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Quantitative analysis of dental microwear in threespine stickleback: a new approach to analysis of trophic ecology in aquatic vertebrates

MARK A. PURNELL*, PAUL J. B. HART†, DAVID C. BAINES* and MICHAEL A. BELL‡

*Departments of *Geology and †Biology, University of Leicester, Leicester, LE1 7RH, UK; and ‡Department of Ecology and Evolution, Stony Brook University, Stony Brook, NY 11794-5245, USA*

Summary

1. The threespine stickleback *Gasterosteus aculeatus* is an important model organism in studies of genomic and phenotypic evolution, adaptation and speciation. Fossil *Gasterosteus* offer the potential to test models derived from studies of extant fishes over true evolutionary time-scales. Competition for food resources, for example, plays an important part in stickleback speciation, causing divergence in food gathering traits and ecological character displacement, but it is not possible to test this model in fossils because evidence of diet is almost never preserved.

2. We demonstrate here that quantitative analysis of dental microwear, a technique previously applied only to mammals, provides a reliable guide to the dietary preferences of stickleback. Teeth from stickleback raised under laboratory conditions exhibit microwear patterns that vary systematically according to substrate coarseness and whether fishes feed on *Daphnia* within the water column, or on chironomid larvae from the bottom. Furthermore, microwear data exhibit a progressive shift in their distribution that tracks differences in experimental feeding treatments.

3. Microwear in wild populations also exhibits a relationship with feeding. In blind assessments of trophic niche based on microwear patterns we were able to correctly assign all but one equivocal population to trophic group. Microwear data from wild stickleback exhibit a shift in distribution comparable with that observed across the range of treatments in the laboratory and these allow populations to be ranked according to the degree to which they approach fully benthic or fully limnetic feeding.

4. Our results demonstrate that microwear has the potential to be a powerful tool in the analysis of fish trophic ecology, particularly in the analysis of species pairs and niche differentiation. It has advantages over the trophic snapshot provided by analysis of stomach contents in that microwear reflects feeding and food preferences over a longer period of time, and can be applied where these data are unavailable. Furthermore, it is applicable to extinct organisms and fossils, allowing the role of trophic ecology, niche partitioning and competition over evolutionary time-scales to be investigated for the first time.

Key-words: competition, feeding, niche differentiation, stickleback, tooth microwear.

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Introduction

The threespine stickleback *Gasterosteus aculeatus* L. (species complex) is increasingly important as a model organism in studies of genomic and phenotypic evolution, adaptation and speciation (Schluter 2000; McKinnon & Rundle 2002; Bell, Aguirre & Buck 2004; Cresko *et al*. 2004; Foster & Baker 2004; Kingsley *et al*. 2004; Colosimo *et al*. 2005). Some of the most exciting and widely cited recent work on the ecological controls on speciation has focused on this fish, perhaps the best known being analyses of species pairs of threespine

Correspondence: M. A. Purnell, Department of Geology, University of Leicester, Leicester, LE1 7RH, UK.
E-mail: mark.purnell@leicester.ac.uk

stickleback in post-glacial coastal lakes in British Columbia. Here, two reproductively isolated but coexisting trophic forms occur ('limnetics' – feed on plankton in open waters; slim bodied; numerous, long gill-rakers; narrow mouths – and 'benthics' – forage on the lake bottom; larger, deeper bodies; fewer, shorter gill-rakers; wide mouths). Field and experimental evidence indicates that the differences between these two forms are the result of competition for food resources causing divergence in food gathering traits and ecological character displacement (McPhail 1992, 1994; Schluter & McPhail 1992; Schluter 1994).

Despite this work and other compelling evidence, it is not yet clear whether competition and ecological character displacement operate over geological timescales to cause speciation and adaptive radiation. In fact, the role of competition in evolution is contentious (Benton 1996; Sepkoski 1996) largely because of the difficulty of scaling-up from field or laboratory results to the much longer periods over which new species evolve. The hypothesis that speciation was caused by ecological character displacement driven by trophic niche differentiation is particularly difficult to test in fossils because shifts in feeding cannot be observed directly. Such functional changes have to be inferred from changes in morphology, and attempts to determine whether morphological changes were caused by shifts in feeding habits thus become trapped in a circular argument. Indeed, feeding in aquatic vertebrates has been used to exemplify the difficulties inherent in inferring function from structure (Lauder 1995).

