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Taihoro Nukurangi

Stock relationships of black oreo in New Zealand waters

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Final Research Report for
Ministry of Fisheries Research Project DEE9801
Objective 1 (*Part two*)

National Institute of Water and Atmospheric Research

November 2000

Final Research Report

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1. Date:	November 2000
2. Contractor:	National Institute of Water and Atmospheric Research Limited
3. Project Title:	Stock relationships of black oreo and smooth oreo in New Zealand waters
4. Project Code:	DEE9801
5. Project Leader:	Peter Smith
6. Duration of Project:	
Start date:	16 March 1999
Completion date:	11 November 2000

7. Executive Summary

Six techniques have been evaluated to determine the stock relationships of black oreo within the New Zealand EEZ. Laboratory analyses were undertaken using all six methods for samples collected from OEO 1, OEO 3A, OEO 4, and OEO 6. Lateral line scale counts and pyloric caeaca counts revealed differences between samples from OEO 6 and the other three OEO management areas. Genetic analyses identified four potential markers in nuclear DNA which showed no significant differentiation among the four management areas. Three regions of mitochondrial DNA were screened for genetic variation; haplotype frequencies showed no significant differentiation among the four management areas. Parasitological examination identified several potential parasite marker species. The relative parasite abundance differed significantly between areas for *Anisakis* sp., *Hepatoxylon trichiuri*, and gill cysts of unknown aetiology. Microchemical analyses of otoliths were carried out by CSIRO, Tasmania. Six elements were measured by electron microprobe analyses (EPMA) at the otolith primordium to obtain juvenile microchemical signatures, and as scans across the otolith to obtain life-history signatures. Fifty two elements were measured by inductively coupled plasma mass spectrometry (ICP-MS), but only six were above the limits of normal detection. Viewed together, the results of the EPMA analyses at the primordium, the life-history profiles and the whole otolith ICP-MS analyses, do not strongly suggest discrete populations of black oreo among the four management areas. Images of whole otoliths were scanned by the Marine & Freshwater Research Institute, Victoria. Twenty-three Fourier descriptors from each end of the otolith were selected to compare the shape of otoliths from each management area. The shape analyses showed that the sample of otoliths from

OEO 3A was morphologically different to those from OEO 1 and OEO 4. Otoliths from areas OEO 1, OEO 6 and OEO 4 were not morphologically different. The same otoliths were subsequently sectioned and the number of zones counted to the settlement zone. A protocol set of black oreo otoliths from previous analyses was used to ensure consistency among readers. The small differences in settlement age (0.1 – 0.2 zones) between areas are obscured by between-reader variation.

Three techniques: genetics, otolith microchemistry, and age at settlement, point to a common pool of juveniles. Further work with these techniques is not warranted for determining black oreo stock relationships within the New Zealand EEZ. Three techniques showed evidence for regional differentiation: the meristic characters, lateral line scale counts and number of pyloric caecae; the abundance of the parasites *Anisakis* sp., *Hepatoxylon trichiuri*, and gill cysts of unknown aetiology; and otolith shape. These techniques measure different biological processes and should be considered for future stock discrimination studies of black oreo.

8. Objectives

Overall Objective:

To determine stock relationships for black oreo (*Allocyttus niger*) and smooth oreo (*Pseudocyttus maculatus*) within the New Zealand EEZ.

Specific objective:

To conduct a pilot study to evaluate a range of techniques for determining stock relationships for black oreo and smooth oreo within the New Zealand EEZ

The specific objective is subdivided into two components with the analyses of smooth oreo samples undertaken during 1998/1999, and the black oreo analyses to be undertaken in 1999/2000. This final research report covers all the laboratory work carried out on black oreo samples during 1999/2000.

9. Methods

Samples of black oreo were supplied from the four oreo management areas in the New Zealand EEZ:

OEO 3A	Chatham Rise west
OEO 4	Chatham Rise east
OEO 1	Snares/Macquarie Ridge, and
OEO 6	Bounty Platform.

Samples had been collected opportunistically, under a previous Ministry of Fisheries project, DEE9701, through the Ministry of Fisheries Observer programme and NIWA research voyages; details are given in Figure 1 and Table 1. Observers were briefed to collect samples from up to 200 black oreo and 200 smooth oreo; ideally based on 2 sets of 25 fish from 4 tows, for each species during the spring spawning season (October-December). Samples were collected from all four oreo fishery areas,

inspite of limited Observer coverage in the oreo fishery, during the 1998–99 fishing year.

The oreo samples were collected and stored under different conditions to allow evaluation of a range of stock discrimination techniques. Formaldehyde was not permitted on commercial vessels. Therefore formaldehyde fixed material, for parasite studies, was collected onboard the *Tangaroa*, or from vessels unloading fresh fish following a short trip. Samples of black and smooth oreo were collected from OEO 1 when the *Amaltal Voyager* unloaded in Dunedin. The chilled fish were subsequently stored in formaldehyde. No formaldehyde samples could be collected from OEO 6 as vessels freeze fish at sea. However parasites can be recovered from frozen fish, and used for stock discrimination studies, provided that preliminary analyses have been undertaken on fixed material to identify new and difficult parasite species. Frozen samples from OEO 6 were supplied for parasite studies.

Table 1: Black oreo sample collection from four management areas in the New Zealand EEZ (W = whole frozen for meristics, otolith shape and parasites; M = muscle frozen for DNA, O = dry otolith for microchemistry; F = whole fish in formalin for parasites)

Area	Vessel	Samples	Numbers	Position
OEO 1	*Amaltal Explorer*	W	25	46 49.0 S 170 39.5 E
		W	25	48 02.1 S 166 04.4 E
		W	25	47 59.7 S 165 07.3 E
		W	25	48 35.5 S 164 52.0 E
		M O	25	48 33.5 S 164 58.5 E
		M O	25	46 49.6 S 170 41.7 E
		M O	25	48 02.1 S 166 04.3 E
		M O	25	47 57.7 S 165 00.7 E
OEO 1	*Amaltal Voyager*	F	100	46 24 S 170 53 E
OEO 3A	*Amaltal Explorer*	W M O	25	45 25.3 S 171 41.1 E
		W M O	25	44 47.0 S 173 15.4 E
		W M O	25	44 58.0 S 174 47.2 E
		W M O	25	44 50.4 S 175 26.1 E
OEO 4	*Tangaroa*	M O	40	44 16.9 S 179 53.4 E
		M O	18	44 18.9 S 178 14.2W
		M O	7	44 30.2 S 177 52.1 W
		M O	9	44 30 S 177 30 W
		M O	15	44 41 S 178 30 W
		W	25	44 15.7 S 179 53.7 E
		W	50	44 16.9 S 179 53.3 E
		W	25	44 18.9 S 178 14.2 W
OEO 6	*Amaltal Explorer*	W O M	25	49 07.4 S 164 17.7 E
		W O M	25	50 16.6 S 163 35.9 E
		W O M	25	50 13.3 S 165 48.5 E

9a. Meristic analyses of black oreo

Lateral line scales were counted in black oreos from the 4 different fishery areas: OEO 1, OEO 3A, OEO 4, OEO 6. Scales were counted in undamaged whole specimens, along the entire lateral line, on the left side. The length and sex of each individual was recorded and the otoliths removed for methods 9e (otolith morphology analyses of black oreo), and 9f (settlement zone analyses of black oreo). The fish were dissected and the pyloric caeca counted.

Differences in scale counts and pyloric caeca counts between sampling areas were tested with by linear regression, controlling for fish length and sex. The analysis used a Poisson error distribution for scale counts and a multiplicative model. The significance of the area effect was tested using an asymptotic χ^2 test on the residual deviance.

9b. Genetic analyses of black oreo

The original method proposed in the NIWA tender was to use microsatellite DNA. This method was replaced by an alternative, and more recent approach, to determining genetic differentiation among populations by screening regions of both the mitochondrial genome and the nuclear genome (in particular non-coding regions, introns) for polymorphism; and applying the variable regions as genetic stock discrimination tools. The change to the proposed method was approved by John Annala, MFish, on 17 April 1999.

While no one genetic method is ideal, the revised approach enables NIWA to screen a wider cross section of the black oreo genome and to focus on the variable regions to determine genetic relationships among regional samples. The overall costs are no greater than those proposed for the microsatellite analyses. The approach remains the same, and still employs the utility of PCR to amplify specific regions of the black oreo genome. Screening mitochondrial and intron regions in toothfish has produced more polymorphisms per unit time than the use of microsatellite DNA, and the mitochondrial DNA markers have revealed greater population differentiation than found with microsatellite DNA in toothfish in the Southern Ocean (Smith and Gaffney, NIWA, unpublished data).

The nuclear DNA intron/mtDNA screening approach is being developed by several fish genetic groups and NIWA have obtained intron primers from Professor Gaffney (College of Marine Sciences, University of Delaware, visiting scientist in NIWA) and from Professor Chow (National Research Institute of Far Seas Fisheries), for evaluation on black oreos. The specific primers are listed in Table 2. Primers for the calmodulin intron (Chow 1998) and the S7 Ribosomal proteins (Chow & Hazama 1998, Chow & Takeyama 1998) amplify homologous regions in a wide range of fishes, and reveal polymorphisms. In addition Dr Moran (Northwest Fisheries Science Center, Seattle) supplied NIWA with the primer sequences for salmonid intron sequences, most of which are currently unpublished. Professor Gaffney supplied NIWA with primers that amplify specific regions of the mitochondrial (mt) DNA in fish, and NIWA have mtDNA primers from previous analyses of orange roughy (Smith et al 1997).

The laboratory analyses were divided into 3 stages. Firstly DNA was extracted from frozen black oreo muscle tissue samples using one of two procedures: a commercial DNA preparation kit based on guanidinium thiocyanate, DNAzol (Cat No 10503-035 GibcoBRL), and Proteinase K digestion and phenol-chloroform extraction, modified from Bruford et al. (1992). Following extraction, DNA was washed with 70% ethanol, precipitated by centrifugation, air dried and re suspended in 40 µl sterile water and stored at –20°C. DNA concentrations were estimated with a Hoefer DyNA Quant fluorometer and a subset of dilutions made to provide 50ngDNA/µl. A suite of primers was used to amplify different regions of the mitochondrial and nuclear genomes (Tables 2 & 3). Amplification reactions were performed in 50 µl volumes overlaid with mineral oil in a Perkin Elmer Cetus DNA thermocycler or in 50 µl volumes in a Perkin Elmer 9600 thermocycler.

For the nuclear genome, primers to conserved exons, of specific single copy nuclear genes that amplify potentially variable introns, were evaluated for black oreos (Table 2). Those primers that amplified successfully and produced single products for mtDNA (Table 3) and single or multiple products for introns were further screened. Some exon primers detect microsatellite or minisatellite inserts within the intron region and appear as one and two band products in ethidium-bromide stained gels, following amplification (e.g., Chow & Takeyama 1998). PCR protocols were modified (annealing temperature, extension temperature, and magnesium concentration) to improve amplification when multiple amplification products appeared in gels. The presence of a single band in the amplified product was taken to indicate optimum amplification conditions.

The second stage of laboratory analyses screened for genetic variation in the amplified products through the use of several methods that included sequencing; targetted RFLP analysis, based on initial sequencing to identify suitable restriction enzymes; random RFLP analysis; and acrylamide gel electrophoresis. Mitochondrial DNA PCR products smaller than approximately 500 bp were screened for polymorphisms with a bank of 5- and 4-base restriction enzymes. Twelve fish (3 fish X 4 management areas) were used in the initial screening. Enzyme digestions were performed in 20 µl volumes for a minimum of four hours, following manufacturers recommendations (New England BioLabs, Beverly, MA). The digested products were separated in agarose gels (from 1.2 to 2 %) and run at 60v for 1.5 to 4 hours. A DNA size ladder was included to estimate size of the amplified fragments. The amplified products were detected with ethidium bromide, which had been incorporated into the gel, and viewed under UV light.

Those introns producing small amplified (<500 bp) regions were screened for polymorphism in (a) acrylamide gels of varying percentage (5, 10, 15%), and (b) acrylamide gradient denaturing gels using acrylamide gradients to separate products differing in size by 2 or more base pairs. In order to determine the optimum gradient gel conditions, one of each pair of primers was re-designed with a GC "clamp", a string of 30 GC bases to the 5' end, to ensure that one end of the nuclear DNA remained paired during gradient electrophoresis. For each sample of amplified product (8 µl), neutral bromophenol blue loading dye was added (2 µl), and loaded into individual wells in a 6.5% acrylamide gel made up with a gradient from 0 to

80% denaturant (7M urea/40% formamide). Gels were run at 150 volts at 65°C for 3-4 hours in TAE buffer. DNA fragments were stained with ethidium bromide and viewed under UV light.

In the third stage of laboratory analyses those regions of the genome revealing polymorphism were tested in larger samples (20–50 fish) from each oreo management area. The aim was to include both mitochondrial and nuclear polymorphisms for population comparisons. Heterogeneity in mtDNA haplotype frequencies in the total data was tested by the χ^2 randomisation test described by Roff and Bentzen (1989) using the REAP package (McElroy et. al. 1992). This method overcomes the problem of a large number of observed haplotypes at low frequency, by comparing χ^2 values in 1000 random rearrangements of the data. Probabilities were estimated from the number of randomisations that were equal to or greater than the observed χ^2 value. Analyses were carried out on individual regions of mtDNA/restriction enzymes and on composite haplotypes. The proportion of haplotype variation due to differentiation between population samples was estimated with Nei's gene-diversity statistic, G_{ST} (Nei, 1973). Sampling error will produce differences in haplotype frequencies, even when samples are drawn from the same population, therefore a randomisation test was used to assess if differences were due to sampling error (Elliott & Ward, 1992). One thousand randomisations were used and the probability was estimated from the number of randomisations that were equal to or greater than the observed G_{ST}.

Genotype frequencies at intron loci were tested for Hardy-Weinberg equilibrium with χ^2 tests carried out on observed and expected numbers of genotypes. A Monte Carlo χ^2 test was used when there were several cells with less than 5 fish (Zaykin & Pudovkin, 1993). Allele frequencies at each locus were tested for heterogeneity among areas with contingency χ^2 tests using the BIOSYS (Swofford & Selander, 1981) and GENEPOP (Raymond & Rousset, 1995) programmes; probability levels were modified by the Bonferroni procedure for multiple tests after Rice (1989). The proportion of intron variation due to differentiation among populations was estimated with Nei's gene-diversity statistic, G_{ST} (Nei, 1973), as described for mtDNA.

9c. Parasite analyses of black oreo

Black oreo were dissected and examined for parasites from each of the 4 management areas: OEO 1, OEO 3A, OEO 4, and OEO 6. Prior to dissection frozen material was thawed overnight while formalin fixed material was washed in water to remove excess fixative. Parasites in the gills and guts were located under a dissecting microscope and the types and numbers of parasites present recorded for each fish. Dissection and examination was undertaken in two stages. The first stage screened 50 fish from each of the 4 areas (total = 200 fish) for all parasites. An additional 178 fish were sampled for all parasites except gut dwelling digeneans in the second stage of the study.

Table 2: Nuclear DNA intron primers evaluated in black oreo. NA = not available for release until the original author has published data in the scientific literature. PCR conditions show the optimum conditions developed for black oreo as at 30 June 2000

Locus	Primer Code	Primer sequences	PCR Anneal temp. ^0C	PCR Extension temp. ^0C	PCR Mg^{++} mM	Source
Glutamate synthetase	Gsex 3F Gsex 4R	NA	50	75	2	Chow unpub.
Crystalline	CRYSex 2F CRYSex 3R	NA	50	72	2	Chow unpub.
Calmodulin	CALMex 4F CALMex5R	CTGACCATGATGGCCAGAAA GTTAGCTTCTCCCCCAGGTT	50	72	2	Chow 1998
Calmodulin	CALMex4FL CALMex5 RL	NA	50	72	2	Chow unpub.
Ribosomal Protein 1	S7ex 1F S7ex 2R	TGGCCTCTTCCTTTGGCCGTC AACTCGTCTGGCTTTTCGCC	50	72	2	Chow & Hazama 1998
Ribosomal Protein 2	S7ex 2F S7ex 3R	AGCGCCAAAATAGTGAAGCC GCCTTCAGGTCAGAGTTCAT	50	72	2	Chow & Hazama 1998
Creatine kinase	CK6F	GACCACCTCCGAGTCATCTC	55	72	2	Chow & Takeyama 1998
	CK7R	CAGGTGCTCGTTCCACATGA				
Lactate dehydrogenase	LDH-F	TACACTTCCTGGGCSATYGGB ATG	58	70	2	Quattro & Jones 1999
	LDH-R	CCCGCCGCCGCCGCCGTAGTC ATATGCTTGTCTC				
Growth hormone	GH2ex4 GH2ex5A	CAGCCTAATGGTCAGAAACT CGTAGTTCCTCCTGACGTTG	52	68	3	Park et al. 1995
Growth hormone	N81	GCNTGTTTTAAGAAGGATATG CAYAA	58	70	2	Gaffney unpub.
	N82	TTNGCNACNNGTNAGGTANGT TTCNACCT				
Dystrophin	N85 N86	TACCAAACGGCGCTAGAGGA ACATTGTCGGAGATGGGAGG	52	72	2	Gaffney unpub.
Neurofibrom-atosis factor 1	N91	TCACNTTYATGCAYCAGGAGT G	52	72	2	Gaffney unpub.
	N92	TACATYTGYTTYTCGTTDATN GTCAT				
Mixed leukemia lineage 25	N67	GCNCGNTCNAAYATGTTYTTY GG	52	72	2	Venkatesh et al. 1999
	N68	ATRTTNCCRCARTCRTCRCTR TT				

Table 3: Mitochondrial DNA primers evaluated in black oreo. PCR conditions show the optimum conditions developed for pairs of primers. The code numbers refer to a list of abbreviated primer names

Region (code)	Primer sequences	Expected Size (kb)	PCR Anneal temp	PCR extension temp. ^{0}C	PCR Mg^{++} mM	Source
ATPase 6	TAAGCRNYAGCCTTTTAAG	0.7	52	72	2	J. Quattro unpub.
(15/16)	GGGGNCGRATRAANAGRCT					
ND1	GCTATTAAGGGTTCGTTTGTTCA	1.1	50	72	2	Gaffney unpub.
(83/84)	CCAAGAGCTTATTTAGCTGACTT TACT					
ND2	AAGCTATCGGGCCCATACCC	1.2	50	72	2	Park et al. 1993
(75/76)	CCGCTTAGYGCTTTGAAGGC					
ND2	AAGCTATCGGGCCCATACCC	1.2	50	72	2	Park et al. 1993
(25/62)	CTGAGGGCTTTGAAGGCCC					Gaffney unpub.
ND3/4L/ 4	AGTATAAGTGACTTCCAATCAC	2.2	50	72	2	Gaffney unpub.
(77/78)	TTAGAATCACAATCTAATGTTTT					
ND4	CAAGACCCTTGATTTCGGCTCA	2.0	50	72	2	Bielawskei & Gold 1996
(73/74)	CCAGAGTTTCAGGCTCCTAAGAC CA					
CO11	AAAGGGAGGAATTGAACCC	0.7	50	72	2	Gaffney unpub.
(79/80)	GCTCATGAGTGWAGGACRTCTT					

The ecological terminology used to describe the distribution of parasites amongst fishes follows that recommended by Bush *et al.* (1997):

- Prevalence = number of infected hosts divided by number of hosts examined,
- Mean intensity = number of parasites found divided by the number of infected hosts, and
- Mean abundance = number of parasites found divided by the number of hosts examined (both infected and uninfected).

The criteria used to determine whether a parasite had potential for use as a stock discriminator followed those described by MacKenzie (1983, 1987) and Lester (1990), with particular emphasis on the following:

- The parasite should have a lifespan, or remain in identifiable form, in the host long enough to cover the time scale of the investigation,
- The parasite should occur at a reasonably high prevalence, arbitrarily set for this study at >10% at one or more sites,
- The parasite should be easily detected and identified, and
- The method of examination should involve a minimum of dissection.

Samples of formalin fixed gills containing cysts of unknown aetiology (CUAs) were examined by histopathology in an attempt to determine their identity. A selection of gill filaments with CUAs were processed and embedded in wax using standard procedures, and 6 μm sections were stained with hematoxylin and eosin and examined under a light microscope.

Statistical analysis

The parasite data were tested for between-area differences in parasite abundances, using a Poisson regression analysis with bootstrapping. Relative risks (which indicate the mean abundance of the parasite in each of the four areas, corrected for the effects of between-area differences in fish length and sex) were estimated for each parasite. Pairwise comparisons of relative risks between areas were carried out.

Parasite abundances were modelled by Poisson regression (a form of GLM). The regression model was:

parasite abundance ~ Poisson (μ = exp(area effect + tow effect + sex effect + length effect)).

Within-area differences in parasite abundance were examined by comparing fish captured in different trawl tows at each of the sites where tow information was available (areas OEO 3A, OEO 4 and OEO 6). Tow effects were nested within area effects and constrained so that the mean tow effect on the linear scale was 1. The length effect was modeled as piecewise linear over the <30 cm, 30-34.9 cm, and 35+ cm length classes, by including terms for length class, length and length x length class interaction. To test whether parasite abundances were affected by within-tow correlation or fish length, the tow and length effects were significance-tested using the standard GLM chi-squared test of residual deviance.

Hypothesis tests of differences between areas were carried out using bootstrapping methods, because of the complex variability structure of fish within tows within areas. For each parasite, the null hypothesis of no between-area differences in parasite abundance was tested using the coefficient of variation (c.v.) of the relative risks as a test statistic. The c.v. was used as a measure of between-area differences because it is scale invariant, so is not dependent on the area chosen as the base. The procedure for estimating the null distribution of the test statistic depended on whether the tow effect had been found to be significant. If the tow effect was significant, then the null distribution was estimated by resampling tows at random, ignoring area, and resampling fish within each tow. If not, then the null distribution was estimated by simply resampling fish at random, ignoring area and tow.

Pairwise comparisons between areas were tested using the ratio of the relative risks as a test statistic (equivalent to the difference between area effects). The calculation of the null distribution of the test statistic used bootstrapping. Tows were resampled from the two areas to be compared, and for the other two areas, and fish were resampled within each tow. In all cases, hypotheses were tested at the $\alpha = 0.05$ significance level and 500 bootstrap iterations were carried out.

Fish sampled from OEO 1 were not divided by tow, and were conservatively assumed to have come from a single tow. This reduced the statistical power of tests of between-area differences for parasites with within-tow correlation, because the

bootstrapped area effect for OEO 1 was based on a single tow and hence was highly variable. Accordingly, OEO 1 was excluded from the calculation of the test statistic when testing between-area differences in parasite abundance.

Canonical analyses

The differences in parasite distribution between areas were also examined using canonical multivariate analysis (Mardia et al. 1979). Canonical analysis identifies the combinations of variables which have the most power to separate groups: in this case it was used to identify the combinations of parasites which have the most power to discriminate between areas, and hence the areas which differ most in terms of parasite abundances. The results are presented as a plot of area means on 'canonical axes' representing linear combinations of parasite abundances, showing the areas which differ significantly from the others.

The first canonical analysis included long lived parasites only, namely CUA's, *Anisakis* sp., dead *Anisakis* sp., liver plerocercoid, *Hepatoxylon trichiuri*, and dead *Hepatoxylon trichiuri*. A second canonical analysis which included data from all parasites was also performed to examine whether parasite community structure varied between fish from different areas. Since fish length had significant effects on the abundances of most parasites, we restricted both analyses to the 30–35 cm length class, which was present in all areas.

Following Lester *et al.* (1985), a log(abundance + 1) transformation was used on the parasite abundance data. A small random number (between -0.05 and 0.05) was added to all abundances, which prevented difficulties with inversion of matrices of rank 0 and did not noticeably affect the results.

The results of the canonical analysis was displayed as a plot of the first and second canonical axes. Following Lester *et al.* (1985), Lester *et al.* (1988), and Sewell & Lester (1995), 95% confidence limits for the points were presented as circles with radius equal to the square root of 5.99 / no. fish in sample (Mardia *et al.* 1979). However these limits are inaccurate in the presence of within-tow correlation. They are based on the incorrect assumption that fish from each site are independently and identically distributed. Tow means are shown on the same axes, to demonstrate the level of between-tow variation.

9d. Microchemistry analyses of black oreo

Otolith samples

Otoliths, sampled specifically for otolith chemistry, were removed from approximately 100 fish from each of the four oreo management areas (Fig.1). The size range of black oreos sampled for otolith chemistry, across all areas, was 25–42 cm (standard length). In an attempt to reduce the influence of temporal variability, the selection of otoliths for chemical analyses was restricted to the size range 33–40 cm. This range was as narrow as possible while allowing sufficient otoliths (minimum of 25) from each area for chemical analyses.

The black oreo were caught on both commercial and research vessels, but post capture treatment of fish and method of otolith extraction was the same for all otoliths. Otoliths (left and right sagittae) were removed from the fish as soon as possible after capture. The otoliths were wiped with paper tissue to remove connective tissue, and then placed into paper envelopes for storage and air-drying.

