



Similar Associations of Tooth Microwear and Morphology Indicate Similar Diet across Marsupial and Placental Mammals

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Abstract

Low-magnification microwear techniques have been used effectively to infer diets within many unrelated mammalian orders, but the extent to which patterns are comparable among such different groups, including long extinct mammal lineages, is unknown. Microwear patterns between ecologically equivalent placental and marsupial mammals are found to be statistically indistinguishable, indicating that microwear can be used to infer diet across the mammals. Microwear data were compared to body size and molar shearing crest length in order to develop a system to distinguish the diet of mammals. Insectivores and carnivores were difficult to distinguish from herbivores using microwear alone, but combining microwear data with body size estimates and tooth morphology provides robust dietary inferences. This approach is a powerful tool for dietary assessment of fossils from extinct lineages and from museum specimens of living species where field study would be difficult owing to the animal's behavior, habitat, or conservation status.

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Introduction

Mammalian teeth, being both obviously relevant to feeding and well preserved in the fossil record, have been the focus of dietary reconstructions for generations [1] and the emerging field of dental ecology [2] takes advantage of the varied tools teeth provide for dietary analyses. One of the most frequently used techniques in recent years has been the quantification of microwear damage incurred by tooth enamel surfaces during mastication. Although the mechanisms involved in tooth wear are complex, microwear is thought to be correlated to the abrasiveness and physical properties of an animal's diet [3–8]. Wear patterns are constantly overwritten and probably reflect dietary habits over a relatively short period of time; this has been estimated at days to weeks for features observable by SEM, depending upon the region of the tooth observed and the abrasiveness of the diet [9]. As such, microwear has been used to track dietary changes in mammals over seasonal as well as paleontological time scales. Such analyses have generally been restricted to comparisons within a single clade, including primates [2,10–16], xenarthrans [17], mammoths [18,19], carnivores [20–23], ungulates [24–29], rodents [30–33], fish [34], bats [23,35] and macropod marsupials [36]. In such studies within lineages, animals with unknown diets can be compared to closely related taxa whose diets are better understood (via gut contents or controlled feeding experiments, e.g. [7,37,38]). The extent to which microwear comparisons might be suitable between more distantly related mammalian lineages, including fossils without close living relatives, has not been assessed.

Microwear damage to teeth is related to the incidental ingestion of hard particles (e.g. phytoliths, bone fragments, and especially exogenous silica) during mastication [22,39–43]. In mammals, different microwear patterns occur because different types of chewing strokes are associated with different diets. Mammals emphasizing compressive chewing, most effective for the processing of hard, brittle foods (nuts, seeds, bone) via crack propagation have proportionally more pits comprising their microwear patterns than mammals emphasizing the grinding or shearing associated with tough or ductile foods (e.g. leaves, grass, flesh), which tend to have microwear signatures dominated by striations rather than pits (e.g. [44–51]). Early microwear studies took advantage of the different proportions of striations (shearing related damage) vs. pitting (crushing related damage) on the teeth of mammals with different diets, quantifying these features from SEM images (e.g. [52–54]). Generally, a higher proportion of scratches relative to pits is interpreted as being reflective of consumption of tough foods, with the reverse reflecting brittle food consumption [55–57]. The time and expense involved in these analyses led to the development of low-magnification light microscopy techniques (LDM) [28], which follow similar methods to SEM but allow quick and low-cost analysis; damage feature frequencies are counted in fields of view ranging from 0.01 mm² [30] to 0.4 mm² [28] depending upon the size of the animal.

Both SEM and LDM techniques rely on observer measurements and therefore direct comparison of results between different methodologies and users can be difficult. Inter-observer error rates have been estimated at 5–6% in users counting from an SEM

image [58,59]. LDM estimates put absolute feature frequency error rates at about 9%, although subsequent statistical testing showed highly significant differences between the assigned dietary categories even when inter-observer error is included in the analysis [60]. Nevertheless, concerns about repeatability between observers led to the development of microwear techniques focusing on the measurement of features from a photograph [61,62]. Achieving uniformly adequate image quality can be limiting [63,64], however, particularly when a wide diversity of tooth morphologies and sizes are involved, often requiring fine adjustments to focus across a field of view. Error rates associated with analyzing LDM photos range from 45% in inexperienced users to 8% in experienced individuals [64].

3D dental microwear texture analysis (DMTA) techniques have been developed to alleviate inter-observer bias by automating the recognition of microwear features [21,65–67], and have been shown to discriminate diets successfully in a variety of mammalian lineages. DMTA employs scale-sensitive fractal analysis (SSFA) or International Organization for Standardization three-dimensional surface texture parameters (ISO 25178-2) derived from surface elevation data. Dietary discrimination is based on multiple parameters related to the topography of the surface, as opposed to the two variables generally measured in SEM and LDM analyses (scratches and pits, although distinguishing of different size classes of pits and scratches may amplify the number of variables). DMTA has shown the potential to have more discriminatory power than 2D methods when assigning diet, especially within a single lineage [35,68], and even to reveal inter-individual dietary differences within a single species [69]. However, the much smaller tooth area sampled with this technique can lead to variability between analyzed patches (e.g. Phase I and Phase II wear facets in primates [70]). While useful if intra-tooth variation is the object of study, this also necessitates great care and uniformity in choosing regions of the tooth to analyze if dietary comparisons between taxa are required, and perhaps limits comparisons between more distantly related lineages with very different tooth architectures.

Different microwear methodologies have different strengths and weaknesses and all have value depending upon the scientific questions being addressed. The goal of this study is to develop a suite of techniques useful for the assignment of very broad dietary categories across the mammals, irrespective of the animal's lineage. LDM microscopy (direct counting through the lens) was chosen as the microwear methodology most suitable for this study due to the high throughput of samples required and the wide availability of LDM to researchers in a variety of fields. Although training in the method is required, no equipment is needed other than a stereomicroscope. LDM studies analyze a larger proportion of the total tooth surface than do DMTA techniques, giving more representative coverage of the tooth and making comparisons across a wide variety of mammalian lineages more feasible. While recognizing that this method allows differentiation of coarser dietary bins than does DMTA, LDM has been shown in numerous studies to discriminate reliably between broad herbivorous dietary categories (i.e. frugivore, grazer, browser) [60] as well as bone- vs. flesh-consuming carnivores [68].

