UTILISATION OF VERTEBRAL MICROCHEMISTRY TECHNIQUES TO DETERMINE POPULATION STRUCTURE OF TWO INSHORE SHARK SPECIES ALONG THE EAST COAST OF QUEENSLAND, AUSTRALIA

Thesis submitted by

Ron Schroeder

BSc in Electrical Engineering, MBA in International Finance, MAppSc in Marine Biology

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James Cook University

Townsville, Australia

Supervisors

Colin Simpfendorfer

Dave Welch

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CONTRIBUTION OF OTHERS TO THIS THESIS

- Australian Government's Fisheries Research and Development Corporation (2007/035) for funding support.
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1 Abstract

As apex predators, sharks have a controlling influence on the marine ecosystem. However, their life history traits of being slow growing, late maturing and slow reproducing make them vulnerable to overfishing. The East Coast Inshore Finfish Fishery on Queensland, Australia's east coast produces 1800 tonnes per year, of which 35% are sharks. Current management is based on quantitative data, largely focused on teleosts. A need exists to include stock structure and population connectivity to more effectively manage the fishery over the long term. The stock structure of two species commonly taken in this fishery was investigated to determine possible patterns of structuring. *Rhizoprionodon acutus* is a small, fast growing, and short lived species that demonstrates limited movement throughout their life. *Sphyrna lewini* is a larger, slower growing species that has some site attachment as juveniles, but travels widely as adults. Several techniques exist to determine stock structure, including genetics, life history variation, and various tagging methods. For teleosts, otolith microchemisry analysis using inductively coupled plasma mass spectrometry (ICPMS) is often utilised to infer stock structure. This project developed a method to utilise ICPMS on shark vertebra to determine stock structure. Upon confirmation of the method, twelve to twenty samples of *S. lewini* and *R. acutus* were collected from each of six locations on the Queensland and northern NSW Australian coast. Vertebrae were analysed by laser ablation ICPMS for Ba, Ca, Cu, Mg, Mn, Sr, and Zn. This data was then used to determine stock structure among both species.

Both solution based and laser ablation ICPMS were applied to shark samples for method development. Microchemical variation between individuals was greater than the inherent variation in the ICPMS processing for both solution and laser ablation methods. The corpus calcareum or intermedalia of vertebral centra gave similar results on the separation of individuals, but their microchemical compositions differed significantly. Difficulty in polishing the soft vertebrae sections led to surface contamination of sections, but this was isolated in the data analysis stage. Microchemical composition was also not affected by vertebral column position or size of the individual from which the sample was drawn. While either approach can be used to determine geographic population structure in sharks, laser ablation provided greater versatility by allowing isolation of various stages of life in the analysis.

Analysing the whole life mean and end of life mean microchemical signatures in both species, principal components analysis (PCA) demonstrated some separation, but with heavy overlap, between six regions along the Queensland coast. MANOVA confirmed separation and Hotellings T^2 pairwise comparisons demonstrated significant differences between all adjacent regions, except Townsville – Mackay. Lack of separation between these two sites may infer significant migration or may be indicative of similar environmental parameters influencing microchemical signature of animals in those two regions. This study supports existing knowledge of *S. lewini* showing some site attachment as juveniles, and *R. acutus* demonstrating limited migration throughout life. From a fishery standpoint, limited connectivity among metapopulations may exist, allowing for some replenishment capability. However, it would be prudent to

manage these species on a regional basis.

Several areas exist to expand this innovative method. Development of a vertebra standard that more closely approximates a shark would improve sensitivity drift correction capabilities. Tuning the ablation parameters such as scan speed, sample frequency and power may improve utility of laboratory time and data resolution for analysis. Improvements may be made by gaining further insight as to the environment from which animals are collected. This may include water sampling of elemental concentrations, temperature or salinity. Collection of these data would also require intensifying fishing effort to minimise overall time window of sample collection in order to maximise benefits from the knowledge. Further method advancement could be made by analysing vertebrae from animals with known histories, particularly those tagged with gps or acoustic telemetry devices.

2 Literature Review – Analysis of Shark Population Structure Techniques and Potential for Utilisation of Microchemistry Techniques

2.1 Introduction

Sharks occur in all oceans of the world, with many species having global distributions (Simpfendorfer and Heupel 2004). As top predators, it is generally expected that they will play a critical role in marine ecosystems, regulating prey and in turn controlling community structure (Heithaus 2004). As knowledge is gained in the status of the world's fisheries, it is becoming apparent that fishing of sharks is not an easily sustainable practice. This is largely due to their life history characteristics of being slow growing, late maturing and having few offspring. As targeted and bycatch fishing pressure on sharks has increased, significant study of life histories has been occurring (Cailliet and Goldman 2004). In order to ensure sustainability of shark fisheries and preserve the various species, it is important to supplement life history and distribution knowledge with an understanding of their population structure and migration behaviours.

Most shark population structure analyses to date have been based on genetic markers or physical tagging. While effective to within their scope, both techniques have limitations. Genetics can link fish to a population, but not to a specific geographic region. Most physical tags link to where they were marked or recaptured only, but this can be logistically challenging and costly (Ashford et al. 2005). A technique that has become commonplace for teleost fish is elemental

analysis of otoliths. This capability was in its infancy in 1990 (Coutant 1990), and by 2005, nearly 20% of the many hundreds of otolith related studies were chemistry based (Campana 2005). Several factors drive elemental variation in fish otoliths, including water hydrodynamics, temperature, diet, ontogenetics, and physiological regulation (Bergenius et al. 2005). Trace element analysis in otoliths has proven invaluable in determining population connectivity and migration, which in turn helps in the design of marine reserves for conservation purposes (Nowlis and Friedlander 2004). The challenge in utilising this technique with sharks is that they do not have otoliths (Edmonds et al. 1996). They do however have cartilaginous vertebrae that tend to accumulate calcified growth material as they age, thus producing concentric areas reflecting the season or time of year in which the material has been deposited (Cailliet et al. 2006). Can this proven teleost otolith technique be adapted to assist in determining stock structure of sharks based on vertebral microchemistry?

This methods focused review explores population structure analysis techniques that have been utilised to date, beginning with microchemistry analysis primarily in teleost otoliths. Then it provides descriptions of genetics and tagging techniques as applied to shark population structure. This is followed with an overview of the technology deployed to actually perform the analysis. The latter part of this review covers shark vertebrae biology and existing vertebrae analysis capabilities. Finally it concludes with specific recommendations for applying microchemistry based population structure analysis techniques to shark vertebrae.

2.2 Established Approaches to Population Structure and Migration Habit Analysis

This section provides an overview of approaches that have been used to determine stock structure in a marine environment, starting with microchemistry analysis. Although methods focused, there are specific studies describing the application of such approaches, as well as summaries of several such population structure studies (Microchemistry studies in Table 1.1, Shark population structure studies in Table 1.2).

2.2.1 Microchemistry

Given that most marine microchemistry analyses have been performed using fish otoliths, and sharks do not have otoliths (Edmonds et al. 1996), sharks have largely been excluded from studies in this area. Otoliths are acellular and metabolically inert, meaning that once elements or compounds are accreted onto its growing surface, they are permanently retained (Campana 1999). Relative to other biological structures, otoliths are relatively pure, being dominated by calcium carbonate ($CaCO_3$) in a non-collagenous organic matrix, but with dozens of lesser trace elements included. Typical composition may be 96% $CaCO_3$, 3% organic matrix, and <1% combined of all other trace elements (Campana 1999). Although analysis of otoliths is most common, the technique has also been demonstrated using scales, fin spines, or eye lenses (Elsdon and Gillanders 2003). However, resorption levels of these structures, or regeneration during

ontogeny, may blur results somewhat (Gillanders 2001). Utilising scales or spines instead of otoliths would have the obvious benefit of being non-lethal, particularly important if dealing with endangered species.

In general, determination of stock structure with microchemistry information is related to determining statistical differences in an element(s) between sample sets that have been taken from different geographic locations. There can be a general relationship between the level of an element in an otolith, spines, scales, or vertebrae and the ambient elemental concentrations. The most commonly used elements used for reflecting ambient conditions would be strontium and barium (Elsdon and Gillanders 2003). However, the relationship is not simple. Physiological processes act as filters, thus preventing incorporation of a given element into the otolith at a directly proportional rate to that which is available in the environment (Campana 1999). The single most commonly used element in analyses is strontium, whose uptake into an otolith varies not only with ambient concentration levels, but also salinity. Strontium levels can also be utilised for age validation in long lived species (Campana and Tzeng 2000). The reasons for strontium reflecting ambient concentrations are that levels can be 10 – 100 times higher in marine over fresh waters (Courtemanche et al. 2005), and can alternate with calcium in the otolith deposition process (Arai et al. 2007). However, many studies have demonstrated conflicting results for the rate of strontium uptake in the presence of salinity variation, suggesting other factors may be interacting with salinity to affect uptake rates (Elsdon and Gillanders 2003). As such, it often becomes necessary to consider multiple elements in an “elemental fingerprint” when considering variations in salinity, temperature, and ambient concentration,

requiring a multivariate analysis. However, if only one of these items vary, a simple linear equation may suffice (Elsdon and Gillanders 2004). Temperature effects can be more difficult to determine than salinity or ambient concentration levels. Various studies in the field and lab have identified either positive, non-significant, or negative relationships between temperature and elemental concentrations, depending on the study or species. This suggests it is very difficult to develop a generalisation regarding temperature (Elsdon and Gillanders 2003). Early promise of using strontium to calcium ratios as a proxy for temperature in non-diadromous species have proven difficult (Campana and Tzeng 2000).

Temporal issues can arise in a stock separation analysis. In an analysis of various coral reef fish considering temporal as well as spatial factors, it was determined that if the temporal aspect was ignored, the result would have indicated no separation, whereas when it was included it suggested separation (Bergenius et al. 2005). Variability can be incurred by the seasonable nature of growth rates (particularly noticeable in younger animals with shorter histories) or changing ambient elemental conditions (Lessa et al. 2006). Unknown temporal related factors may affect results as well. In a study of corals along the Queensland coast, barium spikes occurred that could not be easily determined by environmental factors (river floods, temperature, season, latitude, etc.), or biological factors (Sinclair 2005).

It should be clear from the above paragraphs that one is unlikely to find a clear, simple, or linear relationship between a particular element, or suite of elements, and an environmental factor. In fact, several issues exist that may specifically confound results. For example, strontium uptake is higher in lower salinity environment, but as ambient strontium concentration itself varies, this can lead to challenges in interpretation (Elsdon and Gillanders 2003). Other issues can be interacting as well. Mercury and lead are typically associated with anthropogenic activity. However, regional variability in ambient concentrations can also be driven by river discharge levels, upwellings, volcanic activity, biological activity or inter-annual differences, all of which may confound expected relationships (Campana 1999). The biology of the fish must be considered in that some common elements such as sodium, potassium, sulfur, phosphorous, or chlorine are under heavy physiological regulation and should not be directly considered in the analysis (Campana 1999). There are also indications that the uptake of trace metals may be diet related (Vas et al. 1990). Finally, ontogenetic consideration must be made. In one study comparing ontogenetic variability in otoliths of embryo versus juveniles, differing levels of various elements were detected. This suggests that environmental factors were not the primary cause of variation, but ontogeny may affect physiological regulation (Chittaro et al. 2006). As such, caution is warranted in inferring environmental patterns from microchemistry test results.

An important element of stock structure determination is the migration habits of individuals. To determine whether two stocks are separate, it is not necessary to have detailed knowledge of how environmental variables such as salinity,

temperature, or ambient elemental concentration influence chemical composition of the animal. However, to actually reconstruct aspects of an animal's life, such as migratory behavior or spawning location, one must have more understanding and predictability of how the environment influences otolith composition (Elsdon and Gillanders 2003). While the technologies involved are discussed in more detail below, this also has implications on how the otolith is analysed. To determine stock discrimination across a life time, one can analyse the whole otolith, while the spawning location should include only the core, and the catch location would consider only the edge (Campana 1999). The reconstruction of location assumes that the individual spends sufficient time in the body of water for full incorporation of elements into the appropriate layer of the otolith. This assumption is likely to be broken for species capable of swimming through large gradients over short periods of time. The precise amount of time required is relatively unknown and unstudied (Elsdon and Gillanders 2003). One good example is the Patagonian toothfish in the Southern Ocean. Using microchemistry of the otolith core and edges, approximately 80% of those caught could be classified to their catch areas, while 63% of juveniles could be classified to their natal estuaries (Ashford et al. 2005).

Hundreds of microchemistry based research analyses have been executed on teleost species, but only three studies have been carried out in this field with elasmobranchs. A very basic analysis using an electron microprobe of school shark (*Galeorhinus galeus*) suggested there may be difference between pre-birth region and birth ring areas of the vertebrae in Sr:Ca ratios. However, only two samples were analysed, so there is no statistical hypothesis testing (Stevens and

West 1997). A study of jaw cartilage in the gummy shark (*Mustelus antarcticus*) in southwest Australia suggests population structure between some of the locations. This is particularly interesting since previous genetics based studies of this species found the region to include a single management unit. However, caution was warned in interpretation due to the sample size and lack of temporal factors in the study (Edmonds et al. 1996). The third example is of the round stingray (*Urobatis halleri*) in California. Forty-four elements were measured in an effort to validate vertebrae based age validation techniques. No population structure analysis was included as there was no geographic variation identified (Hale et al. 2006).

2.2.2 Genetics

Population genetics is a massive field in its own right. Here we consider only aspects as relating to sharks. Although on the surface, both genetics and microchemistry analyses identify very similar concepts of “population separation”, it should be noted that microchemistry results are not expected to be a proxy for genetic based stock discrimination (Campana and Tzeng 2000). Generally speaking, large mobile marine species will tend to show relatively little genetic population structure (Duncan et al. 2006), but evidence of significant genetic stock structure exists with several shark species (Heist 2004). Sharks will tend to have lower mutation rates in mitochondrial DNA (mtDNA) than mammals. However, the mtDNA mutation rates are still higher than nuclear DNA. As such, mtDNA is used more frequently for stock structure analysis while nuclear DNA may be more useful over evolutionary time scales (Heist 2004).