Analysis of tooth microwear patterns offers a way out of this impasse. Studies of extant mammals with known feeding habits have revealed that the abrasives in food and the forces that act on tooth surfaces during feeding produce distinctive microscopic wear (Rensberger 1978; Walker, Hoeck & Perez 1978; Teaford 1988a). This microwear on mammal teeth can provide direct evidence of tooth use, diet and feeding and has been applied with great success to extinct mammals, especially fossil hominins (e.g. Scott *et al*. 2005 and references therein). But is this method applicable to fish teeth? Qualitative work on conodonts (extinct aquatic vertebrates) (Purnell 1995; Donoghue & Purnell 1999), exploratory data from cichlid fishes (Purnell 1999; Purnell, Galis & Seehausen, unpublished), and preliminary observations of wear on stickleback teeth (Caldecutt, Bell & Buckland-Nicks 2001) indicate that microwear analysis has potential beyond mammals, but there are significant developmental and biomechanical differences between the teeth, dentitions and jaws of fish and mammals that raise non-trivial questions regarding the broader applicability of microwear analysis. Microwear in mammal teeth is produced by repeated tooth–tooth and tooth–food–tooth contact during biting and chewing (Teaford 1988a), but fish dentitions do not exhibit the interpenetrative occlusion that is characteristic of mammals, and fish do not chew. Also, food acquisition in the majority of fishes (and other aquatic vertebrates) relies to a large extent on suction (Alexander 1967; Lauder & Shaffer 1993) or ram feeding (Liem 1993) and teeth play a limited role in food acquisition. Finally, in contrast to mammals, fish have polyphyodont dentitions, their teeth being shed and replaced repeatedly through life; are they retained in the mouth long enough for distinctive microwear patterns to form? Does the fact that teeth sampled at random will be at different stages in the replacement cycle influence microwear patterns? Given all these differences it may seem unlikely that, if present, microwear on fish teeth would correlate with trophic ecology.

Clearly the first step in developing a microwear-based method for analysis of feeding and trophic niche in fishes is to establish the existence and nature of the relationship between food, trophic ecology and tooth wear. Our aim is to investigate this in threespine stickleback by testing the following hypotheses: (1) that stickleback teeth exhibit microwear patterns; (2) that microwear patterns vary with trophic niche; (3) that microwear can be used to discriminate stickleback occupying limnetic trophic niches from those occupying benthic trophic niches. This is the first time that quantitative analysis of microwear has been attempted for a non-tetrapod vertebrate.

Materials and methods

EXPERIMENTAL FISHES

Laboratory-fed stickleback

To test the hypotheses that stickleback teeth exhibit microwear patterns and that microwear patterns vary with trophic niche we conducted experiments involving controlled feeding of fishes under laboratory conditions, using first-generation laboratory offspring of threespine stickleback from Hogganfield Loch, near Glasgow. At the start of the experiment they were 12–14 weeks post-hatching. One hundred and forty-six fishes were divided into 30 groups and raised under conditions designed to simulate benthic and limnetic trophic niches (four benthic and two limnetic treatments, each replicated five times, see Table 1). 'Limnetic' fishes were fed live free-swimming *Daphnia magna* at an estimated rate of 1200–1500 per tank per day. These fishes fed in the water column and their teeth should have had little contact with the walls or floor of their tank. 'Benthic' fishes were fed thawed frozen red midge (chironomid) larvae (bloodworms). These were placed on the bottom of the tank using a pipette to ensure that fish ate from the substrate. Fish were fed once a day for 6 months. The substrate in tanks was also controlled: two had bare bottoms (no substrate), the others had a 10 mm layer of clean washed coarse, medium or fine quartz sand (see Table 1). Tanks were cleaned regularly to reduce the growth of algae and thus discourage tooth contact with anything other than controlled food. During the 6 months of the experiment 43 fish died, leaving

Table 1. Trophic treatments for controlled feeding of laboratory stickleback. Each regime was replicated five times (total of 30 tanks). Fishes were kept with continuous freshwater flow maintained at 15 °C ± 1 °C

Treatment	Food type	Substrate	Abbreviation
Limnetic	*Daphnia*	None	LNS
	Daphnia	Medium sand (250–500 μm)	LMS
Benthic	Chironomid larvae	None	BNS
	Chironomid larvae	Fine sand (63–250 μm)	BFS
	Chironomid larvae	Medium sand (250–500 μm)	BMS
	Chironomid larvae	Coarse sand (500 μm–2 mm)	BCS

Table 2. Samples of wild populations of stickleback subject to microwear analysis. Because sampling for stomach content analysis employs particular protocols to minimize the effect of sampling methods on the quality of the samples, stomach content samples and those taken for microwear analysis were collected in different field seasons. Fish from which teeth were sampled were collected June 2000; fish sampled for stomach content analysis were collected June 2004 (Mud, Long, Corcoran) and 2005 (Lynda, Kashwitna). For data and methods for calculating gill-raker number and relative littoral area see Walker (1997)

Sample no.	Location	Trophic assessment	Stomach contents and bottom conditions	Microwear determination
00–14	Mud Lake	Lake resident benthic feeders (gr 17·79, rla ca. 100%)	93·4% B 6·6% L (n = 469) Mud, some sand	Benthic
00–17	Lynda Lake	Lake resident plankton feeders (gr 20·70, rla 24·27%)	42·1% B 57·9% L (n = 247) 'organic muck'	Uncertain/mixed
00–18	Kashwitna Lake	Lake resident, questionable diet but more likely benthic feeding (gr 20·85, rla 100%)	28·5% B 71·5% L (n = 698) 'organic muck'	Benthic
00–19	Long Lake	Lake resident plankton feeders (gr 21·9, rla 30·21%)	32·6% B 67·4% L (n = 1939) Cobbles	Possible limnetic
00–20	Corcoran Lake	Lake resident benthic feeders (gr 20·45, rla 100%)	79·2% B 20·8% L (n = 288) 'organic muck' over cobbles, gravel and sand	Benthic

B, benthic food items; L, limnetic food items; n, number of food items; gr, gill-raker number; rla, relative littoral area.

103 for the analysis of microwear. Of these survivors 26 were 'limnetic' and 77 were 'benthic'.

