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RESEARCH ARTICLE

Controlled feeding trials with ungulates: a new application of *in vivo* dental molding to assess the abrasive factors of microwear

Jonathan M. Hoffman[1,*], Danielle Fraser[2] and Mark T. Clementz[1,3]

ABSTRACT

Microwear, the quantification of microscopic scratches and pits on the occlusal surfaces of tooth enamel, is commonly used as a paleodietary proxy. For ungulates (hoofed mammals), scratch-dominant microwear distinguishes modern grazers from browsers, presumably as a result of abrasion from grass phytoliths (biogenic silica). However, it is also likely that exogenous grit (i.e. soil, dust) is a contributing factor to these scratch-dominant patterns, which may reflect soil ingestion that varies with feeding height and/or environmental conditions (e.g. dust production in open and/or arid habitats). This study assessed the contribution of exogenous grit to tooth wear by measuring the effects of fine- and medium-grained silica sand on tooth enamel using a novel live-animal tooth-molding technique. It therefore constitutes the first controlled feeding experiment using ungulates and the first *in vivo* experiment using abrasives of different sizes. Four sheep were fed three diet treatments: (1) a mixture of Garrison and Brome hay (control), (2) hay treated with fine-grained silica sand (180–250 μm) and (3) hay treated with medium-grained silica sand (250–425 μm). We found a significant increase in pit features that was correlated with an increase in grain size of grit, corroborating earlier chewing simulation experiments that produced pits through grit-induced abrasion (i.e. the 'grit effect'). Our results support an interpretation of large silica grains fracturing to create smaller, more abundant angular particles capable of abrasion, with jaw movement defining feature shape (i.e. scratch or pit).

KEY WORDS: Tooth enamel, Grit, Diet proxy, Jaw mechanics

INTRODUCTION

Microwear, the analysis of microscopic tooth wear, has been widely used as a method of paleoecological reconstruction (Grine, 1986; Grine and Kay, 1988; Solounias et al., 1988, 2010; Solounias and Semprebon, 2002; Merceron et al., 2004, 2005; Fraser and Theodor, 2013) and determining selective forces (e.g. climate and diet factors) responsible for various mammalian adaptations (e.g. hypsodonty, associated with open habitats) (Townsend and Croft, 2008; Billet et al., 2009). However, factors driving microwear patterns among taxa and across dietary guilds are not well constrained, a limitation that affects our power to test hypotheses about evolutionary adaptations using this method. For example, differences in microwear patterns may arise as a result of variation in the physical properties of enamel (e.g. hardness, enamel folding, crystallinity), tooth morphology (e.g. selenodont, hypselodont,

bunodont), mastication biomechanics (i.e. jaw movements) and various physical aspects of ingested abrasives (e.g. hardness, structure and angularity), which include biogenic silica from plants (i.e. phytoliths) and abiotic silica from soil and dust. Teasing apart the factors contributing to inter- and intra-individual differences in microwear using wild populations is therefore difficult because the various drivers of microwear patterns may yield similar or even additive effects. In this study, we employed a novel controlled feeding experiment on domesticated sheep (*Ovis aries* Linnaeus) to assess the effects of ingested abiotic silica on the microwear patterns of ungulates.

Microwear was initially quantified with scanning electron microscopy (SEM) at 500× magnification and used largely in anthropological studies (Walker et al., 1978; Grine, 1981, 1986), but a low-magnification (<40×) method (light microscopy for dental microwear, or LDM) developed by Solounias and Semprebon (2002) is now widely used in non-primate paleodietary studies (Merceron et al., 2004, 2007; Rivals and Semprebon, 2006; Green et al., 2005; Semprebon and Rivals, 2010; Fraser and Theodor, 2013). Studies of extant herbivore populations with known diets have revealed a correlation between dietary guild and relative abundance of microwear features such as scratches and pits (Solounias and Hayek, 1993; Solounias and Semprebon, 2002). A commonly employed dietary classification scheme follows that of Hofmann and Stewart (1972), in which browsers (<10% grass consumption) are identified by a predominance of pits over scratches, grazers (>90% grass) show a predominance of scratches over pits, and intermediate or mixed feeders show intermediate numbers of scratches, with some species tending toward the browser or grazer ends of the microwear spectrum (Solounias and Semprebon, 2002). The number of scratches and pits has been used to classify species into dietary categories using two primary methods: bivariate plotting of average scratch against average pit counts for each taxon to create a 'trophic triangle' (Solounias and Semprebon, 2002; Semprebon et al., 2004; Godfrey et al., 2004) and combining microwear and morphological characteristics using multivariate data exploration (i.e. principal components analysis, discriminant function analysis) to partition species among dietary guilds that are defined *a priori* based on observations of extant species (e.g. Merceron et al., 2006; Rivals et al., 2007; Fraser and Theodor, 2011, 2013).

The initial presumption of this classification scheme based on pit and scratch number was that biogenic abrasives are primarily responsible for scratch-dominated wear among modern grazers because of the high abundance of phytoliths in monocotyledonous grasses relative to dicotyledonous browse (Baker et al., 1959; Piperno, 2006). However, several studies examining the effects of biogenic and abiotic abrasives have cast doubt on the central role of biogenic silica in scratch accumulation (Covert and Kay, 1981; Kay and Covert, 1983; Maas, 1991, 1994; Gügel et al., 2001; Mainland, 2003; Sanson et al., 2007; Lucas et al., 2013; Schulz et al., 2013;

[1]Department of Geology and Geophysics, University of Wyoming, Laramie, WY 82071, USA. [2]Department of Biology, Carleton University, Ottawa, ON, Canada K1S 5B6. [3]Program in Ecology, University of Wyoming, Laramie, WY 82071, USA.

*Author for correspondence (jhoffma9@uwyo.edu)

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Müller et al., 2014). Whereas an early study of material properties showed phytoliths were harder than sheep tooth enamel (Baker et al., 1959), subsequent nanoindentation tests have found phytoliths from four globally widespread species of grass to be softer than enamel (Sanson et al., 2007). However, both phytoliths and abiotic silica (e.g. silicon carbide grit) have produced microwear features in chewing simulations (Maas, 1991, 1994; Lucas et al., 2013). The shapes of predominant features (i.e. scratches or pits) were correlated with the direction of chewing motion relative to the occlusal surface; pits resulted from compressive loading (perpendicular movement) and scratches were produced by shearing movement, dragging particles across the surface (Maas, 1991, 1994; Lucas et al., 2013). These results suggest that along with the physical properties of abrasive particles, jaw mechanics play an important role in microwear development. However, mechanical *in vitro* simulations differ from physiologically induced wear in that they suffer from the experimental artifact of unidirectional 'chewing' strokes and may not accurately simulate natural wear accumulation (Gügel et al., 2001).

Exogenous grit is oft-cited as a contributing factor to microwear primarily because ungulates living in semi-arid or arid environments, such as extant camels (*Camelus bactrianus* and *Camelus dromedarius*), pronghorn (*Antilocapra americana*) and vicuña (*Vicugna vicugna*), show a predominance of coarse microwear features (i.e. coarse scratch morphology, higher mean total pit counts and occasional gouging) relative to congeners in more humid regions (Solounias and Semprebon, 2002). Mainland (2003) examined populations of grazing sheep in Denmark and found relative scratch abundance positively correlated to levels of abiotic silica recovered from feces. Conversely, an abnormal predominance of pits in reindeer (*Rangifer tarandus*) has been attributed to grit consumption while feeding on lichens (Merceron et al., 2004). However, the feeding behavior and diets of the sheep and reindeer populations were not controlled.

