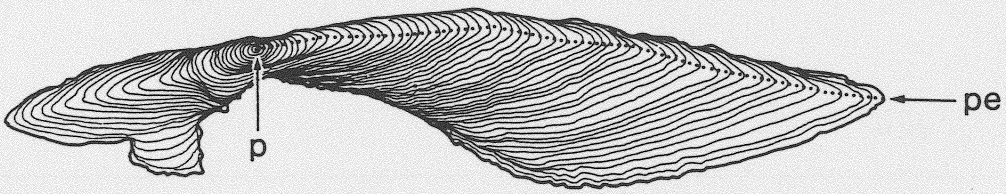
Determination of the Migration Patterns of Juvenile Southern Bluefin Tuna and Jackass Morwong

Fishing Industry Research and Development Council

Final Report

Project 1987/15



CSIRO
AUSTRALIA

Division of Fisheries

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Contents

1. Introduction

Little definite is known about the spatial scales of populations of marine organisms. Movement is pervasive in the ocean. Planktonic larvae, which are nearly ubiquitous among marine fishes and invertebrates, drift or swim for periods varying amongst species from, rarely, a few hours to a year or more and have the potential of wide scale dispersal from spawning areas. We emphasize the word 'potential'. The extent to which larvae are actually transported among habitat patches (or conversely, the extent to which a single habitat patch is self-recruiting) is not known directly for any marine species with a planktonic duration longer than a few hours. Among neritic animals, such as tuna, movement also takes the form of active ontogenetic migrations of juveniles and adults, which can involve periods as long as six years and during which the fish are thought to move thousands of kilometers and through Fishing Zones of numerous countries. The nominally passive movement of larvae and active migration of juveniles and adults define the geographic size of marine populations, and therefore the relevant spatial scales of both ecological study and fisheries management. Although the extent to which apparently distinct populations mix via larval drift or individual migration can have important economic and political implications, our understanding of the movements of any marine animal is, at best, rudimentary.

This is not due to lack of effort. Numerous attempts have been made to document the spatial scales of marine populations and mixing rates among geographic sub-units of those populations. Commonly used procedures include tag-and-recapture programs, analysis of concordant settlement patterns, and differences between areas in gene frequencies or parasite loads. No technique currently available provides even moderately unambiguous results. Tag-and-recapture studies, which provide the only direct evidence of individual movement, nonetheless are difficult to interpret statistically and, parenthetically, cannot be used on small fragile animals, such as larvae. Genetic differences among sites, the most frequently used means of stock discrimination, can result from both post-settlement selection, rarely measured, as well as population differentiation. Genetic differences are also swamped by only slight levels of genetic exchange; populations that cannot be distinguished genetically may, nonetheless, be more than 99% self-recruiting. The degree to which local populations are self-recruiting, the information that ecologists and fisheries biologists need, is virtually unknowable by techniques currently available.

In the early 1970's John Calaprice (and subsequently several co-workers from the Pacific Biological Station at Nanaimo) (Calaprice, 1970, 1971; Calaprice, et al., 1971; reviewed by Calaprice, et al., 1975; Mulligan, et al., 1983) reported on

preliminary studies that used a very different technique to determine the population structure of NW Pacific salmon. Following the lead of some earlier work done on salmonids in Japan (Fisheries Agency of Japan, 1967), Calaprice et al. demonstrated that the trace element composition of bones in salmon from different river systems differed slightly, but consistently. Further, once incorporated into the bones, these differences are permanent and could be used to identify the spawning river used by each individual fish. The technique is based on the premise that the incorporation of trace elements into the calcium carbonate-protein matrices that constitute the bulk of the ossified tissues in fishes (and the skeletal structure of most other marine organisms, including corals and molluscs) varies at least in part with environmental conditions. This premise is supported by studies on, primarily, marine invertebrates, in which variations in, for example, water temperature have well documented effects on the isotopic and elemental composition of calcium carbonate structures, e.g., coral skeletons and foraminiferan tests (e.g., Weber, 1973; Smith, et al., 1973). Recent work on ossified structures in fishes confirms similar effects of the environment on their trace element and isotopic composition (Gauldie, et al., 1980; Radtke, et al., 1987; Behrens Yamada, et al., 1987). There is every indication that ontogenetic variations in trace element and isotope ratios in the skeletal structures of fishes, such as their otoliths, constitute natural phenotypic tags that permanently record an individual's environmental history and that local variations in this history can be used to identify an individual's source location.

The implications of such 'environmental fingerprinting' in fishes are only just being realized. Migration patterns of neritic fishes can be reconstructed for individuals based on ontogenetic changes in composition caused by changing temperature regimes and movement between water masses (Calaprice, 1980-1985). Bays or estuaries used as nursery grounds for coastal fishes can potentially be individually identified by the chemical signatures they leave in otoliths, on the basis of which precise, quantitative links between nursery habitats and parts of the adult population could be developed (Mulligan, et al., 1987). Where larvae or juveniles are highly mobile, but adults much less so (as is usually the case), discriminant analysis based on the composition of calcified structures laid down after the mobile stage can indicate population sub-structuring and provide direct data on the rates and patterns of movement by adults between areas (Edmonds, et al., 1989). If the technology of skeletal microchemical analysis on fishes can be developed to the point where these data can be collected routinely, the potential exists to improve vastly our understanding of the migration patterns of marine organisms, for both fisheries and marine ecology.

Perhaps the most exciting prospect, however, that may develop from this technique is that it may finally be possible to close the gap between the production of pelagic larvae and the recruitment of post-planktonic juveniles. Ossified structures develop early in fishes, in some species even before hatching into planktonic larvae. The strong possibility exists that discrete spawning areas may each produce a diagnostic chemical signature in those initial ossifications, which can be 'read' in the juveniles. Microchemical analysis of ossified structures in marine organisms may allow us for the first time to map directly patterns of larval advection and dispersal and quantify the extent to which sites are self-recruiting, and hence functionally independent ecological units.

We proposed to assess the potential of this technique when applied to Australian finfishes. Specifically, we proposed to determine whether ontogenetic variation in otolith composition could be used to resolve two major questions about stock structure in Australian finfish: is there only one spawning ground/migration route for Southern Bluefin Tuna, and do the bays and estuaries around southern Tasmania constitute the only nursery area for Jackass Morwong in temperate Australia. Both questions are of critical importance to management of the respective species. If all SBT migrate through the AFZ and are subject to the Australian shore-based fisheries, then monoitoring stock recovery by assessing these juveniles is a realistic objective and the conservation of the juvenile stocks in the AFZ crucial from the perspective of management of the global, high seas stock. Similarly, if the bays and inlets of SE Tasmania constitute the only nursery area for jackass morwong, then conservation measures need to be developed and implemented to ensure the continued viability of the fishery in the face of continuing coastal development.

To date, the only evidence to support both hypotheses is negative, that is, no one has caught juvenile SBT outside of the known migration route and juveniles of Jackass Morwong are rarely caught in large numbers outside of SE Tasmanian bays. And in both cases, negative information alone is not sufficient to test the hypotheses, since the lack of information could equally well reflect a lack of sampling in the appropriate habitat. We hypothesised that otolith chemistry could provide a direct test of each hypothesis. Specifically, if juvenile SBT migrated along different routes than the composition of that part of the otolith corresponding to the migration period (roughly 0 to 4+) would differ in consistent ways among individuals, such that groups of chemically similar idividuals would cluster out of the overall population based on the migration route followed. Similarly, we hypothesised that if the bays and inlets of SE Tasmania constituted the only nursery area for Jackass Morwong in Australia, then the chemical signature deposited in that part of the otolith corresponding to the residence

period in the nursery ground a) would be similar for all adults, irrespective of where they were caught, and b) would be similar to the composition of juveniles caught on the nursery ground.

This Final Report reports on 1) our detailed evaluation of current methodologies for otolith chemical analysis, and our recomended procedures when electron probe microanalysis is used for stock delineation in finfish, 2) our assessment of the accuracy and precision of otolith probe microanalysis relative to the scales of natural variability in otolith composition, and an evaluation of the extent to which such variability reflects changing enviromental conditions, 3) a direct test of the hypothesis that SE Tasmanian bays are the sole nursery area for Jackass Morwong, and 4) an evaluation of the liklihood of more than one spawning area/migration route for southern Bluefin Tuna. We will not report here on the potential for ontogenetic variation in chemical composition to be used as a tool for age determination in SBT (based on annual cycles of elemental deposition), as this will be covered in detail in the final report for Project 1989/31.

2. Objectives

1. To determine whether or not all juvenile Southern Bluefin Tuna migrate down the coast of Western Australia and hence, whether the Australian fishery is based on all or only part of each cohort;
2. To validate aging techniques for adult Southern Bluefin Tuna;
3. To determine whether or not bays and estuaries in southern Tasmania are the sole nursery grounds of the Jackass Morwong in Australia

3. Summary of Results

A. Methodological Studies

Electron probe microanalyzers fitted with energy-dispersive (ED) and wavelength-dispersive (WD) X-ray spectrometers are the standard instruments for measuring microscale differences in the chemical composition of the calcified tissues of fishes. We compared, on both empirical and theoretical grounds, the accuracy and sensitivity of the two instruments when used to carry out studies of stock structure on fishes. The results of our methodological studies are given in Gunn, et al. (1992) (appended). In general, we conclude that ED systems on electron probes are prone to produce spectral artifacts, peak overlaps and difficulties in modelling non-linear backgrounds, problems which appear to render current generation ED systems not suitable for quantitative analysis of trace elements (< 5000 ppm) in otoliths. By comparison, for SBT and Morwong, WD systems could measure six elements (Ca, Na, Sr, K, S, Cl) accurately and precisely to levels of several hundred ppm. However, electron probes operating at the beam powers required for WD analysis damage otoliths, causing pitting and chemical change, which increases measurement errors. This damage and the consequent loss in data quality vary directly with the power density of the electron beam, which, in turn, determines in part the rate of data acquisition. Use of a WD spectrometer to analyze otoliths, therefore, requires making a trade-off between acquisition time and data quality, which has to be evaluated against the magnitude of natural variability in the composition of the otoliths of each species studied. In the species we examined, we concluded that beam power densities greater than $3.0\mu W\ \mu m^{-2}$ resulted in unacceptable levels of specimen damage.

Overall, therefore, we drew the following conclusions.

- 1. The quality of data from electron probe microanalysis is critically dependent on the accuracy with which sections can be made and replicated between specimens and on quality of surface preparation. Even minor topographic features (pits, scratches) severely degrade data quality.
- 2. Difficulties in resolving line overlaps, the presence of spectral artifacts and generally low peak-to-background ratios limit the ability of energy-dispersive (ED) spectrometers on electron probes to measure accurately the concentration of even the most abundant trace elements in otoliths (e.g., Na and Sr). We conclude these instruments are not suitable for studies of ageing or stock discrimination in fishes.
- 3. Wavelength-dispersive (WD) spectrometers provide accurate data on at least six elements of potential use in studies of fish biology: Ca, Na, Sr, K, S and Cl. However, this instrument requires high beam powers to generate adequate counting

statistics. At these power levels, damage to the otolith's surface and a loss of data quality are inevitable. The amount of damage is proportional to beam power density (beam power per unit area of beam size) and differs between elements. The investigator must compromise between damage (= noise and error in the data), acquisition time and beam diameter, taking into account both the elements being assessed and the natural variability of their concentrations.

- 4. Inaccuracies due to specimen damage can be minimized most efficiently by using the largest possible beam diameter and by scheduling spectrometers to count first the elements most affected by the beam.
- 5. Until the procedures for probe microanalysis of the hard parts of fishes become routine, studies applying these techniques to analyze fish population parameters must define experimentally the limits of data quality and must specify precisely procedures used for statistically assessing concentrations.

B. Scales of natural Variability and Environmental Sensitivity of Otolith Chemistry

Six elements could be reliably detected in the otoliths of both Jackass Morwong and SBT using wavelength-dispersive electron probe microanalysis; in order of decreasing mean concentration, these were calcium, sodium, strontium, potassium, sulfur and chlorine. The elements in sagittae constitute three distinct sets, separated in concentration from other, less abundant elements by a difference of one to three orders of magnitude: calcium, carbon and oxygen (the latter two not routinely measured due to methodological difficulties) constitute the "macro-constituents", present in concentrations > 10% by weight; sodium, strontium, potassium, sulfur and chlorine constitute a set of 'micro-constituents', which occur in mean concentrations of 100 - 5000 ppm; and a variety of 'trace elements' occur at concentrations < 10 ppm. Only the micro-constituents and Ca can be measured accurately using WD-EPMA. . Measurement error is inversely correlated with mean concentration, varying from 3.7% in sodium to 28% in chlorine. Of the six elements measured, only chlorine occurred occasionally at less than its respective MDL (157 ppm).

Life history scans for both species demonstrate several points. First, all six elements vary ontogeneticly in concentration well in excess of the uncertainty associated with measurement. Second, concentrations of all six elements auto-correlate highly at scales < 100 µm , which suggests that this is the typical scale of ontogenetic variability in composition. Third, absolute variability is highest for strontium, values for which can routinely vary within specimens over half an order of magnitude. However, relative variability is as high or higher in sulfur and chlorine; coefficients of

variation for chlorine in the three specimens depicted range from 37.8 to 92.1, as compared with 18.9 to 37.4 for strontium, 16.3 to 24.9 for sulfur and, at the other extreme, only 1.2 to 1.7 for calcium. And fourth, ontogenetic patterns in the variation are often consistent across specimens. All morwong and SBT we have analysed, for example, show steep gradients in strontium levels in the region immediately around the primordium. Similarly consistent, though less pronounced patterns are evident in sodium and calcium.

The extent to which these ontogenetic variations could be useful for analysis of stock structure depends principally on the extent to which they vary geographically; the working hypothesis is that local environmental differences induce chemical signatures which are site specific. This hypothesis was tested by examining 69 adult morwong from six sites across southern Australia (NSW, two samples each off Victoria and Tasmania, and the GAB) and the composition of the central portion of their otoliths, which presumably reflects environmental conditions specific to the spawning grounds for each fish. Mean concentrations of four of the six elements differed significantly between sites. Post-hoc analysis of the data suggested the samples fall into three groups: (1) eastern and western Tasmania, (2) Victoria and NSW, and (3) the GAB. A step-down procedure applied to successive discriminant function analyses resulted in the same grouping of sites, with the success rate in assigning individuals to groups more than doubling (from 37.6 % to 78.2 %) when the five sites were grouped into the three geographical regions. Neither this success rate nor the degree of separation among groups in factor space improved further when sites were pooled into two groups. We conclude, therefore, that there is significant spatial variation in otolith composition and that this spatial variability groups regionally, which suggests it is related either to local environmental conditions, stock structure or both.

Further examination of the data supported hypotheses based on a genetic or physiological basis for regional differences in otolith composition, as opposed to direct or simple environmental control. Two points were particularly suggestive. First, differences sites evident in analyses of the primordium were, with slight variation, manifest throughout the life history scan of each otolith. Second, settlement of juveniles into nurseryu areas, which should result in a substanntial change in the environmental conditions to which an individual is exposed, had little impact on otolith composition. From these observations, we conclude that the concentrations of most elements are not strongly dependent on environmental factors, but rather vary among individual as a result of genetic or 'locked physiological' differences. The implication is that subtle genetic differences distinguish populations of N. macropterus, which are

manifest in regional differences in otolith composition, but are unlikely to be detected using conventional genetic techniques.

C. Evaluation of nursery area-specific signatures and the links between nursery areas and adult of Jackass Morwong

The links between nursery areas and components of the adult populations can be assessed in two complementary ways: 1) by the determining the source affinities of juveniles collected in each nursery area, and 2) by developing a specific signature for each nursery area and using these to classify adults collected in different regions. In essence, the former assesses the distribution across nursery areas of individuals from each of the putative populations, whereas the latter assesses the contribution of each nursery area to adults collected at each site. With specific regard to N. macropterus, if conventional wisdom is correct, that is, SE Tasmania is the sole nursery area for the species in Australia, then we would expect that 1) elemental patterns similar to those of adults at all sites sampled would be represented in juveniles collected in the nursery area, and 2) adults collected at all sample sites would have a nursery area 'fingerprint' similar to that of the juveniles collected.

The first hypothesis was tested using 116 recently settled N. macropterus and comparing the composition of their otoliths with those of the regional groupings of adults described above. The results indicate that most juveniles examined fall within or close to the areas in discriminant function space defined by the adult groups. Of the 106 juveniles caught in Tasmania, all but 25 classify with the Tasmanian adult samples and of these, about half (13) classify ambiguously, with a probability >25% of being Tasmanian. Overall, only 7% of the Tasmanian-collected juveniles had a probability of <10% of classifying with the Tasmanian-caught adults. The pattern is similar for juveniles collected off Victoria (Port Phillip Bay), although sample sizes are too small to draw strong inferences. Of the 10 individuals examined, six classify with the NSW/Victorian adults, three classify with the Tasmanian-caught adults (at probabilities ranging from 72 to 85%), and one classifies with the GAB-caught adults (at P = 63%). The probability that the Port Phillip Bay juveniles classify with the NSW/Victorian adults is markedly bi-modal, with most individuals having either a very high or very low probability of classifying with the local adults. A similar, though more complex pattern is evident among the Tasmanian-caught juveniles, with evidence of perhaps three modes: one, the largest, peaking at P>95%, an intermediate mode centered near P=35%, and a third at P<5%. The intermediate mode consists of individuals with ambiguous affinities, that is, those distributed at or near the overlap zones of the three adult groups in two-function space.

The second analysis of the link between nursery areas and the adult population requires analysis of that portion of the otolith deposited during occupancy of the nursery areas. Discriminant function analysis of specimens from the six nursery areas arond SE Tasmania and Victoria indicated highly significant differences among all six, which suggests that there are nursery-area specific signals, which could be sought in the adult population. However, further analyses of the data indicated this conclusion to be premature. Analysis of ontogenetic variation in these juveniles indicated that for most elements, recruitment of the planktonic larva into a nursery area had no consistent effect on otolith composition, i.e., there was no indication of convergence upon a common environmentally induced composition for all fish in a particular nursery area, nor any evidence of divergence among nursery areas following recruitment. On this basis, we conclude, therefore, that the apparent discrimination among nursery areas is the manifestation mainly of pre-existing differences among individuals carried over from their larval stages and that locally mean differences in elemental composition reflect differing mixtures of juveniles derived from the three adult groupings described above.

From these analyses, we conclude that there is no nursery-area specific markers in juvenile Jackass Morwong, but that there are markers of stock structure that are manifest in otolith composition from birth. Our data indicate that the vast majority of juveniles in the SE Tasmanian bays and inlets show strong affinities only with the Tasmanian adults, and differ significantly from adults from either the GAB or NSW/Victorian groupings of adults, and similarly, that most of the few juveniles we collected in Victorian waters show strong affinities with the NSW/Victorian adults. We therefore conclude that SE Tasmanian coastal habitats are not the principal nursery areas of the NSW/Vict. or GAB populations of Jackass Morwong, and that there exists one or more, as yet unreported nursery areas for these fishes.

D. Spawning Areas/Migration Routes of Southern Bluefin Tuna

It has long been suspected that some juvenile SBT migrate directly west from WA, rather than east across the GAB, and hence were not subject to the GAB and SE Australian fishery. Whether this happens and what proportion of the stock is involved is not known. Attempts to determine this fraction by conventional techniques, e.g., tagging, have proven unsatisfactory, mainly due to logistical and sampling problems.

We proposed to test an alternative approach, which involved analysis of otolith composition. The approach is based on the hypothesis that 1) composition reflects environmental variables, and 2) that the environment off southern Australia differs from that off the southern Indian Ocean. If both of these are correct, then we would expect to see similar chemical signatures in the early life history of SBT, as they all are subjected to similar environments while migrating down the WA coast, but then a divergence of patterns later in life reflecting the division of the population into an Australian and a non-Australian component. This hypothesis was tested compared by, first, examining ontogenetic variation in juvenile SBT from the known migration route and comparing it with the adults coolected on the high seas fishery, for evidence of adults with radidly different patterns of otolith composition, and second, by comparing the composition of fish collected well outside the AFZ with those of comparable size and age from within the AFZ.

Analysis was based on ontogenetic variation in the composition of sagittal otoliths, as measured using two probe microanalyzers (wavelength dispersive electron probe microanalysis and micro-PIXE), of larvae collected on the single known spawning ground, of juveniles caught at different points along the known migration routes, and of adults caught in the high seas fishery. Fourteen elements were detected in T. maccoyii sagittae, but only six (Ca, Na, Sr, K, S, and Cl) were consistently present at concentrations above minimum detection limits. Comparisons among different samples suggested, first, that variation in the composition of the otolith primordium was unimodal and, generally, normally distributed, second, that this composition varied with individual specimen size or, equivalently, year-class, third, that individuals collected at widely separated locations did not differ significantly in the composition of the most recently deposited sections of their otoliths, and fourth, that all variation in the composition of adult otoliths was encompassed in the range of variation of juveniles collected along the known migration route. On this basis, we conclude that analysis of otolith composition is consistent with the current hypothesis of a single spawning area for T. maccoyii, but also that the range of envirnomentally correlated variation in composition is too low to provide a robust test of the diversity of migration routes. The relative envirnmental homogeneity of the pelagic ocean may result in limited usefulness of analysis of skeletal composition for population discrimination in pelagic fish species, unless this composition has a strong genetic component.

4. Principal Reccomendations for Management

1. Our results suggest three, geographically separated spawning populations of N. macropterus in the southern and southeastern region of Australia: one based in NSW and Victoria, one off Tasmania and one in the GAB. The points of separation of these populations cannot be determined based on the work done to date, though fishermen tell us that the NSW/Victorian population likely migrates annualy along the NSW/Victorian/northern Tasmanian shelf edge. The distinctive nature of the NSW/Victorian and Tasmanian populations strongly suggests that the two stocks be managed separately, particularly given current evidence of overfishing of the NSW/Victorian stock and the risk of serial depletion.

2. The data also indicate the SE Tasmanian nursery areas support primarily the Tasmanian N. macropterus population. There is at least one more major nursery area for N. macropterus in SE Austraina waters and, we expect, based on the NZ observations, that each stock is likely to have a specific nursery area attached to it. It is highly recommended that these nursery areas be identified and appropriate protection for them established. First indications are that nursery areas for the NSW/Victorian stock are likely to be in or near Bass Strait.

3. Juveniles are caught throughout much of the Tasmanian shelf region, indicating that contary to previous expectations the Storm Bay does not support the entire Tasmanian stock, never mind the entire Australian population. This suggests that whereas protection of the Storm Bay nursery area is desirable, it may not be critical for safeguarding the viability of the Tasmanian stock. Unfortunately, critical evaluation of the techniques developed in this study demonstrate that they are not sufficient to allow an assessment of the relative importance of any particular nursery area in SE Tasmania to supporting the adult stock.

4. All data collected on otolith composition of southern bluefin tuna are consistent with the hypothesis of a single spawning area for the species. The stock should be managed on the conservative assumption that all recruitment derives from this spawning ground and that adults migrate to this single ground to spawn.

5. Analysis of otolith chemistry appears to provide a means of assessing population structure for at least one species, N. macropterus, at a scale smaller than that possible using genetic techniques, and at a scale more relevant to the extent of fished stocks. These techniques should be trialled more extensively, both with more data for N.

macropterus to determine the limit of the techniques, and with other fished species, as they could constitute a potentially valuable means of determining stock structure on a space scale relevant to commercial fisheries.

Supporting Details

Document 1

Electron probe microanalysis of fish otoliths -- evaluation of techniques for studying age and stock discrimination

The calcified tissues of teleosts -- their bones, scales and otoliths -- are composed of a calcium carbonate-protein or calcium phosphate-protein matrix into which are bound a large number of microconstituents, elements that are either incorporated into the protein structure or replace calcium, carbon or phosphorus in the crystalline component of the matrix (Carlstrom, 1963; Degens et al., 1969). As early as 1967 (Fisheries Agency of Japan, 1967), preliminary studies suggested that the quantitative analysis of these microconstituents, or trace elements, could provide information on population structure and movements. This suggestion was based on two assumptions and a hypothesis. The assumptions are 1) that the calcified tissues of fish, with few exceptions, are not susceptible to dissolution or resorption and 2) that growth continues throughout life. If these assumptions are correct, calcified tissues are permanent records of the influence of endogenous and exogenous factors on their calcium-protein matrices. The hypothesis is that genetic differences between populations or differences in the environments to which each population is exposed affect the incorporation of trace elements in calcified tissues, which results in chemical compositions specific to each. An extensive fisheries literature supports the initial assumptions for otoliths, which are the calcified structures we have dealt with. The working hypothesis also appears reasonable, given an extensive literature on invertebrates that relates differences in the composition of, for example, mollusc shells and coral skeletons (calcium carbonate matrices similar to those in fishes) to a range of environmental and physiological conditions (Thompson and Livingston, 1970; Weber, 1973; Houck et al., 1977; Buchardt & Fritz, 1978; Smith et al., 1979; Rosenberg, 1980; Schneider and Smith, 1982).

Since 1967, measurements of trace elements in calcified tissues of fishes have been used in three areas of fisheries science. First, as indicators of pollution, principally by heavy metals (Papadopoulou et al., 1978, 1980; Johnson, 1989). Second, as a means of determining the ages of fishes, through analysis of either the rates of decay of 210Pb and 226Ra (Bennett et al., 1982; Campana et al., 1990) or the concentrations of isotopes or elements thought to vary seasonally as a function of water temperature, e.g., oxygen isotopes (Devereaux, 1967; Degens et al., 1969; Mulcahy et al., 1979; Radtke, 1984 a & b; Radtke et al., 1987), iron (Gauldie & Nathan, 1971; Gauldie et al., 1986), phosphorus/strontium ratios (Calaprice, 1985) and strontium/calcium ratios (Radtke & Targett, 1984; Radtke & Cailliet, 1984; Radtke, 1987; Radtke & Morales-Nin, 1989). And third, as an indicator of stock or sub-population identity (e.g., Klokov & Frolenko, 1970; Calaprice, 1971,1983,1985; Calaprice et al., 1971,1975; Bagenal et al., 1973; Behrens Yamada et al., 1979; Lapi & Mulligan, 1981; Mulligan et al., 1983; Mulligan et al., 1987; Edmunds et al., 1989). The analytical techniques used in these studies

have included atomic absorption spectrophotometry (Gauldie & Nathan, 1977; Gauldie et al., 1980), X-ray fluorescence spectro(photo)metry (Calaprice, 1971,1985; Calaprice et al., 1971,1975; Mulligan et al., 1983; Behrens Yamada et al., 1987), neutron activation analysis (Fishery Agency of Japan, 1967; Papadopoulou & Moraitopoulou-Kassimati, 1977; Papadopoulou et al., 1978, 1980), X-ray diffraction (Morales-Nin, 1987), radiochemical analysis (Bennett et al., 1982), stable isotope analysis (Radtke, 1983,1984; Radtke et al., 1987), proton-induced X-ray emission spectrophotometry/ proton-nuclear microprobe analysis (Calaprice,1985; Gauldie et al., 1986), energy-dispersive X-ray electron microanalysis (Lapi & Mulligan, 1981; Mulligan et al., 1987) and wavelength-dispersive X-ray electron microanalysis (Radtke & Cailliet, 1984; Radtke & Targett, 1984; Radtke, 1987; Kalish, 1989; Radtke and Morales-Nin 1989; Townsend et al. 1989). That no single technique has been universally adopted reflects the developing nature of the field and the diversity of research objectives. Moreover, to date there has been no comprehensive analysis of the advantages and disadvantages of each technique for analyzing the calcified tissues in fishes.

We began experiments in 1987 with a view to using ontogenetic variations in the chemical composition of fish otoliths as an indicator of movement/migration patterns. Acquiring such data dictated the use of a probe microanalyser, which could be scanned along a growth axis. Four probe microanalysers are suited for such studies -- electron, proton, ion beam and laser ablation probes. The relatively low cost, easy availability and apparently adequate sensitivity of electron probe microanalysers, based on the literature and the results of our pilot studies, suggested we focus our initial efforts on determining their suitability for analysis of otolith composition.

To acquire and interpret the ontogenetic data obtained with a probe microanalyser, we required two things. First, we needed a method for accurately sectioning otoliths along equivalent growth axes in different individuals, while providing the flat, smooth surface the microanalysers require. And second, we needed to make an informed choice between the two available electron probe techniques (energy-dispersive X-ray spectroscopy [ED] and wavelength-dispersive X-ray spectroscopy [WD]) for analyzing of the aragonite matrices of fish otoliths.

This paper describes the techniques we developed to prepare otoliths for analysis on an electron microprobe, discusses the strengths and weaknesses of ED and WD spectrometers in such analyses, and considers the problems and the compromises involved in acquiring the data.

Description of techniques and experimental results

Preparation of otoliths for electron probe microanalysis

Procedures for sectioning and polishing fish otoliths have been described previously by Pannella (1980), Neilson & Geen (1982), Radtke & Targett (1984), Karakiri & Westernhagen (1988), and Kalish (1989), among others. Relevant studies involving probe microanalysis have dealt with the requirements for single-point analyses; we describe our procedures in detail because they were developed for full life-history scans (sequential analysis at points along the complete growth axis of an otolith, and hence throughout the life of a fish) and because the standards of specimen preparation affect the quality of the data. This section is not a review of the field nor meant to imply that our techniques are superior to those described elsewhere. Rather it summarizes the procedures we have found necessary and sufficient to produce specimens of high enough quality for electron probe microanalysis.

a. Cleaning and storage of otoliths

After extraction, we cleaned each otolith of adhering tissue using fine forceps and a soft bristled brush in millipore-filtered distilled water. The otoliths are then dried in an oven at 40 - 45o C for a minimum of 6 hours. Otoliths taken from specimens stored in ethanol should be transferred into progressively dilute solutions of ethanol prior to drying, to prevent rapid dehydration and consequent fracturing. After drying, the otoliths are stored in small polyurethane capsules (20 mm long, 9 mm diameter, normally used for embedding purposes in transmission electron microscopy) in a moisture-free cabinet or under vacuum.

b. Embedding, sectioning and mounting

The procedure for embedding and sectioning sagittae that we used is as follows: (1) An accurately scaled diagram of the distal surface of the otolith is made with a camera lucida on a stereo dissecting microscope. The position of the primordium must be accurately marked on the diagram. (2) With the otolith oriented dorso-ventrally, the desired plane of analysis in our experiments ran anterior-posterior at the level of the primordium. To achieve a section encompassing this plane, the otolith is fixed firmly upright on its ventral edge to the base of an embedding mould with a drop of quick-drying epoxy resin. We used flat-bottomed polyurethane vials, in diameter slightly greater than the length of the otolith, as moulds. The mould is then filled with a hard-setting polyester resin. (See Mulligan et al. (1987) for a discussion of the desirable qualities of resin used for this purpose.) (3) The otolith is sectioned with a diamond-edged saw blade (350 µm thickness) on a rotary saw. The level of the first cut is determined from measurements made from the scaled diagram in Step 1. Usually, we made the first cut 250 µm below and parallel to the desired plane of analysis. A second cut, 250 µm above the plane of analysis, produces a section 500 µm thick that contains

a complete growth axis (Fig. 1). Ultimately, the thickness of the finished section depends on the accuracy of the scaled diagram and the precision of the cutting equipment. We also found that a small graphite-pencil mark on the distal face of each otolith that indicated the level of the primordium aided accurate sectioning.

Preparatory to grinding, we mounted the section onto a glass round (0.8 - 1.00 mm thick, diameter slightly greater than the section) with a quick-set epoxy resin, so that the side of the section closest to the primordium is against the glass. In thin sections (<300 μm), the primordium can usually be seen with transmitted light microscopy. In thicker sections, or where the primordium is not close to either side, the section is affixed to the glass round with paraffin wax. This wax can be melted, which allows reversimg the position of the otolith and grinding both sides of the section close to the plane of the primordium.

c. Grinding and polishing

Sections have to be ground to expose the growth axis, and then polished to produce the flat, featureless surface required for electron probe X-ray microanalysis. Surface defects can affect data quality by altering the absorption patterns of measured X-ray intensities in ways difficult to quantify (Potts, 1987). We grind the otolith by hand, with the section (on the glass round) held in a suitable grinding tool (an adaptation of that described by Naney, 1984), using 2400 grade silicon carbide wet/dry paper. Final polishing is done using progressively finer grades of diamond paste (6 - 0.5 μm) and/or aluminum oxide powders or pastes (e.g. Linde B, 0.5 μm) on a lapping machine. It is critical that the section and grinding/polishing tools be ultrasonically cleaned between all stages of the process, as cross-contamination of media inevitably scratches the surface of the section, which can adversely affect data quality. We discard data affected by surface irregularities, identified as such by inspection the scan point 'scars' after analysis.

After polishing, the section is ultrasonically cleaned of residual polishing compounds, using ethanol or some other suitable solvent, and then stored in a moisture-free environment, preferably under vacuum.

d. Coating

Non-conductive specimens, such as otolith sections, must be coated with a thin conductive layer to prevent charge build-up. The coat should be of uniform and known thickness, as absorption of X-rays by the coating affects the accuracy of an analysis, particularly when counting long wavelength X-rays. We use a carbon coat of 250 - 300 Å thickness on all standards and specimens. Prior to coating, the specimen is heated under vacuum at 80o C for 5 –10 minutes to remove residual moisture, particularly

from pits and cracks, to prevent bubbling and flaking of the coat during analysis. After coating, the specimen is held under vacuum until it is inserted into the probe.

Choice of the axis for analysis

Species-specific differences in otolith structure to a large extent determine the choice of section required to expose a complete growth axis. The sagitta of Thunnus maccoyii (Scombroidei), for example, has a long rostral process and a deep sulcus. For life-history scans of these otoliths, a section that runs from the primordium to the posterior ventral tip of the otolith provides a long, uninterrupted growth axis along which a complete life-history scan can be run. However, due to the shape of the section and the asymmetrical patterning of the growth axes, programmed probe analysis requires a mapped series of scan lines that track through the curve apices (Fig. 2). In contrast, the finished section from the sagitta of Nemadactylus macropterus, a demersal cheilodactylid from temperate Australian waters, is more nearly linear and may require only one or two such lines (Fig. 2). In both species, tracking through the curve apices results in data acquired along the fastest growth axis. This not only provides the best resolution of growth-dependent variation in composition, but also constitutes a standard scan axis for all individuals, which facilitates comparisons among them.

A comparison of ED and WD X-ray spectrometers for electron probe microanalysis of otoliths

a. Theoretical considerations

An electron probe microanalyser is essentially a scanning electron microscope to which is fitted one or more X-ray detection devices capable of recording X-ray intensity as a function of the photon wavelength or energy. There are two types of X-ray spectrometer on electron probe microanalysers: the energy-dispersive (ED) spectrometer and the wavelength-dispersive (WD) spectrometer. The operation, advantages and disadvantages of each for the acquisition of data from otoliths are now briefly discussed. More detailed treatments of X-ray spectrometry, data acquisition and, in particular, explanations of matrix corrections can be found in the literature on electron probe microanalysis (EPMA) (e.g., Reed, 1975; Heinrich, 1981). For readers not familiar with the field, Goldstein et al. (1981), Moreton (1981), Russ (1984) and Morgan (1985) provide excellent introductions.

The ED spectrometer is a multichannel device that, in normal operation, acquires a spectrum of a broad range of X-ray energies. When an X-ray photon enters the spectrometer's detector (a piece of cooled silicon or germanium), its energy is converted into an electrical pulse with an amplitude proportional to the energy.

According to this amplitude, the number in a particular memory location in a computer is incremented by 1. Commonly, the photon energies are mapped into 1024 memory locations or channels and then a spectrum, or energy histogram, of the photons that have entered the detector over a specified period is displayed. The vertical axis of the histogram represents the number of photons per channel and the horizontal axis is usually calibrated in energy units of thousands of electron-volts (keV).

In contrast to the ED system, a WD 'spectrometer' is, strictly speaking, a monochromator or a single-channel analyser -- it allows photons of only one, very limited, range of wavelengths to pass into its detector. The mean wavelength of this range is set by adjusting the angle at which the photons impact onto a diffracting crystal of a known plane spacing. If a spectrum is required, the crystal is serially and discretely scanned over the appropriate range of angle. At each setting, a count is made of the photons whose wavelength satisfies the constructive interference conditions defined by the crystal plane spacing and the chosen angle of incidence. Acquisition of a broad spectrum using a WD system is possible but very time consuming, so is seldom performed.

The fundamental difference between the two detectors is that the ED spectrometer is an electronic device that processes X-ray energies over an entire spectrum, whereas the WD spectrometer has a narrow operating range defined by the mosaic properties of the diffracting crystal and the width of the counter slit. This fundamental difference has two important consequences.

First, superficially the ED spectrometer is much more efficient at counting the photons entering its input aperture than is the WD spectrometer; because it processes the entire spectrum simultaneously it can acquire data on a range of elements much more quickly than can a WD spectrometer, which collects data on only one element at a time. For this reason, most WD-based EPMA's incorporate three or four spectrometers, each with a crystal with a different lattice plane spacing, among which the task of measuring peaks and backgrounds can then be shared. However, the electronic pulse processing circuitry in an ED spectrometer is much slower than the equivalent circuitry in a WD spectrometer: the former must amplify very accurately small signals from its semiconductor-based detector, whereas the latter only has to count the pulses whose range has been set by the physical properties of the detecting crystal. The high input rates of X-ray photons achievable in a WD spectrometer would saturate ED pulse electronics. As a consequence, WD spectrometers can operate efficiently at primary beam currents an order of magnitude higher than is practical for an ED spectrometer. The net effect is that an electron probe equipped with WD spectrometers can collect, in a given time, many more X-rays for analysis of a particular element than one equipped with a single ED spectrometer, which affects the counting statistics and hence the precision and resolution of the analysis.

Second, electronic amplification in an ED spectrometer also affects data quality and procedures for data processing. In an ED spectrometer, the energy conversion of an X-ray photon into an electrical pulse involves an degree of uncertainty due to random physical events. If sufficient conversions are made, the magnitude of the pulse is normally distributed about a mean value. Because of this conversion uncertainty, a monoenergetic flux of X-rays typically produces a peak 100 eV or more wide when measured by an ED spectrometer. By comparison, the peak on a WD spectrometer is only a few eV wide, since it is set independently of the electronic processing by the lattice structure and orientation of the diffracting crystals.

This difference between the spectrometers affects the way the data are processed. The spectra analysed by both spectrometers are generated, in the main, from photons arising from two processes. First, photons are emitted when atoms excited by the primary electron beam return to their ground state; these photons are referred to as 'characteristic X-rays', have well defined energies or wavelengths, and appear in the generated spectrum as narrow peaks. Their position and magnitude depend on the element, the electron shell(s) stimulated and the number of primary electrons. The second source of photons is the deceleration of electrons in the primary beam by the target. This deceleration produces a low, continuous background X-ray spectrum, known as the 'bremsstrahlung'(braking radiation). The narrow "characteristic X-ray peaks" sit on this background. The relationship between these peaks and the background differs between spectrometers, due to the way the data are processed. Electronic noise associated with data processing in ED spectrometers results in relatively broad peaks, again typically > 100 eV wide, that are rarely more than 3 - 20 times higher than the local background. In contrast, the narrow peaks in a WD spectrometer rise 30 -300 times above the background.

The first step in analyzing the spectrum obtained by EPMA is to remove the background and measure the magnitudes (i.e., height or area) of the peaks. This can be difficult with an ED spectrometer and is prone to error. The wide peaks in an ED spectrum often overlap and sit on relatively wide, often very non-linear regions of the background, all of which reduces the precision of the estimates (for background "stripping" procedures in ED spectrometry, see Ware and Reed, 1973; Schamber et al., 1977; Statham, 1977; Ware, 1981). In contrast, removing the background from WD peaks is usually simple. The background on one or both sides of the peak is measured, the value directly under the peak is calculated by extrapolation or interpolation and this value is subtracted from the peak ordinate. The implicit assumption of background linearity is generally warranted because of the narrow range of the background spectrum.

The differences between the spectrometers in peak-to-background ratios and the accuracy with which the background can be estimated critically affect the precision of

measured concentrations and minimum detection limits. The confidence interval for concentrations measured by EPMA can be found by solving for the roots of the quadratic expression given by Ancey et al. (1978). If one assumes that the background in the standard is negligible relative to the peak count rate, the percentage error (E) in the measured concentration can be expressed as:

(1)

where np is the total number of counts collected in the peak channel(s)

nb is the total number of counts collected in the background channel(s)

a is the ratio of background to peak counting time

2 is determined by the confidence level required. For example, at 95% confidence it is 3.84.

If this relationship is rewritten in terms of the difference between the peak and background counts, Np (=np-nb), and the value of a is 1.0 (which is often the case when measuring low concentrations), then

(2)

That is, the percent measurement error decreases as both the absolute difference and the ratio between peak and background count rates increase. As both absolute and relative differences between peak and background count rate are higher on a WD spectrometer than on an ED spectrometer, the former is more precise. As might also be expected, the detectable concentrations can also be smaller for the WD spectrometer than for the ED spectrometer. Minimum detection limits for WD spectrometers are typically in the range of a few hundred ppm, whereas those on an ED spectrometer are typically an order of magnitude higher and, in extreme instances, can be as high as 1% by weight (Statham 1981, 1982).

b. Experimental Comparisons

To test the relative utility of ED and WD spectrometers when used in EPMA for analyzing elemental variations in otoliths, we compared life-history scans of the same

otolith collected by means of a beryllium-window silicon ED spectrometer and a WD spectrometer. The otolith used was from a morwong, Nemadactylus macropterus. The comparison is based on two closely spaced, parallel paths along the main growth axis of the otolith.

The ED scans were made on a LINK SYSTEMS 860 Series II ED spectrometer fitted to a JEOL JSM 25 scanning electron microscope. The take-off angle of the ED detector was 25° and the beam current was 5.0 nA, giving an overall output count rate of 3200 counts per second (cps) at a dead time of 28% (time constant 20 μs). This is the highest practical output count rate of this model of ED spectrometer for quantitative analytical work. The counting time ('live time') was 240 s, giving a real total counting time very close to that for the WD scans. In this live time, over 750,000 counts were accumulated. Concentrations were determined using the Sr L, Na K and K K series of lines. Spectrum processing to remove the background and to extract k-ratios was by the Filtered Least Squares method (Statham, 1977), in which 'top-hat filtering' is applied to the spectrum. This method is widely used, giving, in general, excellent results when measuring concentrations as low as 0.5% by weight.

The WD scan was made on a CAMECA 'CAMEBAX' electron microprobe at a beam current of 25 nA at 15 KeV and a counting time of 90 s (60 s on peak and 30 s on background) at each point. Three spectrometers were used simultaneously, set, respectively, to the Sr La, Na Ka and K Ka lines.

The results of the comparison are given in Fig. 3. For all three elements tested, both the mean and the variance of estimated concentrations differed significantly between spectrometers. The much higher variance on an ED spectrometer is due to low peak-to-background ratios and low count rates that can be achieved with this spectrometer for all three elements. In the case of Sr, the scatter is also the result of errors in mathematically stripping a strongly non-linear background. The problem is illustrated by examining the region of the ED spectrum for strontium-free calcite several keV either side of the Sr L peak (Fig. 4). Despite the absence of Sr, the background is far from linear. This non-linearity results, in part, from the Sr La line coinciding with the silicon escape peak of the large Ca Ka and, in part, from the 'edge' in the background spectrum derived from the absorption of background X-rays and fluorescence of Si Ka X-rays in the "dead layer" of the detector (Statham, 1981). 'Stripping' the spectrum of the escape peak using the algorithm of Reed and Ware (1972) partly reduces the problem, but also, because the modelling is not perfect, increases the noise level of the background and introduces a small systematic error.

The difference in mean values for Sr is also due in part to the very non-linear background between 1.0 and 2.0 keV. The rapid decrease in detector sensitivity below 2 keV causes a high negative curvature which, when filtered, makes a spurious addition to any peak magnitude. As well, both the Si Ka absorption edge at 1.84 keV and the Si

Ka fluorescence peak from the dead layer of the detector cause local negative curvature in the background near the Sr La peak that is difficult to correct (Statham, 1981). Similar systematic errors, though in this case under-estimates of concentrations due to positive background curvature, are evident in Na and K. Similar differences in accuracy between the two spectrometers are evident when run on standards for each of these elements.

Alternatives to "top-hat filtering" of the ED spectrum, which potentially could correct these errors, run into other difficulties. For example, while it may be possible to model a reasonably accurate background curve, it is difficult to calculate the Si Ka fluorescence peak since the dead layer is not well defined (Statham, 1981). Scaling and fitting the calculated background would, inevitably, add to uncertainty in the peak magnitude. A.T. Marshall (pers. comm.) has suggested that subtracting a model spectrum taken from pure calcite from that acquired on the otolith would reduce systematic errors due to spectral artifacts. This would clearly be a better approach, since the artifacts would be similar in each spectrum. However, scaling and fitting are still required, which will add a small error.

In summary, in the ED detector tested there are systematic errors introduced by all methods of background removal in the region of the characteristic lines for Sr, Na and K. These errors are of a magnitude similar to the real concentrations of elements in the otolith. We conclude that at these concentrations, ED detectors as currently configured for use in EPMA are not sufficiently sensitive or precise to provide suitable data for our otolith-based analyses of life history scans.