Wild-caught stickleback

To test the hypothesis that microwear can be used to discriminate between limnetic and benthic feeding stickleback we analysed teeth from wild-caught fishes. This analysis also allowed us to evaluate how closely laboratory generated microwear compares with naturally generated microwear in stickleback. Samples were obtained by one of us (Bell) from six localities in the Matanuska-Susitna Valley, Alaska (Table 2). Fish were collected using unbaited minnow traps set at depths of 40–75 cm and left for 15–20 h overnight. Individually stored half-jaws from each of 20 fish from each population were shipped to the UK in alcohol. Microwear analysis of these teeth was conducted blind. Assessments of trophic ecology for each sample (by Bell) were based on counts of gill-rakers and calculations of the relative littoral area of lakes (Walker 1997), later supplemented by the results of an analysis of stomach contents (M. Travis, unpublished). These assessments and data were revealed only after microwear analysis was complete and an attempt to assign fishes to trophic niche based on microwear had been made.

MICROWEAR DATA: ACQUISITION AND ANALYSIS

Preparation and imaging of teeth

At the end of the controlled feeding period, laboratory fishes were killed with an overdose of tricaine methanesulphonate (MS222). Standard lengths were measured using Vernier calipers and fish were stored individually in 70% ethanol in numbered plastic specimen tubes. Premaxillae and dentaries of both laboratory and wild fish were removed using a scalpel and fine tweezers under a binocular microscope. Jaws were left to stand in tap water for a few days to soften tissues and ease their removal from jaws using tweezers and a fine mounted needle. At all stages of preparation care was taken to ensure that instruments did not contact tooth surfaces and that teeth were not inadvertently removed.

Only one premaxilla and one dentary from each fish were used for analysis. These were mounted using reversible PVA adhesive on standard 10 mm stubs, coated with silver (240 s), and imaged (Hitachi S-520 SEM). In mammals, microwear patterns develop at specific places where teeth are abraded by food or occlude with other teeth (Teaford 1988b), but because of the differences between mammal and fish dentitions it is not

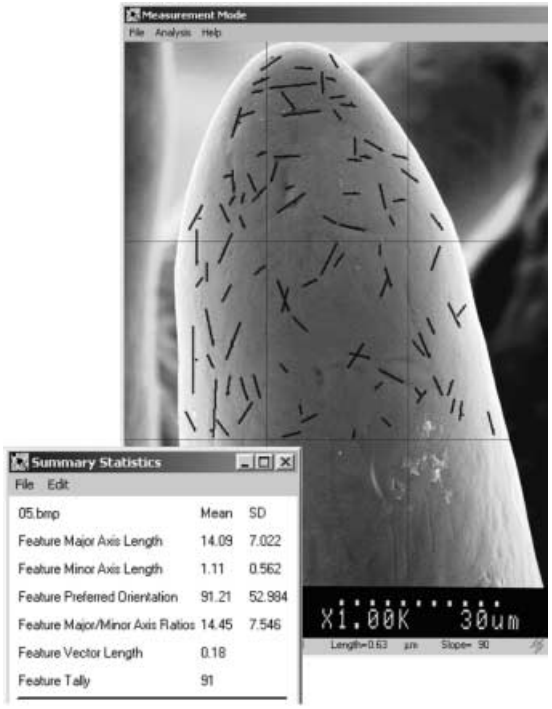


Fig. 1. Screenshot of a marked-up image and the summary data output from Microware 4·0. The summary statistics window is not open during data acquisition. This micrograph shows a premaxilla tooth from a fish raised under benthic (BFS) experimental conditions. Lines marking microwear features have been thickened to make them clear in the figure.

obvious where to obtain micrographs for acquisition of microwear data from fishes. After exploratory imaging of whole dentaries and premaxillae of stickleback and cichlids (Purnell, Galis and Seehausen, unpublished), the distal portion of the labial surface of the second tooth from the symphysis was selected as the standardized site for image acquisition (Fig. 1). This is where teeth are most likely to impinge on food and substrate during feeding. The second tooth was chosen in order to minimize the possibility of analysing wear artefacts arising from preparation of the jaws, the first tooth being more likely to have been damaged when jaws were split at the symphysis. The only occasion when this protocol was varied was if the second tooth was obscured by soft tissue or was absent, in which case the next tooth along was selected. During image capture, the orientation of teeth relative to the electron beam was standardized, and automatic contrast and brightness were employed to standardize images. Standardization of image capture is important for effective comparison of teeth (Gordon 1988). Images were captured at a magnification of 1000, providing a field of view of approximately 80×100 μm, comparable with that commonly used in analysis of occlusal microwear in mammals (Grine, Ungar & Teaford 2002; Scott *et al*. 2005), but smaller than that employed in buccal-dental microwear work (Galbany *et al*. 2005). A larger field of view was not possible because of the small size of stickleback teeth (Fig. 1).

Micrograph negatives were scanned into Adobe Photoshop 5 using a flatbed scanner with transparency adapter. Image size was standardized to 650 pixels high × approximately 450 pixels wide. Each image was analysed using the custom software package Microware 4·02 (Ungar 1995, 2001) designed specifically for analysis of mammal microwear. A recent comparative study of operator error and alternative methods of microwear analysis (Grine *et al*. 2002) advocated use of Microware as a standard approach to quantitative analysis. All analyses were undertaken on a Viglen Contender C800 computer running Windows 2000. This was equipped with a 40·6 cm (16 inch) monitor set at a screen resolution of 1024×768 pixels, resulting in an onscreen magnification of approximately × 1800. For each image, Microware was calibrated using the micrograph scale bar to ensure that dimensions measured on screen were correctly translated into micrometers (separate tests were carried out on micrograph scale bar accuracy).