Controlled feeding trials might be the best means of teasing apart the drivers behind different microwear profiles among species. American opossum (*Didelphis viriginianus*) fed fine-grained pumice showed a prevalence of scratches (Covert and Kay, 1981; Kay and Covert, 1983). Similarly, rabbits (*Oryctolagus cuniculus*) fed grass diets of increasing biogenic abrasiveness (i.e. higher phytolith content) showed higher relative scratch counts, greater variability among individuals (Schulz et al., 2013) and increased rates of tooth wear (Müller et al., 2014). Tooth wear rates also increased when rabbits were fed diets with abiotic abrasives (i.e. sand), relative to diets with biogenic abrasives (Müller et al., 2014). Still, results from these studies do not necessarily bear on microwear in ungulates, which has not been measured under controlled feeding conditions. Furthermore, microwear interpretations are potentially hindered by methodological inconsistencies among individuals and labs. The LDM method was developed as a quick and cheap alternative to SEM-based analyses, although categorization of feature shapes is prone to observational bias and different researchers may record different absolute counts of scratches and pits (Mihlbachler et al., 2012). SEM studies may encounter similar issues in defining parameters for image-analysis software (Grine et al., 2002). However, for either method, the effects of observer bias may only be significant if dietary categorization is impacted.

The objectives of this study were to: (1) develop a method for conducting repeated measures controlled feeding trials with ungulates; (2) quantify the contribution of observer bias to the variation in the dataset; (3) test the hypothesis that abiotic silica has a quantifiable effect on physiologically induced microwear on selenodont teeth; and (4) test the hypothesis that any observed microwear patterns resulting from a 'grit effect' are correlated to grain size. The repeated measures method presented here enables critical evaluation of the causal factors of microwear and controlled evaluation of methodological issues, impacting paleoecological interpretations and further standardizing test design for microwear studies.

RESULTS

Loss-on-ignition analysis

The Garrison–Brome hay mixture used as a control in this study had a significantly higher mean silica content (MSC) than that of washed hay (Mann–Whitney test, $P<0.001$; Table 1). The difference (3.98% of the hay's dried mass) represents the exogenous silica adhered to the hay and accounts for 69.22% of the control treatment's silica content. The sand used in the grit treatments was almost pure silica (Table 1).

We predicted MSCs (and propagated error) for scat from the control ($12.89\pm0.15\%$) and treatments ($30.80\pm0.10\%$) (dashed lines, Fig. 1) using mass balance calculations and the loss-on-ignition (LOI) data of the hay and sand. We used a dry matter digestibility of 58%, based on previously reported values for Garrison hay (58.2%) and Brome hay (57.8%) fed to lambs (Matejovsky and Sanson, 1995). Sheep scat from the sand-free control trial (Table 1) had a significantly lower MSC than the predicted value (one-sample t-test, $P=0.026$) as well as a lower MSC than those measured for the fine and medium sand treatments (Kruskal–Wallis test, $P=0.008$). Aside from one sample from the fine sand treatment (Fig. 1), measured MSC was higher than MSC predicted for both treatments; however, this difference was only significant for medium sand (one-sample t-test, $P<0.001$). No statistically significant difference in MSC was detected between sand treatments (Mann–Whitney test, $P=0.052$). Carbonate content of scat from the control treatment (Table 1) was significantly higher than the carbonate content of the hay and scat from the fine sand and the medium sand treatments (Kruskal–Wallis test, $P<0.001$).

Microwear analyses

Microwear patterns were largely pit dominated (Fig. 2). There was considerable overlap in total scratch and pit counts between observers for each treatment (Fig. 3, Table 2) and the natural grazers (Fig. 4, Table 2). Of the two sand treatments, the medium sand treatment showed higher mean pit counts and greater pit count

Table 1. Summary statistics of loss-on-ignition data

Sample	N	%MSC	%Organic	%Carbonate
Garrison–Brome hay mixture	10	5.75±0.06	92.56±0.10	1.69±0.11
Washed Garrison–Brome hay	5	1.77±0.03	96.76±0.03	1.47±0.05
Silica sand	3	99.79±0.07	0.03±<0.01	0.18±0.07
Scat – control diet (hay)	5	11.03±0.54	82.32±1.45	6.65±1.16
Scat – fine sand diet	3	47.26±7.33	51.36±7.34	1.38±0.02
Scat – medium sand diet	4	62.17±1.42	36.79±1.39	1.04±0.08

N, number of samples analyzed in triplicate; MSC, mean silica content.
Data are given as means±s.e.m. All calculations are relative to dry mass.
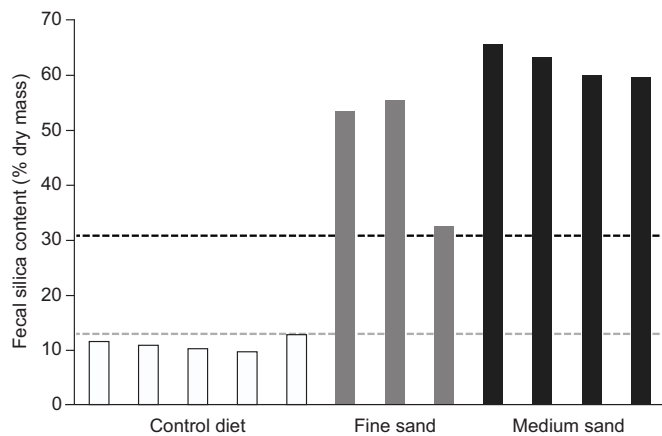
Fig. 1. Silica content of fecal pellets from the controlled feeding trials. Sheep were fed the control diet (no added silica), fine sand (180–250 μm) or medium sand (250–425 μm). Each bar represents the silica content of three fecal pellets produced and collected during anesthetization. The light gray dashed line represents predicted mean silica content (MSC) for scat from control trials (12.89±15%) and the black dashed line represents predicted MSC for scat from treated diets (30.80±0.10%). The thickness of the prediction lines exceeds propagated error.

ranges. Additionally, the fine sand treatment exhibited higher scratch counts than both the hay control and coarse sand treatment (Fig. 3, Table 2).

The first and second principal components (PC1 and PC2) explain most of the variation in the data among treatments (Fig. 5, Table 3). PC1, which explains 46.16% of the variation, reflects differences in pits among treatments. PC2 explains 34.49% of the variation and reflects differences in total scratches among treatments. Together, PC1 (O1P+O2P, where O is observer and P is no. of pits) and PC2 (O1S+O2S, where S is no. of scratches) account for 80.65% of the variation among treatments. These principal components also reveal the increase in scratch counts between the control and fine sand treatment and the increase in pit counts between the fine and medium sand treatments (Fig. 5A, Table 2).

