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Tooth microwear texture in odontocete whales: variation with tooth characteristics and implications for dietary analysis

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Abstract

Understanding the diets and trophic relationships of toothed whales is central to understanding their roles in marine ecosystems, and associated conservation issues. Yet this is problematic because direct observation of what free ranging marine mammals eat is difficult. Quantitative 3D textural analysis of tooth microwear (DMTA) offers a new way of investigating diet in odontocetes and other marine mammals, but the application of this approach requires that we first understand how non-dietary variables affect the texture of microwear in odontocetes. Here we present the first analysis of microwear texture in odontocetes (beluga, *Delphinapterus leucas*) testing null hypotheses that microwear texture does not vary with dental surface tissue type (dentine, cementum), and that microwear texture does not vary with tooth characteristics (location in jaw, degree of wear, wear facet slope and facet orientation). Our results reveal that these variables have a significant impact on microwear textures, and thus have the potential to mask variation in texture caused by dietary differences. This does not mean that microwear texture analysis cannot be used as a tool for dietary analysis in toothed whales, but any future studies should adopt sampling protocols that standardize non-dietary variables to mitigate their effects in DMTA analysis.

Keywords: Microwear; Texture; DMTA; Diet; Cetacean

1. Introduction

The toothed whales (Odontoceti) including dolphins and their relatives are important marine predators with significant impacts on marine food-webs. Diet and trophic relationships are key mediators of ecosystem functioning [e.g. 1] and are thus central to understanding the roles of odontocetes in marine ecosystems, their responses to environmental pressures, and associated conservation issues [2], including conflicts with human fishing activities [e.g. 3]. Yet determining odontocete diets is problematic because of the difficulties of direct observation of what free ranging marine mammals eat.

A range of indirect methods have been developed to estimate diet [4] but none is without issues. Stomach contents analysis [5,6] captures diet only over a short period, typically 24 to 48 h [7], and analyses can be biased by differences in the relative resistance to digestion of organic and mineralized hard parts [8]. Furthermore, in species with a diet that includes different prey items, large numbers of individuals need to be sampled in order to capture a true picture of diet [e.g. 9]. The use of molecular techniques to determine specific prey species in stomach contents [10] is increasing, but isolation of DNA from partially digested prey can be problematic, and methods for determining prey proportions are only just starting to be developed [4]. Furthermore, it is nearly impossible to detect secondary predation or cannibalism [11].

A number of techniques avoid the problems inherent in stomach contents analysis by looking for signatures of prey in the tissues of the consumer. Quantitative analysis of fatty acid signatures can identify prey species and proportions of prey items [12], but this technique is still being developed, and

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calculating the calibrations that account for the difference in the proportion of a specific fatty acid in the prey and the predator is complex and time consuming [11,13]. Analysis of stable isotopes, particularly analyses of $\partial^{13}C$ or $\partial^{15}N$, avoids many of the pitfalls of methods based on stomach contents, but it too is not without methodological limitations [11,14]. Stable isotope analysis of $\partial^{15}N$, for example, provides a measure of the relative trophic position of a species within a specific trophic web, rather than food items consumed [15], and comparison of individuals or populations from different geographical areas is thus problematic unless the isotopic composition of food items in their respective trophic webs has been characterised. Furthermore, multiple dietary combinations can result in the same $\partial^{13}C$ and $\partial^{15}N$ values [16], and different tissues integrate dietary proxy data over different timescales [17]. Nielsen et al. [11] provide a recent review and comparative analysis of different approaches to dietary determination in extant animals.

Dietary analysis of fossil cetaceans is also important if we are to understand their roles in ancient ecosystems and test hypotheses linking diet to evolutionary patterns. Unfortunately, few of the techniques outlined above are applicable to fossil odontocetes, stem cetaceans, or other marine amniotes.

Quantitative 3D textural analysis of tooth microwear (commonly referred to as dental microwear texture analysis, or DMTA) offers a new way of investigating diet in odontocetes and other marine mammals, potentially providing independent evidence of diet, or supplementing other data as part of a multiproxy approach. DMTA was originally designed to investigate the diet of fossil animals, where direct observations of feeding are not possible [18–20] – a comparable issue to the difficulties of observing what free ranging marine mammals eat. DMTA is now a well-established approach to understanding the dietary ecology of many vertebrate groups, primarily terrestrial mammals (including primates, various ungulates, carnivores and small mammals) but also fishes [18,19,21–29]. Dietary signals accumulate over longer intervals than stomach contents or faecal analyses (normally up to a few weeks) [30,31], but turnover on short enough timescales that seasonal variation can still be studied [22]. The approach is able to detect dietary signals in teeth that differ significantly in size, and detect dietary differences where tooth morphology does not differ [27].

The few studies that have applied quantitative 3D textural analysis of tooth microwear to fishes [27,28] highlighted the applicability of the technique to a broader range of aquatic vertebrates. But DMTA has yet to be applied to marine mammals, despite its clear potential to provide a new class of data to enhance dietary analysis of odontocetes, and to be applied to dietary analysis of extinct taxa, including stem cetaceans. Before we can investigate whether DMTA has the power to detect dietary differences between populations and taxa, however, there are differences in the dentition of odontocetes compared to other mammals that mean we must address a number of important null hypotheses. Our aim with this paper is to test these null hypotheses and establish a methodology that will allow robust microwear texture analysis in odontocetes. Our study material is based on tooth samples from the beluga whale, *Delphinapterus leucas,* a member of the largest group of odontocetes, the Delphinoidea. Belugas are the most abundant odontocete in the arctic, and as opportunistic predators, eating a variety of fish and invertebrate species, they play an important role in the structure and function of arctic marine ecosystems [32]. They are subject to the same difficulties of dietary analysis as other marine mammals [e.g. 33].

2. Non-dietary variables and tooth microwear in odontocetes

In odontocetes, texture of microwear on tooth surfaces and how it varies within teeth, within individuals and between individuals is potentially influenced by a variety of non-dietary variables. These variables include the dental material of which tooth surfaces are composed, tooth position, degree of wear, and the characteristics of wear facets (such as facet steepness and facet orientation in the jaw; Fig. 1).

Regarding surface composition and dental material, in many odontocetes, including beluga, teeth bear little enamel and this is rapidly worn way completely after eruption of teeth. At birth, the body of the tooth is composed of dentine (so-called pre-natal or fetal dentine), but after birth more dentine (post-natal-dentine) continues to be deposited incrementally on the inner surface of the tooth cone, and cementum is deposited externally [34]. The functional surfaces of worn adult teeth, typically faceted in some way, therefore expose a central core of dentine surrounded by cementum (Fig. 2). Topography of wear facets suggests cementum is a little more wear resistant than dentine, and this is consistent with previous analyses of relative hardness in the closely related narwhal (*Monodon monoceros*), although the difference is small (0.36 ± 0.05 GPa and 0.33 ± 0.03 GPA for narwhal cementum and dentine respectively [35]).

