) pellet: bison (e), horse (f), goat (g), white-tailed deer (h). Light blue arrows signify contaminating phytoliths; a selected example is magnified in panel (f). The large, dark and irregular polygons in (f) and (g) illustrate rare instances of contaminating organic matter. Scale bar: 100 μm.

the supernatant (Figure S1). Such a supernatant may be the result of soluble tannins, but it could also signal the presence of residual organic matter or suspended clays, contaminants that will produce overestimates of phytolith masses (see Section 4.3.2 for a discussion of remedies). Overall, the results in Table 2 suggest that our protocol is reproducible and well suited to detecting species- or population-level differences in dietary silica.

In our sample, we found that hypsodonty varied positively as a function of exogenous grit in the dung samples of four domesticated species and three undomesticated species (Figure 2b), but such results are underpowered and preliminary. Hypsodonty was unrelated to phytolith concentrations in the dung of domesticates, but positively related to those of undomesticated species (Figure 2c). Last, our bootstrap resampling efforts revealed species-level differences

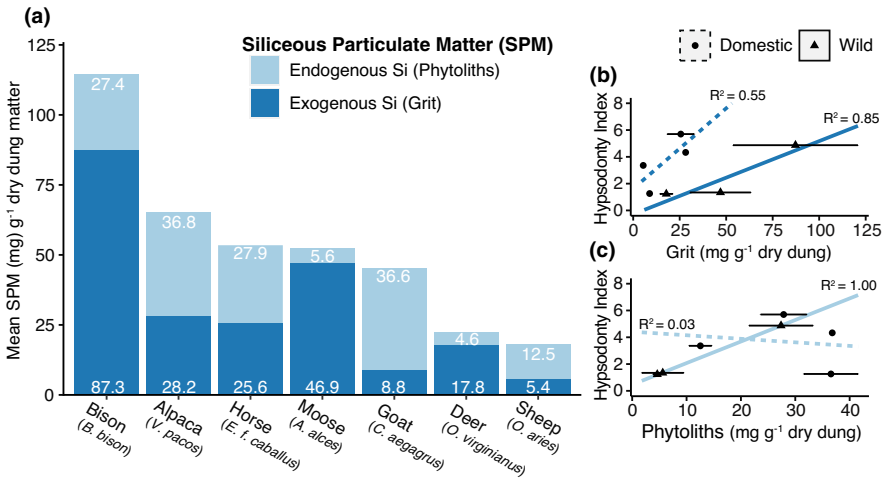


FIGURE 2 Rank order differences in the total amount of exogenous and endogenous faecal siliceous particulate matter (SPM) in our sample of six ungulate species (a). Tooth hypsodonty varied as a positive function of exogenous SPM (grit) in our sample (b). Tooth hypsodonty did not vary as a positive function of endogenous SPM (phytoliths) in our sample of domesticated ungulates, but it did among undomesticated species (c). See Table S2 for hypsodonty data sources.