Electron microprobe analysis (EPMA)

Procedures for embedding, sectioning and preparing otoliths for electron probe microanalysis were similar to those outlined in Gunn *et al.* (1992). Due to the well documented complex and heterogeneous crystalline structure of black oreo otoliths (Davies *et al.* 1988), the sagittae were embedded and sectioned to expose a growth axis from the primordium to the dorsal margin (Fig.2), as the dorsal lobe is considered more 'normal' compared to the ventral lobe. The anterior side of the dorsal and ventral lobes were glued with 5-minute araldite to the base of a 8 mm polyurethane embedding capsule. The capsule was then filled with Araldite D™ resin and placed in an oven at 40°C to aid curing. Once set, embedded otoliths were sectioned using a diamond saw (Struers Accutom), and the sections fixed to round resin backings of the same diameter with Araldite D™. Grinding to the required plane was performed with 400–1000 grade silicon carbide wet/dry papers on a lapping machine. Sections were polished using 6 μm then 3 μm diamond paste, followed by aluminium oxide powder ('Linde B', 0.5 μm. Finished sections were 250–500 μm thick. Sections were ultrasonically cleaned in 100 % AR Grade ethanol between all grinding and polishing stages, and, following final polish, heated at 80 °C for 10 mins to remove any residual moisture. Prior to probing, specimens were coated with a 250 - 300 Å layer of carbon using a Dynavac carbon coater.

Concentrations of the macroelements (> 100 ppm) Na, Sr, K, S, P, and Ca were measured by EPMA using a JEOL JXA-8900R electron probe fitted with 5 wave-length dispersive detectors. Weight fractions of these elements were calculated based on count rates measured from the L_α line for Sr, and the K_α lines for other elements, on standard materials, and the ratios of intensities on standards and otoliths computed using 'PAP' matrix conversion (Pouchou and Pichoir, 1984). Minimum detection limits and confidence intervals for concentration estimates were calculated using the equations provided by Ancey *et al.* (1978). To obtain a spawning ground/larval chemical 'signature', a single-point scan was done adjacent to the primordium on each section, with beam conditions as follows: a defocused spot of 50 μm diameter, 15 kV accelerating voltage, and beam current of 100 nA (= beam power density of 0.76 $\mu W\ \mu m^{-2}$). The edge of the analysis was placed adjacent to the first major growth check surrounding the proteinaceous core of the primordium (Fig.3). On a minority of sections, surface features such as cracks and pits required the scans to be done on the ventral side of the primordium. However, subsequent paired analyses on individual otolith sections (n = 23) were done to examine the variability between dorsal-side and ventral-side analyses and a significant difference was found for both Sr and P (paired *t*-test, $p < 0.05$ for both). As a result data from specimens analysed on the ventral side were excluded. The beam was defocused to reduce specimen damage, and to minimise the probability of misleading results due to very small scale variability in otolith composition around the primordium. Peak counting times for each element were 10 secs for Ca and 60 secs for the other elements, giving a total acquisition time for each analysis of approximately 2 minutes. All samples were

randomly assigned to runs of analyses, so that there was no imbalance in representation of each area in any one run nor at any stage within a run. Standard materials for each element were analysed at the beginning of each day to check for spectrometer drift and this was shown to be negligible over several continuous days of analyses.

On a subset of specimens, single point EPMA analyses were also done on the dorsal margin of the otolith sections to examine the level of heterogeneity in the chemistry of most recent otolith growth. Beam conditions for these scans were as follows: 15 μm beam diameter, 15 kV accelerating voltage, and 25 nA beam current (= beam power density of 2.12 $\mu W\ \mu m^{-2}$)

Life-history traces i.e. lines of point scans across the full section (primordium to dorsal margin, Fig. 4) were done on a subset of sections for left—right sagittae comparisons as part of early "ground-truthing". The elements analysed and beam conditions were the same as detailed above for margin scans, and with 25 μm spacing between points. Analysis time for each point was the same as for the single-point analyses, so a full life-history trace, of say, 120 points on an oreo otolith section took around 4 hours to complete.

Primordium to dorsal margin traces were also done on otolith sections from 4 fish of similar length (and, wherever possible, same sex) from each of the 4 sample areas i.e., a total of 16 life-history traces, using the above mentioned beam conditions and 25 μm point spacing.

ICP-MS analysis

'Sister' otoliths (i.e. opposite member of sagittae pair) to those analysed on the electron microprobe were digested whole and analysed by inductively coupled plasma mass spectrometry (ICP-MS). Otoliths were weighed (to 0.0001 g), placed in new 40 ml polycarbonate vials (precleaned with dilute HCl) and digested with 0.2 g of concentrated HNO_3 (Seastar Chemicals, US) prior to dilution to 20 g final sample mass with ultra-pure water (i.e. 1% HNO_3 final acid concentration, sample dilution factors of 100-2000). Powder-free gloves and plastic forceps were used to reduce possibility of metals contamination. Milli-Q deionised water (≥18 MΩ) was used for otolith pre-cleaning and sample dilution (at CSIRO Marine Lab.), while milli-Q deionised water was further purified in a quartz sub-boiling still prior to use for standard and rinse solution preparation (at University of Tasmania). Indium was also added as an internal standard (concentration 25 $ng\ g^{-1}$) and was prepared from a 1000 $\mu g\ g^{-1}$ single element solution (High Purity Standards, Charleston, US). ICP-MS calibration was performed using standards prepared from a 100 $\mu g\ g^{-1}$ multi element solution (QCD Analysts-Environmental Science Solutions, Spring Lake, US).

Otolith samples were analysed using an *ELEMENT* High Resolution ICP-MS (Finnigan MAT, Bremen, Germany). This instrument has predefined resolution settings ($m/\Delta m$ at 10% valley definition) of 300 (low), 3000 (medium) and 7500 (high). For this study, low and medium resolution modes were used. The ICP-MS was equipped with a Meinhard nebuliser and Scott double pass water cooled spray chamber (standard configuration). Instrument tuning and optimisation was performed

daily using a 10 ng g^{-1} multi-element solution. Aqueous standards covering the concentration range 0-300 ng g^{-1} were used for external calibration. Four standards were used for each element, providing correlation coefficients in excess of 0.995. All standards were prepared in 1% (w/w) HNO_3.

A number of representative acid and water blanks (typically 3) were prepared with each batch of otolith samples digested. Each blank was analysed at the beginning of each sample sequence, and subsequently during the course of the analysis to monitor for increased background levels. Only those elemental concentrations significantly above blank levels (average blank concentration + 3σ) are used in this study for regional comparisons. Early ICP-MS analyses on oreo samples examined concentrations of 52 elements (Sr, Na, K, S, P, Ba, Mg, Fe, Zn, Cu, Al, Rb, Co, Pb, Y, Zr, Nb, Mo, Ag, Cd, Sn, Sb, Te, Cs, La, Ce, Pr, Nd, Sm, Eu, Gd, Tb, Dy, Ho, Er, Tm, Yb, Lu, Hf, Ta, W, Hg,Tl, Bi, Th, U, Sc, Ti, V, Cr, Mn). Of these, the majority were found to be absent or at too low a concentration in oreo otoliths to escape the limitation of nominal minimum detection limits imposed by the blanks. Blank concentrations were found to be the main limiting factor with regard to which elements could be considered in this work. Only strontium (Sr86 andSr88), potassium (K39), sulphur (S32), phosphorus (P31), barium (Ba137 and Ba138), and magnesium (Mg24, Mg26, Mg25) isotopes were able to be measured reliably. Multiple isotopes of some elements were considered to ensure that spectral overlap from isobaric or polyatomic species was minimal. Although scandium (Sc45) and Yttrium (Y89) were routinely above blank + 3σ levels, the concentration of these elements appeared to vary significantly with batch analysed and differences that were too strongly linked to batch to allow confidence in using these elements in an area comparison.

To minimise the effect of instrument drift arising from the high sample Ca content, only small sequences of ~20 samples were analysed at any one time before instrument recalibration. It was intended that samples from collection areas were analysed randomly to help prevent spurious results occurring from day-to-day instrument operation. This did not happen for one batch of analyses, and all specimens analysed on that particular day were from area OEO 1. Subsequent comparisons with other area OEO 1 specimens analysed in batches on other days revealed a strong correlation of variability of some element concentrations to individual batches. For this reason we excluded the data for all elements measured on this day from our discriminant analyses.

Early in the ICP-MS analyses, trials were done comparing whole otolith chemistry to that of sister otoliths pared down to a primordial core. For a pared otolith the exterior layers were ground away, using a hand-held dental drill with grinding tip, to isolate the primordial region and early growth layers. The surface of the remaining portion was wiped with 100% ethanol to remove any residual dust. The distinctive settlement-zone in the otoliths was used as the outer boundary of otolith material to be retained for analysis. Powder-free gloves were worn and plastic forceps used to handle the otoliths during the grinding process to reduce risk of contamination.

9e. Otolith morphology analyses of black oreo

Samples of black oreo otoliths were sent to the Central Ageing Facility (CAF), Marine and Freshwater Research Institute, Victoria in March 2000. These samples had been collected from the four management areas: OEO 1, OEO 3A, OEO 4, and OEO6 (Figure 1 and Table 1). Both sagittal otoliths were supplied in paper envelopes together with biological, trip and station data. The samples registered by the CAF are shown in Table 4.

This project is the second component of the study to use otolith shape as a stock discrimination tool for New Zealand oreo stocks. The first component analysed the shape of smooth oreo (*Pseudocyttus maculatus*) otoliths. From this study it was determined that the clearest indication of stock differentiation was achieved using Fourier analysis on the outline of the shape from the sagittal otolith. The results of the morphometric and otolith circularity analyses were inconclusive, and therefore not applied to the black oreo otoliths.

Table 4: Black oreo otolith samples registered the by CAF

CAF Batch	Area	Trip Date Station No's	Number of samples supplied
748017	OEO 3A	1171/1	100
749018	OEO 1	1171/005,1771/010, 1171/011, 1171/037, 1171/33, 1171/37, 1171/38, 1171/39	99
749019	OEO 6	1171/49, 1171/56, 1171/89	82
749020	OEO 4	1171/020, 1171/022, 171/027	97

A greyscale image of each black oreo otolith was collected using the customised image analysis system developed in the CAF (Morison *et al.* 1998), and saved in tagged image file format (tiff) for subsequent analysis. All images were written to network drives and backed up on compact disk. Images were primarily collected at a magnification of 8.064x (0.63x primary objective, 16x magnification and 0.8x secondary objective). Images of otoliths which were larger than the 640 x 480 pixel display using 8.064x magnification were saved at a magnification of 5.04x (0.63x, 10x and 0.8x).

Otoliths were weighed to the nearest milligram. Biological data, otolith weight and image details, including name and path were combined in a MS Excel spreadsheets. Samples were returned to NIWA for the settlement zone analyses.

Data Collection

The shape analysis of the black oreo otoliths was undertaken using the same program that was developed for the smooth oreo data. The image of the otolith was outlined using an automated pixel gradient tracing routine. The otolith was traced three times. The first trace calculated the perimeter of the otolith and the second trace calculated the area of the otolith. The third trace collected X-Y pairs of coordinate data at 128 equidistant locations around the perimeter of the otolith for the Fast Fourier Transformation (FFT). All automated tracing was started on the apex of the posterio-dorsal lobe (Figure 5) in a counter-clockwise direction.

The X-Y coordinates of marked points and Fourier descriptors written to Excel via Dynamic Data Exchange (DDE). Based on the results of the smooth oreo analysis, the circularity and otolith area data were not used for the black oreo analysis.

A percentage of samples were unusable for Fourier analysis due to breakage of the otolith. The number of samples taken from each batch are shown in Table 5.

Table 5: Number of black oreo otoliths used in analysis of shape by Fourier analysis and morphometrics

Batch	N	Fourier series	% Analysed
748017	100	94	94
748018	99	92	93
748019	82	78	95
748020	97	87	87

Analysis of shape using Fourier analysis

Each of the X-Y pairs of pixel coordinates was expressed as a complex number (real and imaginary numbers) for a Cartesian Fourier transform (Friedland and Reddin 1994). The FFT was calculated in Optimas and the resultant array of 128 complex numbers (Fourier descriptors) saved for later analysis. The 0^{th} descriptor was used to normalise for differences in otolith size and the 1^{st} descriptor to normalise for position and rotation of the image. The remaining Fourier descriptors represented the otolith shape independent of its size, position or rotation.

Determination of the appropriate number of Fourier descriptors

The same number of Fourier descriptors were used to describe the outline of the sagittal otolith for the black oreo as were used to describe the smooth oreo otoliths due to the similar morphology. Because the Cartesian Fourier Transform is asymmetric around the middle frequency, both the 2^{nd} to the 23^{rd} descriptors and the 105^{th} to the 127^{th} descriptors from the original Fourier series were used for subsequent analyses.

Test of differences between areas

The reduced set of twenty-three Fourier descriptors from each end of the series was used to compare the shape of otoliths from each area. The absolute value (harmonic) of each of the Fourier descriptors in the shape vector was calculated as :

$$\text{Harmonic} = (a+b_j)^{0.5}$$

Where :

a = real component of complex number

b_j = imaginary component of complex number

The mean harmonic distance between each of the areas was then calculated. The estimator used in this study to test differences between areas was the square root of the sum of the squared differences in the mean harmonics. This was calculated as:

$$DH_{jk} = \sqrt{\sum_{i=2}^{46} (\overline{H}_{ij} - \overline{H}_{ik})^2}$$

Where :

DH_{jk} = Observed harmonic distance between area j and area k

$\overline{H}_{ij}$ = i^{th} mean harmonic from area j

$\overline{H}_{ik}$ = i^{th} mean harmonic from area k

The matrix of Fourier descriptors for samples from the two areas being compared was thus reduced to one harmonic distance value. The observed harmonic distance was calculated for each of the area comparisons. This was repeated for each of the area comparisons.

A randomization test was then applied to estimate the probability that the observed harmonic distance would occur by chance alone. Samples were randomly allocated to groups and a new harmonic distance calculated. This process was repeated 5,000 times and a distribution of values for randomised harmonic distance was obtained. The probability of obtaining the observed harmonic distance by chance was estimated as the proportion of randomisations for which the harmonic distance was greater than or equal to the observed harmonic distance.

All programs for conducting randomisation tests were written in Visual Basic for Applications (VBA) in MS Excel.

9f. Settlement zone analyses of black oreo

A transition between wide inner and narrow outer zones in the otoliths of black and smooth oreos potentially marks settlement age, at 5–7 years (Doonan *et al.* 1995).

Black oreo otoliths were prepared with standard techniques by embedding in vinyl molds with an epoxy resin. Otoliths were aligned in the mold in the same axis and sectioned with a high speed diamond saw to provide a dorso-ventral section that included the primordium. Otolith sections were polished manually and the polished edge attached to glass slides with thermoplastic resin. Final polishing was undertaken on a Struers Planopol petrographic turntable with a variety of grit sized wet and dry carborundum papers to give approximately 0.3 mm thickness sections. The prepared sections were read under a compound microscope with transmitted light, and the number of zones out to the settlement zone counted to provide an estimate of the age at settlement.

The mean settlement zone ages were calculated for the areas and an ANOVA analysis used to calculate the statistical significance of the between area differences for one reader. Data from the other reader were treated similarly, and the ANOVA analysis repeated. The readings were compared for between reader variation.

9g. Evaluation and overview of data analyses for black oreo

This section reviewed the results on black oreo stock discrimination techniques and provided an interpretation of the statistical analyses. For each technique a significant difference, or lack of significant differences among areas, can be produced by different biological processes, which are summarized below.

Genetic methods: significant differences between area samples indicates that the samples were taken from separate reproductively-isolated stocks. A lack of significant genetic differentiation indicates either one common genetic stock, or two, or more, ecological stocks of similar genetic structure which have not diverged.

Settlement zone: differences between areas for the otolith settlement zone indicates that the samples were taken from different spawning groups, or that the adults are derived from a common larval pool but settle out at different ages by area and remain as discrete adult stocks. No significant differences between areas indicate that samples have been taken from a common stock or two or more discrete stock subject to similar environments.

Parasites: significant differences in parasite distribution between samples of black oreos indicates a lack of mixing among those groups. Differences may be due to the presence of unique parasites or the prevalence and intensity of common parasite species. Lack of differences indicate that either the parasite and its intermediate hosts are widespread, regardless of the stock structure of oreos, or movement of oreos between areas. A knowledge of the parasite life cycle and the distribution of its intermediate hosts would be required to distinguish the biological factors leading to lack of differences.

Otolith microchemistry: area differences in both the primordium and the outer margin demonstrate discrete nursery and adult stocks; a lack of differences in both the primordium and the outer margin demonstrate one stock or several stocks occupying similar environments. No area differences in the primordium, but differences in the outer margin, demonstrate one common nursery stock, or two or more nursery stocks exposed to similar environments, with post recruitment stages exposed to different environments, coupled with limited movement following settlement/recruitment. Differences in the primordium, but no differences in the outer margin, demonstrate different nursery areas, but movement of adult fish among areas or that discrete adult stocks occupy similar environments.

Phenotypic methods: meristic characters that are determined early in the life cycle may show differences between areas resulting from groups of larvae which were derived from the same spawning group then being subject to different environments, or due to larvae derived from discrete spawning groups. For long-lived species it is possible that different year classes, from the same group of spawning adults, could be exposed to different water masses and therefore develop meristic differences. For

morphometric characters that are determined later in the life cycle than meristic characters, such as otolith morphology, differences among samples would indicate that the samples were taken from different stocks exposed to different physical environments. Lack of differences would indicate that the samples were taken from one common stock or two or more stocks exposed to similar physical environments.

10. Results

10a. Meristic analyses of black oreo

The mean lateral line scale counts for areas OEO 1, OEO 3A, and OEO 4 are similar but OEO 6 is higher:

Area	Mean scale count	n
OEO 1	100.8	100
OEO 3A	100.9	100
OEO 4	100.1	99
OEO 6	103.0	82

Similarly, the mean pyloric caeca counts for OEO 1, OEO 3A, and OEO 4 are similar but OEO 6 is lower:

Area	Mean caeca count	n
OEO 1	12.5	98
OEO 3A	12.4	100
OEO 4	12.7	96
OEO 6	11.6	79

As found with the smooth oreo samples, length distributions in the four samples differ widely, but the OEO 6 length distribution is unexceptional, compared to the other areas:

Area	Lower quartile	Mean	Upper quartile
OEO 1	33.3	35.7	37.8
OEO 3A	28.7	31.2	33.2
OEO 4	26.9	28.5	30.2
OEO 6	30.3	33.6	37.1

As found for smooth oreo samples, the counts of scales and pyloric caeca are not closely associated with length. The following data show the correlations with length:

	Scales	Caeca
Males	0.13	-0.05
Females	0.13	-0.04
All	0.14	-0.03

Differences between areas were tested by two linear regressions, regressing scale and pyloric caeca counts on area, length and sex. In both cases, there was a statistically significant difference between areas over and above the effects of length and sex (scales $P = 0.015$, caeca $P = 0.0001$). For scale counts, the length effect was also significant ($P = 0.008$), but not for pyloric caeca ($P = 0.56$).

Both the area effects are driven by the differences between OEO 6 and the other three OEO management areas. The OEO 1 samples were taken from two sub locations within this management area (Table1 and Fig. 1), one sample from the Southland coast (tow 5) and three samples from the Southern Plateau (tows 10, 33, and 37). The mean scale counts by tow for OEO 1 and OEO 6 are:

Area/tow	Mean scale count	n
OEO 1/5*	98.7	25
OEO 1/10	101.4	30
OEO 1/33	100.6	26
OEO 1/37	102.8	19
OEO 6/49	103.4	25
OEO 6/56	102.2	28
OEO 6/81	103.3	29

Removing tow 5 from the OEO 1 area samples then the mean lateral scale count is 101.4. This revised OEO 1 sample is marginally different to OEO 6 ($P = 0.049$)

The mean pyloric caeca counts by tow for OEO 1 and OEO 6 are:

Area/tow	Mean caeca count	N
OEO 1/5*	12.6	25
OEO 1/10	12.5	29
OEO 1/33	12.7	26
OEO 1/37	11.9	19
OEO 6/49	11.9	25
OEO 6/56	11.7	26
OEO 6/81	11.3	28

Removing tow 5 from the OEO 1 samples then the mean pyloric caeca count is 12.4. The revised OEO 1 is significantly different to OEO 6 ($P = 0.001$).

Plotting meristic counts against geographical position there is evidence for a north-south trend among the OEO 1 (excluding tow 5) and OEO 6 samples in pyloric caeca counts (regression $R^2 = 0.825$, $P = 0.012$), but not with lateral line scale counts (regression $R^2 = 0.445$, $P = 0.148$).

10b. Genetic analyses of black oreo

Mitochondrial DNA

Four regions of mitochondrial DNA, ND1, ND2, ND4, and COII, were amplified successfully producing amplified DNA products in the expected size range. Three of these regions of DNA were independently cut with 19 restriction enzymes to find polymorphisms (Table 6). Two other regions of mtDNA (ATPase 6 and ND3/4) produced weak amplification products in black oreo, and were not further tested.

Those regions of mtDNA revealing polymorphism were tested in larger samples from each of the four management areas. The number of haplotypes observed in each management area sample is given in Table 7. Heterogeneity in haplotype frequencies was tested by the χ^2 randomisation test (Roff & Bentzen 1989) by individual restriction enzyme digests on regions of mtDNA (Table 8) and by composite haplotypes (Table 9). Only one region/restriction enzyme revealed a marginal heterogeneity in the data (ND 1 *ScRF* 1); the heterogeneity is non-significant applying a Bonferroni modified *P* for multiple tests. The ND 1 *ScRF* 1 heterogeneity was produced by a rare haplotype that was clustered with 4 observations in OEO 3A and only 1 in the other three OEO management areas. The composite haplotypes are shown in Table 9. There were 3 common haplotypes and a large number of rare haplotypes (Table 9), as frequently observed in marine fishes. There was no significant heterogeneity in the distribution of composite haplotypes among the four management area samples (χ^2 = 101.8, $P = 0.099$). There was no significant heterogeneity in the distribution of composite haplotypes among two pooled management area (OEO 1/OEO 6 and OEO 3A/OEO 4) samples (χ^2 = 32.37, $P = 0.308$). Gene diversity was estimated to be 0.035 which is less than that due to sampling error ($P = 0.208$).

Introns

Results from amplification of black oreo DNA samples with intron primers are summarized in Table 10. Most introns that were successfully amplified produced a single amplification product. One intron produced two or more amplified products that appeared to be the result of a direct allele size polymorphism. The Ribosomal Protein 1 (RP-1) intron showed several different genotypes: a common one band genotype and several two band genotypes, that included the common band; and which were interpreted as the products of a single nuclear genome locus. This locus is weakly polymorphic, with genotype frequencies shown in Table 11.

Table 6: Results of PCR amplifications of specific regions of mitochondrial DNA followed by restriction enzyme digests on a minimum of 12 black oreo (3 fish X 4 management areas) for three regions of mitochondrial DNA. N = number of fish tested; * polymorphism found in larger sample of fish (number of variants/total number of fish tested)

	DNA region (primers)		
Restriction	CO11	ND1	ND2
Enzyme	(Mt 79/80)	(Mt 83/84)	(Mt 75/76)
Alu 1	12	12	*24/124
*Ava*11	12	*5/51	12
Bst NI	12	12	12
*BstU*1	12	24	12
Cfo 1	12	12	12
Dde 1	12	*1/112	12
Dpn 1	12	12	12
*Hae*111	12	*61/127	12
Hinf 1	12	12	*13/150
Msp 1	12	* 13/170	12
*Nci*1	12	*1/48	12
Nde 11	12	12	12
*Nla*111	12	12	12
*Sau*3A	12	12	12
Sau 96 1	12	*57/112	12
*ScrF*1	12	*6/140	12
Rsa 1	12	*3/116	12
Taq 1	12	12	12
Tsp 509 1	*2/60	12	12

Table 7: Numbers of mtDNA haplotypes produced by six restriction enzymes in the four management area samples of black oreo

Restriction enzyme	**mtDNA region**	**OEO 1**	**OEO 3A**	**OEO 4**	**OEO 6**
Alu 1	ND 2	21,2,6	23,1,4	25,2,3	31,3,3
Ava 11	ND 1	11,1	11,2	13, 0	11,2
Hae 111	ND 1	14,6,6,3	24,4,2,5	10,9,5,4	18,6,8,3
Hinf 1	ND 2	25,2,0,2	25,2,0,1	24,4,0,2	29,8,0,0
Msp 1	ND 1	44,2,1,0,1	36,2,3,1,0	32,1,0,0,0	45,0,2,0,0
Nci 1	ND 1	11,1	10,0	12,0	14,0
Sau 96	ND 1	15,10,0,0	8,16,0,1	16,10,0,0	16,19,1,0
ScRF 1	ND 1	35,0,0	40,4,0	25,0,1	34,1,0

Table 8: Black oreo χ^2 randomisation test results and G_{ST} for two regions of mtDNA cut independently with six restriction enzymes. Two restriction enzymes (*Ava* 11 & *Nci* 1) revealing little polymorphism are excluded

MtDNA region/ Restriction enzyme	χ^2	**Probability**	G_{ST}	**Probability**
ND2 *Alu* 1	3.1	0.838	0.014	0.709
ND1 *Hae* 111	11.3	0.263	0.037	0.140
ND2 *Hinf* 1	6.6	0.379	0.022	0.479
ND1*Msp* 1	11.1	0.542	0.023	0.269
ND1 *Sau* 96	10.9	0.199	0.050	0.126
ND1 *ScRF* 1	10.6	0.049	0.036	0.170

Table 9: Black oreo composite mtDNA haplotypes, based on the ND 1 and ND 2 regions of mtDNA cut with six restriction enzymes

		Number of black oreo per area			
Composite	Haplotype	OEO 1	OEO 3A	OEO 4	OEO 6
AAA	AAA	3	1	3	2
AAA	AAB	6	8	4	13
AAA	ABA	1	2	3	4
AAA	ADA	1			
AAC	ABA	2			1
AAC	ABB	1			
AAB	BCB	1			
AAA	ACA	4		2	1
BAA	DBA	1		1	
BAC	DAB	1			
AAC	ADA	2			1
CAA	AAB	1			
ACA	AAA		1		
AAA	ACB		1		
AAC	AAA		1		1
AAA	ADB		1	1	2
AAA	BCA		1	1	3
AAC	ADB		1	1	
AAC	ADA			1	
CBA	AAB		1		
CAA	AAB		1		
AAA	ADA			2	
AAB	DCA				
AAA	ABB			2	
AAA	BAA			1	
ACA	DBB			1	
CBA	BAB				1
AAB	BCA			1	3
AAB	BCB			1	
AAC	ABA			1	
BBA	DBA		1		
AAA	BCC				1

The LDH intron, amplified with clamped primers, appeared as a single locus with one and two band genotypes that appeared to fit a simple genetic model with two alleles (Fig 6 and Table 11). The observed genotypes were in Hardy-Weinberg equilibrium (Table 11). The calmodulin locus, amplified with clamped primers and digested with restriction enzymes, also revealed polymorphism with two restriction enzymes (Table 10). The observed genotypes at the CAM intron revealed with the restriction enzyme *Hinf* 1 were in Hardy-Weinberg equilibrium (Table 11, Calmex *Hinf* 1). A weak polymorphsim was revealed with the restriction enzyme *Alu* 1 (Table 11, Calmex *Alu* 1).