WD-based EPMA; effects of operating conditions on data quality and acquisition

In EPMA the kinetic energy of the primary electrons that strike the aragonite target is dissipated as heat, causing local temperatures to rise rapidly. This heat causes the calcium carbonate, which constitutes the bulk of the target, to decompose to the oxide, losing carbon dioxide. This loss reduces the target's overall mass, thereby increasing the relative abundance (weight fraction) of the remaining elements and reducing the accuracy of measurements. This problem is inherent in all EPMA, but is particularly severe in WD analysis because of the high beam currents required to accumulate sufficient counts in the spectrometers to estimate element concentrations accurately. In theory, long acquisition times using very low beam currents minimize damage and presumably measurement error. In practice, however, such long acquisition times are often not practicable (due to the cost of EPMA and the number of samples needed for population analyses) and still leave unanswered the extent to which even slight levels of target damage affect data quality. Consequently, we examined experimentally the effects of different beam conditions on measurement accuracy (and

minimum spatial resolution of data points) to quantify the trade-offs involved and to determine optimal operating conditions for making life-history scans of our subject species.

Three operating parameters appeared to us to be of paramount significance: (1) the accelerating voltage, which determines both the excitation efficiencies for the spectral lines selected for analysis and the volume of the target from which X-rays are produced, (2) beam power density, which is the combined effects of accelerating voltage, beam current and the area scanned and which in part determines damage rates, and (3) the order in which elements (X-ray lines) are sequentially measured on the available spectrometers.

1a. Effects of accelerating voltage on excitation efficiency and the absorption of X-rays

The accelerating voltage (i.e., beam energy) should be high enough to induce efficiently the most energetic selected characteristic X-ray lines for the elements being analyzed, but low enough to ensure, first, that absorption correction factors for the least energetic lines are reliable when standards and specimen are dissimilar in composition and, second, that the volume of the target from which X-rays are excited is as small as possible in instances where a highly focussed probe is required. We initially tested otoliths at voltages ranging from 5 to 30 kV. Five kV was too low to excite elements such as potassium adequately for detection, and voltages over 20 kV produced very large volumes within which the X-rays were generated without a commensurate gain in measurement accuracy. We preferred to avoid large generation volumes because we occasionally need to use a nearly focussed probe in, for example, parts of the otolith where growth is very slow. Hence our experimental studies were limited to the range of 10 to 20 kV.

We routinely measure five elements present in low concentrations, as well as Ca. Table 1 lists these elements, their characteristic spectral lines and the count rates (i.e., the rate of data acquisition) at these lines that would be measured on our probe at three accelerating voltages, after background subtraction and assuming a concentration of 2000 ppm by weight for each element. Values were calculated from count rates on standard materials for each line and on the ratios of the intensities on standard and aragonite targets computed with the "PAP" (Pichou and Pichoir, 1984) matrix conversion software supplied by CAMECA. To ensure that the damage caused by heating was approximately the same at all voltages, the power dissipated in the target was arbitrarily held constant at 375 μW by adjusting the beam current. In these tests, beam size was 14 μm2.

In general, count rates increase as the accelerating voltage increases, but the rate of increase is uneven among elements (Table 1). The rate actually decreases at higher voltages for Na, due to heavy absorption of the relatively long wavelength Na Ka X-

rays by the aragonite matrix, even though the excitation efficiency increases. We suspect that this heavy absorbtion is due to pitting of the target surface, which often occurs at high voltages. In contrast, K Ka count rates increase substantially between 10 kV and 20 kV. This increase results from a large increase in the generation efficiency of this line at higher voltages and relatively little absorption of the emitted X-rays, which have the shortest wavelength of any element in our analyses. Correction factors for K, due to absorption, as computed for an aragonite matrix based on Philibert's formula (Philibert, 1963), are slight at all voltages tested; even at 20 kV, 95% of the X-ray intensities generated are counted. In contrast, for Na correction factors vary from 0.70 at 10 kV to only 0.39 at 20 kV, i.e., at voltages > 10 kV, the Ka count rates measured are only about half of the X-ray intensities actually generated.

Procedures for collecting data and correcting them for matrix effects at each voltage are illustrated in Table 2. The comparison is based on count rates from parallel analyses with each accelerating voltage in the same area of the same otolith. The peak and background are measured in turn for each line, a subtraction is performed, and concentrations (computed weight-fractions, in p.p.m.) are calculated from the matrix corrections for aragonite. Measurement errors are estimated from equation (1) and the detection limits are calculated by Ancey et al.'s (1978) method.

Two points are evident from Table 2. First, background-corrected count rates show the same trends across elements and voltages as was evident in Table 1. But second, percent measurement errors do not necessarily increase as background-corrected peak count rates decrease (e.g., S Ka at 15 kV and 20 kV). The measurement error decreases in this instance because the error term depends on both peak and background count rates, and the latter is lower for S at 20 kV than 15kV. The optimal accelerating voltage has to be decided by balancing across elements the effects of different voltages on the error terms associated with each element, e.g., percent errors are lowest for Na Ka at 10 kV, but lowest for K Ka at 20 kV.

1b. Effects of accelerating voltage on the volume of X-ray generation

Particularly for focussed electron probes, the voltage chosen should ensure that the volume of the target from which X-rays are generated is small enough to achieve the spatial resolution (e.g., distance between adjacent points) required of the scan or experiment. However, the voltage should also be large enough to ensure that most of the X-rays produced are generated below the surface layer, minimizing effects of any contamination or irregularities in the surface (while also bearing in mind that with the three-dimensional structure of otoliths, increasing the depth of the generation volume also increases the number of increments over which the data are acquired). The lateral diameter of the generation volume is given approximately by :

,(3)

where L is the diameter of the volume (in μm) from which 95% of the generated X-rays are emitted, Eo is the accelerating voltage (in KeV), Ec is the excitation energy for the X-ray line (in KeV) and is the density of the target. This expression follows Reed (1975), although modified slightly based on Heinrich's (1981) suggested relationship between the width and depth of X-ray generation.

An estimate of the depth into the target from which X-rays are received by the detectors is given by Heinrich (1981):

,(4)

where D is the depth (in μm) from which 95% of X-rays are received by the detector, , Eo and Ec are as in equation 3, and f is the absorption correction factor.

It is evident from these equations that, for a given accelerating voltage, L is largest and D smallest for Na, since it has the lowest excitation energy, and L smallest and D deepest for K, as it has the highest excitation energy. Calculated generation diameters and penetration depths for these elements at the three accelerating voltages considered and assuming a conservative otolith density of 3.0 are given in Table 3. These values set the maximum spatial resolution that can be achieved in a life history scan. In many cases, even these values are impractical since it is necessary to degrade the horizontal resolution, by defocussing the beam, to minimize specimen damage (see below). A defocussed 10 μm beam at 15 kV, for example, would generate Na Ka X-rays from an actual disc about 14 μm in diameter. Furthermore, as target density almost certainly varies throughout an otolith (and is usually less than 3.0), even these values must be treated as approximations only.

2. Beam power density and specimen damage

The kinetic energy of the electrons in an EPMA beam is dissipated as heat in the target. In otoliths, the temperature rises sufficiently to decompose some of the carbonate, causing pitting (Fig. 5) and a loss of measurement accuracy. The rate of chemical change depends on the beam power density (BPD), which we define as:

,(5)

where Eo is the accelerating voltage, I is the beam current and A is the area over which the electron beam is spread. (In a focussed beam, the beam spread is Gaussian, whereas in the defocussed condition, the beam is nearly uniformly spread over the sampled area, with only a slight Gaussian roll-off at the margins.)

The effect of changing beam power density on count rates and stability were determined for Ca, Sr and Na through a series of experiments, in which each of the three parameters that define beam power density (Eo, I and A) was varied independently. The details are given in Table 4. The number of counts in 5 s intervals were measured over periods of 120 s for Na and 240 s for Sr. Ca was measured on a second spectrometer at the same time as either the Na or Sr counts were being collected. Five replicates of each treatment were taken to estimate the variance in counts between different points in the same region of an otolith.

Experiment 1. The effects of spot size on count rates of Na Ka, Sr La and Ca Ka, voltage (15kV) and beam current (25 nA) held constant; total dwell time 120 s.

Spot sizes ranged in diameter from a nominal 3 μm (focussed beam) to 20 μm (defocussed), which correspond to beam power densities ranging from nominally 50 μWμm-2 to 1.2 μWμm-2. The spot diameter was set by observing the cathodoluminescent disc through the optical microscope of the electron probe when it was targeted on a piece of polished thorium oxide. The effect of spot size on sodium, strontium and calcium counts in successive 5 s intervals is depicted in Figure 6, and for Na and Sr normalized to Ca in Figure 7. The response patterns for Na, Sr and the normalized data are similar. For all but the 3 μm and 7 μm spots, counts are highly variable, but increase relatively stably over the 120 s. However, under a 3 μm beam, Na counts decreased rapidly to average -72.6% at the end of the full 120 s, most of this decline occurred in the first 30 s and Sr counts decline rapidly over the first 100 s. Calcium count rates also differed in pattern among treatments, increasing rapidly within the first 10 s of counting at 3 μm, increasing asymptotically at 7 and 10 μm and increasing linearly for the 17 μm and 20 μm treatments. The variance among replicate counts was extremely high at 3 μm (120% of the mean), but much less at other spot sizes. Results for Na/Ca and Sr/Ca ratios, in general, paralleled those for the elements without normalization, with the erratic behavior of Ca appearing to have little effect.

The overall change in Ca counts correlated with beam power density up to a certain point (9.7 μWμm-2), above which the relationship appears to breaks down (Fig. 8). Changes in Na, Sr and normalized data are relatively unaffected by beam power densities less than about 2 μWμm-2, but then increase (negatively) at higher values (Fig. 8).

Experiment 2. The effects of voltage on counts of Na Ka, Sr La and Ca Ka, beam diameter (14 μm) and current (25 nA) held constant; total dwell time 120 s.

The voltages tested ranged from 10 to 25 kV, corresponding to a range of beam power densities of 1.6 to 4.1 μWμm-2. The results are depicted in Fig. 9. At the three lower voltages tested, Na and Ca counts and the Na/Ca ratio increased slightly with dwell time. However, changes in Na counts and the Na/Ca ratio are negative at 25 kV, whereas Ca count rates continued to increase.

Changes in Sr counts (Fig. 9) were smaller than those in Experiment 1. Except for the 10 kV treatment, for which Sr counts decreased over the 240 s, all voltages tested resulted in small, linear increases in Sr counts. Calcium counts were stable at 10 kV, but increased asymptotically at other voltages (net mean increases of +9.4, +10.1 and +10.4% for 15, 20 and 25 kV, respectively). Changes in Sr/Ca ratios over 240 s were small and negative.

Experiment 3. The effects of beam current on counts of Na Ka, Sr La and Ca Ka, beam diameter (14 μm) and voltage (15 kV) held constant; counting time 120 s.

Beam currents ranged from 10 to 40 nA, corresponding to beam power densities of 1.0 to 3.9 μWμm-2 . The results are depicted in Fig. 10. Na counts changed little over the total 120 s dwell time, but in all cases changes were positive. Ca counts also increased in all treatments; the extent of the change correlated with the beam power density. Na/Ca ratios increased slightly in the 10 nA treatment, but decreased slightly at 25 and 40 nA.

Mean Sr counts changed minimally at 10 nA, but increased 6.8 and 8.8% at 25 and 40 nA, respectively (Fig. 10). In general, the higher the beam current, the greater the variance in Sr counts. Ca counts increased with time in all three treatments, the extent of change being proportional to the beam power density. Sr/Ca ratios decreased slightly over the 240 s in all treatments.

Summary and Interpretation of Experimental Results

The results of these experiments document that variations in any of the three beam operating parameters (spot size, voltage and current) can change the count rates of the elements that constitute an otolith's aragonite-protein matrix. The nature, direction and magnitude of these changes were not simple, but rather appeared to reflect the influence of several different factors. First, in many tests, count rates increase with time, either abruptly or as long term linear or asymptotic trends. Such an increase, evident in Ca Ka, Sr La and Na Ka, occur when the weight fraction of an element increases due to decomposition of the carbonate and subsequent loss of the target's mass. In all three elements, however, the positive trend eventually breaks down and counts decline markedly (Fig. 8). At high beam power densities (such as when using the

focussed beam), this decline begins almost immediately during acquisition (Fig. 6). We attribute these declines to the effects of pitting. When a pit appears in the surface of an otolith, the emitted X-rays are heavily absorbed, due to an increased path length. All elements are affected by this process, though the magnitude of the effect differs between elements, corresponding to the energy of their respective X-rays (i.e., because Ca Ka X-rays are more energetic than those of Sr La and Na Ka there is less absorption and less attenuation until pitting is severe). It is difficult to determine when in an analysis the effects of pitting outweigh any effects of chemical change on count rates. In the 25 kV treatment in Experiment 2, for example, Na counts increased over the first 90 s only to fall during the final 30 s. Presumably, after 90 s the effects of pitting outweighed those of chemical change. The difficulty lies in measuring the effect of pitting 10 s, 30 s, or 60 s into the analysis. Since the absorption coefficient for Na Ka in CaCO3 is very similar to that in CaO, the Na Ka count rate should change at a rate similar to Ca Ka, if pitting was not a factor. In fact, this was not the case in our experiments: when Na counts increased, they did so more slowly than Ca counts. Sr counts also fell -- presumably due to pitting -- when small spot sizes and high beam power densities were used. We conclude, therefore, that pitting is always a factor when otoliths are analyzed with WD EPMA and always affects perceived X-ray count rates.

3. Selection of counting times and scheduling of spectrometers

As with all of the other parameters that need to be considered in formulating a set of operating conditions, the choice of suitable counting times is a compromise between the accuracy and precision desired and the time available to analyse the required number of points. Elements for which peak-to-background ratios are high (e.g., Ca) generally require shorter counting times than those for which the ratio is low (e.g. Cl or S). The counting times we used in routine analysis of the six elements (other than carbon and oxygen) most abundant in otoliths (Ca, Na, Sr, K, S and Cl) are given in Table 4. These acquisition times were chosen in part on the basis of our pilot studies and in part on indications from the literature of the degree to which the concentrations of these elements vary in otoliths. The time spent in moving spectrometers and doing matrix corrections on-line is about 13% of counting time.

In scans where several elements are to be analysed at each point, scheduling of the serial measurements of the spectrometers depends on the relative stability of the count rates and the precision required for each element. Table 4 shows one of our typical schedules. To save machine time, backgrounds were measured on only one side of each peak and extrapolated to the position of the peak with a measured gradient. Each scan point took about four minutes. The elements that are normally found in lowest concentrations share spectrometer 1. For this spectrometer, we calculated the trade-off between counting times and measurement precision for K and Cl, and chose a

time allocation that gave us the best combination. Ca is measured about half way through the analysis, because all elements are to be normalized to it. On spectrometer 3, sodium was measured before strontium because Na Ka counts are more likely to be severely affected by pitting.

4. Standards and correction for spectrometer drift

When unknown materials are analyzed with EPMA, standards are routinely measured in parallel to check for spectrometer drift (i.e., slight changes in the position of the analyzing crystal resulting from changes in, for example, temperature) during extended runs. The standards used in our studies are given in Table 5. Throughout our studies, Ca, Sr and S standards were measured at the beginning and end of each life history scan, as well as every 60 points throughout "long" scans. Spectrometer drift was generally very slight, in the order of 1-3%, but very occasionally, drift resulted in differences of up to 15% between initial and final standard counts. The reasons for these extreme cases are not known. However, there was some indication that spectrometer drift was highest when room temperatures or atmospheric pressure varied significantly during a scan.

We corrected for spectrometer drift by multiplying estimated weight fraction concentrations by correction coefficients calculated by linear interpolation between the initial and final measurements of the standards. This procedure obviously assumes drift is linear, but this assumption can be tested at any stage of an analysis if it is in doubt. We also arbitrarily discarded life-history scans in which spectrometer drift exceeded 5% for any one of the three standards.

Experimental test of measurement error

The ultimate tests of the procedures developed above are to determine, first, the extent to which they produce consistent estimates of concentrations and, second, whether patterns in the variation of these concentrations can be consistently replicated within and between specimens. In practical terms we undertook these tests by measuring the degree to which parallel life-history scans in two otoliths from the same fish indicated similar ontogenetic variation in element concentrations. Assuming that the chemistry of otolith deposition does not differ between the two sides of the endolymphatic system, otoliths from the same animal should have identical chemical patterns. Hence, differences between the two life-history scans would reflect the limits of our procedures, that is, specifically, the combined effects of the quality of specimen preparation (including the extent to which we could section along the same growth axis in the two otoliths), of the beam power settings we used and of inherent measurement

error. The comparison indicates the limits to which EPMA of elemental variations can be used for assessing biological significance, under our operating conditions.

The comparisons were done with pairs of sagittae from morwong (N. macropterus). A number of comparisons were done; the results from all were similar and those from one chosen at random are depicted in Figure 11. Plots for each element for the two otoliths overlapped closely and, in all elements, indicated similar patterns of ontogenetic variation. Aside from occasional single point "spikes", the estimated absolute concentrations were virtually identical in the two otoliths for all elements except sodium. For sodium, both ontogenetic patterning and mean absolute concentrations were much the same in the two otoliths, but for reasons not yet clear to us the tracks diverged slightly in this individual near the primordium and edge. Interestingly, potassium and sulphus also show edivence, albeit much less than for sodium, at about the same positions, which may indicate differences between the otoliths in their growth characteristics. However, such divergence was not typical in our left/right comparisons, and in general, we conclude that our methods produce a high level of repeatability in both the patterning and absolute concentrations of all six elements assayed.

Discussion

There are clear differences between the abilities of ED- and WD-based EPMA to detect and accurately quantify the elements in the calcium carbonate matrix of an otolith. The conventional ED electron probe is severely limited its ability to detect elements present in concentrations less than 1000 ppm because of difficulties in resolving line overlaps, the presence of spectral artifacts and the generally low peak-to-background ratios and low peak count rates. Nonetheless, some improvements in using these spectrometers for life-history scanning may be possible. Systematic errors, for instance, can sometimes be avoided by compromising on other analytical parameters. For example, when the background is linear and there are no interfering lines or artifacts, Sr could be measured on an ED system using the Sr Ka line at 14.14 kV. However, the accelerating voltage would have to be increased to 35 kV to obtain sufficient excitation efficiency, which may be undesirable because the primary electrons are scattered through a large volume and correction procedures for the other, lighter elements become unreliable. Systematic errors in the measurement of Na Ka could also be reduced if a thin window or windowless detector were used; these detect Na Ka X-rays more efficiently and produce a flatter background spectrum at this line energy than the conventional Be window detector we used. The minimum detection

limits for lines near large peaks (e.g., K Ka) might also fall due to the impressive gain in energy resolution (less than 130 eV for Mn Ka) recently achieved by ED manufacturers, largely without sacrificing counting speed. The new thin-window germanium detector for the soft X-rays used in microprobe analysis avoids the problem of the escape peak overlapping Sr La, although it does create a new overlap between the Ge La and Na Ka lines. However, the energy resolution is high (around 135 eV for Mn Ka) and this type of detector shows promise for studies of "age determination", in which strontium is the primary element of interest (e.g., Radtke and Targett, 1984). Given these recent advances in ED spectrometers, and the fact that they are cheaper and more widely available than WD systems, we are continuing our work on defining the areas of life-history scanning that might be amenable to ED spectrometry.

Until the difficulties in conventional ED-based EPMA can be resolved, we suggest such systems be used cautiously for detecting and measuring trace elements in otoliths, particularly if many elements are likely to be present. In a recent study using an ED spectrometer, Mulligan et al. (1987) determined the presence or absence of 34 trace elements in the otoliths of striped bass (Morone saxatilis) from tributaries of Chesapeake Bay (USA). They did not report the concentrations detected, but it seems likely from our research that, other than carbon and oxygen, only three elements -- Ca, Sr and Na -- routinely occur in concentrations above 500 ppm. Unless the otoliths of M. saxatilis differ markedly from those we have examined, Mulligan et al. (1987) detected elements at concentrations below 0.1 wt % (1000 ppm), which is the practical minimum detection limit of an ED spectrometer (for some elements, the limit can be as high as one or two percent when lines overlap broadly (Statham, 1981)). It is not clear how Mulligan et al. (1987) achieved the sensitivity they reported.

Unfortunately, Mulligan et al. (1987) also give no indication of their criteria of detection, although calculating the minimum detection limit at a given confidence level must precede detection. Ancey et al., (1978) showed that the minimum detectable concentration Cmin is given by

, (6)

where Cs is the concentration of the element in the standard, F is a matrix correction factor, which corrects for effects of absorption, electron backscattering and fluorescence in both standard and unknown , I is the peak count rate on the standard for the line being measured, B is the background count rate on the unknown (in this case the otolith), t is the peak counting time on the unknown, determines the level of confidence (for example, it is 13.0 for a 5% risk of both kinds - that of detecting the element when it is, in fact, not present and also of not detecting it when it is, in fact, present), and a is the ratio of the background to peak counting times (for ED

spectrometers, a = 1.0). Any concentration "measured" less than C_{min} is meaningless and the element has not been detected. For purposes of statistical analysis, we record as zero any "measured" concentration less than the minimum detection limit. It is not clear how Mulligan et al. (1987) treat values less than Cmin. This is particularly worrying in that they report the presence in otoliths of such elements as technetium (Tc), which is a radioactive element produced in fission reactors and released into the environment in minute quantities from medical laboratories. It is likely that the reported "detection" of Tc is due to an inadequate definition of detectability rather than extraordinarily high levels of the element in the environment.

Mulligan et al. also reported detecting the rare earth elements (REE's) Ce, Pr, Sm, Gd, Tb, Dy, Ho, Er, Tm and Yb in M. saxatilis otoliths. The L spectrum of these elements, which is used to assay their presence, contains at least 10 lines with intensities greater than 1% of the respective La line. Because the La lines for all of these elements lie between 4 and 11 keV, overlaps are numerous (even for WD spectrometers), making their individual detection and measurement virtually impossible at low concentrations. With ED spectrometers, the overlaps are particularly severe for atomic number differences between 2 and 5 (Smith and Reed, 1981). Errors in measuring the lighter REEs affect measurements of the less abundant heavier ones (Haskin and Paster, 1984) through the use of overlap coefficients. The heavier, odd-numbered REEs have been measured with an WD spectrometer (Amli, 1975), but only at concentrations around 3000 ppm by weight and only in mineral phases where the lighter REEs are virtually absent. Therefore, Mulligan et al.'s report of detecting Ho and Tm by ED-based EPMA appears incredible, unless these elements have been bio-concentrated to an extraordinary degree in otoliths.

Finally, it should be noted that extensive line overlaps are not confined to the rare earth elements; Ka and Kb lines overlap extensively between neighbors for elements with atomic numbers 22 to 30 (Ti to Zn). Pulse pile-up and silicon escape peaks add to the interference problem. Given such difficulties, it is essential that studies such as Mulligan et al.'s report not only statistical criteria for detection, but also techniques for dealing with line overlaps and spectral artifacts. Without such details, other workers cannot assess the accuracy of the nominally effective stock discrimination of Morone saxatilis reported by Mulligan et al.(1987).

In summary, at counting times over 10 minutes, ED spectrometers can detect concentrations of elements within otolith aragonite matrices as low as around 500 ppm, but only if artifacts, overlaps and non-linearities in the background can be accurately modelled. This will not generally be the case and especially not when large numbers of elements are being assessed. We conclude that the ED spectrometer is not generally effective for measuring trace element levels and for scanning life-histories in otoliths. Whether they are adequate for measuring concentrations in other calcified structures in

fishes, e.g., scales and vertebrae (see, for example, Sauer & Watabe, 1989), remains to be determined.

Operating parameters and data quality in WD-based EPMA

WD-based EPMA is nominally capable of detecting and accurately measuring elements present at concentrations over 100 ppm. In the otoliths we have examined, this effectively limits the elements to six: Ca, Na, Sr, K, S and Cl. Carbon and oxygen, which are also abundant in aragonite matrices, can be measured with a WD microprobe but the concentrations of both are very unstable under the electron beam and hence they are not routinely analysed. We have rarely found other elements, such as magnesium, iron, and zinc, in concentrations greater than 100 ppm. We note, however, that our samples include only a limited number of species (albeit ecologically quite diverse), and that further work on additional species is clearly needed before broad generalizations about element concentrations in otoliths are warranted.

The effect of the electron beam on the otolith must be considered in interpreting data produced by a WD system. Our tests indicate that the extent and nature of the damage incurred, and hence the quality of the data, are closely related to the beam power used in the analysis and the area over which the electron beam is spread, that is, beam power density. The greater the beam power density, the greater the damage and the lower the data quality, but the faster the acquisition rate. As machine time is expensive, individual operators will always have to decide how much damage is acceptable. At the other extreme, very low beam power densities ensure minimal chemical change and pitting, but also require very long counting times to provide viable measurements for elements that are present at low concentrations. Unless very accurate estimates are required, machine time is unlimited or very few points are to be analyzed, beam power densities less than ~ 0.5 $\mu W \mu m^{-2}$ seem to us to be rarely warranted.

We settled upon a maximum beam power density of 3.0 $\mu W \mu m^{-2}$ as an acceptable compromise between damage and acquisition rate. At densities below this, count rates of Ca, Sr and Na counts are relatively stable and the normalized Sr/Ca and Na/Ca ratios decline only slightly with increasing dwell time (up to the limits required to acquire enough counts for measurement errors to be less than about 5%). In practice, of course, the time taken to analyze each point depends upon the desired degree of accuracy and the number of elements sought, both of which depend on levels of natural variability and on the objectives of the study.

Because of the very high beam power densities focussed beams ($\leq \sim 5$ µm diameter) entail, even if scanned, they do not, in our experience, produce adequate stability within an otolith matrix for effective measurement of concentrations of most elements. In addition to producing chemical change and pitting, focussed beams also

often disrupt the surface coating, with resultant charging of the target. Not only does damage of this kind result in erroneous estimates, but also -- and perhaps more importantly -- the extent of this error is difficult to quantify. Unless one is prepared to accept (or ignore) such sources of systematic error, focussed beams do not appear suitable for the analysis of otoliths. Parenthetically, scanning the beam without defocussing does not appear to be an effective means of reducing damage. The heating from a focussed beam scanned once over a given area has a greater, and less predictable, effect on data quality than that from a beam defocussed to cover that same area.

Assuming one operates at a fixed 'safe' beam power density and noting that the counting error is inversely proportional to the square root of the number of counts collected, then it follows from Equations 2 and 5 that this counting error (E) is related to the beam diameter (d) and the counting time (t) by the proportionality:

(7)

That is, counting errors decline rapidly with an increase in beam diameter, but much more slowly as counting time increases. As increasing beam diameter also permits an increase in the beam power without changing the damage rates, it follows that whenever possible the beam diameter should always be set to the highest possible value, that is, the largest consistent with the spatial resolution required by a particular study. Adjacent points in an analysis, however, still need to be separated by at least 4 μm, because of beam spreading in the target.

Measurement Error and Scales of Natural Variability in Otoliths

The aim of any measurement procedure is to reduce measurement error to levels less than the signal being sought. Obviously, the greater the signal-to-error ratio the more confidence one has in the measurement. Assuming that the effects of beam damage, pitting and the like have been taken into account, the resulting measurement error in analyzing otoliths has two very different components: that due to counting statistics and that due to inherent, small-scale variability in the otolith's composition. The first component can be readily quantified by standard analyses, and are summarized in Table 5. Given the count rates we can achieve without undue specimen damage, our estimates of composition range in accuracy from about 4% for Na to about 20% for S and Cl. Not surprisingly, the more abundant the element in an otolith, the better the counting statistics and the more accurate our estimates. Hence, at the concentrations we routinely measure and on the basis of machine properties alone, differences between points or individuals in Na concentrations of less than about 5% and in Cl levels of less than about 20% are noise. Determining the magnitude of

measurement error induced by microscale differences in composition, which is below the scale involved in our life-history scans, is more difficult. Parallel runs on pairs of otoliths (Fig. 11) suggest that error due to this scale of natural variability is of the same order of magnitude as that due to statistical properties of count rate acquisition. We have found that filtering the raw data with a 5 point moving average eliminates much of this noise while still retaining what appears to be the biologically significant signal in the data. We emphasize, however, that this is a wholly subjective and arbitrary decision until the scale of the relevant signal can be determined from suitably designed field and laboratory studies.

For the six elements that we routinely assay, the counting error, approximate sampling error and scale of natural variability of concentrations for the two species of fish with which we have worked extensively are given in Table 6. In all instances, the scale of natural variability in both species exceeded by at least an order of magnitude the combined counting and sampling measurement errors. On that basis, we conclude that our procedures are adequate and sufficient for determining at least the major ontogenetic trends in the deposition of these macroconstituents in the otolith and for using such data in studies of stock discrimination and population structure.

Table 1. Calculated count rates for aragonite containing 2000 ppm, by weight, of the elements listed in Table 1. Beam power is set at 375 μW.

Element and line	Count rates in Hz			Analysing crystal
	10 kV at 37.5 nA	15 kV at 25 nA	20 kV at18.8 nA	
Na K_α	40.1	27.0	21.4	TAP
Sr L_α	32.5	40.0	40.7	TAP
S K_α	16.8	22.7	25.0	PET
Cl K_α	19.2	26.8	30.2	PET
K K_α	27.0	43.0	53.3	PET

Table 2. Count rates, counting errors, and corrected concentrations for the same morwong otolith target at 10 kV, 15 kV and 20 kV. ND = not detected. In this experiment, the beam was defocussed to 50 μm diameter to minimize damage to the target. The total dwell time of the beam on the target at the end of the experiment was 12 minutes (4 minutes at each voltage). Peak and background counting times were 60 s and 30 s respectively. Errors are calculated with χ^2 = 3.841 and the detection limits with λ = 13.0.

Element and line	Count rates in Hz		
	10 kV 37.5 nA	15 kV 25.0 nA	20 kV 18.75 nA
Na K_α			
peak	69.7	56.6	36.7
bgnd	11.0	8.7	5.1
peak-bgnd	58.7	47.9	31.6
Computed weight fraction (ppm)	2880	3100	2970
abs and % errors	(±119, 4.1%)	(±139, 4.5%)	(±162, 5.5%)
Detection limit			
Sr L_α			
peak	69.1	71.7	65.6
bgnd	37.6	40.3	33.7
peak-bgnd	31.5	31.4	31.9
Computed weight fraction (ppm)	1960	1625	1700
abs and % errors	(±189, 9.6%)	(±162,9.9%)	(±156, 9.1%)
Detection limit			
S K_α			
peak	5.5	8.0	6.1
bgnd	2.1	3.8	2.5
peak-bgnd	3.4	4.2	3.6
Computed weight fraction (ppm)	410	380	330
abs and % errors	(±95, 23%)	(±90, 24%)	(±77, 23%)
Detection limit			
Cl K_α			
peak	5.0	6.1	6.1
bgnd	4.0	4.9	5.6
peak-bgnd	1.0	1.2	0.5
Computed weight fraction (ppm)	110 ND	90 ND	30 ND
Detection limit	200	140	130

K K_α			
peak	16.3	28.5	32.7
bgnd	7.9	11.0	10.8
peak-bgnd	8.4	17.5	21.9
Computed weight fraction (ppm)	750	840	880
abs and % errors	(±128, 17%)	(±86, 10.2%)	(±75, 8.5%)
Detection limit			

Table 3. Diameter (L) and depth (D) (in μm) of the generation volume in aragonite from which 95% of X-rays emanate when the beam is focussed (after Reed, 1975 and Heinrich, 1981).

Element and line	Accelerating voltage		
	10 kV L/D	15 kV L/D	20 kV L/D
Na K_α	1.6/0.7	2.9/1.1	4.5/1.3
K K_α	1.3/0.8	2.7/1.8	4.3/2.9

Table 4. Typical scheduling of the three WD spectrometers to measure six elements (time taken to complete each count is given in brackets).

Spectrometer #	3	2	1
Crystal	TAP	PET	PET
	Na K_α pk (60 s)	K K_α pk (60 s)	S K_α pk (60 s)
	background (30 s)	background (30 s)	background (30 s)
	Sr L_α pk (60 s)	Ca K_α pk (10 s)	Cl K_α pk (60 s)
	background (30 s)	background (5 s)	background (30 s)
	On line PAP matrix correction		(12 s)
	Beam current measurement		(10 s)
	Spectrometer movement		(20 s)

Table 5. Standards used in WD life history scans of otoliths.

Element	Line	Standard	
Ca	$K\alpha$	Wollastonite	$CaSiO_3$
Sr	$L\alpha$		$SrTiO_3$
S	$K\alpha$	Pyrite	FeS_2
Na	$K\alpha$	Jadeite	$NaAlSi_2O_6$
K	$K\alpha$	Adularia	$KAlSi_3O_8$
Cl	$K\alpha$		AgCl

The Na, K and Cl standards are unstable under a dwelling point beam, so standard count rates must be acquired with a 50 μm moving beam. As standards for Ca, Sr and S are stable, these are used to check for spectrometer drift during scanning.

Table 6. Minimum detection limits, amount of measurement error and the mean and ranges of estimated concentrations (weight fractions), for morwong and southern bluefin tuna, of the six elements that can be assayed reliably with a WD electron probe. The values for morwong and tuna are based on a random subset of our data, include numerous individuals and positions along the scanned axis of points analyzed in the two species. The values illustrate concentrations in the two species; they are not definitive means and ranges. n = 478 points for morwong and 120 points for tuna. Concentrations are given in ppm, except for Ca which is in percent of the target mass. Note that values below the minimum detection limit are effectively zero.

Element	Minimum detection limit	Morwong		Tuna	
		Mean (range) concentrations	Measurement error (absolute, %)	Mean (range) concentration	Measurement error (absolute, %)
Ca	-	38.8% (35.3 - 44.5)	-	40.6% (39.2 - 41.2)	-
Sr	311	2240 (1430 - 3860)	±157 (7%)	1626 (1070 - 2380)	±153 (9%)
Na	159	3331 (2680 - 4240)	±122 (3.7%)	2916 (1590 - 3790)	±117 (4%)
K	136	729 (280 - 1630)	±72 (10%)	442 (230 - 750)	±67 (15%)
S	149	421 (220 - 1220)	±76 (18%)	392 (210 - 790)	±75 (19%)
Cl	157	255 (0 - 1230)	±72 (28%)	272 (10 - 1170)	±72 (26%)

Figure Legends

Fig. 1. Distal view of a Nemadactylus macropterus sagitta, showing position of the first cut (fc) and second cut (sc) taken to provide a section that includes the primordial region (p) and the full growth axis to the posterior extremity (pe). The solid line at the ventral edge (ve) of the otolith illustrates the region of the otolith that is glued to the base of the mould. Scale bar = 2 mm.

Fig. 2. Diagrammatic representation of a prepared section from a T. maccoyii sagitta (upper figure) and a Nemadactylus macropterus sagitta (lower figure) showing the position of the programmed series of electron probe analyses through the full growth axis, from the primordium (p) to the posterior extremity (pe).

Fig. 3. Life history scans of Sr (taken with the L line), Na (Ka line) and K (Ka line) by ED spectrometer (open circles) and WD spectrometer (solid circles). Note the differences in the trend lines in the Sr scans with the two spectrometers; the positive trend in the ED scan is spurious, due to the high noise levels.

Fig. 4. ED spectra from pure calcite, $CaCO_3$ -- one unmodified and one mathematically stripped of the escape peak with the algorithm of Reed and Ware. The peak at 1.8 keV in the unmodified spectrum is mostly the silicon escape peak due to the loss of photoelectrons from the dead layer of the detector. Note the non-linearity in the stripped spectrum.

Fig. 5. Backscattered electron micrograph of target regions for two beam conditions on a morwong sagitta. The uppermost target is for 14 μm diameter beam at 25 nA and 15 kV; the smaller target is for a focussed (< 5 μm diameter) beam of the same power. The focussed beam produces a central bright region, indicative of extensive decomposition of $CaCO_3$, and severe pitting (the black spots around and in the target), which may be formed by escaping CO_2.

Fig. 6. Changes in counts for Na over 120 s total dwell time and Sr over 240 s total dwell time under constant beam power (accelerating voltage 15 kV, beam current 25 nA) and beam diameters ranging from 3-20 μm. (c) and (d) show the corresponding changes in Ca counts. Each point is the mean and one standard deviation of five replicate counts for each 5 s time interval.

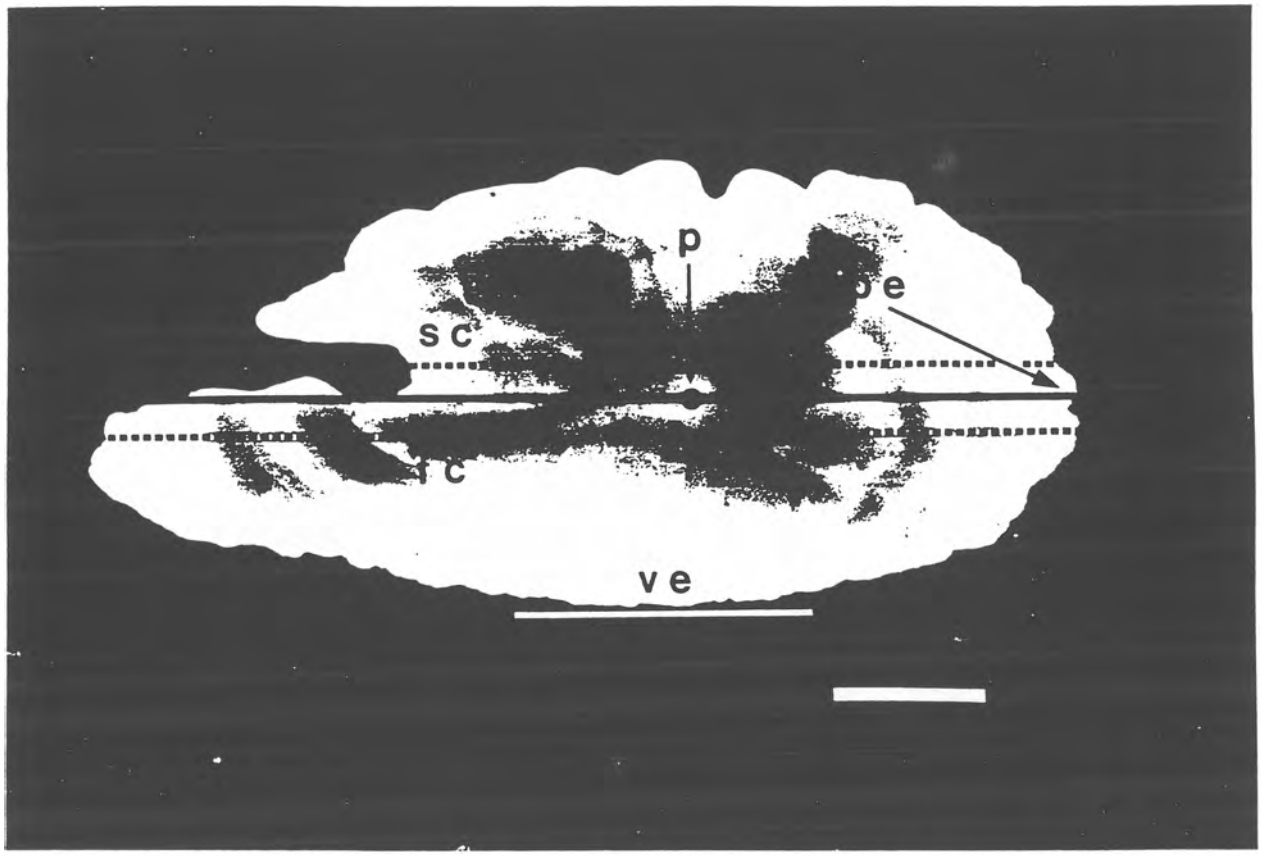
Fig. 7. Change in Na/Ca and Sr/Ca ratios over total dwell times of 120 s and 240 s, respectively, under constant beam power (accelerating voltage 15 kV, beam current 25 nA) and beam diameters from 3 to 20 μm. Each point is the mean and one standard deviation of five replicate counts for each 5 s time interval. For clarity, only one side of the SD bar is shown.

Fig 8. The relationship between BPD and change in counts of Ca, Sr and Na and Sr/Ca and Na/Ca ratios over 120 s and 240 s. Each point represents the mean (and one standard deviation) change in counts between the first counting period (after 5 s) and the last (after either 120 s or 240 s).

Fig. 9. Change in Na and Sr counts over total dwell times of 120 s and 240 s, respectively, under constant beam diameter (14 μm), constant beam current (25 nA) and accelerating voltages from 10 to 25 kV. (c) and (d) show corresponding counts for Ca. Each point is the mean and one standard deviation of five replicate counts for each 5 s time interval. For clarity, only one side of the SD bar is shown.

Fig. 10. Change in Na and Sr counts over total dwell times of 120 s and 240 s, respectively, under constant beam diameter (14 μm), constant accelerating voltage (15 kV).and beam currents from 10 to 40 nA. (c) and (d) show corresponding counts for Ca. Each point is the mean and one standard deviation of five replicate counts for each 5 s time interval. For clarity, only one side of the SD bar is shown.