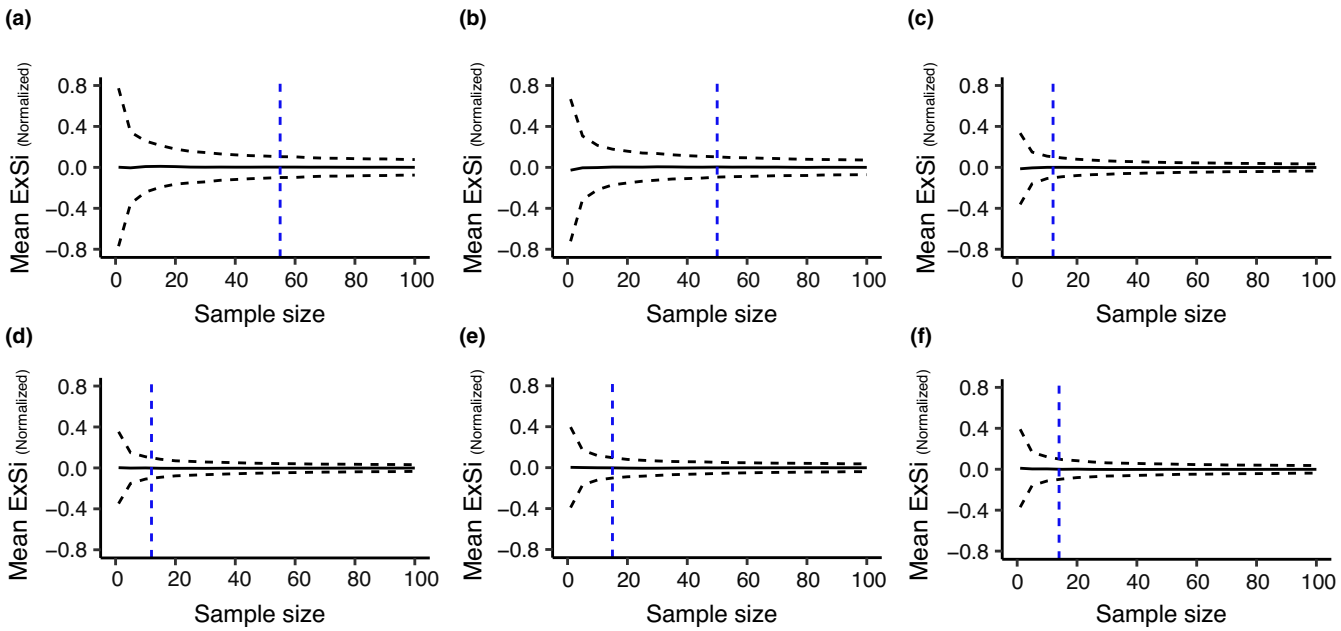


FIGURE 3 Bootstrap resampling curves based on the variation in exogenous grit observed for each study species: bison (a); horse (b); moose (c); goat (d); white-tailed deer (e) and sheep (f). Solid black lines represent the normalized population mean for each bootstrapped sample and dashed lines represent the standard error around the mean. The vertical dashed line (in blue) represents the sample size needed to reduce the population standard error to ±0.1 mg/g of faecal dry matter.

in the number of samples needed to decrease the population standard error (SE) of mean exogenous quartz to within 0.1 mg/g dry matter of the observed mean (Figure 3). We found that a minimum sample of 12 individuals was necessary if the species is considered a browser or as many as 55 if the species is considered a grazer.

### 3.3 | Testing the ruminal fluid washing hypothesis

We measured phytoliths and grit in the food, digesta and excreta of two cannulated cows (Figure 4). When compared to food, digesta in the rumen showed elevated concentrations of phytoliths (dorsal rumen: mean net difference = 0.11% DM; mean percent change = +20%; Figure 4b; ventral rumen: mean net difference = 0.13% DM; mean percent change = +24%; Figure 4c) and lower concentrations of grit (dorsal rumen: mean net difference = 0.13% DM; mean percent change = −43%; Figure 4b; ventral rumen: mean net difference = 0.18% DM; mean percent change = −60%; Figure 4c). These findings are a dual testament to the selective digestion of organic matter in food and the effectiveness of ruminal fluid washing.

## 4 | DISCUSSION

Archaeologists developed a rapid heavy-liquid flotation method for separating phytoliths from soil for the purpose of identifying plant community compositions in the archaeological record (Cabanes, 2020; Katz et al., 2010). The practical innovation here was to modify and repurpose