Our ANOVA results show highly significant differences in PC1 (pits) among the treatments (Table 4, $P<0.001$). Pairwise comparisons revealed highly significant differences ($P<0.001$)

Fig. 2. Photographs of microwear on the occlusal surfaces of sheep molars. (A) The photographed locations (black area) for microwear analysis; examples of microwear resulting from the control (B), fine sand (C) and medium sand (D) diet treatments are shown. Scale bars: 0.5 mm.

between the means of the medium sand treatment and those of both the control and fine sand treatments. There were no significant differences for any of the other principal components.

Observer 2 generally had a higher and larger range of pit counts across treatments than observer 1, and observer 1 generally had higher mean scratch counts than observer 2 (Fig. 3, Table 2). These differences are apparent in PC3 and PC4 (Fig. 5B), which reflect the differences in total scratch and pit counts between observer 1 and observer 2. PC3 explains 12.92% of the variation and PC4 explains 6.42% of the variation. Together, PC3 and PC4 explain 19.34% of the variation and reflect overall observer bias. The observer bias is identified by the opposing directionality of feature eigenvectors between observers (e.g. O1S and O2S in Fig. 5B).

We also found significant differences ($P=0.004$) in PC1 (O1P+O2P) among subjects (Table 5). Pairwise comparisons revealed significant differences in mean PC1 values between sheep 2 and sheep 3 and between sheep 3 and sheep 4. However, these differences likely reflect the incomplete distribution of treatments, not individual variation; sheep 1 and 2 are represented by all three treatments, whereas sheep 3 is lacking the medium sand treatment and sheep 4 is represented by only the medium sand treatment. No significant differences exist for any of the other principal components.

When comparing the two sheep representing all three treatments (sheep 1 and sheep 2), we found significant differences (ANOVA, $P=0.003$) in PC1 (O1P+O2P) among treatments (supplementary material Fig. S1, Table S1). Pairwise comparisons revealed highly significant differences ($P=0.013$ and $P=0.009$, respectively) between the means of the medium sand treatment and those of the control and fine sand treatments. There were no significant differences for any of the other principal components.

The microwear patterns observed for naturally grazing Wyoming sheep were pit dominated and overlapped with counts from all three feeding trials (Fig. 4, Table 2). For both observers, counts for the Red Buttes specimen (represented by four images) overlapped with counts from all three feeding trials. We found a highly significant difference in PC1 (pits) between the means of the Wyoming grazers and those of the medium sand trial (Table 4). Pairwise comparisons also revealed highly significant differences in PC2 (scratches) between the means of the Wyoming grazers and those of both the control and fine sand trials (Table 4).

DISCUSSION

Herein, we developed a live molding technique to allow repeated measures testing of ungulate microwear to reduce the number of subjects needed for feeding trials. We also present a robust computational method for comparing treatments and quantifying observer error. In general, our method is successful, repeatable, and can be used as a guideline for future microwear studies regardless of whether they include a controlled feeding component. Overall, our methods compensate for any repeatability differences among current microwear methods. Furthermore, we used our methods to directly assess the contribution of fine- and medium-grained dietary grit to tooth microwear formation in sheep and found that exogenous dietary grit of this size results primarily in the formation of pits on the enamel occlusal surface.

Our system of introducing grit treatments was particularly effective; sand adhered to the hay and the sheep found the corn syrup–sand mixture palatable, as confirmed by LOI analysis of feces. The MSC for scat from the medium sand treatment was significantly higher than the predicted value, an unexpected outcome that was likely due to the sheep selectively ingesting any
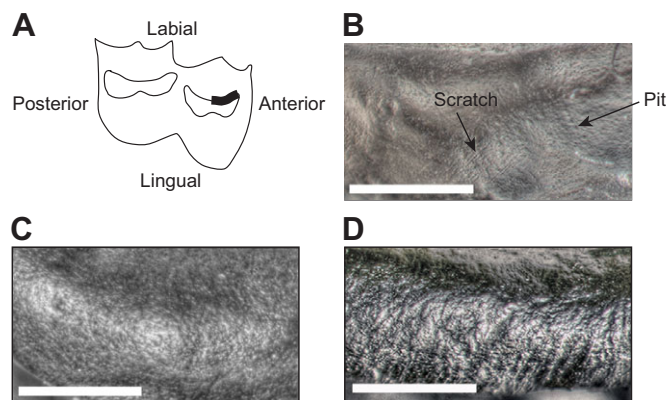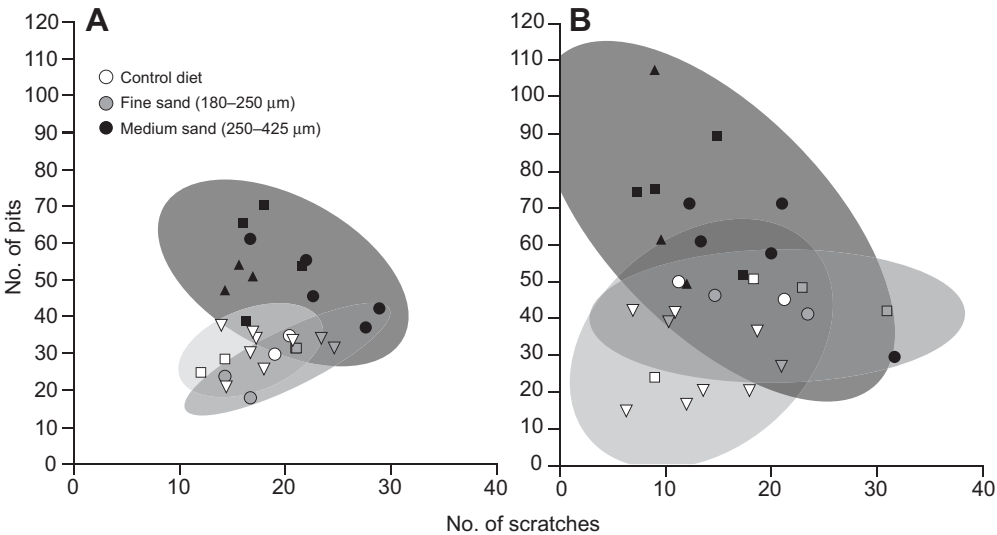
Fig. 3. Bivariate plots of mean total scratches and mean total pits for each treatment. Pits and scratches in each image were counted by two observers (A, observer 1; B, observer 2). Each symbol represents a different test subject and colors distinguish diet; 95% confidence intervals (shaded) are shown for each treatment.

excess corn syrup and sand in the troughs, as well as the additive effects from silica retention time in the rumen.

Our live molding technique was also effective, yielding a 75% success rate at producing molds sufficient for microwear analysis. Inadequate molds may have been primarily due to re-growth of the pellicle, the proteinaceous film produced from animal saliva that coats teeth. To improve success rate, a proteolytic enzyme treatment (Kay and Covert, 1983) can be applied to the occlusal surface after teeth cleaning, but before making the second mold. The molding plate should also be held in place, with the mouth held open, as we found that the anesthetized sheep were prone to moving their jaws and distorting the molds if their mouths were closed. This live molding technique enables us to use repeated measures designs to reduce costs associated with purchase and care of ungulate test subjects.