There is no significant heterogeneity in the data for LDH, RP-1, CAM-*H* and CAM-*A* (Table 12) with both the χ^2 heterogeneity test and gene diversity, indicating that the area samples have been taken from populations with similar gene frequencies.

No variation was found at the ribosomal protein 2 intron when cut with ten restriction enzymes; likewise no variation was found at the glutmate synthetase intron and the calmodulin 2 intron when cut with single restriction enzymes (Table 10). Several other intron regions produced single amplified fragments and require further study in order to detect polymorphism (Table 10).

10c. Parasite analyses of black oreo

Black oreos had a greater parasite species diversity than smooth oreos, with 18 parasite species recorded compared to 10 in smooth oreos. The black oreo parasites included 8 species of digenean worms, one species of adult nematode (*Hysterothylacium* sp.) and cestode (*Bothriocephalus* sp.) inside the stomach, intestine and digestive caecae, a monogenean worm *Eurysorchis australis*, chalimus larvae of a copepod *Caligus* sp., and cysts of unknown aetiology (CUAs) in the gills (Figures 6 and 7), larval nematodes (*Anisakis* sp.), and two types of larval cestodes (*Hepatoxylon trichiuri* and an unidentified plerocercous larvae in the body cavity and mesentries, and the isopod *Elthusa neocytta* in the buccal cavity (Table 13). The prevalence, mean intensity and mean abundance of parasites of black oreo from all areas are shown in Tables 13, 14, and 15, respectively.

Several parasite species identified in black oreo may not be reliable population markers. The gut dwelling digeneans are not considered reliable stock discriminators due to their short lifespan in the host, problems with their identification due to poor fixation, and the possibility of bias due to loss of digeneans when the stomach was everted during capture. Eversion of the stomach due to depressurisation was observed in 51.1% of black oreo examined. The adult nematode *Hysterothylacium* sp. and the adult cestode *Bothriocephalus* sp. were also not considered reliable stock markers because their likely short lifespan in the host means their presence may more reflect the recent diet history of the fishes rather than population differences. Other parasites which were not considered reliable stock markers, due to their very low prevalence, included the copepod *Caligus* sp., the monogenean *Eurysorchis australis*, and the isopod *Elthusa neocytta*. These three ectoparasites were also considered unreliable markers because they could be easily dislodged during stomach eversion, or may abandon their host during the trawl retrieval operation, biasing results (Robinson 1982).

Table 10: Results of PCR amplifications with intron primers on black oreo

Intron	Primer f	Primer r	product size bp	Preliminary results
Glutamate synthetase	Gsex 3F	Gsex 4R	280	no variation: *Hae* 111
Crystalline	CRYSex2F	CRYSex 3R	300	single product
Calmodulin	CALMex 4F	CALMex 5R	250	no variation: *Taq* 1
Calmodulin	CALMex 4FL	CALMex 5RL	280, 350	
CALMex (clamped primers)				polymorhic with *Hinf* 1 and *Alu* 1
Ribosomal Protein 1	S7ex 1F	S7ex 2R	800	size polymorphism
Ribosomal Protein 2	S7ex 2F	S7ex 3R	200	no variation: *Alu* 1, *Ava* 11, *BSA H*1, *Cf0* 1, *Hae* 111, *Hinf* 1, *NcI* 1 *Nla* 111, *Sty* 1, *Xho* 1
Creatine Kinase	CK6F	CK7R	220, 250, 310, 350	two loci?
Lactate dehydrogenase	LDH F	LDH R	1000	one polymorphic locus
Growth hormone	GH2ex4	GH2ex5A	900, 1500	two loci?
Growth hormone	N81	N82	220	single product
Dystrophin	N85	N86	290	single product
Neurofibromatosis factor 1	N91	N92	1500	single product
Mixed leukemia lineage 25	N67	N68	1500	single product

Table 11: Observed genotype frequencies and Hardy-Weinberg equilibrium tests for 4 introns in black oreo: (a) Ribosomal protein -1, (b) LDH-1, (c) Calmex *Hinf* 1, and (d) Calmex *Alu* 1

(a) Ribosomal protein-1

Genotype frequencies

RP-1	CC	AC	BC	DC	EC	HW Probability
OEO 1	18	0	1	0	0	0.906
OEO 3A	23	0	0	1	0	0.917
OEO 4	14	0	0	0	1	0.894
OEO 6	19	1	1	0	1	0.990

(b) LDH-1

Genotype frequencies

LDH-1	FF	FS	SS	HW Probability
OEO 1	19	14	0	0.122
OEO 3A	17	12	2	0.952
OEO 4	20	7	1	0.698
OEO 6	19	7	2	0.271

(c) Calmex *Hinf* 1

Genotype frequencies

CAM-*H*	FF	FS	SS	HW Probability
OEO 1	5	13	13	0.572
OEO 3A	4	14	15	0.794
OEO 4	4	14	9	0.701
OEO 6	6	20	16	0.950

(d) Calmex *Alu* 1

Genotype frequencies

CAM-*A*	FF	FS	SS	HW Probability
OEO 1	0	0	10	-
OEO 3A	0	0	12	-
OEO 4	0	1	11	0.880
OEO 6	0	1	16	0.901

Table 12: Heterogeneity χ^2 tests and genetic diversity at four intron loci in black oreo samples from the four management areas

Locus	χ^2	*P*	G_{ST}	*P*
RP1	9.44	0.665	0.012	0.774
LDH-1	1.75	0.625	0.007	0.599
CAM-*H*	0.74	0.864	0.002	0.932
CAM-*A*	1.66	0.646	0.019	0.722
Overall	13.59	0.886	0.012	0.909

The remaining 4 parasites and pathological markers were considered to have potential as stock discriminators, namely living and dead larvae of the nematode *Anisakis* sp. and the cestode *Hepatoxylon trichiuri* in the body cavity, an unidentified larval cestode encysted in the liver, and CUAs in the gills. Histopathology found that the structure of the CUA's (Figures 7 and 8) was similar to that described by Munday and Brand (1992).

Abundances of most parasites depended on fish length, while within-tow correlation was also present in most cases (Table 16). Of the potentially informative markers, all except the liver plerocercoid exhibited significant (α = 0.05) between-area differences in abundance. The estimated relative risk values (which indicate the mean abundance of the parasite in each of the four areas, corrected for the effects of between-area differences in fish length and sex) of all parasites are given in Table 17 and the significant pairwise differences between areas are summarised in Table 18. The CUA's, live *Anisakis* sp., and pooled live and dead *Anisakis* sp. and *H. trichiuri* were the only potentially informative markers which exhibited significant pairwise between-area differences (Table 18).

Canonical analyses

In both analyses the first (y) and second (x) canonical axes were closely related to *Anisakis* sp. and CUA abundances respectively. In the first analysis which included potentially informative (long lived) markers only, area OEO 6 was separated from areas OEO 1, OEO 3A, and OEO 4, both on the first axis, due mostly to its low levels of *Anisakis* sp., and on the second axis, due to low levels of CUA's (Figure 9). Area OEO 3A was slightly separated from areas OEO 1 and OEO 4 on the second axis, due mostly to slightly lower levels of *Anisakis* sp. The plot demonstrated the high between-tow correlations in the abundances of these markers. Tow 3 from area OEO 4 and tow 3 from area OEO 3A were clear outliers in abundance of CUA's,

while tow 2 from area OEO 6 had relatively low *Anisakis* sp. This supports the assertion that the confidence regions for area means are too small, and hence that the apparent differences between areas based on 95% confidence intervals are not necessarily significant.

The second canonical analysis included all parasites to examine whether parasite community structure varied between fish from different areas. This analysis differed little from the first analysis (Figure 10), suggesting that the best parasite discriminators were already included in the first analysis.

Table 13: Prevalence of parasites recovered from black oreos. ^{F} = formalin fixed, ^{FR} = Frozen. (Prevalence = number of infected hosts divided by number of hosts examined.)

Area	OEO 1 ^{F}	OEO 3A ^{F}	OEO 4 ^{F}	OEO 6 ^{FR}	All areas
Number fish examined	100	100	96	82	378
Mean fish length (cm)	36.7	31.4	30.4	32.4	32.8
< 30 cm	1	23	43	23	90
30 - 34.9 cm	21	69	50	33	173
> 35 cm	78	8	3	26	115
Range (cm)	28 – 42.6	27 – 36.7	26.5 – 36.5	23 – 41.5	23 – 42.6
% stomach everted	79	34	72.9	12.2	51.1
Parasite prevalence (%)					
Monogenea					
Eurysorchis australis	0	3	1	1.2	1.3
Digenea					
Digenean 1*	45	39	63.5	9.8	40.5
Digenean 2*	12	22	56.3	26.8	29.1
Digenean 3	55	29	53.1	19.5	40
Digenean 4	0	7	14.6	3.7	6.4
Digenean 5	2	0	0	13.4	3.4
Digenean 6	1	0	0	0	0.3
Digenean 7	0	1	1	0	0.5
Digenean 8	0	1	0	0	0.3
Nematoda					
Anisakis sp.	99	97	97.9	69.5	91.8
Anisakis sp. (dead)	27	14	5.2	6.1	13.5
Hysterothylacium sp.	0	22	2.1	3.7	7.1
Cestoda					
Bothriocephalus sp.	6	17	30.2	0	13.7
Liver plerocercoid	31	9	5.2	24.4	17.2
Hepatoxylon trichiuri	2	20	6.2	1.2	7.7
Hepatoxylon trichiuri (dead)	0	35	8.3	0	11.4
Crustacea					
Caligus sp.	0	0	1	0	0.3
Elthusa neocytta	0	4	7.3	0	2.9
Pathological markers					
CUA's	76	75	62.5	6.1	57.1

* Stomach dwelling digeneans not examined in the second sampling stage.

Table 14: Mean intensity of parasites recovered from black oreos. [F] = formalin fixed, [FR] = Frozen. (Mean intensity = number of parasites found divided by the number of infected hosts.)

Mean parasite intensity	OEO 1 [F]	OEO 3A [F]	OEO 4 [F]	OEO 6 [FR]	All areas
Monogenea					
Eurysorchis australis	0	1	1	1	1
Digenea					
Digenean 1*	4.2	7.7	10.7	2.7	7.6
Digenean 2*	2.1	2.6	7	5.4	5.3
Digenean 3	2.2	1.9	3.1	2.8	2.5
Digenean 4	0	1.3	1.6	9.3	2.5
Digenean 5	1	0	0	5.8	5.1
Digenean 6	1	0	0	0	1
Digenean 7	0	1	1	0	1
Digenean 8	0	1	0	0	1
Nematoda					
Anisakis sp.	20.1	9.8	12.4	10.4	13.5
Anisakis sp. (dead)	3	1.5	1.4	2.8	2.4
Hysterothylacium sp.	0	1.5	1.5	1.7	1.5
Cestoda					
Bothriocephalus sp.	1	2.1	5	0	3.6
Liver plerocercoid	1.9	1.1	1.6	1.6	1.7
Hepatoxylon trichiuri	1	2.6	1	1	2.1
Hepatoxylon trichiuri (dead)	0	1.8	1	0	1.7
Crustacea					
Caligus sp.	0	0	2	0	2
Elthusa neocytta	0	1	1.4	0	1.3
Pathological markers					
CUA's	2.1	2.2	2.3	1.2	2.2

* Stomach dwelling digeneans not examined in the second sampling stage.

Table 15: Mean abundance of parasites recovered from black oreos. [F] = formalin fixed, [FR] = Frozen. (Mean abundance = number of parasites found divided by the number of hosts examined, both infected and uninfected).

Mean parasite abundance	OEO 1 [F]	OEO 3A [F]	OEO 4 [F]	OEO 6 [FR]	All areas
Monogenea					
Eurysorchis australis	0	0.03	0.01	0.01	0.01
Digenea					
Digenean 1*	1.9	3	7	0.3	3.1
Digenean 2*	0.3	0.6	3.9	1.5	1.5
Digenean 3	1.2	0.6	1.6	0.5	1
Digenean 4	0	0.1	0.2	0.3	0.2
Digenean 5	0.02	0	0	0.8	0.2
Digenean 6	0.01	0	0	0	0.004
Digenean 7	0	0.01	0.01	0	0.01
Digenean 8	0	0.01	0	0	0.004
Nematoda					
Anisakis sp.	19.9	9.5	12.1	7.2	12.4
Anisakis sp. (dead)	0.8	0.2	0.1	0.2	0.3
Hysterothylacium sp.	0	0.3	0.03	0.06	0.1
Cestoda					
Bothriocephalus sp.	0.06	0.4	1.5	0	0.5
Liver plerocercoid	0.58	0.1	0.1	0.4	0.3
Hepatoxylon trichiuri	0.02	0.5	0.1	0.01	0.2
Hepatoxylon trichiuri (dead)	0	0.6	0.1	0	0.2
Crustacea					
Caligus sp.	0	0	0.02	0	0.01
Elthusa neocytta	0	0.04	0.1	0	0.04
Pathological markers					
CUA's	1.6	1.6	1.5	0.1	1.25

* Stomach dwelling digeneans not examined in the second sampling stage.

Table 16: Statistical significance and p-values for area, fish length, and within-tow correlation for each parasite and pathological lesion in black oreo. All results below the $\alpha = 0.05$ significance level are denoted by (*). Area OEO 1 is not included in calculation of area effect test statistic because tow information was not available for that area, and significant within-tow correlation was present

Parasite		Area effect		Fish length effect		Tow effect	
Potentially informative markers							
CUA's		(*)	0.008		0.06	(*)	0.003
Anisakis sp.	alive	(*)	0.004	(*)	<0.001	(*)	<0.001
Anisakis sp.	dead		0.63	(*)	<0.001	(*)	<0.001
Anisakis sp.	all	(*)	0.004	(*)	<0.001	(*)	<0.001
Liver plerocercoid			0.97	(*)	<0.001		0.23
Hepatoxylon trichiuri	alive	(*)	0.04	(*)	<0.001	(*)	<0.001
Hepatoxylon trichiuri	dead	(*)	0.05	(*)	<0.001	(*)	<0.001
Hepatoxylon trichiuri	all	(*)	0.02	(*)	<0.001	(*)	<0.001
Other parasites							
Digeneans (pooled)			0.14	(*)	<0.001	(*)	<0.001
Digenean 1			0.12	(*)	0.001	(*)	<0.001
Digenean 2			0.16	(*)	<0.001	(*)	<0.001
Digenean 3		(*)	0.03		0.052	(*)	<0.001
Digenean 4			0.49	(*)	<0.001	(*)	0.001
Digenean 5			0.17	(*)	<0.001	(*)	0.005
Bothriocephalus sp.		(*)	0.03	(*)	<0.001	(*)	<0.001
Hysterothylacium sp.			0.06	(*)	0.002	(*)	<0.001

Table 17: Relative risk values for each parasite in black oreo in each area. Risk values are expressed relative to area OEO 3A, except where the estimated value for OEO 3A was low. (A relative risk of zero indicates that the parasite was not found in the area)

Parasite		OEO 1	OEO 3A	OEO 4	OEO 6
Potentially informative markers					
CUA's		0.73	1	0.93	0.04
Anisakis sp.	alive	0.96	1	1.72	0.55
Anisakis sp.	dead	0.53	1	0.8	0.17
Anisakis sp.	all	0.95	1	1.7	0.54
Liver plerocercoid		0.9	1	1.27	1.11
Hepatoxylon trichiuri	alive	0	1	0.12	0.01
Hepatoxylon trichiuri	dead	0	1	0.16	0
Hepatoxylon trichiuri	all	0	1	0.15	0
Other parasites					
Digeneans (pooled)		0.69	1	2.37	0.82
Digenean 1		0.73	1	1.66	0.07
Digenean 2		0.08	0.17	1	0.52
Digenean 3		1.52	1	2.84	0.93
Digenean 4		0	0.23	0.52	1
Digenean 5		0.01	0	0	1
Bothriocephalus sp.		0.11	0.22	1	0
Hysterothylacium sp.		0	1	0.08	0.07

Table 18: Statistically significant pairwise differences in the abundance of informative black oreo parasites between areas. All tests are at the $\alpha = 0.05$ significance level; NS = non-significant

Parasite		Area	Significant differences
Potentially informative markers			
CUA's		6 vs 3A, 4	(p = 0.03, 0.004 respectively)
Anisakis sp.	Alive	4 vs 3A, 6	(p = 0.03, 0.02 respectively)
Anisakis sp.	Dead	all pairs	NS
Anisakis sp.	All	4 vs 3A, 6	(p = 0.03, 0.02 respectively)
Liver plerocercoid		all pairs	NS
Hepatoxylon trichiuri	Alive	all pairs	NS
Hepatoxylon trichiuri	Dead	all pairs	NS
Hepatoxylon trichiuri	All	3A vs 4	(p = 0.04)
Other parasites			
Digeneans (pooled)		all pairs	NS
Digenean 1		all pairs	NS
Digenean 2		4 vs 6	(p = 0.03)
Digenean 3		3A vs 4	(p = 0.04)
Digenean 4		1 vs 4	(p = 0.002)
Digenean 5		all pairs	NS
Bothriocephalus sp.		4 vs 6	(p = 0.03)
Hysterothylacium sp.		all pairs	NS

10d. Microchemistry analyses of black oreo

Preliminary data on both black and smooth oreo were presented in the first final research report in August 1999; only the black oreo data are presented here.

10d. Stage 1 — Preliminary analyses to establish technique

Electron microprobe

The concentration ranges of the six elements measured by early EPMA analyses, in otoliths of black oreo, are shown in Table 19. The ranges were all within the typical ranges observed in otoliths of other species of teleosts, and almost all were above the calculated minimum detection limits (MDL). Some analyses for P were below the calculated MDL of 100 ppm but confidence in measuring P on the electron probe was buoyed by the close match of left and right otolith life-history profiles (see below) and by general agreement with ICP-MS analyses of that element. In some previous stock discrimination studies Cl has been measured, but, due to increasing concerns about the labile nature of this element in otoliths (Proctor and Thresher 1998), this element was excluded from the electron microprobe analyses.

Table 19: Concentration range of macroelements, as measured by EPMA, in black oreo otoliths. Ranges represent minimum and maximum concentrations as measured at primordium and across otolith sections in ontogenetic profiles. Nominal minimum detection limits are shown for typical otolith element concentrations (calculated using formulae of Ancey *et al.* 1978). Values are in parts per million for all elements, except Ca (%wt fraction)

Element	Nominal min. detection limit	Range
Ca	0.03 %	36 – 41%
Sr	311	1470 – 6340
Na	159	1980 – 4080
K	136	160 – 770
S	149	100 – 410
P	100	0 – 330

Left - right otolith comparisons

Seventeen left - right pairs of black oreo otolith sections were analysed with single-point EPMA scans at the primordium. No significant differences (paired *t* - tests, *P*'s all > 0.05) were found between left and right otoliths for the six macroelements (Na, Sr, S, K, P, Ca).

Life-history profiles were done on 3 left - right pairs of black oreo otolith sections. In these intra-pair profile comparisons (Fig. 11), there was generally close matching in both the frequency and amplitude of ontogenetic variability. Profiles were filtered with a 3 point running mean to remove high frequency noise and reduce influence of random measurement error. The left-right otolith profile match is particularly impressive for Sr in #A5 and #A259. Lack of continuity between some sections of the left and right otolith Sr profiles of #A16 may be the result of limitations imposed by the line targetting procedure on the probe and the associated difficulty in keeping to an exact standardised path across the otolith section. The S and P profiles within otolith pairs were also close matches and surprisingly good for P, given its low concentration range and relatively high theoretical measurement error by EPMA.

The profiles of Na and K for the otolith pairs show a higher level of discontinuity, particularly in the second half of profiles of #A16 and #A259. Some of this may be the result of the aforementioned limitations in targetting, but, given the closeness of match in left and right otolith Sr profiles, for #A5 and #A259 in particular, this seems unlikely. These profile disparities are more likely inherent differences or that arising from post-capture handling effects (see Proctor and Thresher 1998) or a combination of both. The left-right otolith differences were smallest close to the primordium, so a decision was made to continue EPMA analysis of Na and K but to treat any area differences in concentration of these elements with caution.

The intra-pair closeness of the Sr, S, and P element profiles provided sufficient confidence to use these elements in building a primordial 'chemical signature'. The absence of significant differences in primordium chemistry between left and right otoliths in a pair allowed random choice of one otolith for electron probe analysis and the opposite member of the pair for ICP-MS analysis.

Male-Female otolith comparison

EPMA analyses of black oreo otoliths from male (n = 10) and female (n = 9) fish from management area OEO 3A were done at the primordium. There were no significant inter-sex differences (ANOVAs, *P*'s all > 0.05) for all elements except Sr. Male black oreo otoliths had significantly higher (ANOVA, F = 4.69, *P* = 0.045) Sr at the primordium compared to that in female black oreo otolith primordia. Effort was made to standardise, within the limitations imposed by desired size range and availability of otoliths, on sex ratio across area samples in all subsequent analyses.

ICP-MS analyses

ICP-MS analysis of 52 elements in whole and 'pared' otoliths from black oreo showed some to be at relatively high concentrations (100-6000 ppm for Sr, Na, K, S, and P as measured by electron microprobe), some at lower concentration (100 ppb – 100 ppm for Ba, Mg, Fe, Zn, Cu, Al, Rb, Co, Pb, Sc, and Y) but still well within the sensitivity limits of ICP-MS, and many at very low (< 100 ppb) or zero levels which were unlikely to be worth pursuing as contributors to regional chemical signatures. However, as indicated in the Methods section (9e), high blank levels of elements in the 100 ppb - 1ppm range, translated to high nominal minimum detection levels (using the commonly used and accepted criteria of mean blank concentration + 3) and made the results for all sub-ppm elements unusable in subsequent analyses.

One aim of the initial exploratory ICP-MS analyses was to determine whether whole otoliths or pared otoliths provided sufficient mass of material and contained sufficiently high element concentrations to obtain reliable measurement of those concentrations. Very high dilution factors showed that pared otoliths were of insufficient mass to provide reliable measurement of most elements and subsequent analyses were confined to digestions of whole otoliths.

10d. Stage 2 — Analyses for management area comparisons

Electron microprobe analysis at primordium

The size ranges and sex composition of the black oreo samples, representative of fish caught from the four management areas, and from which otoliths were analysed for EPMA, are shown in Table 20. A total of 112 otolith sections of black oreo were analysed by EPMA adjacent to the primordium.

Table 20: Details of fish, across samples, from which otoliths were used in area comparisons of element concentrations, as measured by EPMA

	OEO 1	OEO 3A	OEO 4	OEO 6
N	28	29	28	27
Size range SL (cm)	34–37	33–37	33.1–39.1	33–37
Sex M:F ratio	13:15	15:15	11:17	24:3

The distribution of individual concentration values across all these samples was normal for Na, and Ca, but non-normally distributed for Sr, K, S and P (Lilliefor's probability < 0.05). The overlap in measured concentrations among areas is extensive for all elements (Fig. 12), and only one element, Sr, differed significantly among the four areas (Kruskal-Wallis test, $P = 0.01$). A similar result was obtained from an ANOVA, and posthoc analysis (Fisher's protected least-significant difference, PLSD) indicated significant pair-wise differences for Sr between samples from OEO 1 and two of the other areas (Table 21), OEO 3A ($P = 0.006$) and OEO 6 ($P = 0.037$). Posthoc analyses also showed a significant difference between areas OEO 1 and OEO 4 for Na ($P = 0.016$). Pair-wise tests of correlation (Z-tests) between all elements measured by EPMA in black oreo otoliths (Table 22) showed highly significant relationships between some pairs of elements, most notably Na and K, S and P, and Sr and K (P's all < 0.0001).