The nature of global distribution of the scalloped hammerhead shark (*Sphyrna lewini*) suggests that trans-oceanic migrations are occurring. However, a global mtDNA analysis of this species determined very little structure along coastlines, but significant structure across oceanic basins. This compares to other species that are known to be more oceanic and have demonstrated less population structure, such as the shortfin mako or soupfin sharks (Duncan et al. 2006). Further regional analysis determined that the separation was deep enough in the northwest Atlantic population that cryptic speciation had occurred, leading to morphological differences in vertebrae count and lack of ability to interbreed (Quattro et al. 2006).

In another global mtDNA study, this time of common blacktip sharks (*Carcharhinus limbatus*), significant separation was found between the western Atlantic (including Gulf of Mexico and Caribbean) and the rest of the world (including eastern Atlantic, indo-pacific, and eastern Pacific). Only moderate structure existed at these later regions. This supports relatively recent dispersal across the Pacific Ocean, but not across the Atlantic (Keeney and Heist 2006).

Nuclear genetic markers indicate movement by both, or either, sex, while mtDNA reflects only maternal lines. Use of both types can highlight differences in behaviour between sexes. Several species have shown evidence of sex based dispersal (Heist 2004). In a more detailed, but regional study of the same blacktip shark species described above, both nuclear microsatellites and mtDNA

were studied in the northwest Atlantic, Gulf of Mexico, and Caribbean. Here it was found to have significant structure at the maternally inherited mtDNA level, but very little at the nuclear DNA level. This could imply site fidelity for females, but migratory behaviour in males (Keeney et al. 2005).

Mixing rates do not need to be very high in order to prevent stock differentiation in genetics analysis. As little as 1% in most cases is sufficient (Bentzen et al. 1996). As such, it is worth differentiating between “management units” and “evolutionarily significant units”. Management units may be represented by shallow population structure while evolutionarily significant units would show deeper structure. Genetics analysis tends to be best suited for evolutionarily significant units but may not detect management units (Avise 2004).

Interesting inferences can be made from combining microchemistry and genetics analyses. In a (non-shark) example considering Sebastes spp. in and around the Gulf of St. Lawrence, otolith elemental analysis indicated stock structure within the gulf, but genetics analysis identified none. This would imply either a single spawning stock, or sufficient mixing between individual stocks to eliminate genetic separation (Campana et al. 2007).

2.2.3 Tagging

Tagging is often deployed for habitat use studies (Simpfendorfer and Heupel 2004), which is slightly different from classic stock structure and migration

analysis, but has some close parallels. Conventional external tagging in a capture-recapture form is simple, but only detects two points of migration. Also, recaptures can be as low as 1.9% and may extend project times frames well beyond what was originally planned (Elsdon and Gillanders 2003). More advanced tagging techniques include acoustic, radio or satellite telemetry tags. However, their applicability varies. For example, a benthic species that never surfaces is not suitable for satellite telemetry techniques (Elsdon and Gillanders 2003). It is possible to benefit from tagging studies you did not originate. Over 100,000 sharks have been tagged in the United States through various studies over the years. Data collected in southern Mexican waters can provide invaluable stock structure and migration behaviour information for the Mexican fisheries management (Bonfil 1997).

2.2.4 Other Approaches

Other lesser used approaches include direct observation, tracking of fish catch records, or deploying video systems (Simpfendorfer and Heupel 2004). Visual census is a form of direct observation that was deployed to track two species of relatively abundant reef sharks by swimming parallel to the reef crest on SCUBA and simply counting the sharks at different locations (Robbins et al. 2006). Other forms of direct observation include monitoring the natural marks on individuals. Sharks are typically large and long lived fish that can accumulate marks on the fins or body. These can be used as a natural tag to determine locations at various times. This combined with satellite tagging has been used to look at

population structure and/or migration habits of various shark species (Bonfil et al. 2005, Castro and Rosa 2005).

2.3 Microchemistry Techniques

While the previous section addressed the high level microchemistry approaches as applied to stock structure discrimination and migration habits, there are a variety of issues related to the technology itself that can have direct affect on how it would be deployed for our purposes. While the physics and detailed procedures behind the techniques are beyond the scope of this review, it is appropriate to describe issues that will affect sampling design or interpretation of results.

2.3.1 ICPMS / ICP-AES / EMP

There are three basic technologies utilised in microchemistry analysis (Summary in Table 1.3). In order of increasing sensitivity, they are the electron microprobe (EMP), inductively coupled plasma – atomic emission spectrometry (ICP-AES), and inductively coupled plasma – mass spectrometry (ICPMS). ICP-AES and ICPMS can perform solution based (SOL) analysis of a whole structure. ICPMS is highly sensitive with detection limits down to the parts per quadrillion or parts per trillion, allowing a wide range of elements to be analysed (Ludsin et al. 2006). This is much more sensitive than the ICP-AES, which will tend to be used for major elements such as calcium or phosphorous (Edmonds et al. 1996). Some studies require analysing only part of the structure in question, such as the core

to represent spawning area, or the edge to represent the location of death. These cases are best suited for either an EMP or a laser ablation (LA) unit attached to an ICPMS, which analyse solids in either a single spot or multiple spots along a transect of the structure. The size of the area being analysed can be down to the scale of micrometres (Elsdon and Gillanders 2003). The sensitivity of the LA-ICPMS exceeds EMP by two to four orders of magnitude (Campana et al. 1994).

A number of issues must be understood with ICPMS technology in order to ensure accurate interpretation of test results. Instrument drift, or change in sensitivity, can occur during analysis of large numbers of samples. Since the concentration readings can be affected by this, it is wise to randomise samples within the run to avoid any drift affecting one group more than others (Campana 1999). To minimise this drift, recalibration of the machine with known external standards periodically during the run of samples must be performed. Additionally it is useful to utilise a major element as an “internal standard” to ensure reliability of abundance measurements. Use of ratios to calcium is useful in this regard (Arai and Hirata 2006). However, in one particular LA-ICPMS application on round stingray vertebrae, some elements, such as strontium or phosphorous varied along with calcium, invalidating the utility of their ratios (Hale et al. 2006). If more than one lab is to be used in the analysis, intercalibration between labs is necessary (Campana et al. 1997). Even with the above precautions being made, precision is relatively low, in the range of 5% to 8%. As such, it is difficult to make accurate comparisons of elemental content between samples. This implies an inherent restriction in the utility of the procedure to qualitatively studying

relative distributions and concentrations within the sample (Durrant and Ward 2005).

2.3.2 Laser Ablation (LA) versus Solution Based (SOL)

Specific trade-offs are made in the decision to use LA versus SOL techniques. SOL, or whole structure, has somewhat of an averaging affect and will be useful in stock separation applications, while LA (and EMP) will be required along multiple spots in the structure for migration reconstruction (Elsdon and Gillanders 2003). With LA-ICPMS, it is possible to detect subtle shifts in elemental concentrations along a transect of the structure crossing from the core to the edge (Campana 1999). It must also be considered if using an elemental fingerprint based on SOL-ICPMS that little can be inferred from differences, other than the stocks are different. Furthermore, if the results do not indicate a difference, it does not necessarily mean the samples come from the same stock (Campana et al. 2000).

Accuracy, precision, and operational issues must be considered when deciding upon which technique to use. SOL has lower limits of detection (LOD) over LA (Ludsin et al. 2006) and is also more precise (Campana 1999). However, contamination in the preparation process can add significant variability or error into the results. LA preparation has less handling than SOL, so will be less prone to contamination (Ludsin et al. 2006). The three largest areas of contamination concern in preparation are: 1) equipment to grind and homogenise samples, 2) lab environment and digestion apparatus, and 3) analytical reagents used during

solution preparation. These are more significant in SOL over LA (Jarvis et al. 1992). As an example, using stainless steel instruments in preparation may introduce iron, chromium, nickel and molybdenum into the materials (Stevens and West 1997). Operational consideration must be given to time and cost factors. In a given time one can quantify more elements in SOL than in LA. However, in the preparation of the samples themselves, LA is much simpler and less time consuming, and multiple samples can be run from a single preparation (Ludsin et al. 2006). If using both LA and SOL in a project, extreme care must be exercised when comparing results between the two techniques (Campana et al. 1997). In a study specifically comparing LA versus SOL, strontium and barium concentrations were similar between the two techniques, but magnesium and zinc showed little similarity. With these two elements, SOL tended to estimate higher levels than LA (Ludsin et al. 2006).

2.4 Microchemistry Application to Shark Vertebrae

2.4.1 Shark Vertebrae Biology

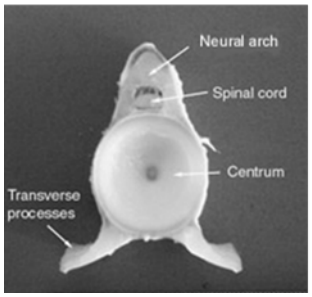
In order to determine whether microchemistry techniques may be successfully applied to shark vertebrae, it is important to have a basic understanding of the relevant biology, with some comparison between elasmobranchs and teleosts. Sharks are grouped as chondrichthyes or cartilaginous fish, meaning they have completely cartilaginous skeleton throughout life (Officer et al. 1995). The cartilage itself behaves and performs similarly to mammalian trabecular bone in stiffness, strength, and mineral fractions (Porter et al. 2006). It has been a widely held view that bony fish continually resorb minerals deposited in the bony skeleton, but chondrichthyans do not (Officer et al. 1995). This is important

because chondrichthyans like sharks lack the calcium carbonate otoliths of teleosts, so non-resoption of cartilage may mean it still has elemental composition that has been accumulated throughout its life (Edmonds et al. 1996).

The cartilage is unmineralised, but with considerable deposits of calcium phosphate within it in the form of a poorly crystalised apatite. Deposits occupy sites such as jaw, fin cartilage, chondrocranium, neural and haemal arches of the spin and, importantly for our purposes, the vertebrae centra (Clement 1992). The calcium to phosphorous ratio in the cartilage suggests that the structure more closely approximates that of apatite rather than bone, and can be approximated as $Ca_{10}(PO_4)_6(OH)_2$. Both the calcium and phosphorous are of structural importance, while other elements, such as strontium or lead, may reflect the environment. (Edmonds et al. 1996). Due to similar chemical and physical properties, strontium (and possibly barium) will replace calcium to some extent in the calcified tissues. The level of replacement may be relative to their environmental availabilities (Edmonds et al. 1996).

Vertebrae centra appear to grow by apposition, but without the need for remodelling. Patterns of crystallisation are very close to those of mammalian endochondrial ossification, with the exception of the final steps where calcified extracellular cartilage matrix is removed and replaced by bone. These final steps are not required for growth to occur and no bone forming osteoclasts have been described in chondrichthyans. Furthermore, there is no histological evidence of resorption. The vertebrae centra accumulate growth rings and the bow-tie shape

of the sagittal cross section allows the appositional growth to occur while the shark is growing with a fixed number of vertebrae (Plate 1.1) (Clement et al. 1992). However, there is some circumstantial evidence for vertebrae cartilage resorption in sharks. One shark was found with an injured spinal column and signs of localised resorption may have been activated temporarily as part of the inflammatory response, stopping once repair to the damaged skeletal tissue was complete (Officer et al. 1995). The uptake of some trace metals has been linked to calcium metabolism, which can get incorporated into the various cartilaginous tissues, such as the vertebrae centra (Vas et al. 1990). Thoracic vertebrae appear to be the most homogeneous, possessing a larger radius and clearer growth bands than other sections. Caudal or cervical bands may miss growth bands (Piercy et al. 2006).

A. 

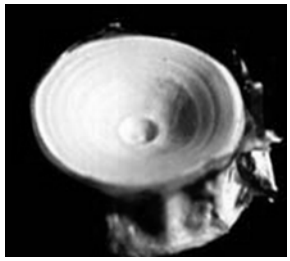
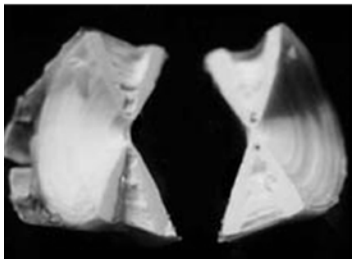
B. 
C. 

Plate 1.1 – Various photos of a porbeagle shark (*Lamna nasus*) vertebra. A: Entire vertebra section, including neural arch, spinal cord, and transverse processes, B: Vertebra centrum showing birth and growth rings, C: Sagittally cut vertebra demonstrating "bow tie" shape used in sectioning for ageing or laser ablation ICPMS preparation. (Canadian Shark Research Lab 2008)

2.4.2 Existing Vertebral Analysis Capabilities

While substantial work has been done in the field of age and growth of elasmobranchs using vertebrae, little has been done at the elemental level. The

one study using vertebral microchemistry for population structure analysis in school sharks previously described was actually a proof of concept approach that only utilised two samples (Stevens and West 1997). An analysis of the round stingray (*Urobatis halleri*) determined that the growth bands from a photomicrograph matched the LA-ICPMS signal for calcium very well, indicating a strong correlation between assessed age and growth bands. However, the correlation was much stronger in younger animals (<5 years) than it was for older ones (>11 years). This is likely due to the physical size of the bands and detectable limits of the LA-ICPMS system. There was occasionally inter-annual variation in bands, which was attributed to stress, temperature change, or food availability. Since only one site was sampled, no stock separation analysis was performed (Hale et al. 2006). Also related to age and growth verification is "bomb dating". Previously caught, long lived, shark species have been tested for radiocarbon 14C in shark vertebrae and comparing them to known levels in the oceans from atomic bomb testing in the 1950's and 1960's. This has helped confirm age and growth estimation processes. Again, no stock structure analysis was performed from this (Campana et al. 2002).

2.4.3 Conclusion: Potential for Vertebrae Microchemistry Based Stock Structure Analysis in Sharks

Based on the extensive microchemistry work that has been performed in teleost fish otoliths, the very small amount of elemental analysis of elasmobranch cartilage, and the biological parallels between otoliths and vertebral cartilage, I would conclude that vertebral microchemistry based stock structure analysis in

sharks is very feasible. Furthermore, it may be highly complementary to genetics and tagging based studies to gain an in depth understanding of the migration and breeding of a species.