Fig. 11. Raw data from life-history scans for the left (open circle) and right (solid circle) sagitta of a Nemadactylus macropterus. Beam power density was 2.4 μWμm-2, beam diameter 14 μm and the spacing between points 25 μm. P = primordium, E = edge of otolith.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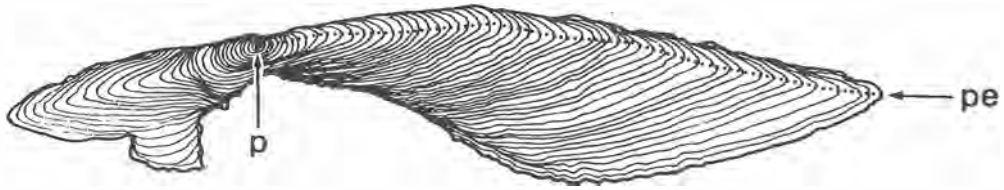
e
s c
c
v e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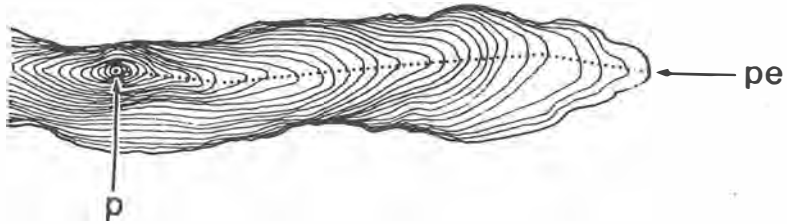
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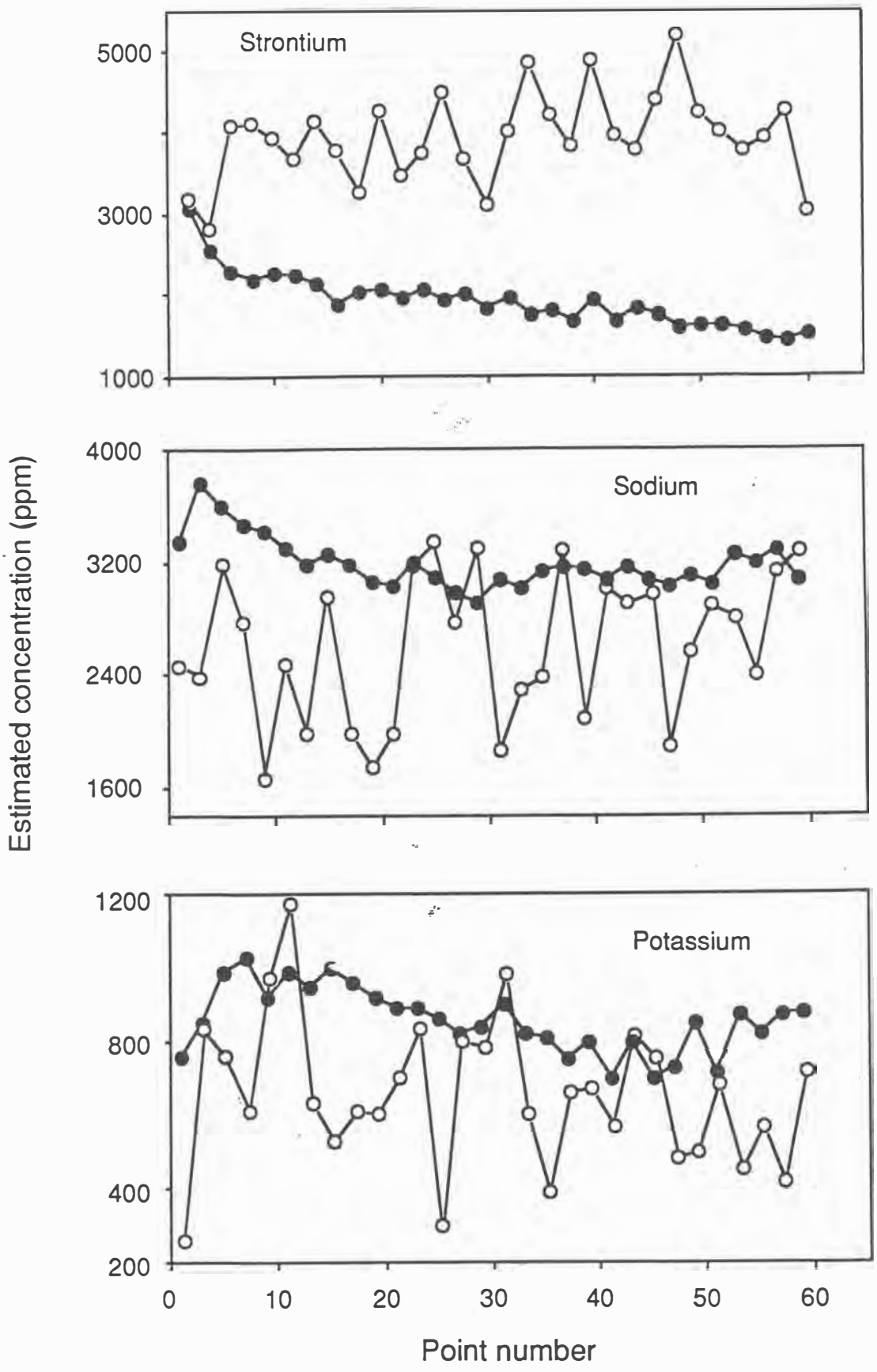
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3000
1000
Sodium
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3200
2400
1600
Potassium
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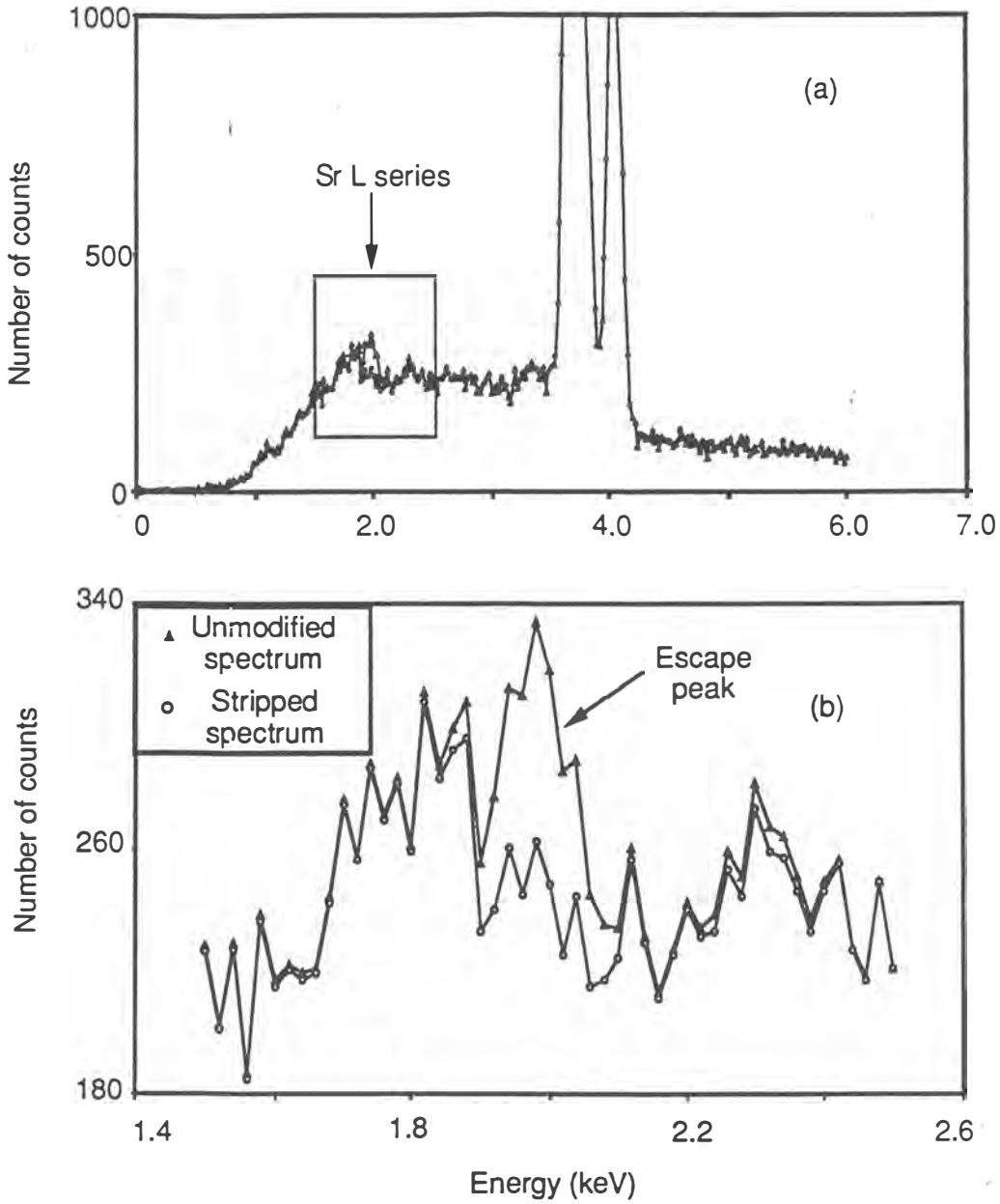
800
400
200
0
10
20
30
40
50
60
Point number
Estimated concentration (ppm)


1000
500
0
Number of counts
(a)
Sr L series
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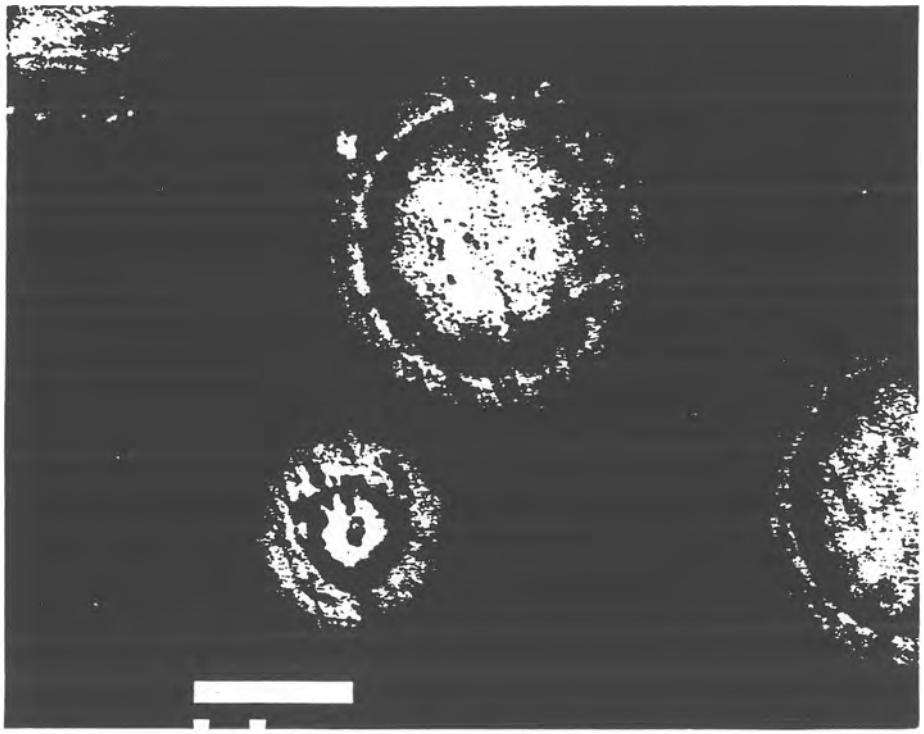
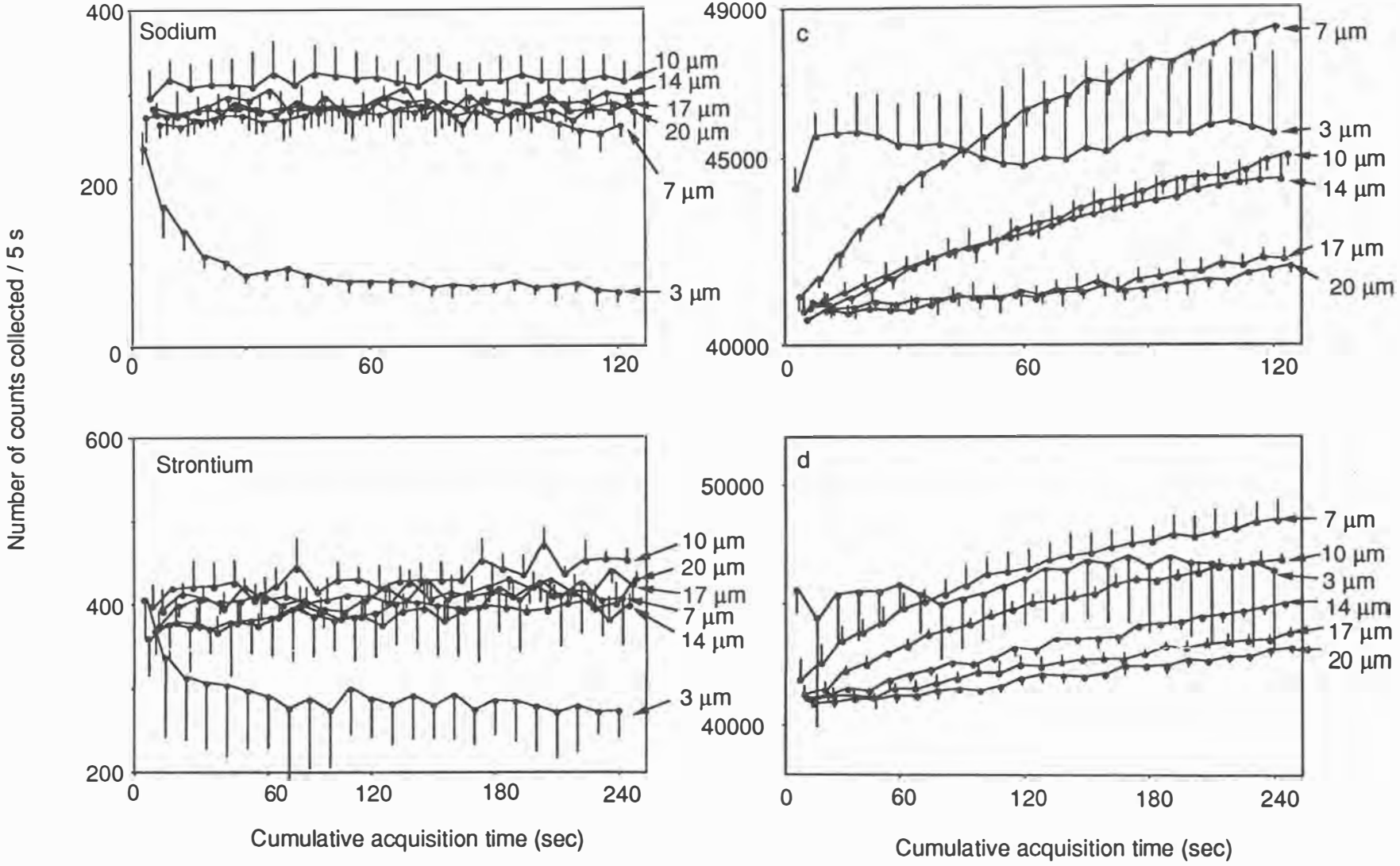
2.0
4.0
6.0
7.0
340
260
180
Number of counts
Unmodified spectrum
Stripped spectrum
Escape peak
(b)
1.4
1.8
2.2
2.6
Energy (keV)




Sodium
400
200
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60
120
10 µm
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60
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Strontium
600
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7 µm
10 µm
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14 µm
17 µm
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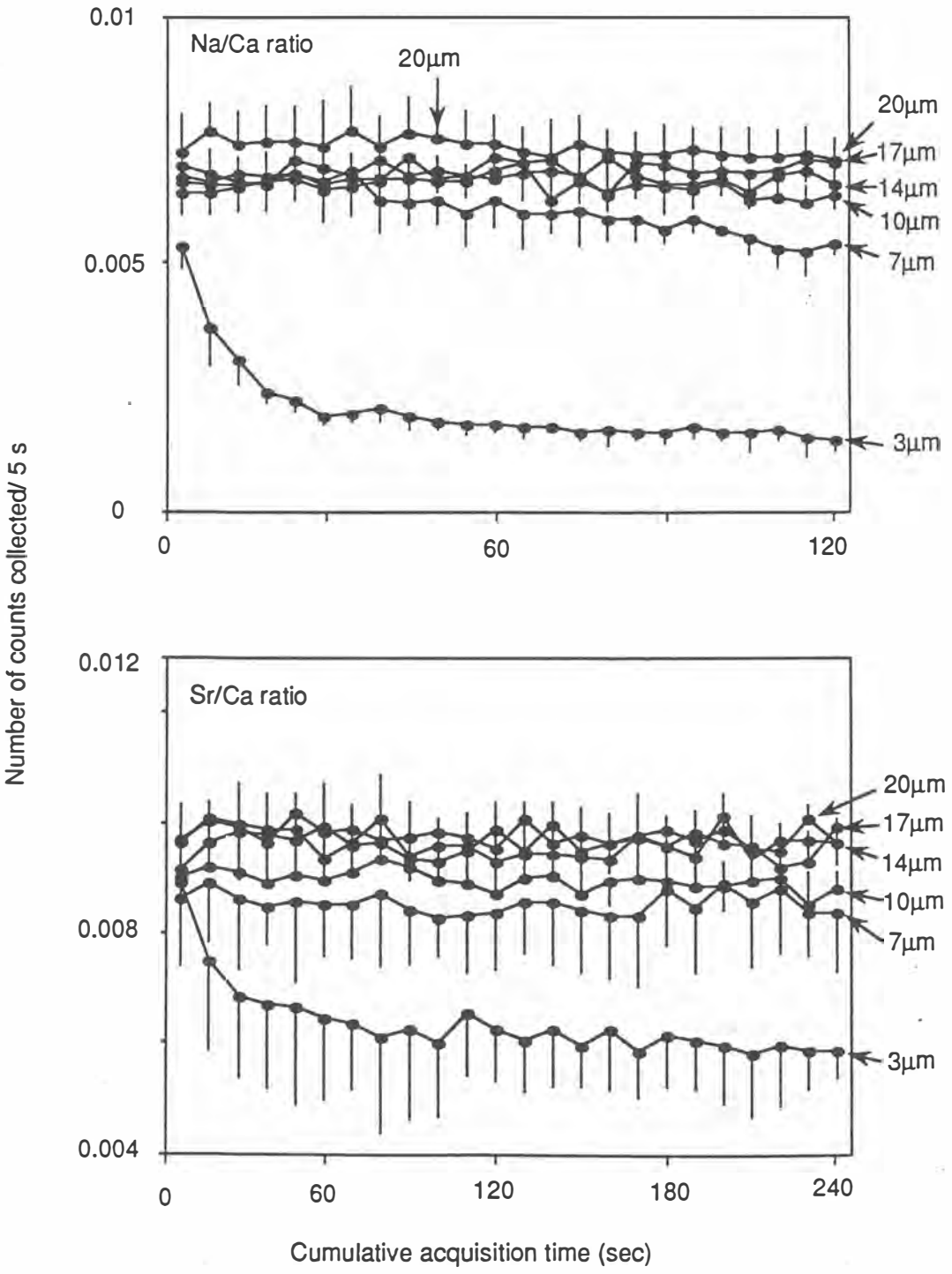
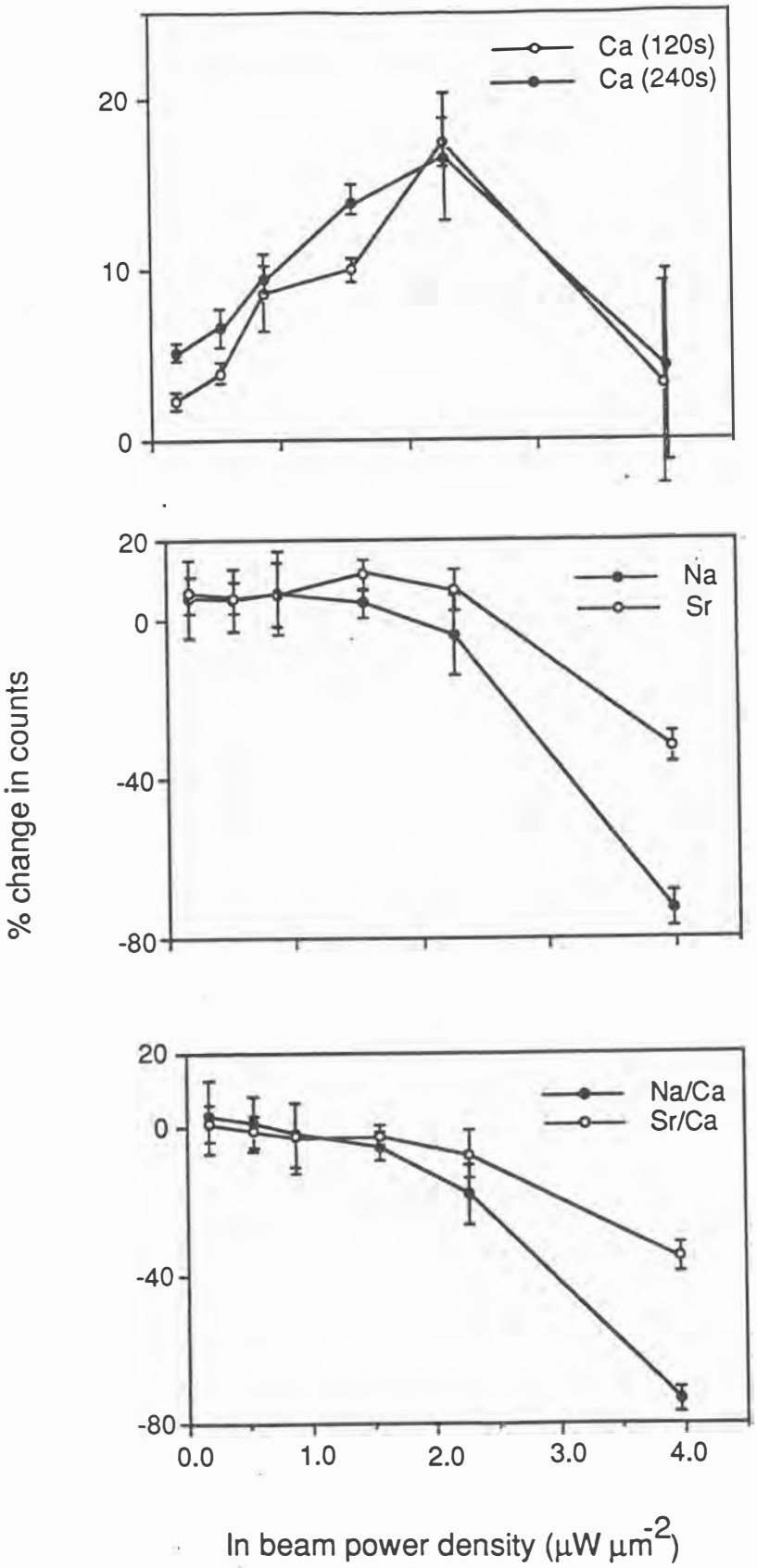
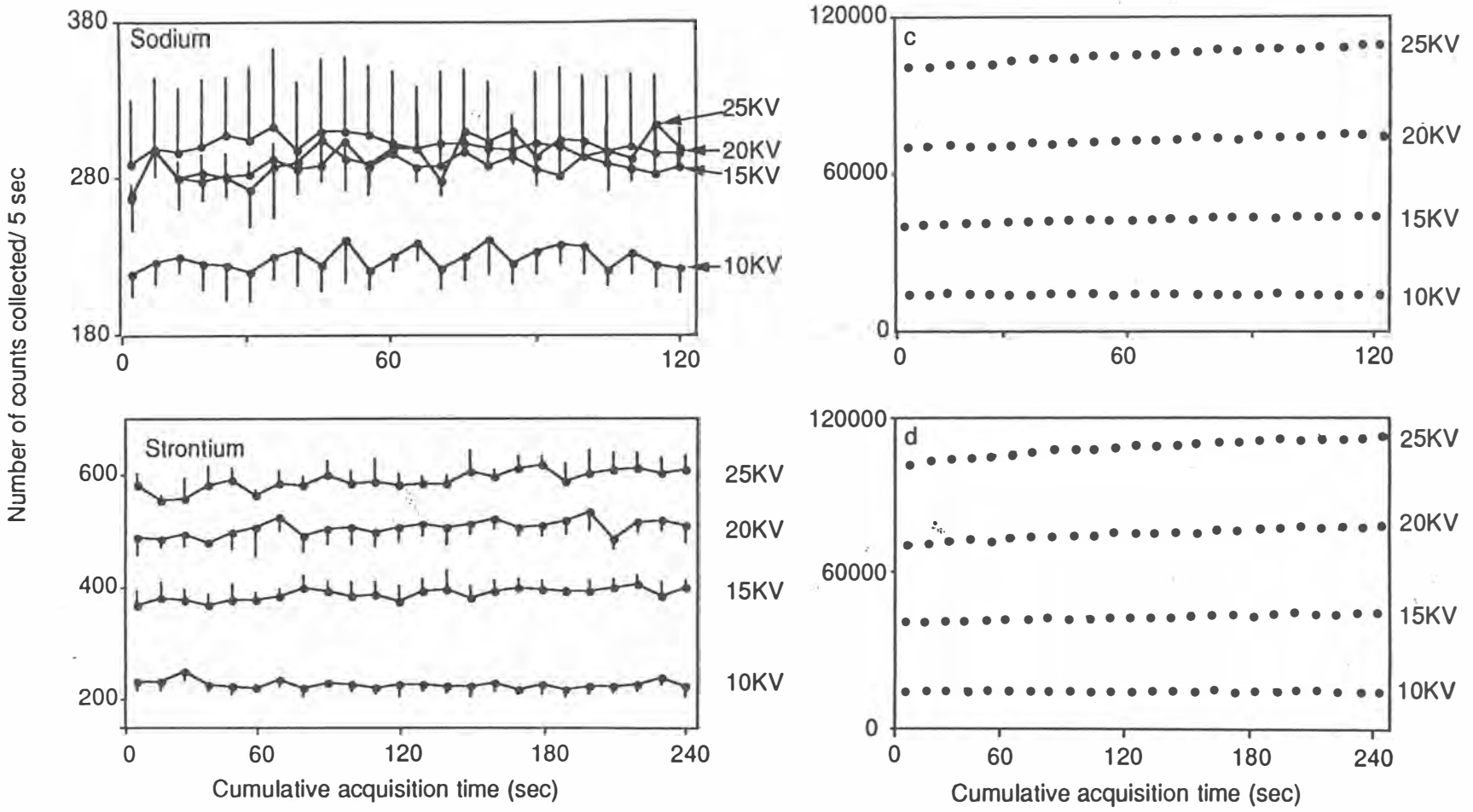
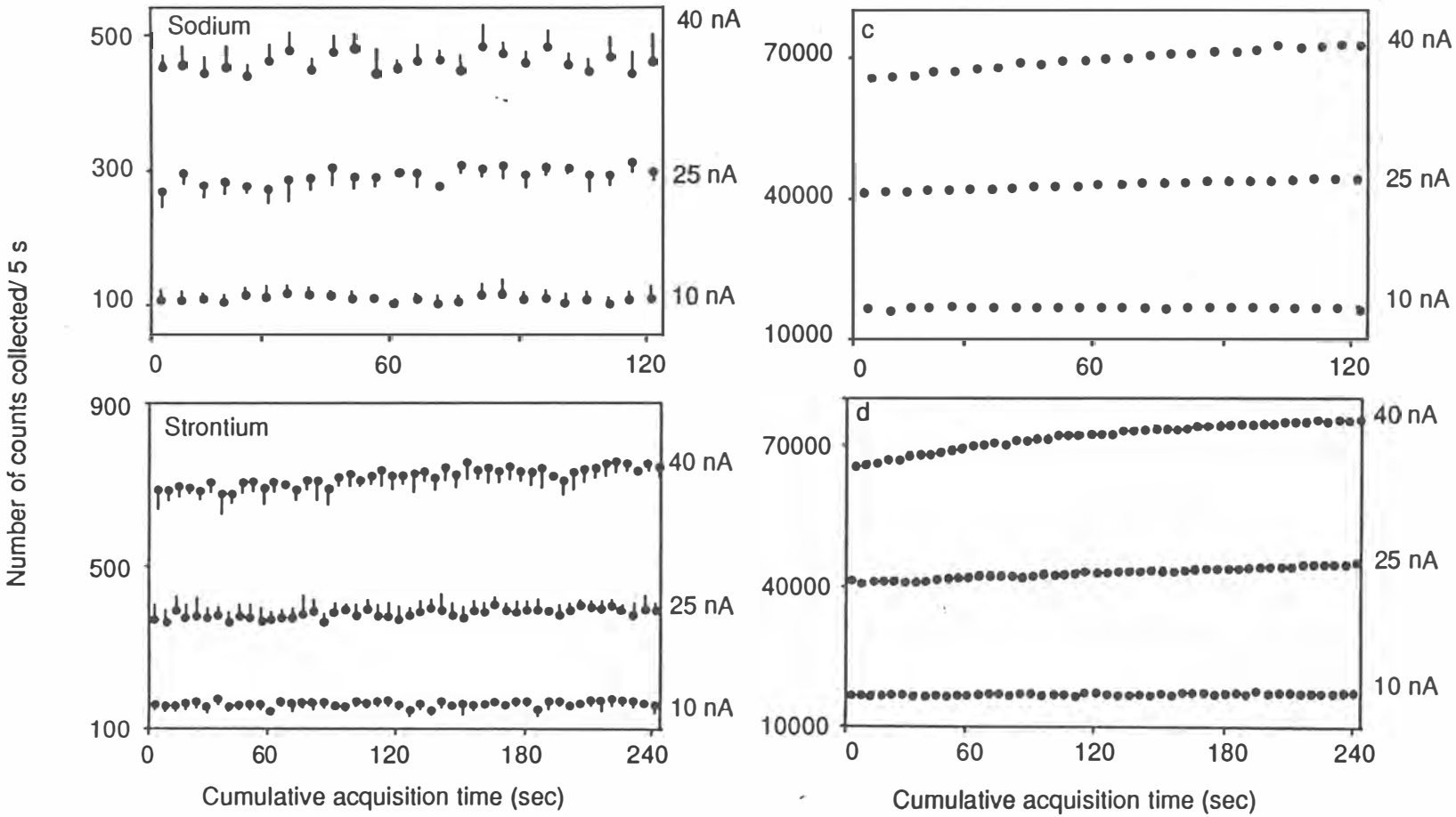
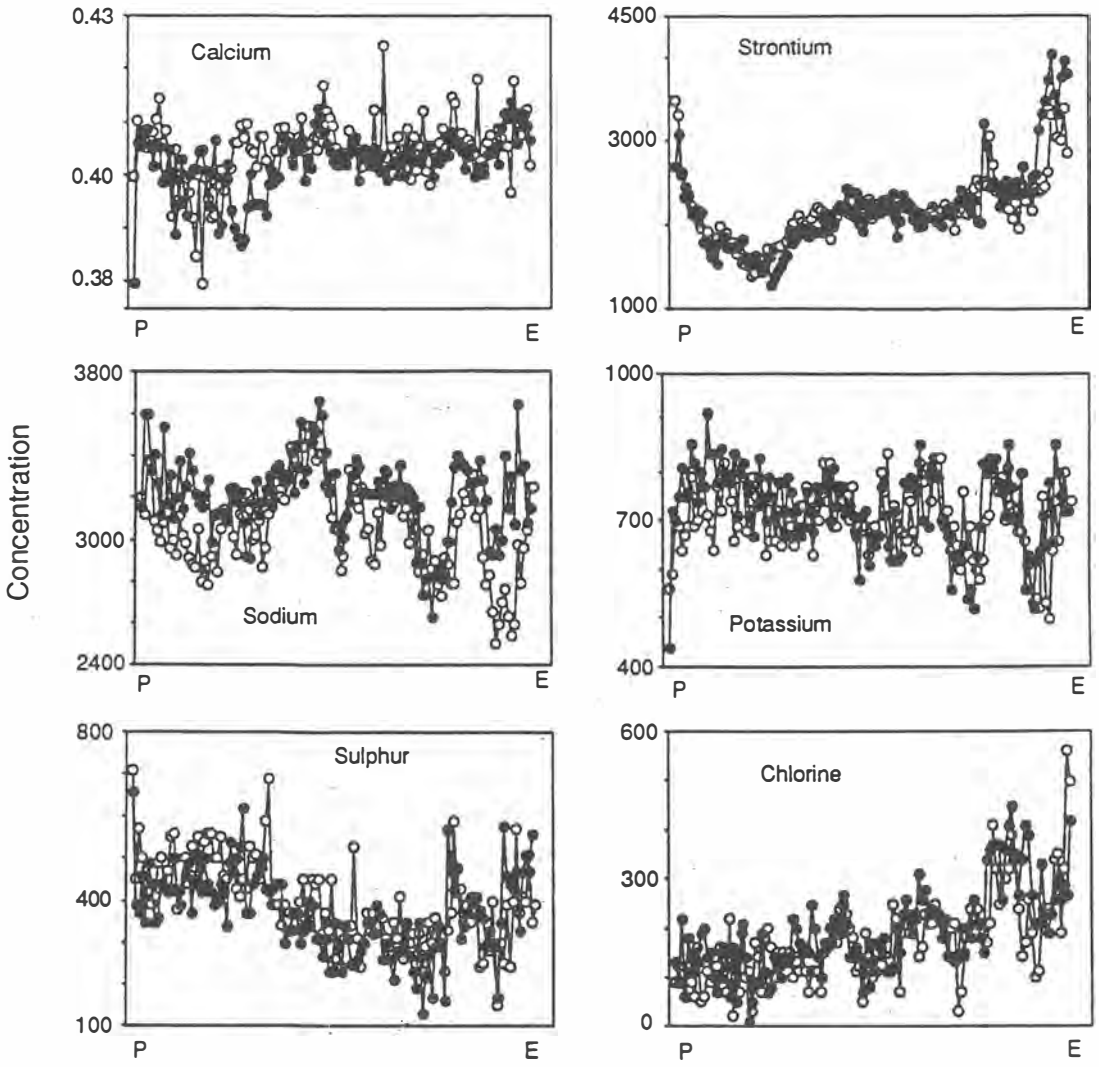
Number of counts collected / 5 s
Cumulative acquisition time (sec)
Cumulative acquisition time (sec)


Na/Ca ratio
20µm
20µm
17µm
14µm
10µm
7µm
3µm
0.01
0.005
0
0
60
120
Sr/Ca ratio
20µm
17µm
14µm
10µm
7µm
3µm
0.012
0.008
0.004
0
60
120
180
240
Number of counts collected/ 5 s
Cumulative acquisition time (sec)


Ca (120s)
Ca (240s)
20
10
0
Na
Sr
20
0
-40
-80
Na/Ca
Sr/Ca
20
0
-40
-80
0.0
1.0
2.0
3.0
4.0
% change in counts
In beam power density (μW μm-2)


Sodium
380
280
180
0
60
120
25KV
20KV
15KV
10KV
Strontium
600
400
200
0
60
120
180
240
25KV
20KV
15KV
10KV
Number of counts collected/ 5 sec
Cumulative acquisition time (sec)
c
120000
60000
0
0
60
120
25KV
20KV
15KV
10KV
d
120000
60000
0
0
60
120
180
240
25KV
20KV
15KV
10KV
Cumulative acquisition time (sec)


Sodium
40 nA
25 nA
10 nA
500
300
100
0
60
120
c
70000
40000
10000
Strontium
900
500
100
180
240
d
Number of counts collected/ 5 s
Cumulative acquisition time (sec)
Cumulative acquisition time (sec)


Calcium
0.43
0.40
0.38
P
E
Strontium
4500
3000
1000
P
E
Sodium
3800
3000
2400
P
E
Potassium
1000
700
400
P
E
Sulphur
800
400
100
P
E
Chlorine
600
300
0
P
E
Concentration
Position along otolith axis

Document 2

An evaluation of electron probe microanalysis of otoliths for stock delineation and identification of nursery areas in the southern temperate groundfish, *Nemadactylus macropterus* (Cheilodactylidae)

Since 1967, a number of studies have investigated the use of calcified tissue composition as an indicator of stock or sub-population identity (eg., Klokov & Frolenko, 1970; Calaprice, 1971,1983,1985; Calaprice et al., 1971,1975; Bagenal et al., 1973; Gauldie & Nathan 1977, Behrens Yamada et al., 1979; Lapi & Mulligan, 1981; Mulligan et al., 1983; Mulligan et al., 1987; Edmunds et al., 1989), using a variety of analytical techniques (see Coutant 1990, Gunn et al., 1992). Results, in general, have been mixed, due in part to methodological limitations which, until recently, dictated analysis of relatively large amounts of tissue. Although results have often been promising (eg., Calaprice et al. 1971, Calaprice 1985, Edmunds 1989), an inability to assess fine scale variation in composition and/or single otoliths from larvae or juveniles of known origin, for example, made difficult a more thorough investigation of the potential of the techniques.

In 1987, we began experiments with a view to using such fine-scale, ontogenetic variations in the composition of fish otoliths as an indicator of movement/migration patterns. The work has developed along three lines: (1) investigation of the operating characteristics of probe microanalyzers as they affect data quality, and the development of techniques for 'life history scans' across otoliths; (2) evaluation of the value of chemical information for stock delineation; and (3) experimental and correlative studies on the effects of environmental and physiological factors on otolith composition. Results of the methodological studies are reported above. The current paper deals with the second line of inquiry. Specifically, we evaluate the extent to which otolith composition in a test species varies ontogenetically, among individuals within sites, and among sites within the geographic range of a species, in order to assess whether variations in otolith composition, as measured using wavelength-dispersive electron probe microanalysis, are of sufficient magnitude to resolve population structure. We then infer the factors that result in variation in otolith composition, at this level of resolution, and comment on the apparent strengths and weaknesses of the technique for resolving population structure.

Methods

Collection details for juveniles and adultys are provided in Fig. 1 and Table 1. Recently settled (0+) juveniles were collected by hand-lining and trawling at six sites off Tasmania and southern Victoria. Adult specimens were obtained from commercial and scientific trawls at six sites across south eastern Australia. In order to minimise possible affects of interannual variation in composition, we minimised the number of

year-classes in the sample by using only adults in the size range of 30 - 35 cm SL. Examination of otolith macrostructure, reference to published length-age keys for the species (Smith, 1982), and enumeration of the episodic, and probably seasonal (see Proctor, et al., in prep.) variation in Sr concentrations along the growth axis indicate that specimens were a mixture of the 1980 to 1984 year classes. Sites overlapped broadly in year class distributions, based on the length-age keys and the Sr data; mean year-class values for the sub-sample of 22 individuals aged by Sr plots were 1982.8, 1981.5, 1982, and 1982.4 for Tasmanian, Victorian, NSW and GAB fish, respectively (ANOVA $F_{3,19} = 1.0$, NS). Juveniles were from the 1987 and 1988 year-classes.

All specimens were frozen shortly after collection and remained frozen until the otoliths were removed. After extraction, each otolith was cleaned of adhering tissue using fine forceps and a soft bristled brush in millepore-filtered distilled water. Otoliths were then dried in an oven at 40-45^{o}C. for a minimum of 6 hrs, after which they were stored in polyurethane capsules.

Procedures for embedding, sectioning and preparing otoliths for probe microanalysis are detailed in Gunn et al (1992). Only sagittae were used in this study, because of their larger size. Prior to embedding, a scaled diagram of the distal surface of each otolith was made in order to guide subsequent sectioning. The otolith was then fixed upright on its ventral edge to the base of an embedding mould with a drop of quick-drying resin. The mould was then filled with a harder-setting resin. After hardening, the otolith was sectioned using a diamond-edged saw blade (350 μm thick) on a rotary saw. Grinding to the plane of the primordium was done by hand using 2400 grade silicon carbide wet/dry paper. Final polishing was done using progressively finer grades of diamond paste (6 - 0.5 μm) and/or aluminum oxide powders or pastes (eg., Linde B, 0.5 μm) on a lapping machine. After polishing, the section was ultrasonically cleaned and stored in a moisture-free environment. Prior to probe microanalysis, the section was coated with a 250-300 Å coat of carbon, using a sputter coater, and then stored in vacuum until insertion into the probe.

The procedures we used to analyse otolith composition are detailed, discussed and justified in Gunn, et al (1992). Again in brief, specimen damage under the electron beam in inevitable. The amount of damage, and hence quality of the data, is proportional to beam power density (ie., beam current X accelerating voltage/ target area). As a result of our experimental studies, we concluded that beam power densities greater than 3.0 $\mu W\ \mu m^{-2}$ resulted in unacceptable levels of specimen damage and data precision and accuracy. Hence, data for the current study were acquired using a defocused beam, a beam power density of 2.4 $\mu W\ \mu m^{-2}$ and a total acquisition time of 3 min, 42 sec per point. The electron probe microanalyzer used was a Cameca

"Microbeam" fitted with three wave-length dispersive detectors. The concentrations (weight-fractions) of the elements detected were calculated based on count rates measured for the $K\alpha$ lines for most elements, and the $L\alpha$ line for strontium, on standard materials and the ratios of the intensities on standards and otoliths, computed using the "PAP" (Pichou and Pichoir, 1984) matrix conversion software supplied by Cameca. Minimum detection limits and confidence intervals for the concentration estimates are based on equations provided by Ancey et al. (1978).

Ontogenetic variation in composition was assessed by scanning a series of points along the longest growth axis of each fish. We refer to these series for each fish as a "life history scan". The finished section of the sagitta of N. macropterus exposes a nearly straight, uninterrupted growth axis, through which we ran a mapped series of programmed scan lines that tracked the slight curvature of the growth axis (Fig. 2). Considerable effort was expended to duplicate this axis as closely as possible in each specimen, in order to maximise comparability of the data sets between individuals. The life history scan line for each fish ran from the primordium to the posterior ventral tip of the otolith. The size of the scan points and their spacing was determined, in part, by logistical considerations and, in part, by results of experimental trials, discussed below. Reproducability of life history scan data was evaluated by comparisons of left-right otolith pairs from the same fish.

Results

Composition of N. macropterus otoliths -- data quality and reproducability

Six elements could be reliably detected in N. macropterus otoliths using wavelength-dispersive electron probe microanalysis (WD-EPMA); in order of decreasing mean concentration, these were calcium, sodium, strontium, potassium, sulfur and chlorine. The elements in N. macropterus sagittae constitute three distinct sets, separated in concentration from other, less abundant elements by a difference of one to three orders of magnitude (Fig. 3): calcium, carbon and oxygen (the latter two not routinely measured due to methodological difficulties) constitute the "macro-constituents", present in concentrations > 10% by weight; sodium, strontium, potassium, sulfur and chlorine constitute a set of 'micro-constituents', which occur in mean concentrations of 100 - 5000 ppm; and a variety of 'trace elements' occur at concentrations < 10 ppm. Only the micro-constituents and Ca can be measured accurately using WD-EPMA. Absolute ranges of concentrations, measurement error (absolute and percent 95%

confidence intervals) and minimum detection limits (MDL's) for each of these elements, at our standard operating conditions, are given in Table 2. Measurement error is inversely correlated with mean concentration, varying from 3.7% in sodium to 28% in chlorine. Of the six elements measured, only chlorine occurred occasionally at less than its respective MDL (157 ppm). Although the microanalyzer reports value less than the MDL, these values are noise and were set equal to zero for all subsequent analyses.

Life history scans for three fish chosen casually from the data set are depicted in Fig. 4. The results are typical of our data and demonstrate several points. First, all six elements vary ontogeneticly in concentration well in excess of the uncertainty associated with measurement. Second, concentrations of all six elements auto-correlate highly at scales < 100 μm (e.g., Fig. 5), which suggests that in N. macropterus this is the typical scale of ontogenetic variability in composition. Comparison of "small spot-closely spaced" analyses (6 μm beam diameter spaced at 8 μm intervals, center-to-center) with "large spot-widely spaced" analyses (14 μm diameter, at 16 μm spacing) suggests that a sampling scale finer than our standard analyses reveals few, if any major variations in otolith composition that would not be detected at the coarser sampling scale (Fig. 6). Third, absolute variability is highest for strontium, values for which can routinely vary within specimens over half an order of magnitude. However, relative variability is as high or higher in sulfur and chlorine; coefficients of variation for chlorine in the three specimens depicted range from 37.8 to 92.1, as compared with 18.9 to 37.4 for strontium, 16.3 to 24.9 for sulfur and, at the other extreme, only 1.2 to 1.7 for calcium. And fourth, ontogenetic patterns in the variation are often consistent across specimens. All N. macropterus we have analysed, for example, show steep gradients in strontium levels in the region immediately around the primordium. Similarly consistent, though less pronounced patterns are evident in sodium and calcium.

The quality of these data were assessed by comparing 'replicate' life history scans from the left and right otoliths from the same fish, using the three representative specimens above. Among the three specimens, the quality of the match within each otolith pair differs markedly (Fig. 7). The comparisons suggest two principal sources of error. First, there is consistent evidence of the difficulties in tracking identical growth trajectories even within a pair of otoliths from the same individual. In all three pairs, the match between left and right otoliths deteriorates as the otolith margin is approached, which we attribute to the decline in otolith growth rate with age, the correspondingly larger effect of differences in tracking through the growth apices in the two otoliths, and slight differences the size of the otoliths (and hence different spacing of scan points relative to the length of the otolith). Most of the left/right differences in

specimens #304 and #312 appears to result from these tracking errors; that is, the same ontogenetic patterns and mean concentrations are generally evident, but variously expanded or compressed along the growth axis.

The second source of error is more difficult to account for. In four of the six elements examined (sodium, potassium, sulphur and chlorine), mean concentrations occasionally differ between left and right otoliths over relatively large portions of the otoliths. The mis-match is most evident for Cl in #339 and S in #312. In both instances, the scale of the mis-match exceeds machine-induced measurement error. At this stage we cannot account for the difference. There is no consistency among the samples we examined of either elements that invariably match poorly between otolith pairs, or elements that mis-match in parallel, e.g., Cl levels match well in #304, intermittently match poorly in #312, and differ markedly near the margin of #339, whereas S matches very poorly in #312, but is in good agreement between otoliths in #304 and #339. Given this variability, it is difficult at this stage to identify a single root cause of the problem. Comparisons of parallel life history scans across a single otolith, e.g., Fig. 6, suggest that differences between otoliths in elemental concentrations of the scale observed cannot easily be attributed to either measurement error or differences between otoliths in the position of the scan line relative to the main growth axis. Indeed, life history scans along quite different growth axis in a single otolith produce fundamentally similar mean concentrations and ontogenetic patterns (aside from the compression and expansion problem, noted above)(Fig. 8). We conclude, therefore, that the differences between otolith pairs are real, appear to be more common in some elements than others (e.g., evident in Cl, but not Sr), and may be more common near the otolith margin than closer to the primordium. The data are thus far very sparse, but clearly indicate that slight differences in, particularly, Cl concentrations be used with caution for purposes of stock delineation.

Evaluation of stock structure

We hypothesised that stock structure in N. macropterus would entail spawning grounds or times diagnostic for each stock, and be manifest as unique patterns of elemental composition in the first forming part of the otolith. However, in practice compositional data for the primordium itself proved of low quality, due to the specimen preparation required for EPMA and the incremental structure of otoliths. In most adults prepared for analysis, either or both minute cracks or a pit several microns in diameter developed at the primordium during specimen preparation, the latter due to the 'plucking' of the primordium out of the otolith center during polishing. Our previous

work (Gunn, et al., 1992) indicates that such topographic irregularities degrade EPMA data due to unpredictable patterns of x-ray absorbtion. A comparison of data for point 1 (on the primordium) and point 2 (centered 14 μm from the primordium) for the adults supports this point: for all elements except chlorine the variance in estimated concentrations is 36 (sodium) to 270 % (sulfur) higher for point 1 than point 2 (Fig. 9). Nevertheless, concentrations at point 1 correlate highly for all elements with those at point 2, at r^2 values that range from 0.09 (sulfur) to 0.85 (chlorine). On the basis of these data, we conclude that differences among specimens evident in the primordium are also evident adjacent to the primordium and can be assessed more accurately at the latter.

Use of data from only one point, even if free from conspicuous distortions due to irregular surface features, still risks high error rates due to measurement noise (Gunn, et al., 1992). Consequently, we analysed stock structure in two ways: based on comparisons of point 2 data only, and based on mean concentrations of each element for points 2 - 6 inclusive. The latter filters out high frequency measurement noise, but risks low discriminant power by including information from relatively late in larval life. Point 6 is about 125 μm from the primordium; increment counts suggest this corresponds with a larval age of about 45-55 days.