Table 21: Summary of ANOVA pair-wise comparisons between areas for element concentrations, measured by EPMA at otolith primordium. Fisher's protected least-significant difference (P values in parentheses). ns = no significant element concentration differences

OEO1	Sr (0.037)		
OEO3A	Ns	Sr (0.006)	
OEO4	Ns	Na (0.016)	Ns
	OEO6	**OEO1**	**OEO3A**

Table 22: Results of tests for significant correlation between elements measured, across all samples, by EPMA. Values are P values from correlation Z-tests. ns = not significant

Sr	0.0036				
K	< 0.0001	0.0001			
S	Ns	Ns	Ns		
P	Ns	Ns	Ns	< 0.0001	
Ca	Ns	0.009	Ns	Ns	Ns
	Na	**Sr**	**K**	**S**	**P**

Linear discriminant function analysis (LDFA), using the elements measured by EPMA, suggested very little between-area discrimination (Wilk's lambda = 0.83, $F_{15,287} = 1.34$, $P = 0.18$) and canonical loadings did not give rise to any factors showing significant differences across areas (Fig. 13). Plots of samples in discriminant factor space (Fig. 14) show a broad overlap across all areas, with no clear separation by any one group. This lack of separation is reflected in low 'correct-classification' rates for individuals from all four areas; only 46%, 24%, 46%, and 30%, from areas OEO 1, OEO 3A, OEO 4, and OEO 6 respectively, were correctly assigned by the LDFA to their source group.

Life-history profile comparisons

The length of life-history profiles varied between otoliths (see Table 23), which was partly the result of differences in otolith size, but also due to the aforementioned limitations on achieving a standard growth plane in the final otolith section. Even sections produced from otoliths of identical size may vary slightly in length, depending on the angle of initial embedding and subsequent section. The assumption was made that fish within the sample size range (33 - 35cm) for life-history traces were of similar age, and, for comparison purposes (Figs. 15 and 16), scaled the profiles to fit the same length (2.5 mm), which was close to the mean length of all profiles (2.56 mm, n = 19). In doing this scaling, it was also assumed that the amplitude of the ontogenetic variations across a section varies little with minor differences in the absolute length of the profile i.e. there will be no significant difference in peak heights and trough lows in a life-history profile of say 94 points (2.35 mm) compared to a profile of 100 points (2.5 mm) across the same growth trajectory.

Overall, there was a high level of variability in ontogenetic patterns both within and across areas for Sr, P, and S (Fig. 15a-c). For none of these elements were the 4 profiles of one area clearly distinguishable as a group from the 4 profiles of other areas. Means of concentrations at arbitrarily fixed intervals (every 5 points) along the profiles for each area showed no consistent significant differences for Sr and P (Fig. 16). For sulphur (Fig. 16), the profile of OEO 4 does appear consistently lower than the other 3 areas for the majority of the profile length and this difference is confirmed by an ANOVA (p = 0.0089) on the means for each profile for each area i.e. a comparison of the mean of concentration across the length of each profile. Such differences must be viewed cautiously given the small sample size (n = 4) of profiles for each area. The differences that were evident from post-hoc results (Fisher's PLSD) of the ANOVAs of point means (grouped by area) along the profiles were generally small, and where significant, were usually the result of one profile having an atypical feature e.g. very large peak in Sr early in the profile of #A300. Na and K were excluded from area profile comparisons because of the aforementioned concerns over left - right otolith disparities in their concentrations.

Table 23: Details of black oreos and respective life-history profiles measured by EPMA; 25 μm steps, primordium to dorsal margin. Fish length = Standard length

Specimen	Fish length (cm)	Sex	Area	Profile length Number of analyses	mm
A107L	34	M	OEO 1	114	2.850
A160R	34	F	OEO 1	83	2.075
A163L	35	M	OEO 1	103	2.575
A166L	34	F	OEO 1	102	2.550
A297L	34	F	OEO 3A	98	2.450
A300R	34	F	OEO 3A	111	2.775
A319R	34	M	OEO 3A	97	2.425
A365R	35	M	OEO 3A	132	3.300
A5L	34	M	OEO 4	104	2.600
A5R	34	M	OEO 4	106	2.650
A16L	34	M	OEO 4	94	2.350
A16R	34	M	OEO 4	94	2.350
A57R	33	F	OEO 4	100	2.500
A77R	34	M	OEO 4	113	2.825
A211L	34	M	OEO 6	94	2.350
A235R	34	M	OEO 6	101	2.525
A250L	35	M	OEO 6	111	2.775
A259L	34	M	OEO 6	97	2.425
A259R	34	M	OEO 6	94	2.350

Electron microprobe analysis at margin

EPMA analysis was to be undertaken on the dorsal margins of all black oreo otolith sections, to obtain a chemical signature for most recent otolith growth and determine whether these signatures were sufficient to characterize the area from which the fish were caught. However, due to the fragile nature of the black oreo otoliths, the majority of otoliths had suffered minor damage along the margin, which probably occurred both during otolith extraction from the fish, but also possibly during the grinding and polishing stages in their preparation for EPMA. Although this damage was minor, in the form of small chips and nicks, it was sufficient in many sections to make it difficult, and often impossible, to target the EPMA scan on otolith growth layers that could be judged as definitely true margin growth.

EPMA analyses at the margin, for the same six elements analysed at the primordium, were therefore done only on 41 black otolith sections, spread across the four management areas. These sections had clearly defined, undamaged dorsal margins. An area comparison of these margin analyses for Sr, S and P showed no significant heterogeneity for the latter two elements, but Sr was significantly higher in the margins of OEO 3A sections compared to those of OEO 1 and OEO 6 (ANOVA, Fisher's PLSD, $P = 0.0334$, and 0.0063 respectively).

Whole otolith ICP-MS analysis

A total of 118 whole black oreo otoliths (excluding 16 specimens which were part of the suspect batch mentioned in Methods), representative of fish caught from the four management areas (Table 24), were analysed by solution based ICP-MS. The majority of these were 'sister' otoliths to those analysed with EPMA at the primordium.

Table 24: Details of fish numbers and sex, and their otolith weights, across samples, used in area comparison of otolith composition, as measured by ICP-MS of whole otoliths

	OEO 1	OEO 3A	OEO 4	OEO 6
N	21	30	30	37
Sex M:F ratio	10:11	15:15	13:17	31:6
Otolith weight range (g)	0.0164 – 0.0877	0.0211–0.0621	0.0229–0.0698	0.0164–0.0643
Otolith weight Mean ±SD	0.0339 ±0.0162	0.0347 ±0.0112	0.0381 ±0.0020	0.0349 ±0.0120

In common with the EPMA analyses at the primordium there is broad overlap in the individual values of concentrations within each of the six elements across regions (Fig. 17). The distribution of individual values across all these samples were normal for Sr, K and S but non-normally distributed for P, Ba and Mg (Lilliefor's probability <0.05). Kruskal-Wallis tests show significant differences across areas for 4 of the 6 elements (Sr, Ba, S, and Mg, P for all < 0.05). Potassium shows no significant differences across samples in both Kruskal-Wallis tests and ANOVA (P values > 0.05). Phosphorus also shows no significant differences across samples in Kruskal-Wallis tests ($P = 0.16$) but is significant in an ANOVA ($P = 0.03$), but this latter result is generated by one unusually high value in the OEO 1 sample. Post-hoc analysis of the ANOVAs (Table 25) reveals the OEO 6 sample to be clearly different in chemistry to the other 3 management areas in several of the elements; lower Sr compared to all other areas, lower Ba compared to OEO 3A and OEO 4, lower Mg compared to OEO 1 and OEO 4, and lower S compared to OEO 4 (Fisher's PLSD, $P < 0.01$ in all pair-wise comparisons). The OEO 1 sample is also significantly lower in Ba and P compared to OEO 3A, and significantly higher in Mg to compared to OEO 3A.

Not surprisingly, black oreo otoliths were, in general, very similar in chemical make-up to those of smooth oreos (*Pseudocyttus maculatus*) which were analysed in the first stage of this project. However, there was one striking difference between the chemistry of the two species; Ba concentrations of black oreo otoliths were significantly lower than in smooth oreo otoliths (mean ± SD, 25.6 ± 11.1 ppm for black compared to 78.8 ± 27.2 ppm for smooth).

Pair-wise tests of correlation between all elements measured by ICP-MS in black oreo otoliths (Table 26) raise the expectation that some of these multi-element patterns of variability, particularly Sr and Ba ($P < 0.0001$), may be useful for discriminating stocks.

Table 25: Summary of ANOVA pair-wise comparisons for element concentrations measured by ICP-MS among area samples of black oreo otoliths. Fisher's protected least significant difference (*P* values in parentheses)

OEO 1	Mg (0.0016) Sr (0.0002)		
OEO 3A	Ba(<0.0001) Sr(< 0.0001)	Ba (0.0015) Mg (0.0008) P (0.0150)	
OEO 4	Ba(< 0.0001) Mg(< 0.0001) S (0.0045) Sr (0.0002)	Ba (0.0065) P (0.0410)	Mg(<0.0001)
	OEO 6	**OEO 1**	**OEO 3A**

Table 26: Results of tests for significant correlation between elements measured, across all samples of black oreo otoliths, by ICP-MS. Values are *p* values from correlation Z-tests. ns = not significant

Sr	0.0443				
S	Ns	0.0244			
P	0.0006	ns	Ns		
Ba	Ns	< 0.0001	Ns	ns	
Mg	0.0043	ns	Ns	< 0.0001	Ns
	K	**Sr**	**S**	**P**	**Ba**

As expected from the above ANOVA results, the LDFA (Wilk's lambda = 0.37, $F_{18, 308} = 7.14$, $P < 0.0001$) shows highly significant differences for all 3 discriminant factors (Fig. 18). In canonical space this translates to a weak separation of OEO 4 and OEO 6 individuals from the majority of those from OEO 1 and OEO 3A (Fig.19), and a strong separation of OEO 4 and OEO 6 from each other. This latter separation is largely driven by Factor 1, which has high canonical loadings for Sr, Ba, and Mg. There is a weak separation of OEO 1 individuals from those of OEO 3A, largely driven by Factor 2, which also has a high canonical loading for Mg. Four individuals from OEO 4 were conspicuous outliers, well removed in canonical space; the result of these otoliths having significantly higher Mg concentration compared to all other specimens analysed (mean of 27.2 ppm compared to mean of 8.9 ppm respectively). One individual in the OEO 1 sample was a conspicuous outlier in the Factor 1 vs Factor 3 canonical plot; the result of this otolith having the unusually high P concentration (376 ppm compared to mean of 123 $\pm$ 20 ppm for all other otoliths). The correct-classification rates from the LDFA, for OEO 1, OEO 3A, OEO 4, and OEO 6 were 43%, 57%, 60%, and 78% respectively. Twenty-nine of the 37 individuals from OEO 6 were correctly assigned to their source group, whereas 18/30 from OEO 4, 17/30 from OEO 3A, and only 9/21 from OEO 1 were correctly classified.

There was significant variation across areas in size of fish sampled for otoliths used in ICP-MS analysis. The fish in OEO 3A and OEO 4 samples were significantly smaller (ANOVA, $P = 0.0003$) than those from OEO 1 and OEO 6 (Fig. 20). Ba and Mg concentrations were significantly negatively correlated with fish size (correlation z-test, $P < 0.0001$ and = 0.031 respectively, areas pooled). However, even though there was a weak, but significant positive relationship between fish size and otolith weight, there was no significant difference across areas for otolith weight (ANOVA,

P = 0.612) (Fig. 20). This suggests that the significant Ba and Mg differences between area samples are not driven by the differences in fish size.

Of the four area samples, that of OEO 6 had by far the largest imbalance in sex ratio (Table 23), with 84% being otoliths from male black oreo. However, sex was not significantly correlated with any of the elements analysed (Correlation Z-test, P's all > 0.05) both within this sample and within all samples pooled.

10e. Otolith morphology analyses of black oreo

Fourier analysis of otolith shape

The observed harmonic distances between the management zones calculated between the four management areas are shown in Table 27.

Table 27: Black oreo observed harmonic distances between each of the four management zones

Area	OEO 3A	OEO 4	OEO 6
OEO 1	0.019846	0.008015	0.015986
OEO 3A		0.019425	0.017359
OEO 4			0.010615

The results of the randomization tests of harmonic distance suggest that the shapes of the black oreo evidence complex stock structuring. Only one management area (OEO 3A) shows significant differences at P<0.05 between areas OEO 1 (P=0.0142) and OEO 4 (P=0.018). Management area OEO 3A is not significantly different to OEO 6 (P=0.055). The probabilities of obtaining the observed harmonic distance by chance, between all management zones are shown in Table 28, Figures 21 and 22.

Table 28: Probability that the randomized harmonic distance was greater than or equal to the observed harmonic distance in each of the black oreo management zones

Area	OEO 3A	OEO 4	OEO 6
OEO 1	0.0142	0.740	0.459
OEO 3A		0.018	0.055
OEO 4			0.070

The distribution of randomized harmonic distances for each of the management area comparisons are shown with the observed harmonic distance presented in Figure 21.

10f. Settlement zone analyses of black oreo

Otolith sections were prepared for 378 black oreo otoliths from areas OEO 1, OEO 3A, OEO 4 and OEO 6. The sections were viewed with transmitted light using a compound microscope at 63 or 100 times magnification. Counts were made blind (without knowledge of fish length, area or of the count made by the other reader) by two experienced oreo otolith readers. Otolith sections with agreed interpretations from a previous study ("protocol set") were examined before starting on the new otolith sections. Readers counted only the inner dark pre-settlement zones. Pre-settlement zone counts ranged from 3 to 6, normally 4 or 5.

Mean and standard deviations of counts by reader by area were calculated and an ANOVA analysis of between area differences in zone counts was carried out (Table 29). Both readers found small differences between area OEO 3A and the other three areas. The zone counts by Reader 1 showed a difference of 0.1 zone between OEO 3A and the other three areas, which was not significantly different. The zone counts by Reader 2, however, showed a statistically significant difference of 0.2 zones between OEO 3A and the other three areas (Table 29).

The two readers were well-calibrated; there was very little difference between their overall (all areas) mean zone counts, which were 4.27 and 4.29 respectively. The between-reader variability was low; the variance of the differences between the two readers was 0.27, as compared to 1.08 for the smooth oreo (Smith et al 1999). For 75% of otoliths, the two readers returned the same reading. The differences between readers did not vary by area to a statistically significant extent, as they did for the smooth oreo readings.

There is a small difference in pre-settlement zone count between OEO 3A and OEO 1, 4, and 6, of the order of 0.1–0.2 zones. The two readers differ as to whether the difference is statistically significant, due to between-reader variation, which is minor but sufficient to obscure the small observed difference. The readers appear to be consistently well-calibrated and the small differences between them are due to random variation only.

Table 29: ANOVA results for pre-settlement zone counts in black oreo otoliths from 4 fishery areas. Each otolith was read independently by two readers

Reader 1

Area	Number otoliths	Mean settlement zone	Standard Deviation
OEO 1	85	4.21	0.49
OEO 3A	92	4.35	0.52
OEO 4	83	4.27	0.56
OEO 6	74	4.24	0.49

Non-significant between-area differences ($P = 0.34$)

Reader 2

Area	Number otoliths	Mean settlement zone	Standard deviation
OEO 1	91	4.23	0.42
OEO 3A	93	4.45	0.58
OEO 4	87	4.22	0.44
OEO 6	80	4.25	0.54

Significant between-area differences ($P = 0.005$)

10g. Evaluation and overview of techniques for determining stock relationships of black oreo

Meristic overview

Most meristic characters have a genetic basis but the environment may modify the expression of the character. Variation in meristic characters is determined during the early larval stages and characters are influenced by temperature, salinity, oxygen, pH and food availability (Taning 1952, Barlow 1961, Lindsey 1988). Significant differences in meristic characters may occur among year classes when environmental conditions fluctuate between years (Schmidt 1921, Blouw *et al.* 1988). It was this environmental component, that can produce temporal variation in meristic characters, that encouraged researchers to apply selectively neutral genetic markers for stock discrimination in the 1980s and 90s.

The lateral line scale counts and the pyloric caeca counts show significant regional differentiation, with OEO 6 different to all other area samples. The differences imply that the OEO 6 sample was taken form a different biological group of black oreo to all other areas, and that this group of fish had experienced different environmental conditions during their early larval stages.

Stock discrimination of black oreo in Australian waters found evidence for regional differentiation in lateral line scale and pyloric caeca counts (Ward *et al.* 1996), but was based on small sample sizes (see Tables 30 and 31). A sample from southern Tasmania (55 fish, mean scale count 102.3) had higher counts than small samples from Western Australia (10 fish, mean 96.8) and the Chatham Rise (9 fish, mean 97.8). The lateral line scale counts for Southern Tasmania (Ward et al. 1996) fall within the range found in the New Zealand EEZ (Table 30). The New Zealand EEZ scale counts are higher than the count reported for the Chatham Rise (Ward et al. 1996), but the Chatham Rise count was based on only 9 fish. Pyloric caecae counts also showed significant regional differentiation, but were again based on small sample sizes (Table 31). It seems likely that the regional differences reported by Ward et al (1996) were due to sampling error.

Table 30: Lateral line scale counts in black oreo from New Zealand and Australia (ST = southern Tasmania, and WA = Western Australia). Australian and Chatham Rise (CR) data from Ward *et al.* (1996)

Area	mean	Stdev	N	Min	max
OEO 1	100.8	5.8	100	88	117
OEO 3A	100.9	4.9	100	90	113
OEO 4	100.1	5.5	99	90	115
OEO 6	103.0	6.0	82	91	118
ST	102.3	4.9	55	93	116
WA	96.8	5.77	10	90	107
CR	97.8	5.33	9	88	106

Table 31: Pyloric caecae counts in black oreo from New Zealand and Australia (ST = southern Tasmania, and WA = Western Australia). Australian and Chatham Rise (CR) data from Ward *et al.* (1996)

Area	mean	Stdev	N	Min	max
OEO 1	12.5	1.4	98	9	17
OEO 3A	12.4	1.6	100	9	17
OEO 4	12.7	1.5	96	9	18
OEO 6	11.6	1.8	79	7	17
ST	11.6	1.4	198	9	16
WA	11.7	1.4	7	10	14
CR	13.3	0.9	9	12	15

Ward *et al.* (1996) concluded that the meristic differences might represent year class differences rather than stock differentiation, as has been reported in several marine species in the northern hemisphere (e.g., Harden Jones 1966). It seems unlikely that the observed meristic differences could be driven by year class differences, due to the very slow growth rates of black oreo (Doonan *et al.* 1995). The observed differences in scale and pyloric caeca counts, in our larger samples, would argue against one common pool of larvae and juveniles in the New Zealand EEZ, unless the OEO 6 sample was dominated by fish from different year classes with significantly different counts. There is a significant effect for length with scale counts ($P = 0.008$), but not pyloric caeca counts ($P = 0.56$). However the mean size of black oreo in the OEO 6 sample was intermediate between OEO 1 and OEO 3A and so the meristic differences are not due to a simple length, and presumably age effect.

The major hydrological features of the Southern Plateau are the subtropical front and the subantarctic water mass. It is possible that the samples from OEO 1 and OEO 6 were collected from different water masses, with the more northerly samples from OEO 1 influenced by the subtropical convergence water and the more southerly samples from OEO 6 by subantarctic water. The water masses on the Southern Plateau may act as barriers to species distributions of some southern species such as southern blue whiting *Micromesistus australis* and the Antarctic cod *Notothenia* spp. In black oreo there is evidence for a significant north-south decrease in pyloric caeca counts in the OEO 1 and OEO 6 samples from the Southern Plateau, but not in the lateral line scale counts. However it is unlikely that this level of population sub-division is produced by sub-groups of black oreo that remain as discrete units from larval to adult stages and the north south differences in one of two meristic characters may represent sampling error.

Genetics overview

The lack of population differentiation at the nuclear intron loci, and with mtDNA markers, indicate little genetic differentiation among the black oreo samples. Ward *et al.* (1998) reported a lack of genetic differentiation in smooth oreo samples from Western Australia, Tasmania, and the Chatham Rise with a different suite of allozyme and mtDNA markers. Such results are typical of a marine species with a potentially long pelagic juvenile stage and opportunity for gene flow, even if post recruits show little movement among areas (e.g., the armorhead, *Pseudopentaceros wheeleri* Martin *et al.* 1992; and spiny lobster, *Panulirus argus* Silberman *et al.*

1994). The lack of overall genetic differences could be due to present levels of gene flow, particularly by juvenile movement, or to historical gene flow between stocks which are currently isolated but have not evolved genetic differences. Thus the overall genetic data support, and do not reject the null hypothesis of a single stock of black oreo within the New Zealand EEZ.

Ward *et al.* (1998) reported weak evidence for genetic differentiation between black oreo samples from the Chatham Rise and Tasmania with a mtDNA marker. The difference was just significant with a heterogeneity χ^2 analysis (P = 0.034), but not with a gene diversity analysis (P = 0.223). A similar genetic marker found no significant genetic difference among smooth oreo samples from Western Australia, southern Tasmania and the Chatham Rise (Ward *et al.* 1998). Genetic differentiation in black oreo, over this wider spatial scale, would imply restricted ocean wide gene flow allowing the evolution of discrete stocks. Only 23 black oreo juveniles have been recorded from the New Zealand EEZ and these exhibited similar pelagic characteristics to smooth oreo juveniles (James *et al.* 1988, McMillan unpub. obs.). An additional 6 juveniles came from the stomach of a butterfly tuna (*Gasterochisma melampus*) caught between New Zealand and South America, 45° 46'S, 113° 32'W, (McMillan unpub.obs.) Radiocarbon data on black oreo otolith cores led Morison *et al.* (1999) to conclude that black oreo juveniles must live at high latitudes, although none have been reported from the Southern Ocean. The weak genetic differentiation among black oreo from the Chatham Rise and Tasmania stocks needs to be tested with additional samples throughout the range of the species and additional genetic markers, but is outside the scope of this project.

If the limited juvenile collection is indicative of black oreo juvenile distribution then it is possible that there is a wide scale movement during the pelagic phase. Given the west to east circulation of the subtropical convergence then the longevity of the juvenile pelagic phase may promote long distance gene flow creating a single genetic stock of black oreo, especially within the New Zealand EEZ. However there may be discrete ecological stocks with restricted movement post recruitment.

In shallow water teleosts a negative correlation between genetic differentiation and dispersal ability has been reported (Waples 1987). A similar relationship probably applies to deepwater species. The lack of genetic differentiation in black oreo within the New Zealand EEZ contrasts with orange roughy *Hoplostethus atlanticus* which shows significant genetic differentiation among spatially isolated spawning groups from the Chatham Rise, east coast South Island, and Puysegur (Smith *et al.* 1997). Orange roughy eggs hatch near the bottom (Zeldis et al 1994) and it is assumed that the early juveniles are demersal reducing the potential for extensive gene flow. In hoki *Macruronus novaezealndiae* there is little genetic differentiation among spatially isolated spawning groups within the New Zealand EEZ (Smith *et al.* 1996), and hoki juveniles remain in the pelagic environment for several months (May and Blaber 1989) promoting gene flow.

Parasites overview

The parasite diversity of black oreo was greater than that of smooth oreo, with 18 species of parasite and cysts of unknown aetiology (CUA's) compared to 10 for smooth oreo (Smith *et al.* 1999). This could be a result of their more diverse prey, which include a larger proportion of amphipod and decapod crustaceans compared to smooth oreos, which feed predominantly on salps (Clark *et al.* 1989). Crustaceans are well known as intermediate hosts for nematode and cestode parasites of marine fishes (Rohde, 1984), and the increased prevalence and abundance of *Anisakis* sp., *Hysterothylacium* sp., *Bothriocephalus* sp., *Hepatoxylon trichiuri* and cestode plerocercoid larvae in black oreo are likely to be related to a greater proportion of crustaceans in the diet.

Only 4 parasites and pathological markers were considered to have potential as stock discriminators. These were living and dead larvae of the nematode *Anisakis* sp., living and dead larvae of the cestode *Hepatoxylon trichiuri* in the body cavity, an unidentified larval cestode encysted in the liver, and CUAs in the gills. Larval *Anisakis* sp. and *H. trichiuri* have been used as biological tags in deepwater fish (e.g., Lester *et al.* 1988, Sewell and Lester 1995). The presence of apparently dead and degenerating *Anisakis* and *Hepatoxylon* larvae in some of the larger black oreos suggests that these parasites are potentially useful for stock discrimination even after their death, because they remain identifiable post mortem. The larval cestode also appears to fulfill the criteria of a useful parasite marker. Histopathology of the CUA's (Figures 7 and 8) was similar to that described in red cod *Pseudophycis bachus* from Tasmania and English sole *Paraphrys etulus* and flathead sole *Hippoglossoides elassodon* from British Columbia (Munday and Brand 1992). These enigmatic bodies are common in marine fish, but their cause remains unknown (Munday and Brand 1992). However CUAs appear to fulfill the criteria for stock discriminators (longevity suited to the time scale of the investigation, based on histopathological evidence; occurrence at a reasonably high prevalence; easily detected and identified; and requiring a minimum of dissection).

Uncertainty with identification could limit the use of *Anisakis* sp. as a stock discriminator. Four types of morphologically distinct *Anisakis* larvae are found in marine fishes (Smith & Wootten 1978). All of the *Anisakis* larvae found in the previous smooth oreo parasite study had an oblique ventriculo-intestinal junction and a rounded mucron bearing tail and hence conformed to the morphology of *Anisakis* type 1 larvae. Genetic studies have shown that there are morphologically indistinguishable sibling species of *Anisakis* (Mattiucci *et al.* 1997, Nascetti et al. 1986). Two species of *Anisakis* have been identified in samples of barracouta *Thyrsites atun*, red cod *Pseudophycis bachus* and blue cod *Parapercis colias* from New Zealand waters (Mattiucci *et al.* 1997). Preliminary results with allozyme markers in *Anisakis* from black oreos have shown that there are two types of *Anisakis* present (Smith unpublished data). However the allozyme technique is limited to frozen material, and many enzymes were denatured in the frozen-thawed specimens of *Anisakis* removed from black oreo.