In typical microwear analyses of mammals, standardizing tooth position, facet type and orientation is relatively trivial because of the secure evidence of homology provided by the heterodont dentition. Odontocete dentitions on the other hand are homodont, and the number of teeth can vary to some degree between individuals. Consequently, there are no unambiguously homologous tooth positions, and no homologous wear facets. Without these landmarks, the approach to sampling homologous facets on homologous teeth typically used for analysis of dental microwear in mammals is inapplicable to odontocetes, and the effects of tooth position and wear facet characteristics need to be understood before microwear can be used for dietary analysis. As they wear, odontocete teeth exhibit a number of different forms of faceting (Fig. 1). Blunting of tips and general rounding of teeth is common, but so is the development of oblique facets of various forms, the latter reflecting occlusal interactions between upper and lower teeth. Facet type can vary between the teeth within an individual, as can the orientation of oblique facets, presumably reflecting variation along the jaw in the nature of occlusal relationships between teeth. Breakage of tooth tips is also common.

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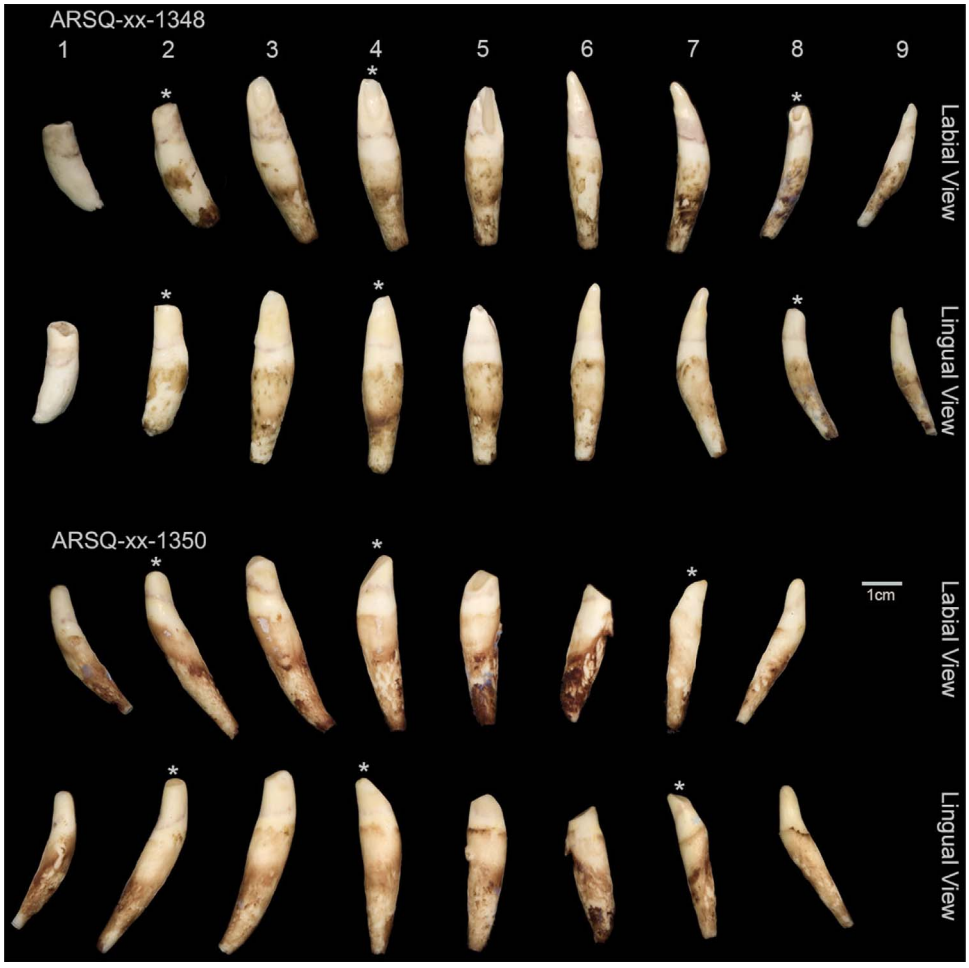

Fig. 1. Teeth of specimens ARSQ-xx-1348 and ARSQ-xx-1350 sampled for study. All are from lower left jaws. Mesial is to the left, and tooth position is identified by numbering them in sequence from mesial to distal. See Supplementary Figure S1 for orientation of teeth in the jaw. For all teeth, apical is oriented towards top of page. Asterisks indicate the teeth sampled for microwear texture analysis.

Finally, the degree of tooth wear must also be taken into account in analysis. Wear in odontocetes has been studied in few species [36], but is known in delphinids to vary between teeth within an individual, and between individuals. It also varies between populations and this is thought to reflect differences in diet [37]. Because teeth are not shed and replaced, wear accumulates over the lifetime of the animal.

Specifically, we test the following null hypotheses associated with non-dietary variables:

That microwear texture does not vary with dental surface tissue type (dentine within wear facet, cementum within wear facet, cementum off facet).

That microwear texture does not vary with tooth characteristics (tooth location in jaw, degree of tooth wear, degree of overall tooth wear, facet slope and facet orientation).

3. Material and methods

3.1. Specimens sampled

Teeth were extracted from the lower left jaws of beluga obtained through subsistence hunting by Inuit communities in Cumberland Sound and Hudson Bay, Nunavut (Table 1). All teeth were removed (by CJDM), and were identified by numbering in sequence from mesial to distal, LL1, LL2 etc. Specimens are housed in the Freshwater Institute, Fisheries and Oceans Canada (Winnipeg). Various characteristics of the teeth were scored for analysis. Tooth location within the jaw was quantified as tooth position divided by the total number of teeth. Wear facets were characterised as light tip wear or tip rounding (scored as 1 for quantitative analysis), shallow oblique facets ($<45^\circ$ to long axis of tooth; scored as 2), and steep oblique facets ($>45^\circ$ to long axis of tooth; scored as 3). Facet orientation was recorded as mesial (scored as 1 for quantitative analysis), labial (2), distal (3), lingual (4). The degree of wear and faceting for teeth, and the total amount of wear in an individual, was characterised following the method of Foote et al. [37] (modified from [38]), where the index of wear (*Id*) for each individual was calculated as follows:

$$Id = (\Sigma q.kq)/n$$

Where q is the degree of wear of a tooth (0 = none; 1 = wear with up to a quarter of the crown height removed; 2 = wear up to half the crown height removed; 3 = wear with more than