The distributions of individual mean concentrations of the six detected elements for the 68 adult N. macropterus analysed are depicted in Fig. 10. Three of the six (K, S, and Cl) are not normally distributed, due to a significant skew to higher concentrations. Ca also shows evidence of a weak skew, and Sr evidence of a weak bimodality. Differences in the concentrations among adult sampling sites are depicted in Fig. 11. Mean concentrations of four of the six elements differ significantly among sites, the exceptions being strontium and sulfur. The differences are manifest in both point 2 data and point 2-6 data, and are of similar pattern and comparable magnitude in both data sets. For most elements, error bars are smaller in the filtered data, which presumably reflects the removal of random measurement errors. Differences among sites are greatest for chlorine, in which mean values for three sites (eastern and western Tasmania and the Great Australian Bight) do not differ significantly from the minimum detection limit (MDL), whereas means for three other sites (western and eastern Victoria and New South Wales), all contiguous, are not only well above the MDL for chlorine, but also do not differ significantly. A similar, though less pronounced grouping of sites in also evident in Na and K (concentrations in the Victorian and NSW fish higher than in those from Tasmania and the GAB) and Ca (lower concentrations in Victorian and NSW adults).

The grouping of sites was examined further by plotting Na/CL and Sr/Ca ratios for specimens from each of the six sites (Fig. 12). K was not analysed, as its concentrations tend to follow Na, both among individuals (ms. in preparation) and sites. Again as expected, the scatter of points for individual fish is greater for the single point data as compared with the filtered data, but the patterns of regional groupings and the relationships between elemental concentrations are virtually the same for the two data sets. Overall, the data indicate a regional grouping of the Victorian and NSW samples, based on both Na/Cl and Sr/Ca ratios, and suggest a mean difference between the both Tasmanian samples and specimens from the GAB on the basis of the Sr/Ca ratio.

These apparent groupings were tested and quantified by means of linear discriminant function analysis (LDFA), using the SYSTAT package. Results of a three group discrimination, using both point 2 data only and point 2-6 averaged data, are shown in Fig. 13. The rationale for the 3 group discrimination will be discussed below. Several conclusions can be drawn from these analyses. First, the overall pattern of site separation is similar for the single point and averaged data, but, second, the scatter of points and the overlap among sites are greater for the single point data. Third, the three groups are statistically separable, but at a relatively low rate of successful classifications. For the single point data, only 66% of individuals were accurately classified into their three respective 'source populations'; for the filtered data, the success rate increases to 78%. The relatively poor separation is due, in part, to overlap along the edges of the three groups (NSW/Victoria, GAB, and Tasmania) and in part to a few individuals located in discriminant function space well outside of the areas defined by other individuals collected at the same place and time.

The statistics supporting the first (six site) LDFA for the averaged data are given in Table 3. The analysis developed five discriminant functions to classify the six sites. However, only the first three are significant. Examination of the canonical loadings indicates that discriminant function 1 correlates with Na, K, and Cl concentrations, and hence represents mainly the initial separation of sites along the Na/Cl axis indicated in Fig. 12a. The second discriminant function loads heavily only on Sr, whereas the third is mainly a K residual from the first discriminant function. Step-down procedures, in which sites are sequentially pooled, raises the contribution of Ca to the second discriminant function, identifying it with the Sr/Ca axis in Fig. 12b. The nature of the site separations is indicated in Fig. 14, which shows the distributions of each of the discriminant functions among the six sites sampled. Function 1 separates the two Tasmanian and the GAB samples from the two Victorian and the NSW samples; function 2 distinguishes (albeit poorly) between the GAB sample and the remainder; and function 3 tends to separate the east and west coast Tasmanian samples. The

remaining two functions do not clearly distinguish among any sites. The primacy of the first three functions remains in a step-down procedure, as the sites are sequentially pooled based on their degree of overlap. The final step, at which all functions contribute significantly ($P < 0.001$) to the discrimination, is at the level of three groups and two discriminant functions (ie., Fig. 12); the third function, separating the two Tasmanian samples, is not quite significant in the final step ($p = 0.057$). Post-hoc analyses (Steffe's F-test) of the discriminant functions indicates the samples from the Victorian and NSW sites do not differ significantly and consistently in any of the three functions, the GAB sample differs from all other sites (which do not differ significantly) in function 2, and none of the sites differ significantly in function 3, though the western Tasmanian sample nearly differs significantly from the other sites.

We draw three general conclusions from these analyses. First, there are significant differences among samples from different sites in terms of the elemental composition of the primordial region of their otoliths. Second, analyses of the primordium itself, of a point 20 μm exterior of the primordium, and of the mean value for the region between 20 and 120 μm exterior to the primordium produce similar results, indicating that site-specific differences are manifest through at least the first 120 μm of otolith growth. And third, sites pool on the basis of common patterns of elemental composition into three groups - one composed of the NSW and the two Victorian samples, a second consisting uniquely of the GAB sample, and a third consisting of the two Tasmanian samples. In both cases where sites are pooled, the pooled sites are geographically contiguous and nearest neighbors.

Site-specific differences and similarities in ontogenetic variation in composition

That differences in elemental composition among sites are maintained out to at least 125μm from the primordium and an age of 45-55 d post-hatching suggest that delineation among samples is not dependent on environmental conditions specific to only a spawning site, assuming that the elemental data reflect environmental conditions at all. At the minimum, these hypothesised environmental differences must encompass several weeks and perhaps months of larval development. To assess the ontogenetic patterning of these elemental differences, we compared the concentrations of apparent key elements for specimens from the three pooled areas (NSW/Victoria, the GAB, and both Tasmanian sites) at several points along their respective long growth axes. Five-point filtered data were assessed at four positions : pts. 2-6, 6-10, 36-40 and 80-84. The

first position is immediately adjacent to the primordium, the second immediately exterior to it (and presumably encompassing the second 2-3 months of planktonic larval development), the third we estimate to correspond to the approximate age when the pre-juveniles recruit to the nursery areas, and the fourth, outermost position is the farthest along the growth axis at which we had data for all specimens (maximum number of points differed depending upon the length of the axis) and, we estimate, corresponds to otolith deposition at an age of 6-7 years.

The results of the comparison for four elements are shown in Fig. 15. Three points are evident. First, the mean pattern of ontogenetic change in composition is very similar for samples from all three pooled sets of sites, eg., Na and Sr concentrations decline between points 2 and 6, and then increase towards the otolith margin in samples from all three sites. Second, the pattern of ontogenetic variation in concentrations differs among elements. And third, virtually identical differences are evident among sites irrespective of where the analysis was done in the otolith. NSW/Victorian specimens, for example, at all stages of their apparent life histories have Cl levels higher than those of fish collected elsewhere. A discriminant function analysis based on mean concentrations at point 36 and point 80 results in site delineations virtually identical to those derived from concentrations measured near the primordium.

Discussion

Effects of Data Quality on Stock Delineation

Electron probe microanalysis using WD-spectrometers reveals extensive variability in the concentrations of six elements in N. macropterus otoliths. Some of this variability is induced by the inherent, small scale compositional heterogeneity of otoliths and some is noise that reflects the limits of detectability and precision of the electron probe. However, comparisons of life history scans along similar growth axis of left and right otolith pairs indicates significant ontogenetic variability for all elements. For most elements, there is also evidence of geographic variability in composition.

The extent to which this ontogenetic and geographic variability can be used to assess either life history variations or population structure is critically dependent on the scale of the life history or population 'signal' relative to analytical 'noise', of whatever source. In that regard, our results suggest that data quality varies widely among elements. Two principal, identifiable sources of this 'error' are the effects of beam conditions, which differ among elements (eg, Na more sensitive to effects of pitting than Sr)(see Gunn, et al., 1992), and the interaction of counting statistics with mean

elemental concentrations (eg, concentrations of Cl and S near the limits of detectability using EPMA result in wide confidence intervals around each estimation). The effects of these factors can be estimated from standard formulae, to provide a 'minimum significant difference' between point estimates (whether in the same or different otoliths) for each element, as discussed in Gunn, et al. (1992). Empirically, however, this 'minimum significant difference' can also be estimated from parallel life history scans done on the same otolith (eg, Fig. 5), by assuming that paired points in two parallel life history scans are replicates and assessing the quantifying the. This is, in some respects, a worse case analysis, in that it ignores both small scale differences in composition (by treating each of the paired set of points as true replicates, despite a 25 µm distance between them) and the effects of beam power density, which differed between the 'small spot' and 'large spot' scans and which can be expected to have a marked effect on estimated concentrations of some elements, e.g., Ca (Fig. 5). For the five elements other than Ca, differences between 'replicate points' are largest for Sr (maximum difference 964 ppm) and lowest for Cl (maximum 210 ppm)(Table 1). Operationally, therefore, a conservative approach would be to accept differences between point estimates as 'real' only if the difference between them exceeds these maxima. This criterion is very draconian, however, and a more realistic, but still conservative one would perhaps be the upper boundary of the 99% CI about the mean difference in the 'replicate' data set. This criterion suggests a 'safe' difference between point analyses ranging from 331 ppm for Sr to 73 ppm for Cl, with the other elements falling between the two extremes (Table 1). In passing, these empirically derived values are similar to the estimates of analytical precision for the various elements as determined from theoretical considerations (Table 1 and Gunn, et al., 1992).

To a large extent this geographic variation is driven by local differences in the mean concentrations of one element -- chlorine. Tasmanian specimens, with few exceptions, had chlorine concentrations below detection limits, whereas NSW and Victorian samples consistently showed concentrations well above the Cl minimum detection limit. Why chlorine would prove such a marked discriminator among sites and regions is not clear, particularly since it's concentrations in blood plasma, if not also endolymph, can reasonably be expected to be heavily buffered by the osmo-regulatory system. J. Calaprice (in Thresher, et al., in prep) discounted Cl as a stock discriminator in his studies on Atlantic bluefin tuna because the element is widely considered a 'universal contaminant' and hence could be an artefact in otoliths, derive from any of a number of sources during specimen preparation and analysis. The issue of contamination is a major one, particularly at the sub-ppm level, but we think it an unlikely explanation for its distribution in our samples, for four reasons. First, Cl does not vary independently; its concentrations in otoliths co-varies among specimens with

Na and K. If Cl concentrations are principally the effect of contamination, one needs to invoke the same sources of contamination for both Na and K, which is unlikely. Second, samples collected in the same region but at different times, different places, and using different types of gear (eg., juveniles and adults from the Tasmanian sites) nonetheless exhibit similar concentrations of Cl (in this example, virtually none), suggesting the observed variability in Cl among regions does not derive from any simple effect of specimen capture and handling. Third, the order of specimen preparation and analysis was, in several instances during the study, deliberately randomised in order to check for systematic error; comparisons of randomly inter-mixed with remaining specimens showed no conspicuous effects of specimen preparation on detected element concentrations. And finally, left-right comparisons involving pairs of otoliths from the same individual show a high correlation for Cl concentrations, suggesting that specimen preparation and analysis post-removal of otoliths from the specimen are unlikely to be a source of much measurement error. As noted in the discussion of left-right comparisons, however, we do see more consistent evidence of left-right differences in the outer margins of otoliths for Cl than we do for other elements. We cannot yet account for this left-right difference, which may well be due to an as yet unidentified source of contamination (E. Brothers, in Thresher, et al., in prep., suggests that even the side on which a fish lies on a dock after capture can alter marginal otolith composition). Although the magnitude of the effect is much less than that involved in discriminating among regions, it does suggests Cl data be applied cautiously until such issues are resolved.

The observations of significant ontogenetic variability in otolith composition and regional differences in mean concentrations are consistent with previous studies using either probe microanalysis (eg., Radtke, 1987; Kalish, 1989) or bulk (whole otolith) analysis (eg, Gaudie & Nathan, 1987; Edmunds, 1990). Our data, however, permit a more detailed evaluation of the interaction between the ontogenetic and geographic components of variability than has been previously possible, and appears to allow a direct evaluation of whether otolith chemistry can be used to distinguish among fish from different spawning areas and different nursery grounds. Because we know so little about the reproductive biology of the studied species, however, the assessment of the utility of otolith chemical analysis for purposes of stock delineation at the spawning ground level has had to be inferred, rather than tested directly. That is, it would have been preferable to collect larvae (or at least adults) from known spawning grounds, assess the extent to which these fish differed in otolith composition, and then, if possible, map the subsequent dispersal and mixing of individuals from each spawning ground. This is not yet possible for the species, and evaluation has instead had to be based on, 'are there consistent regional differences in otolith chemistry?' and "are these

differences consistent with what we might expect of the stock structure of this species?'. The value of otolith chemistry for distinguishing among nursery areas is more direct, in that we can test for a nursery ground-specific signal in N. macropterus and seek evidence of this signal among the adult population.

With regard to the spawning ground aspects of the study, four of six elements measured near the primordium differed significantly among the six sites sampled for adults. Moreover, the pattern of differences appeared to be regionally based; the two Tasmanian samples pooled together in the LDFA, as did the geographically adjacent NSW and Victorian samples. Such a grouping of sites could derive any of four very different mechanisms: (1) all sites differ, and the grouping is a statistical artefact of the small number of sites sampled; (2) regional differences result from retrospective modification of otolith chemistry in response to the latest conditions encountered by each adult and adjacent sites pool because their environmental characteristics are more similar than those of widely separated sites, (3) the sites pool because each regional set derives uniquely from a common spawning ground or spawning population; and (4) each set is derived from a number of spawning grounds or populations that have similar chemical fingerprints, within which individuals mix widely and the boundaries of which are set by constraints on adult or larval mixing.

The possibility that the regional groupings are an artefact is difficult to evaluate without a priori information about the range of chemical fingerprints possible and their likelihood of occurrence. If you assume, however, three chemical phenotypes randomly distributed among six individuals (= sites), then the probability that at least two adjacent sites will have identical characteristics is extremely high. However, the probability that all pooling of sites will be only among nearest neighbors is less than 0.01. Therefore, we discount the hypothesis that the apparent regional groupings are a statistical artefact. We also think it unlikely that the groupings (and similarity of fish within sites) are the result of retrospective modification of otolith chemistry. It is a consistent assumption of otolith-based aging studies that otolith structure, at least, is not modified after deposition. A similar assumption underlies chemical studies, although there are no experimental data to verify the point (as opposed to studies on scales, which are modifiable retrospectively, eg, Sauer and Watanabe, 1989). Indeed, there is evidence that at least some water- and alcohol- soluble compounds are transported into or out of otoliths during preservation (R. Gauldie, pers. comm.). Despite this, our analyses indicate that juvenile morwong collected at the same site and time show little evidence of convergence on a common marginal composition, which implies that recent environmental history not has little or no effect on the composition of the otolith interior, but even has relatively little effect on marginal composition. Where there was

some evidence of convergence, eg., in Sr levels, there was no indication of convergence retrospectively beyond what appeared to be age at settlement, ie., when common environmental conditions were encountered. For these reasons, the assumption remains reasonable that chemical composition is not grossly distorted retrospectively when dealing at concentrations of > 100 ppm. Whether the assumption remains valid when dealing at trace element concentrations, ie., > 10 ppm, is less clear.

Distinguishing between the other two hypotheses -- a single spawning ground for each regional phenotype or multiple spawning grounds with regionally restricted mixing --is not possible without additional information. As noted, information on the reproductive biology of Australian N. macropterus is sparse. Smith (1989) found running ripe individuals in autumn (February/March) off NSW and Holdway (unpublished) found larvae apparently of N. macropterus (B. Bruce, pers. comm.) in small numbers off Victoria. Our own collections off Tasmania (see Thresher, et al., 1989 for sampling sites and protocol) indicate large numbers of relatively young larvae present all along the east, but not the west coast of Tasmania and B. Bruce (pers. comm.) reports similar larvae off South Australia (in the GAB). From these scattered observations, it is clear that N. macropterus spawn at a number of sites along the SE Australian coast, and certainly spawn in each of the three regional groupings of sites identified by otolith chemical analysis. Sampling is not sufficiently complete, however, to determine whether these are discrete spawning areas, or represent samples along a continuous band of activity off the coast. Genetic data provide little additional information. Richardson's (1982) samples for allozyme analysis are drawn from Tasmanian and NSW/Victorian sites, and hence appear to bracket two otolith-based regional groupings. Richardson's data nonetheless indicate no fixed genetic difference between regions, a result recently been confirmed by Elliott and Ward (in press) for allozymes and Grewe, et al. (ms.) for MtDNA.

The lack of genetic differentiation in SE Australian N. macropterus populations at face value is consistent with our observations of apparent examples of larval mixing. Probe micro-analysis of otoliths of juveniles from Victorian and Tasmanian coastal habitats indicates most are similar in composition to adults collected at the same sites, which appears to be consistent with expectations based on regional, self-recruiting populations. The distribution of the probabilities that each juvenile originated in the region where it was collected (based on adult composition) was conspicuously bi-modal, however, after discounting a small number of individuals of uncertain origin. Four out of ten juveniles caught off Victoria had chemical phenotypes more typical of Tasmanian (3) or GAB (1) origin, whereas 8 of 106 Tasmanian-caught juveniles classified with mainly the NSW/Victorian adult sample. Although the data are

obviously preliminary, these mis-matched individuals could be the first direct evidence of mixing in the larval stage for a marine fish species. If so, the apparent mixing rate, about 7-8%, is high enough to prevent genetic divergence among samples from the NSW, Victorian and Tasmanian sites.

It appears, therefore, that in our test species probe micro-analysis of otoliths may resolve population structure at a scale finer than is possible with current genetic techniques. The initial working hypothesis of why this is possible is that the chemical phenotype is largely determined by environmental factors (), which presumably differ at relatively fine space scales. The data to support this environmental sensitivity, however, are not abundant, and to a large extent are drawn from the invertebrate literature (eg.,). Among studies involving teleosts, data are ambivalent. To date, all reported effects have involved strontium, which has been reported as sensitive to changes in salinity (Radtke, 198-; Kalish, 199-) and temperature (Radtke et al., 1990; however, see Kalish, 199-; ? & Kingsford, 1992). There are, in fact, two reasons to suspect a lower level of environmental responsiveness among most elements than is widely assumed.

The first reason is theoretically based. Most of the elements detected in our study (Ca, Na, K, Cl and S) are physiologically important and well documented to be highly buffered in plasma, and hence presumably in the endolymph (see Kalish, 199-). Hence, an expectation that, as an example, relatively slight changes in salinity significantly alter the incorporation of Na and Cl in otoliths is unrealistic in the light of well developed teleost osmo-regulatory mechanisms. Of the six elements detected, only Sr is likely to be relatively unaffected by such physiological controls, though it is presumably affected by many of the same factors that constrain variation in Ca concentrations and may well be subject to a suite of other physiological constraints (see Kalish, 1990, for a discussion of possible controls on Sr incorporation into otoliths). The effects of endogenous constraints on otolith composition are apparently reflected in the correlated distributions of the elements among specimens: concentrations of Sr and Ca correlate among specimens, as do, as a separate group, Na, K and Cl. Only S varies among specimens more or less independently, though ontogenetically its concentrations often vary in parallel with Sr (Thresher, et al., in prep.). Not suprisingly, factor analysis identifies two significant underlying variables, one each related to the Sr/Ca and the Na(or K)/Cl axis. The environmental, physiological or genetic factor which broadly set the relative abundances of the elements within each set are not yet known, though as noted, there are indications for at least some species that Sr/Ca ratios may be indirectly affected by temperature and salinity. Na, K and Cl are all involved in osmo-regulation and Na and K, in particular, are critical in neurological activity and abundantly linked

in the physiological literature. Sulfur is bound into the protein component of otoliths, and its concentrations may be growth rate-related.

The second reason we suspect a weak effect of the environment on otolith composition is empirical: where we expected to see such an effect, it was not immediately evident. Two observations are particularly relevant: (1) settlement into nursery areas had no apparent effect on otolith chemistry, other than a slight effect on strontium, and (2) differences among regional groupings are manifest from the primordium to the otolith margin, and hence apparently unaffected by life history stage, irrespective of habitat occupied. Regarding the transition to the nursery areas, for elements other than Sr there was no indication of convergence on a common chemical phenotype by individuals in a given nursery area, nor evidence of divergence among nursery areas in response to local conditions. This suggests that the concentrations of 5 of the 6 elements we measured do not vary in response to environmental conditions in the nursery areas in any simple or direct way. The nursery areas sampled ranged from mid-shelf to shallow, coastal embayments and differed markedly in terms of temperature and salinity histories, water column chemistry, depth, substratum, turbidity, and invertebrate composition (and hence presumably the diets of the juveniles). The lack of a demonstrable impact of any of these on otolith composition suggests their effects at the >100 ppm level are weak and/or indirect, except possibly for effects on strontium. That the regional differences among fishes are apparently consistent through life suggests the same conclusion. Although the concentrations of several elements (Sr, Na, K, S) vary ontogenetically in otoliths, this variation is superimposed on and apparently separate from whatever determines regional differences in composition. Ontogenetic variation in N. macropterus may be a function of a life history that spans environments from high seas nekton to shallow coastal embayments and the open continental shelf, but the regional differences, at least, appear to be largely unaffected by this ontogenetic migration and habitat shift.

Exactly what determines these apparent regional differences in 'base' composition is not clear. There are four classes of hypotheses, however. 1) Retrospective modification of chemical phenotype, based on the adult habitat; for reasons discussed above, we think this mechanism unlikely. 2) Life cycles for each region that are closed within areas of a uniquely diagnostic environmental feature. This seems unlikely given the diversity of habitats occupied by the species during its life history, but cannot be falsified until the factors that affect otolith composition are determined. 3) The base composition is set by environmental influences early in larval development, and then maintained, albeit overlayed by ontogenetic modification,

throughout the subsequent life and environmental history of each individual. 4) The base composition is determined genetically.

The information currently available is not sufficient to discriminate between a 'locked environmental effect' and a genetic hypothesis. A key datum that would permit such discrimination is a measure of year-class effects on otolith composition. Although robust falsification is not possible, regional differences in otolith composition that vary widely among year-classes, and perhaps can be correlated with conditions on or near spawning grounds, argue against a genetic basis for regional differences and support the effect of an environmental influence early in larval development. Sample sizes within years and within regional groupings are too small in the current study for any statistically powerful test of year-class differences; a detailed examination of year-class effects in N. macropterus is in progress (Proctor, et al., in prep). Preliminary results, however, suggest only small differences among years for most sites, which is consistent with the similar classifications of adults and juveniles for both the NSW/Victorian and Tasmanian regional groupings. Despite a mean difference of five years between adults and juveniles (adults from the 1980-84 year-classes, juveniles derived from the 1987 and 1988 year-classes), juveniles and adults from each regional grouping overlap broadly in concentrations of the regionally diagnostic elements.

We tentatively conclude, therefore, that the regionally diagnostic 'base' concentrations of most measured elements probably have a genetic basis. If so, then this conclusion apparently conflicts with both genetic analysis of the species in Australia (Richardson, 1982; Elliott & Ward, ms.; Grewe and Smolenski, in prep.), which indicate no regional differences, and with our preliminary estimate of mixing among regional groupings during the larval stage -- circa 7-8%/annum. An exchange rate this high should prevent regional genetic differentiation. At this point, the data are not adequate to resolve this apparent contradiction. Its resolution, however, critically affects the way compositional data obtained from WD-based electron probe microanalysis is used for stock delineation. If the regional differences are primarily genetically determined, then year-class effects are relatively unimportant, which simplifies analysis, but the usefulness of the approach will vary widely depending upon the extent and pattern of genetic differentiation among populations. However, if regional differences in 'base' concentrations are primarily determined by environmental effects, perhaps via a 'locked phenotype' mechanism of some kind, then variability among year-classes could be a critical co-variate in an analysis of population structure and the electron probe microanalysis is likely to be useful wherever significant environmental differences between spawning grounds are known or suspected.

We conclude therefore that otolith composition, in the > 100 ppm range, exhibits high levels of individual, ontogenetic and regional variability, that this variability exceeds substantially the scale of measurement error, that co-variance among elements suggests this variability results from only a few underlying physiological variables, and that the regional component of the variability is largely 'fixed' within individuals, and may be under genetic control. The conclusions have several implications about the utility of elemental analysis of otoliths.

First, the high levels of significant (ie., greater than measurement error) variability at all scales and in all elements examined confirm scope for possible 'de-coding' of the variability to reconstruct either or both the physiological/environmental history of an individual and its migration history/stock structure. The next step is to determine experimentally the causes and correlates of observed elemental configurations, and specifically to determine the roles of environmental, physiological and genetic effects on composition. Some of this work has been undertaken (eg., Kalish, 1989; Radtke, et al., 1990), but given the complexity of elemental variability, a good deal more is required. Second, the regional and ontogenetic co-variance among elements, however, suggests that the amount of information that can be gleaned from a six element analysis may be less than at first appearance, that what is 'reconstructable' is likely to be an integrated physiological history rather than a set of unambiguous indicators of specific life history features, and that multi-variate and higher order statistics will often be required to extract available life-history correlates. And third, the 'fixed' nature of regional differences in composition implies that simple efforts to use life-stage specific, environmentally induced signals to identify migration patterns, eg., nursery areas in N. macropterus, are not likely to be successful, due to confounding effects of regional and, perhaps, ontogenetic variability. This 'fixed' difference also implies, however, that full life history traces are not required for stock delineation based on mean elemental composition; the required 'base differences' can be assessed equally well at any consistently examined section along the growth axis, or could even be determined using whole otolith (bulk) analysis, though probably with some loss of resolution because of ontogenetic variability. Such limited analyses are much cheaper and quicker than conducting a full life history scan, and may make feasible analysis of the large numbers of specimens typically required for a population analysis.

Table 1.

	Location	Date collected	Collection method	Sampl size
Adults				
	Kiama, NSW (Lat.34°40'S Long.151°10'E)	12 Jan 90	Trawl	11
	W.Bass Strait, Vic. (Lat.38°34'S Long.141°10'E)	30 Aug 87 - 6 Sep 87	Trawl	6
	W.Bass Strait, Vic. (Lat.37°48'S Long.139°45'E)	6 Dec 89	Trawl	17
	Maria Is., E Tas. (Lat.42°40'S Long.148°15'E)	16 April 89	Trawl	2
		15 Nov 89	Trawl	11
	Port Davey, SW Tas. (Lat.43°07'S Long.145°32'E)	21 Mar 89	Trawl	5
	GAB, S.Aust. (Lat.33°25'S Long.125°56'E)	15 Oct 89	Trawl	16
Juveniles				
	Cygnet, SE Tas. (Lat.43°10'S Long.147°05'E)	19 Dec 87	Hand-line	27
		3 Dec 88	Hand-line	13
	CSIRO Wharf, SE Tas. (Lat.42°53'S Long.147°20'E)	25 Sep 87	Hand-line	9
		9 Nov 87	Hand-line	11
	Port Davey, SW Tas. (Lat.43°07'S Long.145°32'E)	21 Mar 89	Trawl	26
	Maria Is., E Tas. (Lat.42°31'S Long.148°10'E)	7 April 88	Trawl	11
	Nutgrove Beach, SE Tas. (Lat.42°55'S Long.147°21'E)	29 Mar 88	Trawl	9
	Phillip Is., Vic. (Lat.38°35'S Long.145°10'E)	13 Dec 87	Trawl	10

Figure Captions

1. Sample locations for adult and juvenile N. macropterus

2. Diagramatic representation of N. macropterus sagitta, indicating plane of analysis and scan lines used to track main growth axis for life history scans.

3. Mean concentrations of 14 elements detected in the sagittae of N. macropterus.

4. Representative life history scans of three N. macropterus chosen casually from our data set.

5. Auto-correlation plots for Sr concentrations in life history scans of the three representative specimens depicted in Fig. 4.

6. Comparison of short life history scans near the margin of an N. macropterus otolith taken using large beam spots (14 μm diameter, at 16 μm intervals) and small beam spots (6 μm diameter, at 8 μm intervals).

7. Comparison of life history scans for three elements for each of the three representative N. macropterus.

8. Comparison of elemental concentrations at Point 1 (immediately on the primordium) with Point 2 (20 microns radial to the primordium) for adult N. macropterus.

9. Distribution of estimated concentrations of the six micro-constituent elements at the primordium of adult N. macropterus. P_N is the probability that the distribution is normal.

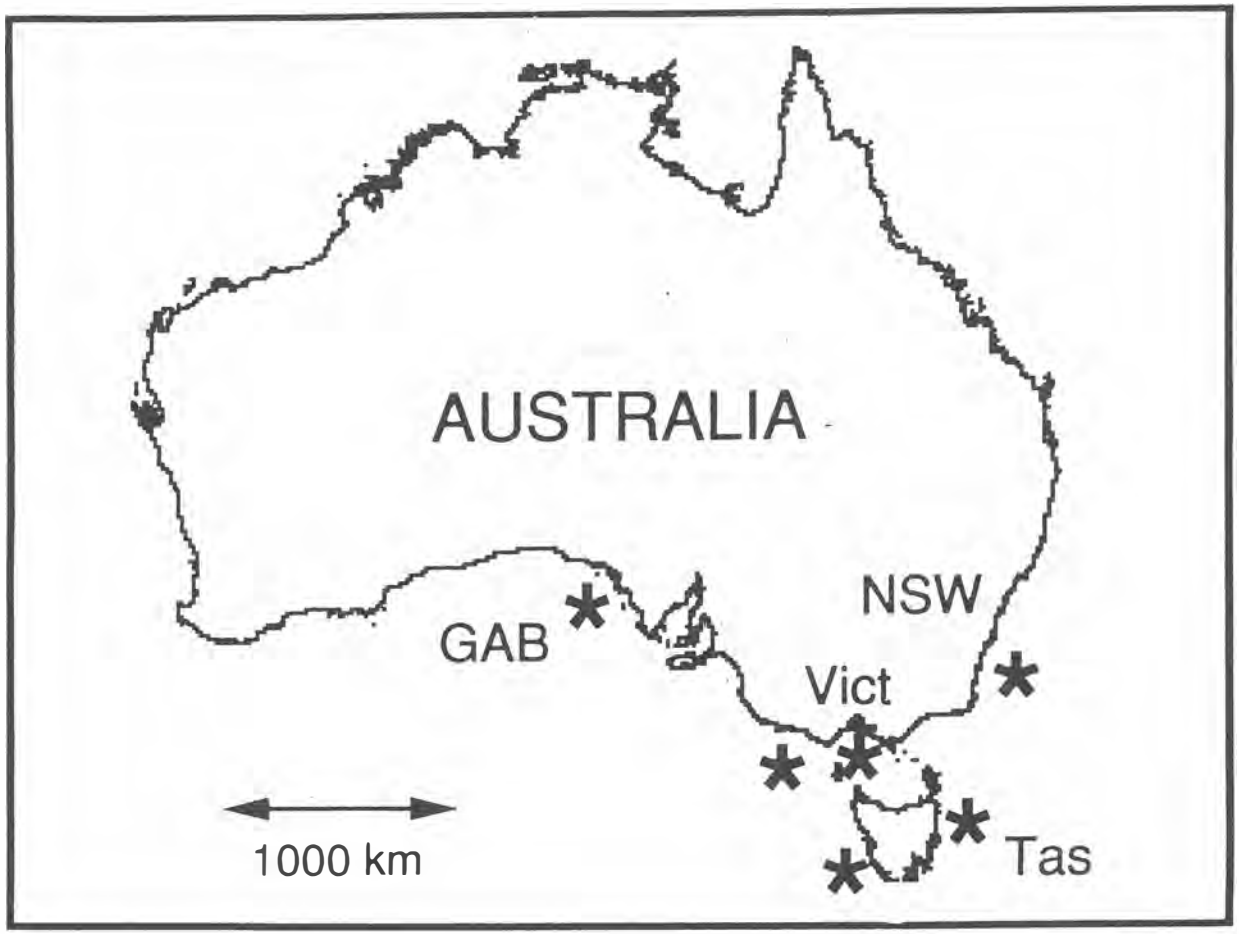
10. Distribution of the mean concentrations of elements among adults collected at six sites in SE Australia. P = probability means differ among sites.

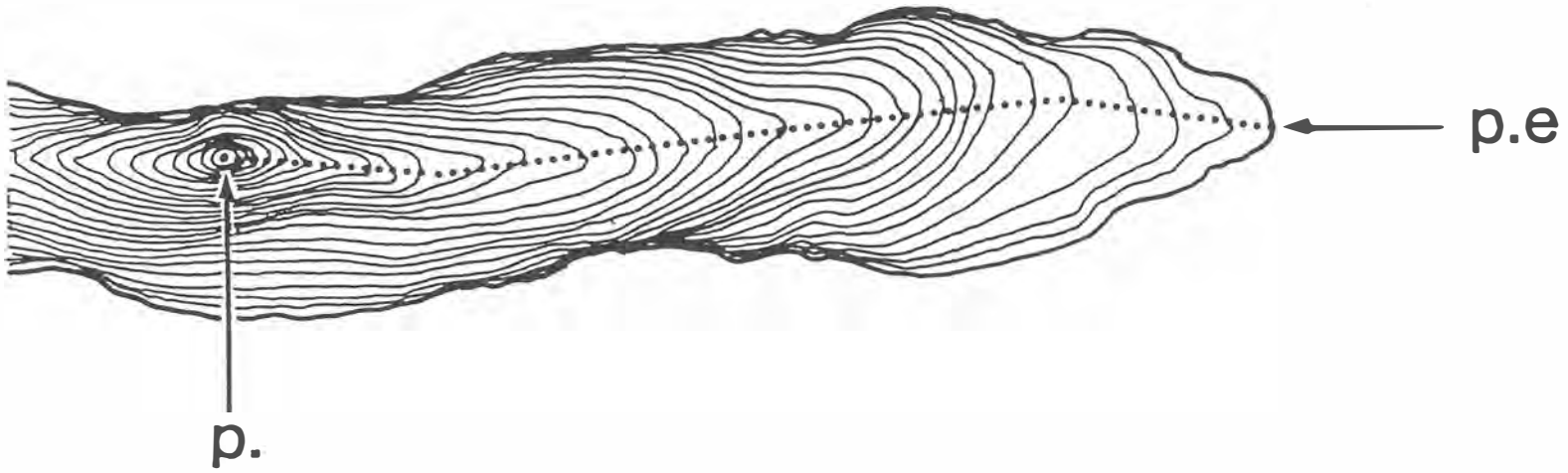
11. Na/Cl and Sr/Ca plots for concentrations at the primordia of adult N. macropterus, split by sample site. Data are for Point 2 only, and for the mean concentrations of Points 2-6.

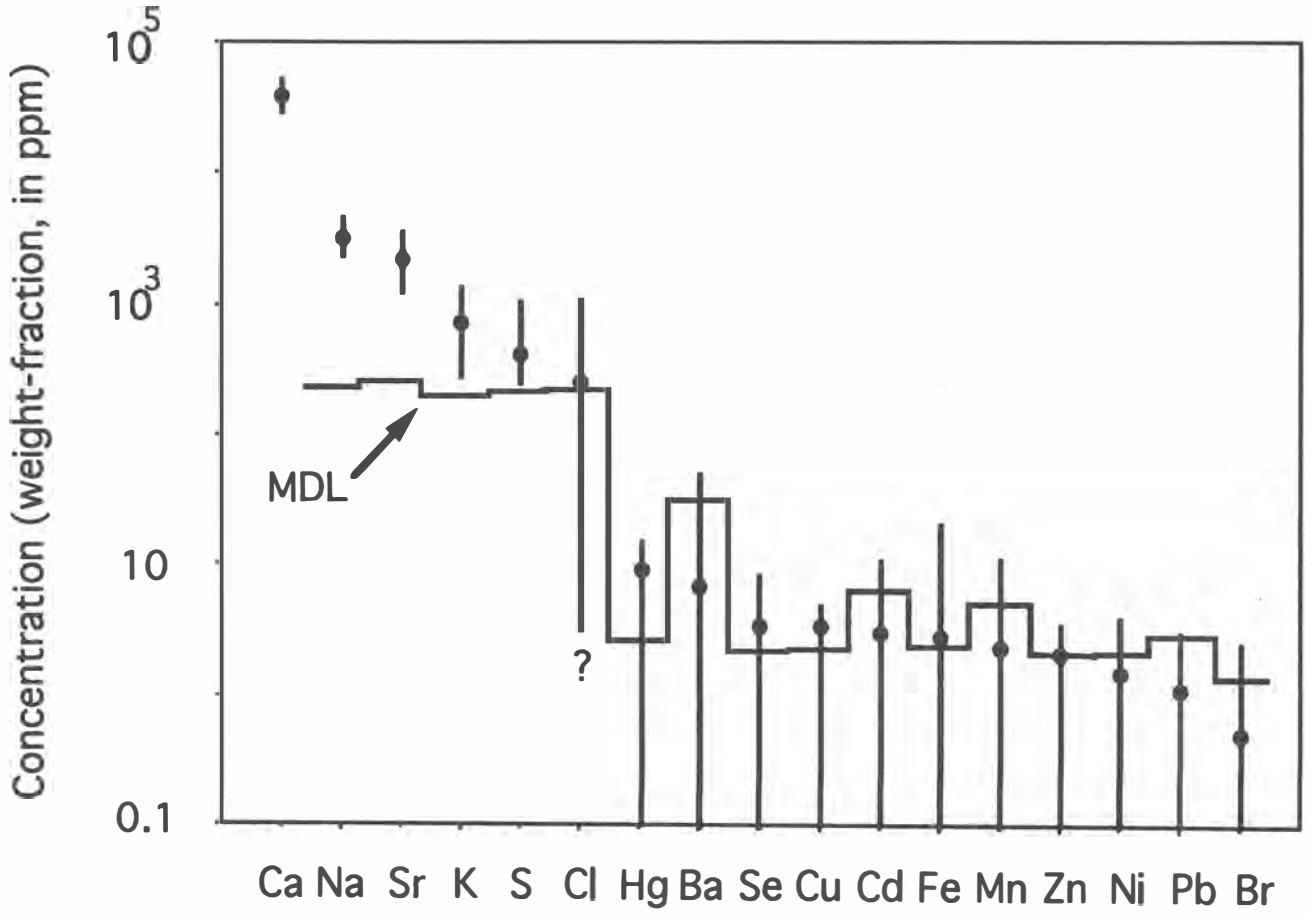
12. End result of a Linear Discriminant Function analysis based on mean elemental concentrations of Pts. 2-6 for adult N. macropterus collected at six sites in SE Australia.

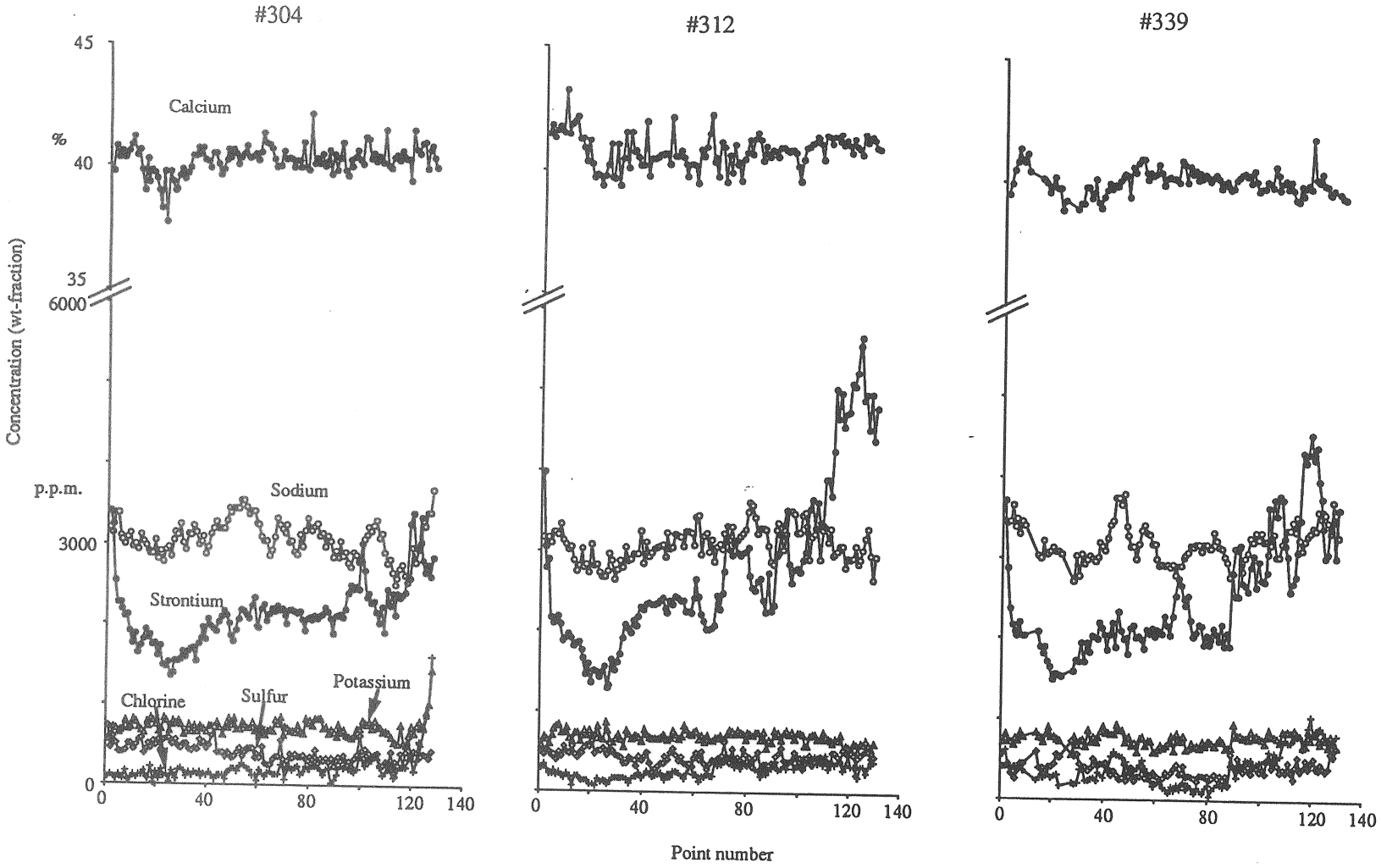
13. Distribution among samples of Factor values derived from linear discriminant function analysis. P = signicance level of factor in delineating among samples; horizontal lines indicate sites that do not differ significantly based on Fisher's PLSD test.

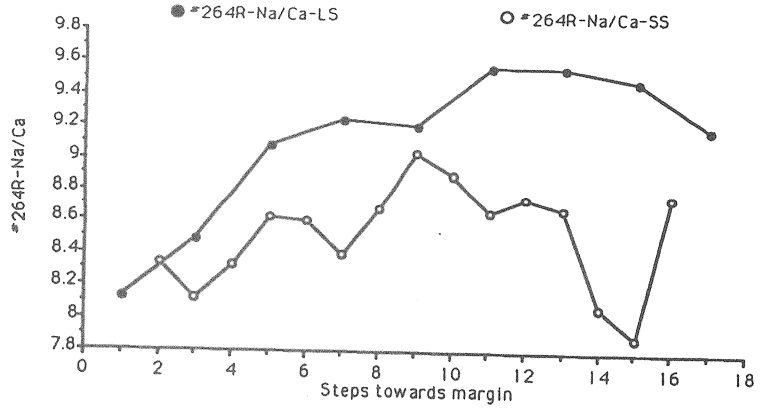
14. Mean and standard errors of the concentrations of four elements at four positions along the life history scan of adult N. macropterus, split by groupings of fites identified in the discriminant function analysis.

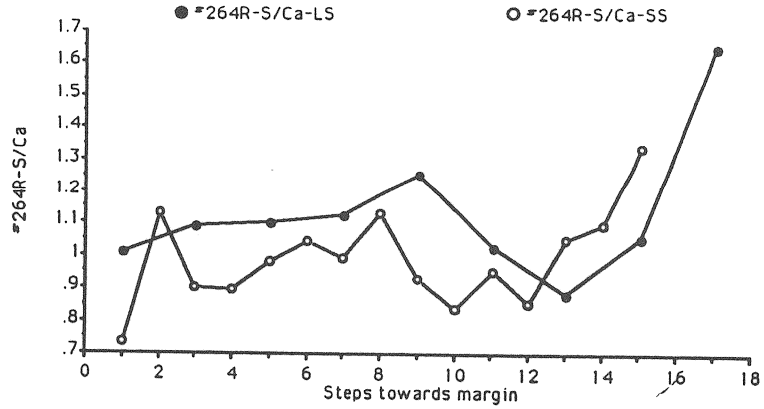

AUSTRALIA
GAB
NSW
Vict
Tas
1000 km

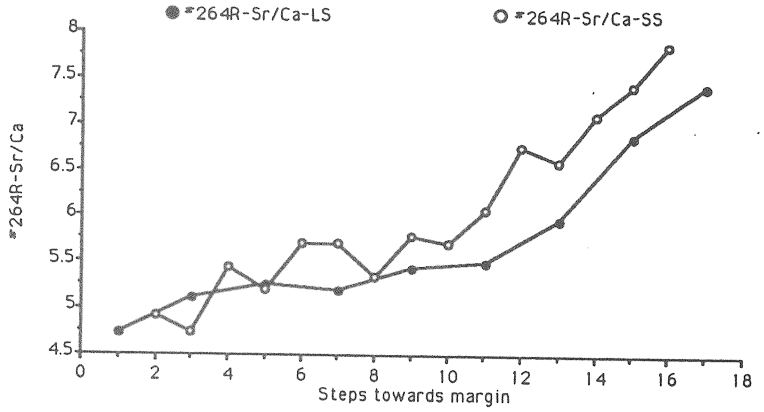

p.e
p.