All parasites, except type 3 digeneans, showed significant variation in abundance with fish length, while all, except the liver plerocercoid, exhibited significant variation in abundance between trawl tows. Jones and Gibson (1993) also reported

tow to tow variation in numbers of parasites of orange roughy *Hoplostethus atlanticus*. Therefore determination of between area differences must account for variation due to both fish length and tow. When this was done, 3 of the 4 potentially informative parasites displayed significant pairwise variation in abundance between sampling areas. These included CUA's, *H. trichiuri*, and *Anisakis* sp. CUA's were significantly less abundant in OEO 6 than in OEO 3A and OEO 4, with OEO 1 appearing similar to OEO 3A and OEO 4 but with too few samples to show a statistically significant result. *Anisakis* sp. were significantly more abundant in OEO 4 than in OEO 3A and OEO 6, and appeared less abundant in OEO 6 than in OEO 3A and OEO 1, although there were too few samples to show a significant result. *H. trichiuri* was significantly more abundant in OEO 3A than in OEO 4, and was not present in quantity in areas OEO 1 or OEO 6, although the latter results were not significant.

Some of these differences were depicted graphically in the first canonical analysis, which included data only from long lived, potentially informative parasites. However, the canonical analysis was weakened by it's incapacity to incorporate tow information into the 95% confidence intervals generated. Incorporation of tow means into the graphical representation of the analysis highlighted some of the large between-tow correlations in the abundances of the potentially informative markers used. The outlying tow means depicted in the analysis for areas OEO 3A, OEO 4 and OEO 6 clearly demonstrated that the 95% confidence regions for the area means in the canonical analyses were too small, and hence the apparent differences between areas are not necessarily significant. The statistical significances of these differences are better tested by the randomisation tests (results given above). The results of the second canonical analysis, which included data from all parasites to examine for differences in parasite community structure, were not visibly different to those of the first, suggesting that the long lived parasites included in the first analysis were the best discriminators.

In light of the significant tow to tow variation found in black oreo, future sampling should be structured around small samples of fish from a large number of tows from each area. For a sample size of 100 fish per area, a design of 10 fish from 10 tows would be a suggested starting point.

Otolith microchemistry overview

The composition of otoliths of black oreo is similar to that of most marine species (Campana *et al.* 1994, Edmonds *et al.* 1995, Thresher *et al.* 1994). The constituent elements could be grouped into three broad categories based on mean concentration. The first, at concentrations > 10% by weight includes calcium, carbon and oxygen, the second covering the range of 100–5000 ppm includes Na, Sr, K, Cl, S, and P (sometimes referred to as macroelements), and a third includes trace elements present at concentrations 10–100 ppm (e.g., Ba, Mg, S, Y) plus many < 10 ppm (e.g., Zn, Cu, Rb, Fe, Ni, Co, Sc).

Electron microprobe analyses in the primordial area of black oreo sections revealed few significant differences in element concentrations across the samples from the four management areas. Strontium concentrations, from post-hoc tests of the ANOVA, suggested that the OEO 1 sample was significantly different from OEO 3A

and OEO 6 samples. Similarly, Na concentrations suggested that the OEO 1 sample was significantly different from the OEO 4 sample. However, the results of the LDFA on primordial chemistry analyses, suggest no significant separation across area samples.

The comparison of profiles across the full life-history of a sub-sample of otoliths from similar sized fish from each fishery area also showed little between-area heterogeneity. There was as much variation among profiles for each area as there was between areas. Comparisons of the early portions of the profiles did not suggest different geographic origins of these fish, and, apart from a significantly lower concentration of S in the OEO 4 profiles compared to other areas, there were no consistent profile differences that would suggest dispersal to different areas and separation into discrete stocks.

Compared to EPMA analyses at the primordium and EPMA life-history profiles, ICP-MS analysis of whole black oreo otoliths showed more significant differences in constituent chemistry across area samples, particularly with respect to areas OEO 6 and OEO 4, compared to the other area samples. The separations in canonical space and correct classification rates, arising from the LDFA, were not compelling, but do suggest moderate to weak separation of OEO 4 and OEO 6, and with OEO 1 and OEO 3A showing most overlap. However, the separation of OEO 4 from the other area samples is largely the result of the high Mg concentrations of 4 otoliths in a sample of 30.

Strontium and sodium were the only elements to show any across area differences at the primordium and only for OEO 1 compared to the other area samples. Of all the elements that have been used in previous teleost stock structure studies, strontium is the one element that appears to be unambiguously linked to the environments which fish experience in their lifetimes (Secor 1992, Tsukamoto *et al.* 1998). Many of the other elements, including sodium and potassium, are now viewed with caution, because of their apparent lability during post-capture treatment of fish and handling of otoliths (Proctor and Thresher 1998), and/or because of the difficulties associated with accurate and reproducible measurement of them (Campana 1999, Thresher 1999). The disparities shown in the left - right otolith profile comparisons of sodium and potassium in this study corroborate the need for caution in using them in stock structure studies.

Accepting the Sr differences at the primordium as being significant, albeit based on only one set of samples at one point in time, one conclusion might be that the OEO 1 fish had different origins to those of the other areas. However, the separation of OEO 1 fish is not strong enough to be compelling, and furthermore, the life-history profiles do not support such a conclusion. Accepting common origins of some fish, the higher level of heterogeneity seen in whole otolith chemistry suggests some divergence into different environments later in life. Of the four area samples, those of OEO 6 approach the greatest level of similarity among themselves and strongest separation relative to the other three groups.

The lack of significant consistent differences between areas in the EPMA life-history profiles, compared to the results of the whole otolith ICP-MS, is surprising. Sr differences in whole otolith chemistry are a major contributor to the separation of

area OEO 6 from the other three areas and yet no such differences are evident from the life-history profiles. This disparity may be largely the result of the sample of life-history profiles being too small to achieve a meaningful picture, but it may also be influenced by differences in Sr loadings across different growth axes in the otolith structure. All growth axes, both from the dorsal and ventral halves of the otolith, are amalgamated in the whole otolith analyses, whereas our life-history profiles only sample one specific growth axis in the dorsal half. The preferred method for comparisons would be life-history profiles along the standard growth axis, but on a much larger sample from each area (e.g. on all the 112 otolith sections prepared for EPMA analyses). However, the resources required to do this were beyond the scope of this project.

Viewed together, the results of the EPMA analyses at primordium, the life-history profiles and the whole otolith ICP-MS analyses, do not strongly suggest discrete populations for any of the four management areas. The lack of heterogeneity in chemistry at the primordium is suggestive of the black oreo sampled having had common geographic origins, but this rests on two important assumptions: (1) the physical and/or chemical environment of spawning grounds/larval habitats influence the rate of element incorporation into the otolith growing surface; and (2) there is sufficient heterogeneity in the physical and chemical characteristics of the Southern Pacific Ocean, the Tasman Sea, and the Southern Ocean to impart between-area differences in the otoliths of black oreos if they were spawned in different areas. The first assumption is now accepted as truth for some elements, most notably for Sr (Secor 1992, Tsukamoto *et al.* 1998, Thresher 1999), and to a large extent for Mg and Ba (Campana 1999, Thresher 1999). Judging the validity of the second assumption is difficult, as there is little current knowledge of where black oreos are spawned. However, given the known heterogeneity in, at least, the temperature of water masses of the four management areas and their surrounds (see discussion in Smith *et al.* 1999), this assumption seems justified. Also, the relatively higher level of heterogeneity seen across management zones in the primordia of smooth oreo otoliths (Smith *et al.* 1999) suggests there is certainly potential for habitat ('geographic') specific chemical signatures in oreo otoliths.

Whereas the EPMA life-history profiles did not clearly depict common origins, followed by migration to the four management zones, the pattern of heterogeneity of whole otolith ICP-MS analyses do suggest some geographic separation through the post-larval stages. In addition, EPMA analyses at the otolith margin, albeit small in sample size, showed a significant difference in Sr concentration for area OEO 3A fish compared to the other areas which corroborates mature-age geographic separation of at least this sample.

Otolith chemistry is largely only informative for stock structure if regional heterogeneity in the environment is significant. Homogeneity in chemistry is not proof of commonality, unless it can be shown that the habitats that support black oreo populations are sufficiently diverse in environmental characters to expect different chemical signatures, if discrete stocks exist.

A more definitive picture of black oreo origins, migration history, and stock structure may be possible through examination of otolith chemistry, but it could only come after analysis of replicate samples from each management area. Due to resource

constraints, this study has not attempted to examine interannual nor seasonal variability in chemical signatures of otoliths from fish in the management zones, and a previous study on another deep-water teleost, *Hoplostethus atlanticus* (Thresher *et al.* 1999), has shown that temporal variability can be substantial and can have major implications for conclusions on stock relationships.

Otolith morphology overview

The results obtained from randomized harmonic distances from the shape analysis of the sagittal otoliths from black oreo otoliths from each of the four management zones indicate that the OEO 3A sample was taken from a separate stock from OEO 1 and OEO 4 (Fig. 22). The similarities in otolith shape between areas OEO1, OEO 6 and OEO 4 do not suggest differences in stock structure based on the Fourier – Randomization technique.

The results are comparable to the stock structuring found in the smooth oreo using the same discrimination technique (reported in Smith *et al.* 1999). Samples from area OEO 3A were significantly different from samples from OEO 1 in both species. However black oreo differ from smooth oreo in terms of stock discrimination using otolith shape, in that black oreo show a significant difference between OEO 3A and OEO 4. This was not found in the study of smooth oreo otolith shape. Management areas OEO 1 and OEO 4 show no differences in otolith shape, in both species of oreo. The difference between OEO 3A and OEO 6 was highly significant in the smooth oreo, but not in black oreo. The smooth oreo OEO 3A sample was taken from the northern Chatham Rise.

Otolith shape may differ among ages, sexes and year-classes (Castonguay *et al.* 1991; Campana & Casselman 1993) and variation in growth rates may be the most significant cause of significant variation in otolith shape (Campana & Casselman 1993). The age, and hence the growth rate, of black oreo used in these analyses is unknown, as is the extent to which the observed differences can be attributed to these factors. The Fourier descriptors were standardized for otolith size before analysis, which will at least partially remove any age effect on the differences. However, the extent to which otolith shape reflects growth rate rather than just age, may still contribute to the observed differences. The mechanisms which drive the differences in otolith morphology between stocks are still poorly understood (Smith *et al.* 1998). Further sources of variability may be dependant on where the samples are taken from within each of the management areas.

Morphological differences are determined later in the life cycle than meristic characters and the observed differences in otolith shape are likely to be determined post recruitment, so that the technique describes ecological rather than genetic stocks. The ecological stocks of black oreo determined by otolith shape could be derived from a common pool of juveniles, but which exhibit little geographical movement following recruitment

Settlement zone overview

The differences in settlement ages (marked by the wide inner and narrow outer zones in the otolith) between areas are small, 0.1–0.2 zones. The differences in settlement age between areas, in particular OEO 3A and the other areas are minor and are

obscured by between-reader variation. Therefore the technique of testing age of settlement between management areas is not appropriate for stock discrimination of black oreo within the New Zealand EEZ. A lack of significant differences between areas indicate that samples have been taken from a common stock or two or more discrete stocks subject to similar environments or of similar genetic background.

In smooth oreo the age at settlement, based on mean zone counts, was 6.38 and 6.52 for two readers (Smith *et al.* 1999). These data suggest that smooth oreo settle at a greater age than black oreo, and remain in the pelagic environment for about 2 years longer than black oreo. A longer pelagic phase may promote greater juvenile advection in smooth oreo than black oreo, reducing the opportunity for genetic differentiation among regional smooth oreo stocks. The distributions of both smooth and black oreo juveniles are known from relatively few specimens, but most of the smooth oreo juveniles have been found between 60–68°S, while no black oreo juveniles have been found south of the Antarctic convergence zone (James *et al.* 1988, McMillan unpub. obs.). The longer pelagic phase in smooth oreo is not reflected in wider adult distribution, as adults of both species have similar overall distributions around New Zealand and Australia.

11. Conclusions

Meristics The lateral line scale counts and the pyloric caeca counts show significant regional differentiation, with OEO 6 different to all other area samples. The differences imply that the OEO 6 sample was taken from a different biological group of black oreo to all other areas, and that this group of fish had experienced different environmental conditions during their early larval stages.

Genetics There is no evidence for genetic sub division among the samples from the four management areas, based on intron loci and mitochondrial DNA. The data support and do not reject the null hypothesis of a single genetic stock. The results are typical of marine species with long pelagic juvenile stages.

Parasites Significant pairwise between-area differences in abundance were recorded for 3 potentially informative parasites and suggest that each area sample was taken from a different biological group. However significant tow to tow variation has hindered the analysis and must be accounted for in future experimental designs. Ideally future sampling should be based on small samples of fish from a large number of tows; 10 fish from 10 tows is a suggested starting point.

Otolith microchemistry The results of the EPMA analyses at the primordium, the life-history profiles and the whole otolith ICP-MS analyses, do not strongly suggest discrete populations of black oreo among the four management areas. There is weak evidence that black oreo in OEO 3A have higher strontium levels in the outer margin of the otolith than black oreo in other areas.

Otolith morphology The shape analyses showed that the sample of otoliths from OEO 3A is morphologically different to those from OEO 1 and OEO 4. Otoliths from areas OEO 1, OEO 6 and OEO 4 are not morphologically different.

Settlement zone The small differences in settlement age (0.1–0.2 zones) are obscured by between-reader variation. Therefore the technique of testing age of settlement between management areas is not a useful tool for determining stock relationships of black oreo within the New Zealand EEZ, and no further work should be undertaken to determine black oreo stock relationships with this technique. Black oreo settle at a lower age than smooth oreo, indicating different life history strategies between these two species.

Stock relationships of black oreo within the New Zealand EEZ No consistent stock differences were found among the four management areas with the six different techniques, but this might be expected because the techniques measure different biological processes. Otolith shape suggests that the sample from OEO 3A was different to those from other areas, in particular geographically neighboring samples from OEO 1 and OEO 4. There was weak support for OEO 3A being different to other areas in strontium levels in the outer otolith. The two meristic characters both suggested a difference between the sample from OEO 6 and the other 3 area samples, again there was weak support for this differentiation with strontium levels in the whole otolith analyses. The parasite data are more complex and show tow to tow variation but suggest that there are differences among all four areas.

The limited information on black oreo juveniles demonstrate a wide distribution, which coupled with longevity in the pelagic phase (determined from settlement zone counts), and the west to east circulation of the sub Antarctic convergence, suggest extensive gene flow to produce one genetic stock of black oreo, particularly within the New Zealand EEZ. Within this single genetic stock there may be isolated ecological stocks with restricted movement of post settlement adults. The lack of heterogeneity in chemistry of the otolith primordium provides support for a single stock hypothesis, but equally supports a multi stock hypothesis in which the physical and/or chemical environment of the spawning grounds and larval habitats are similar.

An evaluation of the six techniques used to determine stock relationships of black oreo, shows that three techniques: genetics, otolith microchemistry, and age at settlement, point to a common pool of juveniles. Further work with these techniques is not warranted for determining black oreo stock relationships within the New Zealand EEZ. Three techniques showed evidence for regional differentiation: the meristic characters, lateral line scale counts and number of pyloric caecae; the abundance of the parasites *Anisakis* sp., *Hepatoxylon trichiuri*, and gill cysts of unknown aetiology; and otolith shape. These techniques measure different biological processes and should be considered for future stock discrimination studies of black oreo. Two techniques, otolith morphology and parasite abundance, are likely to be responding to environmental differences during the post settlement period, while the meristic differences are determined during the larval stages.

12. Publications

Progress report September 2000.

13. Data storage

Meristic data. Scale count data are stored in an Excel spreadsheet on the NIWA Greta Point computer P drive.

Genetic data. Hard copies are stored in laboratory record books at NIWA, Greta Point. Population genetic data are stored in the GENEPOP and REAP programmes, and in EXCEL files on the NIWA Greta Point computer H: drive.

Otolith microchemistry data. Data consist of 4 Excel spreadsheets. These data have been burnt to CD and stored in the MFish Data Management offline data archive system, and supplied to NIWA. In addition all data are stored on Craig Proctor's Power PC Macintosh 7600/120. As a backup, electron microprobe data is stored on the PC attached to the JEOL JXA-8900R electron microprobe at CSIRO Division of Minerals, Clayton, Victoria (under the care of Mr. Ian Harrowfield) and as hard-copy printout in Craig Proctor's office at CSIRO Marine Research in Hobart.

Parasite data. All data for population analyses are stored on the NIWA Greta Point computer P drive; hard copies of parasite descriptions are stored in laboratory record books at Greta Point.

Otolith shape data. All data, images and programs written for the delivery of the otolith shape analysis are saved on a network drive at the Central Ageing Facility (MFRI); this network is fully backed up on a tape drive once a week. Further backups are done on an incremental basis as any data changes on the drives on a nightly basis. The otolith images have been stored on two Kodak CD-R's, original and backup. The original has been supplied to NIWA for long term storage.

Otolith settlement zone data. Otolith sections are stored in the NIWA fish ageing laboratory at Greta Point; otolith reading data are stored in an Excel spreadsheet on the NIWA Greta Point computer P drive.

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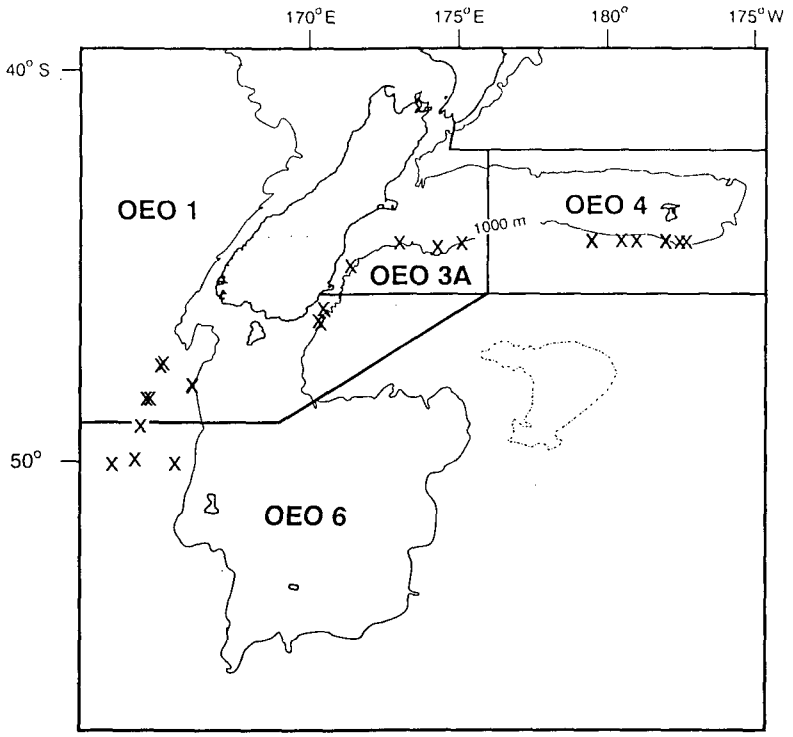


Figure 1. Location of black oreo samples collected from four management areas for evaluation of stock relationships

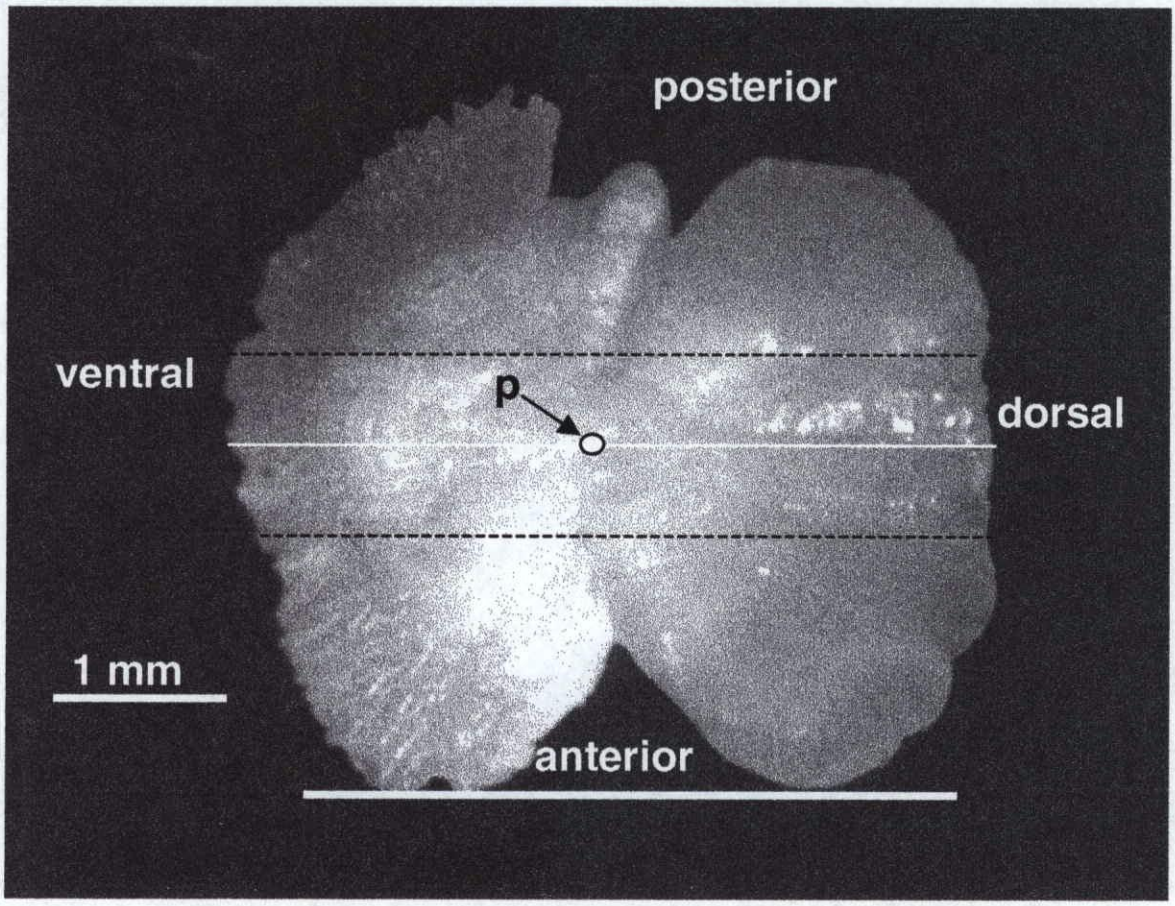


Figure 2. Sagitta otolith of a black oreo showing the orientation in which otoliths were embedded (thick white line represents base of embedding mould). Dotted black lines indicate level of cuts with diamond saw, and thin white line is the final growth plane achieved after grinding and polishing the section. p = primordium

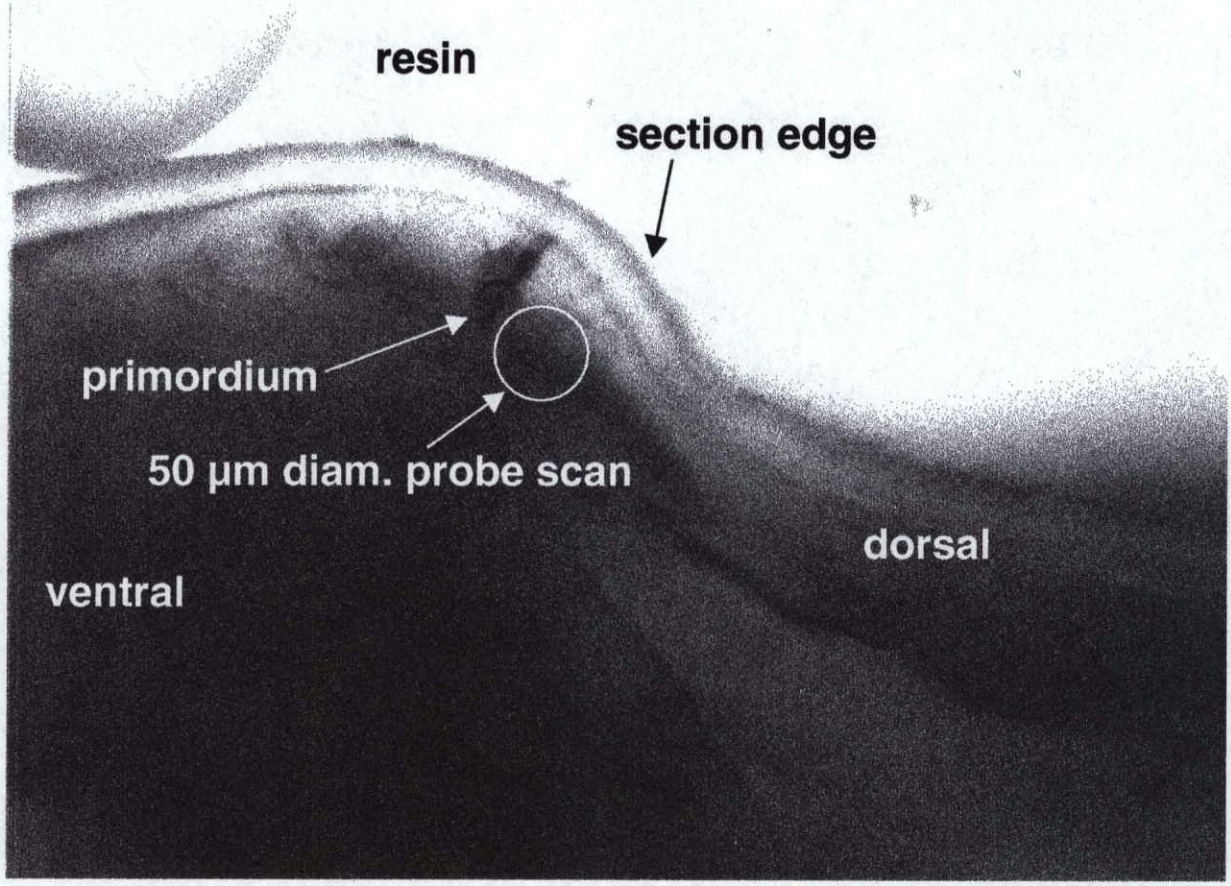


Figure 3. Transmitted-light microscope image of the primordial area of a section of a black oreo otolith showing position of probe scan.