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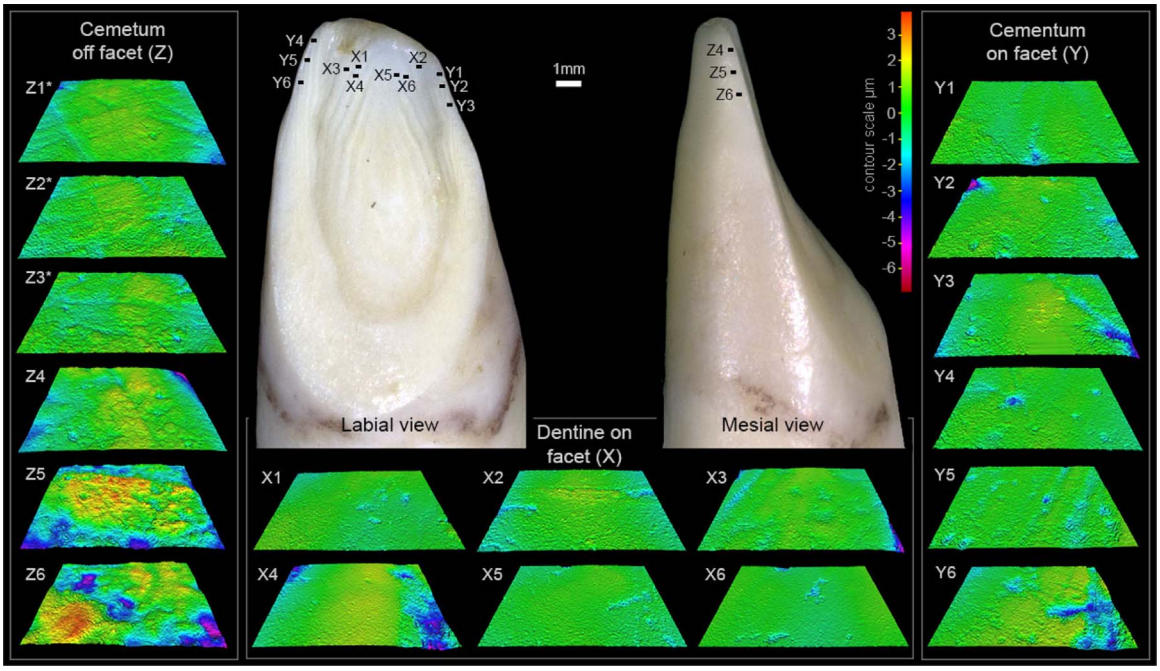

Fig. 2. Tooth LL4 from specimen ARSQ-xx-1348 illustrating sampling of surfaces with different composition (cementum and dentine). Labial and mesial views are shown with locations from which texture data were collected (X = dentine on facet, Y = cementum on facet, and Z = cementum off facet). Digital elevation models (DEMs) show the data files collected with codes matching their location on the tooth surface. All DEMs have lateral dimensions 146 x 111 µm, and vertical dimensions shown in false colour (see scale bar). Locations marked with (*) are collected from cementum off facet locations on the distal surface, equivalent in position to those from the mesial off facet surface.

Table 1
Specimens of beluga (*Delphinapterus leuca*) from which teeth were sampled. Specimens were harvested by the Sanikiluaq communities of Hudson Bay, Nunavut. The amount of wear is averaged from two independent scores (MAP and RHG). Teeth are housed in the collections of the Freshwater Institute, Fisheries and Oceans Canada (Winnipeg).

Specimen number	Community	Year of harvest	Sex	*Id* (total wear)
ARSQ-xx-1348	Sanikiluaq	2013	male	1.94
ARSQ-xx-1350	Sanikiluaq	2013	male	1.81

half the crown height removed), k_q is the number of teeth worn to level q, and n is the total number of teeth in the individual. Independent scoring of wear for six beluga individuals (by MAP and RHG) yielded strongly correlated values (r = 0.8949, p = 0.016) and values for *Id* reported in the text are an average of these two scores for each individual. Fig. 3 summarizes the characteristics of teeth from each individual.

After extraction, remnants of soft tissue and microbial films adhering to teeth were removed by soaking in water (individual falcon tubes heated in water bath at 50 °C for 24 h), followed by 2 min ultrasonic cleaning, followed by soaking in 15% methanol for 30 min. Remaining material was brushed off with cotton buds dipped in 15% methanol, and teeth were left to air dry overnight.

To test our hypotheses, we sampled surfaces composed of cementum and surfaces composed of dentine from within the same facets, and cementum from the outer surfaces of teeth adjacent to sampled facets (see Fig. 2). For analysis of how microwear texture varies with tooth characteristics, we sampled dentine from within wear facets.

3.2. Surface texture data acquisition

Data were acquired from high fidelity surface replicas of teeth. Molds were prepared using President Jet medium body polysiloxane dental moulding compound, and cast using EpoTek 320 LV black epoxy. Both were mixed and applied following the manufacturer's instructions. Analysis of accuracy and precision of moulding compounds indicates that replicas made this way compare favourably with the most accurate and precise moulding compounds, with very small absolute differences in parameter values between replica and original [39]. Casts were cured under elevated pressure (2 bars, 24 h) to reduce bubbles and increase cast quality. To reduce data capture time and maximise data quality, replica surfaces were sputter coated with gold (Emitech K500X sputter coater, four minutes).

High-resolution 3D surface data were captured following standard lab protocols [26,27,39] using an Alicona Infinite Focus microscope G4b (IFM; Alicona GmbH, Graz, Austria; software version 2.1.2). A x100 objective was used for data capture, providing a field of view of 146 x 111 µm. The Alicona Infinite Focus microscope G4b has a CCD of 1624 x 1232 pixels; in theory for a field of view of 146 µm, this equates to a lateral sampling distance of 0.09 µm, but the limits imposed by the wavelength of white light mean that lateral optical resolution is between 0.35–0.4 µm. For all samples, vertical and lateral resolution were set at 20 nm and 440 nm respectively. For each

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	1348 LL2 shallow upper labial	1348 LL4 steep lower labial	1348 LL8 steep lower labial	1350 LL2 shallow upper lingual	1350 LL4 steep middle mesial	1350 LL7 shallow upper distal
1348 LL2 shallow upper labial		steep lower mid	steep lower rear	lingual	steep middle mesial mid	distal rear
1348 LL4 steep lower labial	3		rear	upper lingual front	middle mesial	shallow upper distal rear
1348 LL8 steep lower labial	3	1		shallow upper lingual front	middle mesial mid	shallow upper distal
1350 LL2 shallow upper lingual	1	3	4		steep middle mesial mid	distal rear
1350 LL4 steep middle mesial	4	2	3	4		shallow upper distal rear
1350 LL7 shallow upper distal	2	4	3	2	4	