A study using LDM to distinguish a broader range of dietary categories (i.e. grazer, browser, carnivore, insectivore) in the same analysis, however, has not yet been undertaken and complications arise when considering different types of foods that possess similar physical properties. For example, flesh-consuming carnivores and browsing ungulates have been found in some cases to have overlapping microwear patterns [71]: both eat tough or ductile foods that require a shearing motion for breakdown and that

incorporate relatively little grit. Complications stemming from the relatively coarse discriminatory power of LDM microwear analysis, such as the difficulty of identifying specific food items with similar fracture modes, demonstrate that additional lines of evidence are required when developing a proxy for the determination of diet in a mammal with a truly unknown biology.

The two main strategies for oral processing (shearing and crushing) are also reflected in tooth shape [51,72–78]. Mammals with diets requiring vertical crushing for breakdown have molars characterized by low, rounded cusps and few enamel ridges. A diet composed of tough foods, by contrast, requires transverse shearing movements and fosters the evolution of molars with ridges of enamel (shearing crests) that come into contact between upper and lower teeth during mastication to serve as cutting surfaces [73–78]. Mammals emphasize shearing vs. crushing surfaces on their cheek teeth (relative to other members of the same clade) depending on diet, shown in a number of studies of living and fossil mammals [78–84]. Even foods with similar physical properties can in some cases be associated with distinct tooth morphologies. Both leaf and meat eaters require shearing forces to process their food, however reduction of ingested foods to small particle size is of particular importance for herbivores [85,86] due to the difficulties involved in breaking down plant cell walls. Thus, herbivore and carnivore teeth are distinct, with more and/or longer shearing crests typically present in herbivores relative to carnivores of similar sizes. Since tooth shape reflects selection for efficient processing of a particular type of diet over evolutionary timescales versus the microwear damage directly caused by foods recently consumed by the individual, tooth shape and microwear can provide independent sources of complementary information. This increases the discriminatory power of dietary analysis and can also reveal cases in which microwear and morphological data are seemingly non-correlated, providing additional information about an animal's ecology (e.g. [87]). An adaptation of a combined microwear/morphological technique to comparison of dietary information across different mammalian clades, however, has not yet been undertaken.

Body size—as can be estimated from molar tooth size [88,89]—also places important constraints on diet. Whereas carnivores, insectivores, and hard-object feeders (fruits, nuts, seeds) can rely on “auto-enzymatic” digestion, molecular breakdown by enzymes produced by the animal itself, herbivores eating high fiber plant matter require bacterial symbionts to break down plant cellulose (e.g. [90–93]). Thus, the lower quality of high-fiber plant matter (relative to meat, fruit, or seeds) requires either a long residence time in the gut to increase the digestive yield or a high throughput at low yield. Both of these alternatives would be limited by small body size, with the minimum estimated to be at about 500 g for extant mammals subsisting entirely on leaves (e.g. [94,95]). Below this threshold, increasing degrees of omnivory are required. Thus, body mass can constrain ecology and also be a useful indicator of an animal's diet in addition to the tooth characteristics discussed above.

The goal of this study is to develop a widely applicable and widely available analytical protocol for the assignment of diet in mammals by augmenting LDM microwear with additional dietary metrics. Like microwear, neither body mass nor tooth morphology is diagnostic of diet when standing alone, but each can narrow the pool of potential dietary guilds to which a mammal might belong. Analytical techniques individually evaluating LDM microwear, body mass, and tooth morphology have been used effectively in numerous dietary studies, however these analyses are typically limited to comparisons within a single extant mammalian clade, and rarely used in combination with one another. It is unclear how

consistent microwear patterns and shearing crest lengths should be in more distantly related lineages with similar diets but different jaw mechanics, and whether microwear and morphological analyses should be trusted in extinct lineages without close extant relatives. To fill this gap in current knowledge, an analytical protocol is developed for the assignment of broad dietary categories (grazing, browsing, hard-object feeding, insectivory, bone-dominated carnivory, flesh-dominated carnivory) in mammals using the combined information available from body mass, microwear, and tooth morphology. The extent to which these variables may be used as predictors of ecological niche in phylogenetically divergent lineages is tested by taking advantage of the frequent convergence in diet between extant placental (eutherian) and marsupial (metatherian) mammals despite at least 100 million years of separate evolution [96].

Methods

Sampling Strategy

All of the animal species chosen for analysis were dietary specialists: browsers (leaves of trees and shrubs), grazers (grass and forbs), hard-object feeders (fruits, nuts, seeds), insectivores (cuticle-bearing), bone carnivores, or flesh carnivores. Dietary information was taken from the literature [97], based on observations in the wild; all specimens were wild-shot. Specialized feeders on a single food type minimize the number of unknown variables affecting the observed tooth morphology and microwear.

Casts of tooth crowns were made on-site at the Field Museum of Natural History (Chicago, IL) and the American Museum of Natural History (New York, NY). Following standard techniques [60], 3M ESPE Express vinylpolysiloxane molding compound (light body, regular set) and clear Buehler Epo-Kwik epoxy resin casting material were used to replicate tooth crowns. The second molar is generally preferred for morphological and microwear analyses in most animals due to its intermediate degree of wear

(less than M1, more than M3). However, the molars are often reduced or absent in carnivores where instead the carnassials (equivalent to the upper P4 in extant carnivorans) are modified for food processing. Thus, the upper left second molar was sampled for all taxa except carnivores, for which the upper left carnassial was substituted, following literature convention [20,43,98]. In all, 153 extant species belonging to 9 orders were analyzed, including 111 placental and 42 marsupial species (see Table S1 in Supporting Information S1 for complete list). A maximum of eight individuals were sampled from each species, resulting in an overall sample of 247 eutherian and 146 metatherian teeth.