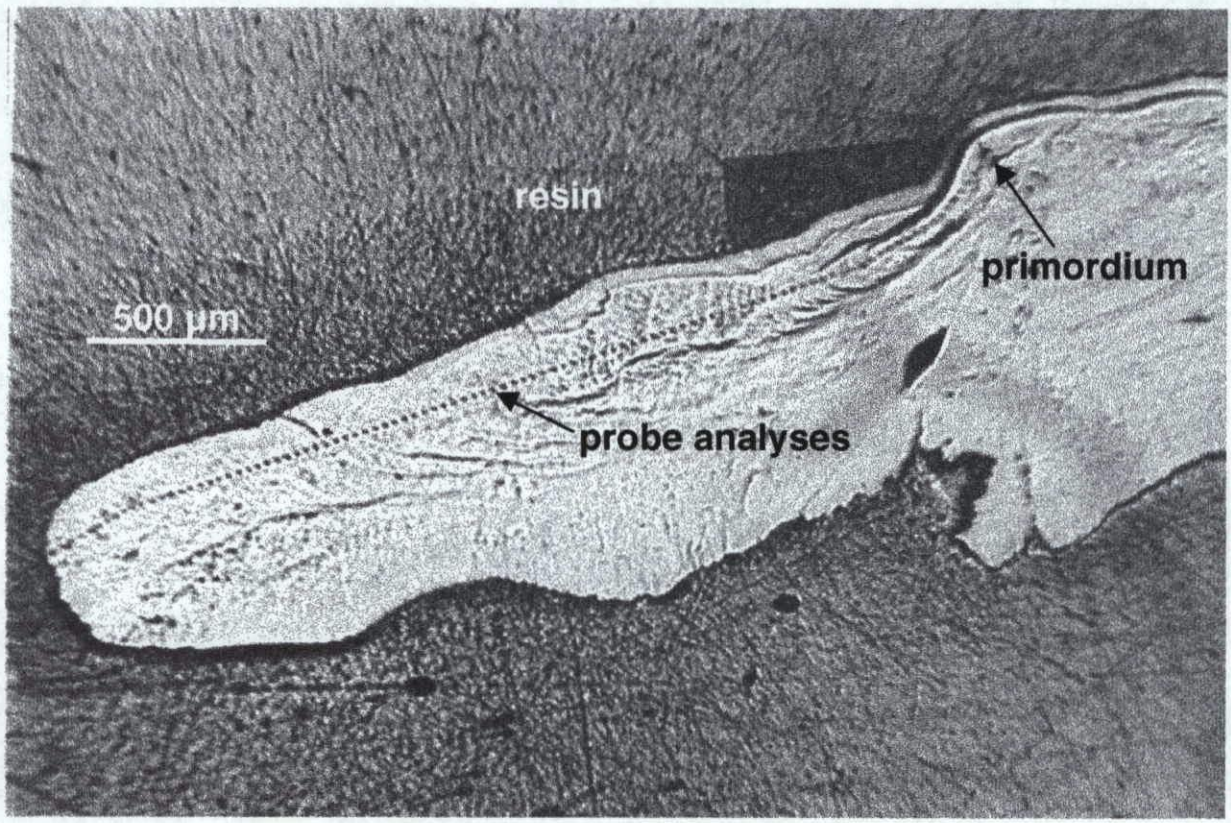


Figure 4. Incident-light micrograph of section of a black oreo otolith showing lines of electron microprobe analyses from primordium to dorsal margin.

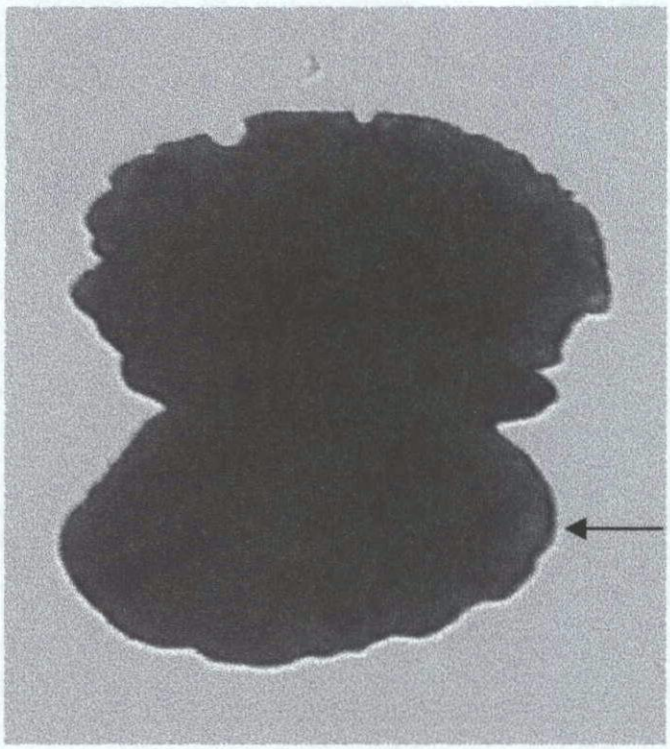


Figure 5. Black oreo otolith. The arrow indicates the starting point of the Fast Fourier Transformation (FFT).

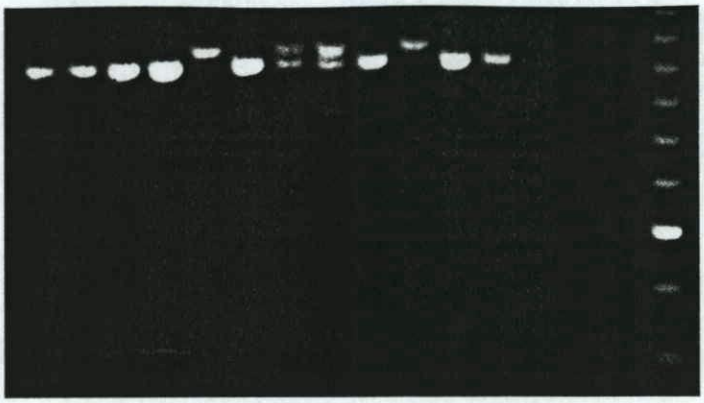


Figure 6. LDH intron polymorphism in black oreo. Amplified products have been separated in an agarose gel. The right hand column contains a DNA size ladder.

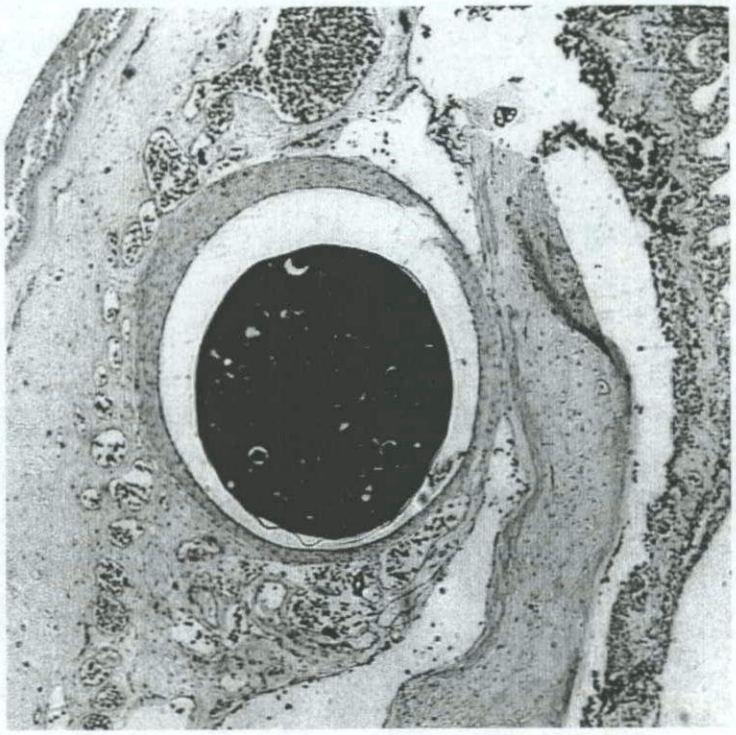


Figure 7. Histopathology of a cyst of unknown origin (CUI) from the gills of a black oreo, showing distortion of the gill lamellae caused by a fibrous or cartilaginous capsule which surrounds a dense, intensely basophillic cyst.

Figure 8. Higher magnification of the dense body which comprises the CUI, showing basophillic appearance interdispersed with swirls of eosinophillic material in black oreo.

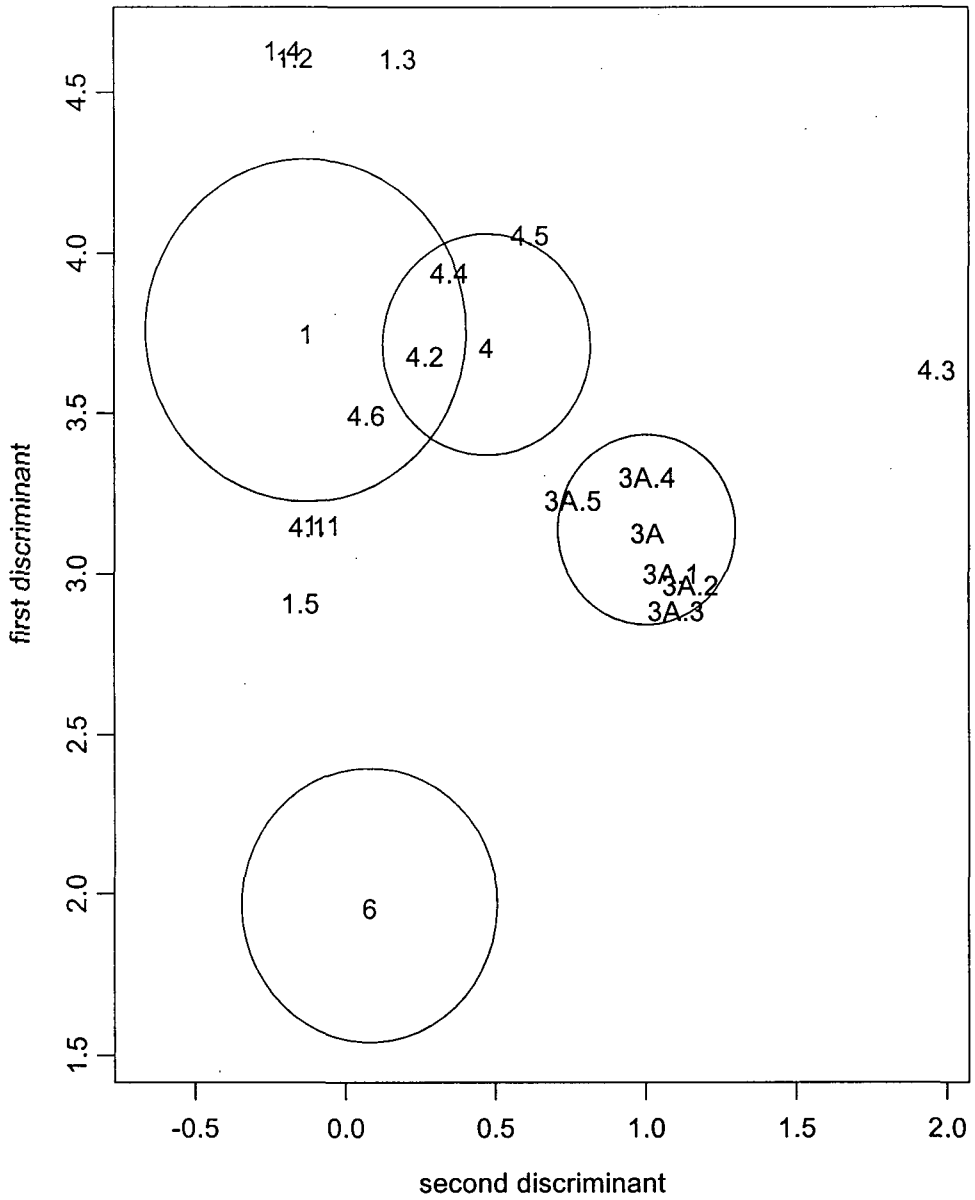


Figure 9. Results of the first canonical multivariate analysis of black oreo. Parasites included were gill cysts, gill metacercaria, *Anisakis* sp., dead *Anisakis* sp., liver plerocercoid, *Hepatoxylon trichiuri*, and dead *Hepatoxylon trichiuri*. Analysis restricted to fish in the 30-35 cm length range. Area means and tow means on the first two canonical axes are plotted. Circles indicate 95% confidence intervals but are too small due to the presence of within-tow correlation.

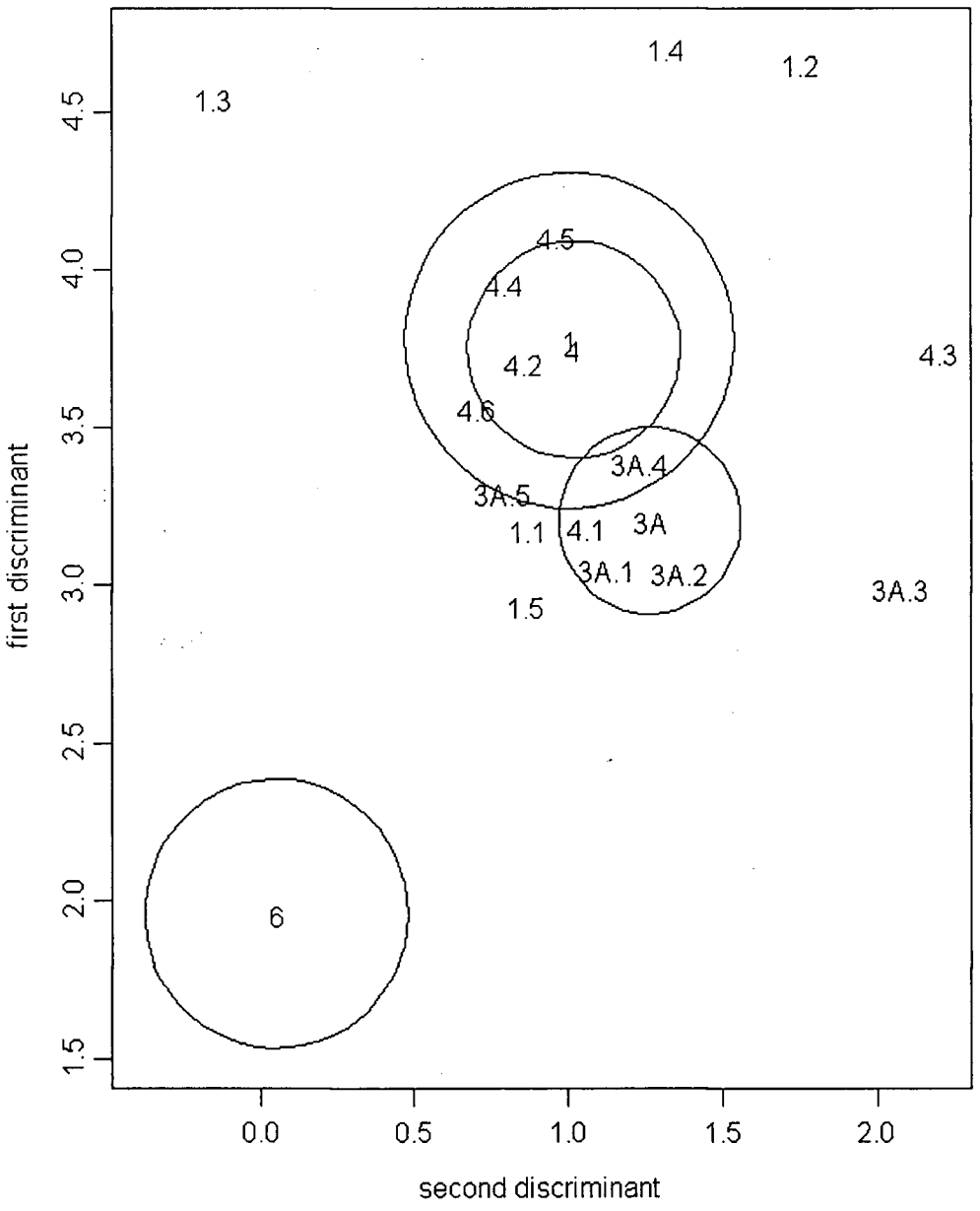


Figure 10. Results of the second canonical multivariate analysis which included all parasites in black oreo. Analysis restricted to fish in the 30-35 cm length range. Area means and tow means on the first two canonical axes are plotted. Circles indicate 95% confidence intervals but are too small due to the presence of within-tow correlation. Adding all the parasites into the analysis makes no real difference, suggesting the best discriminators between sites are included already.

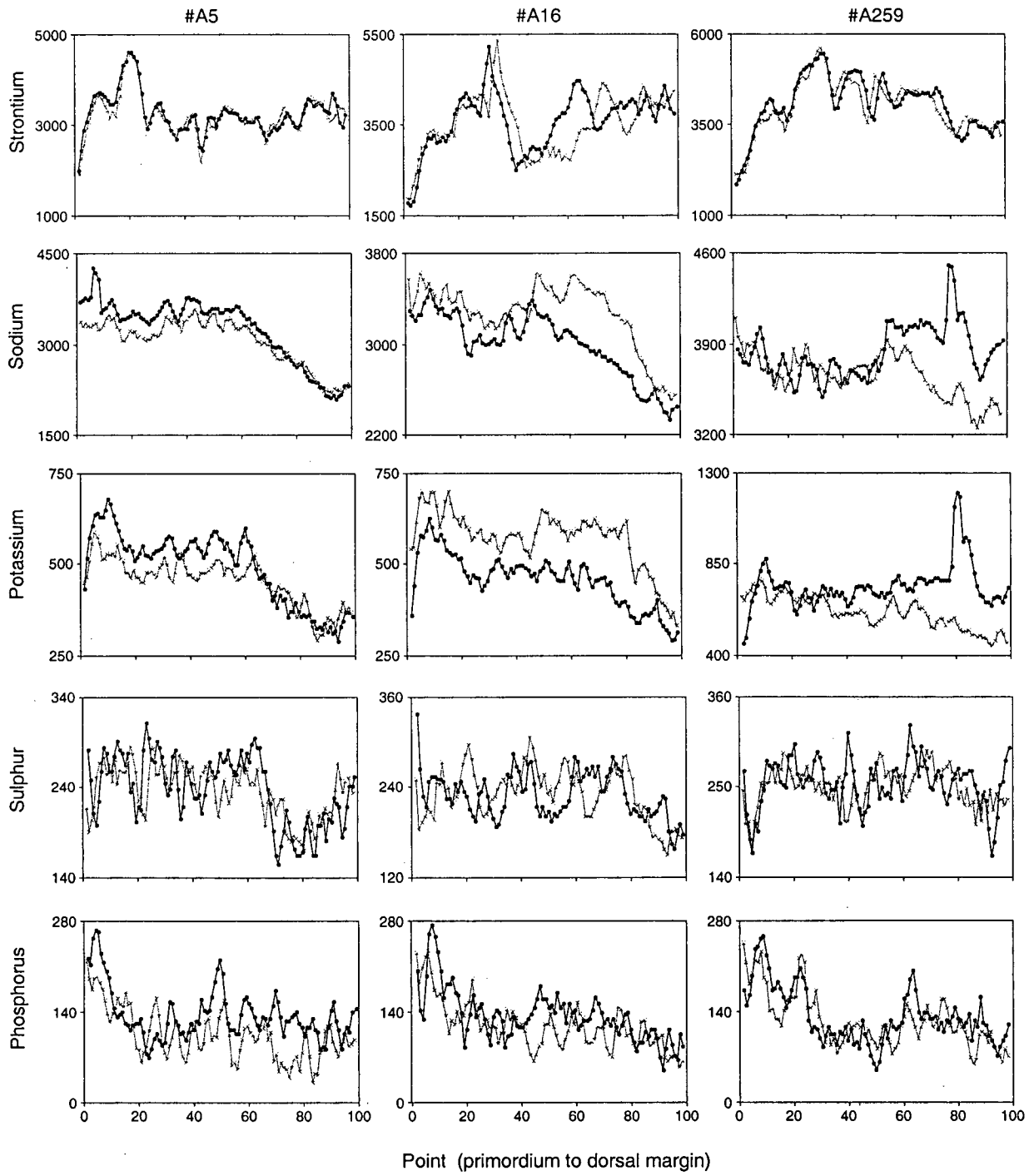


Figure 11. Element concentrations (ppm), as measured by EPMA, across sections of three pairs of black oreo otoliths. Filled circles — left otolith, crosses — right otolith. Profiles have been filtered with a 3 point running mean.

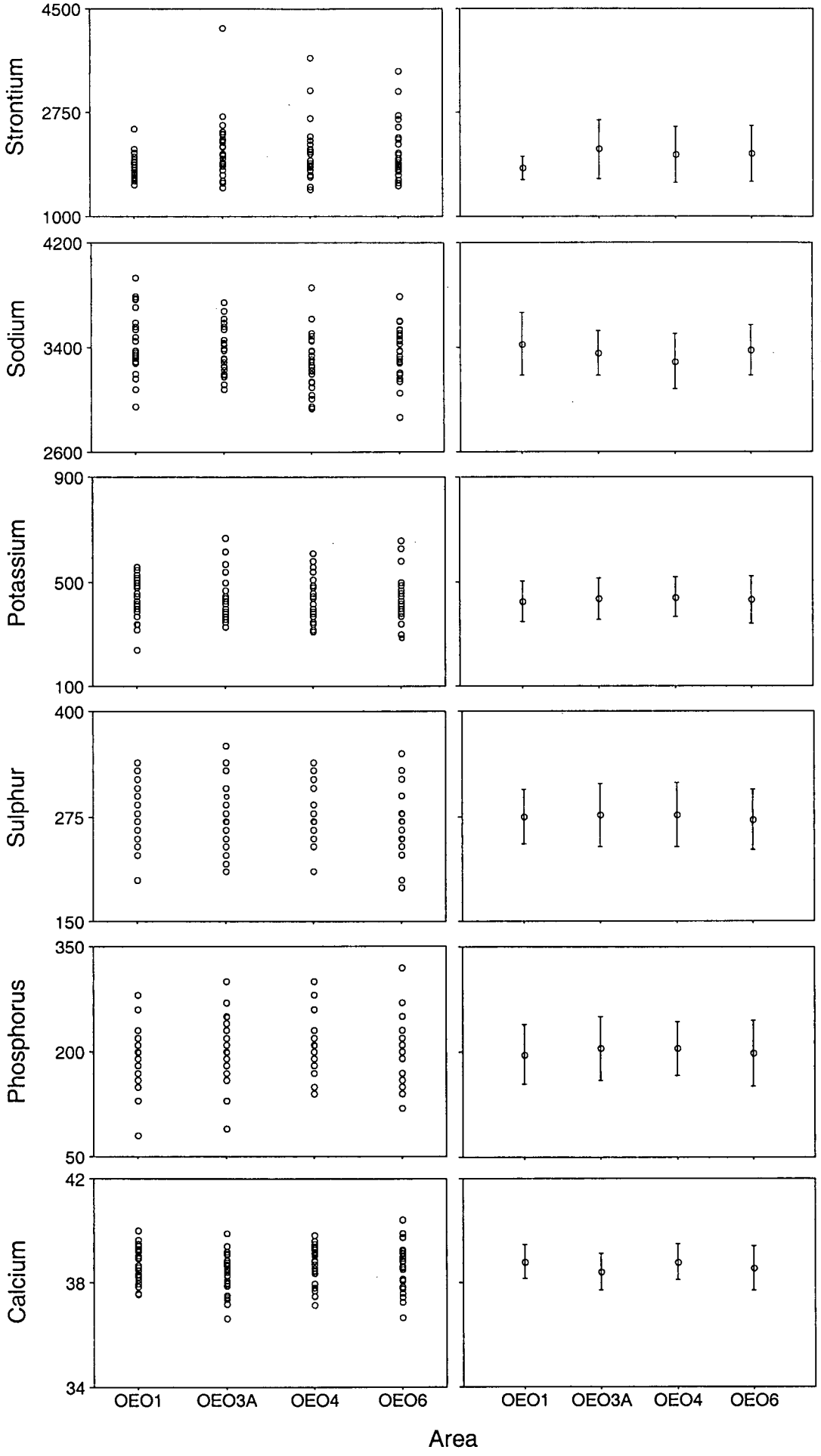


Figure 12. Distribution of concentration of elements in all area samples, measured by EPMA adjacent to the primordium in sectioned black oreo otoliths. Error bars are $\pm$ 1 standard deviation. Concentrations are in ppm, except for Ca which is in Wt %.