Fig. 3. Tooth characteristics in specimens ARSQ-xx-1348, and ARSQ-xx-1350 (tooth positions LL2, LL4, and LL8 in ARSQ-xx-1348, and tooth positions LL2, LL4, and LL7 in ARSQ-xx-1350). Lower left side of matrix tallies differences, upper right shows the characteristics that differ.

sample exposure settings were manually adjusted to maximize data quality in terms of measurement repeatability (this is estimated automatically by the IFM software during data capture) for each sample. Adjusting exposure and contrast do not affect the values for 3-D measurements. The resulting point clouds were edited manually to delete measurement errors (e.g. single point data spikes) and extraneous dirt and dust particles from the surface. They were then levelled to remove variation in 3D orientation of surfaces arising from manual positioning of the sample under the microscope (fit to a least squares plane via rotation around all three axes). After editing, point clouds were exported as .sur files and imported into SurfStand for analysis (software version 5.0; restore bad data option selected, which fills missing data by growing edges inwards rather than using mean values or other planes). Imported surfaces were levelled again, followed by generation of scale limited surfaces. For texture analysis of tooth microwear using ISO characterization of texture (ISO 25178-2 [40]) standardized protocols for the generation of scale limited surfaces have yet to be developed, so three sets of surfaces were generated for analysis through application of the following filters and polynomial operators to remove gross form: second order polynomial (which finds and

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removes the least squares second-order polynomial surface from the levelled data) combined with a spline filter (λ_c 0.025 mm); fifth order polynomial combined with a spline filter (λ_c 0.025 mm); fifth order polynomial combined with a robust gaussian filter (λ_c 0.025 mm). More details, including short definitions of ISO parameters can be found in Supplementary Table S1. The analysis presented here is based on scale limited surfaces resulting from application of a 5th order polynomial and a robust gaussian filter, which can be compared directly with the ISO-based microwear analysis of Purnell et al. [26] and Gill et al. [41]. Surfaces produced using a 5th polynomial and a spline filter, and a 2nd order polynomial and a spline filter, produced results that differ little from these (see Supplementary Information). Analysis based on surfaces produced using a 2nd order polynomial can be compared with the results of Purnell and Darras [27], also based on surfaces generated with a 2nd order polynomial.

3.3. Analytical design, microwear sampling and statistical analysis

For testing of the hypothesis that microwear texture varies with dental surface tissue type, 6 samples were taken from each of the three different types of surface (dentine within a wear facet, cementum within the facet, and cementum from the tooth surface adjacent to the facet) from lower left tooth 4 in specimens ARSQ-xx-1348 and ARSQ-xx-1350 (Fig. 1, Fig. 2). These two individuals are both male and exhibit a similar index of overall wear (*Id*) (Table 1). Clearly, if texture varies with tissue type it is important that this is not a confounding variable in analysis, and the results of this testing for variation between tissue types were used to inform the sampling strategy applied to other teeth. For subsequent analyses only dentine from within wear facets, toward the apex of the tooth was sampled.

The hypotheses that tooth microwear texture varies with tooth position, degree of wear, facet type, and facet orientation was the focus of subsequent analysis. Ideally, with unlimited material, this analysis would be based on a sampling regime that allowed one variable to differ while holding all others constant. For example, an analysis of how microwear texture varies with the degree of wear, should sample multiple teeth that in all other respects are the same (surface material, tooth position, facet type and orientation). With limited resources and access to specimens, however, this was not possible and we adopted a more pragmatic approach to hypothesis testing involving comparisons of microwear texture between teeth that encompass the range of variation in all the variables to test for differences, combined with analysis of how differences in texture relate to characteristics of the teeth (based on comparison of tallies of pairwise differences between teeth and correlations between texture and quantified tooth characteristics). Analysis was based on data collected from three teeth each from ARSQ-xx-1348 and ARSQ-xx-1350 (Fig. 3; Table 1).

Prior to statistical analysis the distributions of texture parameters were tested for normality (Shapiro-Wilks test; $\propto = 0.05$). In addition, because multiple tests were carried out on each sample (e.g. 22 parameters and 6 samples for each surface type in the dental surface tissue type dataset) the results of these tests were subject to a Benjamini-Hochberg procedure (see below; [42]). This analysis revealed that for a number of parameters in both the dental surface tissue type data and the data for broader comparison of teeth we could not reject the hypothesis that the distribution was non-normal. It also revealed that one sample (51551, cementum, off-facet, specimen 1350) was an outlier to other samples of this tissue, and it was excluded from subsequent analysis. Log transformation and exclusion of the outlier reduced the number of distributions for which we could reject the null hypothesis of normality for the dental surface tissue type dataset (5th order polynomial with robust Gaussian) to zero. (Including the additional datasets reported in the Supplementary Materials (2nd and 5th order polynomial with spline datasets), 1 out of 126 comparisons for each dataset was non-normal; in both cases the parameter is Str). For the dataset used in the broader analysis of differences between teeth, log transformation again reduced the number of distributions for which we could reject the null hypothesis of normality to zero. (Including the additional datasets reported in the supplementary materials only 1 parameter deviated from normality: in the fifth order polynomial with spline dataset, Str is non-normal). Consequently, log transformed data were used for all subsequent analyses.

All analyses were performed with JMP Pro 12 (SAS Institute, Cary, NC, USA), except for the Benjamini-Hochberg procedure, which used Microsoft Excel ([43]; www.biostathandbook.com/multiplecomparisons.html). Data were explored using t-tests, analysis of variance (ANOVA), pairwise testing (Tukey HSD), correlations, and principal components analysis (on correlations; PCA). Where homogeneity of variance tests (Bartlett and Levene tests) revealed evidence of unequal variances, Welch ANOVA is reported. Where data were subjected to multiple comparisons (e.g. ANOVA and correlations) a Benjamini-Hochberg procedure was employed to take into account the possibility that some results are significant purely by chance [42]. False Discovery Rate was set at 0.05. The only exception to this was pairwise testing using Tukey HSD; the Benjamini-Hochberg procedure is not required because Tukey HSD is designed to correct for the inflated Type I error rates associated with multiple comparisons.

4. Results

We can reject the null hypothesis that microwear texture does not vary with dental surface tissue type. ANOVA of ARSQ-xx-1350 LL4 reveals that 11 texture parameters exhibit significant differences (Table 2). Pairwise testing reveals that dentine and cementum surfaces within the wear facet differ only in one parameter (Vvc) whereas 11 parameters exhibit differences when off-facet cementum is compared with on-facet cementum and dentine (Tables 3 and 4). Similar results were obtained from ANOVA and pairwise testing of ARSQ-xx-1338 LL4, although differences are slightly less clear cut (Tables 5–7). Five parameters differ between dentine surfaces

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Table 2
Results of ANOVA comparing samples from different tissue types in tooth 4 of specimen ARSQ-xx-1350 (cementum off facet, cementum on-facet, and dentine on facet; scale limited using 5th order polynomial and gaussian filter, data log transformed), including results of Benjamini-Hochberg procedure. [W] indicates Welch ANOVA; significant differences (Benjamini-Hochberg) in bold.