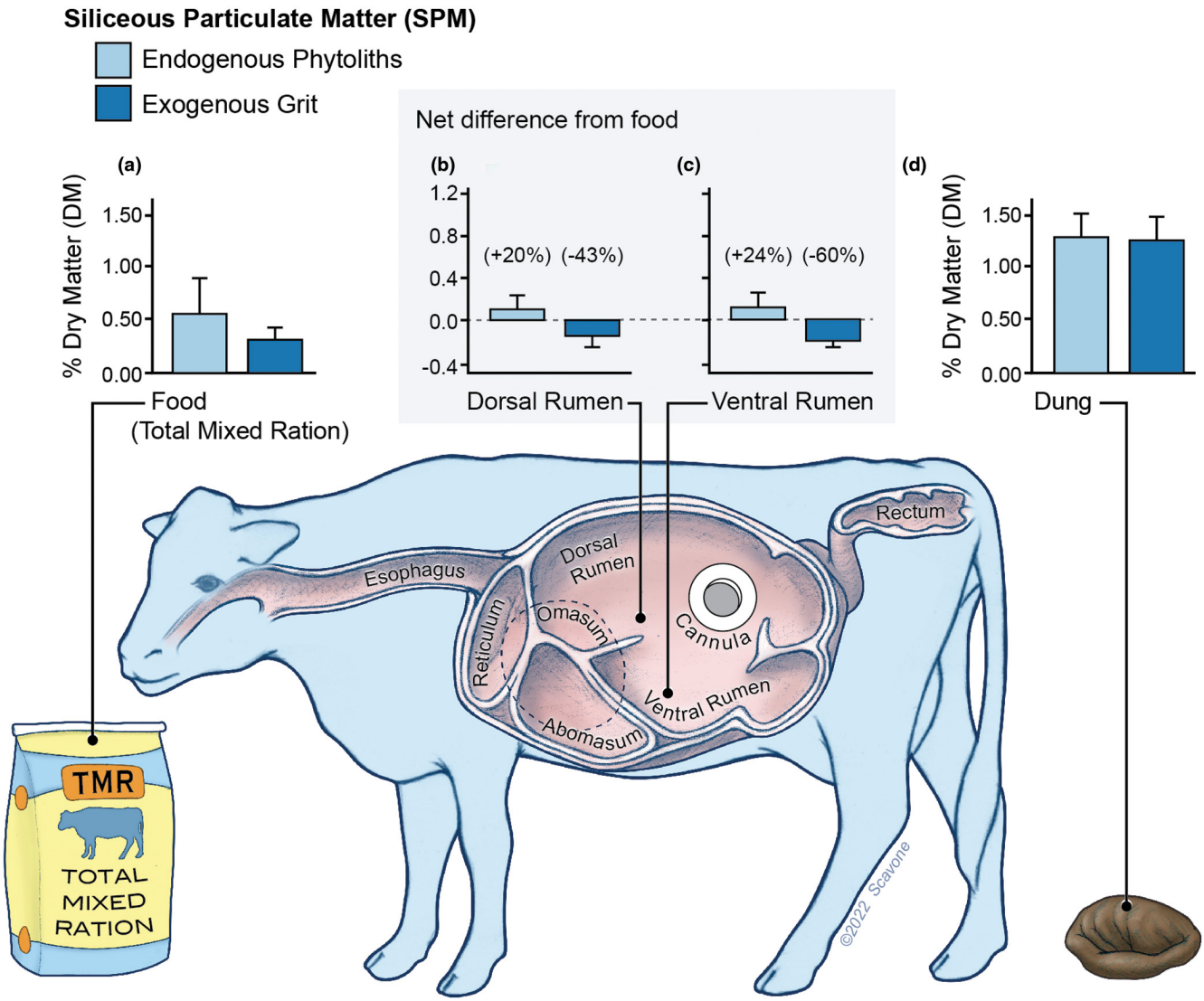


FIGURE 4 Concentrations of endogenous phytoliths and exogenous grit (quartz) in samples of food (a) and dung (d), together with net differences in digesta, relative to food, sampled from the dorsal rumen (b) and ventral rumen (c) approximately 5 hr after morning feeding. Parenthetical values represent percent differences in mass relative to food intake. Ruminal fluid 'washing' of grit is readily apparent in panels (b) and (c). Values averaged from two cannulated cows (see Figure S2 for individual values). Illustration by William Scavone

this protocol for use with animal food and dung samples—to separate and quantify two forms of siliceous particulate matter and calculate their ratio. Our motivation follows in the footsteps of others (Kolesnikov & Abaturov, 1997), but we did not compare the two methods. Instead, we validated the effectiveness of heavy-liquid flotation through microscopy, and, to illustrate the promise of this method for paleobiologists and functional ecologists, we differentiated and compared silica in the diets of wild and domestic herbivores in northeastern United States.