Tooth measurements

Morphometric analysis followed modifications of previous techniques [83,99]. Tooth casts were imaged with a flatbed scanner in occlusal view, except in the case of teeth too large to be fully cast, which were instead photographed *in situ* in occlusal view. Tooth length and width were measured in ImageJ (available at <http://rsbweb.nih.gov/ij/>), as was the total length of the shearing crests (Figure 1). Analysis of the three-dimensional crests from a two-dimensional image will underestimate their total length, but two dimensions are nonetheless adequate to distinguish ecological guilds, as demonstrated below. The measured shearing crest length was then divided by the square root of the molar crown area (length×width) to calculate the Shearing Crest Score (SCS) (see Table S2 in Supporting Information S1 for complete taxon list). This new variable was developed because the more established Shearing Quotient (crest length divided by molar length) [99,100] was developed for use only with lower teeth. Because opposing molars are generally the same size, taking the square root of the crown area provides a linear unit of measure that is independent of shape. As one of the main goals of this study is to develop a proxy for use in the mammalian fossil record, which is composed largely of isolated teeth and in which sample size is usually an issue, a technique that would be applicable to both

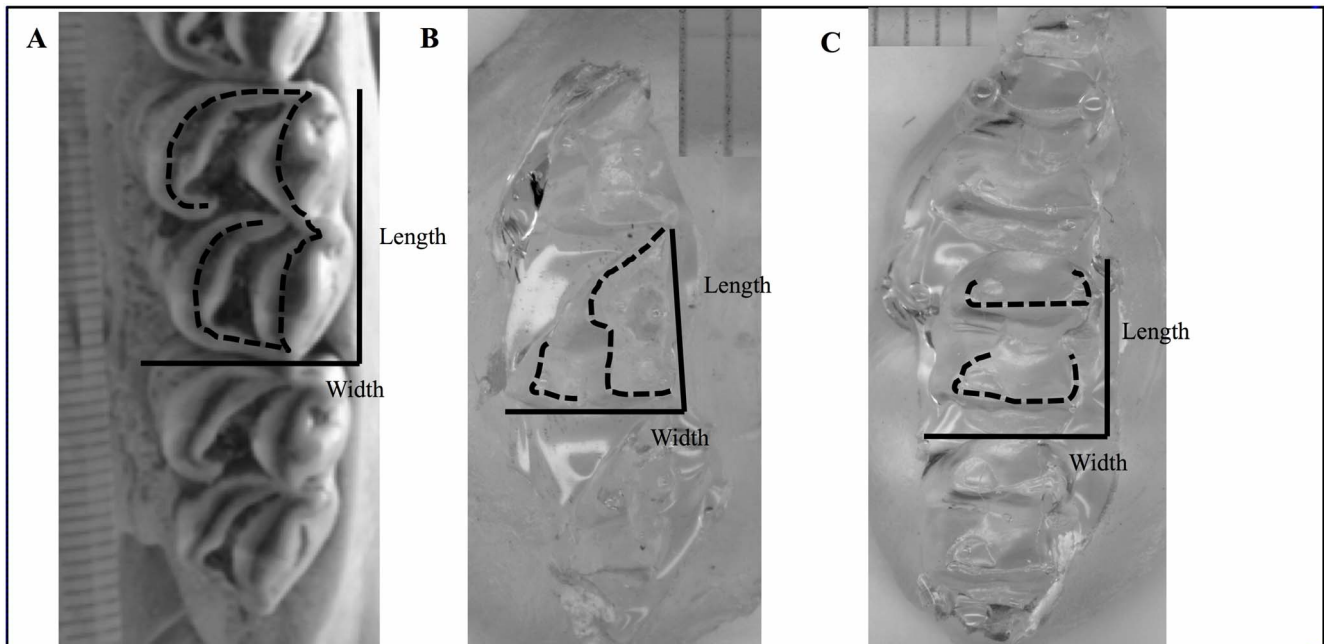


Figure 1. Variables measured for morphometric analysis. Length, width, and total shearing crest length were measured on upper second molars. Morphology varies with phylogeny; examples shown here are A) *Rangifer tarandus* (tooth *in situ*), B) *Perameles nasuta* (tooth cast), and C) *Dorcopsis hageni* (tooth cast). Tic marks are 1 mm. doi:10.1371/journal.pone.0102789.g001

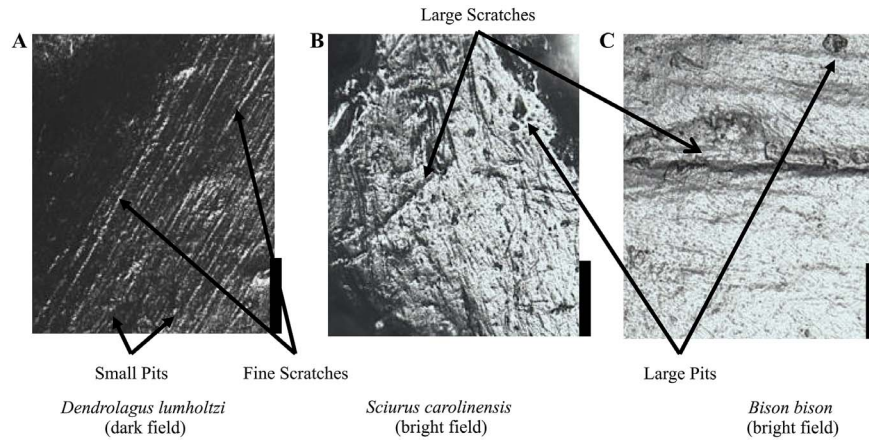


Figure 2. Microwear features tallied during analysis. Large features show up dark on a light field (A); small scratches and small pits show up light on a dark field. (B and C) Dark field versus bright field illumination is achieved by adjusting the light source angle (see also Figure S1 in Supporting Information S1). Scale bars are 0.5 mm.
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upper and lower molars is preferable. Average species body masses were taken from the literature [101]; the published mass is the average between males and females in cases with noticeable dimorphism.