Previous researchers have found LDM inter-observer error to be high but to have little impact on trophic identifications of populations (Mihlbachler et al., 2012; DeSantis et al., 2013). Similarly, we found that relative differences in microwear patterns among dietary treatments were consistent across two observers (Fig. 3). Our multivariate approach also enabled us to quantify the relative contribution of observer bias to our dataset; only 20% of the variation within our dataset was due to inter-observer error (Fig. 5, Table 3).

The success of our methodology allowed us to assess the effect of exogenous grit on microwear formation. We found a clear exogenous grit effect with the consumption of medium sand,

Table 2. Summary statistics of microwear data

Diet	N (n)	No. of S	No. of P
Observer 1			
Control (no sand)	3 (11)	16.8±4.6	31.1±8.0
Fine (180–250 μm)	3 (6)	20.8±4.8	29.4±7.9
Medium (250–425 μm)	3 (12)	19.6±5.9	52.4±12.1
Natural	4 (20)	16.8±4.8	44.4±12.9
Observer 2			
Control (no sand)	3 (11)	13.3±5.2	32.9±13.7
Fine (180–250 μm)	3 (6)	20.4±7.1	40.5±7.9
Medium (250–425 μm)	3 (12)	14.8±6.9	66.5±20.4
Natural	4 (20)	17.2±7.1	29.9±10.1

N, number of subjects; n, number of images analyzed. Data for number of scratches (S) and number of pits (P) are given as means±s.d.

which resulted in a highly significant increase in the number of total pits (Table 4) relative to the control and fine sand treatments as well as larger ranges of pit counts among individual sheep. We also did not find any evidence of a significant grit effect on scratch counts.

Generally, enamel pitting is attributed to attrition (tooth-on-tooth contact), during which pieces of enamel prisms are plucked out (Walker, 1984; Teaford and Runestad, 1992). However, analyses of wild primate and ungulate populations indicate a correlation between higher pit density and dietary hardness (Teaford and Runestad, 1992; Teaford and Walker, 1984; Teaford, 1988; Teaford and Robinson, 1989; Strait, 1993; Crompton et al., 1998; Solounias and Semprebon, 2002; Merceron et al., 2004). This interpretation is further supported by in vitro chewing simulations that produced pits through compressive loading of abiotic silica particles (Maas, 1994; Lucas et al., 2013). Our results agree with those from in vitro experiments and show that natural chewing of medium-grained silica sand contributes to pitting, and attrition is not the sole contributor to pit formation.
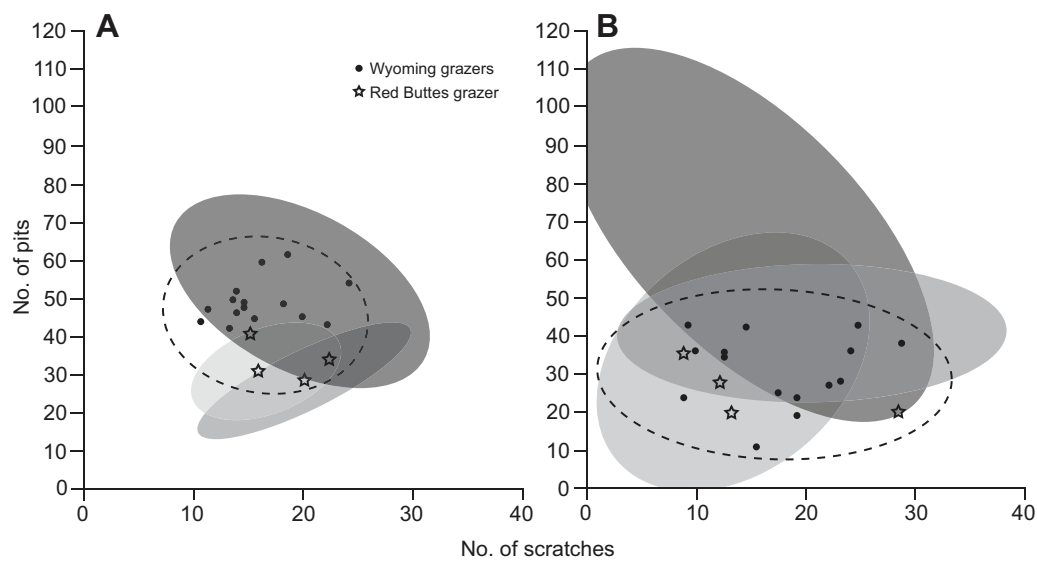
Further, our results contradict the hypothesis that pit abundance is correlated with the relative abundance of abrasive particles contacting the wear facets during chewing, an effect that is inversely proportional to particle size (Maas, 1994). This hypothesis was supported by in vitro simulations that suggested a negative correlation between pit density and particle size (14–73 μm) observed at high magnification (500×), although the negative correlation was not quantified (Maas, 1994). At low magnification (32×), the negative correlation between particle and pit abundance would presumably cause the fine sand treatment to result in a significant increase in pits relative to both the control and medium sand treatments at low magnification. However, we did not see such an effect from the fine sand treatment.

We consider two explanations for the lack of a 'grit effect' from the fine sand treatment. First, the fine sand treatment had the smallest sample size and reduced statistical power for comparison as a result of rejected molds. However, for the two sheep that represent all three diet treatments (sheep 1 and sheep 2), we saw the same pattern among treatments: the control and fine sand treatments were statistically indistinct and the medium sand treatment yielded a significantly higher number of pits (supplementary material Table S1). Second, we suggest the observed 'grit effect' is controlled, in part, by grain size. Previous studies have shown a correlation between particle size and the size of microwear features

Fig. 4. Bivariate plots of mean total scratches and mean total pits for natural grazers. Pits and scratches in each image were counted by two observers (A, observer 1; B, observer 2). Images were from naturally grazing Wyoming sheep and a grazer from Red Buttes, WY, USA; the 95% confidence intervals are shown for each treatment (shaded; see Fig. 3) and the natural grazers (dashed line).

(Wright, 1969; Ryan, 1979; Maas, 1994), with the widths of features ~10% of the particle diameter (Wright, 1969). In accordance with the general practice of LDM as a quicker method than SEM and texture analysis, we did not measure the dimensions of each feature observed in this study. However, we visually compared features to a scale bar of 25 μm, the theoretical threshold between features