Microwear data acquisition

The first stage of data acquisition involved superimposition of a 3×3 grid on the image. Only those microwear features observed in the upper two rows of this grid were recorded, equivalent to measuring all features on the labial surface within approximately 75 μm of the tooth tip. Microware uses a cursor-based interface to record microwear features, the operator marking the end-points of the longest and shortest axis of each feature. The software then automatically calculates the mean values for a range of standard parameters for the features of each tooth (including the lengths of the major and minor axes of features, feature orientation and vector length, and feature tally). Figure 1 shows a screen shot of a marked-up image and summary data output from Microware. Feature tally was normalized to the area measured so as to give features per mm^2. Care was taken to highlight only true microwear features and not artefacts such as large, sharp-edged gashes or chips, possibly caused during preparation (Teaford 1988b). During data acquisition it was found that a few teeth in the image data set were newly erupted and unworn. They had zero values for all microwear features and were excluded from further analysis.

Microwear analysis

The Microware software is designed to generate standard data for analysis of occlusal microwear in mammal teeth, and as such some of its output is unsuited to our purposes. For example, because of the size and nature of the features on the stickleback teeth, minor axis dimensions cannot be measured reliably and were not used in our analysis. Consequently, all minor axis data were excluded, as were data for pits and striations (which are automatically differentiated by the software based on a user defined threshold value for feature length to

width ratio). A further complication arises because, in contrast to the relatively planar facets where microwear generally develops in mammals, the labial surface of stickleback teeth is curved. Preferred orientation data for features is likely to be affected by this, and these data were also excluded from analysis. Exploratory analysis of the remaining data using a series of bivariate plots indicated that feature density (mean number of features per mm^2) and feature length (μm) were the most informative. Mean vector length (= *R*) was also found to be informative; it provides a measure of angular dispersion (Zar 1999), or inversely, a value for the degree of parallelism in the orientation of features (where $R = 1$ features are orientated in the same direction, where $R = 0$ feature orientation is so dispersed that a mean angle cannot be described). Statistical testing and analysis of microwear data was conducted using JMP IN 5·1·2.

Analysis of operator error

Quantitative microwear analysis is essentially a comparative technique, and in order to maximize its potential, data acquisition and analysis should, as far as possible, be standardized (Grine *et al*. 2002; Galbany *et al*. 2005). Because data acquisition is only semiautomated, and relies on human recognition of feature boundaries, it is also crucial to assess the degree to which results are operator dependent and repeatable. In order to do this, 10 micrographs were randomly selected from among the images of teeth from stickleback raised in the laboratory. Three of us (Purnell, Hart, Baines) then independently extracted microwear data from this set of micrographs using Microware, and repeated the process five times, using the same set of images, over a period of weeks. These data were then subjected to analysis of variance in order to test whether microwear data varied between operators, between replicate data sets generated by the same operator, and to test for a relationship between the two.

The results (Table 3) indicate that measurements of number of features (*n*) and mean vector length (*R*) do not vary significantly between replicate data sets for the three operators. Mean feature length does vary when all three operators were taken into account, but it does not vary significantly between the replicate sets of the operator (Baines) who generated the data set upon which our main analyses are based. Measurements of *n*, mean feature length and *R* all vary significantly between operators (Table 3).

Importantly, the results for intraoperator error indicate that the data upon which our analysis is based are reliable. The significant differences between operators, however, are of some concern if microwear analysis of the sort presented here is to be applied more widely (Grine *et al*. 2002; Galbany *et al*. 2005 presented similar results for interoperator error in quantitative analysis of dental microwear in mammal teeth). The causes of these differences require further research, but we suspect that in our analysis they arose primarily from differences in the way different operators applied sharpening filters and adjusted contrast and brightness of images within the Microware software package. Most of the data did not vary significantly between replicate sets by the same operator, and analysis of interactions between operators and replicates indicate that each operator detected the same pattern of variation in *n* and *R* between micrographs, the ranked data generated by the different operators being strongly correlated (r_s 0·84–0·91 for *n*; r_s 0·7–0·89 for *R*). Taken together, these results suggest that with greater standardization of sharpness, brightness and contrast, combined with operator training (we made no attempt to train operators to a consistent standard of measurement), it may be possible to reduce interoperator error to the point where it is no longer significant. Alternatively, fully automated approaches to microwear analysis (Ungar *et al*. 2003; Scott *et al*. 2005) avoid operator-related problems, but these have yet to be widely applied.