However, a number of recommendations arise from this review. From the animal sampling process, the size/age of all samples should be similar, particularly if using SOL-ICPMS techniques. Both sexes should be collected and their relevant data analysed for sex based differences. If looking at younger sharks, the samples should be collected over as short a period as possible to minimise temporal issues. In the preparation and processing of samples, thoracic vertebrae should be utilised and substantial caution is warranted to minimise contamination. When using the ICPMS equipment, it would be preferred to run all samples through a single run on a machine in a single lab. Randomising samples within the run and calibrating the machine against external standards will help minimise issues with instrument drift. With appropriate care due to any project analysing trace elements, I believe the technique can be successfully applied to shark populations, adding to the tool set to help us gain further insight into the behaviour of these challenging species.

Table 1.1 – Selected Population Structure Studies Using Microchemistry Analysis. This list is intended to show reasonable representation of species being studied, but is not a comprehensive view of all microchemistry based studies.

Author	Year	Species Tested	Body Part	Comment on Approach Used
Arai & Hirata	2006	Japanese eels (*Anguilla japonica*)	Otolith	Separate marine and freshwater life phases - migration habits
Arai et al	2007	Chum salmon (*Oncorhynchus keta*)	Otolith	Use SO, LA ICP-MS and Microprobe together to determine spawning and habitat in two Japanese rivers
Arkhipkin et al	2004	Patagonian longfin squid (*Loligo gahi*)	Statolith	Samples from Falkland Islands area to study structure based on geography and cohorts spawned at different times of year
Arslan & Secor	2005	American eel (*Anguilla rostrata*)	Otolith	Analysis of heavy metal uptake to determine stock structure against pollution levels of the Hudson River
Ashford et al	2005	Patagonian toothfish (*Dissostichus eleginoides*)	Otolith Edge	Identify location of capture by otolith edge elemental analysis
Bacon et al	2004	Chinook salmon (*O. tshawytscha*)	Otolith	Strontium analysis to determine rearing and migration history in Skagit River, Washington USA
Bergenius et al	2004	Three coral reef fishes	Otolith	Solution based comparison of three species along four sites of GBR
Campana et al	1994	Atlantic cod (*Gadus morhua*)	Otolith	One of the earlier pioneering efforts to utilise the technique, performed in the northwest Atlantic
Campana et al	1999	Atlantic cod (*Gadus morhua*)	Otolith	Test for stock mixing of populations in overwintering grounds at mouth of Gulf of St. Lawrence
Campana et al	2007	*Sebastes* spp.	Otolith	Tracking seasonal migration
Chittaro et al	2005	French grunt (*Haemulon flavolineatum*)	Otolith	Samples from Belize mangroves and reefs to analyse habitat as well as local spatial scales
Chittaro et al	2006	*Stegastes partitus*	Otolith Edge	Comare embryo otoliths with edge of juvenile. More ontogenetic variability than pop structure, but can lead to connectivity.
Courtemanche et al	2005	Brook trout (*Salvelinus fontinalis*)	Scales	Nonlethal use of microprobe analysis on scales to determine anadromous behaviour
Coutant & Chen	1993	Striped bass (*Morone saxatilis*)	Scales	Pioneering study to identify environmental influences from various samples collected through south-east USA
Edmonds et al	1996	Gummy Shark (*Mustelus antarcticus*)	Jaw Cartilage	Elemental analysis of jaw cartilage at various locations
Fowler et al	2005	Snapper (*Pagrus auratus*, Sparidae)	Otolith	Compare otoliths along transverse section to origin and life movement
Fry	1981	Brown shrimp (*Penaeus aztecus*)	Abdomen	Natural stable carbon isotope analysis to determine migration and structure along Texas coast
Gauldie et al	1995	*Trachyrincus murrayi* & *Coryphaenoides mediterraneus*	Otolith	Proton microprobe on two deep water species to determine temperature profile using oxygen isotope
Gillanders et al	2001	Two-banded bream (*Diplodus vulgaris*)	Otolith	Determination of stock structure at relatively small scales of 100m - 10km in Spanish Mediterranean Sea
Gillanders & Kingsford	1996	*Achoerodus viridis*	Otolith Core	Compare recruit otolith with core of adult otoliths to determine settlement behaviour
Gillanders & Kingsford	2003	3 Sparid spp. (*P. auratus, R. sarba, A. australis*)	Otolith	Analysed from several sites along NSW, Australia coastal estuaries to determine connectivity
Halden et al	1995	Arctic charr (*Salvelinus alpinus*)	Otolith	Microprobe analysis of otolith to determine Sr and Zn content to infer anadromy
Jonsdottir et al	2006	Icelandic cod (*Gadus morhua*)	Otolith	Analysis of 12/17 sites at 2 different years to determine stock structure on geographic and temporal scales
Kennedy et al	2000	Atlantic salmon (*Salmo salar*)	Otolith	Various fish tissues compared with stream water to test strontium isotopes as marker for environmental history
Kimura et al	2000	YOY bay anchovy (*Anchoa mitchilli*)	Otolith	Strontium analysis to infer up-estuary dispersal of larvae in known salinity gradient of Chesapeake Bay, USA
Kraus & Secor	2004	White perch (*Morone americana*)	Otolith	Sr:Ca ratio used to determine timing of movements between various salinities
Miller	2007	Staghorn sculpin (*L. armatus*)	Otolith	Consider three locations on otolith of juveniles collected from five sites at three estuaries in USA west coast
Miller & Shanks	2004	Black rockfish (*Sebastes melanops*)	Otolith	Combining physical otolith characteristics with microchemistry to larval dispersal on Oregon coast
Milton & Chenery	2001	Hilsa (*Tenualosa ilisha*)	Otolith	Bangladesh and elsewhere in the range analysed for structure and comparison to previous genetic and morphological studies
Milton & Chenery	2003	Tropical shad hilsa (*Tenualosa ilisha*)	Otolith	Compare $^{87}Sr/^{86}Sr$ isotope ratios and Sr/Ca ratios to determine diadromous movement patterns
Patterson & Kingsford	2005	*Acanthochromis polyacanthus*	Otolith	Two latitudes on Great Barrier Reef samples to consider structure at spatial, temporal and brood levels
Patterson et al	1999	Nassau grouper (*Epinephelus striatus*)	Otolith	Analysing otoliths for stock discrimination between various locations in Belize and Bahamas
Patterson et al	2004	*Pomacentrus coelestis*	Otolith	Compare natal and post-settlement region of otolith to determine settlement behaviour
Patterson et al	2004	*Pomacentrus coelestis*	Otolith	Controlled experiment at One Tree Island to study effects of plume and inter-reef waters
Patterson et al	2005	*Pomacentrus coelestis*	Otolith	Near natal sections of otolith used to determine variation in source reef
Rooker et al	2001	Pacific bluefin tuna (*Thunnus orientalis*)	Otolith	Assessed differences between three nursery areas in Asian Pacific Ocean across three year period
Secor & Piccoli	1996	Striped bass (*Morone saxatilis*)	Otoliths	Strontium analysed as proxy for salinity to determine age and sex based migration habits on Hudson River estuary
Secor et al	2002	Bluefin tuna (*Thunnus thynnus*)	Otolith	Test between two labs of slightly varying ICP-MS techniques to compare Atlantic and Mediterranean 1 year olds
Stevens & West	1997	School and Gummy Sharks (*G. galeus & M. antarcticus*)	Vertebrae	Very basic analysis of vertebrae using microprobe techniques to determine if useable for stock discrimination in south eastern Australia
Swan et al	2006	*H. dactylopterus* & *M. merluccius*	Otolith	Both solution and laser ablation used to determine stock separation in northeast Atlantic and Mediterranean
Thresher & Proctor	2007	Orange roughy (*Hoplostethus atlanticus*)	Otolith	Identify population structure and life history from elemental composition
Vasconcelos et al	2007	5 teleost spp] in Portugal	Otolith	Estimate connectivity between estuarine nursery grounds
Zlokovitz et al	2003	Striped bass (*Morone saxatilis*)	Otolith / fillet	Comparison of Sr levels and PCB in fillet tissue to reconstruct migration habits in Hudson River, USA

Table 1.2 – Population Structure Studies Including Shark Species

Author	Year	Species Tested	Body Part	Technique	Comment on Approach Used
Bonfil	1997	36 species in Gulf of Mexico/Caribbean	n/a	Fishery records	Approximation of distribution and stock identification from fishery related records, primarily tagging from US studies
Bonfil et al	2005	White shark (Carcharadon carcharias)	Fin	Natural marks and satellite tagging	Satellite transmitting tags and high resolution photos of individuals used to track migration in Indian Ocean
Castro & Rosa	2005	Nurse shark (*Ginglymostoma cirratum*)	Individual appearance	Natural marks	Used distinctice physical marks to determine population characteristics in Brasil
Castro et al	2007	Whale shark (*Rhincodon typus*)	Molecular	Genetics	mtDNA analysed across the global distribution to look for structure
Duncan et al	2006	Scalloped hammerhead (*Sphyrna lewini*)	Molecular	Genetics	Global phylogeographic analysis of mtDNA inferring evolutionary timescales
Edmonds et al	1996	Gummy Shark (*Mustelus antarcticus*)	Jaw Cartilage	Microchemistry	Elemental analysis of jaw cartilage at various locations
Feldheim et al	2001	Lemon shark (*Negaprion brevirostris*)	Molecular	Genetics	Microsatellite structure analysis of samples collected across west Atlantic
Gaida	1997	Pacific angel shark (*Squatina californica*)	Molecular	Genetics	Genetics analysis around relatively small geographic location of California Channel Islands
Gardner & Ward	1998	Gummy Shark (*Mustelus antarcticus*)	Molecular / Vertebrae	Genetics / Vertebrae Count	Allozyme and mtDNA as well as vertebrae counts
Heist & Gold	1999	Sandbar shark (*Carcharhinus plumbeus*)	Molecular	Genetics	Microsatellite analysis complementing 1995 study of same species that used mtDNA
Heist et al	1995	Sandbar shark (*Carcharhinus plumbeus*)	Molecular	Genetics	mtDNA analysed at sites from US Atlantic and Gulf of Mexico to determine stock structure
Heist et al	1996	Sharpnose shark (*Rhizoprionodon terraenovae*)	Molecular	Genetics	mtDNA analysed at sites from US Atlantic and Gulf of Mexico to determine stock structure
Heist et al	1996	Shortfin mako (*Isurus oxyrinchus*)	Molecular	Genetics	mtDNA analysis to consider structure of sites across the world
Holts & Bedford	1993	Shortfin mako (*Isurus oxyrinchus*)	n/a	Tagging	Acoustic telemetry tags to determine horizontal and vertical movements off California
Keeney & Heist	2006	Blacktip shark (*Carcharhinus limbatus*)	Molecular	Genetics	Global phylogeographic analysis of mtDNA inferring evolutionary timescales
Keeney et al	2003	Blacktip shark (*Carcharhinus limbatus*)	Molecular	Genetics	mtDNA analysed at sites from US Atlantic and Gulf of Mexico to determine philopatry for femals in nursery areas
Keeney et al	2005	Blacktip shark (*Carcharhinus limbatus*)	Molecular	Genetics	Microsatellite and mtDNA analysis of nurseries in northwestern Atlantic, Gulf of Mexico, and Caribbean Sea
Kerr et al	2006	White shark (*Carcharadon carcharias*)	Vertebrae	Radiocarbon / growth rings	Radiocarbon age validation compared to vertebral growth rings to determine habitat, age, and trophic structure
Lessa et al	1999	Whitetip Shark (*Carcharhinus longimanus*)	Vertebrae	Age, growth and size	Compare size and growth rates for given age at various locations
Lewallen et al	2007	Leopard shark (*Triakis semifasciata*)	Molecular	Genetics	mt and nuclear DNA population structure along California coast
Punt et al	2000	School shark (*Galeorhinus galeus*)	n/a	Tagging / Genetics	Developed model based on previous tagging and genetics studies
Quattro et al	2006	Scalloped hammerhead (Sphyrna lewini)	Molecular / Vertebrae	Genetics / Vertebrae Count	Through mtDNA, nuclear DNA and vertebrae counts, cryptic speciation is determined to have occurred with the western north Atlantic populations
Robbins et al	2006	Whitetip & gray reef (*T. obesus & C. amblyrhynchos*)	n/a	Visual census	Visual census of two species at two locations in Great Barrier Reef
Schrey & Heist	2003	Shortfin mako (*Isurus oxyrinchus*)	Molecular	Genetics	Microsatellite analysis to complement previous mtDNA study on global scale
Sims et al	2001	Dogfish (*Scyliorhinus canicula*)	n/a	Tagging	Acoustic and number tags deployed to determine short term activities and long term philopatric behaviour
Stevens & West	1997	School and Gummy Sharks (*G. galeus & M. antarcticus*)	Vertebrae	Microchemistry	Very basic analysis of vertebrae using microprobe techniques to determine if useable for stock discrimination in south eastern Australia
Stow et al	2006	Grey nurse shark (*Carcharias taurus*)	Molecular	Genetics	mtDNA analysis of structure between South Africa, east coast and west coast Australia
Weng et al	2007	White shark (*Carcharadon carcharias*)	n/a	Tagging	Satellite telemetry study of 20 adults in the eastern Pacific Ocean migration habits

Table 1.3 – Elements and Techniques of Microchemistry Based Studies. LA: Laser Ablation ICPMS, Sol: Solution Based ICPMS (eg. Whole otolith), Prb: Electron microprobe. (note: the Stevens & West, 1997, and Hale et al, 2006, studies include additional elements not shown in table)