Dental microwear analysis was carried out with low magnification light microscopy, following previously developed protocols [28,60] modified to accommodate a wider range of tooth sizes. Features were counted in a 0.04 mm² reticle grid at 70x (a 3 mm diameter field of view) on an Olympus SZX16 stereomicroscope. Microwear features tallied included number of small pits (P_s), number of large pits (P_l), number of fine scratches (S_f), and number of coarse scratches (S_c) (Figure 2). The total number of scratches (S_t) and total number of pits (P_t) were calculated by adding the two size classes together. Adjustment of the unidirectional light source provided a qualitative measure of feature depth:

small features appear light on a dark background, while large features appear dark on a light background (see Section 3 and Figure S1 in Supporting Information S1 for details or [60] for a more detailed review). Each measurement consisted of light and dark background counts on the same area done back-to-back in order to ensure that identical areas were counted and to prevent double-counting features.

Most molar measurements were made on the M2 protocone, a shared morphological character among all mammals studied and a different area than is generally analyzed in LDM studies. Because of the great variety of tooth morphologies and chewing mechanisms encompassed by the animals in this study, choosing a single Phase I or Phase II facet for analysis (as is commonly done in primates [70]) was impossible. Where permitted by tooth size, up to four measurements were taken on each tooth (within a single

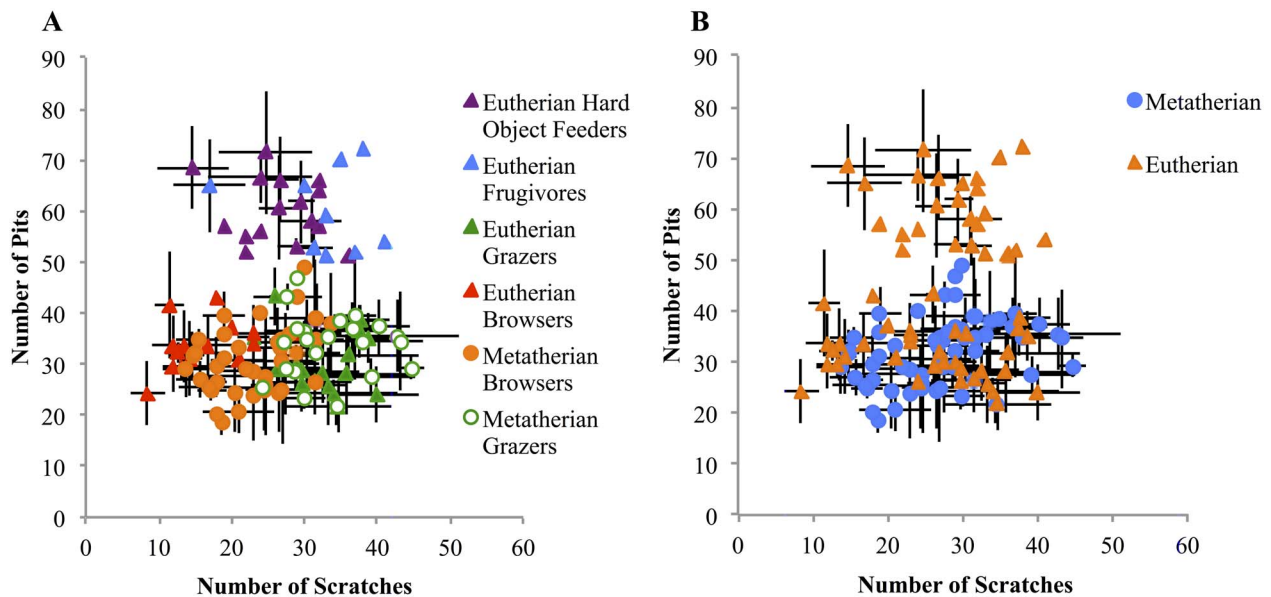


Figure 3. Microwear patterns of marsupial and placental herbivores plotted according to feeding guild (A) and phylogeny (B). Note, the partial separation between marsupials and placentals in B is only based upon the absence of obligate hard object feeders (and, thus, of elevated pit counts) among marsupials in the data set. The three dietary groupings in A are significant ($p < 0.01$). Marsupial and placental herbivores of comparable guilds are statistically indistinguishable ($p = 0.84$).
doi:10.1371/journal.pone.0102789.g003

Table 1. Microwear differences between feeding guilds.

Variable	F	d.f.	p	LSD Post-Hoc Results: p-values											
				C-HO	C-B	C-G	C-I	HO-B	HO-G	HO-I	B-G	B-I	G-I		
Microwear: Scratches	12.85	4	<0.001	0.011	0.868	<0.001	<0.001	0.003	0.061	0.081	<0.001	<0.001	<0.001	<0.001	0.869
	27.26	4	<0.001	0.005	<0.001	0.019	0.285	<0.001	0.425	0.023	<0.001	<0.001	<0.001	<0.001	0.099
	28.40	4	<0.001	<0.001	0.133	<0.001	<0.001	<0.001	0.185	0.615	<0.001	<0.001	<0.001	<0.001	0.353
Microwear: Pits	52.06	4	<0.001	0.001	<0.001	<0.001	0.672	<0.001	<0.001	0.001	0.243	<0.001	<0.001	<0.001	<0.001
	10.60	4	<0.001	0.002	0.145	0.07	0.691	<0.001	<0.001	0.001	0.608	0.016	0.005	0.005	0.005
	49.41	4	<0.001	<0.001	<0.001	<0.001	0.754	<0.001	<0.001	<0.001	0.568	<0.001	<0.001	<0.001	<0.001
Body Mass	49.35	4	<0.001	<0.001	0.156	0.662	<0.001	<0.001	<0.001	0.037	0.005	<0.001	<0.001	<0.001	<0.001
Shearing Crest Score	12.73	4	<0.001	<0.001	<0.001	<0.001	0.063	<0.001	0.495	0.894	0.016	0.045	0.016	0.045	0.572

Statistical results of univariate ANOVA tests for all variables. LSD post-hoc tests reveal significant pairings between dietary group cases. doi:10.1371/journal.pone.0102789.t001