Parameter	F	p	df	B-H significance
Sq (μm)	31.64	**< 0.0001**	2, 14	significant
Sku	0.61	0.5579	2, 14	
Sp (μm)	0.14w	0.8726	2, 6.81	
Sv (μm)	3.71	0.0509	2, 14	
Sz (μm)	1.34	0.2940	2, 14	
Sds (1/mm^2)	4.42	0.0325	2, 14	
Str	2.80w	0.1223	2, 7.67	
Sdq	18.30	**0.0001**	2, 14	significant
Ssc (1/μm)	8.52	**0.0038**	2, 14	significant
Sdr (%)	19.72	**< 0.0001**	2, 14	significant
Vmp (μm^3/mm^2)	0.64w	0.5503	2, 8.35	
Vmc (μm^3/mm^2)	31.46	**< 0.0001**	2, 14	significant
Vvc (μm^3/mm^2)	39.23	**< 0.0001**	2, 14	significant
Vvv (μm^3/mm^2)	13.49	**0.0005**	2, 14	significant
Spk (μm)	0.48w	0.6310	2, 8.96	
Sk (μm)	29.10	**< 0.0001**	2, 14	significant
Svk (μm)	11.28	**0.0012**	2, 14	significant
Smr1 (%)	2.60	0.1097	2, 14	
Smr2 (%)	0.76	0.4878	2, 14	
S5z (μm)	5.95	**0.0135**	2, 14	significant
Sa (μm)	35.96	**< 0.0001**	2, 14	significant

(Table 6), with only 1 pairwise difference between the two on-facet samples. Comparing on facet samples with off facet samples, dentine on-facet and cementum off facet exhibit the greatest number of differences. The analyses based on different scale limited surfaces yield almost identical results (Supplementary Tables S2–S13).

The null hypothesis that microwear texture does not vary with tooth characteristics can also be rejected. ANOVA of teeth LL2, LL4, LL7 from ARSQ-xx-1350 and LL2, LL4 and LL8 from ARSQ-xx-1348 reveals that all but two texture parameters exhibit significant differences (Table 8). Pairwise testing (Tables 9 and 10) indicates that the tally of differences between teeth varies between zero (1350 LL2 and 1348 LL8; 1350 LL2 and 1350 LL4; 1350 LL2 and 1350 LL7; 1350 LL7 and 1348 LL8) and 11 (1348 LL4 and 1350 LL4). Comparing teeth within ARSQ-xx-1348, LL2 exhibits 17 pairwise differences, LL4 14, and LL8 13. Comparing teeth within ARSQ-xx-1350, LL2 exhibits zero pairwise differences, LL4 and LL7 each exhibit five. Comparing teeth from ARSQ-xx-1348 to those from ARSQ-xx-1350, LL2 exhibits 9 pairwise differences, LL4 exhibits 24, and LL8 exhibits 8. Pairwise differences between teeth within ARSQ-xx-1348 are thus greater in number than the differences between the two individuals, and between teeth within ARSQ-xx-1350. Tooth 1348 LL4 exhibits the highest total number of pairwise differences (38; Table 10), tooth 1350 LL2 exhibits fewest (7). The analyses based on different scale limited surfaces yield the same pattern of differences, but with small differences in the tallies (Supplementary Tables S14-S19).

Comparing the pattern of pairwise differences in texture parameters to a tabulation of pairwise differences in tooth characteristics (Fig. 3) no discernible pattern emerges. Teeth that exhibit the highest tallies of pairwise differences in texture differ in very few characteristics (e.g. 1350 LL4 compared to 1348 LL4), those that exhibit few pairwise difference in texture exhibit the highest tallies of differences in characteristics (e.g. 1350 LL2 compared with 1350 LL4 and 1348 LL8), a few teeth have relatively low tallies of differences in both parameters and characters, and few are high in both. More detailed multivariate analysis of the relationship between texture and tooth characteristics is presented below.

In terms of texture parameters, based on both the effect size in ANOVA testing (Table 8) and the total number of pairwise differences (Table 10), seven parameters emerge as those that consistently differ between teeth. These are Sq, Sku, Sds, Vmc, Vvc, Sk, and Sa. In order to further investigate the pattern of differences between teeth we carried out a multivariate analysis (PCA) based on these seven parameters, followed by analysis of correlations with tooth characteristics.

The results of PCA accord well with the pattern of differences in texture parameters between teeth (Fig. 4; Supplementary Table S20). Components 1 and 2 capture 79% and 17% of the variance respectively, with the first three components collectively capturing 99%. Vmc, Vvc, Sk, and Sa all load strongly and positively onto axis 1 (> 0.95), while Sds has a strong negative loading. Sku and Sq load most heavily (both positive) onto axis 2. The samples that exhibit the highest tally of pairwise differences (1348 LL4 and 1350 LL4) occupy opposite ends of the PC1 scale (Fig. 4), while those samples that exhibit zero or very few differences cluster together and overlap in the centre of the plot (e.g. 1350 LL7 and 1348 LL8; 1350 LL7 and 1350 LL2).

Correlation of PC axes 1 and 2 with quantified tooth location, wear, facet type, and facet orientation provide clearer insights into the relationship between tooth characteristics and texture (Supplementary Table S21). PC 1 exhibits moderately strong

Table 3
Pairwise differences (Tukey HSD) between samples from different tissue types in tooth 4 of specimen ARSQ-xx-1350 (cementum off facet, cementum on-facet, and dentine on facet; scale limited using 5th order polynomial and gaussian filter, data log transformed). Tallies include only those parameters that exhibit significant differences after application of the Benjamini-Hochberg procedure to ANOVA results.