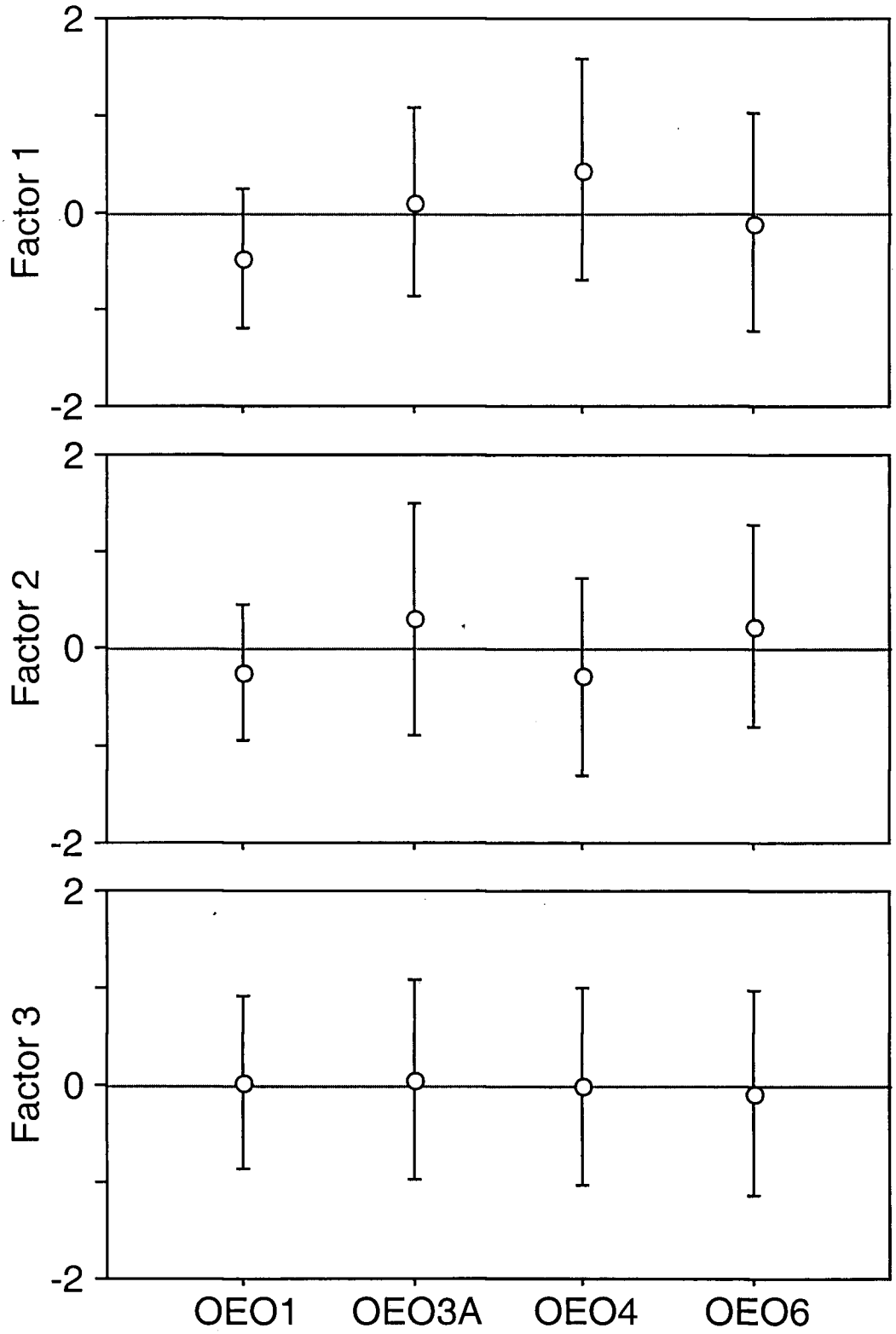


Figure 13. Means of linear discriminant factors, from LDFA of element concentrations measured by EPMA adjacent to the primordium of black oreo otoliths, across area samples. (Error bars are $\pm$ 1 standard deviation). None of the factors were significantly different across samples (p values; Factor 1 = 0.18, Factor 2 = 0.43, Factor 3 = 0.96)

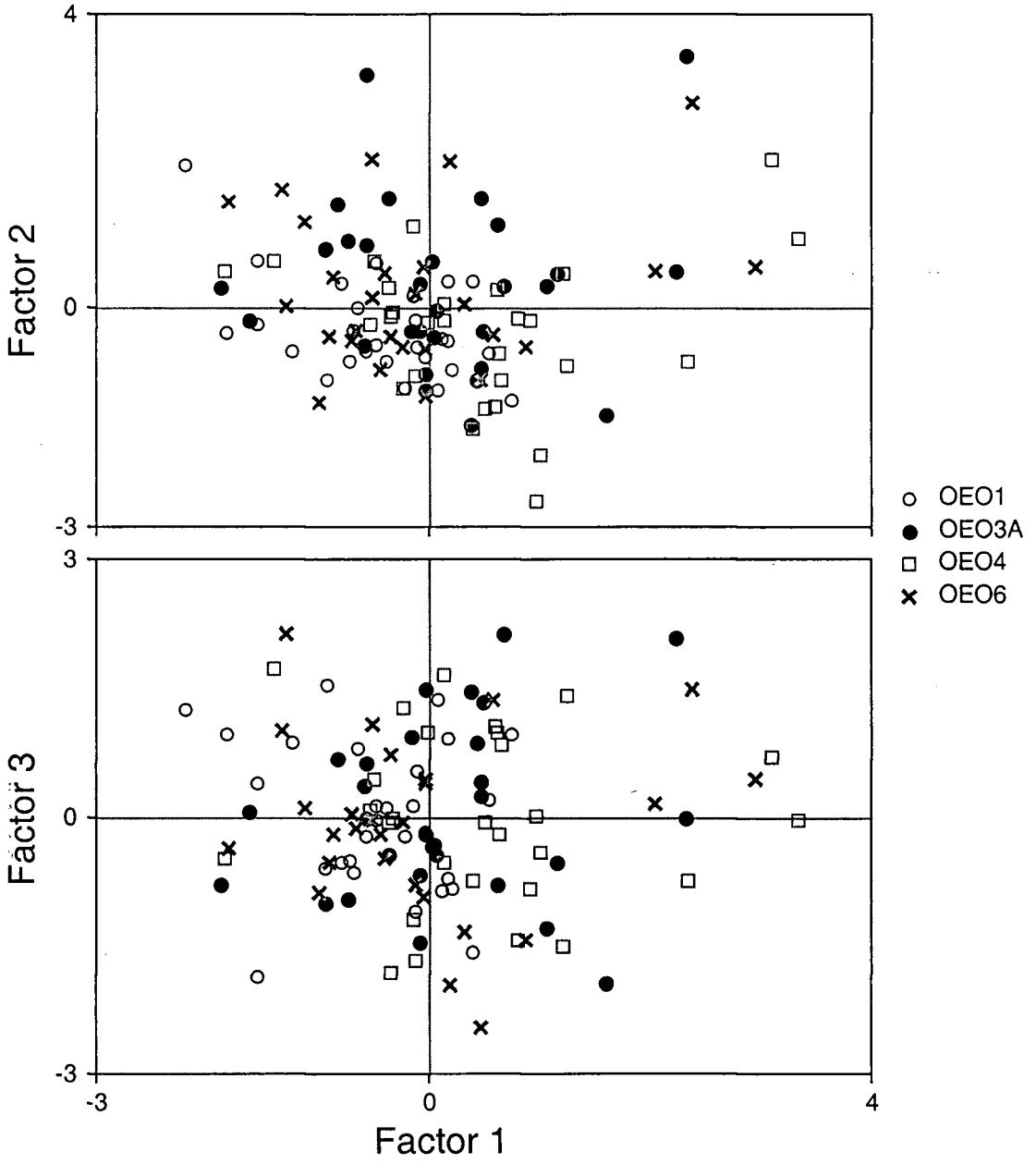


Figure 14. Distribution of black oreo from area samples in linear discriminant factor space, based on LDFA of elements measured by EPMA adjacent to the primordium.

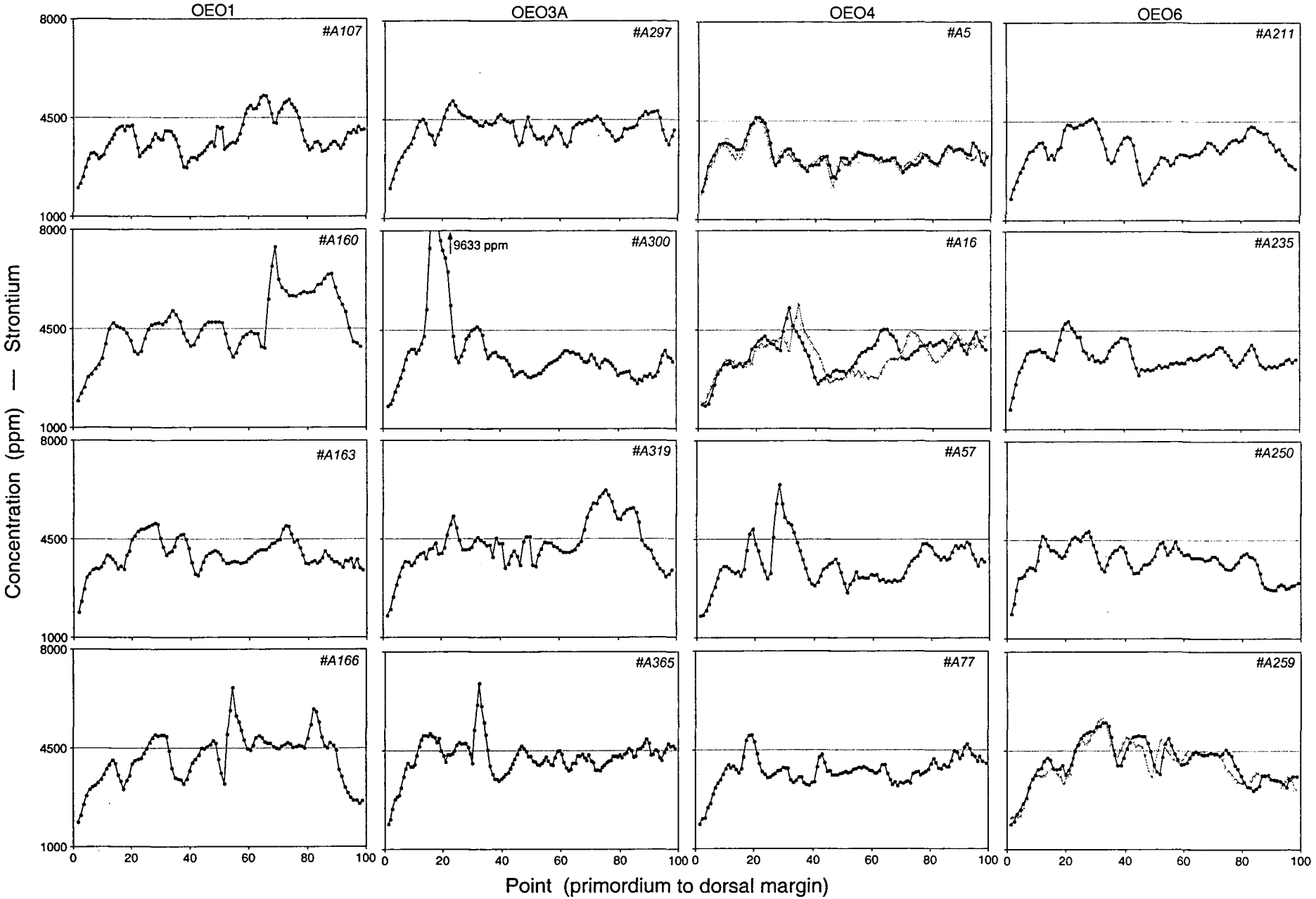


Figure 15 (a). Life-history profiles of strontium concentration (ppm) across sections of black oreo otoliths, as measured by EPMA.. Step size 25 μm. Profiles have been scaled to fit 2.5mm. Left and right otolith profiles are shown for #A5, #A16, and #A259. Horizontal lines at mid-y axis are included as an aid for comparisons between profiles.

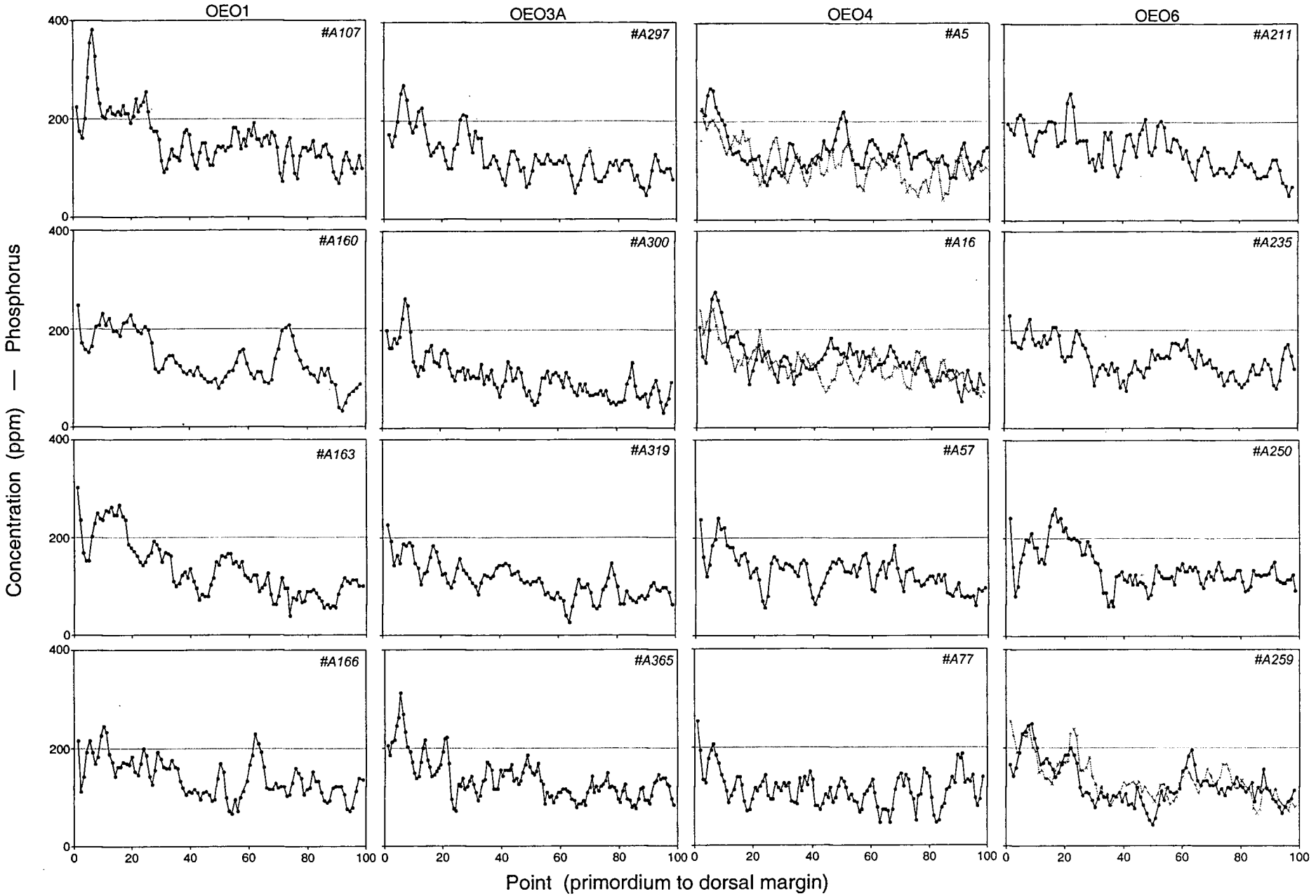


Figure 15(b). Life-history profiles of phosphorus concentration (ppm) across sections of black oreo otoliths, as measured by EPMA.. Step size 25 μm. Profiles have been scaled to fit 2.5mm. Left and right otolith profiles are shown for #A5, #A16, and #A259. Horizontal lines at mid-y axis are included as an aid for comparisons between profiles.

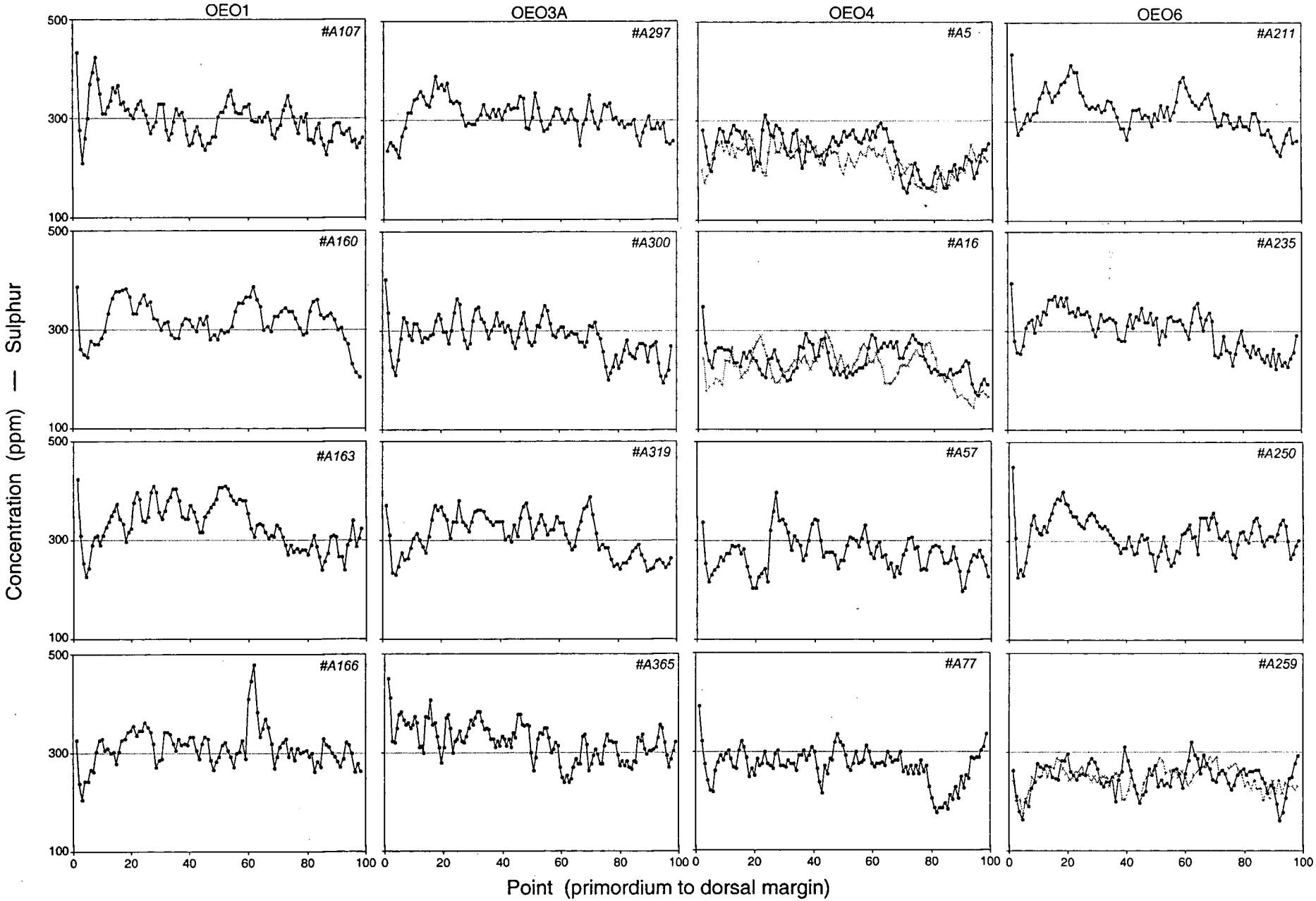


Figure 15 (c). Life-history profiles of sulphur concentration (ppm) across sections of black oreo otoliths, as measured by EPMA.. Step size 25 μm. Profiles have been scaled to fit 2.5mm. Left and right otolith profiles are shown for #A5, #A16, and #A259. Horizontal lines at mid-y axis are included as an aid for comparisons between profiles.

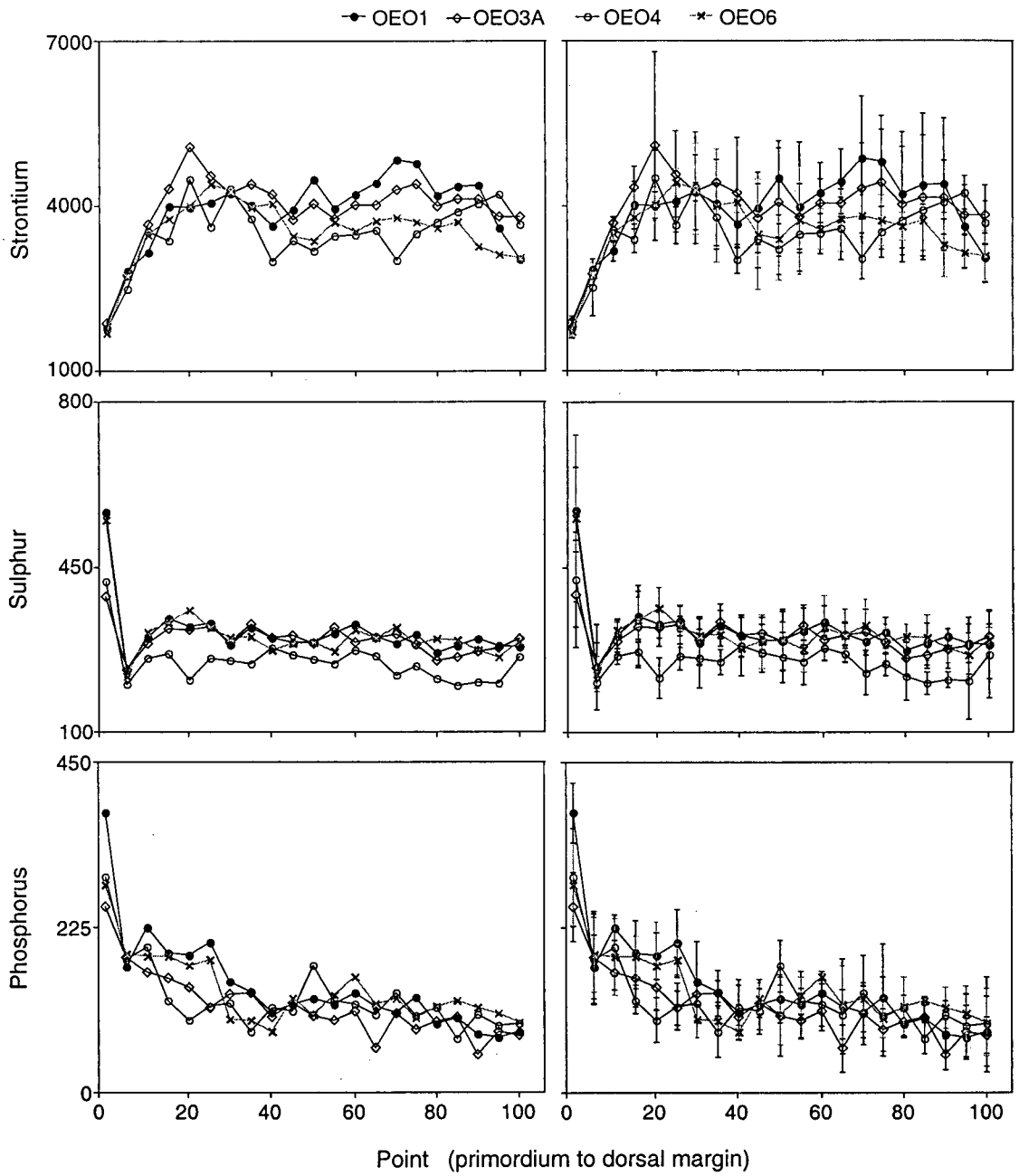


Figure 16. Mean profiles for each area for Sr, S, and P across black oreo otoliths. Data points are mean values at 5 point intervals across profiles of Fig. 9 (i.e. n = 4 for each point). Profiles are shown with and without error bars (± 1 SD) for comparison purposes.