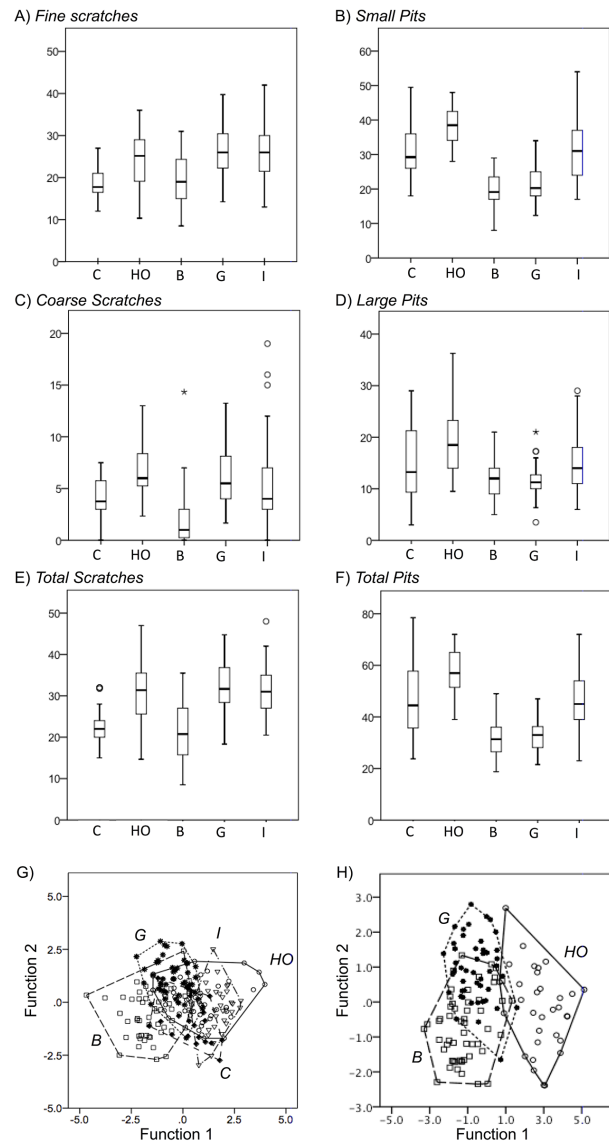


Figure 4. The relative frequencies of microwear variables within feeding guilds. A) Fine scratches (S_f), B) Small Pits (P_s), C) Coarse Scratches (S_c), D) Large pits (P_l), E) Total scratches, F) Total pits. Box plots show the median (center line), interquartile range (boxes), 1.5 times the interquartile range (whiskers), and outlier points. Discriminant analysis using the four independent variables (S_f , S_c , P_s , P_l) as dependents is depicted: G) all five feeding guilds included. Functions 1 and 2 are plotted and account for 71.8% and 24.6% of the variance, respectively. Wilks' Lambda = 0.252, Chi-Square = 250.51, $p < 0.001$. The discriminant function coefficients are S_f 0.039/0.578, S_c 0.499/0.688, P_s 0.845/-0.416, P_l 0.071/-0.166. F) Herbivores groups only (grazers, browsers, hard-object feeders). Functions 1 and 2 are plotted and account for 75.1% and 24.9% of the variance, respectively; Wilks' Lambda = 0.194, Chi-Square = 192.80, $p < 0.001$. The discriminant function coefficients are S_f 0.005/0.379, S_c 0.324/0.844, P_s 0.822/-0.330, P_l 0.270/-0.276. HO = Hard-Object Feeders, B = Browsers, G = Grazers, I = Insectivores, C = Carnivores. Hulls surround each dietary group. doi:10.1371/journal.pone.0102789.g004

wear facet) in order to calculate intra-tooth variation. All measurements included were made by the author over a period of three months, eliminating inter-observer bias and minimizing intra-observer variability over time.

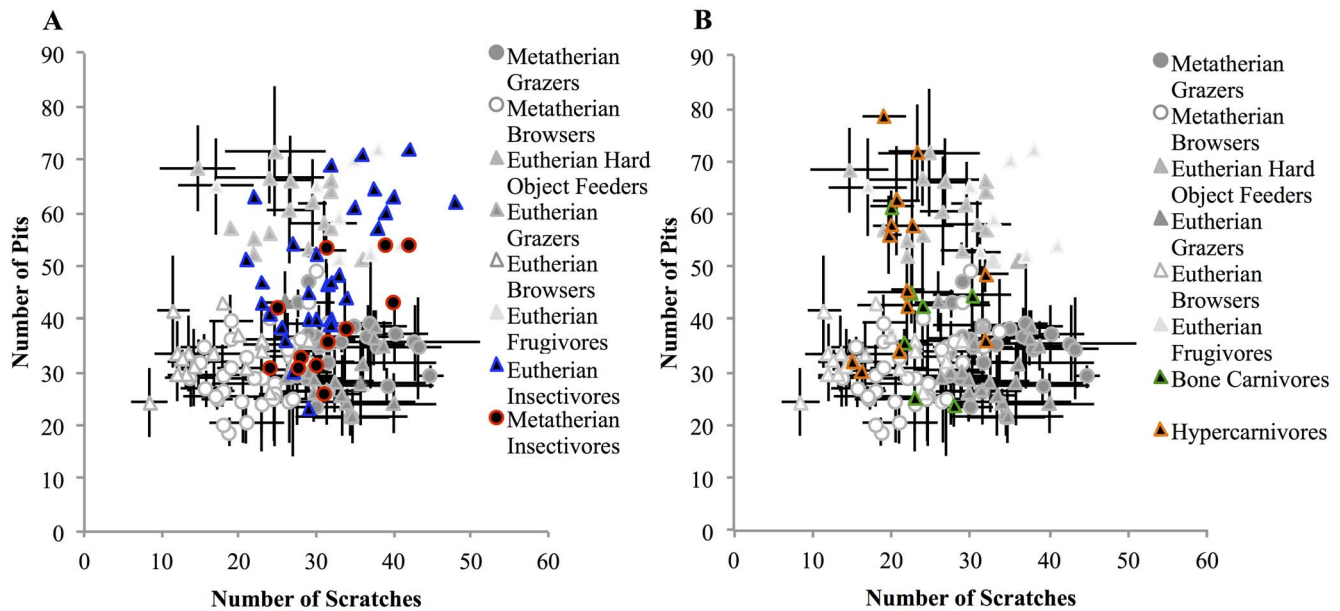


Figure 5. Microwear results of insectivores (A) and carnivores (B) plotted with herbivorous taxa. Marsupial and placental insectivore groups are not significantly different ($p = 0.65$), nor are hypercarnivores (flesh eaters) and bone carnivores (chew and consume bone) (see Table S5 in Supporting Information S1 for details). Regardless of feeding preferences, both flesh and bone specialists consume both types of food, depending on availability, the season, the animal’s status in social groups, and other factors, resulting in a lack of further differentiation. doi:10.1371/journal.pone.0102789.g005