Cementum off differs from cementum on and dentine on	Sq, Sdq, Ssc, Sdr, Vmc, Vvc, Vvv, Sk, Svk, S5z, Sa
Dentine on differs from cementum on and cementum off	Vvc
Cementum on differs from dentine on and cementum off	Vvc

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Table 4
Pairwise differences (Tukey HSD) between samples from different tissue types in tooth 4 of specimen ARSQ-xx-1350 (cementum off facet, cementum on-facet, and dentine on facet; scale limited using 5th order polynomial and gaussian filter, data log transformed). Lower left side of matrix tallies differences, upper right shows the parameters that differ. Pairwise tallies include only those parameters that exhibit significant differences after application of the Benjamini-Hochberg procedure to ANOVA results.

	dentine on	cementum on	cementum off
dentine on		Vvc	Sq, Sdq, Ssc, Sdr, Vmc, Vvc, Vvv, Sk, Svk, S5z, Sa
cementum on	1		Sq, Sdq, Ssc, Sdr, Vmc, Vvc, Vvv, Sk, Svk, S5z, Sa
cementum off	11	11	

Table 5
Results of ANOVA comparing samples from different tissue types in tooth 4 of specimen ARSQ-xx-1348 (cementum off facet, cementum on-facet, and dentine on facet; scale limited using 5th order polynomial and gaussian filter, data log transformed), including results of Benjamini-Hochberg procedure.
[W] indicates Welch ANOVA; significant differences (Benjamini-Hochberg) in bold.

Parameter	F	*p*	df	B-H significance
Sq (μm)	0.95	0.4102	2, 15	
Sku	7.89	**0.0046**	2, 15	significant
Sp (μm)	0.80	0.4690	2, 15	
Sv (μm)	3.58w	0.0696	2, 9.457	
Sz (μm)	0.04	0.9608	2, 15	
Sds (1/mm^2)	15.35w	**0.0013**	2, 8.82	significant
Str	4.93w	0.0438	2, 7.35	
Sdq	0.08	0.9247	2, 15	
Ssc (1/μm)	0.19	0.8262	2, 15	
Sdr (%)	0.41	0.6704	2, 15	
Vmp (μm^3/mm^2)	0.90	0.4269	2, 15	
Vmc (μm^3/mm^2)	8.63	**0.0032**	2, 15	significant
Vvc (μm^3/mm^2)	8.83	**0.0029**	2, 15	significant
Vvv (μm^3/mm^2)	0.76	0.4849	2, 15	
Spk (μm)	1.06	0.3691	2, 15	
Sk (μm)	13.88	**0.0004**	2, 15	significant
Svk (μm)	0.78	0.4751	2, 15	
Smr1 (%)	0.16	0.8508	2, 15	
Smr2 (%)	4.99w	0.0398	2, 7.88	
S5z (μm)	0.54	0.5914	2, 15	
Sa (μm)	2.99	0.0810	2, 15	

significant correlations with the degree of wear of teeth, and with *Id*, the total wear in the individuals. PC2 exhibits moderately strong significant correlations with the location of teeth in the jaw. No other tooth characteristics are correlated with texture.

Further exploration of the relationship between Principal Components and tooth characteristics was carried out through ANOVA (Table 11). Texture as captured by PC1 exhibits significant differences within ARSQ-xx-1348 and ARSQ-xx-1350 when the two facet types (steep and shallow) are compared, but when data from ARSQ-xx-1348 and ARSQ-xx-1350 are combined, correlations are not significant. The same pattern is exhibited in comparisons of texture between teeth that differ in their degree of wear. The PC1 measure of texture differs between facets with different orientation, both when ARSQ-xx-1348 and ARSQ-xx-1350 are combined, and when ARSQ-xx-1350 is considered alone (sampled teeth in ARSQ-xx-1348 all have the same facet orientation). Texture also differs between teeth from different locations in the jaw: PC2 measures of texture differ when ARSQ-xx-1348 and ARSQ-xx-1350 are considered together (texture on rear teeth differs from middle and front teeth); PC1 measures of texture differ between tooth locations in ARSQ-xx-1348 and ARSQ-xx-1350 (in both, texture on middle teeth differs from front and rear teeth).

5. Discussion and conclusions

Our null hypotheses are unequivocally rejected. Microwear texture varies significantly between the different tissues making up the functional surfaces of worn teeth, but there are few differences between cementum and dentine within a wear facet, probably reflecting similarities in hardness of the tissues [35]. Microwear texture varies significantly between teeth with different characteristics of location and wear, and this is evident from pairwise tallies of significant differences in texture between teeth, and in correlations and ANOVA based on the principal components that together capture 96% of the significant textural variation between teeth.

Of the seven parameters that consistently differ between teeth, 4 capture aspects of the distribution of heights of points measured in surfaces (Sa, average height, Sq, root-mean square height; Sku, kurtosis of height distribution). Sds is the number of summits per unit area making up the surface. The remaining three parameters capture aspects of the core surface. The core surface lies between the heights of the surface delimited by the extrapolated intercept of the minimum slope of the bearing area curve (see Supplementary Table S1). The intercepts provide the thresholds to define peaks and valleys in the surface, and the core

Table 6
Pairwise differences (Tukey HSD) between samples from different tissue types in tooth 4 of specimen ARSQ-xx-1348 (cementum off facet, cementum on-facet, and dentine on facet; scale limited using 5th order polynomial and gaussian filter, data log transformed). Tallies include only those parameters that exhibit significant differences after application of the Benjamini-Hochberg procedure to ANOVA results.

Cementum off differs from Cementum on and dentine on	Sds, Sk
Cementum on differs from Cementum off and dentine on	Sds
Dentine on differs from Cementum off and cementum on	Sds
Cementum off differs from Dentine on	Sku, Vmc, Vvc

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Table 7
Pairwise differences (Tukey HSD) between samples from different tissue types in tooth 4 of specimen ARSQ-xx-1348 (cementum off facet, cementum on-facet, and dentine on facet; scale limited using 5th order polynomial and gaussian filter, data log transformed). Lower left side of matrix tallies differences, upper right shows the parameters that differ. Pairwise tallies include only those parameters that exhibit significant differences after application of the Benjamini-Hochberg procedure to ANOVA results.

	dentine on	cementum on	cementum off
dentine on		Sds,	Sku, Sds, Vmc, Vvc, Sk
cementum on	1		Sds, Sk
cementum off	5	2	

Table 8
Results of ANOVA comparing data from different tooth positions in specimens ARSQ-xx-1348, and ARSQ-xx-1350 (tooth positions LL2, LL4, and LL8 in ARSQ-xx-1348, and tooth positions LL2, LL4, and LL7 in ARSQ-xx-1350; scale limited using 5th order polynomial and robust Gaussian filter, data log transformed), including results of Benjamini-Hochberg procedure. W indicates Welch ANOVA; significant differences (Benjamini-Hochberg) in bold.