Table 3. Null hypotheses and test results (one-way ANOVA) to evaluate intra- and interoperator error. Data derived from five rounds of microwear data generation from a single set of 10 images by three operators (Baines, Hart, Purnell). Table also includes analysis of interactions between operator and replicates; where values for operator × replicate are nonsignificant, different operators' measurements differed in the same way

	F	d.f.	*P*
Intra-operator – between rounds of data generation:			
number of features does not differ: *cannot reject*	0·98	4,120	0·421
feature major axis length mean does not differ: *reject*	20·83	4,120	< 0·001
feature *R* does not differ: *cannot reject*	1·40	4,120	0·238
feature major axis length mean does not differ for Baines: *cannot reject*	0·74	4,45	0·568
Inter-operator – between rounds of data generation:			
number of features does not differ: *reject*	19·05	2,120	< 0·001
feature major axis length mean does not differ: *reject*	13·15	2,120	< 0·001
feature *R* does not differ: *reject*	4·77	2,120	0·01
Operator × replicate:			
number of features	0·63	8,120	0·753
feature major axis length mean	22·60	8,120	< 0·001
feature *R*	0·19	8,120	0·991

Data acquisition, tooth size and fish length

We were concerned that tooth size (linked to fish length) may have affected the data through its influence on the area of the tooth measured, particularly the number of features detected (n). Partly for this reason, our analyses of microwear used feature density (n mm^{-2}) rather than n, and in no case was this significantly correlated with area ($P > 0{\cdot}05$). We were unable to reject the null hypothesis of no correlation ($P < 0{\cdot}05$) between two other measured parameters and area measured (lab dentary feature length, $r = 0{\cdot}25$, $P = 0{\cdot}01$; lab premaxilla R, $r = -0{\cdot}33$, $P < 0{\cdot}01$), but correlations were weak, and ANOVA revealed that area measured does not differ between feeding regimes or substrates. The null hypothesis of no correlation between microwear and fish standard length could not be rejected for feature length, density, n or R ($P < 0{\cdot}05$). Results of a t-test showed that there was no significant difference between standard lengths of fish from limnetic and benthic treatments ($t = -1{\cdot}80$, $P = 0{\cdot}077$, d.f. = 59).

Results

MICROWEAR ANALYSIS OF LABORATORY-FED STICKLEBACK

Both substrate and feeding regime have a significant effect on Microwear. Table 4 summarizes the results of testing our null hypotheses (results for both dentary and premaxilla teeth are shown; the two data sets were analysed independently in order to avoid problems of pseudoreplication).

Microwear clearly varies with substrate. In dentary teeth, for example, feature density and major axis length differ significantly between substrates; R differs between benthic and limnetic treatments with medium and no substrate, but not between different substrates of all benthic treatments. A Tukey–Kramer procedure reveals more details of how feature density in benthic treatments varies: all pairwise comparisons indicate significant differences except that the fine sand treatment (BFS) does not differ from the no substrate treatment (BNS), and coarse sand (BCS) does not differ from medium sand (BMS). Microwear also varies with feeding regime: feature density and major axis length differ significantly between benthic and limnetic treatments (in dentary teeth and premaxilla teeth). For premaxilla teeth R also differs between treatments.

Discriminant function analysis reveals that 83% of limnetic dentary teeth and 77% of benthic dentary teeth can be correctly classified using microwear data alone. Results for premaxilla teeth were almost identical (83% and 78%, respectively).

Bivariate plots of feature density and feature length (Fig. 2) show that teeth from benthic and limnetic treatments fall into different but overlapping fields: teeth from limnetic treatments exhibit a broader range of feature lengths, with values extending beyond those generally seen in benthic teeth, combined with a narrower range and low values for feature density; teeth from benthic treatments tend to exhibit a narrower range and lower values for feature length combined with a greater range in feature density, with higher mean and maximum values. The data are somewhat noisy, and density ellipses (Fig. 2A) help to clarify these patterns

Table 4. Hypotheses and tests for relationships between, substrate, feeding regime (limnetic vs. benthic) and microwear on dentary and premaxilla teeth of laboratory-raised stickleback (unworn, newly erupted teeth with zero values for microwear were excluded from analysis). Two-way ANOVA of data for LNS and LMS (limnetic, no substrate and medium substrate) and BNS and BMS (benthic, no substrate and medium substrate) treatments was used to test relationships between microwear and substrate and microwear and feeding regime. One-way ANOVA of data from the four benthic treatments was used to test for relationship between microwear and substrate. One-way ANOVA of pooled limnetic vs. pooled benthic treatments (no substrate and medium substrate) was used to test for relationship between microwear and feeding regime

		Dentary		Premaxilla	
Null hypothesis (reject at $P < 0{\cdot}05$)	d.f.	F	P	F	P
Two-way ANOVA, LNS, LMS, BNS, BMS					
Feature density does not vary between substrates (dp)	1,60	21·41	< 0·001	24·71	< 0·001
Feature major axis length mean does not vary between substrates (d)	1,60	4·81	0·032	0·15	0·7
Feature R does not vary between substrates (d)	1,60	4·60	0·036	0·96	0·331
Feature density does not vary between feeding regimes (dp)	1,60	31·40	< 0·001	18·37	< 0·001
Feature major axis length mean does not vary between feeding regimes (dp)	1,60	6·48	0·014	7·94	0·007
Feature R does not vary between feeding regimes (p)	1,60	3·82	0·055	5·34	0·024
One-way ANOVA, 'benthic' treatments					
Feature density does not vary between substrates (dp)	3,74	13·44	< 0·001	11·47	< 0·001
Feature major axis length mean does not vary between substrates (d)	3,74	3·17	0·029	1·42	0·25
Feature R does not vary between substrates (p)	3,74	1·59	0·197	7·18	< 0·001
One-way ANOVA, 'benthic' vs. 'limnetic' treatments					
Feature density does not vary between feeding regimes (dp)	1,61	22·18	< 0·001	12·85	0·001
Feature major axis length mean does not vary between feeding regimes (dp)	1,61	5·75	0·020	8·08	0·006
Feature R does not vary between feeding regimes (p)	1,61	3·35	0·072	5·28	0·025

d, null hypothesis rejected for dentary teeth; p, null hypothesis rejected for premaxilla teeth.