Data analysis

Statistical analyses were carried out in SPSS v. 22.0.0 (SPSS Inc., Chicago, US). All count data were log-transformed to achieve homoscedasticity (evaluated with residual linear regression plots) and normality (evaluated with Shapiro-Wilk tests) prior to analysis. An independent-samples t-test (comparing two groups) or a univariate ANOVA (more than two groups) was coupled with a post-hoc least significant difference (LSD) test to determine the significance of individual variable pairings. To test for the combined influence of multiple variables, nested ANOVA (two variables) and Linear Discriminant Analysis (LDA) (more than two variables) were then performed using variables found to have discriminating power between dietary guilds. Post-hoc leave-one-out classification results were employed with LDA to evaluate the degree to which the resulting clusters could be distinguished. The significance of LDA was assessed using Wilks’ Lambda. Statistical tests were performed on both the global data set, which includes all sampled teeth, as well as a set comprised only of species with three or more individuals represented (referred to hereafter as the “limited data set”). The limited data set serves as a check on microwear variability sourcing from species represented by limited numbers of individuals, and yielded the same results as the global data set for all statistical tests performed (for details, see Section 4 (Table S3 and S4 in Supporting Information S1)); thus, the results presented below represent the more conservative global data set.

Results

Plotting the total pits vs. total scratches for three herbivorous guilds (browsers, grazers, and hard-object feeders) resulted in the “dietary triangle” typically observed in LDM analyses [60] (Figure 3). Grazers form the lower right corner, with many scratches but few pits; leaf browsers have a similar, slightly more variable number of pits and fewer scratches. The apex is formed by the hard-object feeders, which have many more pits than either grazers or browsers. These three groups, based on total pit and

scratch counts, are significant (Nested ANOVA, total pit and scratch counts as dependent data, type of measurement and diet as nested factors, Grazers:browsers $p = 0.022$, browsers:hard-object $p < 0.0001$, grazers:hard-object $p = 0.004$; limited data set, Grazers:browsers $p < 0.0001$, browsers:hard-object $p < 0.0001$, grazers:hard-object $p < 0.0001$). Obligate hard-object feeding or frugivory is extremely uncommon among Australidelphids, so this dietary habit could not be directly compared. Eutherian hard-object feeders were removed from analyses testing for differences based on phylogeny. A nested ANOVA (total pit and scratch counts dependent, type of measurement and lineage as nested factors) showed the metatherian and eutherian groups to be not significantly different ($p = 0.84$).

Adding the carnivore and insectivore guilds reveals overlap in feature frequency between animals with plant- and animal-based diets. Flesh- and bone-consuming carnivores were treated as a single group because no statistical difference could be found between the two using the LDM methods employed (see Table S5 in Supporting Information S1). Figure 4 shows box plots of all variables tallied during microwear analysis. Results of univariate ANOVA and post-hoc LSD tests for each variable are shown in Table 1. Carnivores and browsers generally have a lower frequency of scratches, S_f , S_c , and S_i . Pit frequencies, on the other hand, are generally higher in carnivores, hard-object feeders, and insectivores. The discriminatory power of LDM microwear alone as a diagnostic dietary feature begins to fail when considering animals feeding on items that can have similar physical properties, such as carnivores vs. browsers (LSD Post-Hoc, $S_{C-B} = 0.868$, $S_{I-C-B} = 0.133$, $P_{I-C-B} = 0.145$), or carnivores vs. insectivores (LSD Post-Hoc, $S_{C-I} = 0.285$, $P_{S-C-I} = 0.672$, $P_{I-C-F} = 0.691$, $P_{I-C-F} = 0.754$). Insectivory, carnivory, and hard-object feeding are particularly problematic because of the variation involved (Figure 5A, B); all rely on patchily distributed resources, and animals belonging to any of these guilds may encounter a variety of food items. LDA of the four microwear parameters (S_f , S_c , P_s , and P_l) reveals separation between the three herbivorous