Parameter	F-ratio	p-value	d.f	B-H significance
Sq	5.6858	**0.0010**	5, 33	Significant
Sku*	16.8894[W]	< **0.0001**	5, 11.86	Significant
Sp	3.3204	**0.0177**	5, 33	Significant
Sv	2.8883	**0.0317**	5, 33	Significant
Sz*	8.8229[W]	**0.0008**	5, 12.79	Significant
Sds	26.3240	< **0.0001**	5, 33	Significant
Str	1.3366	0.2779	5, 33	
Sdq	3.7960	**0.0095**	5, 33	Significant
Ssc	3.2756	**0.0188**	5, 33	Significant
Sdr	5.0617	**0.0020**	5, 33	Significant
Vmp*	12.1187[W]	**0.0003**	5, 12.27	Significant
Vmc	23.2996	< **0.0001**	5, 33	Significant
Vvc*	29.0932[W]	< **0.0001**	5, 10.93	Significant
Vvv	3.9682	**0.0076**	5, 33	Significant
Spk*	13.316[W]	**0.0002**	5, 11.33	Significant
Sk	30.2817	< **0.0001**	5, 33	Significant
Svk	3.7024	**0.0107**	5, 33	Significant
Smr1	1.7800	0.1495	5, 33	
Smr2*	8.3233[W]	**0.0015**	5, 11.57	Significant
S5z*	7.1563[W]	**0.0023**	5, 12.54	Significant
Sa*	18.8968[W]	< **0.0001**	5, 11.80	Significant

is what lies below the peaks and above the valleys. With respect to the core, Sk captures its depth, Vmc the volume of material making up the core, and Vvc the volume of voids in the core.

Comparison of the results from analyses of how texture varies between teeth indicates that the influence of tooth characteristics on wear is complex. Spearman Rank tests of principal components and tooth characteristics indicate that degree of wear of both teeth and dentitions, and location in the jaw are correlated, and ANOVA reveals that texture differs significantly with facet steepness, facet orientation, degree of wear, and tooth location. Yet the pattern of differences between teeth within the two individuals sampled is not the same, and is not the same as when data for the two are combined. Furthermore, patterns of variation in tooth characteristics and microwear texture as captured by tallies of pairwise

Table 9
Pairwise differences (Tukey HSD) between samples from different tooth positions in specimens ARSQ-xx-1348, and ARSQ-xx-1350 (tooth positions LL2, LL4, and LL8 in ARSQ-xx-1348, and tooth positions LL2, LL4, and LL7 in ARSQ-xx-1350; scale limited using 5th order polynomial and robust Gaussian filter, data log transformed). Tallies include only those parameters that exhibit significant differences after application of the Benjamini-Hochberg procedure to ANOVA results.

1348LL2	differs from	1348LL4	Sq, Sds, Sdq, Ssc, Sdr, Vmc, Vvc, Sk, Sa
1348LL2	differs from	1348LL8	Sq, Sku, Sp, Sv, Sz, Vvv, Svk, S5z
1348LL2	differs from	1350LL2	Sku
1348LL2	differs from	1350LL4	Sku, Sds, Vmc, Vvc, Sk
1348LL2	differs from	1350LL7	Sku, Vvv, Smr2
1348LL4	differs from	1348LL8	Sku, Sds, Vmc, Vvc, Sk
1348LL4	differs from	1350LL2	Sku, Sds, Ssc, Vmc, Vvc, Sk, Sa
1348LL4	differs from	1350LL4	Sq, Sku, Sp, Sds, Sdr, Vmp, Vmc, Vvc, Spk, Sk, Sa
1348LL4	differs from	1350LL7	Sds, Vmc, Vvc, Sk, Smr2, Sa
1348LL8	differs from	1350LL2	
1348LL8	differs from	1350LL4	Sq, Sds, Vmp, Vmc, Vvc, Spk, Sk, Sa
1348LL8	differs from	1350LL7	
1350LL2	differs from	1350LL4	
1350LL2	differs from	1350LL7	
1350LL4	differs from	1350LL7	Sds, Vmc, Vvc, Sk, Sa

Table 10
Pairwise differences (Tukey HSD) between samples from different tooth positions in specimens ARSQ-xx-1348, and ARSQ-xx-1350 (tooth positions LL2, LL4, and LL8 in ARSQ-xx-1348, and tooth positions LL2, LL4, and LL7 in ARSQ-xx-1350); scale limited using 5th order polynomial and robust Gaussian filter, data log transformed). Lower left side of matrix tallies differences, upper right shows the parameters that differ. Pairwise tallies include only those parameters that exhibit significant differences after application of the Benjamini-Hochberg procedure to ANOVA results.

	1348 LL2	1348 LL4	1348 LL8	1350 LL2	1350 LL4	1350 LL7
1348 LL2		Sq, Sds, Sdq, Ssc, Sdr, Vmc, Vvc, Sk, Sa	Sq, Sku, Sp, Sv, Sz, Vvv, Svk, S5z	Sku	Sku, Sds, Vmc, Vvc, Sk	Sku, Vvv, Smr2
1348 LL4	9		Sku, Sds, Vmc, Vvc, Sk	Sku, Sds, Ssc, Vmc, Vvc, Sk, Sa	Sq, Sku, Sp, Sds, Sdr, Vmp, Vmc, Vvc, Spk, Sk, Sa	Sds, Vmc, Vvc, Sk, Smr2, Sa
1348 LL8	8	5			Sq, Sds, Vmp, Vmc, Vvc, Spk, Sk, Sa	
1350 LL2	1	7	0			
1350 LL4	5	11	8	0		Sds, Vmc, Vvc, Sk, Sa
1350 LL7	3	6	0	0	5	

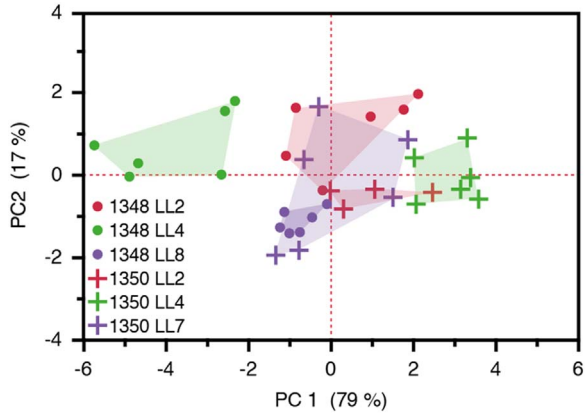

Fig. 4. PCA analysis (on correlations) of the 7 texture parameters that consistently differ between teeth based on both the effect size in ANOVA and the total number of pairwise differences. These are Sq, Sku, Sds, Vmc, Vvc, Sk, and Sa. For details of loadings see Supplementary Table S20.

differences exhibit no discernible correspondence. This suggests that the different characteristics of the teeth interact in how they influence the development of microwear texture.