created by fine (180–250 μm) and medium (250–425 μm) sand. Nearly all observed features were clearly smaller than 25 μm, suggesting fine-grained or smaller particles (<250 μm) produced most of the pits and scratches observed in all three feeding trials. The size discrepancy between the medium sand and features produced during that trial is likely the result of the size-dependent
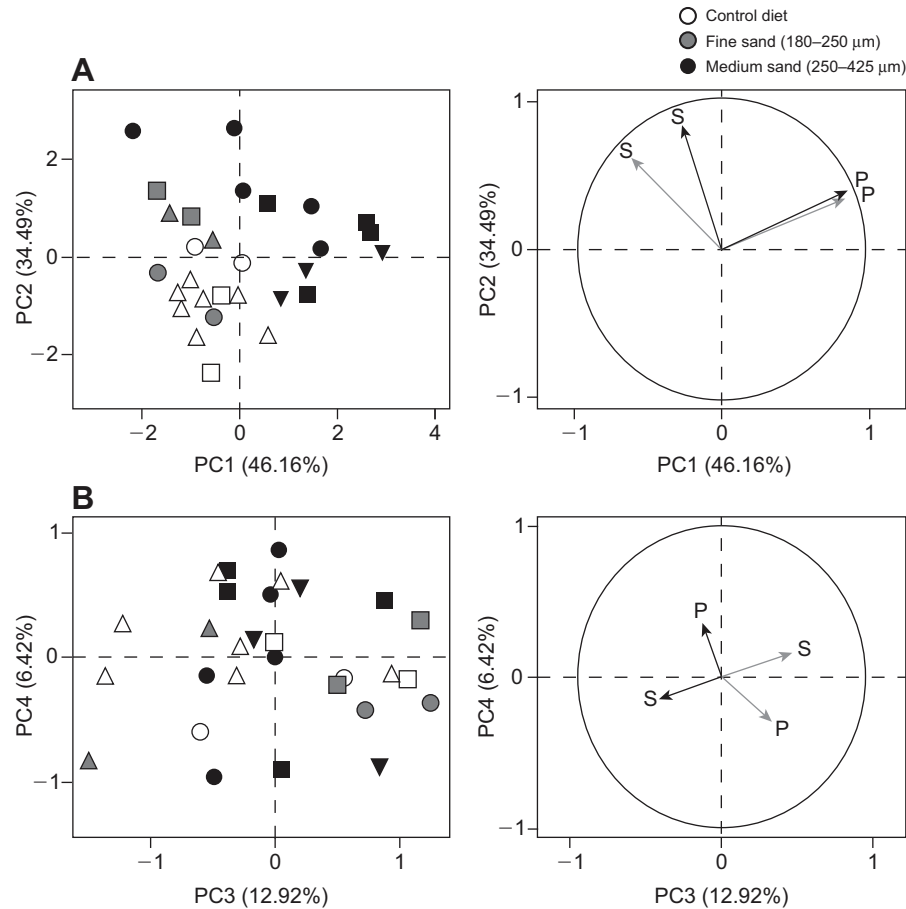
Fig. 5. Bivariate plots and eigenvectors showing variation among diets. (A) Principal components 1 (PC1, pits) and PC2 (scratches) and (B) PC3 and PC4 (observer bias). Black arrows, observer 1; gray arrows, observer 2; S, total scratches; P, total pits; different symbols represent different subjects.

Table 3. Eigenvalues and eigenvectors resulting from PCA of microwear variables

Diet	PC1	PC2	PC3	PC4
Eigenvalue	0.810	0.110	0.066	0.014
Percent	46.16%	34.49%	12.92%	6.42%
Cumulative percent	46.16%	80.65%	93.57%	99.99%
Eigenvectors				
O1P	0.842	0.392	−0.121	0.350
O1S	−0.279	0.850	−0.419	−0.156
O2P	0.831	0.342	0.326	−0.294
O2S	−0.607	0.622	0.470	0.155

PC, principal component; O1, observer 1; O2, observer 2. Pit (P) and scratch (S) counts are mean values.

physical properties of quartz grains, specifically the peak fracture stress.

To chip enamel, grit particles must be harder than enamel and possess a high angle of attack (Atkins and Liu, 2007; Lucas et al., 2013). Also, because the peak fracture stress of quartz particles increases as particle size decreases (Lucas et al., 2014), medium sand grains are more likely than fine sand grains to break into smaller, angular and more abundant grains capable of abrading enamel. Therefore, we suggest the medium sand 'grit effect' (an increased abundance of pits smaller than 25 µm) resulted from fracturing medium sand grains through mastication, with masticatory movement (i.e. shearing versus compression) as the determining factor for feature shape (i.e. pit or scratch). Further, the smaller particles of the fine sand treatment have higher fracture stress and are harder to break, providing fewer angular abrasives to cause wear and increase the pit quantity relative to the control.

Every et al. (1998) described sheep mastication as primarily a cutting process, as opposed to grinding, with compression of the cheek teeth followed by shearing movements. Additionally, the shearing jaw movement at the end of the sheep masticatory sequence does not require much force (Every et al., 1998). Our results support this mastication model where more force is exerted during the compressive phase than during shearing, creating a pit-dominated microwear pattern for all three treatments.

The pit-dominant occlusal surfaces of our control casts resemble the common patterns observed in our Wyoming grazing sheep as well as previously reported patterns for naturally grazing domesticated sheep (Mainland, 2000, 2001, 2006). One exception to this pattern was observed for grazing sheep in Denmark, which exhibited scratch dominance correlated with ingestion of soil

particles less than 180 µm in diameter (Mainland, 2003). Mainland (2006) maintains that soil particles, and not phytoliths, are primarily responsible for striations in sheep and goats and pit-dominated patterns reflect low soil ingestion and not necessarily a browse diet. This hypothesis is not supported by our study; while our fine sand treatment (180–250 µm) resulted in higher scratch counts relative to the control and medium sand treatment, the difference is not statistically significant. Additionally, many of the striations we observed across treatments were thin enough to be created by abrasives smaller than 180 µm (i.e. <18 µm), the minimum diameter of our smallest-grained silica treatment.

Given this size range, the observed fine scratches are attributable to either phytoliths in the hay and/or very fine sand grains adhered to the hay via previous cultivation and processing. The washed hay had significantly lower MSC than the unwashed hay (Mann–Whitney test, $P < 0.001$), with exogenous grit comprising 69.22% (by dry mass) of the total hay silica. To further elucidate the process of scratch formation, it will be necessary to conduct controlled feeding trials with rinsed hay. It will also be necessary to expand feeding trials taxonomically to include grazing taxa with predominantly lateral jaw movement (e.g. cattle).

Numerous studies have documented the amount of soil consumed by different taxa (Table 6), but there is little research describing the different size fractions of ingested soil. The counts for our feeding trials all overlapped with the counts for naturally grazing sheep from Wyoming (Fig. 4). However, we found significant differences in mean scratches and mean pits from natural grazers relative to different feeding trials (Table 4). As we did not have detailed dietary information for most of the grazing sheep, it is difficult to interpret the potential causes of these statistical differences, although smaller grit particles (i.e. <180 µm) may play a role. More grain size analyses of soil in wildlife fecal matter are required to further explore how ingested soil particles may vary with habitat, particularly in arid, more open environments.

For both observers, a pasture-grazing Wyoming sheep (UW 50590) showed considerable variation in feature counts along the shear facet (i.e. between different images), overlapping with the confidence ellipses of all three feeding trials (Fig. 4). This illustrates the heterogeneous nature of tooth wear and reinforces the necessity for averaging counts from multiple search fields along the shear facet rather than relying on a single search field. Using multiple search fields is particularly important at high magnification and may result in skewed interpretations if only a single search field is analyzed.

Table 4. ANOVA results for extracted PC1 and PC2 with Tukey multiple comparison of means for assessing differences among diet treatments

Source	PC1 – pits (O1P+O2P)					PC2 – scratches (O1S+O2S)				
	d.f.	SS	MS	F-ratio	P	d.f.	SS	MS	F-ratio	P
Diet	3	9905	4953	19.66	<0.001	3	386.3	193.1	2.72	0.084
Residual	44	6551	252			44	1846	70.99		
Tukey					P adjusted					P adjusted
Control–fine					0.803					0.246
Control–medium					<0.001					0.709
Fine–medium					<0.001					0.069
Control–natural					0.989					<0.001
Fine–natural					0.925					<0.001
Medium–natural					<0.001					0.140

Control, control diet (hay with no added silica); fine, fine-grained sand treatment (180–250 µm); medium, medium-grained sand treatment (250–425 µm); natural, grazing Wyoming sheep from various localities.