Author	Year	Species Tested	Body Part	LA	Sol	Prb	Ag	Al	B	Ba	Bi	Ca	Cd	Ce	Co	Cr	Cu	Fe	Gi	Hg	K	Li	Mg	Mn	Na	Ni	O	P	Pb	Rb	S	Sn	Sr	V	Zn	Zr	Total
Arai & Hirata	2006	Japanese eels (*Anguilla japonica*)	Otolith	x			x			x		x	x		x	x		x					x	x					x			x	x	x	x		14
Arai et al	2007	Chum salmon (*Oncorhynchus keta*)	Otolith	x	x	x				x		x				x							x	x									x		x		7
Arkhipkin et al	2004	Patagonian longfin squid (*Loligo gahi*)	Statolith		x					x		x	x										x	x					x				x				7
Arslan & Secor	2005	American eel (*Anguilla rostrata*)	Otolith		x			x			x		x		x		x		x					x		x			x					x	x		11
Ashford et al	2005	Patagonia toothfish (*D. eleginoides*)	Otolith Edge	x						x													x	x									x				4
Bacon et al	2004	Chinook salmon (*O. tshawytscha*)	Otolith			x						x																					x				2
Bergenius et al	2004	Three coral reef fishes	Otolith		x					x		x												x									x				4
Campana et al	1994	Atlantic cod (*Gadus morhua*)	Otolith	x					x	x		x						x					x							x		x	x		x		9
Campana et al	1999	Atlantic cod (*Gadus morhua*)	Otolith		x					x												x	x	x									x				5
Campana et al	2007	*Sebastes* spp.	Otolith		x					x		x	x								x	x	x	x	x								x				9
Chittaro et al	2005	French grunt (*Haemulon flavolineatum*)	Otolith	x						x							x					x	x					x	x		x	x		x		9
Chittaro et al	2006	*Stegastes partitus*	Otolith Edge	x						x			x	x			x					x	x	x				x	x		x	x		x		12
Courtemanche et al	2005	Brook trout (*Salvelinus fontinalis*)	Scales			x						x																					x				2
Coutant & Chen	1993	Striped bass (*Morone saxatilis*)	Scales	x																													x				1
Edmonds et al	1996	Gummy Shark (*Mustelus antarcticus*)	Jaw Cartilage		x			x	x	x		x	x				x	x			x		x	x	x			x	x		x		x		x		16
Fowler et al	2005	Snapper (*Pagrus auratus, Sparidae*)	Otolith	x						x			x		x	x	x						x	x					x				x		x		10
Gauldie et al	1995	*T. murrayi & C. mediterraneus*	Otolith			x																					x						x				2
Gillanders et al	2001	Two-banded bream (*Diplodus vulgaris*)	Otolith		x					x			x									x		x									x				
Gillanders & Kingsford	1996	*Achoerodus viridis*	Otolith Core		x			x		x		x			x		x			x			x	x		x			x			x	x			x	13
Gillanders & Kingsford	2003	Sparid (*P. auratus, R. sarba, A. australis*)	Otolith		x					x												x		x									x				4
Halden et al	1995	Arctic charr (*Salvelinus alpinus*)	Otolith			x																											x		x		2
Hale et al	2006	Round stingray (*Urobatis halleri*)	Vertebrae	x				x		x		x		x	x	x	x	x					x	x	x	x			x	x		x	x	x	x	x	44
Jonsdottir et al	2006	Icelandic cod (*Gadus morhua*)	Otolith		x					x												x	x	x									x				5
Kennedy et al	2000	Atlantic salmon (*Salmo salar*)	Otolith		x																												x				1
Kimura et al	2000	YOY bay anchovy (*Anchoa mitchilli*)	Otolith			x						x																					x				2
Kraus & Secor	2004	White perch (*Morone americana*)	Otolith			x						x																					x				2
Miller	2007	Staghorn sculpin (*L. armatus*)	Otolith	x						x		x											x	x					x				x				6
Miller & Shanks	2004	Black rockfish (*Sebastes melanops*)	Otolith	x						x		x											x	x					x				x		x		7
Milton & Chenery	2001	Hilsa (*Tenualosa ilisha*)	Otolith	x				x		x		x										x	x	x	x								x		x		9
Milton & Chenery	2003	Tropical shad hilsa (*Tenualosa ilisha*)	Otolith	x								x																					x				2
Patterson & Kingsford	2005	*Acanthochromis polyacanthus*	Otolith		x					x		x	x				x						x	x									x				7
Patterson et al	1999	Nassau grouper (*Epinephelus striatus*)	Otolith		x					x		x																	x				x		x		5
Patterson et al	2004	*Pomacentrus coelestis*	Otolith	x						x		x											x	x									x				5
Patterson et al	2004	*Pomacentrus coelestis*	Otolith	x						x		x											x	x									x				5
Patterson et al	2005	*Pomacentrus coelestis*	Otolith	x						x		x											x	x									x				5
Rooker et al	2001	Pacific bluefin tuna (*Thunnus orientalis*)	Otolith		x					x		x										x	x	x									x				6
Secor & Piccoli	1996	Striped bass (*Morone saxatilis*)	Otolith			x						x																					x				2
Secor et al	2002	Bluefin tuna (*Thunnus thynnus*)	Otolith		x					x		x									x		x	x	x								x				7
Stevens & West	1997	*G. galeus & M. antarcticus* sharks	Vertebrae			x				x		x	x		x	x	x	x		x	x			x		x		x			x		x		x		19
Swan et al	2006	*H. dactylopterus & M. merluccius*	Otolith	x	x					x		x										x	x	x					x	x			x		x		9
Thresher & Proctor	2007	Orange roughy (*H. atlanticus*)	Otolith			x											x			x									x				x		x		5
Vasconcelos et al	2007	5 teleost spp of Portugal	Otolith		x					x		x	x				x				x	x	x	x	x	x			x				x		x		13
Zlokovitz et al	2003	Striped bass (*Morone saxatilis*)	Otolith / fillet			x																											x				1
		% Studies Using This Element		40%	42%	23%	2	12	5	70	2	65	26	5	14	12	26	12	2	7	12	26	58	65	14	12	2	5	35	12	5	14	95	7	40	5	

3 Method Development – Determining Stock Structure in Shark Populations Using Inductively Coupled Plasma Mass Spectrometry (ICPMS) Microchemistry Techniques

3.1 Introduction

Sharks play an important role in the world's oceans as apex predators (Stevens et al. 2000a, Heithaus 2004); and recent declines in some populations have highlighted the need for improved management to ensure ongoing provision of ecosystem services and the maintenance of biodiversity (Heithaus et al. 2008). Sustainable management for sharks is especially important because many species have K-selected life history that result in low rates of population increase (Musick et al. 2000). The development of sustainable management systems depends on the availability of sound knowledge of several aspects of the biology and ecology of shark populations, including life history, population structure, changes in abundance and susceptibility to fisheries gear (Simpfendorfer and Donohue 1998). While the life history of sharks has been increasingly well studied (Carrier et al. 2004), stock structure has been poorly investigated even though knowledge of it dictates the spatial extent of management units. Improved knowledge of stock structure in shark populations will help improve the management of shark populations through identifying the appropriate spatial scales at which actions are applied.

Most shark population structure analyses to date have been based on genetic markers (Keeney and Heist 2006, Ovenden et al. 2009), physical tagging (Stevens et al. 2000b), life history (Lenanton et al. 1990) or electronic tagging (Hunter et al. 2006). While effective within their scope, all techniques have limitations. Genetics can link fish to a population, but not to a specific geographic region (Ashford et al. 2005) because of the small amounts of migration between regions that can result in homology (Bentzen et al. 1996). Genetics thus reflects structure on a more evolutionary time scale as opposed to short-term scales relevant to day-to-day management (Avise 2004). Physical tagging on the other hand provides information on short time scales, but it can be limited by the distribution of release and recapture effort and can be logistically challenging and costly (Ashford et al. 2005). Electronic tagging can provide data independent of recapture effort, but is expensive at the scale required to reliably identify stock structure (Sibert and Nielsen 2001).

A technique for examining stock structure that has become commonplace for use with teleost fishes is elemental analysis of their calcified structures using inductively coupled plasma mass spectrometry (ICPMS). Although usually applied to otoliths, several other structures of fish have been analysed using this technique, including scales, fin spines, eye lenses, etc. (Elsdon and Gillanders 2003). This approach was in its infancy in 1990 (Coutant 1990), and by 2005 nearly 20% of the many hundreds of otolith related studies were chemistry based (Campana 2005). Several factors drive elemental variation in fish otoliths,

including water hydrodynamics, temperature, diet, ontogenetics, and physiological regulation (Bergenius et al. 2005). Otoliths provide an ideal structure for examining stock structure because material deposited within them is not reworked and so provides a permanent record of elemental composition (Campana and Neilson 1985). For examining stock structure, a suite of elements is examined to determine if individuals from different locations carry different "elemental fingerprints", thus potentially representing separate stocks.

Two ICPMS approaches have been used in otolith studies: whole structure solution analysis (SOL-ICPMS) where the whole otolith is dissolved and analysed to give an elemental composition representative of the whole of an individual's life; and laser ablation (LA-ICPMS) where a laser is used to sample along a transect of the otolith to provide a time series of elemental composition or a composition at a specific point in life. Both approaches have proven useful in determining stock structure in teleosts. In one analysis of otolith edges of Patagonia toothfish using LA-ICPMS, 80% of samples could be classed back to their catch site, while 63% of juveniles could be classed to their natal estuaries (Ashford et al. 2005). In a SOL-ICPMS whole otolith analysis of five estuarial fish species in Portugal, success rate of predicting estuaries was between 70% and 92%, depending on species (Vasconcelos et al. 2007).

Given the utility of ICPMS approaches in the study of teleost stock structure, similar application to sharks may provide a useful tool for improving management of shark populations. One of the challenges in adapting this technique for sharks

is that they do not have otoliths to serve as a permanent record of elemental composition (Edmonds et al. 1996). They do however have cartilaginous vertebrae that accumulate minerals (mostly Ca and P) over time, thus producing concentric areas reflecting the season or time of year in which material has been deposited (Cailliet et al. 2006).

To be effective as a stock structure technique, shark vertebrae must provide a permanent record of mineralisation. Sharks have completely cartilaginous skeleton throughout life (Officer et al. 1995) and this behaves and performs similarly to mammalian trabecular bone in stiffness, strength, and mineral fractions (Porter et al. 2006). It has been a widely accepted that bony fish continually resorb minerals deposited in the bony skeleton, but chondrichthyans do not within their cartilaginous vertebrae (Clement 1992, Officer et al. 1995). This non-resorption of minerals within the cartilage means that it retains elemental composition that has been accumulated throughout its life (Edmonds et al. 1996). The Ca to P ratio in the cartilage suggests that the structure more closely approximates that of apatite rather than bone, and can be approximated as $Ca_{10}(PO_4)_6(OH)_2$ (Edmonds et al. 1996). Both the Ca and P are of structural importance, while other elements, such as Sr or Pb, may reflect the environment (Edmonds et al. 1996). Due to similar chemical and physical properties, Sr, and possibly Ba, will replace Ca to some extent in the calcified tissues. The level of replacement may be relative to their environmental availabilities (Edmonds et al. 1996). The vertebral centra appear to grow by apposition, but without the need for remodelling (Clement 1992). Patterns of crystallisation are very close to that of mammalian endochondrial ossification, with the exception of the final steps

where calcified extracellular cartilage matrix is removed and replaced by bone. These final steps are not required for growth to occur and no bone forming osteoclasts have been described in chondrichthyans (Clement 1992). The vertebrae centra accumulate growth rings and the biconcave shape allows the appositional expansion to occur while the shark is growing with a fixed number of vertebrae (Plate 2.1). Thus shark vertebrae may provide a suitable tissue for conducting elemental studies.

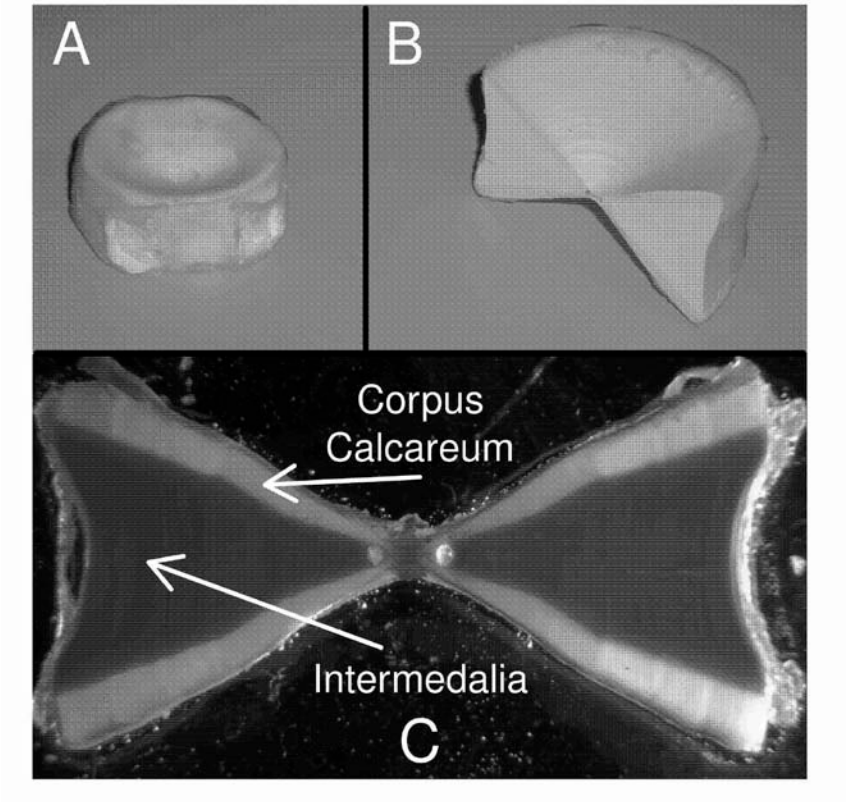


Plate 2.1 – Vertebrae of sharks. A: Whole vertebra centrum of a pig eye shark (*Carcharhinus amboinensis*), B: Sagitally cut vertebra centrum of great hammerhead shark (*Sphyrna mokarran*) demonstrating "bow tie" shape used in sectioning for laser ablation, C: Thin section of Australian blacktip shark (*Carcharhinus tilstoni*) vertebra prepared for LA-ICPMS, identifying the corpus calcareum at the light coloured edges and the intermedalia in the dark coloured centre.

While substantial work has been performed in the field of age and growth of elasmobranchs using vertebrae (Cailliet and Goldman 2004), little has been done at the elemental level. (Stevens and West 1997) demonstrated variation in Sr:Ca ratios in pre-birth sections of vertebrae between pupping areas of school sharks (*Galeorhinus galeus*) using very limited samples in a proof of concept study. An analysis of the round stingray (*Urobatis halleri*) vertebrae determined that the growth bands from a photomicrograph matched the LA-ICPMS signal for Ca, indicating a correlation between assessed age, growth bands and elemental composition (Hale et al. 2006). This further supported the lack of remodelling of minerals with the vertebrae, but since only one site was sampled, no stock separation analysis was performed. The most comprehensive use of ICPMS to study stock structure in sharks was conducted by Edmonds et al (1996) using jaw cartilage. This study demonstrated variation in elemental fingerprints of gummy sharks (*Mustelus antarcticus*) among three locations in southwestern Australia, suggesting the possible separation of stocks.