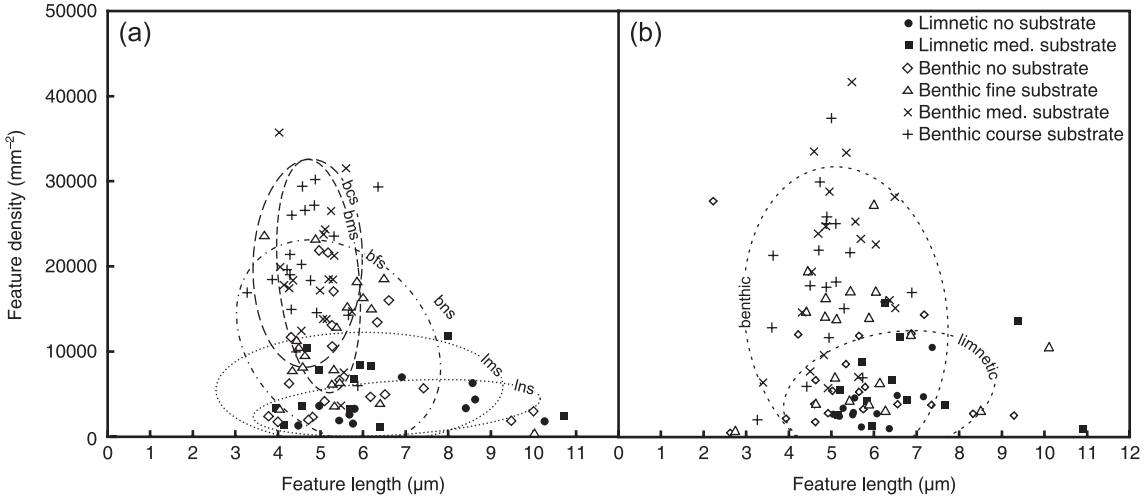


Fig. 2. Mean microwear feature density and length for dentary teeth (a) and premaxilla teeth (b) from stickleback raised under controlled feeding conditions. Density ellipses, intended only as a guide to the pattern of distribution of the data on the plot, are drawn to include 80% of the data for each treatment, assuming a bivariate normal distribution.

in the distribution of the data. The ellipses also highlight the fact that the distribution patterns of the data for the different treatments show a gradual transition, from LNS at one end of the spectrum to BCS at the other.

The data for premaxilla teeth show essentially the same overall pattern of distribution as those from dentaries (Fig. 2B), even though microwear data for dentary and premaxilla teeth from the same individual fish may differ.

MICROWEAR ANALYSIS OF WILD-CAUGHT STICKLEBACK

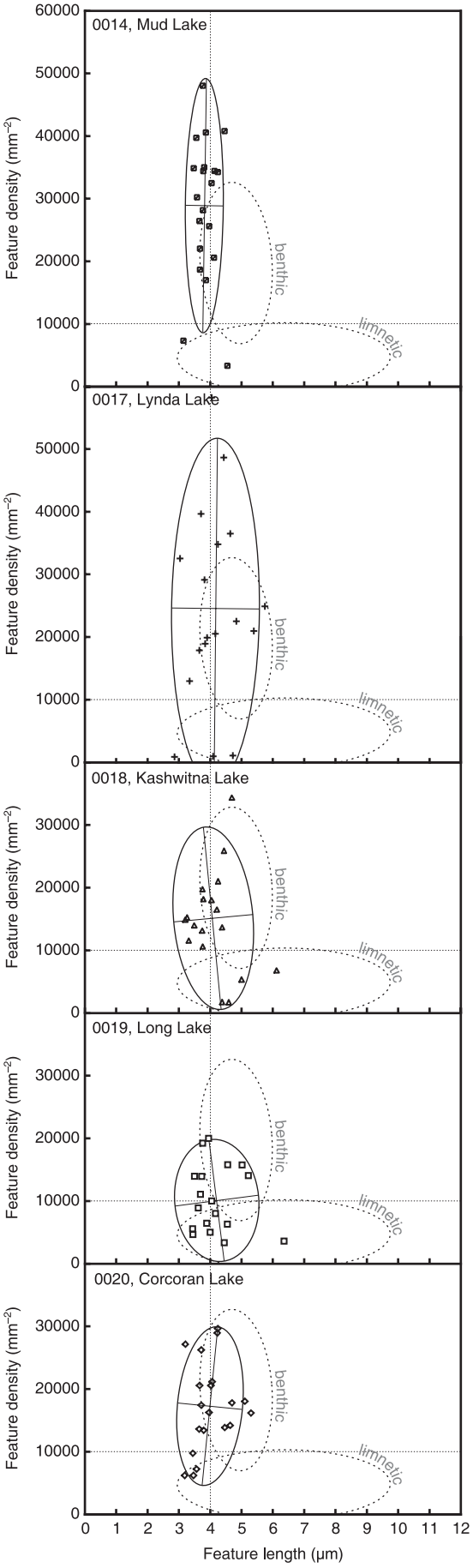
Because the analysis of laboratory fishes revealed that the pattern of microwear data obtained from dentary teeth is closely comparable with that obtained from premaxilla teeth, analysis of microwear in wild-caught stickleback used dentaries only. ANOVA reveals that feature density differs significantly between populations ($F = 9{\cdot}83$, $P < 0{\cdot}0001$; d.f. 4,89), but feature major axis length and R do not (respectively, $F = 1{\cdot}07$, $P = 0{\cdot}375$; $F = 0{\cdot}84$, $P = 0{\cdot}505$; d.f. for both 4,89). (Nonparametric tests and Welch ANOVA gave the same results.) The results of a Tukey–Kramer procedure indicate that feature density differed significantly ($P < 0{\cdot}05$) between the following pairs of lakes: Lynda–Long, Lynda–Kashwitna, Mud–Long, Mud–Kashwitna, Mud–Corcoran.