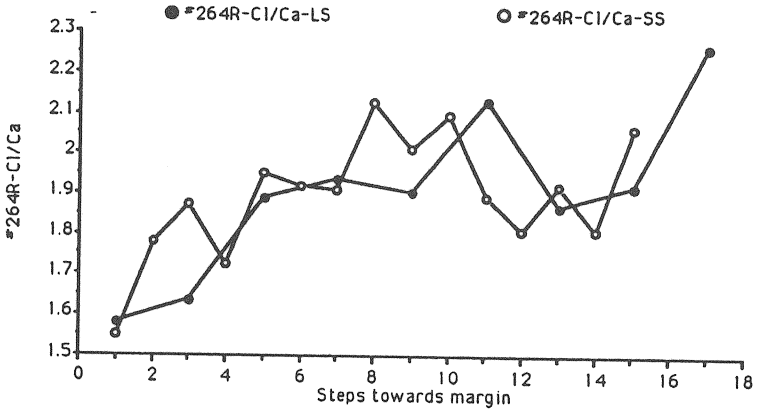

Concentration (weight-fraction, in ppm)
10^5
10^3
10
0.1
MDL
?
Ca Na Sr K S Cl Hg Ba Se Cu Cd Fe Mn Zn Ni Pb Br

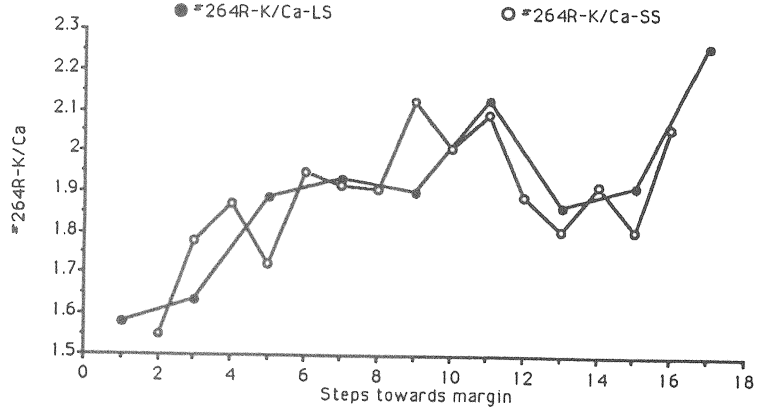

#304
#312
#339
45
Calcium
%
40
35
6000
Concentration (wt-fraction)
p.p.m.
Sodium
3000
Strontium
Potassium
Chlorine
Sulfur
0
0
40
80
120
140
Point number







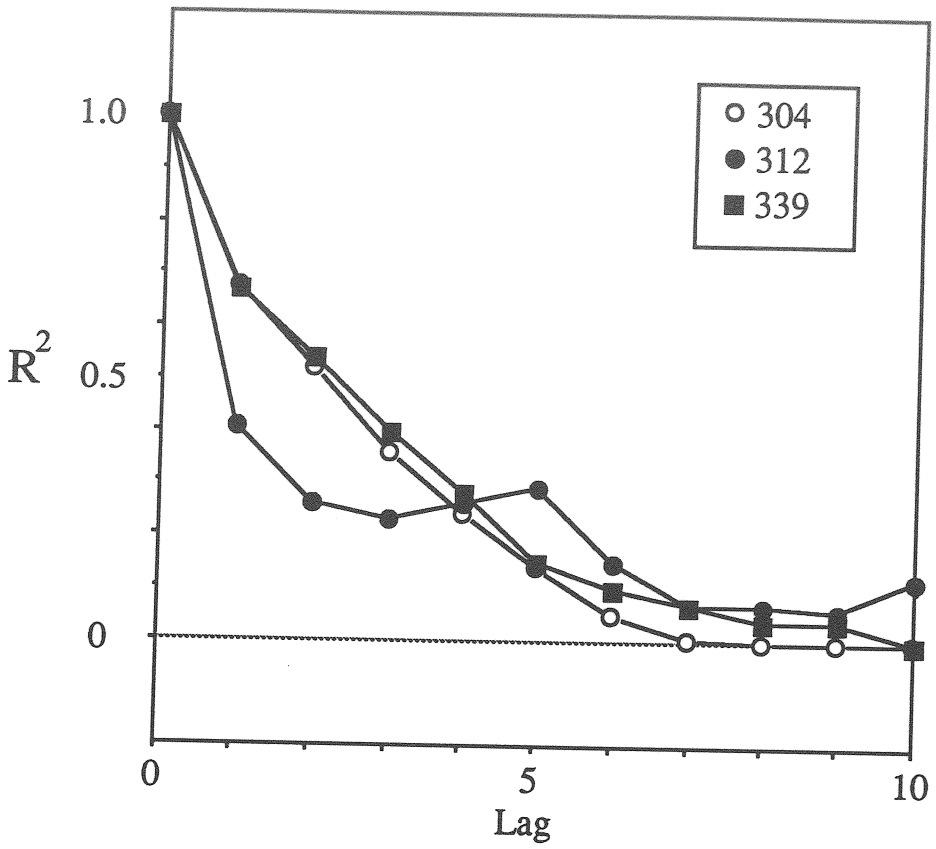




Nm#264R

LS = Large Spot (14 µm diam., 15kV, 25nA
16µm steps)

SS = Small Spot (6 µm diam., 15kV, 5.5nA
8µm steps)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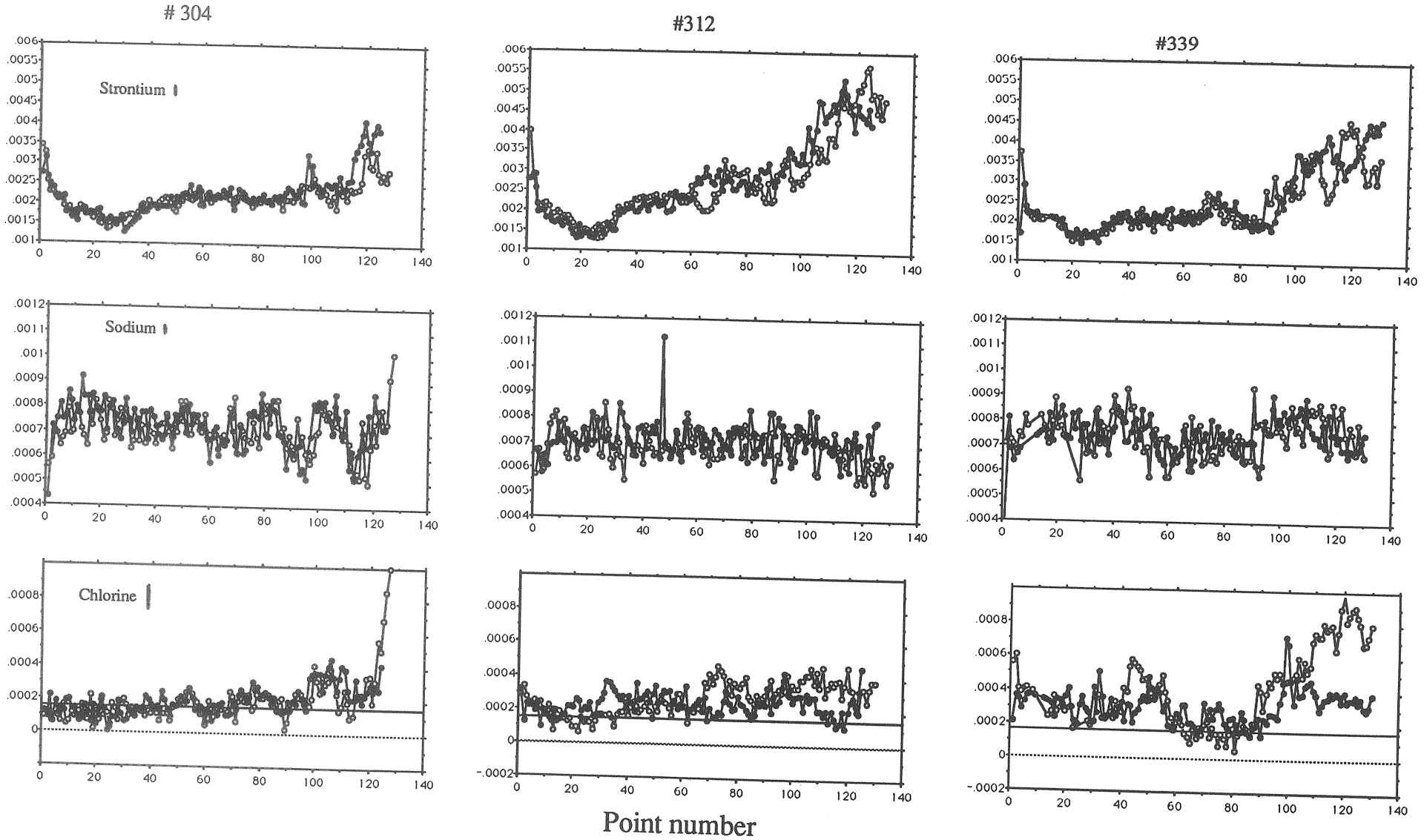
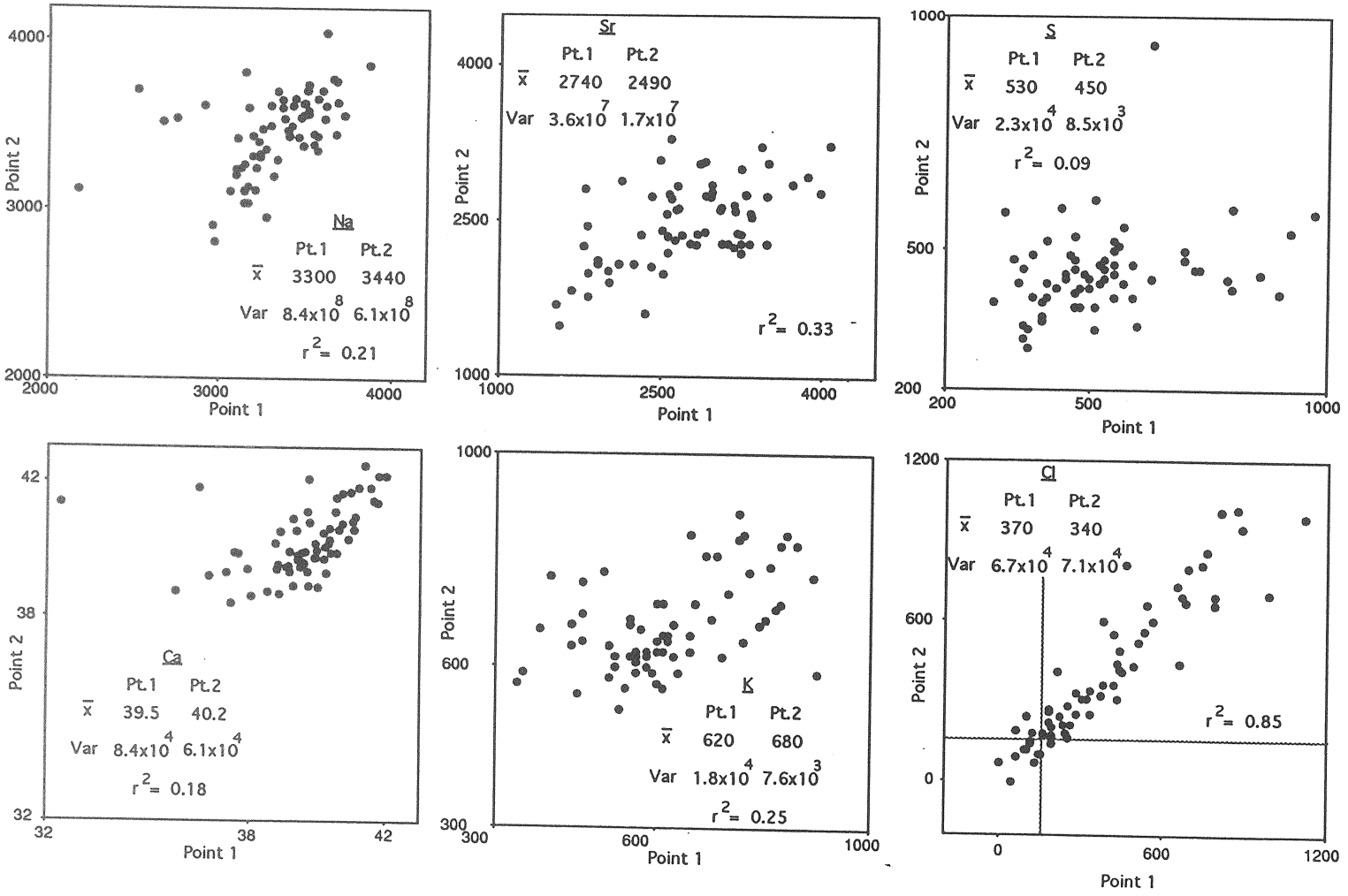
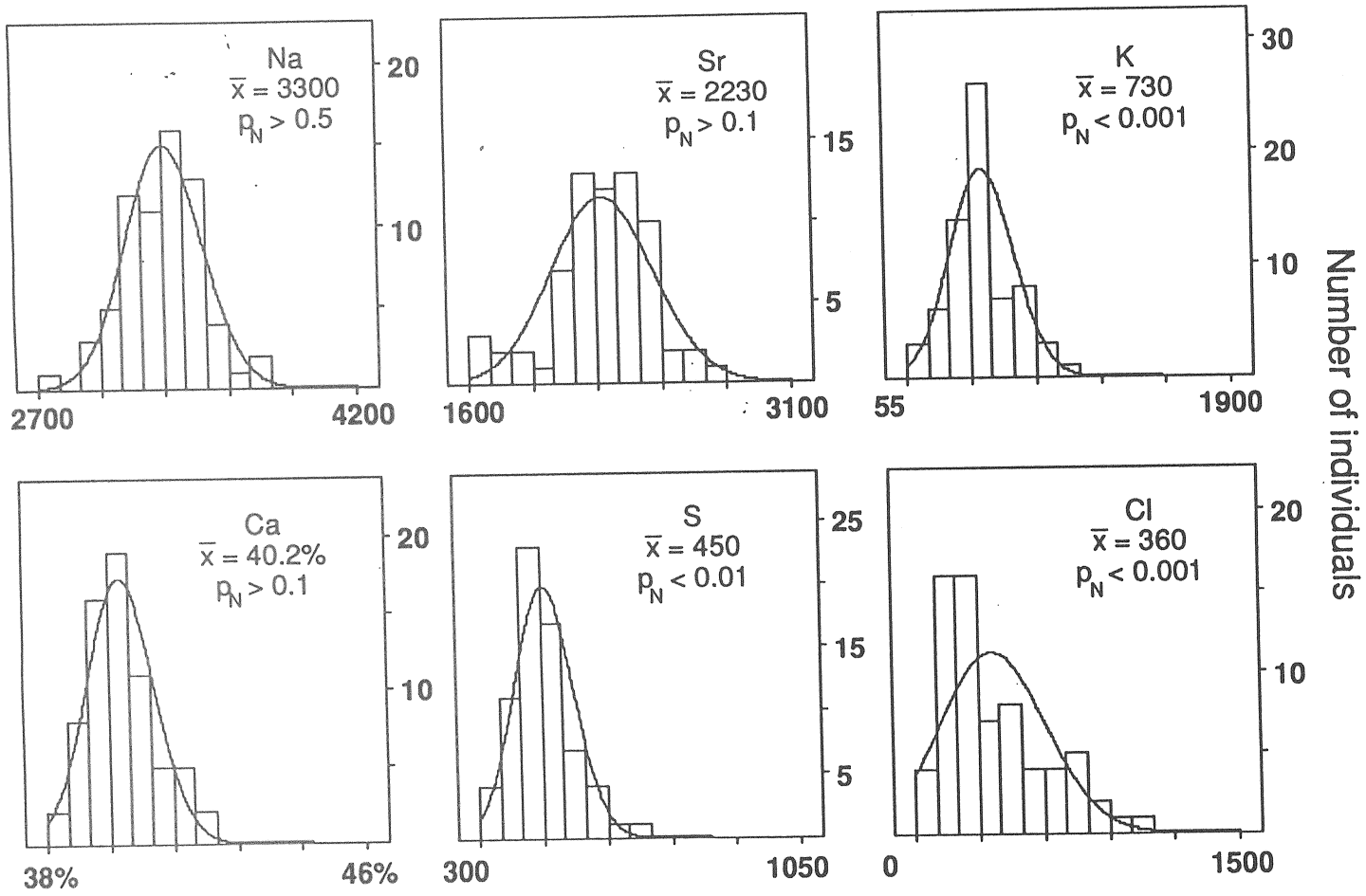
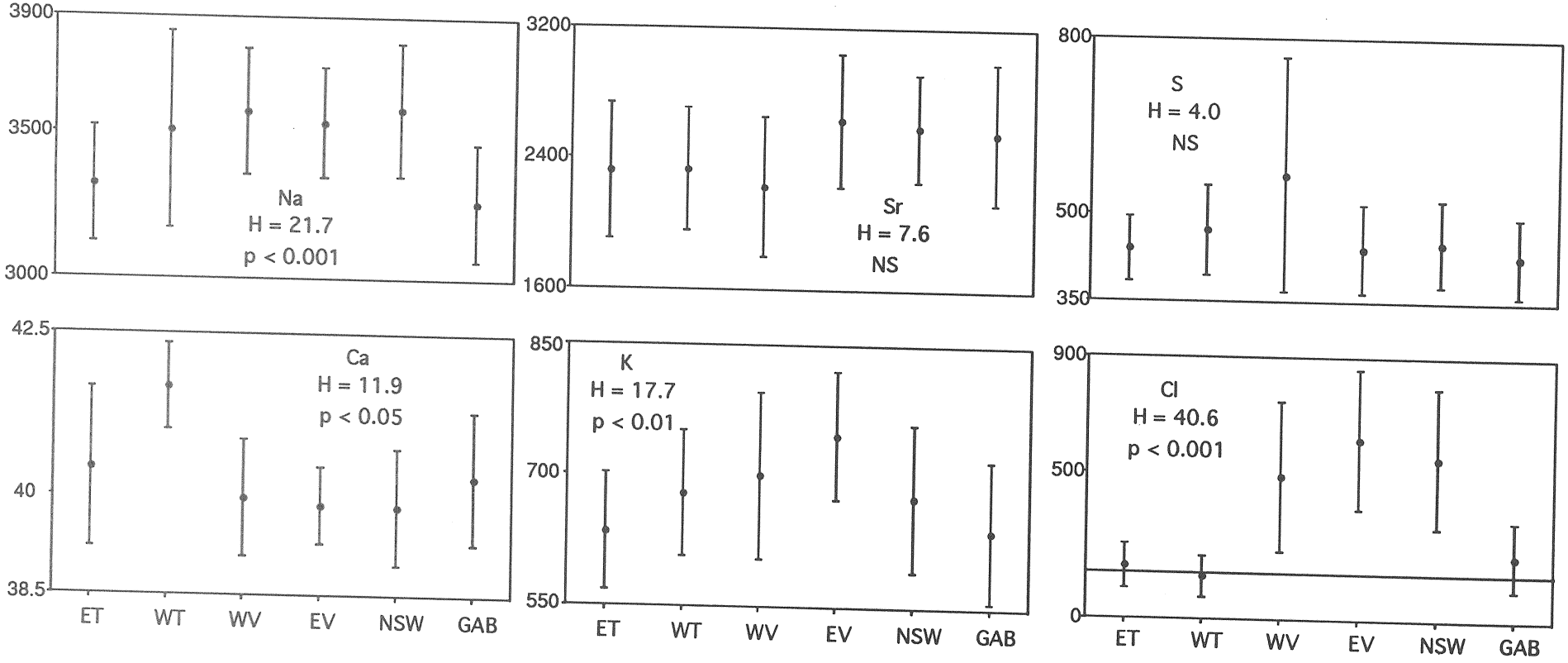
0.5
0
R^2
304
312
339
0
5
10
Lag


304
#312
#339
Strontium
Sodium
Chlorine
Point number


Na
Pt.1 Pt.2
x̄ 3300 3440
Var 8.4x10^8 6.1x10^8
r^2 = 0.21
Sr
Pt.1 Pt.2
x̄ 2740 2490
Var 3.6x10^7 1.7x10^7
r^2 = 0.33
S
Pt.1 Pt.2
x̄ 530 450
Var 2.3x10^4 8.5x10^3
r^2 = 0.09
Ca
Pt.1 Pt.2
x̄ 39.5 40.2
Var 8.4x10^4 6.1x10^4
r^2 = 0.18
K
Pt.1 Pt.2
x̄ 620 680
Var 1.8x10^4 7.6x10^3
r^2 = 0.25
Cl
Pt.1 Pt.2
x̄ 370 340
Var 6.7x10^4 7.1x10^4
r^2 = 0.85
Point 1
Point 2


Na
x̄ = 3300
p_N > 0.5
2700
4200
Sr
x̄ = 2230
p_N > 0.1
1600
3100
K
x̄ = 730
p_N < 0.001
55
1900
Ca
x̄ = 40.2%
p_N > 0.1
38%
46%
S
x̄ = 450
p_N < 0.01
300
1050
Cl
x̄ = 360
p_N < 0.001
0
1500
Number of individuals


3900
3500
3000
Na
H = 21.7
p < 0.001
3200
2400
1600
Sr
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NS
800
500
350
S
H = 4.0
NS
42.5
40
38.5
Ca
H = 11.9
p < 0.05
850
700
550
K
H = 17.7
p < 0.01
900
500
0
Cl
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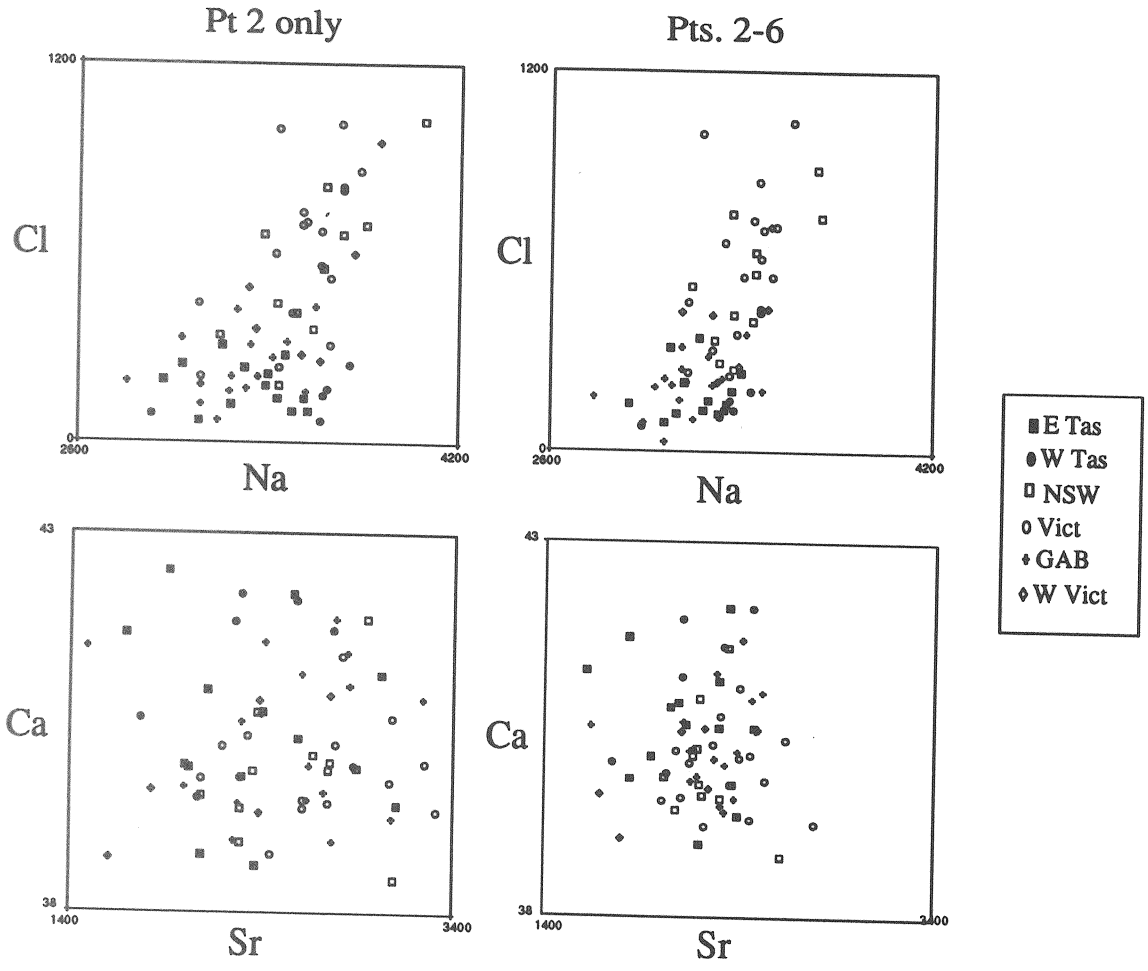
p < 0.001
ET
WT
WV
EV
NSW
GAB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Pts. 2-6
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Na
Ca
Sr
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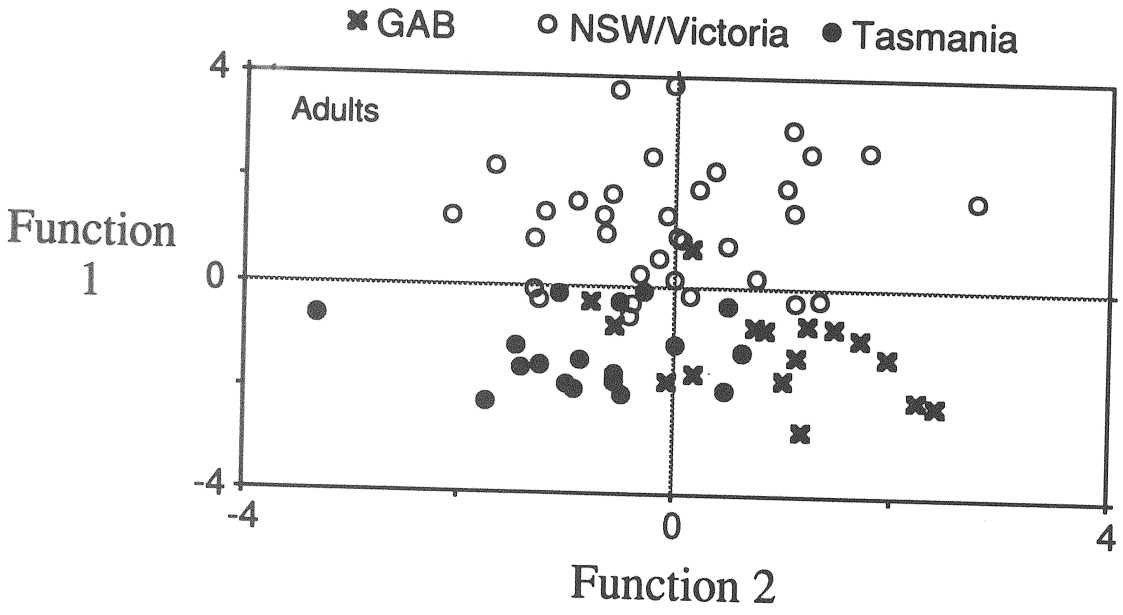
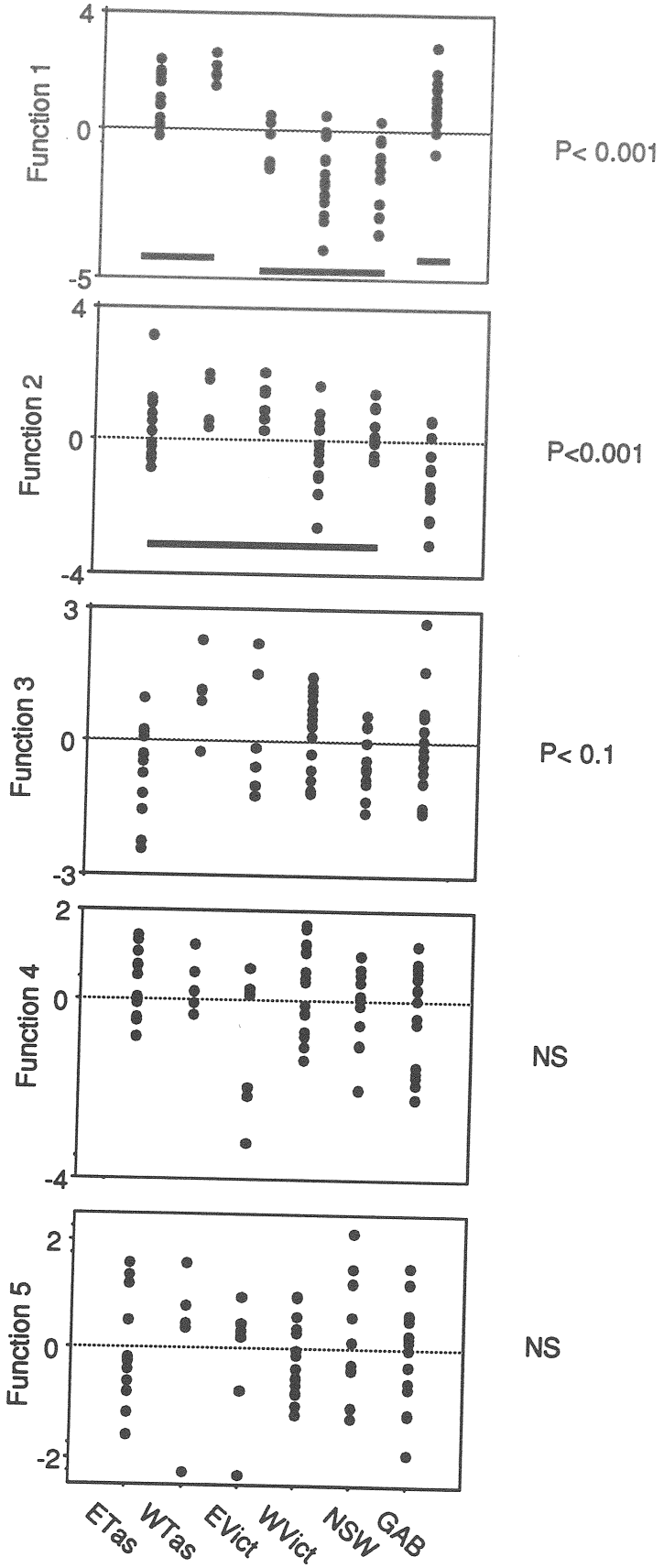
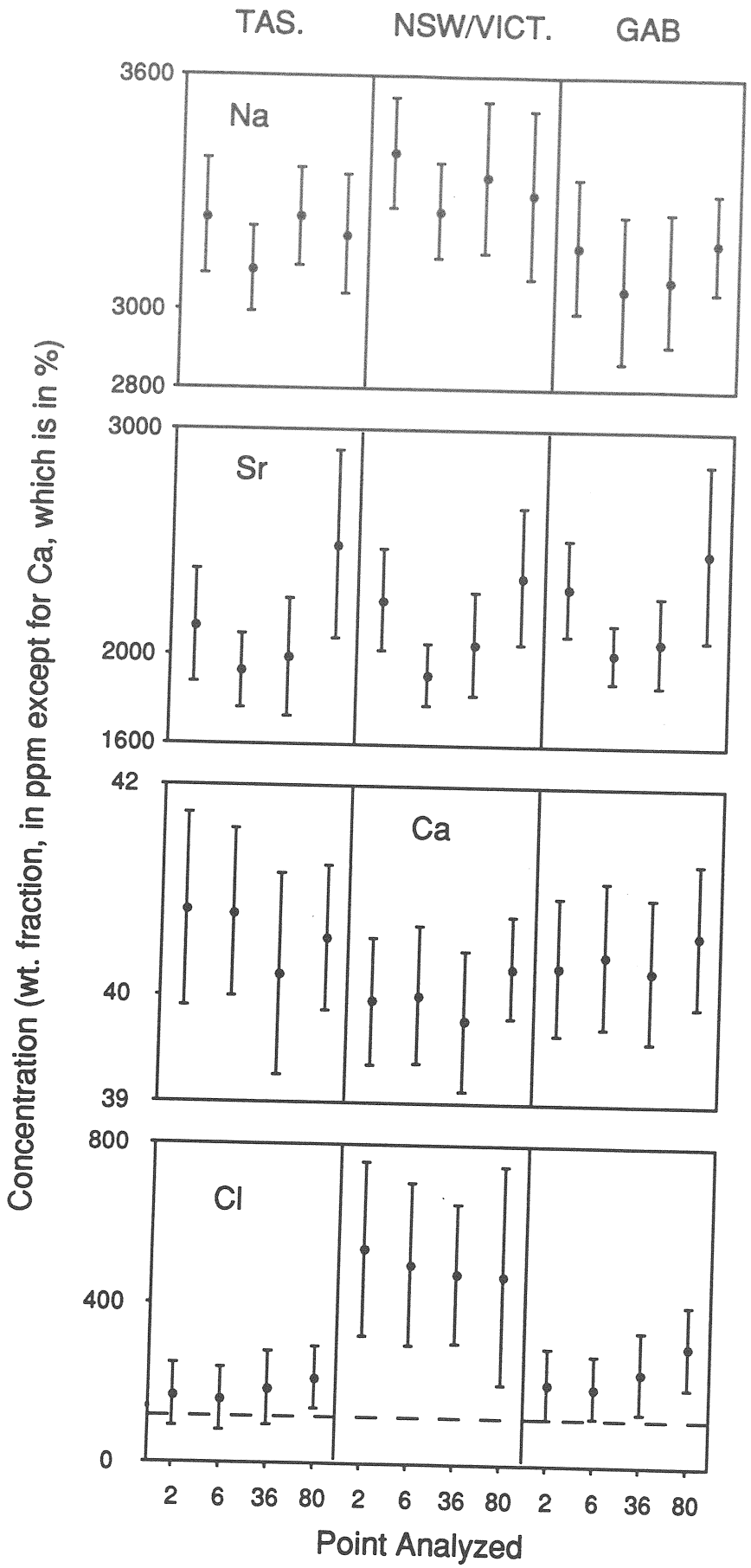
2600
4200
43
38
1400
3400
E Tas
W Tas
NSW
Vict
GAB
W Vict


GAB
NSW/Victoria
Tasmania
Adults
Function 1
Function 2
4
0
-4
-4
0
4


Function 1
4
0
-5
P< 0.001
Function 2
4
0
-4
P<0.001
Function 3
3
0
-3
P< 0.1
Function 4
2
0
-4
NS
Function 5
2
0
-2
NS
ETas
WTas
EVict
WVict
NSW
GAB


TAS.
NSW/VICT.
GAB
Na
3600
3000
2800
Sr
3000
2000
1600
Ca
42
40
39
Cl
800
400
0
2 6 36 80 2 6 36 80 2 6 36 80
Point Analyzed
Concentration (wt. fraction, in ppm except for Ca, which is in %)

Document 3

Evaluation of nursery area-specific signatures and the links between nursery areas and adult groups in Jackass Morwong, Nemadactylus macropterus

Nemadactylus macropterus (Cheilodactylidae) is a moderate sized, bottom-dwelling fish common on the middle and outer continental shelf off southern Australia, New Zealand, South Africa and the Pacific coast of South America (Robertson 1978). At present, the population structure of the species in Australian waters is contentious. A small amount of tagging data for adults (Smith, 1989) and allozyme data for specimens collected at several sites along the south-eastern part of the country (Richardson 1982) have been interpreted as suggesting a single, broadly distributed population in Australian waters (Smith 1989, Tilzey et al. 1990). This interpretation appears justified based on the early life history of the species. Spawning apparently occurs along the middle continental shelf (Vooren 1972). The eggs are planktonic, incubate for about 3 days (Robertson 1978) and hatch into larvae that have a planktonic stage lasting 9-12 months (Vooren 1972). Morphologically specialised late-stage larvae, known as 'paper fish', are neustonic and generally caught offshore of the continental shelf (Vooren 1972, Furlani, et al. in prep). The long planktonic duration of the species and the off-shore distribution of the larvae have been interpreted as implying high rates of larval mixing among sites, and hence a single Australian population. However, the population structure of the species has been studied in some detail in New Zealand, through tagging studies, examination of the distributions of eggs, larvae and juveniles (Robertson 1978) and analysis of otolith chemistry (Gauldie and Nathan 1977). These data indicate strongly that the species forms geographically discrete populations, each with a specific spawning area and nursery ground. There are three populations in New Zealand, which jointly occupy a range similar in size that inhabited by the species in Australia. There is no reason to assume that the behavior of the species in Australia differs from that in New Zealand, and hence that the weak information currently available on the Australian stocks is incorrect.

There is also considerable uncertainty about the distribution and importance of nursery areas of N. macropterus in Australia. Following its planktonic stage, juveniles settle into shallow coastal habitats and then, as they grow, migrate offshore over a three to four year period. The only place where large numbers of juveniles have thus far been found in Australia is south eastern Tasmania, which has lead to the suggestion that the shallow bays and inlets of SE Tasmania are a critical habitat supporting the entire Australian population (Tilzey et al 1990). If this is true, then conservation measures need to be developed and implemented to ensure the continued viability of the fishery in the face of continuing coastal development.

Hence, the present study was designed to obtain information relative to these hypotheses, in two ways. First, if the species forms discrete spawning populations, each at a different site, we hypothesised that the composition of the central portion of the otolith, which forms early in larval development, would differ among populations

due either to genetic differences among populations of differences in the spawning environments. Second, we further hypothesised that if the bays and inlets of SE Tasmania constituted the only nursery area for the species in Australia, then the chemical signature deposited in that part of the otolith corresponding to the residence period in the nursery ground a) would be similar for all adults, irrespective of where they were caught, and b) would be similar to the composition of juveniles caught on the nursery ground.

Methods and Materials

Procedures used for preparation and analysis of otoliths are detailed above. Collection details for juveniles and adults are provided in Document 2 (above) and in Fig. 1. Recently settled (0+) juveniles were collected by hand-lining and trawling at six sites off Tasmania and southern Victoria. Adult specimens were obtained from commercial and scientific trawls at six sites across south eastern Australia. In order to minimise possible affects of interannual variation in composition, we minimised the number of year-classes in the sample by using only adults in the size range of 30 - 35 cm SL. Examination of otolith macrostructure, reference to published length-age keys for the species (Smith, 1982), and enumeration of the episodic, and probably seasonal (see Proctor, et al., in prep.) variation in Sr concentrations along the growth axis indicate that specimens were a mixture of the 1980 to 1984 year classes. Sites overlapped broadly in year class distributions, based on the length-age keys and the Sr data; mean year-class values for the sub-sample of 22 individuals aged by Sr plots were 1982.8, 1981.5, 1982, and 1982.4 for Tasmanian, Victorian, NSW and GAB fish, respectively (ANOVA $F_{3,19} = 1.0$, NS). Juveniles were from the 1987 and 1988 year-classes.

Results

The links between nursery areas and components of the adult populations can be assessed in two complementary ways: 1) by the determining the source affinities of juveniles collected in each nursery area, and 2) by developing a specific signature for each nursery area and using these to classify adults collected in different regions. In essence, the former assesses the distribution across nursery areas of individuals from each of the putative populations, whereas the latter assesses the contribution of each

nursery area to adults collected at each site. With specific regard to N. macropterus, if conventional wisdom is correct, that is, SE Tasmania is the sole nursery area for the species in Australia, then we would expect that 1) elemental patterns similar to those of adults at all sites sampled would be represented in juveniles collected in the nursery area, and 2) adults collected at all sample sites would have a nursery area 'fingerprint' similar to that of the juveniles collected.

Results of the first analysis are depicted in Figs. 2 and 3, in which the three-site discriminant functions developed from the adults (see Document 2) were used as a training set to classify each of 116 recently settled N. macropterus. Procedures for data acquisition for the juveniles were identical to those used for the adults. Analysis is based on the mean values for points 2-6 from the primordium. Most juveniles were collected in SE Tasmania; a small number were also collected at Port Phillip Bay, Victoria.

Most juveniles examined fall within or close to the areas in discriminant function space defined by the adult groups; only one juvenile, with an exceptionally high value on the Function 2 axis, does not match at least approximately the characteristics of at least one of the three adult groups (Fig. 2). Moreover, most, albeit not all, juveniles classify with the adults collected in the same area. Of the 106 juveniles caught in Tasmania, all but 25 classify with the Tasmanian adult samples and of these, about half (13) classify ambiguously, with a probability >25% of being Tasmanian. Overall, only 7% of the Tasmanian-collected juveniles had a probability of <10% of classifying with the Tasmanian-caught adults (Fig. 4). Samples from the five Tasmanian sites were distributed similarly in two-function space (Fig. 3), though the variance is conspicuously higher at one site, Cygnet.

The pattern is similar for juveniles collected off Victoria (Port Phillip Bay), although sample sizes are too small to draw strong inferences. Of the 10 individuals examined, six classify with the NSW/Victorian adults, three classify with the Tasmanian-caught adults (at probabilities ranging from 72 to 85%), and one classifies with the GAB-caught adults (at P = 63%). The probability that the Port Phillip Bay juveniles classify with the NSW/Victorian adults is markedly bi-modal (Fig. 18), with peaks at >95% and between 5-10%, that is, most individuals had either a very high or very low probability of classifying with the local adults. A similar, though more complex pattern is evident among the Tasmanian-caught juveniles, with evidence of perhaps three modes: one, the largest, peaking at P>95%, an intermediate mode centered near P=35%, and a third at P<5%. The intermediate mode consists of individuals with ambiguous affinities, that is, those distributed at or near the overlap zones of the three adult groups in two-function space.

The second analysis of the link between nursery areas and the adult population requires analysis of that portion of the otolith deposited during occupancy of the nursery areas. The otolith of the smallest juvenile we found had a long growth axis almost exactly 500 μm in length; several other small fish had long growth axes in the range of 570 - 650 μm. Correspondingly, we examined a standard region approximately 600 - 800 μm exterior from the primordium along the main growth axis as otolith deposited early in the nursery area stage of development. Specifically, we used as the datum of interest for each specimen the mean composition of points 35-39, inclusive, in a standard life history scan, which covers the region between 680 - 780 μm from the primordium.

Discriminant function analysis of specimens from the six nursery areas sampled (Table 4) indicated highly significant differences among all six. The weakest discriminator (the fifth root of the LDFA) was significant at $P< 0.01$ ($Chi^2 = 12.1$, 2 DF). The preliminary conclusion then is that there are nursery-area specific signals, which could be sought in the adult population. Further analyses of the data, however, indicated this conclusion to be premature. Specifically, if there are nursery area-specific environmental signals in the otolith, then we would expect them to be manifest ontogenetically in either or both of two ways. First, we would expect that at the end of the larval period (approximately points 25-30) mean concentrations of various elements would diverge among sites, reflecting the specific environment at each, ie., the nursery area 'fingerprint'. Second, we would also expect that among individuals within sites concentrations of these same elements would converge, reflecting recruitment into a common environment. Again, this convergence should occur at approximately points 25 - 30. These predictions are tested in Figures 5 and 6. For the second prediction, we analysed in detail one site (Cygnet) for which the sample size of juveniles was large enough that we could reduce possible variability due to differences in date of recruitment, by examining juveniles all caught on the same day and falling within a narrow size range.

For most elements there is no indication from either data set of a nursery area-specific effect on elemental concentrations. Differences among nursery areas, which the LFDA interpreted as nursery-area specific signals, in fact are evident throughout the larval stage for 5 of the 6 elements examined; for these, there is little or no indication of either divergence among sites, in the case of mean concentrations, or convergence among individuals, in the case of variation within the single site, at or near points 25-30. The only element showing such an effect is strontium: mean strontium concentrations overlap broadly among juveniles from the different nursery areas during the larval stage, but diverge markedly (and significantly) beginning about point 25, and

Cygnet juveniles show evidence of converging on, in this case, two different post-recruitment trajectories, also beginning about point 25. We conclude, therefore, that with the exception of strontium, the apparent discrimination among nursery areas is the manifestation mainly of pre-existing differences among individuals carried over from their larval stages. Specifically, it appears that the mean differences between nursery areas in the concentrations of elements other than Sr is the result of differential mixing of individuals that conserve throughout early life individual differences of the concentrations of these elements.

We tested this conclusion by re-analysing for "nursery-area-specific signals" using as input elemental data for points 2-5, i.e., early larval life, rather than points 35-39, those specific to the early juvenile stage. In general, the results were similar to those obtained using points 35-39, with good discrimination among most nursery grounds and a comparable level of overall site separation (Pillai Trace statistic = 1.22 for points 2-5 vs. 1.21 for points 35-39, $p < 0.001$ in both cases). The accuracy of correctly assigning juveniles to nursery areas was less in the point 2-5 analysis (51% vs. 82%), however, which reflects the divergence of strontium concentrations in the nursery areas and its increased importance as a discriminator; two discriminant functions (2 and 3) load onto strontium at $r > 0.5$ in the original analysis, whereas it does not achieve this load level for any function in the point 2-5 analysis. Reflecting this, mean strontium concentrations differ among nursery areas at only $p < 0.02$ ($F_{5,110} = 2.82$) for the point 2-5 analysis, versus at $p < 0.001$ ($F_{5,110} = 6.21$) in the analysis based on otolith material deposited during residence in the nursery areas. By comparison, differences among nursery areas for four of the other five elements are significant at a comparable, if slightly higher level for the point 2-5 analysis than for the point 35-39 analysis. The exception is sodium, which differs among nursery areas in both analyses ($P<0.001$ for both), but for which F values increase from 9.0 (point 2-5 analysis) to 13.4 (point 35-39 analysis). Inspection of mean life history traces in Figure 5 suggests the higher F value for sodium in the latter analysis is due mainly to one site (Port Phillip Bay), which begins to diverge from the remaining sites around point 20, ie., well before apparent age at recruitment into the nursery area.