Perhaps the easiest question to ask in this context is whether the parameters that differ between surfaces of a tooth, and between teeth in our study are the same as those that have the power to discriminate between populations and species with different diets. In the absence of data from odontocetes, the only possible comparison is with previous DMTA analysis that have used ISO parameters. Analysis of specialist and opportunist durophagous fish (*Anarhichas lupus* and *Archosargus probatocephalus*) [27], found that Sdq, Sdr, Vmc, Vvv, Sk and Sa discriminated best between the specialist durophagous and more opportunist durophagous fish, and between populations of the opportunist durophage with different proportions of hard prey in their diets. Of these parameters only Vmc, Sk and Sa are among the parameters that exhibit the greatest differences in our analysis (largest effect size in ANOVA and total number of pairwise differences). Vmc and Sk were also found to differ with diet (amount of hard-shelled prey) in cichlids [28]. The other parameters found to discriminate between fishes with different diets (Sdq, Sdr, Vvv) also exhibit significant differences between teeth in beluga, but the statistical effect size, and the magnitude of differences between mean parameter values for samples that differ are greater in the dietary analysis than between beluga

Table 11
Results of ANOVA comparing tooth characteristics with Principal Components derived from analysis of surfaces texture parameters (Sq, Sku, Sds, Vmc, Vvc, Sk, Sa, surfaces scale limited using 5th order polynomial and gaussian filter, data log transformed). Benjamini-Hochberg procedure was applied to the results for each tooth character. ^{W} indicates Welch ANOVA; significant differences (Benjamini-Hochberg) in bold. Facet orientation for ARSQ-xx-1348 is not included because sampled teeth have the same facet orientation.

Comparison	F	*p*	df	B-H significance	Pairwise differences
Facet type – PC1, 1348 & 1350	1.64^{W}	0.2126	1, 23.41		
Facet type – PC2, 1348 & 1350	0.92	0.3450	1, 32		
Facet type – PC3, 1348 & 1350	2.24	0.1439	1, 32		
Facet type – PC1, 1348	**9.95**	**0.0061**	**1, 16**	**significant**	
Facet type – PC2, 1348	6.29	0.0233	1, 16		
Facet type – PC3, 1348	1.58^{W}	0.2289	1, 14.66		
Facet type – PC1, 1350	**19.81**	**0.0005**	**1, 14**	**significant**	
Facet type – PC2, 1350	0.30	0.5907	1, 14		
Facet type – PC3, 1350	0.40	0.537	1, 14		
Facet orientation – PC1, 1348 & 1350	**9.50**	**0.0001**	**3, 30**	**significant**	**Mes dif lab & dist**
Facet orientation – PC2, 1348 & 1350	2.24^{W}	0.1359	3, 12.16		
Facet orientation – PC3, 1348 & 1350	0.85	0.4797	3, 30		
Facet orientation – PC1, 1350	**11.26**	**0.0015**	**2, 13**	**significant**	**Mes dif dist & ling**
Facet orientation – PC2, 1350	1.16^{W}	0.3622	2, 7.72		
Facet orientation – PC3, 1350	0.53	0.5999	2, 13		
Level of wear – PC1, 1348 & 1350	**18.46**	**< 0.0001**	**2, 31**	**significant**	**3 dif 1 and 2**
Level of wear – PC2, 1348 & 1350	0.81	0.4536	2, 31		
Level of wear – PC3, 1348 & 1350	1.47^{W}	0.2691	2, 12.07		
Level of wear – PC1, 1348	**9.95**	**0.0061**	**1, 16**	**significant**	**Only level 2 & 3**
Level of wear – PC2, 1348	6.29	0.0233	1, 16		
Level of wear – PC3, 1348	1.58^{W}	0.2289	1, 14.66		
Level of wear – PC1, 1350	**9.45**	**0.0082**	**1, 14**	**significant**	**Only level 1 & 2**
Level of wear – PC2, 1350	0^{W}	0.9984	1, 5.79		
Level of wear – PC3, 1350	1.14	0.3027	1, 14		
Tooth location – PC1, 1348 & 1350	2.16^{W}	0.1430	2, 18.58		
Tooth location – PC2, 1348 & 1350	**4.55**	**0.0184**	**2, 31**	**significant**	**Rear dif mid & front**
Tooth location – PC3, 1348 & 1350	0.14	0.8675	2, 31		
Tooth location – PC1, 1348	**13.85^{W}**	**0.0027**	**2, 7.77**	**significant**	**mid dif front & rear**
Tooth location – PC2, 1348	17.14	**0.0001**	2, 15	significant	Rear dif mid & front
Tooth location – PC3, 1348	1.62^{W}	0.2535	2, 8.56		
Tooth location – PC1, 1350	**11.26**	**0.0015**	**2, 13**	**significant**	**mid dif front & rear**
Tooth location – PC2, 1350	1.16^{W}	0.3622	2, 7.72		
Tooth location – PC3, 1350	0.53	0.5999	2, 13		

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teeth. Other studies, although focussed on terrestrial rather than aquatic vertebrates, have also found Vmc and Sa to vary with more abrasive diets in grazing ungulate mammals [24], and Vmc, Sa and Sk to vary with increasingly 'hard' prey in insectivorous bats [26]. This partial overlap between the parameters that offer the best potential for dietary discrimination and some of the parameters (Vmc, Sk and Sa) that exhibit non-dietary differences between teeth and between individuals suggests that future analyses of microwear texture in odontocetes will need to scrutinise carefully the significance of differences exhibited by these parameters.

Clearly, our results have significant potential impacts on future studies of microwear texture in toothed whales. Analysis of how microwear varies with tooth characteristics in a sample of more individuals with the same diet would be a worthwhile test of our conclusions, but our results have obvious implications for the design of sampling strategies for DMTA dietary analysis. Such analysis must ensure they sample the same tissue type (we would recommend dentine or cementum from within wear facets) in teeth from a consistent region of the jaw. Variation in facet type, steepness and orientation, and degree of wear should also be reduced to the minimum practicable, to mitigate the effects of non-dietary differences in texture.

Our results must not be interpreted to mean that microwear texture analysis cannot be used as a tool for dietary analysis in toothed whales – we do not test this hypothesis. But our analysis does sound a note of caution. Significant differences in texture can arise from the characteristics of the teeth and tooth surfaces sampled, and these differences have the potential to mask variation in texture caused by dietary differences, and thus limit the discriminatory power of DMTA in odontocetes. However, the degree to which this is true will depend on the magnitude of differences in parameter values, the number of differences in texture, and the statistical effect size associated with dissimilarity in diet. These may prove to be greater than the differences in texture associated with tooth characteristics.

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Author contributions

MAP designed the study with input from RHG, ST and CJDM. CJDM provided teeth, and ST prepared them for analysis, collected data, and conducted initial statistical tests. MAP and RHG analyzed and interpreted the microwear results and statistical tests. MAP wrote the paper with input from RHG, ST and CJDM.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bsbt.2017.11.004.

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