MICROWEAR-BASED INTERPRETATIONS OF TROPHIC NICHE IN WILD STICKLEBACK

Analysis of laboratory fishes showed that teeth from benthic and limnetic treatments fall into different fields on a bivariate plot of feature density vs. feature length (Fig. 2), and we used these plots as the basis for a blind test of the hypothesis that microwear can be used to discriminate between limnetic and benthic feeding populations of wild stickleback (Fig. 3). The distribution of wild stickleback's microwear data and how it compared with the laboratory data were used to interpret trophic ecology (carried out in Leicester), and the reliability and accuracy of our interpretations were tested by comparing them with the trophic assessments based on gill-raker number and lake littoral area (known to Bell, but revealed only after microwear-based assessment was completed). These data were subsequently supplemented with stomach content data. In two cases (Lynda, Kashwitna), stomach content data were not available until the final stages of analysis, and as a further test we made predictions based on microwear patterns, of what stomach content data would show. Interpretations and data are summarized in Table 2.

Sample 0014, from Mud Lake, exhibits a large range, high maximum, and the highest mean value for feature density, with very few low values. Combined with a very narrow range and relatively low values for feature length this microwear suggests that this is a benthic-feeding population. Our blind assessment was 'probable benthic' and this matched assessments of trophic ecology based on gill-raker number and lake littoral area, and those based on stomach contents. This sample had the highest proportion of benthic food items.

Sample 0017, from Lynda Lake, exhibits high maximum and mean values and a large range for feature density, suggestive of benthic feeding. However, feature length also exhibits a relatively broad range of values, extending into the field of limnetic values, and several teeth have low feature density, all suggestive of limnetic feeding. Our blind assessment was 'uncertain, possibly mixed'. Gill-raker number and lake littoral area suggest that this is a population of plankton feeders, but based on the pattern of microwear data we predicted that stomach contents would indicate mixed benthic and limnetic foods and this proved to be the case (Table 2): of all the samples the Lynda Lake fishes had the most evenly balanced mix of benthic and limnetic prey.



Sample 0018, from Kashwitna Lake, exhibits moderately high maximum feature density combined with a moderately narrow range and mostly low values for feature length. This pattern is mixed, but our blind assessment was 'benthic'. Assessments, based on gill-raker number and lake littoral area, of 'likely benthic feeding' are not unequivocal, and our blind assessment notwithstanding, some aspects of the microwear data, such as mean feature density being relatively low, led us to predict that stomach contents would include a greater proportion of limnetic food items than some of the other benthic feeding populations. This proved to be the case: 72% of prey items were classified as limnetic (Table 2).

Sample 0019, from Long Lake, exhibits a low maximum, a limited range and low mean values for feature density, suggesting limnetic feeding, and although the relatively broad range of values for feature length is consistent with this, most of the observed feature lengths were shorter than the range of values produced in the laboratory work. For these reasons our blind assessment was 'possible limnetic', and this was in accord with the assessment based on gill-raker number and lake littoral area. Stomach contents, however, reveal that benthic organisms made up a significant component (33%) of the food. Stomach contents are derived from samples collected at a different time to those used for microwear analysis, and provide only a snapshot of feeding over the few hours prior to capture, leading us to be cautious in interpreting these data. Nevertheless, it is noteworthy that both stomach contents and microwear give a similar signal of mostly limnetic feeding with a benthic component.

Sample 0020, from Corcoran Lake, exhibits a relatively high mean value and no low values for feature density, suggesting benthic feeding. A few teeth exhibit feature lengths that range up into the field of limnetic values, but the feature density for these teeth is higher than observed in limnetic laboratory fishes. Our blind assessment was 'benthic'; gill-raker number, lake littoral area, and stomach content data all provide strong corroboration of this (Table 2).