Figure Captions

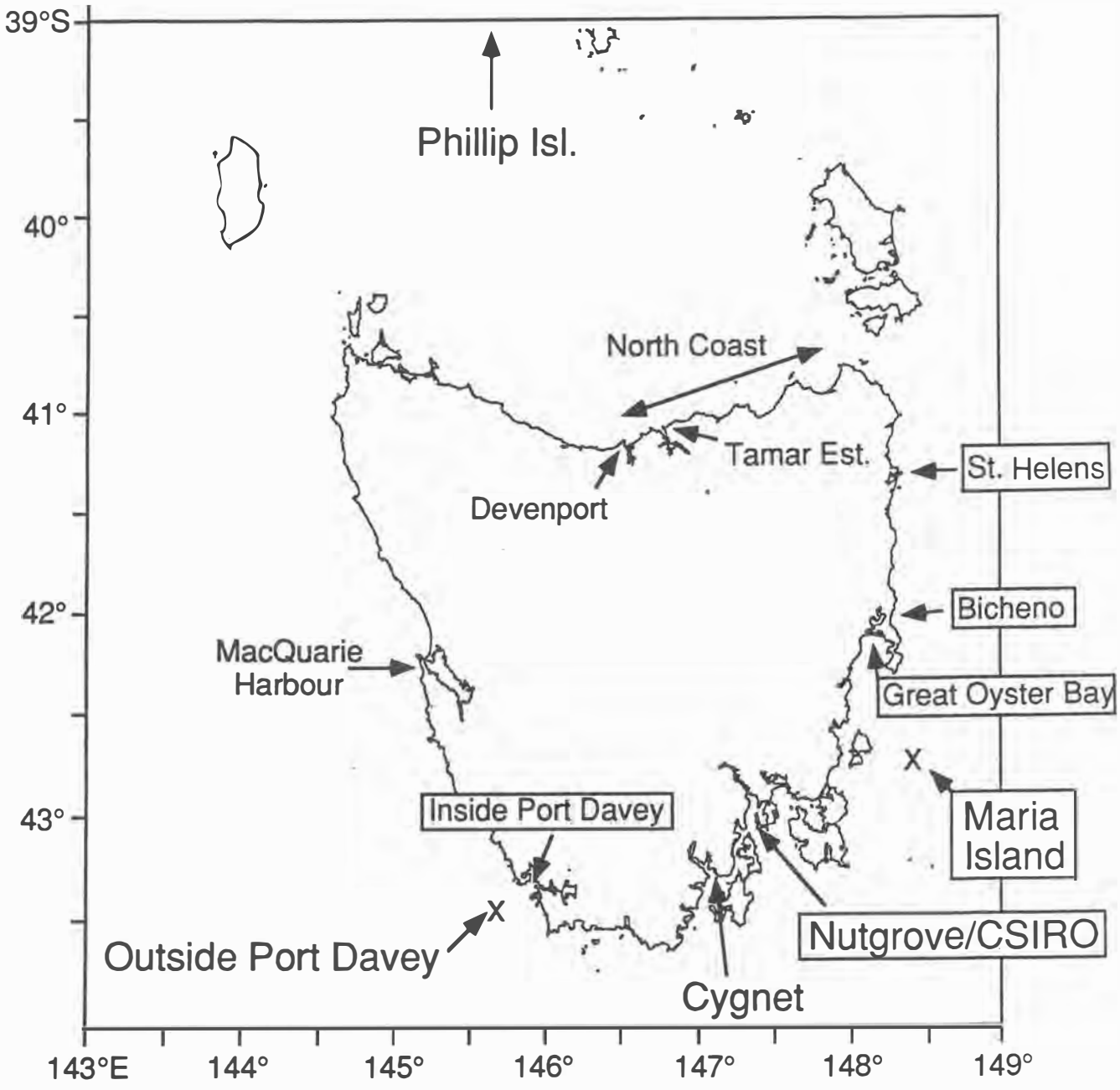
1. Sampling locations for juvenile N. macropterus in Tasmanian coastal waters. Sites indicated with large lettering indicate where juveniles were found and collected.

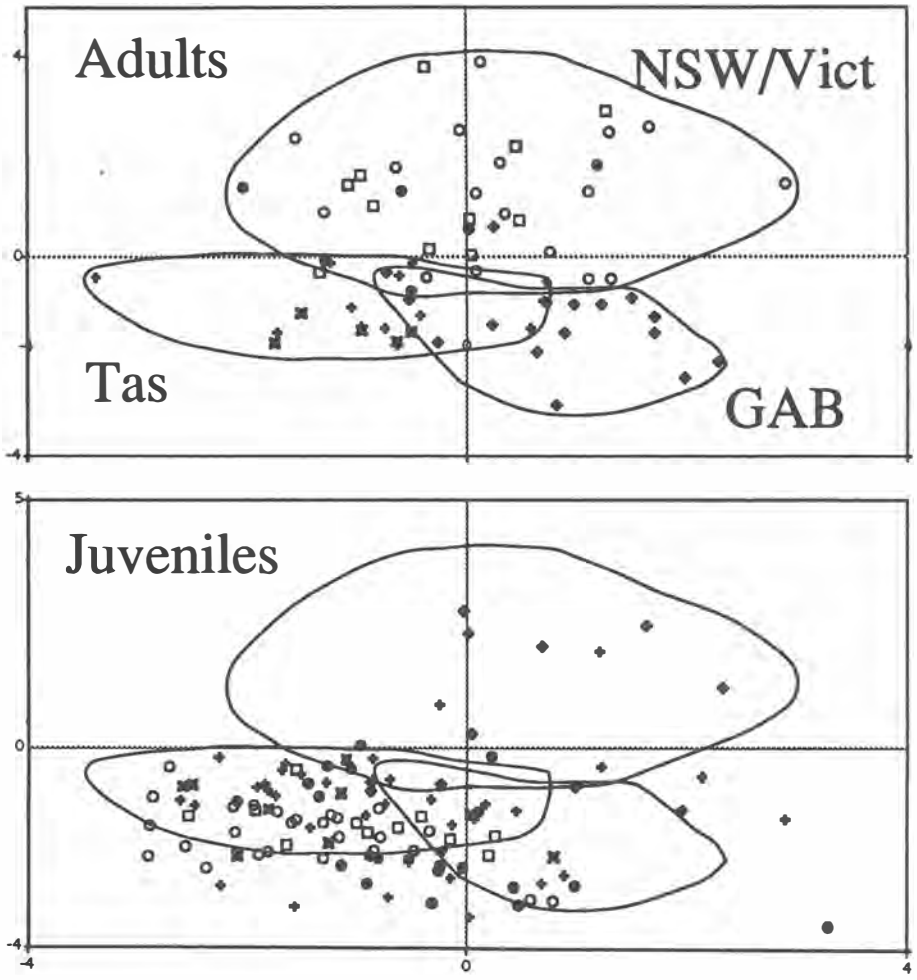
2. The distribution of juveniles in two factor space defined by the adult groupings of N. macropterus. Symbols indicate different sampling locations for the juveniles.

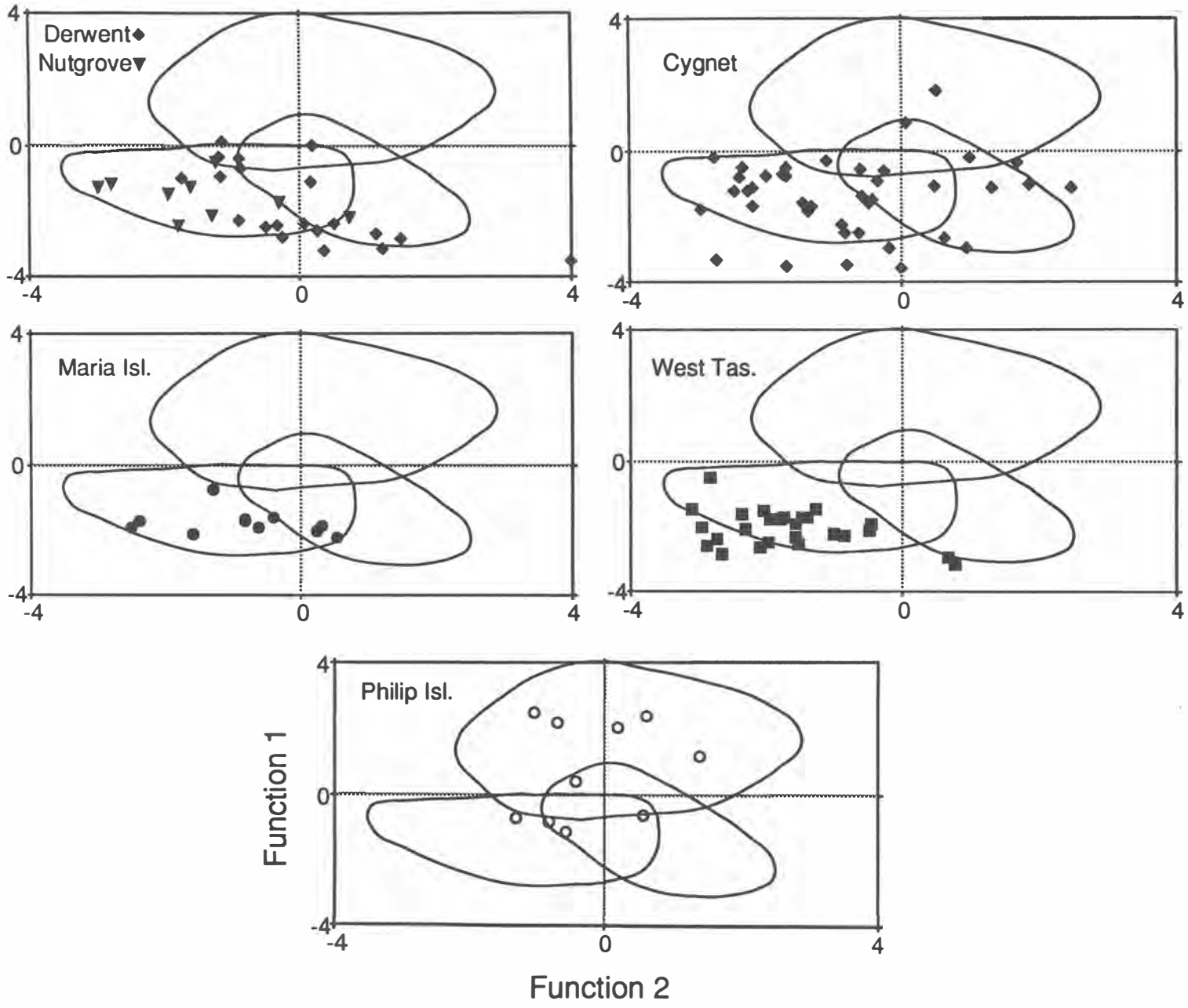
3. Distribution of juveniles from each nursery area in two factor space defined by the adult groupings of N. macropterus.

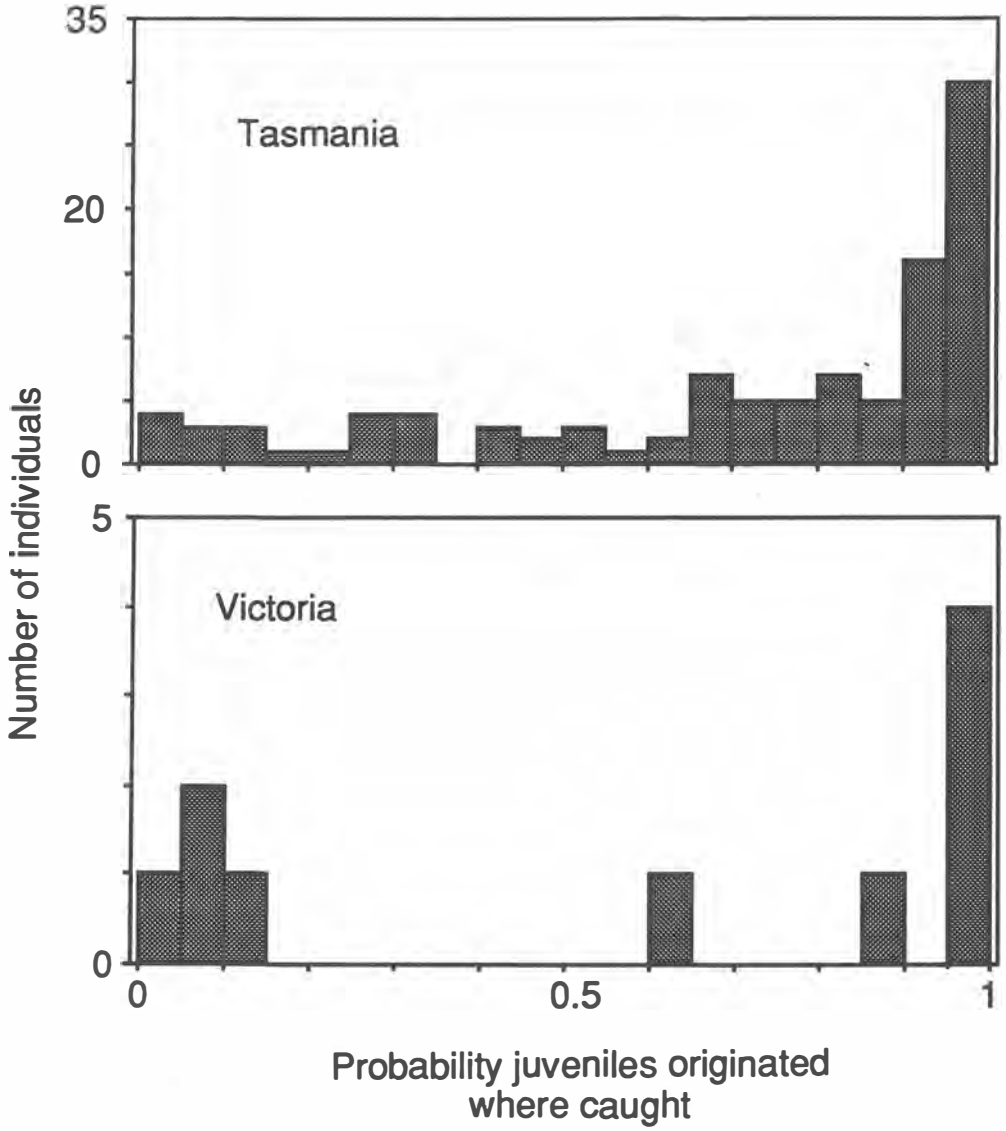
4. Mean life history scans (5 point running means) for each of the six elements for juveniles from each of the nursery areas, showing divergence of Sr traces at approximately the age (= otolith radius) of settlement of post-larvae into benthic nursery areas.

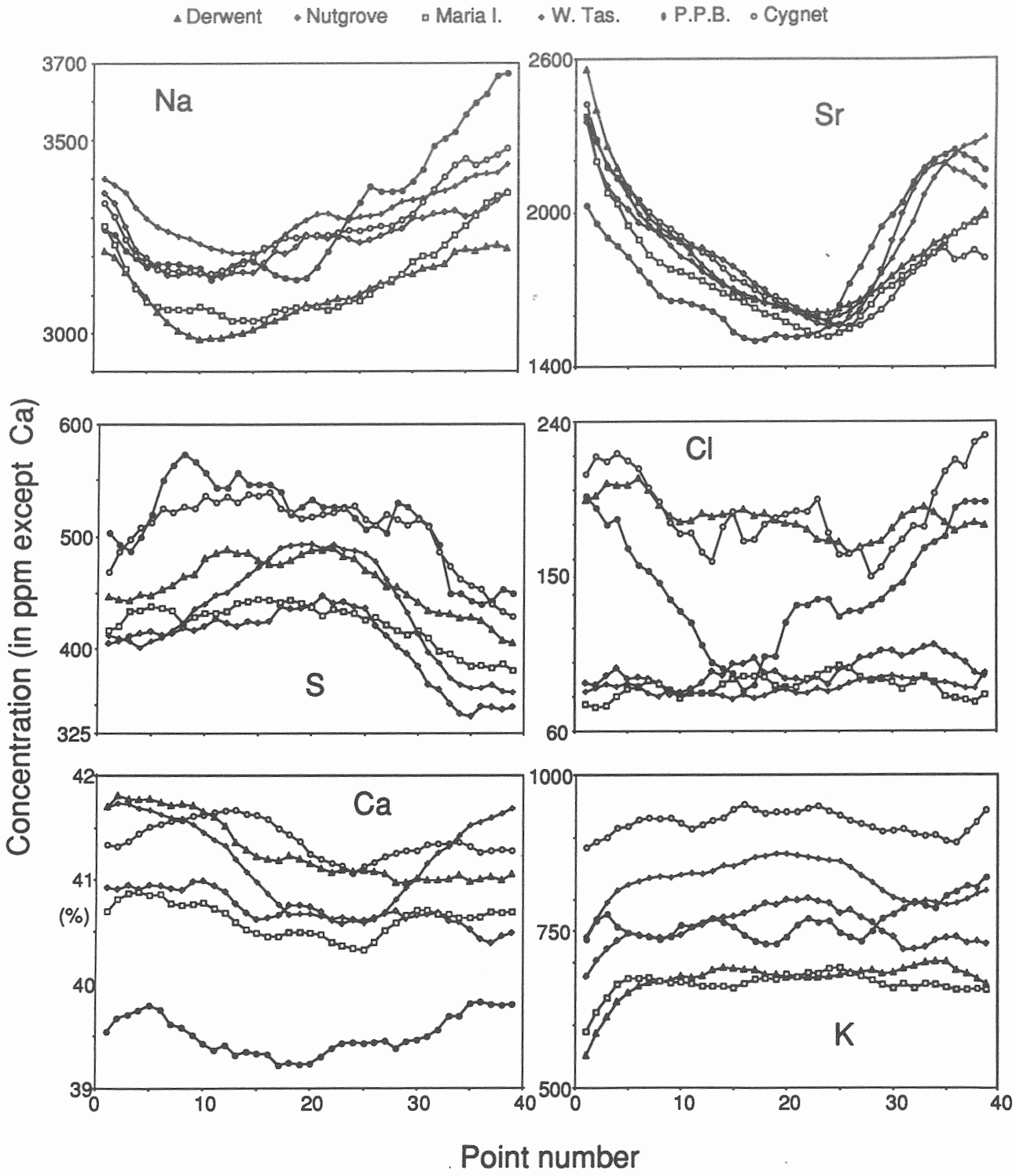
5. Individual life history scans (5 point running means) of individual N. macropterus juveniles collected at Cygnet (SE Tasmania).

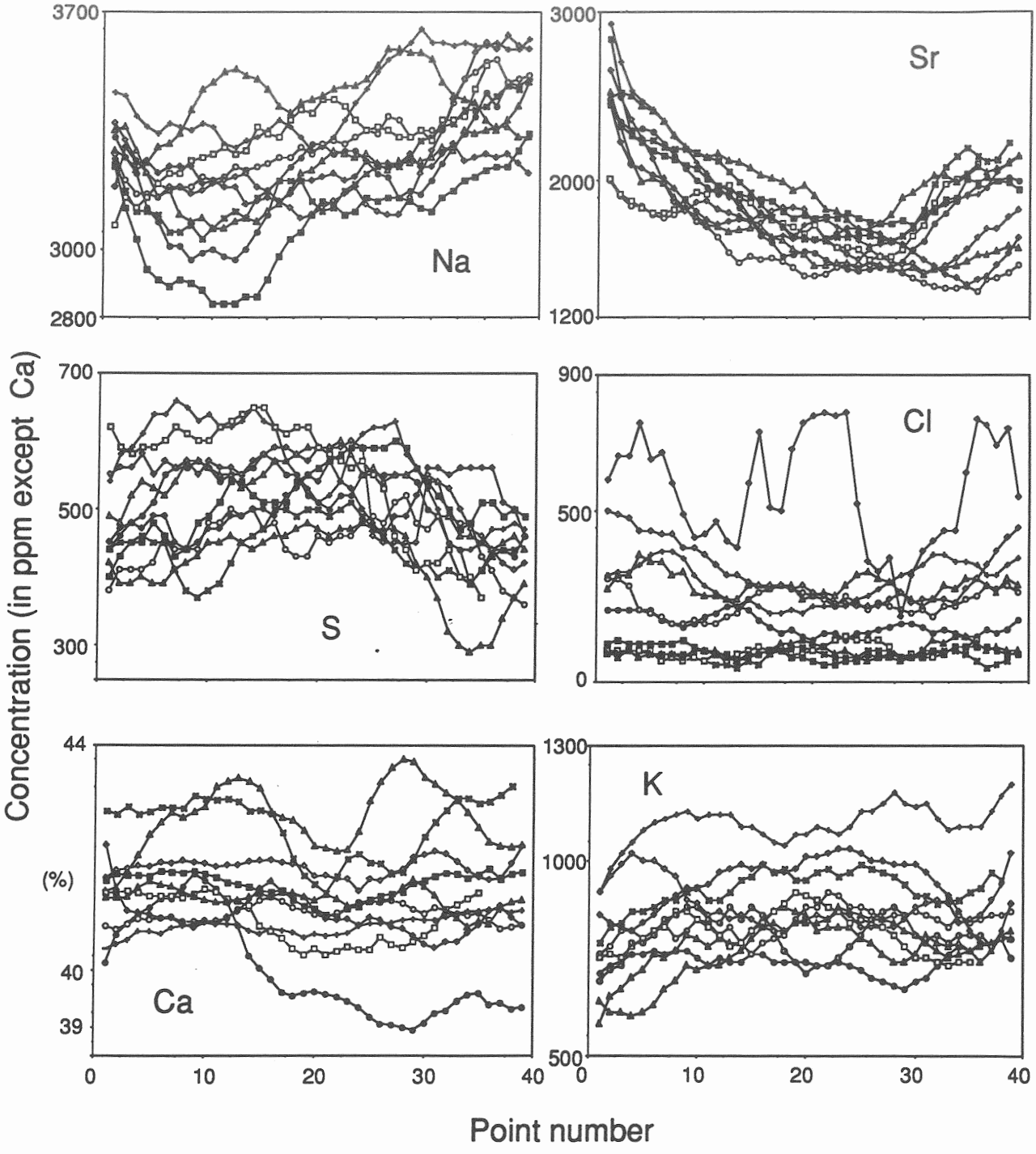

39°S
40°
41°
42°
43°
143°E
144°
145°
146°
147°
148°
149°
Phillip Isl.
North Coast
Tamar Est.
Devenport
St. Helens
Bicheno
Great Oyster Bay
MacQuarie Harbour
Inside Port Davey
Outside Port Davey
Maria Island
Nutgrove/CSIRO
Cygnet


Adults
NSW/Vict
Tas
GAB
Juveniles
Factor 1
Factor 2


Derwent◆
Nutgrove▼
Cygnet
Maria Isl.
West Tas.
Philip Isl.
4
0
-4
Function 1
Function 2


35
20
0
Tasmania
5
0
Victoria
0
0.5
1
Number of individuals
Probability juveniles originated where caught


▲ Derwent ◆ Nutgrove ▫ Maria I. ◆ W. Tas. ◗ P.P.B. ○ Cygnet
Na
Sr
S
Cl
Ca
K
Concentration (in ppm except Ca)
(%)
Point number
3700
3500
3000
2600
2000
1400
600
500
400
325
240
150
60
42
41
40
39
1000
750
500
0
10
20
30
40


Concentration (in ppm except Ca)
3700
3000
2800
Na
3000
2000
1200
Sr
700
500
300
S
900
500
0
Cl
44
(%)
40
39
Ca
1300
1000
500
K
0
10
20
30
40
Point number

Document 4

Stock structure of the southern bluefin tuna, *Thunnus maccoyii:* an evaluation based on probe microanalysis of otolith composition

Information on the stock structure and migration patterns of tunas (Scombridae) is sparse (see Hunter, et al., 1986; Schaefer, 1987). The large size of the animals, the scale of their movements and, for many species, the value of the landed catch, has made difficult resolving even the basics of population structure for many species. Genetic techniques have been applied to a few species of tuna, but have generally proven of limited value, presumably due to mixing of the highly mobile adults among populations. Other approaches, such as tagging of juveniles and adults, analysis of parasite loads, and locating and enumerating discrete spawning areas, all provide useful information, but are limited in scope and in the strength of the inferences that can be drawn from them. As a result, there remains considerable uncertainty about the population structures of virtually all tunas, which has prompted continuing research into alternative, and perhaps more definitive, techniques for evaluating their population structure.

One such possibility is the analysis of the chemical composition of skeletal structures. As early as 1967 (Fisheries Agency of Japan 1967), preliminary studies suggested that the quantitative analysis of the microconstituents, or trace elements, in otoliths, vertebrae and scales could provide information on population structure and the movements of individual fish. This suggestion was based on two assumptions and a hypothesis. The assumptions are 1) that the calcified tissues of fish, with few exceptions, are not susceptible to dissolution or resorption and 2) that growth continues throughout life. If these assumptions are correct, calcified tissues are permanent records of the influence of endogenous and exogenous factors on their calcium-protein matrices. The hypothesis is that genetic differences between populations or differences in the environments to which fish in each population are exposed affect the incorporation of trace elements in calcified tissues, which results in chemical compositions specific to each. An extensive fisheries literature supports the initial assumptions for otoliths, if perhaps not for scales and vertebrae (Sauer, et al., 1984). The working hypothesis also appears reasonable, given an extensive literature on invertebrates that relates differences in the composition of, for example, mollusc shells and coral skeletons to a range of environmental and physiological conditions (Thompson and Livingston, 1970; Weber, 1973; Houck et al., 1977; Buchardt & Fritz, 1978; Smith et al., 1979; Rosenberg, 1980; Schneider and Smith, 1982).

Since 1967, a number of studies have investigated the use of calcified tissue composition as an indicator of stock or sub-population identity (eg., Klokov & Frolenko, 1970; Calaprice, 1971, 1983, 1985; Calaprice et al., 1971,1975; Bagenal et al., 1973; Gauldie & Nathan 1977, Behrens Yamada et al., 1979; Lapi & Mulligan, 1981; Mulligan et al., 1983; Mulligan et al., 1987; Edmunds et al., 1989), using a variety of analytical techniques (see Coutant 1990, Gunn et al., 1992). Results, in

general, have been mixed, due in part to methodological limitations which, until recently, dictated analysis of relatively large amounts of tissue. Specific application of these techniques to scombrids has also had a mixed history. J. Calaprice, in a series of brief 'grey literature' publications (e.g., Calaprice, 1986; summarised by Calaprice, in press) and summaries, found preliminary evidence using proton probe analysis of regional differences in the composition of vertebrae of northern Atlantic bluefin tuna (Thunnus thynnus) and eastern Pacific yellowfin tuna (Thunnus albacares), which prompted Hunter, et al. (1986) to encourage further research into the field. Subsequently, Ianelli (in prep) used energy dispersive electron probe microanalysis to test for regional differences in the composition of otoliths of the Pacific skipjack (Katsuwonus pelamis), and again reported preliminary evidence of regional differences in the concentrations of some elements.

In 1987, we began experiments with a view to using such fine-scale, ontogenetic variations in the composition of fish otoliths as an indicator of movement/migration patterns. Results of the methodological studies that underpin this work are reported in Gunn, et al. (1992) and Sie & Thresher (1992). The current paper deals with application of these techniques to investigating the population structure of the large pelagic scombrid, Thunnus maccoyii (southern bluefin tuna). Two aspects of the migration history of T. maccoyii are uncertain, and can have significant impacts on the management of the fishery for the species.

Southern bluefin tuna are known to spawn in the NE Indian Ocean, south of Indonesia and off the NW Australian coast (Nishikawa, et al., 1985; Davis , et al., 1990). Eggs and larvae are assumed to drift south in the Leeuwin Current, until the fish, as young-of-the-year juveniles, reach the SW corner of Australia and migrate in large schools east along the temperate shelf. The juveniles are thought to spend 4-6 years in the temperate region, slowly migrating across to the SE Australian coast, before turning south as young adults into sub-Antarctic waters (Murphy & Majkowski, 1981; Caton, et al., 1990). Adults migrate to the Indian Ocean spawning area between September and January, though the duration of the spawning period and frequency of spawning by individual adults is not known.

The two aspects of this migration route in principal doubt are 1) that the Indian Ocean spawning area is the sole spawning ground for the species, and 2) the proportion of juveniles that migrate entirely across the temperate Australian southern shelf. With regard to the former, most tunas spawn either over broad areas, e.g., Pacific yellowfin (T. albacares), or have several discrete spawning areas, north Atlantic bluefin (T. thynnus). A single spawning area is sufficiently unusual for the large scombrids that there have been persistent suggestions that other areas exist, but have not been identified due to lack of sampling for eggs and larvae. With regard to the migration route, it has long been suggested that juveniles turn south from the temperate Australian

coast and move into sub-Antarctic waters over a range of ages and at points all along the southern coast of Australia. Some juveniles are clearly remain along the coast the Australian coast until ages of 5-6 years (and remain subject to the Australian shore-based fishery), but fraction these represent of the total juvenile population is not known.

We speculated that analysis of otolith composition could help resolve both unknowns. Specifically, we hypothesised, first, that if all T. maccoyii were derived from the sole known spawning area, the composition of the otolith primordium (which is deposited in the first few days of larval life) would be similar for all individuals and, further, be similar to the composition of otoliths of larvae and small juveniles collected on or near the spawning ground. Individuals with markedly different patterns of primordium composition could constitute positive evidence of additional spawning areas. And second, we hypothesised that alternative migration routes, involving different sets of water mass characteristics, would induce diagnostic changes in otolith composition during ontogeny, such that individuals known to have migrated along the southern Australian coast would be distinguishable from those which migrated at an earlier age into the sub-Antarctic region. This hypothesis was tested by 1) comparing the composition of otoliths of similar aged juveniles caught off Australia and from a location well outside the 'dominant' migration route (off South Africa), and 2) by determining the range of variation in otolith composition of Australian juveniles as comparing with this the variability of adults, for evidence of outlying individuals.

Materials and Methods

Five sets of samples were used to examine geographic variability in the composition of T. maccoyii otoliths: (1) larvae collected on the known NW Australian spawning ground, (2) small (approximately 25 cm FL) juveniles caught by long-line off the west coast of Australia, (3) larger juveniles taken from the commercial catch off southern Australia, (4) large juveniles taken from the commercial catch off South Africa, and (5) adults taken from the commercial catch off SE Australia. Sample locations are depicted in Fig. 1, and details of the samples are given in Table 1. The larval sampling is described by Davis, et al. (1990). The western Australian juveniles were caught by long-lining off the RV Shoyo Maru in March 1990. Eight juveniles were examined, drawn from three schools (as indicated by simultaneous hook-ups). The southern Australian juveniles were collected at points all along the southern Australian coast in 1988 and 1990. The large adults were taken by commercial long-liners working off the SE Australian coast, also during 1988 and 1990.

Larvae were preserved in ethanol; all other specimens were frozen shortly after collection and remained frozen until the otoliths were removed. After extraction, each otolith was cleaned of adhering tissue using fine forceps and a soft bristled brush in millepore-filtered distilled water. Otoliths were then dried in an oven at 40-45°C. for a minimum of 6 hrs, after which they were stored in polyurethane capsules.

Procedures for embedding, sectioning and preparing otoliths for probe microanalysis are detailed in Gunn et al (1992). Only sagittae were used in this study, because of their larger size. Prior to embedding, a scaled diagram of the distal surface of each otolith was made in order to guide subsequent sectioning. The otolith was then fixed upright on its ventral edge to the base of an embedding mould with a drop of quick-drying resin. The mould was then filled with a harder-setting resin. After hardening, the otolith was sectioned using a diamond-edged saw blade (350 μm thick) on a rotary saw. Grinding to the plane of the primordium was done by hand using 2400 grade silicon carbide wet/dry paper. Final polishing was done using progressively finer grades of diamond paste (6 - 0.5 μm) and/or aluminum oxide powders or pastes (eg., Linde B, 0.5 μm) on a lapping machine. After polishing, the section was ultrasonically cleaned and stored in a moisture-free environment. Prior to probe microanalysis, the section was coated with a 250-300 Å coat of carbon, using a sputter coater, and then stored in vacuum until insertion into the probe.

The procedures used to analyse otolith composition are detailed in Gunn, et al (1992) and Sie & Thresher (1992) for electron probe and proton probe (micro-PIXE) microanalysis, respectively. The electron probe microanalyzer used was a Cameca "Microbeam" fitted with three wave-length dispersive detectors. The inevitable specimen damage due to electron probe microanalysis was minimised through use of a defocused beam, a beam power density of 2.4 $\mu W\ \mu m^{-2}$, for a total acquisition time of 3 min, 42 sec per point. The concentrations (weight-fractions) of the elements detected were calculated based on count rates measured for the K_α lines for most elements, and the L_α line for strontium, on standard materials and the ratios of the intensities on standards and otoliths, computed using the "PAP" (Pichou and Pichoir, 1984) matrix conversion software supplied by Cameca. Minimum detection limits and confidence intervals for the concentration estimates are based on equations provided by Ancey et al. (1978). Micro-PIXE analysis was done on the CSIRO proton microprobe (Sie & Ryan, 1986; Ryan, et al. 1990) . The beam used during analysis had a diameter of 15-20 um and a current of 5-7 nA, which was accumulated for a total charge of 3 μC for each point. The X-rays were detected in an EDS (Si(Li)) detector, subtending 50 msr solid angle at the target. A 100 um Al filter was used in all measurements, in order to

attenuate the Ca K lines. Concentrations (weight-fractions) and minimum detection limits were calculated based on Ryan, et al. (1990), and normalised to 40% Ca.

Ontogenetic variation in composition was assessed using EPMA by scanning a series of points along the longest growth axis of each fish (a "life history scan"). The finished section of the sagitta of T. maccoyii exposes an nearly uninterrupted growth axis, through which we ran a mapped series of programmed scan lines that tracked the slight curvature of the growth axis (Fig. 2). Considerable effort was expended to duplicate this axis as closely as possible in each specimen, in order to maximise comparability of the data sets between individuals. The life history scan line for each fish ran from the primordium to the posterior ventral tip of the otolith. Data were filtered to remove high frequency noise and reduce random measurement error by using a 5 point running mean. Reproducability of life history scan data was evaluated by comparisons of left-right otolith pairs from the same fish. For logistical reasons, full life history scans were not done using micro-PIXE. Rather, analysis was based on point estimates made at the otolith center (the primordium) and at the otolith margin. More detailed examination of otoliths using micro-PIXE was not considered justified given the relative dearth of elements detected (see below).

To test the hypothesis that there is more than one spawning area for T. maccoyii, we examined variability in the composition of the otolith primordium. In practice analysis was based the mean of the first five point analyses out from the primordium for all individuals other than larvae (for which only a single point analysis was possible due to the small size of the otolith). As these points were 20 μm apart, the datum for each individual was the mean composition over the innermost 80 μm of material, which corresponds to approximately the first 20 d of larval life (see Jenkins & Davis,1990).

Results

Fifteen elements were detected in T. maccoyii otoliths, six using EPMA, and nine using micro-PIXE (Fig. 3). As in other species we've examined (Thresher & Sie, 1990) the elements in T. maccoyii sagittae constitute three distinct sets, separated in concentration from other, less abundant elements by a difference of one to three orders of magnitude (Fig. 3): calcium, carbon and oxygen (the latter two not routinely measured due to methodological difficulties) are present in concentrations > 10% by weight; sodium, strontium, potassium, sulfur and chlorine occur in mean concentrations of 100 - 5000 ppm; and a variety of 'trace elements' occur at concentrations < 10 ppm. .

Of the macro- and micro-constituents, only Cl was occasionally recorded at concentrations below its minimum detection limit. In contrast, there were relatively few 'trace elements' in T. maccoyii otoliths and their mean abundance was, in all elements, below their respective minimum detection limits.

Typical life history scans, for three fish drawn casually from our data set of adult tuna, are depicted in Fig. 4. All elements measured using EPMA exhibit significant ontogenetic variation in concentration, the relative magnitude of which correlates inversely with mean concentration. Several elements exhibit consistent trends across the otoliths (e.g., Na concentrations invariably decline from the otolith primordium towards the margin, whereas Sr concentrations decline sharply immediately exterior to the primordium but then gradually and irregularly increase towards the margin). However, the exact patterning of the variability around those trend lines and mean concentrations of elements differ considerably among individuals.

Variation in Composition of the Otolith Primordium

The hypothesis that there is more than one spawning are for T. maccoyii was tested by comparing the composition of the primordium and adjacent region between juveniles collected near the single known spawning area (off the west coast of Australia), older juveniles caught off southern Australia (which almost certainly derived from the Australian spawning area), similarly aged juveniles caught off South Africa (of unknown origin) and large adults caught in the high seas fishery (also of unknown origin). The generic hypothesis was tested by means of two specific hypotheses: first, that individuals from the known spawning area would group together in a cluster analysis, along with other individuals derived from that spawning area, and be distinct from one or more additional groupings indicative of derivation from one or more additional spawning areas; and second, that discriminant function analysis would distinguish between individuals from the known spawning area and significant outliers, the grouping of which might also provide evidence of additional spawning areas.

Results of the first analysis are depicted in Fig. 5. Prior to analysis, elemental concentrations were standardised to eliminate effects of scale differences among elements. Clustering is based on a farthest neighbour paradigm using Euclidean distances. Results of the analysis indicated one outlying individual (from southern Australia), which had an unusually high concentration of Cl at the primordium, and three major groupings of individuals. Within these groups, individuals were highly

variable, however, such that there are few obvious clusters of very similar individuals and no obvious, well defined separation points between clusters. Rather the three major groupings constitute an arbitrary break-point, which could as easily be revised to define two, four or more clusters. The numbers of individuals from the three source groups (W. Aust., S. Aust., S. African and adult) in each of the three arbitrary selected 'major clusters" are indicated on Fig. 5. Western Australian juveniles, unambiguously from the known spawning area, are distributed across two of the three groups. The third group differs from the other two more than they differ from each other, lacks altogether juveniles unambiguously derived from the known spawning ground, and hence indicate a previously unidentified spawning area. However, this third group includes a large number of southern Australian juveniles, which, as noted, have a high probability of deriving from the known spawning area.

Alternative clustering routines, e.g, centroid and nearest neighbour paradigms and use of a Pearson coefficient similarity measure, produce results leading to conclusions similar to those above, ie., there are few, if any, major discontinuities in the cluster trees and Western Australian juveniles characteristically are distributed across all major groupings of individuals, however identified.

Discriminant function analysis applied to the four source groups of individuals resulted in a weak, but significant separation of groups (Fig. 6) (Wilk's lambda = 0.46, $F_{18,105}$= 1.83, $p<0.05$), in which 30 of the 46 individuals were accurately assigned to their source group. Separation was based on one significant discriminant factor, onto which two elements loaded significantly (canonical coeficients = 0.81 for K , and 0.53 for Cl). The two additional, much weaker discriminant factors were loaded onto primarily by Sr, K and Ca, and S, respectively. ANOVA and post-hoc analysis (Fisher's PLSD) of the factor 1 values indicated significant differences between all pair-wise combinations of groups except for the western Australian juveniles and the adults.

Two observations suggest that these differences are not related to spawning area, however. First, the overall distribution of both factors 1 and 2 are normal (Lilliefor's probability = 0.1 for factor 1, and 0.60 for factor 2), ie., there is no indication of a bimodality which might reflect signatures of two discrete spawning areas. Second, it is highly probable that most, if not all of the southern Australian juveniles derive from the same spawning area as the western Australian juveniles, despite a significant difference between the groups in distributions of factor 1 values. That these two groups nonetheless differ implies an alternative explanation for the observed discrimination between sample groups.

One possibility is temporal variation. The western Australian and southern Australian juveniles were collected in different years, consist of very different age groups and hence derive from different year-classes. For these reasons, they also differ significantly in mean size. The possibility that discrimination between sample groups relates to temporal variation in the elemental composition of the otoliths was tested by plotting factor 1 and factor 2 values for each individual against fork length and against apparent year-class. The latter was determined based on date of capture and age estimated from the number of apparently annual peaks and troughs in strontium concentrations along the life history scan (see Proctor, et al., in prep.). Results of the analyses are given in Figs. 7 and 8. There is no indication that factor 2 varies significantly as a function of either year-class or individual size; however, factor 1 varies as functions of both (which, because of the few years over which the samples were collected, are highly correlated). Multiple regression analysis (after excluding the Western Australian juveniles in order to linearize the relationship) indicates only one significant correlate, ie., after size effects are removed, apparent year-class does not account significantly for any residual variability, and vice versa. The high correlation between apparent year-class and fork length, due to the few years of sampling, does not provide enough power to resolve the relative importance of the two variables. To the extent that both indicate time, however, they suggest that values of factor 1 increased from 1970's into the 1980's and then declined abruptly between 1987 and 1990. Sample sizes are to small to justify an ANCOVA on the south African, southern Australian and adult material, but there is no conspicuous difference between these groups in factor 1 values after effects of apparent year-class/fork length are taken into account.

Variations in Life History Scans

To test the hypothesis that most T. maccoyii migrate along the southern Australian shelf before entering the high seas population as adults, we undertook two comparisons of ontogenetic variability in otolith composition, comparing juveniles collected in the AFZ with 1) juveniles caught off South Africa and 2) adults. The first comparison assesses whether alternative migration routes results in different patterns of otolith composition; the latter assesses whether the range of variability exhibited in the adult population is fully encompassed by the variability of juveniles found in the known migration route.

Results of the first comparison are depicted in Figs. 7 a-d and summarised in Table 2. Ca and K are not depicted, as the former tends to vary only slightly and the latter typically varies in parallel with Na. The plots emphasise two points: that

individual variability in most elements is extreme, and that overall, ontogenetic variability in elemental concentration is similar for both sets of juveniles. Statistical comparisons of the means and variances of elemental concentrations for each set of juveniles, made at arbitrarily fixed intervals along the life history scans, indicate few consistent significant differences between South African and Australian juveniles for any element (Table 2). Those differences that do occur tend to be small and predominate at the primordium. Differences associated with the primordium are discussed further above. The only other consistent pattern of differences among the two sets of juveniles is in the variability of chlorine concentrations, which is consistently higher for the Australian juveniles. Mean chlorine values are also higher for the Australian-caught juveniles, albeit not significantly so. Comparison of Cl concentrations at the primordium for not only the two sets of juveniles, but also adults, smaller juveniles caught off western Australia and larvae caught in the known spawning area (Fig. 8) suggests that the difference between the two, older juvenile groups rest primarily on the Australian sample, which shows a higher level of variability than any of the other groups of individuals.

To test robustly for evidence of divergence in otolith composition with age in the two groups, discriminant function analyses were conducted using elemental data at 25 point intervals along the life history scans of both sets of juveniles, and at the otolith margin. Results are depicted in Fig. 9. Two points are evident. First, despite occasional differences between the two groups in mean concentrations of individual elements, at no point along the otoliths was it possible to generate a significant discrimination between the South African and Australian juveniles. The best discrimination occurred at point #1 (the primordium and adjacent region), but even there Wilk's lambda was only 0.69 and the significant level only 0.32. The second conclusion from the analysis is that there is no indication of increasingly marked differences between the two sets of juveniles with increasing distance along the otolith axis. Even at the margin of the otolith, where deposition unambiguously involves the two sets of juveniles moving along different migration routes, discrimination between the groups is no better than early in juvenile development.

The second test of the presence of alternative migration routes was a comparison of ontogenetic variability between juveniles collected along the Australian migration route and high seas adults. These comparisons are given in Fig. 10 a-f. Overall the pattern of ontogenetic variability for the adults is similar to that of the Australian juveniles and is of a comparable magnitude. With the exception of one adult with consistently high Cl values, there is no indication that any of the adults exhibit

variations in elemental concentrations that would not be consistent with those of the Australian juveniles.

Comparisons of Trace Element Concentrations

Micro-PIXE analysis was carried out on a sub-set of the South African and southern Australian juveniles, to assess whether trace element concentrations would distinguish between the two groups at either the primordium or at the otolith margin. The same ten trace elements were detected at the primordium and at the otolith margin, but individual variability was high, none were ever all detected in the same otolith, and most estimated concentrations were below detection limits (Table 1). None of the ten elements differed significantly between the South African and southern Australian juveniles, at either the primordium or margin (largest difference was for Cu at the outermost point, which differed at $p = 0.26$). To test whether a multi-variate statistic might distinguish between the two groups of juveniles, the elemental data were examined using cluster analysis (Fig. 11). Analysis at the primordium identifies two groups (plus an outlier with very high Mn levels), but as in the parallel analysis above for the micro-constituents Australian juveniles are present in both clusters. Similarly, analysis of the outermost point on the otolith also revealed two clusters of juveniles, but again both clusters involved both southern Australian and South African individuals.