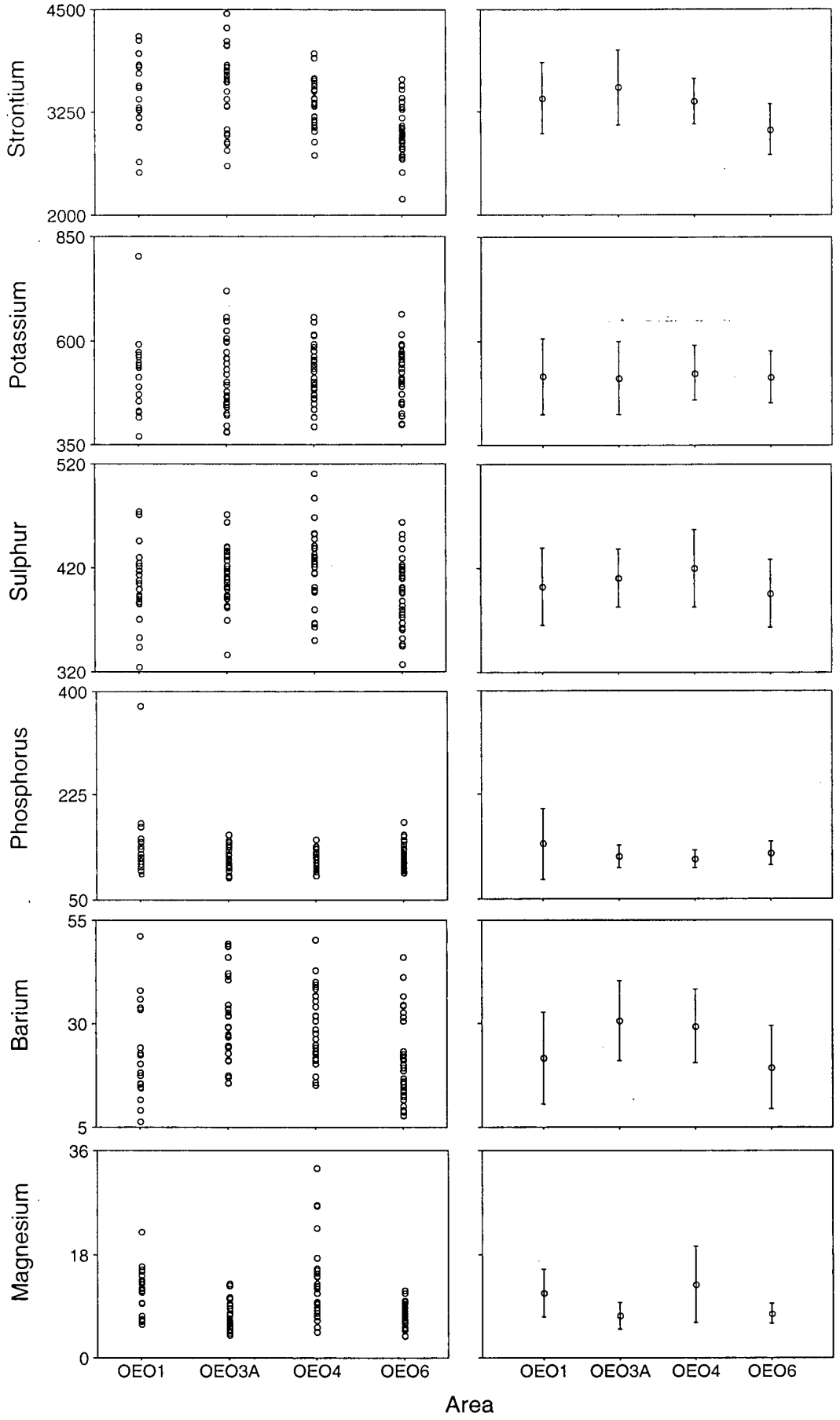


Figure 17. Distribution of concentration of elements in all area samples of black oreo, measured by ICP-MS of whole otoliths. Error bars are ± 1 standard deviation. Concentrations are in ppm.

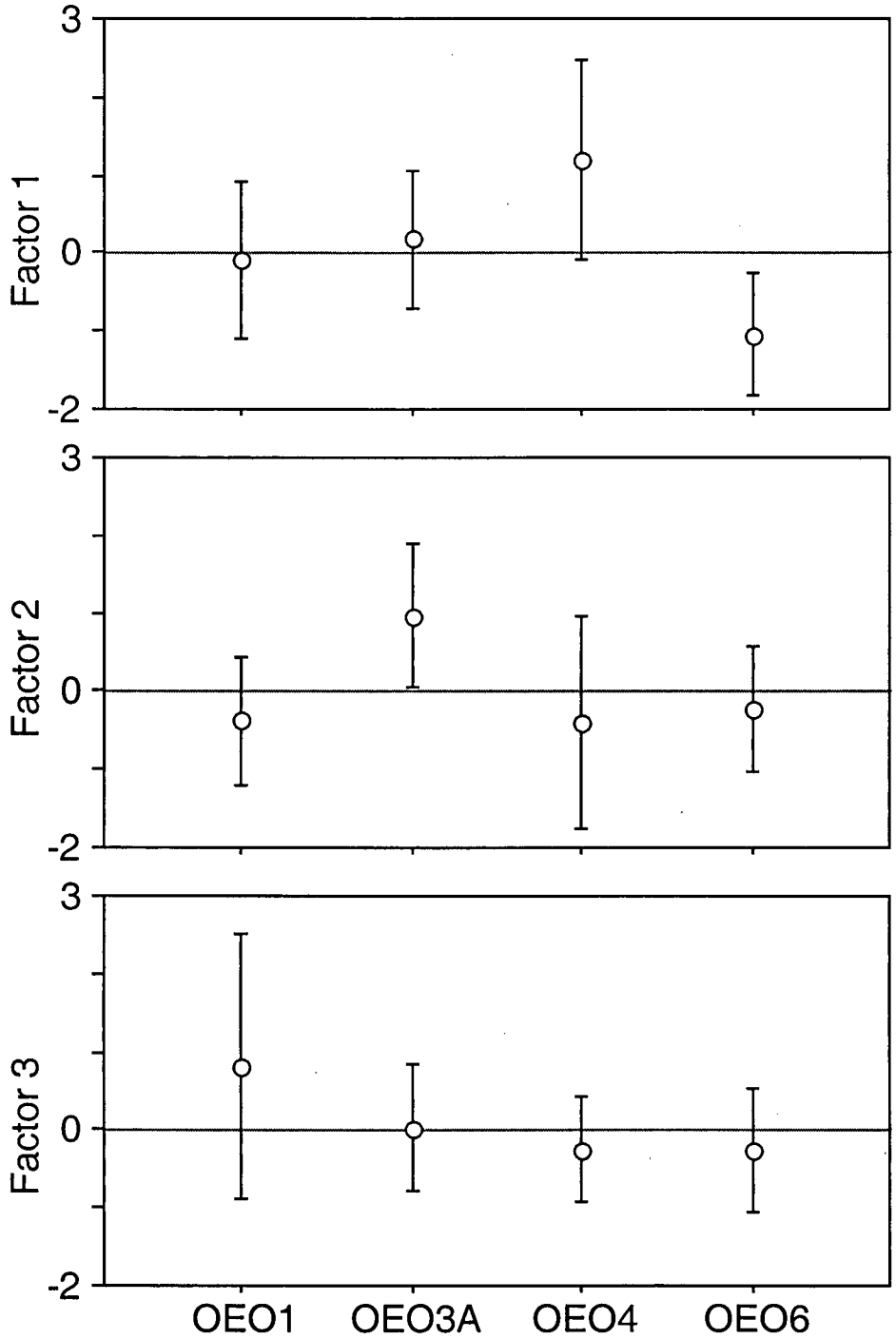


Figure 18. Means of linear discriminant factors, from LDFA of element concentrations measured by ICP-MS of whole black oreo otoliths, across area samples. (Error bars are $\pm$ 1 standard deviation). All three factors were significantly different across samples (p values; Factor 1 < 0.001, Factor 2 < 0.001, Factor 3 = 0.002)

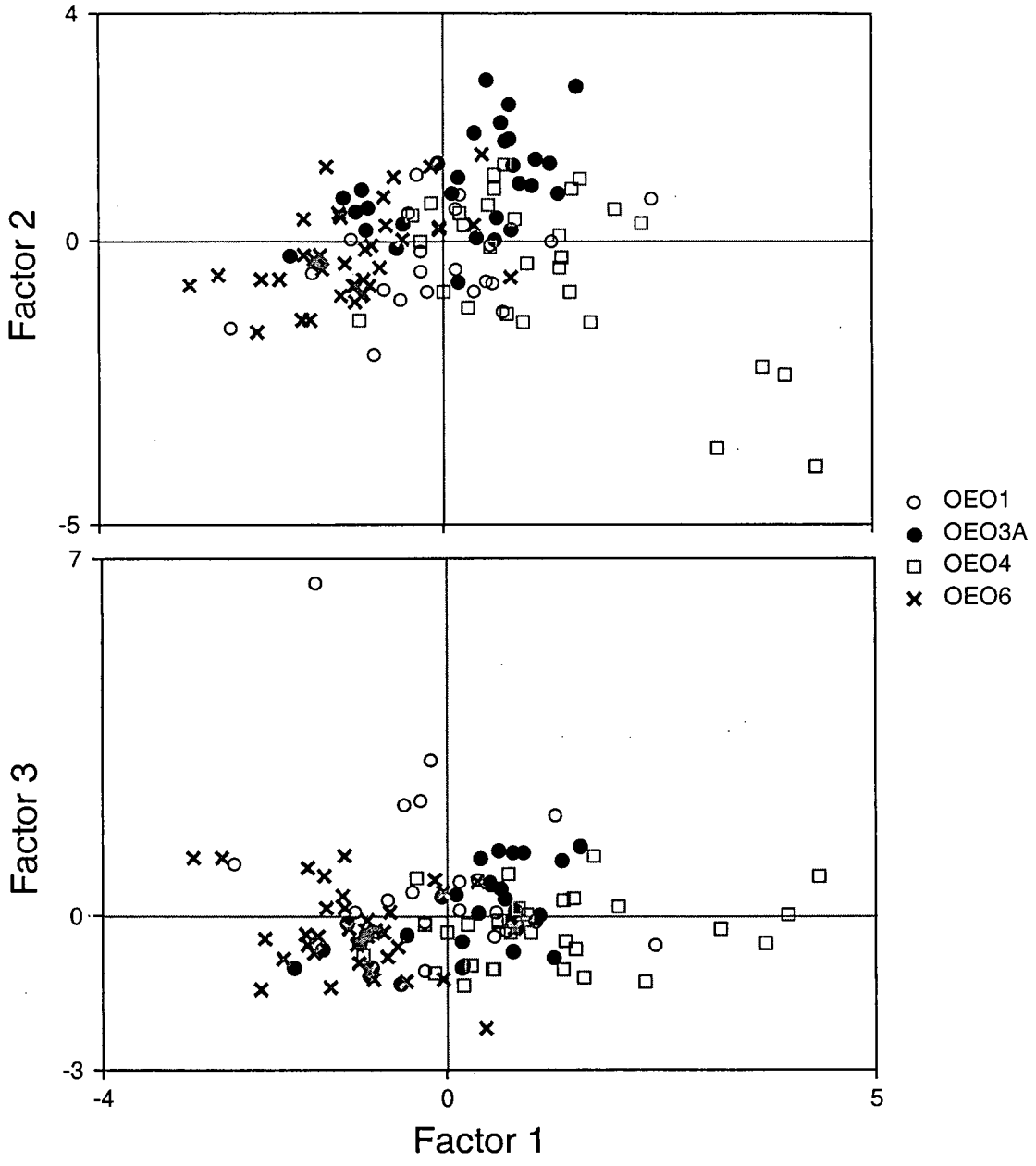


Figure 19. Distribution of black oreo from four management areas in linear discriminant factor space, based on LDFA of elements measured by ICP-MS of whole otoliths.

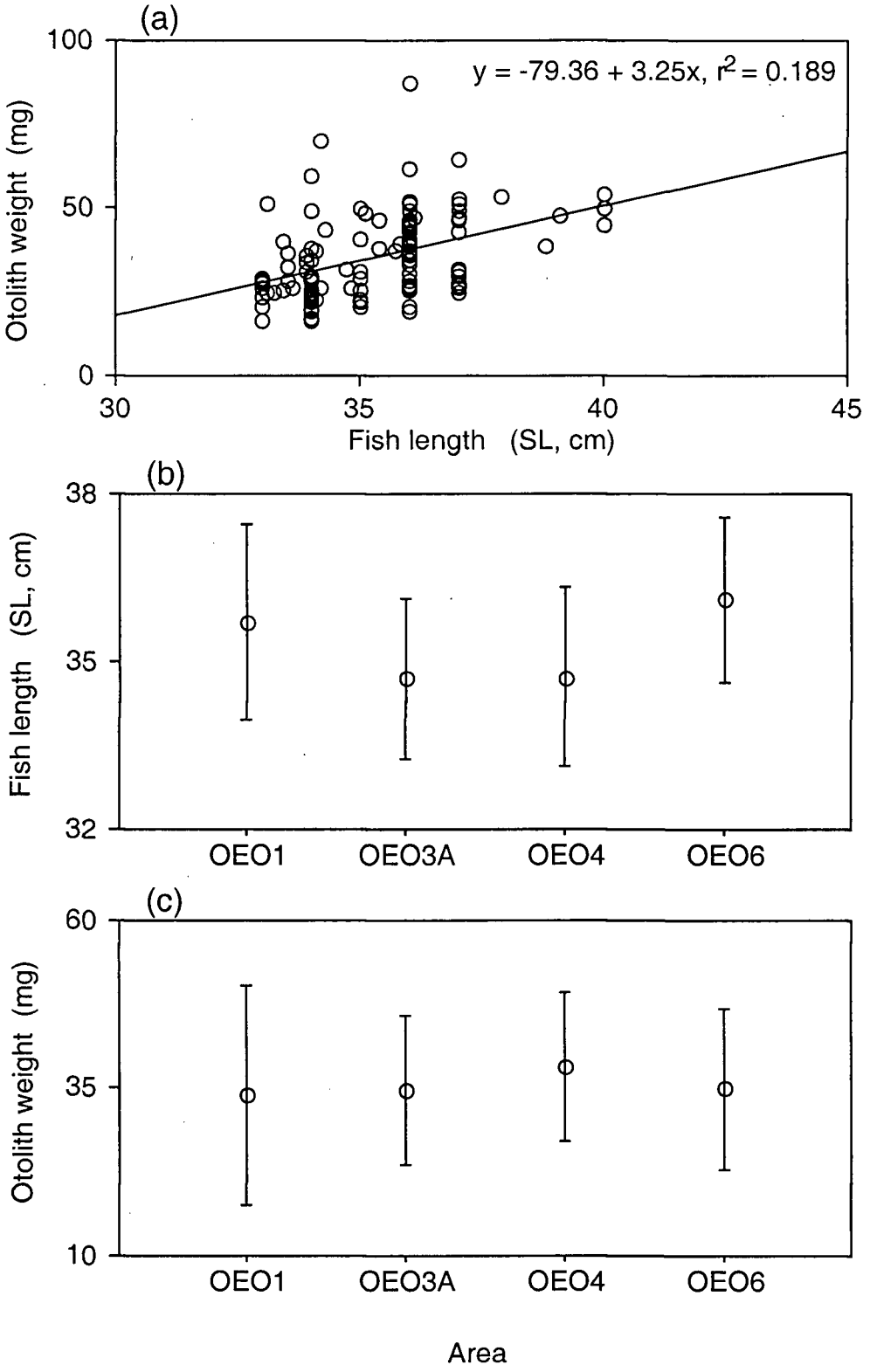


Figure 20. (a) Relationship between otolith weight and fish length for all samples analysed by ICP-MS of whole otoliths for area comparisons (b) Mean size of black oreos (standard length) across samples, (c) Mean weight (mg) of otoliths across samples.

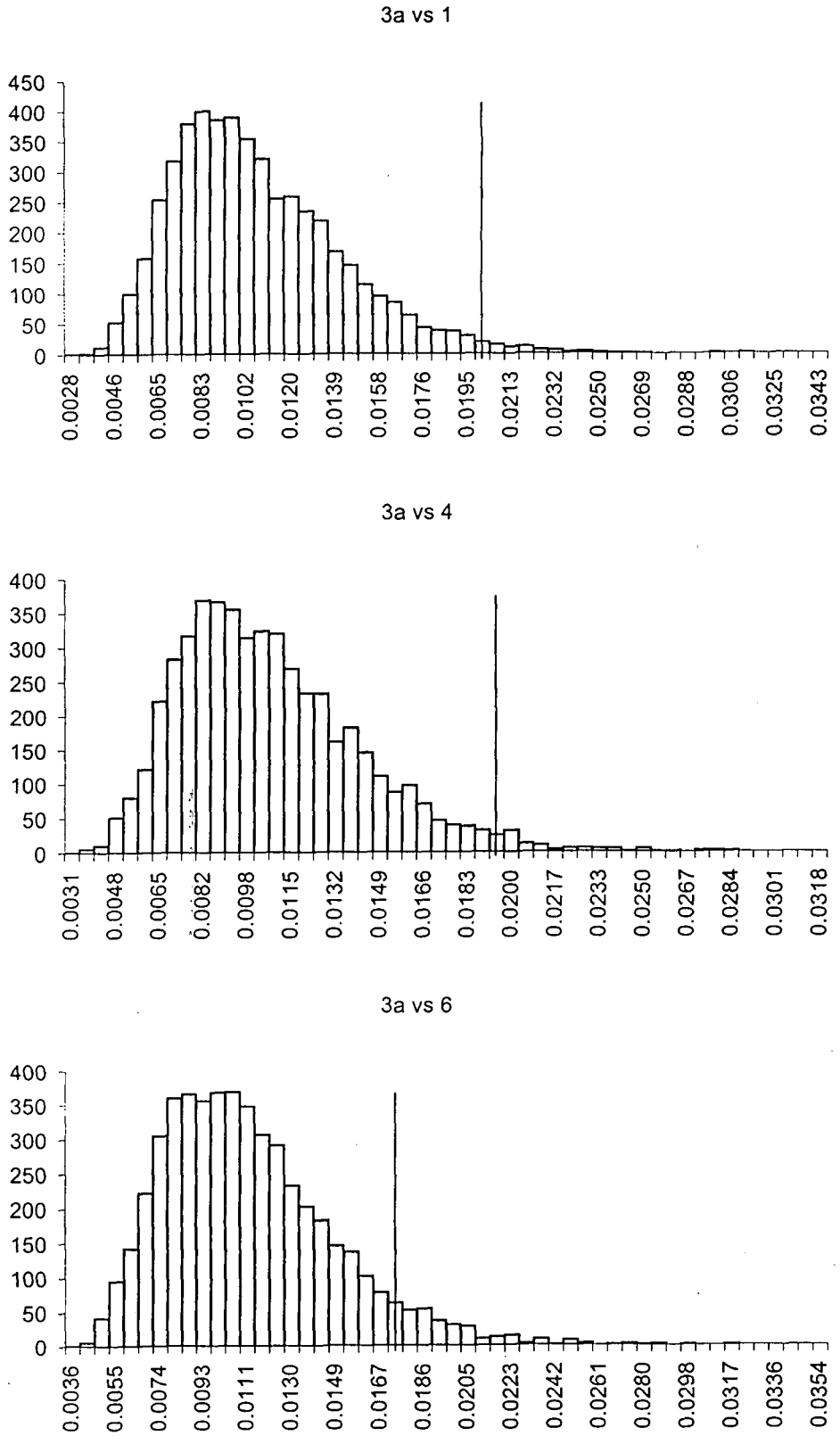
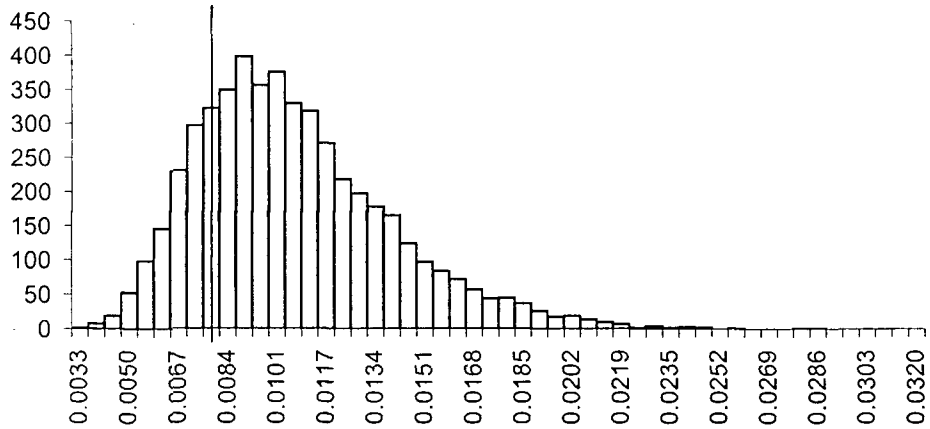
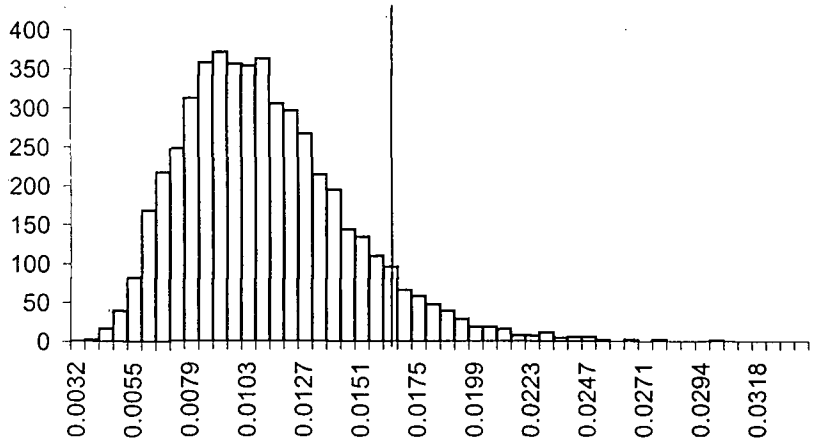


Figure 21. Frequency distribution of randomized harmonic distances between each of the black oreo management areas, from analyses of otolith shape. The thin lines represent the observed harmonic distance for each pairwise comparison.

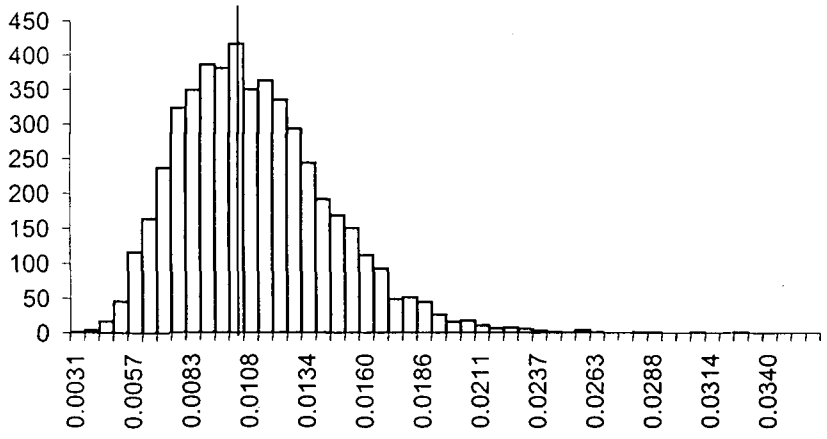
1 vs 4



1 vs 6



4 vs 6



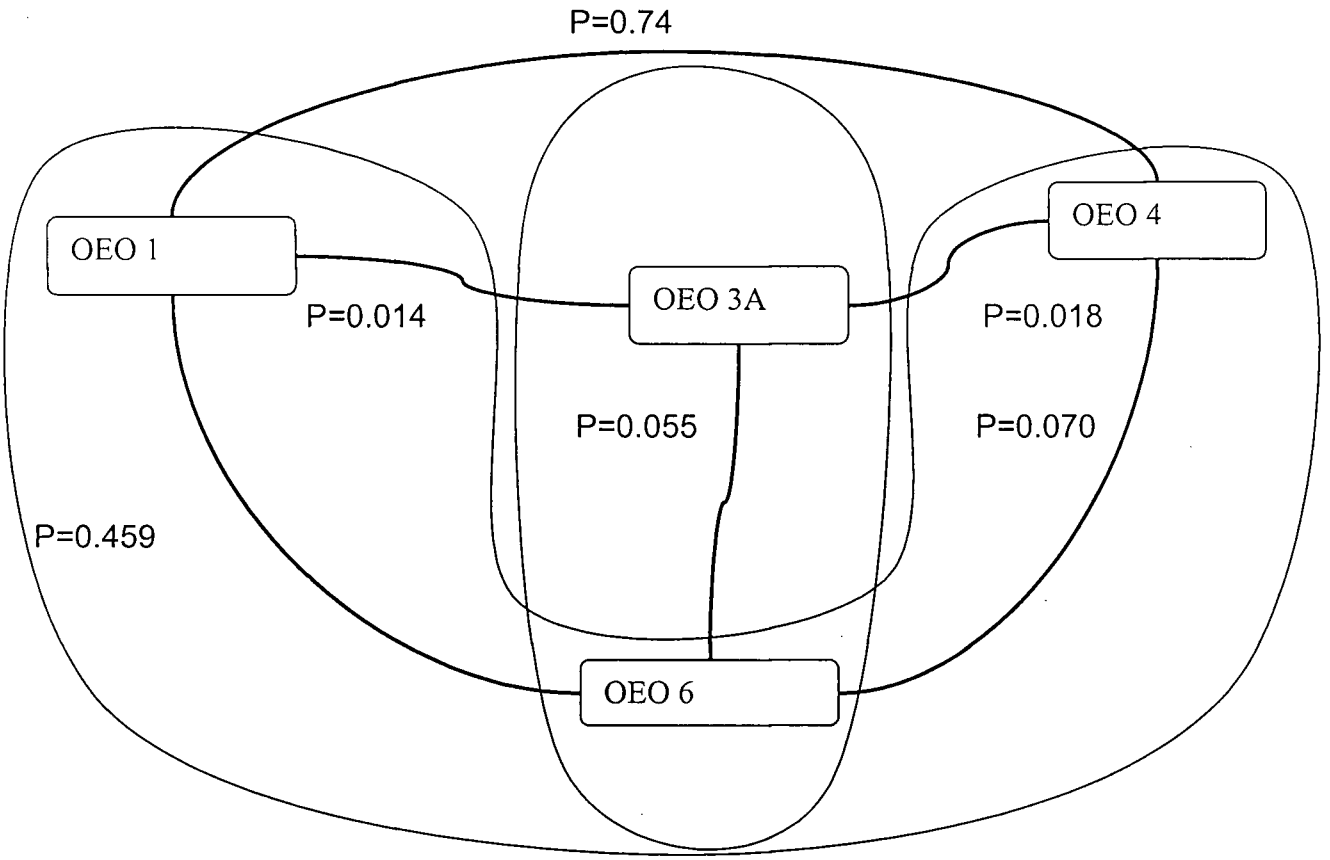


Figure 22. Schematic representation of suggested black oreo stock relationships from otolith shape analyses. Thick lines connecting management areas indicate test with probability of being same stock, based on harmonic distance randomisation test. The groupings are indicated by thin lines.