Discussion

Analysis of microwear in stickleback raised under controlled laboratory conditions provides strong support for both of our initial hypotheses: stickleback teeth

Fig. 3. Mean microwear feature density and mean microwear feature length for dentary teeth from wild-caught stickleback. Solid density ellipses, intended only as a guide to the pattern of distribution of the data on the plot, are drawn to include 80% of the data for each population, assuming a bivariate normal distribution. For comparison, boundaries of the dotted ellipses are 80% density contours of the laboratory dentary data for pooled benthic (coarse and medium substrate) and pooled limnetic treatments. Data for coarse and medium substrate benthic treatments were used because conditions similar to fine and no-substrate benthic treatments are unlikely to be encountered in the wild.

exhibit microwear patterns and those patterns vary systematically according to substrate coarseness and whether fishes feed 'limnetically' or 'benthically'. Furthermore, microwear patterns exhibit a progressive shift in the distribution of data that tracks the different experimental feeding treatments.

Our results support the hypothesis that microwear can be used to discriminate stickleback occupying limnetic trophic niches from those occupying benthic trophic niches, but this is a more complex issue. Microwear in wild populations exhibits a relationship with feeding, and our blind assessments of trophic niche, a stringent test of the use of microwear as a method, were successful in that we correctly predicted the trophic ecology of all but one equivocal wild population. In several cases, based on microwear, we correctly predicted how stomach contents would indicate deviations from fully benthic or fully limnetic diets. What is particularly encouraging about these results is that wild populations exhibit a shift in the distribution of microwear data that is comparable with that observed across the range of limnetic and benthic treatments in the laboratory. The absolute values of wild stickleback's microwear data differ slightly from laboratory data, but the patterns of data distribution allow the wild samples to be ranked according to the degree to which they approach fully benthic or fully limnetic patterns. This makes perfect sense, given that these wild populations are not benthic-limnetic species pairs as such, and is nicely illustrated by the data for the three populations where interpretations based on gill-raker number, lake littoral area and stomach content data are in accord. Mud Lake, Corcoran Lake and Long Lake, in this order, exhibit microwear that is decreasingly benthic in character. This is congruent with the stomach content data for these populations, which contained 93·4%, 79·2% and 32·6% benthic prey items, respectively. Considering all the populations, based on the patterns of microwear distribution Mud Lake is the most benthic, with Corcoran Lake the next most benthic. Stickleback from Long Lake are the most limnetic. Kashwitna, based on microwear, is more intermediate, but closer to the limnetic end of the spectrum, and Lynda has the most mixed signal. This microwear-based ranking of populations is strongly correlated with percentage of benthic prey in stomach contents (Spearman rank correlation $r_s = 0{\cdot}9$, $P < 0{\cdot}037$) and negatively correlated with gill-raker number ($r_s = -1$). Discriminant function analysis lends some support to this ranking: 85% of teeth from the benthic end member and 86% of teeth from the limnetic end member were correctly classified based on microwear data alone.

DIFFERENCES BETWEEN MICROWEAR IN WILD-CAUGHT AND LABORATORY-FED STICKLEBACK

Laboratory fishes were raised under conditions designed to discover whether stickleback teeth exhibit microwear patterns, what the effects of substrate might be, and whether benthic/limnetic feeding influences microwear. The limited range of variables involved is obviously essential for a laboratory investigation of this kind, but will not simulate the conditions that a stickleback would experience in the wild, and it is perhaps surprising that microwear data derived from wild-caught stickleback are comparable with data derived from laboratory stickleback to the degree that they are. Clearly there are differences: microwear feature lengths in wild populations are generally shorter than those found in our experimental fishes, especially when limnetic populations and treatments are compared, and feature density tends to range into higher values in wild-caught fishes. We are uncertain of why this may be, but possible explanations include the fact that unlike the laboratory limnetics, the teeth of wild limnetic-feeding fishes are unlikely never to come into contact with abrasive substrate, especially given that the fishes used in this work were collected in relatively shallow water (in experiments sandy substrate had an effect on microwear in limnetic fish). Laboratory fish were fed particular food, requiring minimal handling, whereas wild fish, especially larger ones with larger gape, will encounter a much larger range of prey items, and given their well known voraciousness, are likely to attempt to eat prey that requires much more handling and repeated biting (Gill & Hart 1994). They may also tackle prey with a harder exoskeleton with the capacity to induce greater microwear. These factors are all likely to increase the number of features per tooth (and hence feature density).

The rate at which teeth are replaced, how that compares with the rate at which microwear patterns form, and how rapidly one pattern may be overprinted by another, should a fish change its diet, are all obviously of central importance in understanding variation in microwear, but all are currently unknown. Work is underway to address these uncertainties, and we are optimistic that this will lead to much better understanding of the relationship between feeding, food and microwear in fishes.

Our results demonstrate that microwear has the potential to be a powerful tool in the analysis of fish trophic ecology, particularly in the analysis of species pairs and niche differentiation. It has advantages over the trophic snapshot provided by the analysis of stomach contents in that microwear reflects feeding and food preferences over a longer period of time. Perhaps most excitingly, microwear has the potential to be applied to extinct organisms and fossils, allowing, for example, the role of trophic ecology, niche partitioning and competition over evolutionary time-scales to be investigated for the first time. This is of special significance to stickleback. Not only are they important as model organisms in studies of evolution and speciation (Schluter 2000; McKinnon & Rundle 2002; Bell *et al*. 2004; Cresko *et al*. 2004; Foster & Baker 2004; Kingsley *et al*. 2004; Colosimo *et al*. 2005), they have a fossil record that is amenable to exactly this kind of long-term

microevolutionary analysis (Bell, Baumgartner & Olson 1985; Bell 1988).

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