The purpose of this study was to determine if ICPMS microchemistry techniques could be used to examine stock structure in shark populations by testing assumptions necessary to achieve positive results. Specific aims were to (1) demonstrate if ICPMS provided repeatable results (i.e. results of repeated analysis of samples were within the bounds of machine accuracy), (2) determine if results were consistent between different regions within individual vertebrae (i.e. corpus calcareum versus intermedalia) and different positions on the vertebral column (i.e. different vertebrae from the same individual give the same results), (3) quantify the effect of surface contamination from the preparation

process, (4) examine the effect of size (and hence age) on elemental composition, and (5) compare elemental composition between two geographic regions to investigate if the technique can provide evidence for separate stocks.

3.2 Materials and Procedures

3.2.1 Sample collection

Three species of shark were examined as part of this study: scalloped hammerhead (*Sphyrna lewini*), Australian blacktip (*Carcharhinus tilstoni*), and milk sharks (*Rhizoprionodon acutus*). All sharks were collected at inshore locations along the Queensland east coast by observers on commercial fishing vessels between September 2007 and August 2008. Collections occurred at three locations: Princess Charlotte Bay (PCB; 14.0º S, 144.0º E), Bowling Green Bay (BGB; 19.3º S, 147.2º E), and Brisbane (BNE; 27.0º S, 153.5º E). Attempts were made to minimise size variation of animals where possible, with *C. tilstoni* ranging from 520 mm to 1,330 mm, *R. acutus* 415 mm to 720 mm, and *S. lewini* from 510 mm to 1,350 mm. Sections of vertebral columns were cut from animals onboard vessels and frozen for future sample preparation.

3.2.2 Sample preparation and analysis

3.2.2.1 SOL-ICPMS

Shark vertebrae were defrosted and the neural arch and lateral processes removed prior to cleaning away as much organic tissue as possible. Individual

vertebra were separated and soaked in 5% sodium hypochlorite solution for ~30 minutes. Samples were then dried for ~18 hours in a drying oven at 50ºC. In order to eliminate any maternal effects on the elemental composition, the pre-birth section was eliminated by drilling out material inside the birth ring with a cobalt alloy drill bit. Diameters of birth rings were measured on whole and sectioned vertebrae using an optical micrometer. For *C. tilstoni*, *R. acutus*, and *S. lewini*, the diameters removed were 3.97mm, 2.84mm, and 4.76mm, respectively. Following removal of pre-birth material each vertebra was crushed and homogenised for 90 seconds in a zirconium based ceramic dish using a Rocklabs Benchmill 1A. The crushing dish was scrubbed after use for 30 seconds and rinsed thoroughly with Milli-Q water to minimise possible cross-contamination between samples. To digest this powder into a nitric acid solution, 0.10 gram was measured and placed in an acid washed 25ml beaker with 2.0ml of 65% Suprapure HNO_3 nitric acid. These were covered and heated to boil for ten minutes. In order to dissolve residual organic material, 0.5ml H_2O_2 hydrogen peroxide was added to each beaker and simmered for an additional sixty minutes. The entire beaker contents were combined with Milli-Q water to create 100ml of store solution for each sample. Blank samples were prepared in identical fashion, but without inclusion of vertebra powder. All digestions occurred under a laminar flow hood.

A slight variation of the above process was utilised when preparing for processing duplicate sample analyses. For the digestion process, 0.10 gram of the crushed and homogenised vertebra powder was placed in a 25ml beaker with 6.0ml of 32.5% Suprapure HNO_3 nitric acid. However, instead of heating the sample, it

was left covered and undisturbed for ~ 72 hours. This allowed time for full dissolution of sample, but left residual organic material either floating on the surface or settled on the bottom of the beaker. 3.0ml of the solution was carefully drawn from the centre, leaving any organic matter behind, and added to a 50ml volumetric flask. The flask was filled with Milli-Q water to create the store solution for the analysis.

An initial, semi-quantitative scan using SOL-ICPMS of four random samples was made for sixty elements to determine which samples were consistently above the limit of detection (LOD). From this activity, and based on the literature, it was determined that elements of interest that would most likely have biological significance included Ba, Ca, Cu, Mg, Mn, P, Sr, and Zn (Edmonds et al. 1996, Patterson and Kingsford 2005, Swan et al. 2006, Campana et al. 2007, Vasconcelos et al. 2007). Given the likelihood of instrument drift, and to minimise effects of any calibration errors, all samples for both SOL- and LA-ICPMS were randomised (Campana 1999).

After semi-quantitative scans, the samples were analysed by a Varian 820-MS (Melbourne, Australia) Inductively Coupled Plasma Mass Spectrometer (ICPMS). ^{137}Ba, 43Ca, 65Cu, 24Mg, ^{55}Mn, ^{31}P, ^{88}Sr and ^{66}Zn were monitored for quantification. Ga, Y and In were added into all solutions including the blanks to act as the internal standard in order to correct for matrix effects and the instrumental drift. A series of commercially available multi-element standard solutions were used to externally calibrate the instrument. Detection limits (3

standard deviation of 10 blank measurements) for these elements were 0.05 µg/g (Ba), 6 µg/g (Ca), 0.03 µg/g (Cu), 0.25 µg/g (Mg), 0.05 µg/g (Mn), 10 µg/g (P), 0.02 µg/g (Sr), and 2 µg/g (Zn).

Repetitive sample testing of SOL-ICPMS was achieved by processing multiple (between seven and nine) adjacent vertebrae on five individual sharks (two *C. tilstoni* from PCB, two *C. tilstoni* from BGB, and one *S. lewini* from PCB). To determine the importance of position on the vertebral column from which the sample was selected, vertebrae were analysed via SOL-ICPMS from the cervical, thoracic and caudal regions of five *C. tilstoni* of similar sizes from PCB.

3.2.2.2 LA-ICPMS

For LA-ICPMS, each vertebra centrum was cleaned and bleached as with the SOL-ICPMS preparation. A thin section of 500 microns was cut sagittally using a Buehler low speed IsoMet diamond tipped rotary saw (series 15HC wafering blade with tap water as coolant), then secured on a glass slide (25mm X 45mm) with clear polyester casting resin. The samples were lightly buffed for ~5 seconds with 3 micron lapping film while being rinsed with tap water. They were not polished due to potential for scratching the relatively soft sample material.

To analyse elemental composition of the vertebrae sections, a Coherent Geolas Pro 193 nm ArF Excimer laser unit was connected to a Varian 820-MS (Melbourne, Australia) Inductively Coupled Plasma Mass Spectrometer (ICPMS)

via 3 metre of Tygon tubing (inner diameter 3.2mm). Vertebrae section slides were set on a chamber insert (55mm x 8mm height) and loaded into the circular sample chamber (55mm diameter x 15mm height). He was used as the carrier gas, flowing at 235 ml/min. The instrument was optimised to the maximum sensitivity (238U signal > 2 million cps for NIST 610) while keeping the oxide ratio low (ThO/Th ~0.3%) and 238U/232Th ~ 1. Other instrumental parameters were: RF Power 1300 W, sampling depth 5mm, plasma flow 16.5 l/min, auxiliary flow 1.65 l/min, carrier gas flow 0.97 l/min, and dwell time 20 ms. The laser repetition rate was fixed at 10 Hz on energy density of 6 J/cm^2. A 31 µm laser beam was used and the scanning speed was set at 62 µm/s in a step repeat pattern. National Institute of Standards and Technology (NIST) 610 glass standard was measured before and after each sample slide, the data was used to correct for instrumental drift. Prior to measuring each transect, a cleaning run was made to remove surface contamination from the sample.

To measure the possible surface contamination effects from the wafering blade being in contact with the samples, a JEOL JXA 8200 electron probe micro-analyser (EPMA) was used to determine approximate elemental composition. A semi-quantitative energy dispersive spectrometry (EDS) was performed on both a polished blade and a used one.

The output from LA-ICPMS was a time series for each element being analysed, with units of counts per second at the ICPMS detector (Figure 2.1). To process the raw data, several steps were undertaken. First, individual outlier points were

identified as any point more than 40% above or below the ten point simple central moving average, ignoring the current point. Outlier values were replaced with the calculated simple central moving average value. Second, the entire time series was then smoothed by a simple central moving average of width 11 points. Third, start and end points in the sample sequence were identified by visual inspection, determining at which point the signal moved substantially above the background signal or began dropping off of the sample signal at the end. Fourth, the background signal inherent in LA-ICPMS was removed by subtracting the simple mean of points 5 through 20 (before the sequence start) from the entire time series. Fifth, the birth ring sequence location was calculated and based on the diameters for each species as listed in the SOL-ICPMS preparation above, laser scan speed, ICPMS detection measurement period and the physical geometry of each individual sample. Sixth, the catch location influence section was defined as the outer 0.2mm lateral distance from the centrum edge, accounting for LA-ICPMS scan parameters and centrum geometry. Seventh, the whole life mean was calculated as the simple mean of the values between the birth ring and sequence end. Eighth, the catch location influence mean was the mean of the values between the catch location influence point and sequence end. Finally, each of the elements investigated was divided by the corresponding Ca level as an internal standard.

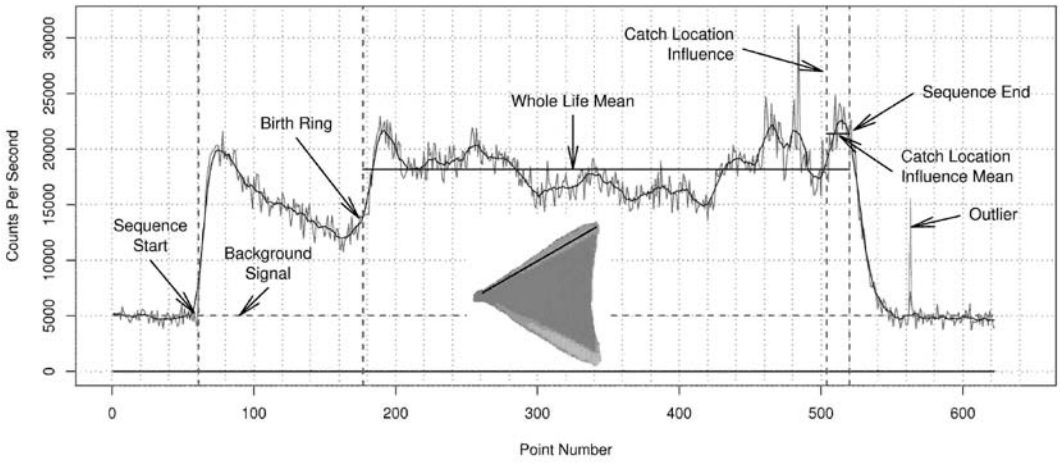


Figure 2.1 –Typical raw data plot from a LA-ICPMS scan, graphically indicating important values defining the sequence. Vertebra photo inset indicates transect location along corpus calcareum. Note: the substantial direction change near the birth ring does not consistently occur in all samples, although it is not a rarity.

To account for the inherent drift in ICPMS sensitivity over time NIST 610 standards were used for external calibration (Jarvis et al. 1992) of whole life and catch location influence mean ratio values. Each LA-ICPMS processing day began and ended with a pair of NIST 610 standard transects, with periodic pairs of standards run throughout the day at 60 – 120 minute intervals. Data from each standard sample was processed by taking the mean of each element for the middle 60 points of the time series and calculating the ratio to corresponding Ca values. A linear interpolation model was created by using the mean of each pair of standard samples with run times as the independent variable, normalised to the first standard run of the first day of LA-ICPMS processing (Figure 2.2). An elemental ratio correction factor for each sample was calculated based on the actual sample run and date. Appropriate drift corrections for whole life mean and catch location influence mean for each sample were made by dividing the measured value by the corresponding correction factor.

Repetitive sample testing of LA-ICPMS was achieved by taking a single vertebral centrum from five individuals (all *C. tilstoni* from PCB) and completing four scans in the corpus calcareum (one each of the four segments visible on a sagittal section) and two from the intermedalia. To determine the effects of surface contamination and running multiple identical scans across a given transect for cleaning purposes, 2 scans were programmed on the NIST610 standard and each of the corpus calcareum and intermedalia of a *C. tilstoni* sample and run four times. To determine the effect of size of individuals, 24 *R. acutus* cervical vertebrae of varying sizes from BNE were analysed using LA-ICPMS on the corpus calcareum considering the whole life segment of the transect. Drawing all samples for this analysis from a single region eliminated geographic location as a possible confounding source of variability. The ability of LA-ICPMS to separate populations based on geography was tested using 24 *R. acutus* cervical vertebrae of varying sizes from BNE (South) and PCB (North) using LA-ICPMS on the corpus calcareum. The same samples and transects were utilised to test for stock separation based on geography using both the whole life and catch location influence time series segments.

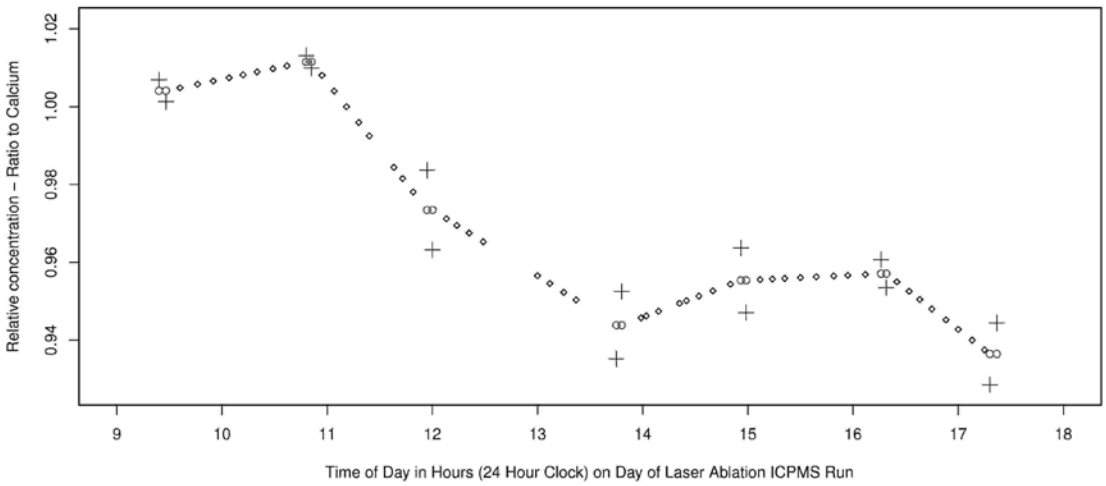


Figure 2.2 – Typical LA-ICPMS drift correction model. Seven pairs of NIST 610 standards were run through the day (crosses). Means were used for creating linear interpolation model (circles). Correction factors calculated from model for each sample run time (small diamonds).