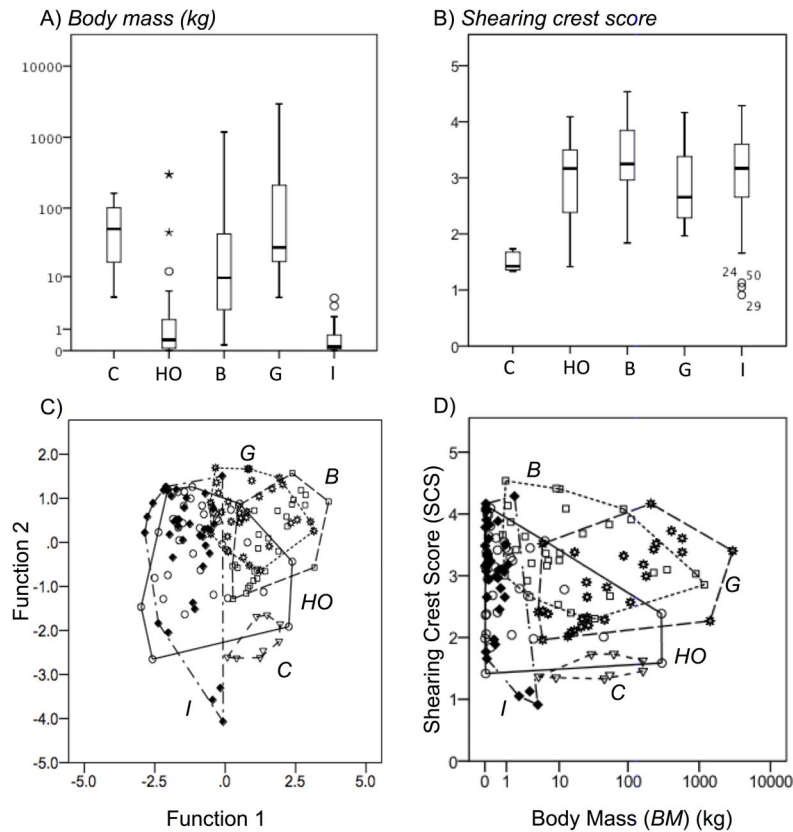


Figure 6. The relative frequencies of body mass and Shearing Crest Score (SCS) within feeding guilds. The distribution of A) body mass (BM) and B) Shearing Crest Score (SCS) among all specimens and feeding guilds is shown. Box plots show the median (center line), interquartile range (boxes), 1.5 times the interquartile range (whiskers), and outlier points. C) Discriminant function analysis using BM and SCS as dependent variables. HO=Hard-Object Feeders, B=Browsers, G=Grazers, I=Insectivores, C=Carnivores. Function 1 (75.2%) and Function 2 (24.8%) are largely synonymous with BM/P_s and SCS, respectively. Wilks' Lambda=0.686, Chi-Square=51.76, $p<0.001$. The discriminant function coefficients are $BM_{-0.157/0.989}$, $SCS_{0.994/0.116}$, $P_{s0.845/-0.416}$, $P_{i0.071/-0.166}$. D) Bivariate plot, BM vs. SCS, showing polygonal dietary morphospaces. Hulls delineate dietary groups.
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guilds in dietary space (Figure 4H). Canonical discriminant function 1 (75.1% of variance) is strongly correlated with small pits ($P_s = 0.892$), while function 2 (24.9% of variance) is strongly correlated with coarse scratches ($S_c = 0.798$). Hard-object feeders, which have a higher proportion of pits to scratches, have positive function 1 values while browsers and grazers tend to have more negative values. Grazers generally have a higher proportion of coarse to fine scratches (=positive values) than browsers do (negative values), although there is some overlap between these two guilds on function 2. The “dietary space” defined by this analysis is therefore hallmarked by increasing numbers of small pits on function 1 and increasing numbers of coarse scratches on function 2. This LDA test of the three herbivorous guilds resulted in defined clusters and a post-hoc leave-one-out correct assignment rate of 82.8% (limited data set 84.2%), however the same test including all five guilds had a correct assignment rate of only 57.2% (limited data set 57.1%) and substantial overlap between insectivores, carnivores and hard object feeding herbivores (Figure 4G). Because of this, reliable dietary assignment for an animal not known for sure to be an herbivore requires additional lines of evidence, as outlined below.

Consistent with previously recognized correlations [89,91,102,103], some aspects of diet may be inferred from body size alone. The minimum body size for obligate marsupial and placental browsers is ~500 g [94,95]. Obligate insectivores

require body sizes smaller than the threshold for browsers due to the size and patchiness of their food sources, with the exception of specialists feeding on colonial insects (i.e. anteaters and aardwolves, not included here due to reduced dentition). As a result, insectivore body mass is significantly different from all four of the other guilds (Table 1), allowing this group to be reliably distinguished (Figure 6A) despite the variability in its microwear patterns.

Calculating a Shearing Crest Score (SCS) for each individual by plotting total shearing crest length against the square root of first molar area (length×width) yielded significant differences between carnivores, hard-object feeders, and browsers/grazers of all orders, marsupials included (Table 1—see also Figure S2 in Supporting Information S1). Carnivores, relying on a maximum force applied to a short cutting blade (carnassial teeth) for prey subdual [45], have significantly shorter SCS than any of the other groups (Figure 6B). Plotting SCS and body mass against one another shows the five feeding guilds occupy polygonal morphospaces of varying overlap (Figure 6D). On the lowest end of the body mass spectrum there is a wide range of SCS scores, reflecting varied diets on the parts of these animals. The insectivores are distinct from high-fiber herbivores, although not from hard-object feeding herbivores: there is considerable overlap in fracture properties in foods used by mammals with small body masses (discussed above), resulting in teeth adapted for similar function in breaking them