Table 5. ANOVA results for extracted PC1 and PC2 with Tukey multiple comparison of means for assessing differences among individual test subjects

Source	PC1 – pits (O1P+O2P)					PC2 – scratches (O1S+O2S)				
	d.f.	SS	MS	F-ratio	P	d.f.	SS	MS	F-ratio	P
Sheep	3	6739	2246	5.78	0.004	3	172.6	57.53	0.698	0.562
Residual	25	9717	388.7			25	2570	82.38		
Tukey					P adjusted					P adjusted
Sheep 1–sheep 2					0.915					0.956
Sheep 1–sheep 3					0.076					0.492
Sheep 1–sheep 4					0.302					0.963
Sheep 2–sheep 3					0.022					0.816
Sheep 2–sheep 4					0.574					1.000
Sheep 3–sheep 4					0.007					0.950

In regards to paleodietary and paleoenvironmental analyses, our results are significant in that they support the interpretation that increased rates of pitting observed in ungulates from semi-arid and arid environments reflect grit consumption (Solounias and Semprebon, 2002), confirming that microwear can be affected by environment as well as diet. Our results also suggest that hypsodonty (i.e. high-crowned teeth), which appeared within multiple North American ungulate lineages during the middle Miocene (~15.8 Ma), may have been a co-evolutionary adaptation to the consumption of grit in open environments (Stirton, 1947; Janis, 1988) rather than with the appearance of grassland savannahs (Kovalevsky, 1874; Webb, 1983). The hypothesis that grit consumption was an evolutionary driver is further supported by the asynchronous timing of North American grassland expansion and hypsodonty acquisition in Glires (i.e. rodents and lagomorphs) and ungulates (Jardine et al., 2012). Similarly, the increasing occurrence and degree of hypsodonty and hypselodonty (i.e. ever-growing teeth) in South American herbivorous mammals, from the middle Eocene (40 Ma) to the early Miocene (20 Ma), is coincident with a period of dry, open environments lacking grasslands but exposed to frequent volcanic ashfall (Strömberg et al., 2013; Dunn et al., 2015). Abiotic grit, in the form of dust or volcanic ash, likely drove hypsodonty adaptations and can potentially be identified in the microwear of fossil populations.

Abiotic grit consumption within grassland ecosystems may also be important for the interpretation of hominid paleoecology and craniodental evolution. High pit densities on extant primate molars and early hominids such as *Australopithecus africanus* have been largely associated with feeding on 'hard objects' (e.g. nuts and seeds) (Teaford and Walker, 1984), although high feature density characterized by plentiful small pits has also been attributed to abrasion from grit ingestion in a dusty habitat (Nystrom et al., 2004). Although the sheep in our study have different tooth morphology (e.g. selenodont versus bunodont) and jaw mechanics, our results suggest that grit can lead to increased pit density by *in vivo* compression loading and may offer support or revision to previous interpretations of hominid microwear.

MATERIALS AND METHODS
Feeding trials
This study was conducted in strict accordance with the guidelines proposed by the Institute for Laboratory Animal Research (National Research Council, 2011). The protocol was approved by the University of Wyoming's (UW) Institutional Animal Care and Use Committee (IACUC). All anesthetizations used isofluorane and all efforts were made to minimize suffering. We used four individual domestic sheep at the UW Red Buttes Environmental Biology Laboratory as test subjects in our controlled feeding trials using repeated measures. These sheep were subjects in a parallel, non-dietary study and were made available to our study for a short period of time (~1 month). All four sheep were female, approximately 2 years old, and obtained from Colorado livestock markets.

We were able to use a repeated measures research design as a result of overwriting of earlier microwear patterns by subsequent meals (i.e. the 'last

Table 6. Estimated soil in diets of wildlife and domesticated ungulates

Taxon (location)	% Soil (DMI)	Diet	Method	Reference
Bighorn sheep (AB, Canada)	10	Grazer	AIA	Skipworth (1974)
Sheep (UK; seasonal high)	>30	Grazer	AIA; Ti	Thornton (1974); Abrahams and Steigmajer (2003)
Sheep (NZ; seasonal high)	33	Grazer	AIA	Healy and Ludwig (1965a,b)
Sheep (NZ; yearly mean)	5–9	Grazer	AIA	Healy and Ludwig (1965a,b)
Cattle (ID, USA; summer)	14–20	Grazer	Ti	Mayland et al. (1977)
Cattle (MT, USA)	14	Grazer	Ti	Mayland et al. (1975)
Cattle (UK)	>10	Grazer	AIA	Thornton (1974)
Cattle (NZ; seasonal high)	32	Grazer	Ti	Healy (1968)
Bison (WY, USA)	6.8	Grazer	AIA	Beyer (1994)
Feral horses (OR, USA)	5	Grazer	Ti	Sneva et al. (1983)
Pronghorn (WY, USA)	5.4	Mixed	Ti	Arthur and Gates (1988)
Elk (WY, USA)	<2.0	Mixed	AIA	Beyer (1994)
White-tailed deer (MD, USA)	<2.0	Browser	AIA	Beyer (1994)
Mule deer (WY, USA)	<2.0	Browser	AIA	Beyer (1994)
Moose (WY, USA)	<2.0	Browser	AIA	Beyer (1994)
	<2.0	Browser	AIA	Beyer (1994)

DMI, dried matter intake; Ti, titanium concentration; AIA, acid-insoluble ash; NZ, New Zealand.

supper effect') (Grine, 1986). Increasing dietary abrasiveness is correlated with decreasing turnover time of features (i.e. the time over which microwear features are replaced). Sheep have high molar wear rates (5.57–10 µm day^{-1}) due to high dietary abrasiveness (Solounias et al., 1994). For sheep, the mean microwear scratch depth was 7.7 µm, meaning microwear scratches would be erased within 3 days of feeding (Solounias et al., 1994). Molar wear rates for ruminant browsers and mixed feeders are significantly lower: 0.91 µm day^{-1} and 2.54–3.87 µm day^{-1}, respectively (Solounias et al., 1994). A repeated measures design also allowed us to control for individual variation in chewing style and behavior.

The sheep were held in an open-air, mesh fence enclosure devoid of plant material. Plants within 2 ft (∼60 cm) of the fence were removed to prevent opportunistic feeding through the fence. Diet treatments (described below) were administered in a plastic-lined trough, which was cleaned between feedings. We were unable to control for the introduction of wind-blown dust and geophagy.

Over the course of approximately 1 month, we fed all individuals three treatments: (1) a mixture of locally grown Garrison and Brome hay (C$_3$ grasses) with an MSC of 5.75% (Table 1) by dry mass (control), (2) hay from the control treatment with added silica ranging in grain size from 180 to 250 µm (fine sand), and (3) hay treated with silica ranging in grain size from 250 to 425 µm (medium sand). Grain size classes were chosen in correspondence with the Wentworth (1922) grain size classification scheme for clastic sediments.