3.2.3 Data analysis

Results from repetitive sample testing (SOL-ICPMS and LA-ICPMS) were analysed by calculating coefficient of variation (CV) for each of the elements. Principal components analysis (PCA) and MANOVA was used to provide visual and hypothesis based tools for comparison of within and between individual variation. In addition to the repeatability analysis for LA-ICPMS, the scans described above were also used to compare elemental fingerprints between the corpus calcareum and intermedalia regions using PCA and MANOVA. All data analysis was executed using R scripts. Due to scale differences in concentrations of various elements, PCA's were based on correlations matrix (Quinn and Keough 2002). Pillai's trace was used as the MANOVA test statistic for its robustness (Quinn and Keough 2002). All data was first examined for normality and power transformed as appropriate. Post hoc univariate ANOVAs

were run after each MANOVA to explore the contribution of each response variable to the overall variability.

Elemental ratios between different positions on the vertebral column were analysed with PCA and MANOVA to demonstrate if within-individual variation was greater or less than between-individual variation. PCA was used to visually determine if there was any size based effect. Median fork length of the shark was used to put vertebra into two equal size classes. MANOVA was used to determine significant differences in elemental composition between the two size classes. Differences in elemental composition between geographic locations was examined using PCA and statistical differences were tested using MANOVA.

3.3 Assessment

3.3.1 Repeatability of measurements

The CV for SOL-ICPMS repetitive samples were within 7% variation for all elements except Cu and Zn (sample B only) (Table 2.1). PCA showed strong grouping of samples from the same individuals suggesting the variation between individuals was greater than the variation inherent in the SOL-ICPMS processing (Figure 2.3). The two main principal components explained 40% and 36% of the overall variability. Significant difference existed among the SOL-ICPMS repetitive samples (MANOVA, Table 2.2). Post hoc summary ANOVA indicated that all 7 elements were significant in contribution to variability. The CV for LA-ICPMS repetitive samples were within 5% variation for all elements except Cu and Zn for

both the corpus calcareum (Table 2.3a) and intermedalia testing (Table 2.3b). The PCA for the whole life segment of the corpus calcareum demonstrated reasonable grouping among the five samples (Figure 2.4), although two samples had a high degree of overlap. The two main principal components explained 46% and 22% of overall variability. Significant difference existed among the LA-ICPMS whole life repetitive samples (MANOVA, Table 2.2). Post hoc summary ANOVA indicated that all tested elements except Cu were significant in contribution to variability. Analyses for the LA-ICPMS of intermedalia suggested similar groupings, but are not included here for brevity.

Table 2.1 –Coefficient of Variation for 7-9 repetitive SOL-ICPMS samples from five individuals (*Carcharhinus tilstoni* from PCB or BGB, and one *Sphyrna lewini* from BGB) and seven elements of interest.

Sample	n	Ba:Ca	Cu:Ca	Mg:Ca	Mn:Ca	P:Ca	Sr:Ca	Zn:Ca
A	7	2%	7%	2%	1%	2%	1%	6%
B	9	3%	21%	2%	3%	2%	1%	13%
C	9	1%	18%	2%	1%	2%	1%	5%
D	7	3%	27%	3%	3%	2%	1%	5%
E	9	7%	22%	2%	2%	1%	1%	5%
Mean		3%	19%	2%	2%	2%	1%	7%

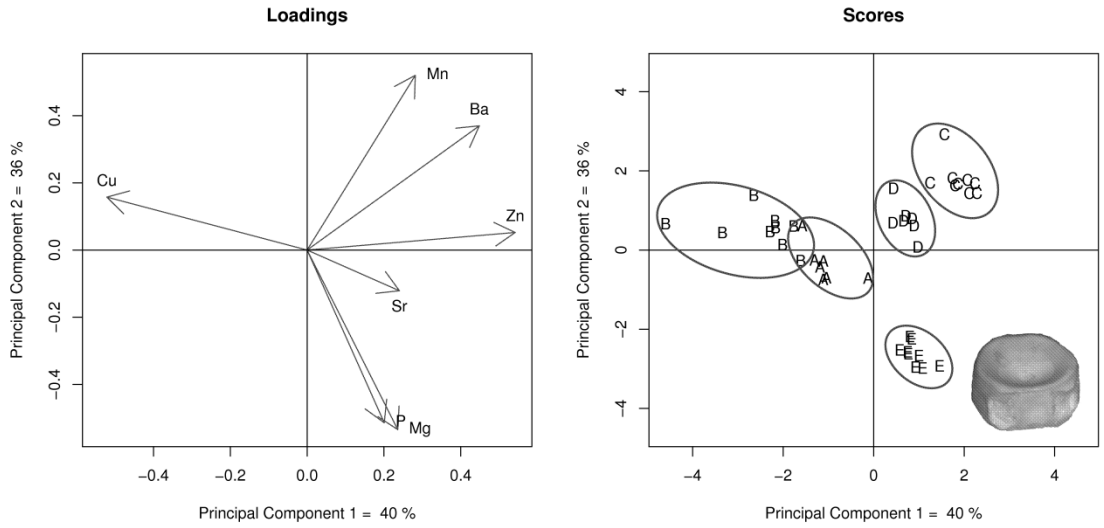


Figure 2.3 – Principal components analysis of SOL-ICPMS results indicating grouping of repetitive samples of five individuals, 2 *Carcharhinus tilstoni* from PCB (B & E), 2 from BGB (C & D), and 1 *Sphyrna lewini* from PCB (A). Inset photo indicates solution based processing.

Table 2.2 –MANOVA and post hoc ANOVA results for each of the seven analyses performed. Significance levels: * P<0.05, ** P<0.01, *** P<0.001.

Analysis	MANOVA	Mg	P	Mn	Cu	Zn	Sr	Ba
Repeatability (SOL-ICPMS)	Pillai=3.73 *** F(28,132)=63.7 p<.0001	F(4,36)=276 *** p<.0001	F(4,36)=13.0 *** p<.0001	F(4,36)=1744 *** p<.0001	F(4,36)=33.7 *** p<.0001	F(4,36)=24.4 *** p<.0001	F(4,36)=39.0 *** p<.0001	F(4,36)=1173 *** p<.0001
Repeatability (LA-ICPMS)	Pillai=2.62 *** F(28,48)=3.26 p<.001	F(4,15)=5.31 ** p=.007	F(4,15)=3.86 * p=.024	F(4,15)=50.4 *** p<.0001	F(4,15)=2.12 p=.129	F(4,15)=3.08 * p=.049	F(4,15)=16.5 *** p<.0001	F(4,15)=20.3 *** p<.0001
Corpus Calcareum v Intermedalia (LA-ICPMS)	Pillai=.988 *** F(7,22)=262 p<.0001	F(1,28)=423 *** p<.0001	F(1,28)=.042 p=.840	F(1,28)=85.6 *** p<.0001	F(1,28)=4.58 * p=.041	F(1,28)=140 *** p<.0001	F(1,28)=8.83 ** p=.006	F(1,28)=17.7 *** p<.001
Vertebral Column Position (SOL-ICPMS)	Pillai=1.15 F(14,14)=1.35 p=.291	F(2,12)=1.35 p=.296	F(2,12)=.062 p=.940	F(2,12)=.039 p=.962	F(2,12)=.681 p=.525	F(2,12)=2.55 p=.119	F(2,12)=.096 p=.909	F(2,12)=.082 p=.921
Effect of Size (LA-ICPMS)	Pillai=.246 F(7,16)=.744 p=.639	F(1,22)=1.40 p=.250	F(1,22)=.077 p=.784	F(1,22)=.034 p=.856	F(1,22)=.526 p=.476	F(1,22)=1.11 p=.304	F(1,22)=2.24 p=.148	F(1,22)=2.13 p=.158
Geographic Separation Whole Life (LA-ICPMS)	Pillai=.758 *** F(7,40)=17.9 p<.0001	F(1,46)=48.3 *** p<.0001	F(1,46)=2.29 p=.137	F(1,46)=.049 p=.826	F(1,46)=28.5 *** p<.0001	F(1,46)=1.28 p=.263	F(1,46)=.222 p=.640	F(1,46)=12.0 ** p=.001
Geographic Separation Catch Location Influence (LA-ICPMS)	Pillai=.887 *** F(7,40)=45.0 p<.0001	F(1,46)=128 *** p<.0001	F(1,46)=11.7 ** p=.001	F(1,46)=5.18 * p=.028	F(1,46)=41.9 *** p<.0001	F(1,46)=.120 p=.731	F(1,46)=74.6 *** p<.0001	F(1,46)=35.6 *** p<.0001

Table 2.3 –Coefficient of Variation for five repetitive LA-ICPMS samples from five individuals (all *Carcharhinus tilstoni* from PCB) and seven elements of interest: (a) four scans from each sample represent all corpus calcareum segments from the "bow tie" section of vertebrae; (b) two scans from each sample represent centre of intermedalia on both sides of the "bow tie" section of vertebrae.

(a)

Sample	n	Ba:Ca	Cu:Ca	Mg:Ca	Mn:Ca	P:Ca	Sr:Ca	Zn:Ca
1	4	1%	8%	3%	8%	8%	1%	27%
2	4	4%	39%	3%	4%	3%	1%	17%
3	4	6%	46%	1%	7%	3%	2%	8%
4	4	5%	8%	2%	2%	5%	2%	13%
5	4	10%	32%	1%	5%	5%	2%	19%
Mean		5%	27%	2%	5%	5%	2%	17%

(b)

Sample	n	Ba:Ca	Cu:Ca	Mg:Ca	Mn:Ca	P:Ca	Sr:Ca	Zn:Ca
A	2	5%	11%	0%	6%	1%	1%	14%
B	2	2%	22%	2%	5%	2%	2%	16%
C	2	3%	1%	1%	0%	4%	1%	13%
D	2	2%	18%	3%	6%	12%	1%	20%
E	2	0%	38%	2%	4%	5%	1%	8%
Mean		3%	18%	2%	4%	5%	1%	14%

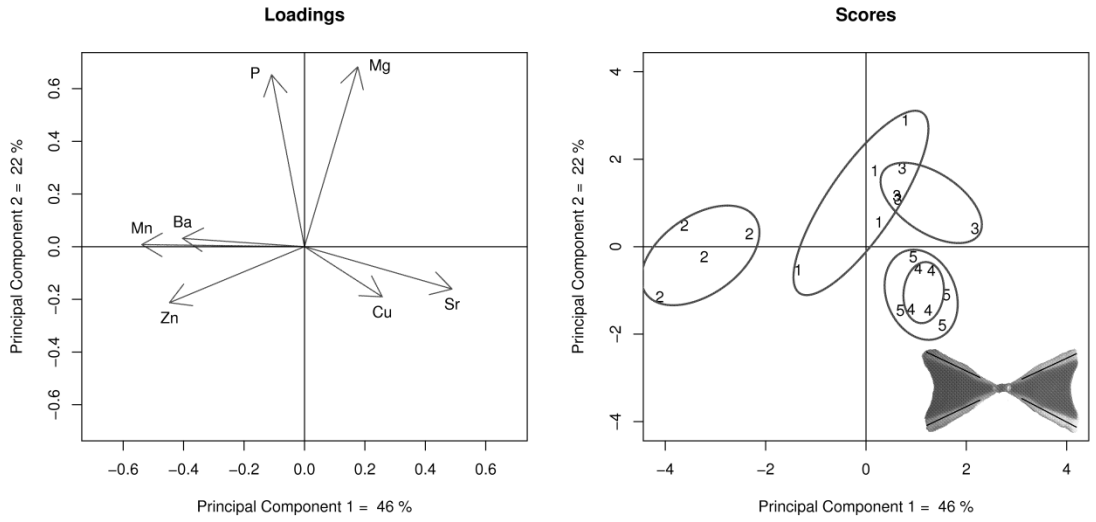


Figure 2.4 – Principal components analysis for LA-ICPMS indicating grouping of repetitive samples of five individual *Carcharhinus tilstoni* (1 - 5) from PCB. Inset photo indicates laser ablation tracks used in the analysis.

3.3.2 Corpus calcareum – intermedalia comparisons

The PCA for comparison of the corpus calcareum and intermedalia showed clear separation between the groups (Figure 2.5). The two main principal components explained 51% and 22% of overall variability. Elemental composition of the two vertebral regions are significantly different (MANOVA, Table 2.2). Post hoc summary ANOVA indicated that all elements except P were significant in explaining variability.

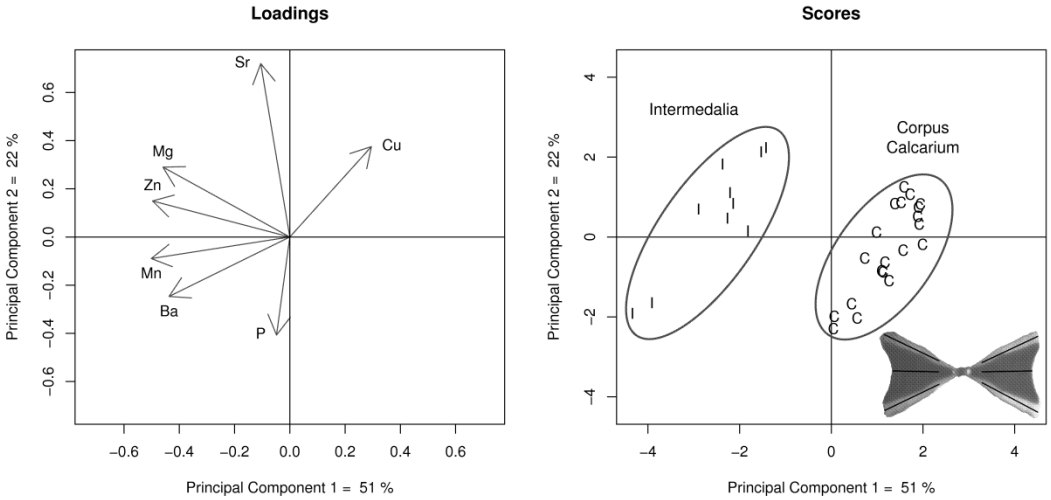


Figure 2.5 – Principal components analysis for LA-ICPMS indicating grouping of repetitive samples of intermedalia and corpus calcareum (I and C, respectively) of five individual *Carcharhinus tilstoni* from PCB. Two intermedalia and four corpus calcaria samples were used from each individual. Inset photo indicates laser ablation tracks used in the analysis.