### 4.1 | Comparative analyses

Prior research reported total silica levels in the dung of herbivores (Beyer et al., 1994; Healy & Ludwig, 1965; Hummel et al., 2011; Vandevenne et al., 2013). Such findings were instructive for their purposes, but measures of total silica (phytoliths and quartz combined) are inadequate for testing competing hypotheses for the evolution of specialized dental traits, such as ungulate hypsodonty (Damuth & Janis, 2011). Our comparison of ungulates is germane to this debate insofar as it shows proof of concept—Figure 2b,c illustrate trends, but definitive hypothesis-testing would need to account for species-specific variation in total dung mass, frequency of defecation, and diet-dung integration times, all extrapolated over years of feeding behaviour. Such investigations could prove instructive, informing views on the functional ecology and evolution of dental morphologies.

### 4.2 | Ruminal fluid washing

Another consideration is that dung samples may overestimate the extent to which crystalline quartz is visited on teeth, at least when comparing ruminants to other hypsodont ungulates. Necropsies

of goats, sheep and llamas, all ruminants, found excess accumulations of exogenous grit in the posterior chambers of the stomach, suggesting that some fraction of it was 'washed' from food surfaces before regurgitation and remastication (Hatt et al., 2019, 2020, 2021). Such washing could explain why non-ruminant equids (horses) express greater hypsodonty than ruminants with similar diets (Damuth & Janis, 2011); they probably encounter more exogenous grit per bolus (Dittmann et al., 2017). On balance, our findings in vivo support these arguments: digesta from the rumen of cannulated cows showed net reductions in exogenous grit, especially in the ventral rumen where immersion is greatest (Figure 4).

Although preliminary, our findings invite some back-of-envelope calculations. On average, TMR-fed Holstein dairy cows ingest 24.8 kg of dry matter daily, relying on bouts of initial chewing, or 'feeding chews' (19,255 per day), and remastication, or 'ruminating chews' (28,946 per day; Dado & Allen, 1994). Given that *c*. 53% of ingested dry matter is remasticated (Kennedy, 1985), and that feeding and ruminating chews will encounter different concentrations of grit—0.30% DM and 0.15% DM, respectively (Figure 4)—and assuming a density of 8.7 x 10−8 particles/g (Sanson et al., 2017), we can estimate 20,881 grit particles per feeding chew and 7,827 grit particles per ruminating chew. Assuming that 5.5% of grit particles produce a scratch, and that each scratch removes $3.4 \times 10^{-8}$ $mm^3$ enamel (Sanson et al., 2017), we calculated yearly enamel losses of 270 and 153 $mm^3$ from feeding and ruminant chewing, respectively. Estimating a total postcanine tooth volume of 7305 $mm^3$—based on zebu *Bos indicus* (Janis, 1988)—and a tooth surface of *c*. 40% enamel (Sanson et al., 2017), we calculated ~7 years of enamel life. But in the absence of ruminal fluid washing, the functional life of enamel would be reduced to ~5 years, a difference of two interbirth intervals (Ratnayake et al., 1998). It is, therefore, tempting to suggest that ruminal fluid washing confers significant fitness advantages.

Setting aside the evolutionary importance of ruminal fluid washing—a topic that invites further study—another notable outcome of our experiment concerns the correspondence between silica input and output. Dung was enriched in phytoliths and grit relative to food—mean net differences of 0.7% and 1.0% DM or mean percent changes of +130% and +316%, respectively (Figure 4d). Greater concentrations in dung are expected for two reasons: first, digestive attrition of plant matter will concentrate phytoliths in the gastrointestinal tract; and second, dung represents hours of grit accumulation and integration (62–79 hr for cows; Van Soest, 1994). Accounting for differential enrichment in dung will be essential for any field biologist intent on using dung to reconstruct silica–animal interactions during feeding.