The Garrison and Brome grass hays were grown and processed at the UW Livestock Farm and used here as a proxy for natural grazing. Hay is an appropriate comparison for natural grazing and has been shown, in domestic sheep and goat (*Capra hircus*) populations in Denmark, to result in microwear counts that are not significantly different from those of grazing populations (Mainland, 1998). It is worth noting that grass hay does acquire grit during cultivation and processing, and local climate factors may correlate to grit acquisition (i.e. the wind and aridity of Wyoming may cause more grit to be acquired than in Danish hay). These climate factors, however, would presumably affect natural grazing in different regions in a similar manner.

We separated commercial sandblasting silica (30–60 grit) into two size classes (fine and medium sand) using mesh sieves. These size classes were chosen to represent realistic grit consumption and were large enough to avoid silicosis, a health hazard resulting from inhalation of particulate matter of diameters less than 15 µm (Quality of Urban Air Review Group, 1996). Our grit treatments were applied to the hay using a diluted corn syrup mixture to yield treatments that were ∼15.44% inorganic by dry mass (added abiotic silica was ∼10.28%), which is comparable to ingested soil estimates for natural grazers (Table 6). The corn syrup–sand mixture also compensated for potential palatability issues.

To confirm that the grit was being ingested by the sheep, we used sequential LOI analyses (Dean, 1974) of pre- and post-feeding trial fecal matter. We sampled fecal pellets produced and collected during anesthetization, with each sample composed of three fecal pellets. Sample dry mass ranged from ∼100 to ∼270 mg and was analyzed along with inorganic and organic standards that bracketed pellets in mass. Samples were dried at 105°C for 24 h, then combusted in a muffle furnace at 500°C for 4 h, and then roasted at 1000°C for 4 h. We cooled and weighed samples after each step. The final ash was devoid of organics (combusted at 500°C) and carbonates (combusted at 1000°C), and consisted primarily of silica. These masses were used to calculate the proportional content of silica (both abiotic and biogenic), carbonates and organics, relative to the dry mass of the scat. We also analyzed samples of the Garrison–Brome hay, the silica sand and hay rinsed in DI water (to remove grit and calculate biogenic silica).

We fed sheep each treatment (control, fine sand, medium sand) for 3 days before they were anesthetized (administering 7% isofluorane through a face mask) for tooth molding. The 3 day feeding period was based on estimated wear turnover rates of 1–3 days for ruminants (Solounias et al., 1994). Following each molding session (described below), the sheep were fed a control treatment of hay with no added sand for 5 days to 're-set' the occlusal surface and empty the rumen of silica from previous treatments. The latter is important as hay-fed sheep have been observed spending an average of 8.3 h day^{-1} ruminating (Domingue et al., 1991) and the mineral composition of cattle boluses is similar to diet with ∼88% of dietary silica returning to the

mouth during rumination (Little, 1975; Mayland and Lesperance, 1977). Furthermore, specific gravity of particles is inversely correlated to the particles' retention time in the ruminant digestive system (Poncet, 1991; Schettini et al., 1999). As particles with a specific gravity of less than 1.04 pass through cow digestive tracts within 72 h (Hristov et al., 2003), 5 days was sufficient for quartz grains (specific gravity=2.65) to pass through the sheep reticulo-rumen and avoid treatment overlap. The lined troughs were cleaned between feedings.

After we anesthetized the sheep, we removed the gas mask and gently used a toothbrush to remove food and debris from between teeth while taking care to avoid dragging the brush and debris across the occlusal surface. Kay and Covert (1983) reported the complication of the pellicle, a proteinaceous film on teeth that can obscure dental impressions, in their live feeding trials with opossums. An enzyme can be applied to remove the pellicle; however, abrasives also obliterate the film. We therefore molded the teeth within a few hours of the most recent feeding to avoid the re-growth of the pellicle. After cleaning, we took two molds of the cheek teeth using Sultan Genie$^©$ regular body polyvinylsiloxane dental impression material administered in a fitted mouth plate. For each mold, the sheep's mouth was held open by a researcher while another researcher held the molding plate in place against the upper cheek teeth. The first mold was used as an additional cleaning step to remove any remaining debris. The second mold was cast for microwear analysis.

We made a total of 12 molds but, because of the difficulties of the live molding process (e.g. movement of the molding plate, possible pellicle re-growth), rejected three. There was one defective mold for each round of treatments (the molds for the control and fine sand treatments for sheep 3 and the medium sand treatment for sheep 4 were excluded). For the nine remaining treatments, first or second upper molars were cast using Epo-Tek$^©$ epoxy and resin material in a vacuum chamber to remove bubbles (Nielsen and Maiboe, 2000).

For comparison with naturally feeding sheep, we also made tooth molds of the upper second molars from the skulls of four modern sheep from the UW Comparative Osteology Museum (UWA) and UW Collection of Fossil Vertebrates (UW). These sheep fed in different areas in Wyoming, including the Red Buttes pastures (UW 50590), Big Horn Basin of Washakie County (UWA 8158B), Red Desert of Carbon County (UWA 9272B) and along the Green River in Sweetwater County (UWA 345B). Cleaning and molding procedures followed Solounias and Semprebon (2002) and casts were made using the method described above.

Microwear data collection and analysis

We photographed tooth casts using a SPOT Flex digital camera mounted onto an Olympus$^©$ SZX10 stereomicroscope at low magnification (32×) (as per Solounias and Semprebon, 2002). We took all pictures along the anterior half of the buccal enamel band of the shearing facet (Fig. 2) at a resolution of approximately 0.866 pixels µm^{-1}. We used High Dynamic Range (HDR) imaging to enhance the visibility of microwear features in the photographs as per Fraser et al. (2009). Images were tone mapped using Photomatix Pro 3$^©$. For a detailed description of photographic methodology, see Fraser et al. (2009).

For each cast, we selected at least two distinct 0.4 mm^2 search fields and cropped them from digital microwear images using Adobe Photoshop CS5$^©$ software. Each search image was sized at 365×365 pixels (1.71×1.71 in, 4.34×4.34 cm) and was assigned a random identification number. Further, we created two additional sets of images by randomly rotating these fields (90, 180 or 270 deg clockwise) and re-ordering the set. We took these steps to: (1) create image anonymity and reduce the possibility of biased replicate counts; (2) compensate for potential variation in counts associated with an image's sequence within a counting session; and (3) test for bias between counts by the same individual, which are all symptoms of intra-observer bias, as discussed by Mihlbachler et al. (2012). While all microwear methods (i.e. LDM, SEM and texture analysis) suffer from inter-observer bias in absolute feature counts, LDM counts made by different observers are highly correlated and reflect consistent relative differences between specimens (Mihlbachler et al., 2012). For each set of images, two observers (J.M.H. and D.F.) independently marked and tallied microwear counts on each image using ImageJ$^©$ software. We recorded four quantitative variables for each image: total scratch (S) and total pit (P) counts for each observer (O1, O2). Pits were identified as circular to subcircular features (length:width≈1) with distinct borders, with smaller

pits brighter than larger pits. Scratches were identified as elongate features. (length:width>1.0). We also qualitatively compared the features to a 25 µm scale bar to assess the correlation between particle and feature size.