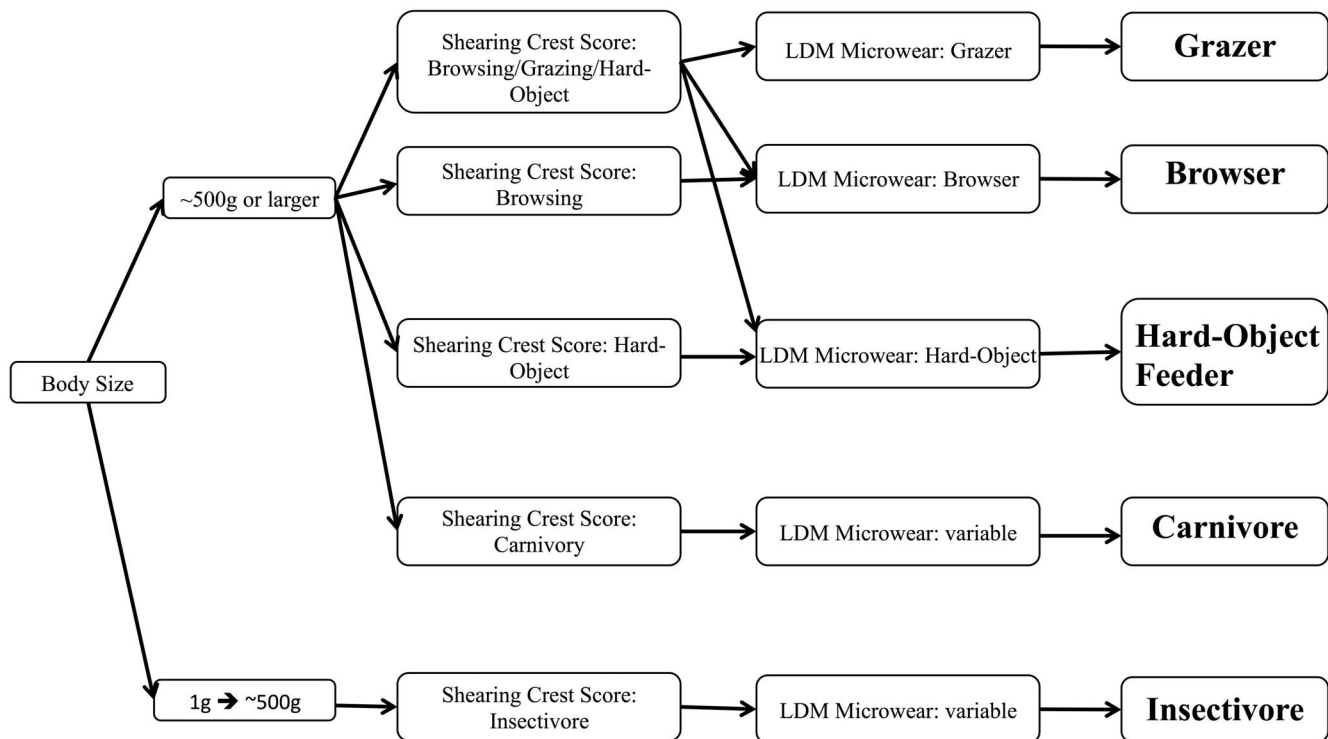


Figure 7. Flow chart representing the path taken toward the assignment of feeding guild using the three different analyses (body size, tooth morphology, LDM microwear) in succession.
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down. At larger sizes, there is a clear distinction between herbivores (which generally have much more complexly ridged teeth) and carnivores. The three main types of herbivores cannot be confidently distinguished from each other with SCS results, however herbivores, carnivores, and insectivores can be separated out, which is impossible using LDM microwear alone.

Each of the three independently derived lines of evidence (LDM microwear, SCS, and body mass) is informative in different ways for the assignment of diet among the five broad feeding guilds discussed here. None of these techniques on its own is able to discriminate reliably between all five, however the complementary strengths in discriminatory power from all three analytical methods can be taken into account (Figure 7). Body mass is key for certain physiological thresholds related to what food a mammal is able to digest. Once minimum body sizes are surpassed, SCS scores can distinguish carnivores and herbivores. Once herbivory has been indicated by SCS results, LDM microwear analysis can provide assignment to a more specific herbivorous niche.

The following of this algorithm results in correct assignment of dietary guild 92% of the time, determined by re-assigning diet to all specimens used in this study in the context of Figure 7. The uncertainty stems from the overlapping physiological and morphological parameters of insectivores and hard-object feeders, discussed above. These two groups have similar body masses (Figure 6A), and take advantage of patchy resources with similar physical properties (i.e. hard-shelled insects and seeds, both of which are hard and brittle) in an opportunistic fashion; many insectivores supplement their diets with seeds and vice versa. This makes obligate insectivores difficult to distinguish from obligate hard-object feeders of similar size.

Discussion

Low-magnification microscopy of dental microwear [25,28,60,104,105], relative shearing crest length [83,94,99,100,106–108], and body mass [90,94] estimates represent established techniques for the determination of diet in mammals. The results here indicate that, when combined, these characteristics can be used to infer mammalian diets across a remarkable range of phylogeny and animal form. Neither LDM microwear, shearing crest morphology, nor body mass is fully diagnostic as to feeding guild independently: LDM microwear can readily distinguish grazing, hard-object feeding, and browsing, while SCS in combination with body mass delineates herbivores, carnivores, and insectivores. When used in this workflow, however, the techniques outlined here are a powerful tool for the partitioning of dietary habits into the coarse categories evaluated here (Figure 7).

The specimens used in the analysis are drawn from a global sample of extant mammals, collected in different seasons and over several decades. Because microscopic damage records diet over a relatively short period of time [9], the results presented here are noisier than would be derived from sampling a single locality or a single season. That the outcome is nonetheless robust is testimony to the strength of the correlations described. Dietary similarities clearly trump individual morphologies and jaw mechanics, so that distantly related species specializing on a similar set of food objects have comparable shearing crest and microwear parameters.

The method presented here is a powerful tool for assigning a general dietary category to an animal with a truly unknown ecology. Specific dietary distinctions beyond the five categories discussed are outside the scope of this paper, although a variety of studies have used different microwear techniques such as DMTA to subdivide diet (generally within a single mammalian clade) on a

much finer scale, which can include looking for evidence of fallback foods and even inter-individual differences [27,65,69,109,110]. Further work would be needed to know whether the highly specific measurements used as evidence of extremely specific diets in these and other studies have the same universality as the basic scratch and pit counts used here. Such highly specific techniques could certainly be applied after use of the coarse assignment workflow described here to identify appropriate targets for future investigation with finer analyses like DMTA.

Having been tested using extant mammals with known diets and demonstrated to be successful across the mammalian phylogeny, this workflow is also suitable for studies on the evolution and ecology of extinct mammals as well as museum specimens of living species where field study would be difficult owing to the animal's behavior, habitat, or conservation status. Importantly for the wide application of this diagnostic approach, no significant difference was found between the LDM microwear patterns or SCS distributions of eutherians and metatherians belonging to the same feeding guilds. Marsupial and placental mammals, separated by more than 100 million years of evolution, have different chewing cycles and use their teeth in different ways [76,111–117]. Despite this marsupial and placental mammals belonging to the same dietary niche have statistically indistinguishable microwear and SCS—a remarkable example of the influence of food material properties on oral processing. Thus, evolutionary changes in the diets of the earliest mammals, the ecological selectivity of the end-

Cretaceous extinction, and the re-emergence of herbivory in the Paleocene recovery would all be appropriate targets for tooth-based dietary research. Current work is being directed toward the study of the post-Cretaceous mammalian diversification in the western North American interior [118].

Supporting Information

Supporting Information S1 Additional data tables and statistical analyses. This file contains specimen information and data used in the microwear/morphological analyses, as well as the results of statistical analysis of the limited data set. (DOCX)

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Author Contributions

Conceived and designed the experiments: HC. Performed the experiments: HC. Analyzed the data: HC. Contributed reagents/materials/analysis tools: HC. Wrote the paper: HC.

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