3.3.3 Repeated transect variation

When running repeated identical scans across a transect with no surface contamination, it would be expected that all resultant time series of elements in ratio to Ca would be near identical to the first scan. This proved to be the case with Mg, Mn, P and Sr (Figure 2.6A). However, for Ba, Cu, and Zn, each successive scan decreased in detected concentration relative to Ca (Figure 2.6B). In these diagrams, Scan 1 would be the equivalent of the cleaning run from other transects and Scan 2 would be the actual data used for time series processing and statistical analyses. This phenomena did not occur for the NIST 610 glass standard scans, where all elemental Ca ratios appeared consistent with previous identical scans. The reduction in signal for the corpus calcareum was greater than that of the intermedalia (Figure 2.7). By the fourth scan, signal reduction was up to 80% for Cu, 55% for Zn, and 20% for Ba. The effect was

less for the intermedalia than the corpus calcareum, but for Ba there was no intermedalia signal reduction. The semi-quantitative analysis in the EPMA EDS to identify possible contaminants revealed wafering blade composition to be approximately 85.5% Cu, 8.5% Fe, and 5.0% Sn, with small amounts of Si near the diamond tipped edge. Analysis of a used blade, that has been used for sectioning, included Ca and Zn as well.

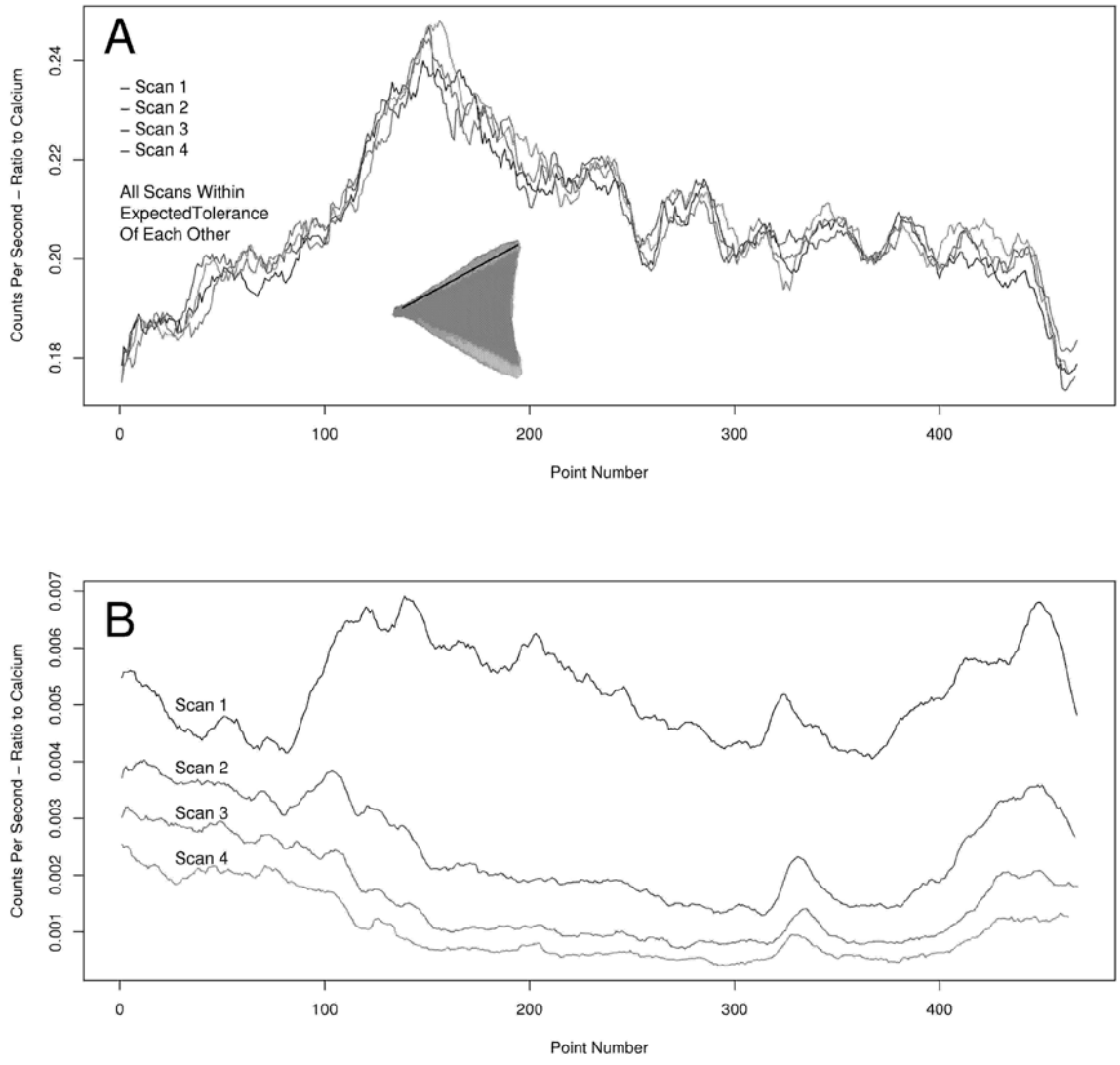


Figure 2.6 – Typical time series plots of four identical repeated LA-ICPMS scans for Mg (A) and Cu (B) from a *Carcharhinus tilstoni* from PCB. Inset photo indicates laser ablation track used in the analysis.

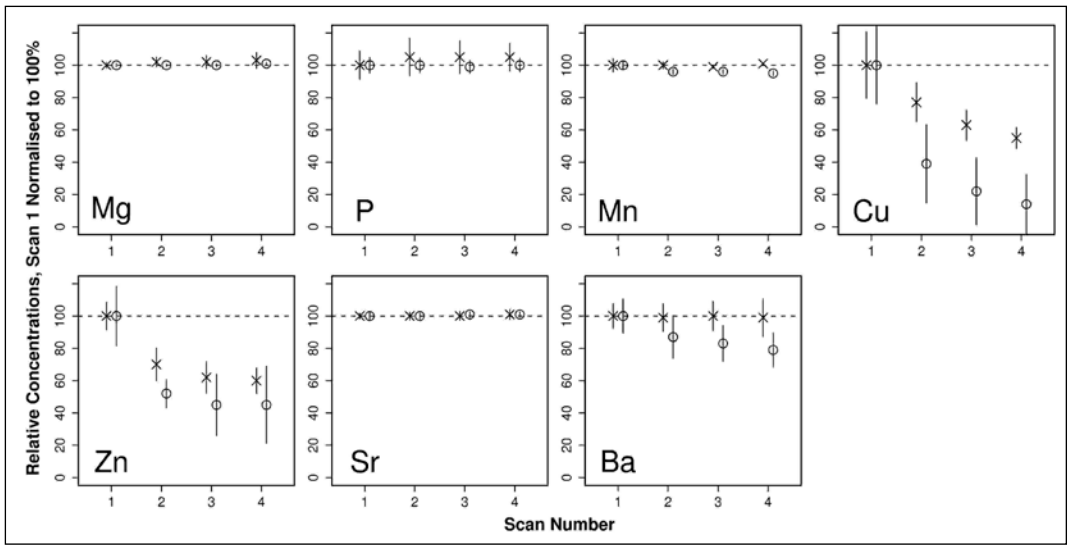


Figure 2.7 – Mean relative concentrations (+/- 1 RSE) of each element in ratio to Ca over four consecutive and identical LA-ICPMS scans for the corpus calcareum (O) and intermedalia (X).

3.3.4 Variation between vertebral column position

The PCA for the vertebral column position analysis indicated strong grouping for the three locations within each individual (Figure 2.8). The two main principal components explained 51% and 23% of the overall variability. Variation among vertebral column positions was not significant (MANOVA, Table 2.2). On the post hoc summary ANOVA test, none of the individual elements were significant.

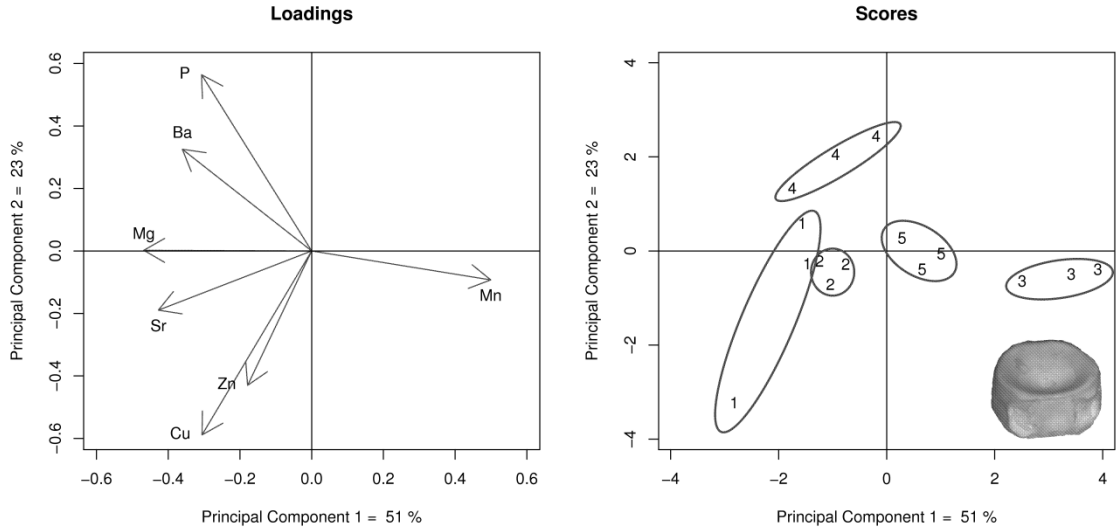


Figure 2.8 – PCA for SOL-ICPMS indicating grouping of samples from three positions along the vertebral column (cervical, thoracic, caudal) of five individual *Carcharhinus tilstoni* sharks from PCB (1 – 5). Inset photo indicates solution based processing.

3.3.5 Effect of size

The PCA for the individual size sensitivity analysis indicates no pattern or grouping (Figure 2.9). The two main principal components explain 25% and 23% of the overall variation. Analysis with greater resolution of three size classes instead of two indicate similar lack of grouping but is not included here for brevity. Variation among size classes was not significantly greater than variation among individuals (MANOVA, Table 2.2). The post hoc summary ANOVA analyses showed no significance in any individual elements for size based variation.

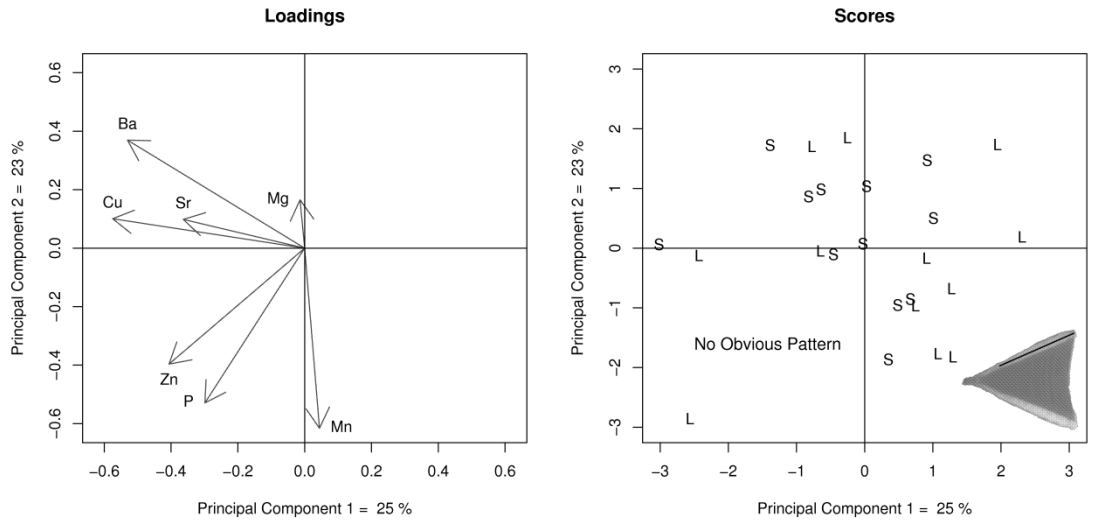


Figure 2.9 – Principal components analysis for LA-ICPMS results indicating lack of grouping of samples based on two size classes (S – smaller than median, L – larger than median) of 24 individual *Rhizoprionodon acutus* from BNE. Inset photo indicates laser ablation transect used in the analysis.

3.3.6 Geographic separation

The PCA for overall stock separation between PCB and BNE for LA-ICPMS whole life mean showed reasonable groupings, but displayed overlap (Figure 2.10). The two main principal components explain 27% and 17% of overall variability. Interestingly, the similar PCA that considered only catch location influence means (i.e. outer section of centra only) showed similar groupings, but without overlap (Figure 2.11). The two main principal components were stronger, with 43% and 17% of variability explained. BNE samples are associated with the same elements as whole life analysis, with the addition of Sr. The two locations had significantly different elemental compositions for both the whole life and catch location influence analyses, but distinction was greater for the catch location influence (MANOVA, Table 2.2) than for the whole life analysis

(MANOVA, Table 2.2). For the catch location influence post hoc summary ANOVA analyses, Mg, Cu, and Ba were significant in showing univariate differences, whereas for the whole life analysis, all elements except Zn were significant.