For statistical analyses, the mean counts for each image were categorized by observer (e.g. O1S) to quantify the observer bias. Statistical analyses were performed using R 2.15.3 statistical software (R Development Core Team, 2014). We applied Shapiro–Wilk tests to each variable to test for deviations from normality and the counts for each image were condensed into four principal components (PC1, PC2, PC3 and PC4) using principal component analysis. The extracted principal components were then used in ANOVA to test for differences among treatments (control, fine sand and medium sand) and among animal subjects to assess individual variation. We conducted Tukey honest significant difference tests to identify differences among individual and treatment means (α=0.05).

Conclusions

We present the first application of live animal tooth molding for studying microwear on ruminant teeth. Live animal tooth molding enables repeated measures design as well as decreased cost and number of required test subjects. The level of control offered by our method allows future study of various diets, the effects of different abrasives and inter-individual variation. Further, our method allowed us to quantify the contribution of inter-observer bias to the variation in our dataset as well as to find consistent relative differences among treatments.

Finally, we present the first *in vivo* experimental test of the effects of exogenous grit ingestion and of differently sized abrasives on microwear in ruminants. While the shapes of microwear features are likely controlled by jaw movement, our controlled feeding trials show considerable evidence for a 'grit effect' on feature abundance that is influenced by grain size and subsequently fracture stress. More work is needed to evaluate the effects of smaller abiotic particles (<180 µm) and quantify the role of jaw movement in determining feature shapes.

Author contributions

J.M.H. and M.T.C. conceived and designed the feeding trial experiments and the method of silica application to the diet treatments. J.M.H. performed the feeding trials, LOI data collection, and molded and cast the sheep teeth. Tooth casts were photographed by J.M.H. and analyzed by J.M.H. and D.F. Data analysis was led by J.M.H. and D.F. with contributions from M.T.C. All authors contributed to the interpretations of results. J.M.H. drafted the manuscript with substantial contributions from D.F. and M.T.C.

Supplementary material

Supplementary material available online at
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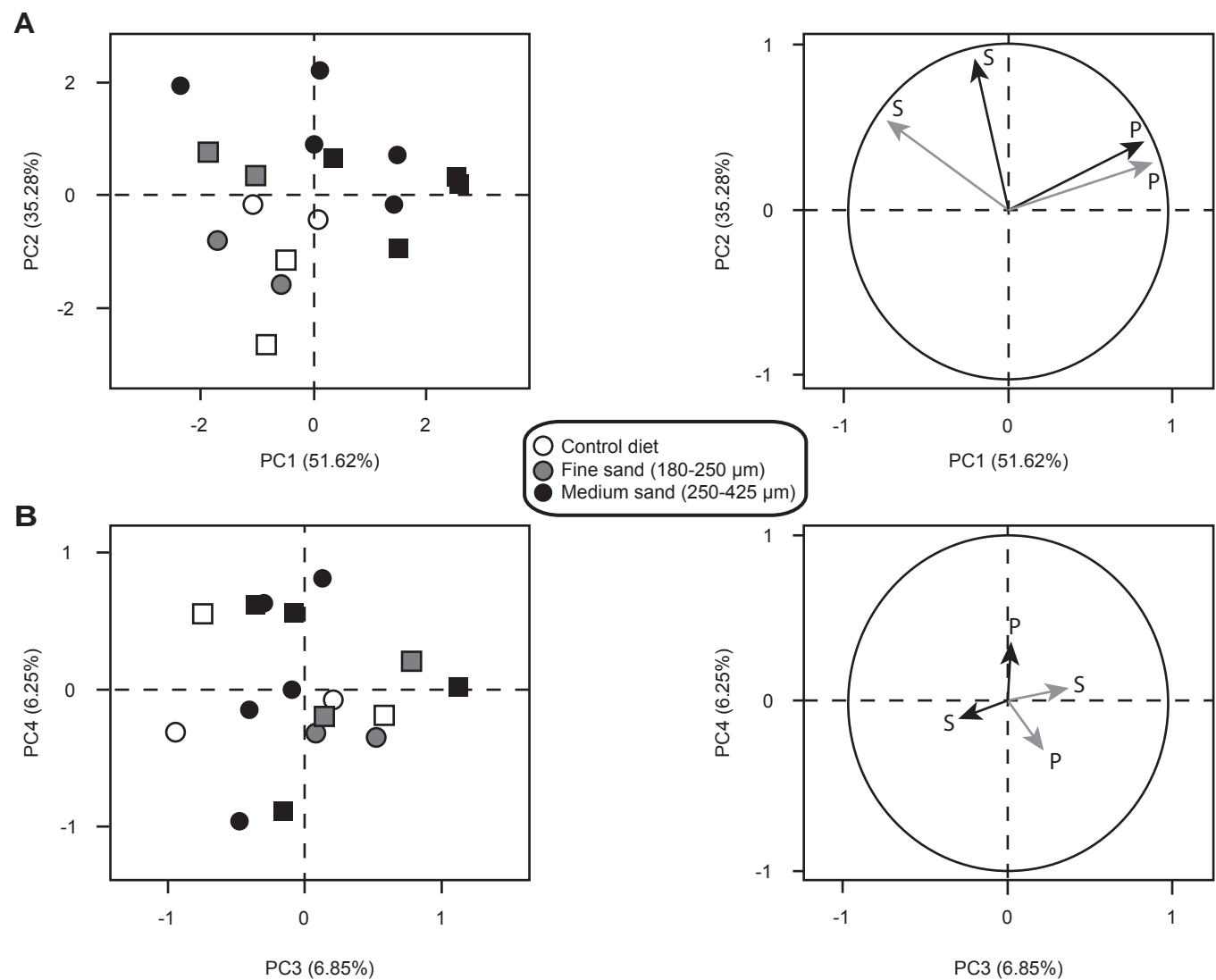
Figure S1. Bivariate plots and eigenvectors showing variation among diets (control, fine sand, and medium sand) for sheep 1 and 2. A) Principal components 1 (pits) and 2 (scratches) and B) principal compents 3 and 4 (observer bias). Black arrows=Observer 1; grey arrows=Observer 2; S=total scratches; P=total pits; different shapes represent different subjects; different colors represent different diets.

Table S1. Analysis of Variance results for extracted principal components 1 and 2 from sheep 1 and sheep 2 with Tukey comparisons for assessing differences among diet treatments.

Principal Component 1 - Pits (O1P+O2P)						Principal Component 2 - Scratches (O1S+O2S)					
Source	df	SS	MS	F-ratio	P	Source	df	SS	MS	F-ratio	P
Diet	2	4,391	2195	9.035	0.003	Diet	2	141.2	70.60	0.963	0.406
Residual	14	3,402	243			Residual	14	1,026	73.28		

Tukey Multiple Comparisons of Means	P adj.	Tukey Multiple Comparisons of Means	P adj.
Control-Fine	0.982	Control-Fine	0.990
Control-Medium	0.013	Control-Medium	0.567
Fine-Medium	0.009	Fine-Medium	0.474

Control=control diet (hay with no added silica); Fine=fine-grained sand treatment (180-250 μm); Medium=medium-grained sand treatment (250-425 μm).