Discussion

T. maccoyii, like other species investigated to date (e.g., Kalish, 1989; Thresher, et al., in prep.), exhibits high levels of individual and ontogenetic variability in the composition of its otoliths. At the levels of sensitivity we employed (to about 4 ppm, depending upon the element), this variability is manifest primarily in the micro-constituents, present on average in concentrations of approximately 100 to 5000 ppm. Again like those of other species we've examined, T. maccoyii otoliths contain very few trace elements detectable using micro-PIXE. Even by the standards of other fish species, however, southern bluefin tuna otoliths are remarkably 'clean', to the extent that the mean concentrations of all trace elements detected were below minimum detection limits. In that regard, it is perhaps not surprising that trace elements, measured using micro-PIXE, contributed little to resolution of stock structure and migration routes in T. maccoyii. Examination of T. maccoyii held in sea-cages suggests that concentrations of

Cu, at least, in otoliths is environmentally sensitive (Proctor, et al., in prep), but for it, and perhaps other trace elements, to be useful in stock structure analysis in T. maccoyii will require application of more sensitive analytical methods, e.g., laser probe analysis. J. Calaprice (pers. comm.) drew similar conclusions in his examination of the otoliths of T. thynnus, and for that reason shifted the emphasis of his studies to vertebral composition.

Among the micro-constituents, the magnitude of individual and ontogenetic variability in T. maccoyii otoliths appears comparable to those of other tunas (Radtke & Morales-Nin, 1989; Ianelli, in prep) and other fish species (Kalish, 1989; Thresher, et al., in prep). Three observations suggest this variability would be useful in resolving stock structure in the species. 1) Regional differences in micro-constituent concentrations, apparently indicative of spawning populations, have been noted in other fish species, e.g., Thresher, et al., in prep.; Ianelli, in prep.). 2) Many of the elements involved in Calaprice's (1986) discrimination among apparent northern bluefin tuna populations, albeit in vertebrae, occur at micro-constituent concentrations in T. maccoyii otoliths. And 3) variation in the concentrations of at least some micro-constituents in otoliths appear to be environmentally linked, e.g., Radtke (1989); Kalish (1989).

Yet, despite this, we found little evidence of either stock structuring or geographic variability in the composition of T. maccoyii otoliths. With regard to a potential discriminator among spawning areas, all southern bluefin tuna we examined had similar patterns of primordium composition, the variability observed was unimodally and, in most cases, normally distributed, and apparent differences among source groups, e.g., South Africa versus southern Australian juveniles, were small, even where significant, and appear related less to regional differentiation than variation between year-classes. With regard to differences in ontogenetic variability that reflect alternative migration routes, we found no adults whose range of variation markedly exceeded the ranges established by examination of juveniles collected along the southern Australian migration route, juveniles collected outside this migration route (e.g., off South Africa) showed no evidence of consistent divergence from the Australian juveniles in the concentrations of any element or suite of elements, and, perhaps more importantly, direct comparison of the most recently deposited otolith material, at the margin, indicated no significant differences between South African and southern Australian juveniles, despite having been caught in two widely separated and oceanographically different regions.

There are fundamentally three reasons why individual variation in otolith composition did not assist in resolving population structure. First, the variation might

be too small relative to measurement error to produce a biologically useful signal. This does not appear to be the case with most elements, in which individual and ontogenetic variation exceeds considerably both empirically measured and theoretically determined measurement error (see as well Gunn, et al., 1992; Thresher, et al., in prep). Another source of measurement error, high levels of specimen contamination, are more difficult to assess and could obscure otherwise significant differences among individuals. Contamination problems have been linked in the past with measurement of Cl (J. Calaprice, pers. comm.) and trace metals (Sie & Thresher, 1992), but for both of these, at least, we think we have reduced the problems by controlling contaminant sources. Nonetheless, irregularly occurring contamination of samples cannot easily be discounted (see discussion in Thresher, et al., in prep).

A third source of measurement error clearly does have an impact on the current analysis, though adjusting for it does not appear to alter fundamentally the conclusions drawn. The linear discriminant analysis based on primordium composition easily distinguishes between very small juvenile T. maccoyii, caught just south of the known spawning area, larger juveniles, caught off South Africa and Australia, and large adults. As discussed above, this discrimination appears related less to regional differences or multiple spawning areas as it does to either body size or, more likely, variation among year-classes in composition of the primordium. The narrow period of sampling, over 3 years (1988-1990), provides inadequate power to resolve whether body size or year-class is the principal correlate of differences among individuals in factor 1 scores. Retrospective modification of composition of the primordium as a function of age or body size, however, is unlikely; as opposed to scales and vertebrae (e.g., Sauer and Watabe, 1984), there is no indication or obvious physiological mechanism for routine modification of otolith structure and composition once material has been deposited. In contrast, the hypothesis that otolith composition is influenced by environmental conditions is entirely consistent with temporally correlated variation in composition. What factors affect otolith composition remains very unclear (Kalish, 1989), but one clear implication is that in T. maccoyii, at least, evaluation of stock structure needs to either be adjusted for year-class effects or confined to comparisons within year-classes. Our sample sizes are too small to factor in year-class variation robustly, but initial indications are that after effects of year-class are removed differences among the four groups of tuna sampled disappear or are very slight.

The second reason why variation in otolith chemistry might provide little information relevant to stock structure is that the structure may lack variation, ie., there may well be only one spawning area for T. maccoyii. The unimodal and normal distribution of discriminant factor values suggests this is a plausible explanation, given

that in at least some other species the distributions of micro-constituent concentrations and factor values for the primordium are conspicuously bi-modal (e.g., Thresher, et al., in prep.). Such a mono-morphic population structure, however, can not account for the overall concordance of composition later in juvenile life, by which stage individuals have clearly diverged into alternative migration routes. Although the proportion of individuals remaining along the southern Australian shelf may be uncertain, the presence of some juveniles off South Africa, for example, testifies to the migration of at least some individuals as juveniles west from Australian waters as opposed to east along the coast.

Hence, the third reason why otolith composition could reflect poorly stock structure is that the concentrations of the elements involved are not environmentally labile, or that the environment itself, the pelagic ocean, is not sufficiently heterogenous to induce detectable variations in otolith composition. The inability of micro-constituent analysis to discriminate between South African and southern Australian juveniles at the otolith margins argues strongly for these hypotheses. Nonetheless, it is unlikely that this poor discrimination results from a lack of environmental sensitivity of the pattern of elemental deposition. As noted earlier, our experimental data for T. maccoyii suggest that the concentrations of at least some elements, e.g., Cu and S, are environmentally sensitive; work on other species, although still subject to dispute over interpretation (see discussions in Thresher, et al., in prep), are also consistent with a degree of environmental sensitivity for, for example, Sr concentrations; and the apparent correlation between year-class and Factor 1 values at the primordium, described above, is also consistent with a discernible effect of the environmental on otolith composition. For these reasons, we hypothesise that the inability of micro-constituent analysis to discern between South African and Australian juveniles or, between these juveniles and the adults, lies less in the insensitivity of these micro-constituents to environmental conditions as it does to the relative homogeneity of oceanic conditions. Sie & Thresher (1992) note that the variability in trace element concentrations in the fish species they examined (which included T. maccoyii) generally declined along an inshore-offshore axis. The number of species examined was small, but the conclusion appeared to be consistent with expectations given the heterogenous nature of coastal environments relative to the homogeneity of the pelagic ocean. We suspect that in the case of southern bluefin tuna, the movement of individuals within and along the edges of the circum-global Southern Ocean results in relatively similar environmental conditions accessible by tuna off both South Africa and southern Australia.

In a broader context and despite Calaprice's (1986) conclusions for northern bluefin tuna, we suspect that analysis of otolith composition may not be a fruitful means of stock delineation in pelagic fish species, to the extent that this composition is environmentally dependent. The relative homogeneity of the pelagic ocean suggests that the environmental signals available for detection are slight and may well be difficult to resolve from variation in micro-constituent concentrations. The key question, however, is the extent to which regional differences derive from a genetic, as opposed to a purely environmental basis. The determinants of skeletal composition in fishes are far from clear, and are likely to differ widely depending upon the element and the environment involved. Preliminary examination of our data for sea-caged T. maccoyii suggests strongly that a simple or direct relationship between environmental variability and the concentrations of any any elements detectable using electron or proton probe microanalysis is unlikely. Rather, a range of physiological and genetic factors are likely to play as large, or a larger role than the environment in determining the concentration of most elements. If so, then regional differences in otolith composition could well reflect population sub-structuring even in the face of little or no regional environmental diversity. Calaprice (1986) presents preliminary information indicative of an ontogenetic change in vertebral composition related to migration history, very much along the line of that for which we sought in otoliths in this study. If this is indeed the case, then an environmental effect in northern bluefin tuna is likely. Such an ontogenetic effect may well be the most effective way of discriminating between genetic and environmental effects on regional differences in skeletal composition.

Acknowledgments

We thank N. Manning, P. Rummel, C. Ryan, and G. Suter for their assistance in probe microanalysis and C. Bobbi and D. Mills for their assistance in the preparation of material for analysis and for discussing with us the results of the work. We particularly thank T. Davis, M. Excel, W. Hearn, B. Jeffries, H. Kono, H. Nakano, A. Penny, the Australian Fisheries Service Observer program, the Funakawa and Uwajima Fisheries High Schools, the Japanese Far Seas Fisheries Research Laboratory,, the South African Sea Fisheries Research Institute, the Tuna Boat Operators Association of Australia, the Federation of Japan Tuna Fisheries Co-operative Associations, and the National Federation of Fisheries Co-operative Associations for their invaluable assistance in obtaining the specimens on which this study is based. This study was funded in part by grants 1987/15 and 1989/30 from the Australian Fishing Industry Research and Development Committee and in part by a Prime Minister's Environmental Research Grant.

Table 1. Capture details of T. maccoyii used in study

Code	Location		Date Caught	Number of fish	Size range (F.L. cm)
Larvae					
L:	Lat.16°43'S	Long.115°8'E	16 Jan 87	4	<1
	Lat.16°30'S	Long.115°51'E	28 Jan 87	6	<1
Immature (<140cm FL.)					
WA:	Lat.24°45'S	Long.112°37'E	9 Dec 90	3	all 25
	Lat.29°49'S	Long.114°27'E	13 Dec 90	3	27 - 28
	Lat.33°11'S	Long.114°48'E	16 Dec 90	2	44, 44
O:	Lat.34°10'S	Long.121°55'E	11 Dec 83	1	56
	Lat.33°0'S	Long.131°0'E	10 Mar 88	4	56 - 61
	Lat.34°49'S	Long.134°42'E	7-8 Apr 88	3	75 - 85
	Lat.43°3'S	Long.148°4'E	12 Jun 88	2	125, 134
	Lat.34°50'S	Long.134°40'E	18 Mar 90	2	98, 98
	Lat.38°0'S	Long.151°0'E	4 - 8 Jul 90	4	134 - 138
	Lat.37°58'S	Long.151°15'E	5 Jul 90	1	131
S:	Lat.42°0'S	Long.23°0'E	20-25 Jul 90	2	111, 124
	Lat.44°0'S	Long.25°0'E	20-25 Jul 90	4	122 - 131
	Lat.44°0'S	Long.3°0'E	20-25 Jul 90	2	126, 139
Mature (>140cm FL.)					
A:	Lat.43°3'S	Long.148°4'E	12 Jun 88	1	179
	Lat.44°48'S	Long.145°35'E	28 Nov 88	2	175, 180
	Lat.45°8'S	Long.145°17'E	4 Dec 88	1	165
	Lat.44°0'S	Long.147°10'E	13 - 14 Apr 89	2	177, 178
	Lat.43°0'S	Long.148°0'E	14 - 16 Jul 89	2	178, 178
	Lat.15°0'S	Long.114°0'E	15 Oct 89	2	176, 184
	Lat.43°10'S	Long.148°10'E	5 - 6 Apr 90	2	175, 177
	Lat.37°58'S	Long.151°15'E	10 - 12 Jul 90	2	175, 178

Table 2. Comparison between mean and standard deviations (mean/sd) of estimated concentrations of six elements at points along the life history scans of juvenile T. maccoyii caught off Australia (A)(n=16) and South Africa(S)(n=8). Vertical pairs of numbers in bold face differ significantly ($p<0.05$), based on an unpaired t-test (means) or F-test (SD).

point	source	Na	Sr	K	Ca	S	Cl
1	A	**3480**/239	**1970/244**	506/100	40/1.1	**436**/61	592/**538**
	S	**3180**/377	**1430/605**	468/66	41/1.0	**326**/59	266/**124**
25	A	3290/384	1410/110	471/60	41/0.9	**350**/83	490/**710**
	S	3110/371	1350/162	498/49	41/0.9	**272**/55	228/**55**
50	A	**3360**/363	**1340**/968	527/66	40/0.4	296/**84**	445/**469**
	S	**2900**/379	**1530**/1570	508/59	41/0.9	295/**37**	221/**60**
75	A	3110/307	1670/274	531/87	40/**0.4**	324/89	381/326
	S	2980/365	1650/157	519/90	41/**1.0**	334/90	300/169
100	A	3050/379	1760/191	497/80	41/0.6	380/112	338/**254**
	S	2970/421	1620/241	507/84	41/1.1	391/107	261/**126**
125	A	3150/443	1510/174	509/107	41/1.0	334/130	329/**211**
	S	2850/304	1510/189	477/76	41/0.9	352/112	243/**99**
150	A	3110/498	1600/243	456/62	41/0.9	383/121	259/**134**
	S	2830/249	1820/215	467/57	41/0.9	383/107	200/**59**
175	A	3010/**552**	1820/141	457/73	41/0.8	378/134	277/**157**
	S	2670/**185**	1820/167	429/77	41/1.1	404/106	189/**73**

Figure Captions

1. Sampling locations for T. maccoyii used in this study

2. Schematic diagram of a T. maccoyii sagitta, showing the plane of analysis and the programmed scan lines required to obtain a life history scan.

3. The distribution of mean (solid circles) and range (vertical lines) of concentrations of elements detected in T. maccoyii sagittae, relative to the minimum detection limits for each element (irregular horizontal line).

4. Typical life history scans, filtered using a 5 point running mean, for three adult T. maccoyii drawn casually from the data set.

5. Clustering tree generated by cluster analysis of micro-constituent concentrations in the region of the primordium of small western Australian juveniles (W), southern Australian juveniles (O), South African juveniles (S) and adult (A) T. maccoyii. Clutering is based on a complete linkage (farthest neighbour) algorithm and calculation of Euclidean Distances based on standardized concentrations of each micro-constituent in each otolith.

6. Distribution of western Australian, southern Australian and South African juveniles and adult T. maccoyii individuals in discriminant factor space based on linear discriminant function analysis of micro-constituent concentrations in and near the otolith primordia.

7. The relationships between discriminant factor values for individuals and their size (caudal fork length) and apparent year-class.

8. a-d. Life history traces for four elements of southern Australian and South African juvenile T. maccoyii.

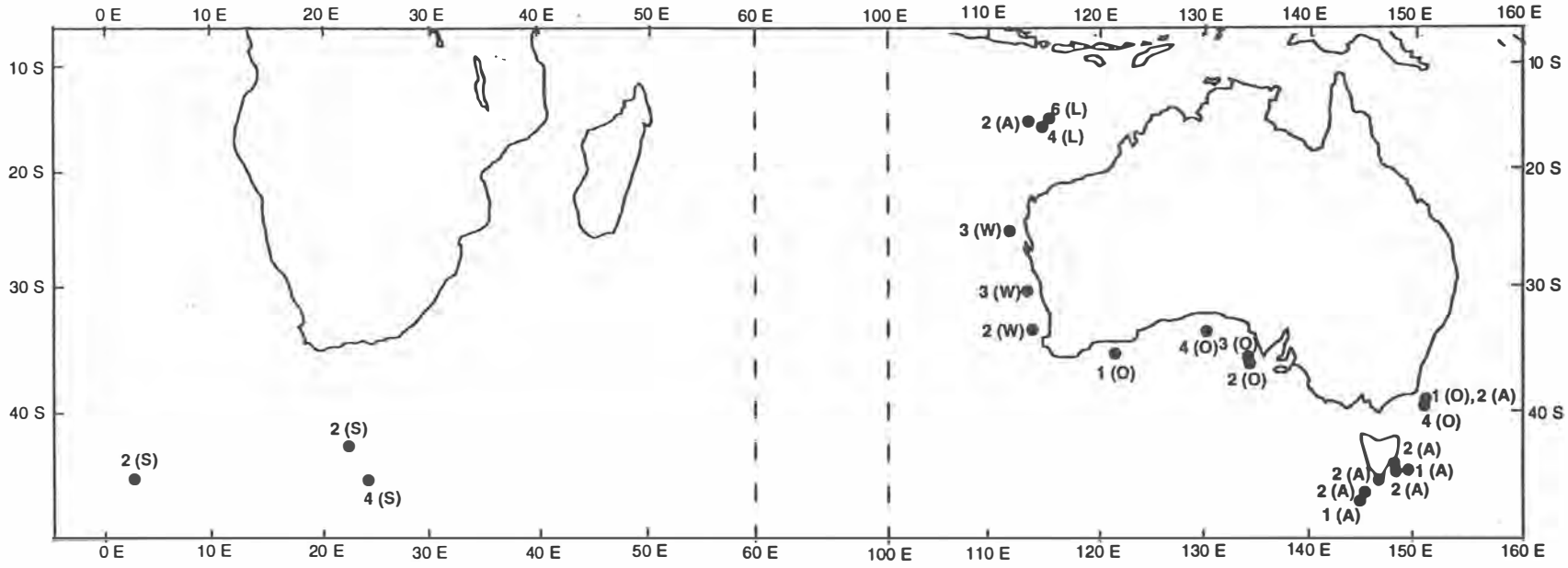
9. The distribution of Cl concentrations in the primordium of sagittae of T. maccoyii larvae from the known spawning ground, juveniles collected off western Australia, southern Australia and South Africa, and adults.

10. The relationship between the effectiveness of linear discriminant analysis to separate South African and southern Australian juvenile T. maccoyii as a function of

probe positions along the life history scan of each otolith, including an analysis based on the margin of the otolith. Wilk's lambda declines as discrimination between samples increases.

11. Micro-constituent life history traces (symbols) for adult T. maccoyii plotted against the 95% confidence intervals (irregular solid lines) of the mean life history trace for T. maccoyii juveniles caught off southern Australia.

12. Cluster tree of South African and southern Australian juvenile T. maccoyii generated by complete linkage analysis of standardized trace element concentrations at the primordium and otolith margin.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30 E
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50 E
60 E
100 E
110 E
120 E
130 E
140 E
150 E
160 E
10 S
20 S
30 S
40 S
2 (S)
2 (S)
4 (S)
2 (A)
5 (L)
4 (L)
3 (W)
3 (W)
2 (W)
1 (O)
4 (O)
3 (O)
2 (O)
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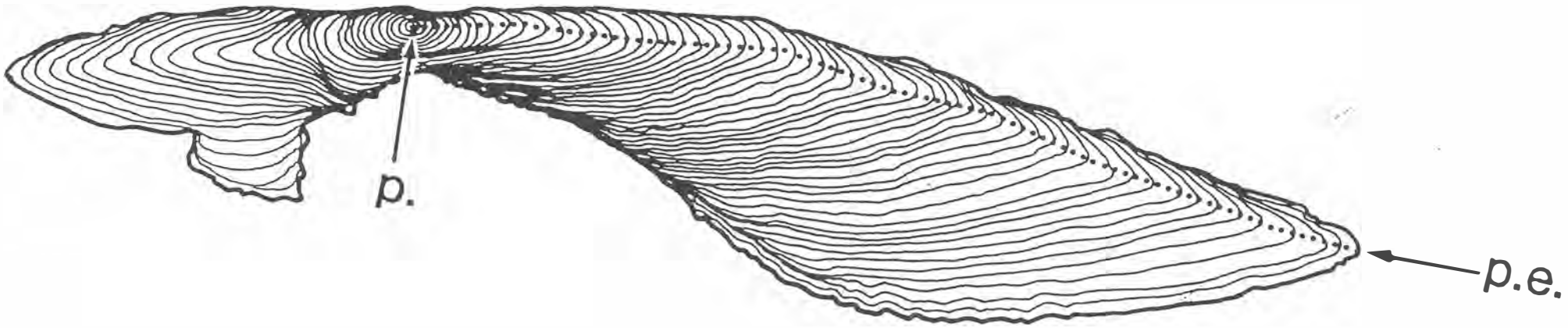
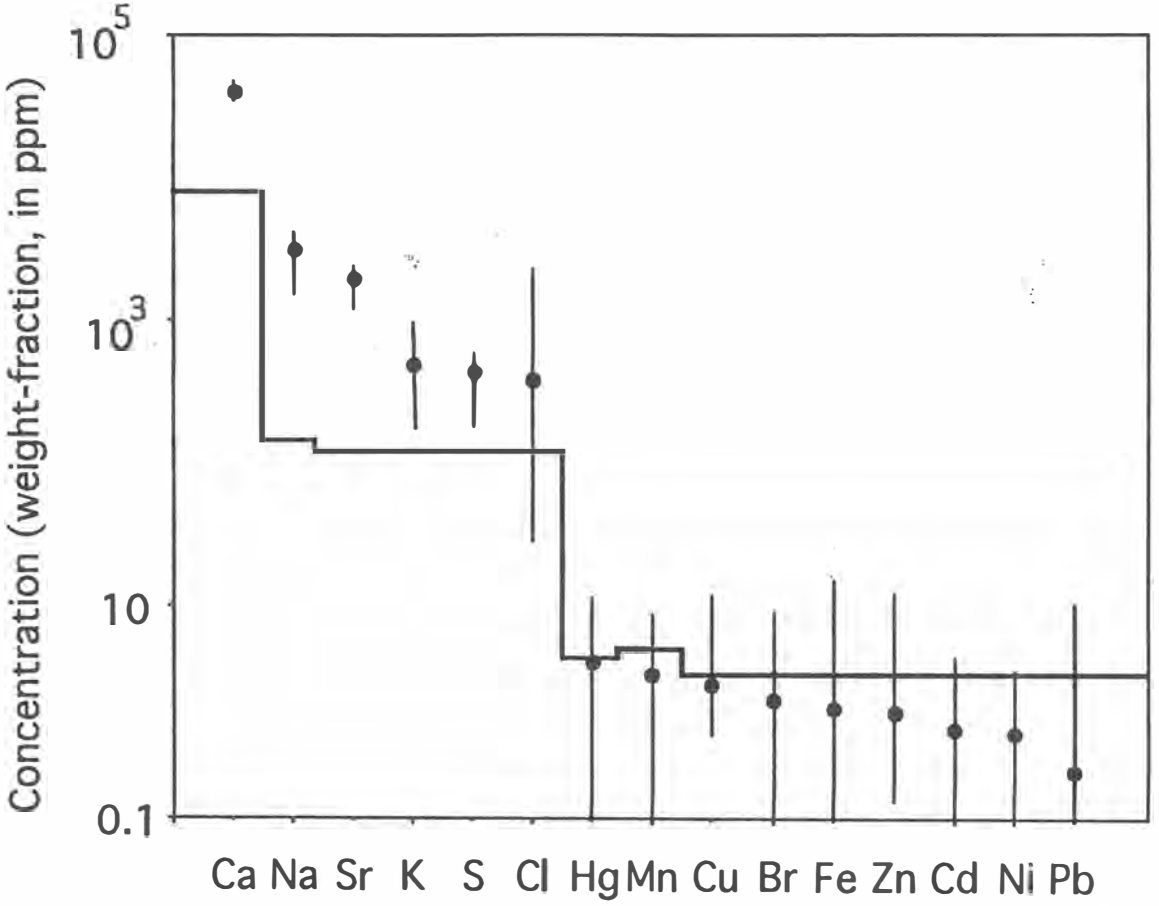
2 (A)
1 (A)
2 (A)
2 (A)
1 (A)


p.
p.e.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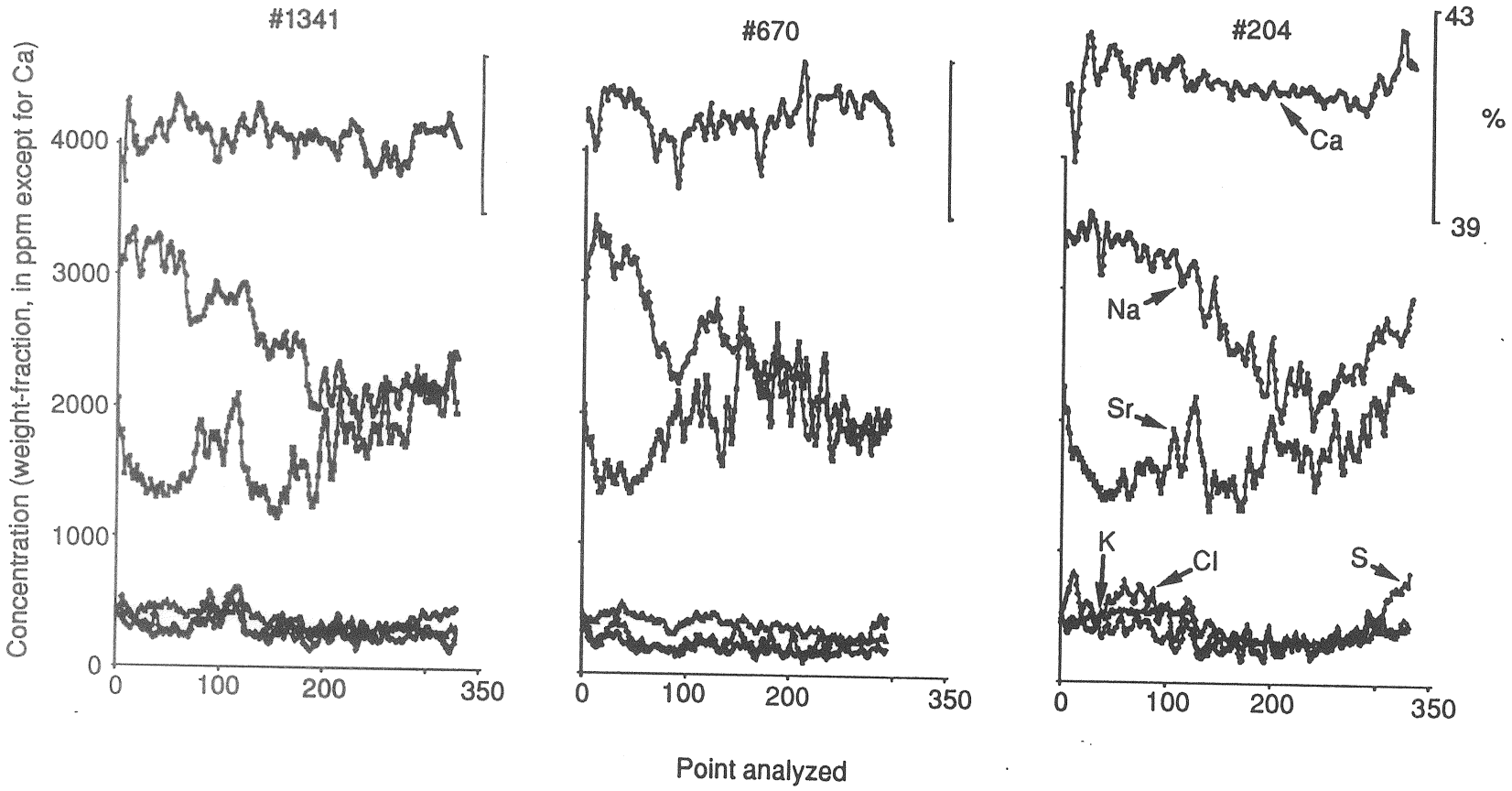
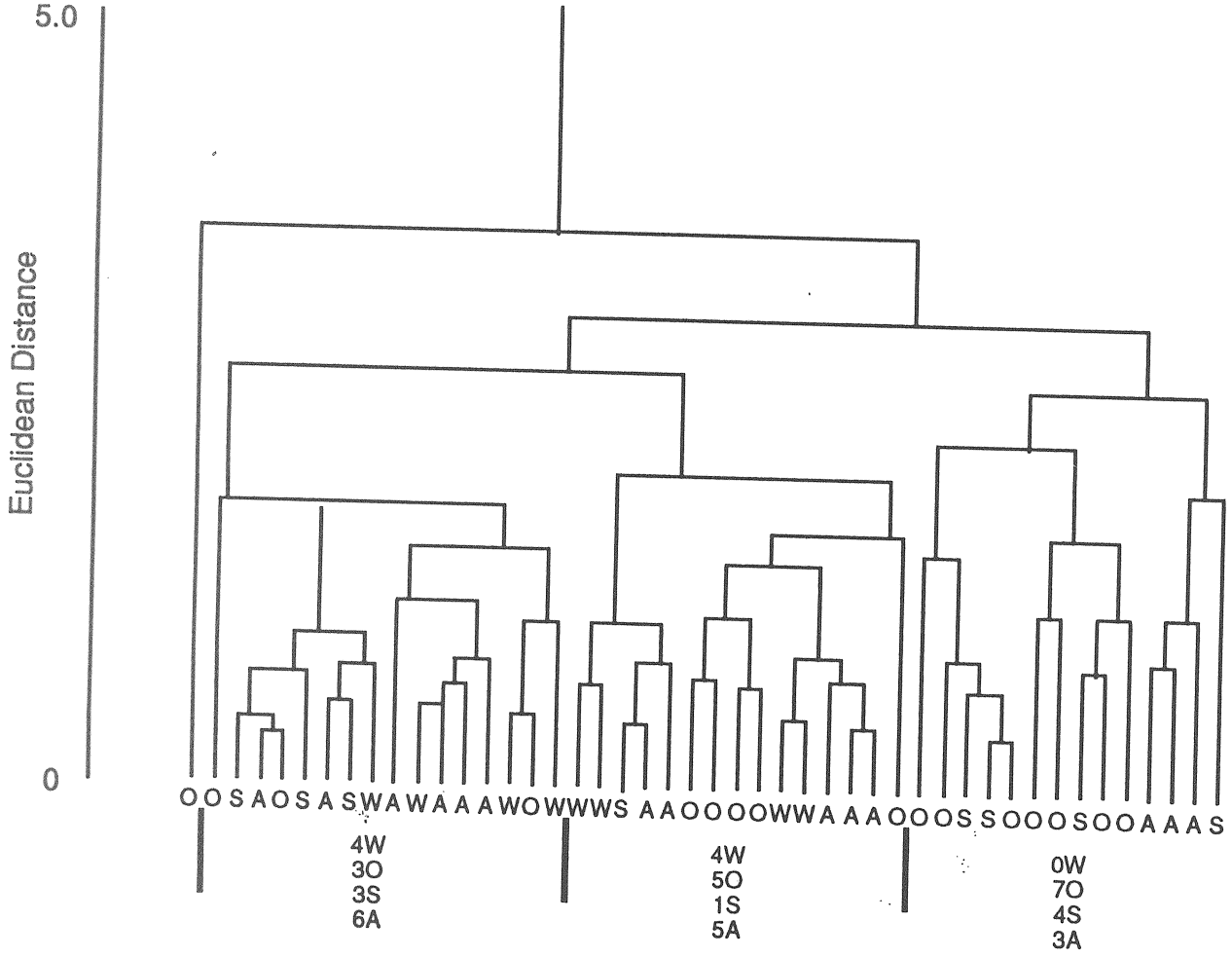
10^3
10
0.1
Ca Na Sr K S Cl Hg Mn Cu Br Fe Zn Cd Ni Pb


#1341
#670
#204
Concentration (weight-fraction, in ppm except for Ca)
4000
3000
2000
1000
0
0
100
200
350
43
%
39
Ca
Na
Sr
K
Cl
S
Point analyzed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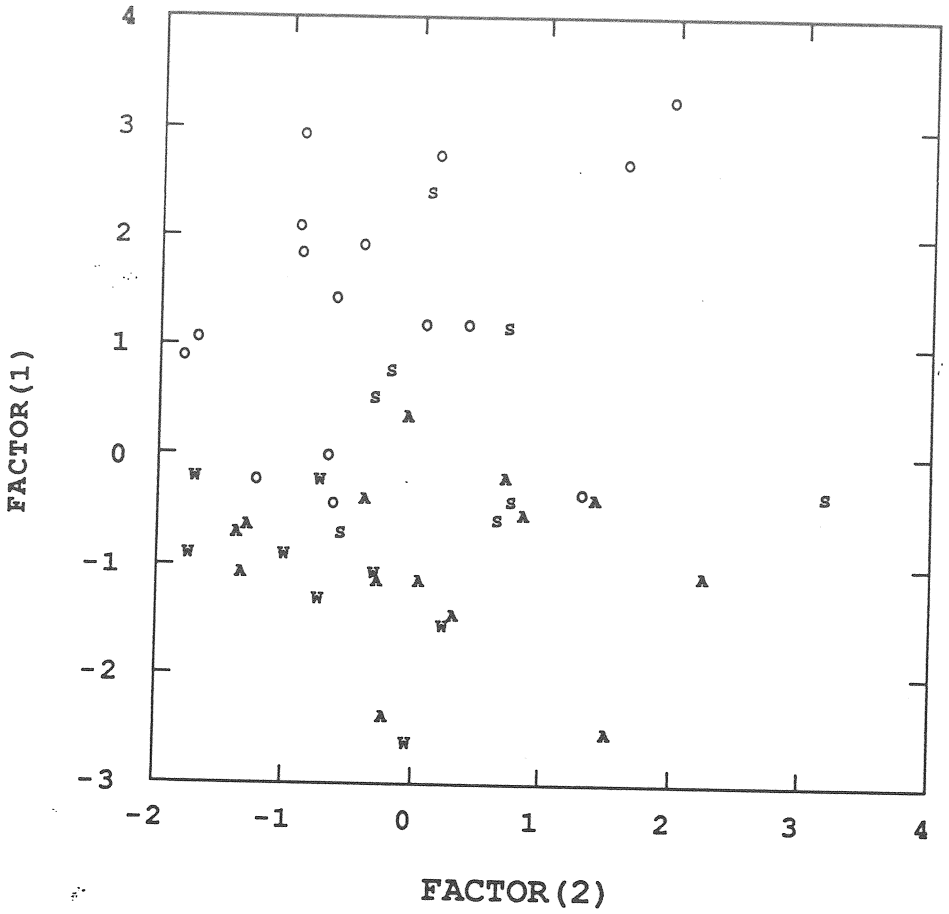
7O
4S
3A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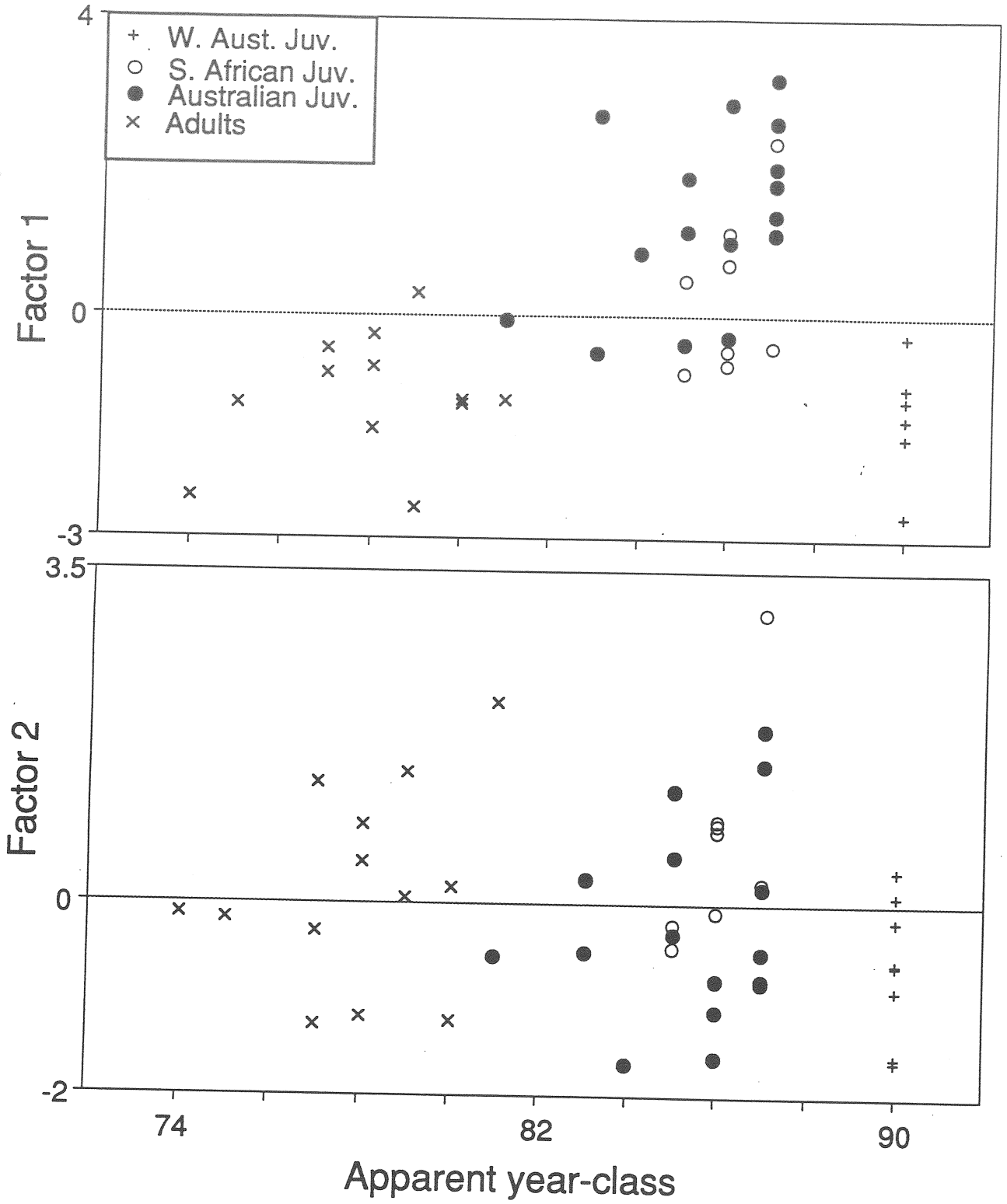
3
4
FACTOR(1)
FACTOR(2)