## 4.3 | Caveats and future directions

### 4.3.1 | Sampling strategies

The mean (±1 *SE*) amount of silica that we measured in the dung of bison (11 ±3.0%), moose (5.3 ±2.0%) and eastern white-tailed deer (2.2 ±0.4%) agrees favourably with values from wild conspecifics in Wyoming (bison: 15 ±0.91%; $n = 4$; moose: 5.4 ±0.12%; $n = 3$) and Maryland (deer: 2.7 ±0.4%; $n = 16$; Beyer et al., 1994). Still, bootstrap resampling speaks to the need for much larger sample sizes to compare species, populations or individuals in space and time. To reduce SEs to ±0.1 mg/g faecal dry matter, the minimum sample ranged from 12 (moose) to 55 (bison) individuals (Figure 3). This latter sample size is extremely conservative, however, and it should be viewed as impractical. Our analysis suggests that a minimum sample size of at least 20 individuals is adequate for reducing the population standard error by a factor of four for even the most variable species examined.

Another limitation of our study is that it represents a temporal snapshot with small samples. Our estimates should be considered proof of methodological promise, rather than representative of species-specific patterns. Collecting larger cross-sectional samples has the potential to inform debate on whether the evolution of hypsodonty is related to species-level differences in silica ingestion (Damuth & Janis, 2011; Hummel et al., 2011). At the same time, it will be important to capture seasonal and annual variation in silica ingestion across individuals and species. Silica ingestion can vary as much as 30-fold over the course of year, as observed among sheep in New Zealand (range: 2%–60% of faecal DM; Healy & Ludwig, 1965) and wild bighorn sheep *Ovis canadensis* (range: negligible to 30% of faecal DM; Skipworth, 1974).

### 4.3.2 | Contamination

A murky supernatant (Figure S1) hints at incomplete dry-ashing and excess organic matter, a factor that may have inflated phytolith masses in samples from goats and sheep (Table 1) and account for the intra-sample variability of exogenous grit among goats (Table 2). Indeed, we detected trace organic matter in at least one sample (Figure 1). To dissolve contaminating organic matter, the sample can be re-ashed at 500°C for 4 hr and treated with 50 µl hydrogen peroxide ($H_2O_2$) immediately after the addition of 6 N HCL (see Section 2.3). These steps were not performed here, and care must be taken to adjust the initial concentration of SPT so that the final density remains 2.26 g/ml. If the contaminant is unequivocally clay, the remedy is an additional flocculation step with a common clay-dispersing agent, for example, sodium hexametaphosphate ($Na_6[(PO_3)_6]$; cf. Mercader et al., 2019). To determine the mass of clays removed, the clay-laden supernatant can be withdrawn and put into another centrifuge tube, washed with Milli-Q water, and centrifuged to produce a pellet.

### 4.3.3 | Dry- versus wet-ashing pre-treatment steps

If measuring the material properties of siliceous particles is desirable, then lower-temperature ('wet' ashing) treatments could be preferable to dry-ashing, which may affect the shape and hardness of phytoliths

(Sanson et al., 2007). Parr et al. (2001) found no discernible differences in phytolith shape when comparing dry- versus wet-ashing treatments, but Kaiser et al. (2018) found that dry-ashed phytoliths were marginally harder than their wet-ashed counterparts (but with significant overlap). On balance, wet-ashing methods with oven-dried samples (Hummel et al., 2011) are advised if the study aim is to compare the material properties of phytoliths (Erickson, 2014) or explore the biomechanics of phytolith–enamel interactions (Lucas et al., 2013, 2014; Rodriguez-Rojas et al., 2020; Winkler et al., 2019, 2020).

## AUTHORS' CONTRIBUTIONS

L.D.F., E.J.L., A.v.C., S.L.G. and N.J.D. conceived the project, supplied reagents and designed the methodology; L.D.F. and E.J.L. collected the data; L.D.F. and N.J.D. analysed the data; L.D.F. and N.J.D. wrote the initial draft of the manuscript. All authors contributed critically to ensuing drafts and gave final approval for publication.

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### CONFLICT OF INTEREST

The authors have no competing interests to declare.

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### DATA AVAILABILITY STATEMENT

Data deposited in the Dryad Digital Repository https://doi.org/10.5061/dryad.j6q573ndx (Fannin et al., 2022).

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