W. Aust. Juv.
S. African Juv.
Australian Juv.
Adults
Factor 1
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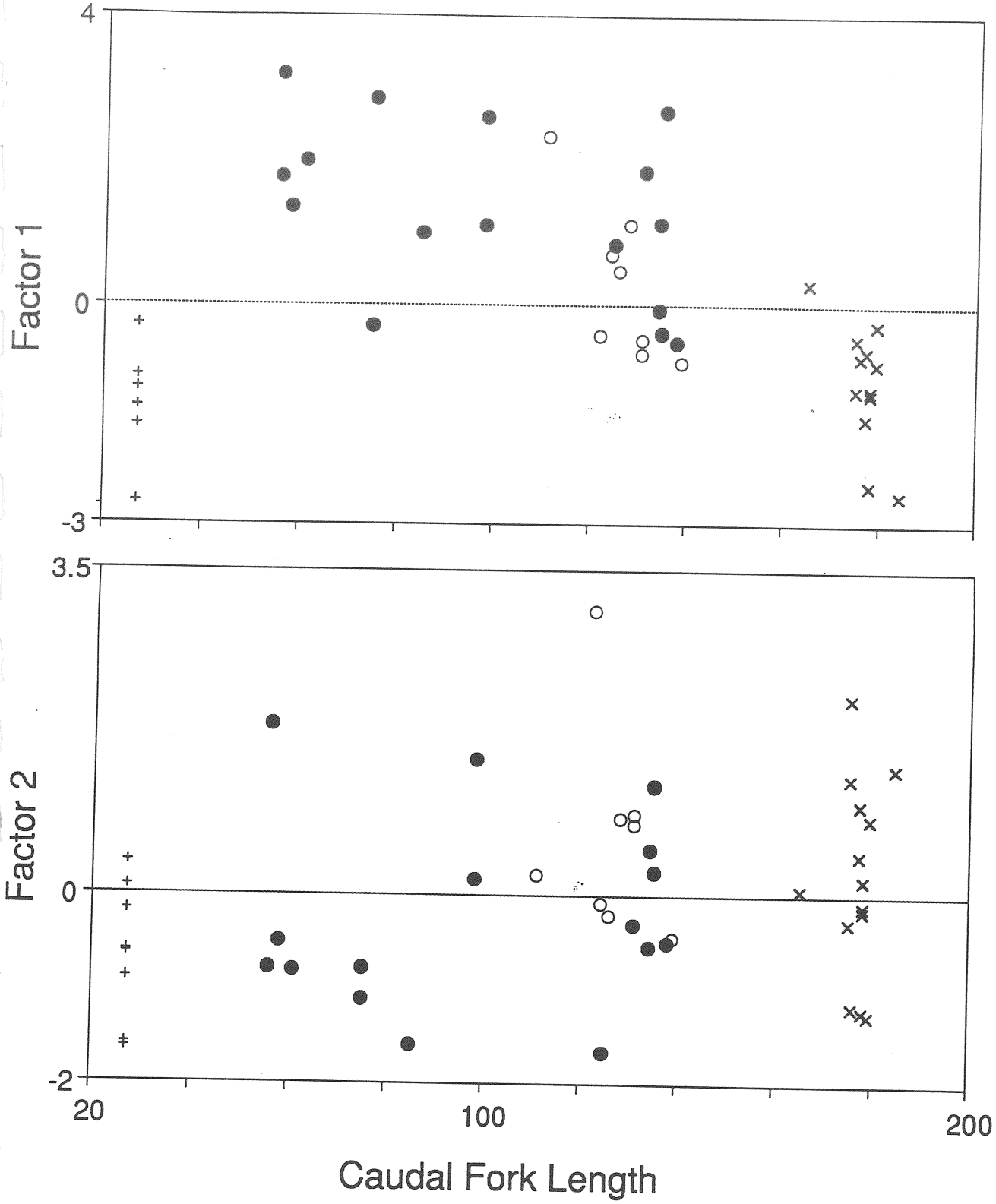
-3
Factor 2
3.5
0
-2
74
82
90
Apparent year-class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Factor 2
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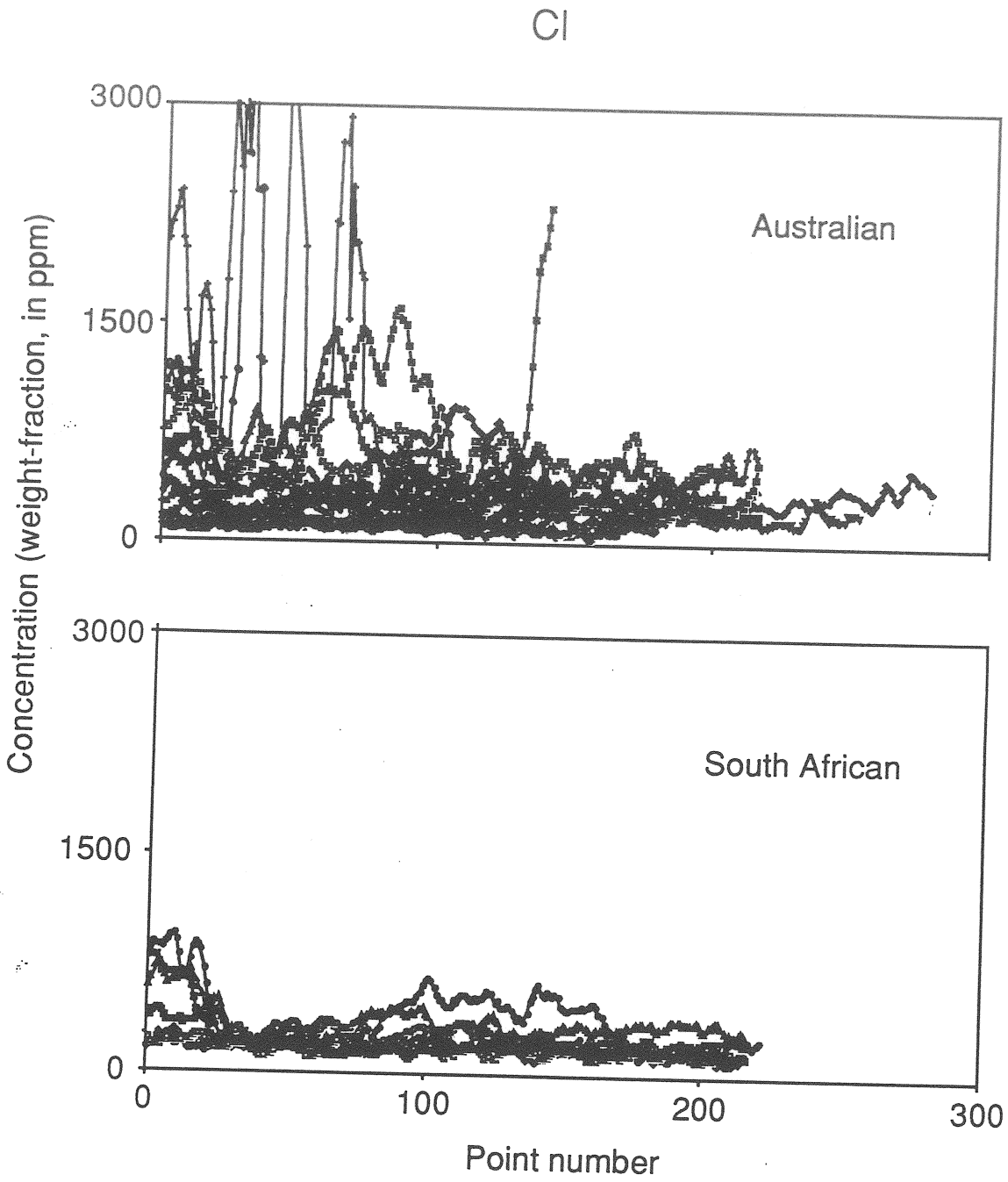
100
200
Caudal Fork Length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1500
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Australian
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1500
0
South African
Concentration (weight-fraction, in ppm)
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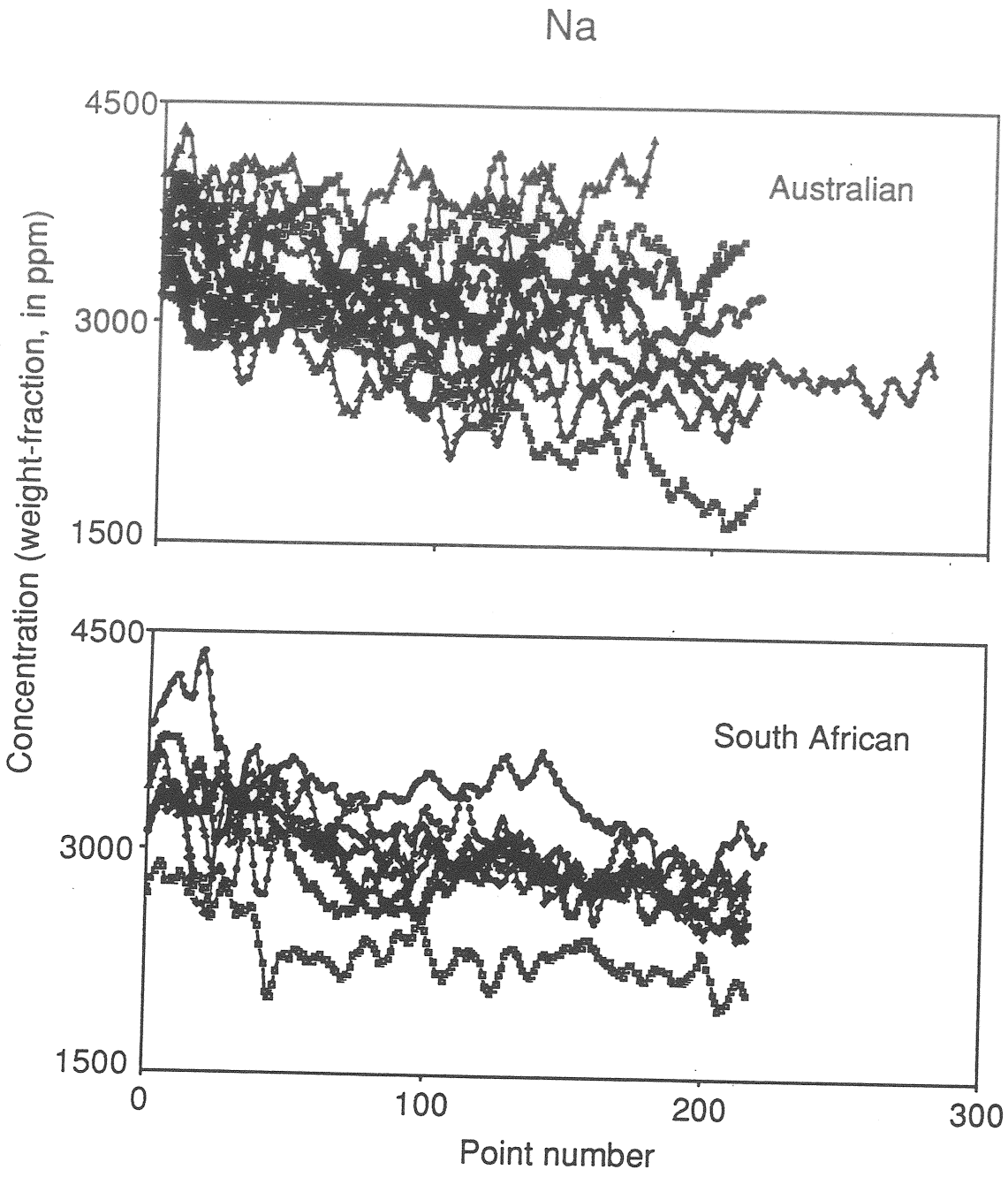
100
200
300
Point number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3000
1500
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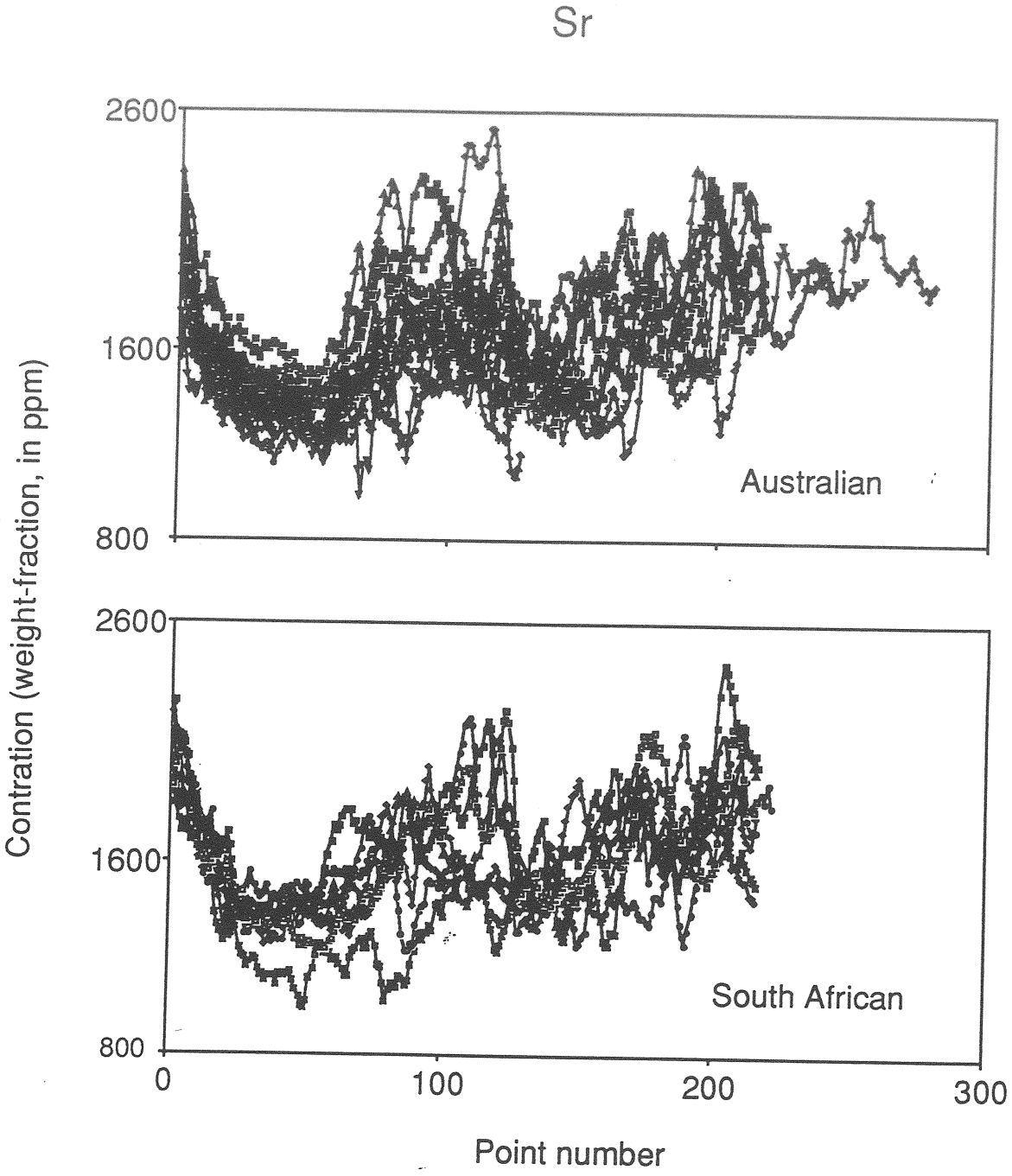
200
300
Point number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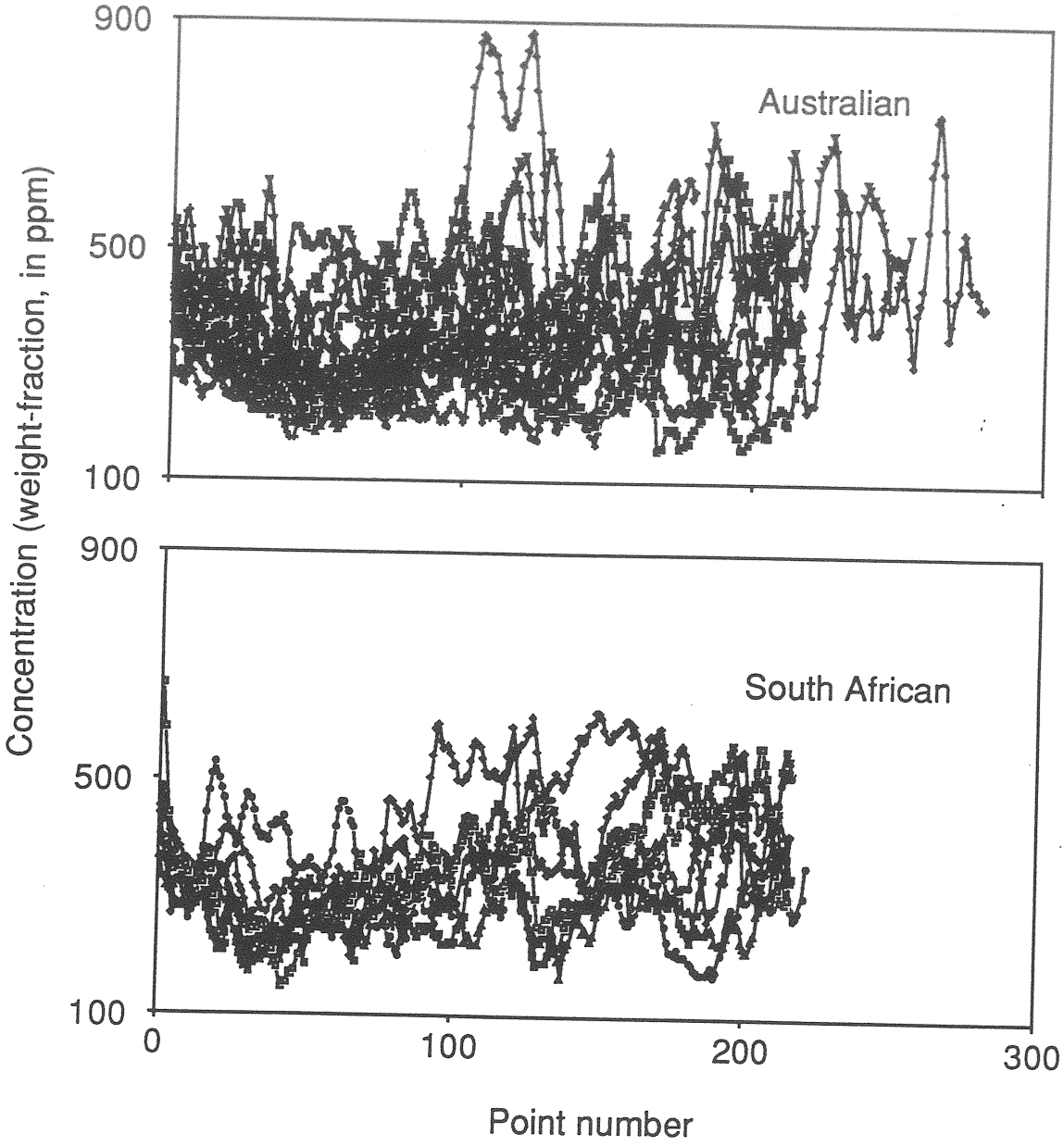
Australian
2600
1600
800
South African
0
100
200
300
Point number
Contration (weight-fraction, in ppm)

S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100
Australian
900
500
100
South African
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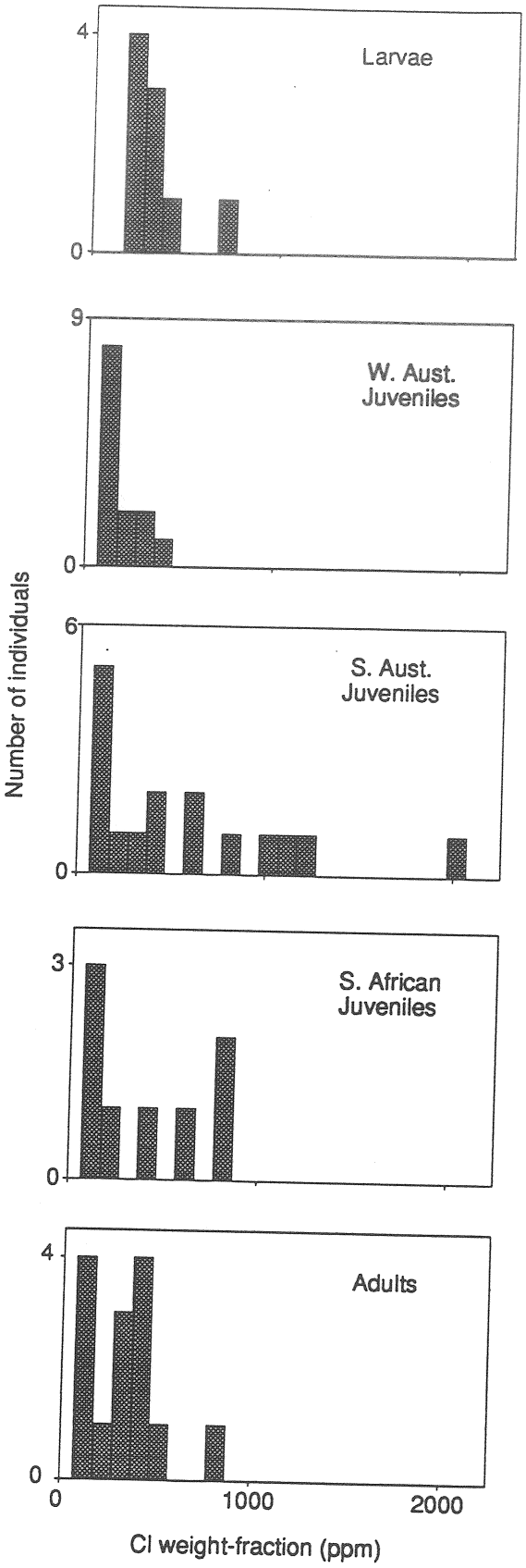
100
200
300
Point number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Juveniles
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S. Aust.
Juveniles
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0
S. African
Juveniles
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0
Adults
4
0
Number of individuals
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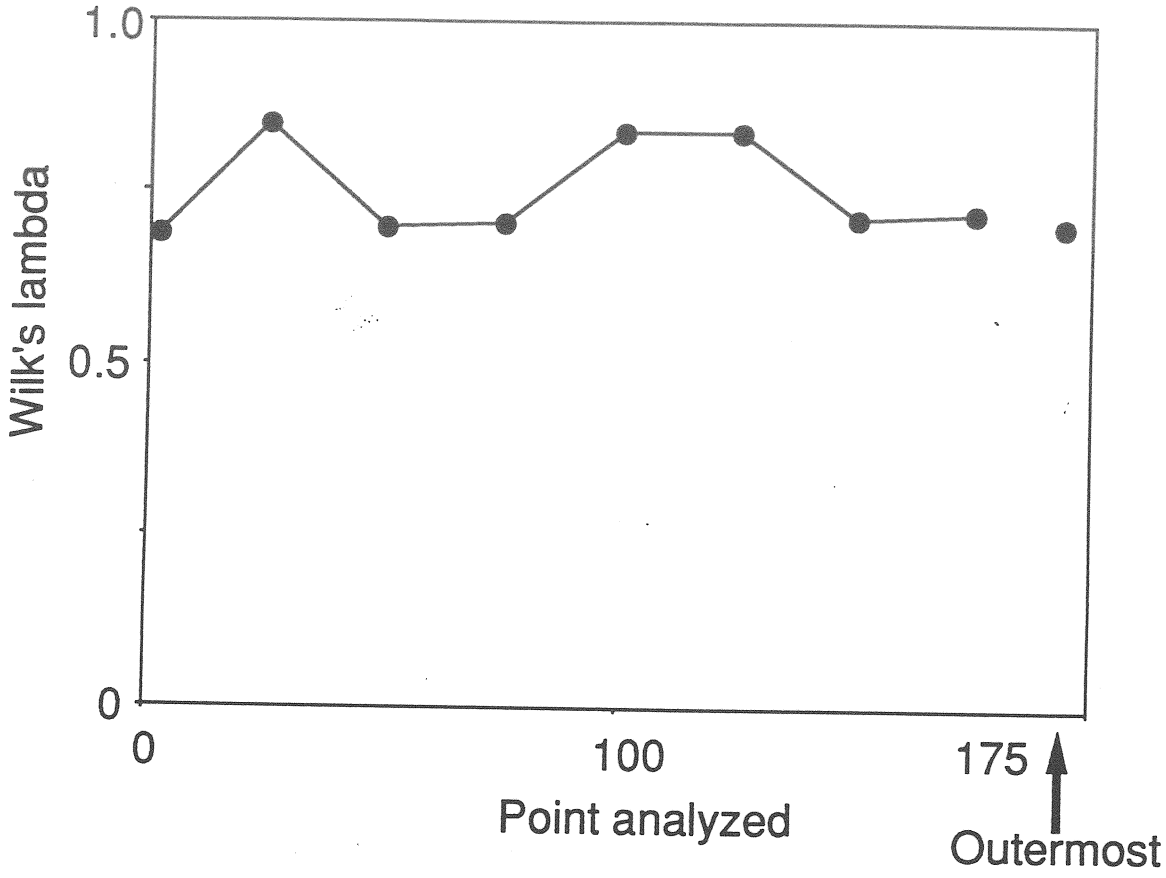
1000
2000
Cl weight-fraction (ppm)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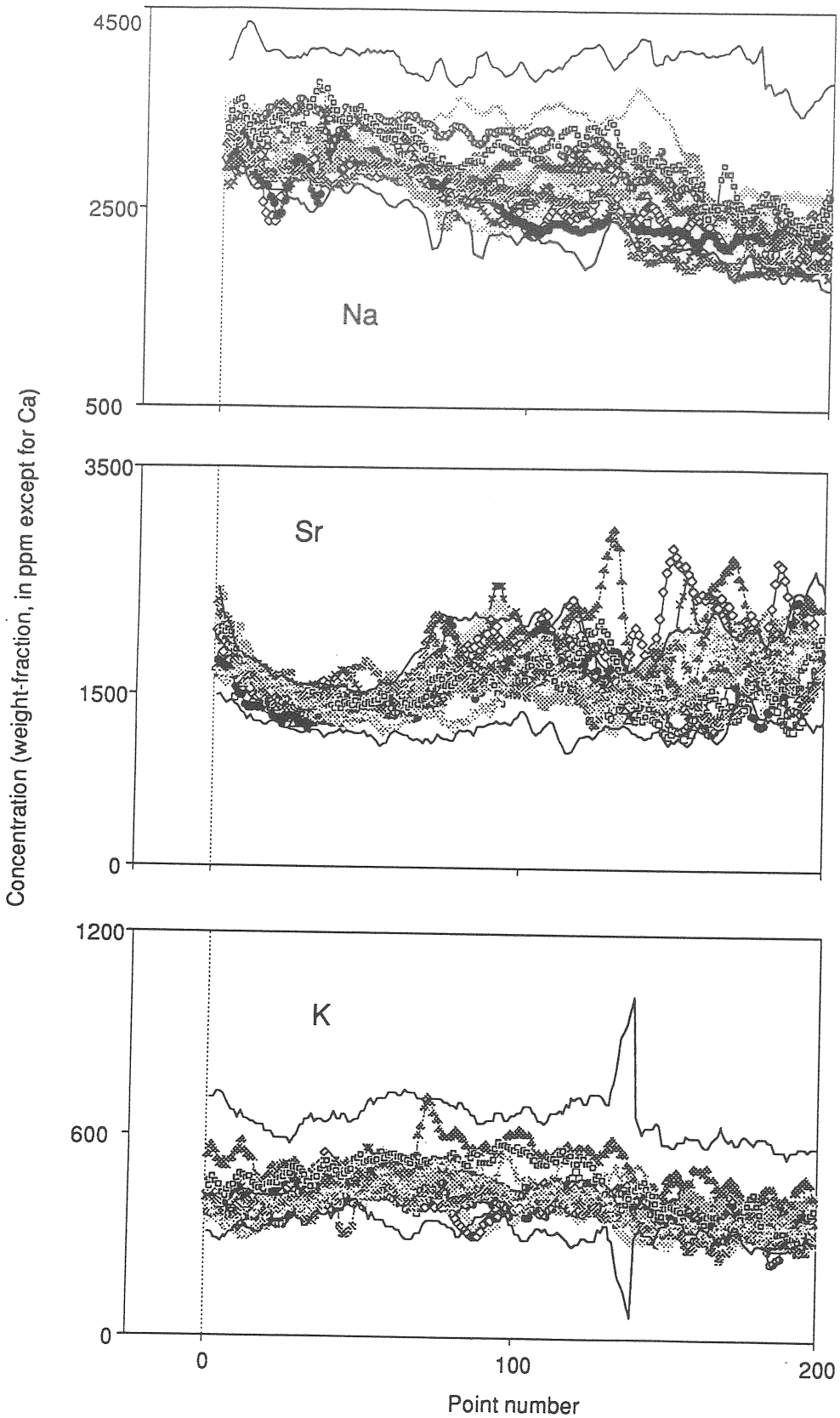
Point analyzed
Outermost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Sr
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1200
K
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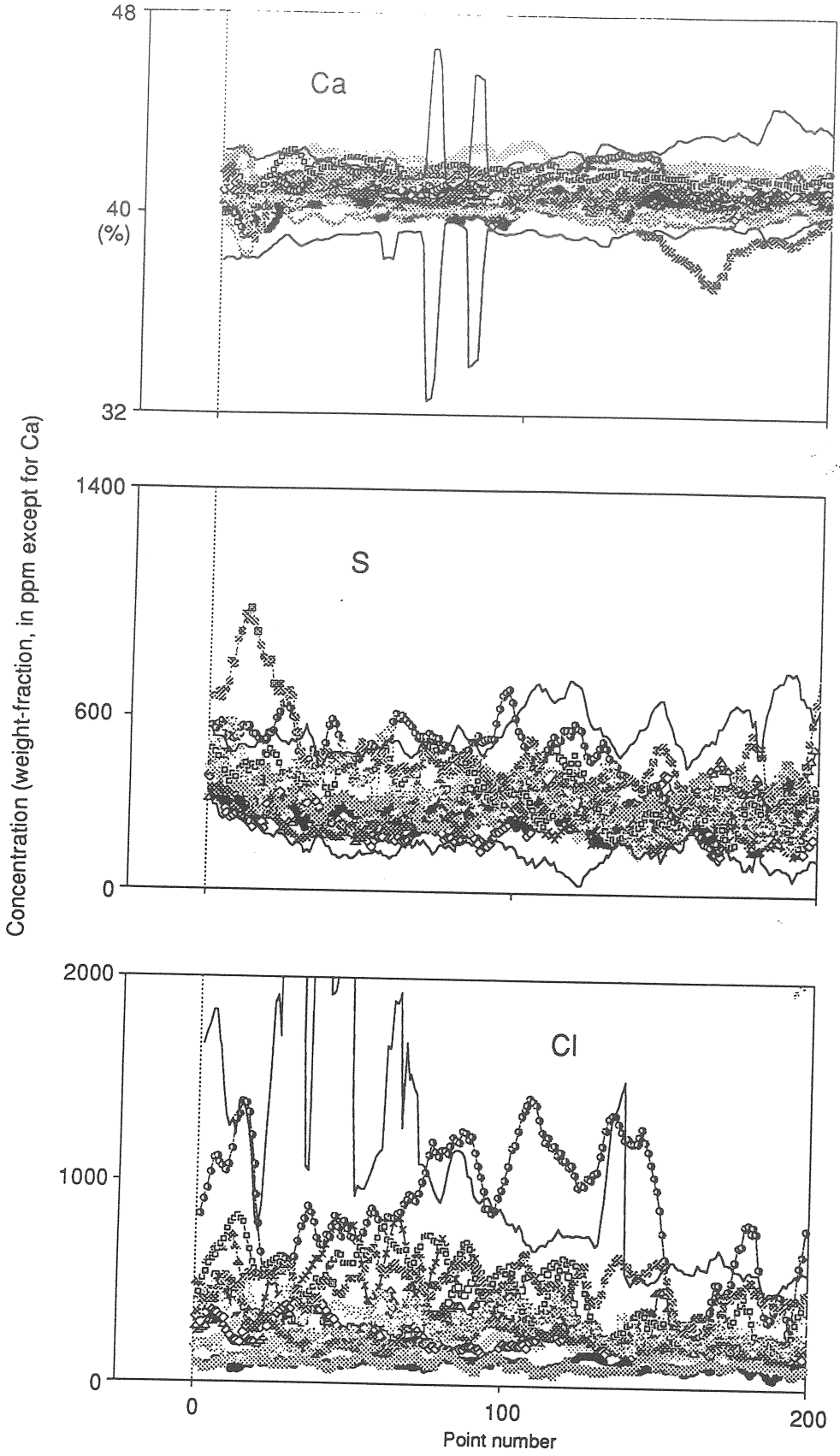
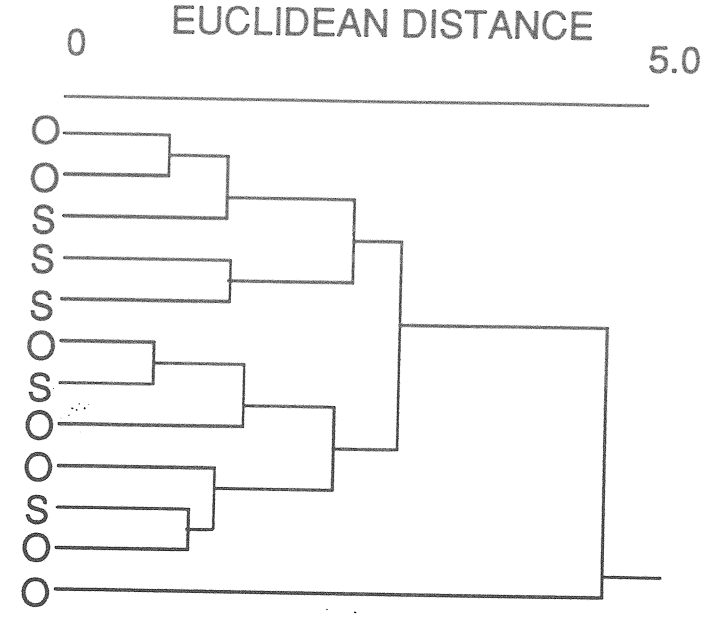
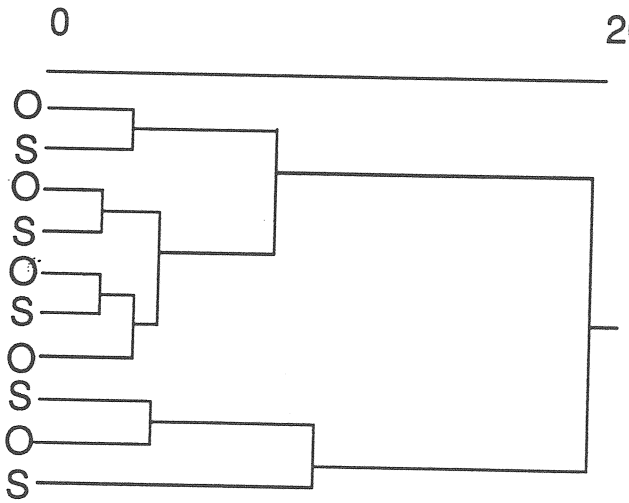
0
0
100
200
Point number
Concentration (weight-fraction, in ppm except for Ca)


48
Ca
40
(%)
32
1400
S
600
0
2000
Cl
1000
0
0
100
200
Point number
Concentration (weight-fraction, in ppm except for Ca)



PRIMORDIUM



OUTERMOST POINT

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Appendix 1. Original Application

Fishing Industry Research Trust Account
Application for Grant
86/87

1. Title of Proposal

Determination of the migration patterns of juvenile Southern Bluefin Tuna and Jackass Morwong.

2. Name of Applicant

CSIRO

3. Division : Fisheries Research, CSIRO Marine Laboratories, GPO Box 1538, Hobart, Tasmania 7001

4. Proposal:

It is proposed to determine the migration patterns and number of nursery areas of larval and juvenile Southern Bluefin Tuna and Jackass Morwong, by analyzing natural variation in the composition of their otoliths.

5. Name of Person Responsible for Program:

F.R. Harden Jones, Ph.D., Chief,
Division of Fisheries Research,
CSIRO Marine Laboratory,G.P.O. Box 1538,
Hobart Tasmania 7001
Telephone: (002) 206 222 Telex: 57182

6. Qualifications of Staff to be Employed on the Program:

		% of Time on Project
R.E. Thresher	Ph.D. - Project Leader	(70)
N. Elliott	Ph.D.	(30)
J. Gunn	B. Sc.	(70)
Experimental Scientist (to be appointed)		(100)

7. Objectives:

1. To determine whether or not all juvenile Southern Bluefin Tuna migrate down the coast of Western Australia and hence, whether the Australian fishery is based on all or only part of each cohort;
2. To validate aging techniques for adult Southern Bluefin Tuna;
3. To determine whether or not bays and estuaries in southern Tasmania are the sole nursery grounds of the Jackass Morwong in Australia.

8. Justification, including Practical Application:

The majority of Australia's commercially important finfish species undertake extensive reproductive migrations. The data linking spawning areas, nursery areas and adult distributions are often sparse, however, even for species where the locations of spawning and nursery areas are known. Traditional approaches to linking the areas used during different stages of a fishes' life cycle involve either extensive surveys for drifting larvae and migrating juveniles or tagging large numbers of larvae and small juveniles. These approaches are often expensive and difficult to apply.

Two of the most striking gaps in our information on larval drift and juvenile migrations concern the Southern Bluefin Tuna and the Jackass Morwong. The Southern Bluefin Tuna is the single most important commercial finfish in Australia and is also caught in large quantities by Japanese and New Zealand fishermen in international waters. The Australian fishery is based on 2-7 year old fish and is concentrated along the Western Australian and South Australian coasts of the Great Australian Bight. The Japanese and New Zealand catch consists of mature fish. Current multilateral management plans for the Southern Bluefin Tuna are based on the assumption that following spawning, in the Java Sea, larvae and juveniles move down the Western Australian west coast, before entering the Australian fishery as 2 year olds. If some juvenile tuna, in fact, migrate to the Southern Ocean along routes other that the western Australian coast, the structure of management plans for the fishery might need to be altered, as would current procedures for monitoring recruitment to the stock. The hypothesis that some juvenile Southern Bluefin Tuna migrate, for example, into the western Indian Ocean, has never been tested, even though it is consistent with oceanographic data for the spawning grounds.

Similarly, movements by the larvae and juveniles of the Jackass Morwong are not known. Jackass Morwong are of interest not only because it constitutes one of the major components of the SE Trawl fishery, with current landings of approximately 2000 tonnes annually, but also because it is a locally available and relatively robust species, which can be used to validate experimentally the analytic techniques employed in the project. Historical data, recently summarized by D. Smith (NSW State Fisheries), suggest that year-class strength of Jackass Morwong varies substantially, for reasons that, while unclear, presumably relate to rates of larval and juvenile mortality. Studies of Jackass Morwong in New Zealand document discrete spawning and nursery grounds for the species, and it is assumed this is also true in Australian stocks. Neither spawning nor nursery grounds have been located, however. Recently, the Tasmanian Division of Sea Fisheries has discovered the presence of juvenile Jackass Morwong in bays and estauries in southeastern Tasmania. It has been suggested that these coastal habitats constitute the primary, and perhaps only nursery grounds for the species in Australia, and hence support the entire fishery. The evidence for this hypothesis is entirely negative, that is, juveniles have not yet been caught in other areas. Positive determination that these coastal areas are a critical habitat for juvenile morwong is neccesary in order to ensure responsible management of the areas and maintain the long term viability of the fishery.

We propose to determine the migration patterns of juvenile Southern Bluefin Tuna and Jackass Morwong, using a technique that avoids the difficulties of tagging fragile larvae and juveniles. This technique takes advantage of the effects of a seasonally and spatially variable environment on the chemical composition of fish otoliths. Considerable work has demonstrated that the isotopic and elemental composition of calcified structures in marine organisms (e.g., molluscs, foraminiferans, corals and fishes) is dependent upon environmental conditions such as water temperature. To the extent that habitats differ, the composition of otoliths of fishes living in those habitats will also differ. Hence, if juvenile Southern Bluefin Tuna migrate along two different routes, each of which traverses different water masses, then otoliths of fishes in the

adult population should show evidence of two different patterns of composition. Similarly, if all of the Jackass Morwong caught in the SE Trawl fishery originate in the same nursery ground, then not only should their otoliths all have a similar composition, but also this composition should match that of juveniles collected in the known nursery area. In either species, if there are adults that fall outside the range of composition found in juveniles collected from the known nursery areas, then it implies strongly that we have not identified all of those juvenile habitats.

Hence, the two major practical applications of this study are as follows:

1. determination of whether or not the Australian shore-based fishery for Southern Bluefin Tuna is based on all, or only part of the juvenile and sub-adult stock;

2. positive identification of specific nursery areas for Jackass Morwong, estimation of the number of different nursery areas, and detailing of the links between nursery areas and portions of the adult stock.

Ancillary information likely to develop from the study include:

1. confirmation of the age of recruitment of Southern Bluefin Tuna to the Australian fishery (based on the number of seasonal cycles of water temperature evident in the otolith);

2. independent assessment of procedures for aging Southern Bluefin Tuna (by comparison of the number of seasonal cycles of water temperature with (a) the known age of tetracyclin-tagged individuals and (b) ages as determined by traditional procedures based on counting annuli); and

3. prediction of the likely location of the spawning grounds of the Jackass Morwong (based on temperature information derived from the composition of that part of the otolith formed during the early larval stage).

9. Location of Operation:

The base for the program will be the CSIRO Marine Laboratories in Hobart. Samples of Southern Bluefin Tuna otoliths are already on hand or, for the adults, can be obtained from other FIRTA-funded projects. Samples of juvenile Jackass Morwong will be obtained from coastal habitats in Tasmanian and Bass Strait waters, whereas adult specimens will be obtained from the commercial fishery. X-ray microanalysis will be conducted at the CSIRO Division of Mineral Chemistry, in Melbourne.

10. The Proposal in Detail, including Procedures:

(a) Plan of Operation

(1) Method of Procedure

(A) Southern Bluefin Tuna

Otoliths of large numbers of juveniles are already at hand, and may be supplemented, as needed, from the commercial catch. Between 15 and 30 otoliths will be analyzed for juveniles in each of at least three year-classes in order to establish both the range of overall composition and the seasonal and ontogenetic patterning of that composition for fishes from the known migration route. Otoliths for adult tuna are available based on FIRTA grant 85/76, "Otolithic age determination of mature southern bluefin tuna (Thunnus maccoyii)" (J. Thorogood, project leader). All otoliths that can be obtained for the adults will be analyzed (one otolith per adult), using procedures identical to those used on juveniles.

(B) Jackass Morwong

Samples of juvenile Jackass Morwong will be collected in Tasmanian coastal habitats using a small boat and trawl and by diving . Along with the currently suspected nursery areas, juveniles will also be sought in other coastal habitats of Tasmania and Bass Strait. Between 15 and 30 otoliths will be analyzed for each year class present in each apparent nursery area, to determine the range of variation in composition of each. Samples of adults will be obtained from the commercial fishery,

to cover the geographic range of the species. Depending on intial results, up to 200 otoliths of the adults will be examined.

(C) Analytic Techniques

Two primary analytic techniques will be applied to otoliths: optical microscopy, which provides data on number and patterning of growth increments, and wave dispersive x-ray microanalysis, which provides data on zonal variations in otolith composition (and hence changes in composition with growth). All otoliths studied will be examined optically prior to chemical analysis, in order to obtain data on specimen age (based on number of daily growth increments) and variation in otolith size at age. The latter is likely to reflect variation in larval and juvenile growth rates, and may differ between juvenile habitats. Optical examination will be done using a high resolution optical microscope coupled with an image enhancing closed circuit television and microcomputer-based digitizer. Scanning and transmission electron microscopy will be used to supplement optical examination, where increment widths dictate use of the higher resolution system. X-ray microanalysis will be conducted using a Cameca Camebax x-ray microprobe. The fine resolution of the microprobe (minimum beam size approximately 3 square microns) permits detailed analysis of changes in otolith composition with age. Ten juveniles each of Southern Bluefin Tuna and Jackass Morwong will be analyzed at the finest spatial scale possible, in order to document fully ontogenetic and seasonal changes in otolith composition. Based on these data, a coarser scale of analysis, which is both far less time consuming and less expensive, will be determined for routine examination of the bulk of the samples. Results of x-ray microanalysis will be ground-truthed by comparison with both atomic absorption spectrophotometry and isotopic analysis; for each, pairs of otoliths from twenty Southern Bluefin Tuna will be split into matched groups, with one otolith from each pair subjected to x-ray microanalysis and the second to the alternative technique.

Data for temporal changes in both optical and chemical features are likely to be inherently noisy, and will be smoothed using standard statistical procedures. Quantitative comparison of juveniles and adults will be done using pattern recognition and cluster analyses.

(D) Validation of temperature-dependency of otolith composition

Groups of twenty juvenile (0+) Jackass Morwong will each be maintained in the laboratory for three months in five different, constant temperature environments. At the end of the three month period, otoliths will be removed and examined at fine scale resolution using x-ray microanalysis. Element ratios for that part of the otolith laid down in the laboratory will be compared for fishes in each treatment group using analysis of variance, both to verify the effects of temperature on composition for a species of interest and to determine the form of the relationship between temperature and composition. Validation for the Southern Bluefin Tuna, which cannot be conveniently maintained in the laboratory, will be sought be comparing otolith composition of larvae, obtained in a forth-coming CSIRO DFR program on distribution of larval Southern Bluefin Tuna, with temperatures at the point of collection, and by correlating otolith composition at later stages of the life cycle with probable water temperatures in which those stages occur.

(ii) Facilities Available

(a) At sea: F.R.V. "Ophelia" - a 6 m Shark Cat, fully equipped for near-shore operations, will be used to trawl coastal habitats for juvenile morwong and to support diver operations. The vessel, owned by the Division of Fisheries Research, is trailerable, and hence can be used to obtain samples from inshore habitats all along the Tasmanian coast.

(b) On Land: CSIRO Marine Laboratories, Hobart provide office aand general laboratory facilities and a full back-up of computing, technical and library services. Optical microscopy, scanning and transmission electron microscopy and chemical analysis of otoliths will be done at the marine laboratories (atomic absorbtion spectrophotometry). Isotopic analysis and X-ray microanalysis will be done at the CSIRO Division of Mineral Chemistry, in Melbourne.

(b) Supporting Data: The CSIRO Division of Fisheries Research has a longstanding record of fisheries research in areas of direct importance to the fishing industry, and has a commitment to research which will be of long-term value to Australia.

In addition, the CSIRO Division of Fisheries Research has been engaged for many years in analysis of otolith structure. For the last three years, studies undertaken by the proposed project leader have focussed on the microstructure of the otoliths of juvenile and larval fishes. Techniques developed for these studies will be applied to preparation of the otoliths of Southern Bluefin Tuna and Jackass Morwong for both optical and chemical analysis.

Data on the effects of temperature on otolith microchemistry have been provided for several species of fishes, including cod (Gadus morhua), striped mullet (Mugil cephalus) and the Antarctic cod (Notothenia larseni). Work conducted in New Zealand has also documented a correlation between iron content and developmental temperature for the Jackass Morwong. In a study similar in concept to the one proposed, environmentally induced variations in the composition of calcified structures (vertebrae) were used successfully to indentify stocks of the sockeye salmon, Oncorhynchus nerka, in Canadian rivers.

Distribution of juvenile Jackass Morwong has been studied in some Tasmanian bays and estuaries by the Tasmanian Division of Sea Fisheries; seasonal cycles of recruitment have been documented for the Derwent Estuary and for Frederick Henry Bay. These data will be used to plan and guide collection of juveniles for otolith examination, and to collect specimens for validation studies in the laboratory.

11. Proposed Commencement Date and Anticipated Completion Date

1 July 1987 - 30 June 1989

12. Funds Requested

	Year 1	Year 2
(a) Total Salaries and Wages	33623	36894
(b) Total Travel Expenses	11848	8429
(c) Total Operating Expenses	41900	40000
(d) Total Capital Item	0	0
Total Gross Cost	87371	85278
Estimated Income	0	

13. Funds to be provided by the Applicant or sought from Other Sources

	Year 1	Year 2

1. Salaries of Existing CSIRO Staff		
1 Research Scientist (70% of time)	37476	43401
1 Scientific Services Officer (30% of time)	12110	15148
1 Experimental Scientist (70% of time)	28254	29314
Total Salaries	77840	87863
2. Operating Expenses	Nil	Nil
3. Capital Equipment		
Leitz Orthoplan Microscope and Associated Image Analysis Equipment	55000	55000
Electron Microscope Unit	275000	275000
F.R.V. Ophelia (6 m Shark Cat) with tow vehicle and trailer	37000	37000
X-Ray Microanalysis Unit (Division of Mineral Chemistry)	350000	350000
Total Capital	717000	717000

14. Cooperating Agencies and their Functions:

Otoliths of mature Southern Bluefin Tuna will be obtained through cooperation with the Commonwealth Department of Primary Industry.
With regards to the Jackass Morwong, close ties will be maintained with the Tasmanian Division of Sea Fisheries throughout the program, but no formal links with the DSF will be required for this program to be completed. Assistance will be sought from South Australian Fisheries, the Victorian Institute of Marine Science, and New South Wales State Fisheries in obtaining otoliths from commercially caught Jackass Morwong.

The CSIRO Division of Oceanography will assist in the analysis of otolith chemistry by means of atomic absorbtion microspectrophotometry. The CSIRO Division of Mineral Chemistry has agreed to assist with x-ray microanalysis and isotopic analysis of otoliths, for which the program will be charged only the cost of operating the equipment.

15. Is similar work being undertaken in Australia

Tasmanian Sea Fisheries has investigated the distribution of juvenile Jackass Morwong in southeastern Tasmanian estauries. Work has also been conducted in Victoria and New South Wales, by the Victorian Institute of Marine Science and NSW State

Fisheries, respectively, to locate juvenile Jackass Morwong locally. Neither effort was successful.

A preliminary project to examine the microchemical composition of Australian Salmon otoliths has been completed by J. Kalish, at the University of Tasmania.

16. Plans for Reporting or Publishing Results

(a) Detailed reports to FIRTA
(b) Popular accounts of significant progress in "Australian Fisheries" and at relevant trade meetings.
(c) Publication of scientific results in suitable journals and discussions of results and the techniques used with other fisheries research organizations in Australia.

Details of Funds Requested

	Year 1	Year 2
(a) Salary and Wages		
Experimental Scientist 1.M (to assist in collection of field samples, preparation of otoliths for analysis, and construction and operation of experimental apparatus)	27023	29700
Employer's Superannuation Contribution (20.5%)	5540	6089
Leave Loading	400	400
Diving Allowance	660	660
Total Salary	33623	36849
(b) Travel Expenses		
Return Airfare (Hobart-Melbourne) (to use microprobe, based on four trips in Year 1, five trips in Year 2)	2000	2500
Return Airfare (Hobart- Cleveland) (to obtains details of constructing controlled temperature aquaria)	650	0
Travel Allowance (in Melbourne, based on four trips of 14 day duration at $84.70 per day in Year 1, and five trips of 14 day duration in Year 2)	4743	5929
Travel Allowance (in Cleveland, based on one trip of three days		

duration)	255	0
Travel Allowance (in Tasmania, based on two field trips of ten days duration each, involving three staff at $70 per day)	4200	0
Total Travel Expenses	11848	8429

(c) Operating Expenses

Cost of Operating X-ray Microprobe (based on estimate provided by Division of Mineral Chemistry)	25000	30000
Fuel and operating costs of F.R.V. Ophelia (based on 44 days at sea Year 1, 20 days in Year 2, at $100 per day)	4400	2000
Self-contained, controlled temperature Aquarium Unit (to be built)	5000	0
Diving Equipment (Purchase and Maintanence)	2000	500
Purchase and Transport of Specimens	500	500
Computing (CSIRONET, for data reduction and analysis)	2000	3000
Supplies for Field Sampling, Chemical Analysis, Experimental studies, and Preparation of otoliths for X-ray microanalysis	3000	4000
Total Operating Expenses	41900	40000

(d) Capital Items

	0	0
Gross Total Cost	87371	85278
Estimated Net Income	Nil	Nil