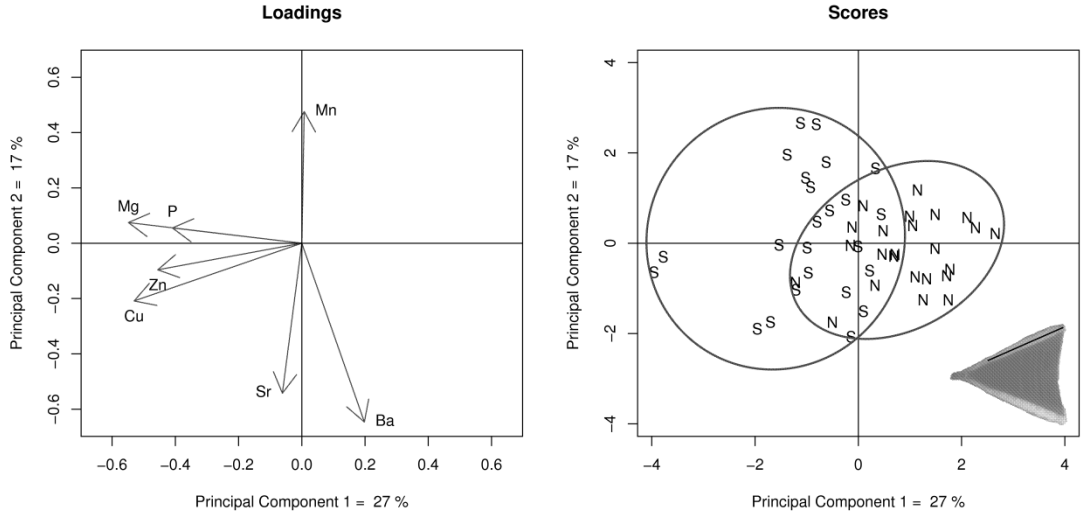


Figure 2.10 – Principal components analysis for LA-ICPMS indicating grouping for *Rhizoprionodon acutus* from two regions (N – north, PCB, S – south, Brisbane). Inset photo indicates laser ablation track used in analysis (whole life).

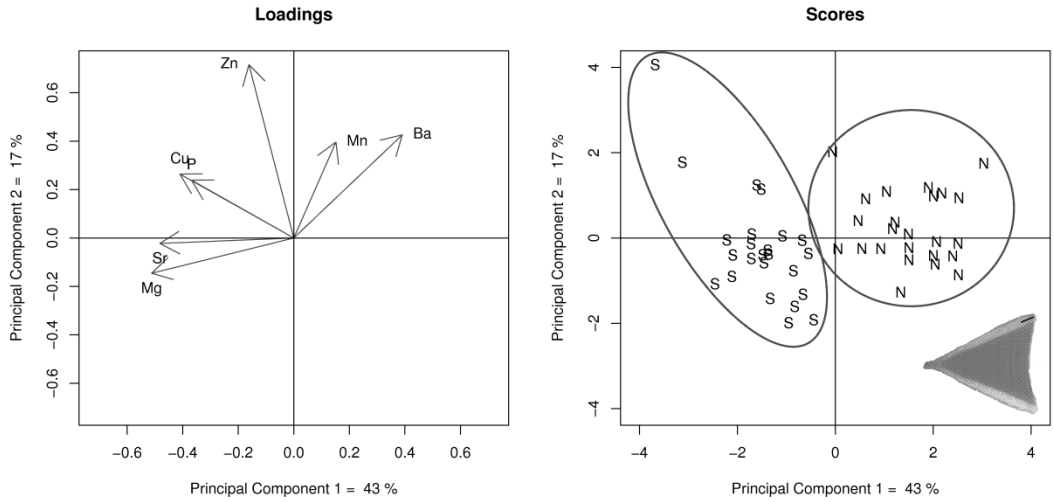


Figure 2.11 – Principal components analysis for LA-ICPMS indicating grouping for *Rhizoprionodon acutus* from two regions (N – north, PCB, S – south, Brisbane). Inset photo indicates laser ablation track used in the analysis (catch location influence).

3.4 Discussion

The results of this study demonstrate that shark vertebrae are a suitable tissue for use in ICPMS-based elemental composition studies from which inferences about stock structure can be made. While the assumptions investigated are fundamental to the ability to use vertebrae for reliable microchemical studies they have not previously been tested. The results provide the basis for continued research in this field and its application to stock structure studies.

The initial requirement in answering the question of applicability of ICPMS techniques to sharks is understanding variability. Variability between individuals must be greater than the combined variability inherent in ICPMS processing itself, including effects of handling, storage, and sample preparation. In otoliths, measured concentration of various elements can vary due to handling and preparation techniques (Proctor and Thresher 1998) as well as sample storage (Milton and Chenery 1998). Cartilaginous vertebrae, being a softer structure, may be prone to similar issues. All of the most abundant elements tested here (Ba, Mg, Mn, P, and Sr) demonstrated low variability with CVs being 5% or less. For SOL-ICPMS, these figures included handling and storage of each sample separately. For both LA-ICPMS methods (along corpus calcareum and intermedalia), the measurements were taken off separate sections of the same sample so no replication in handling or preparation existed. These results indicate that either technique will provide for sufficient repeatability, generally

within anticipated ICPMS precision expectations, and with the ability to demonstrate separation of the elemental fingerprint between individuals.

The much greater CV's for Cu in all three tests (SOL-ICPMS, intermedalia LA-ICPM and corpus calcareum LA-ICPMS) and Zn for the two laser ablation tests may be due to the much lower abundances and being closer to the LOD, causing greater apparent noise in the signal. Zn having much lower CV for SOL-ICPMS than for LA-ICPMS is consistent with the suggestion by Ludsin et al. that LA-ICPMS will show higher LOD than SOL-ICPMS (Ludsin et al. 2006). However, their suggestion that LA-ICPMS, being less susceptible to contamination, may provide more precise estimates than SOL-ICPMS is not confirmed in the present study. Contamination during preparation and processing is a major concern, particularly for SOL-ICPMS. The three largest areas for contamination are in the equipment to grind and homogenise samples, lab environment for digestion apparatus, and the analytical reagents used during solution preparation (Jarvis et al. 1992). These points consider only the variation within individual sharks and related processing, but not among them. The exploratory PCA technique utilised suggested reasonable grouping within, and separation among, individual sharks. Further consideration of contamination issues for LA-ICPMS for some elements is considered below.

SOL-ICPMS processing yielded precise concentrations of various elements of interest through extensive use of calibration and correction techniques, spiked samples, standards, and blanks. However, care must still be exercised if

comparing results produced from different labs or using different preparation methods (Campana et al. 1997). The method described here for LA-ICPMS processing will not yield actual concentrations of the samples. We have not corrected for isotope ratios or matrix effects between the sample and the NIST 610 standard. Furthermore, the phenomena discovered in our samples with Ba, Cu, and Zn reducing intensities with repeated scans would make resolving to actual elemental concentrations highly challenging and risky. For Cu this was likely due to surface contamination effect from Cu and Zn in the wafering blade used to section the vertebrae. However, the reasons for the declining intensities for Ba are less clear, but may be related to their proximity to LODs or other sources of contamination. A possible secondary cause for the intensity reductions is that they were related to inter-elemental fractionation, which is a known challenge for LA-ICPMS in situ analysis. Chen (1999) identified similar issues where Zn and other elements, in ratio to Ca, decreased in intensity over scan time of two geological samples. The effect was not found in the NIST 613 silicate standard, suggesting there may be a matrix dependent effect (Chen 1999). While approaches may exist to correct for this kind of contamination and possible fractionation, our primary interest for stock separation purposes is in relative concentrations.

With the only correction conducted in this study being the external NIST 610 based sensitivity drift, it is important to note that only relative concentrations between samples may be utilised for LA-ICPMS using the method described here. Furthermore, it is important to ensure consistency in scan repetitions, in that exactly one cleaning scan is run prior to the actual data collection scan. This

surface contamination or fractionation effect clearly adds another source of variability to Cu, Zn, and Ba, but all three elements still had significant univariate effects in various post-hoc ANOVAs. Not resolving to actual concentrations for LA-ICPMS means direct comparison can not be made with results from SOL-ICPMS on a sample to sample basis. Even if this issue were overcome, additional challenge would be presented due to the physical geometry of the vertebrae centra. SOL-ICPMS samples represent a mix between the corpus calcareum and intermedalia material as well as being more heavily weighted towards the outer radius of the vertebrae where greater amount of material exists.

Several points need consideration in deciding whether LA-ICPMS or SOL-ICPMS is most appropriate for a given application. From a practical aspect, trade-offs exists between relative costs, sample preparation time, data quality, data type, and data analysis time. Sample preparation for SOL-ICPMS is substantially more involved than for LA-ICPMS, but ICPMS lab time is greater in processing the LA-ICPMS samples. Various parameters, such as scan speed or physical size of samples, can affect sample processing efficiency for LA-ICPMS. For example, increased scan speed or smaller samples will allow for shorter sample turnaround time. Smaller physical samples may also allow greater density of samples on the test slide, reducing overhead involved with changing slides in the ablation chamber. With parameters as defined in the present study, forty to sixty samples per day were processed. Consideration must also be given to analysis of data output from the ICPMS process. SOL-ICPMS provided concentration for each

sample element, whereas LA-ICPMS yields a time series requiring substantial software development effort to process the raw data (see appendix).

The lack of variation in elemental fingerprints between vertebral positions means that there is little importance placed on where along the vertebral column samples are collected from. However, it is prudent to maintain positional consistency in studies with large sample sizes. Besides eliminating variability that may not have been identified in the present study, it allows the relative size of a shark to be inferred by vertebra radius or time series length in LA-ICPMS. Vertebrae from thoracic or cervical regions are larger than those from the caudal region, and larger vertebrae are preferred in that greater resolution of elemental fingerprint would be available in LA-ICPMS. Practical aspects of obtaining large number of shark samples for the present study required the use of observers on commercial fishing operations. In this type of sample collection, it is possible that sharks get trunked and processed prior to an observer obtaining measurement of an animal. If all samples are cut from the cervical region, drawing the sample is easiest and size inferences may be possible if measurements are missed.

The analysis suggests that there are significant differences in the elemental composition of the corpus calcareum and intermedalia. Despite these differences either can be used in LA-ICPMS studies, so long as the two are not directly compared. The results in the present study suggest a slight preference for the use the corpus calcareum as there may be slightly less variation than intermedalia samples. Importantly, practical laboratory benefits exist in that the

corpus calcareum is easier to focus the laser on, making LA-ICPMS processing more time efficient and reducing potential for focus based errors.

Several possible applications exist for ICPMS analysis of shark vertebrae, but the underlying motivation behind development of this particular method is for determination of stock structure. It is not necessary to obtain detailed knowledge of how environmental variables (temperature, salinity, ambient elemental concentrations) influence chemical composition of the animal, but one needs to compare multivariate elemental fingerprints of animals from various regions (Elsdon and Gillanders 2003). When considering multivariate analyses such as those executed in the present study, little can be inferred from differences, other than the stocks are different. Furthermore, if the fingerprints are the same, it does not necessarily indicate they are from the same stock. Consequently, elemental fingerprinting can not be used as a proxy for genetic separation (Campana et al. 2000). The analyses for the milk sharks presented here are intuitively what one might expect, with much greater separation between stocks when considering the catch location influence mean as compared with the whole life mean. However, caution is warranted in inferring that this indicates there may be mixing of individuals over the life span and not during the final weeks or months of their life, indicating moderate levels of migration. While multi-elemental fingerprints are effective for stock separation, migration or environmental reconstruction may require LA-ICPMS analysis of specific elements, such as Sr (Elsdon and Gillanders 2003). For non-diadromous species occupying an area with low temperature gradient and unpredictable ambient elemental concentrations, making inferences on migration from elemental profiles

would provide substantial challenge, requiring physical confirmation of several animals' location through time in order to develop the methodology. This might require collecting electronic tag data for much of the life span prior to LA-ICPMS in order to develop an understanding of environmental influences on vertebral chemical composition.

While effort was made in the present study to draw samples caught within a very limited time frame, temporal effects were not specifically analysed. In otolith studies of teleost fishes, it is possible that ignoring temporal effects lead to no stock separation while including them suggests separation (Bergenius et al. 2005). It may also be possible that interannual variability in elemental composition can confound spatial interpretations, such as in a study spanning several estuaries in New South Wales, Australia (Gillanders 2002). Even within a catch year, unexplained variations may occur. In corals sampled along the Queensland, Australia coast undergoing LA-ICPMS, Ba spikes were noted in some, but not all, samples that could not be easily explained by environmental conditions (river floods, temperatures, season, latitude, etc.) or biological factors (Sinclair 2005). In one *C. tilstoni* sample processed via SOL-ICPMS simultaneously to those of the present study, one sample had a Ba level approximately five times the expected value. It was treated as a contamination affected outlier, but analysis of the preparation process could not determine the possible source of contamination. These issues indicate it is simpler to eliminate temporal effects in the sampling process than to correct for it in the data analysis stage.

Size and sex of the individual sharks need consideration as well. While sex has not been directly considered in these analyses, many species have been determined to have sex based dispersal through genetics studies (Heist 2004). It is logical that if a portion of animals from one region migrate differently to the remaining animals, irregular results are possible. Consistency is the priority, but preferred sampling is for equal and representative mix of sexes for each location, possibly executing a sub-analysis based on sex if sufficient samples exist. Likewise with size, the preference is for similar size distributions from each location to eliminate size as a potential source of variation. In teleost fishes, smaller individuals tend to have higher concentration of trace elements, so it is important not to have confounding differences in elemental concentrations among groups (Campana 1999). Lack of PCA grouping in the *R. acutus* from Brisbane region suggests individual shark size did not affect the outcome of LA-ICPMS. However, the PCA for stock structure included sharks that were beyond the size range of the Brisbane collected sharks. It would be risky to extrapolate the lack of size effect beyond the size range tested. Further analysis is warranted to determine size effect on a set of sharks from the same region that include a wide size distribution.

3.5 Comments and Recommendations

This study demonstrated that assumptions regarding the use of shark vertebrae in the study of stock structure using ICPMS-based elemental analysis can be met. While both SOL-ICPMS and LA-ICPMS can be used effectively in analysing

shark vertebra, each method has advantages and disadvantages. SOL-ICPMS has the advantage of providing true concentrations and having slightly lower LOD, but is contamination prone, time consuming in preparation, and yields only a single value for each element. LA-ICPMS allows analysis of various sections of the vertebrae and is simple to prepare, but is difficult to draw actual elemental concentrations from, is time consuming in the data analysis, and has slightly higher LOD. Given the various benefits involved, the versatility of having access to time series of data for LA-ICPMS should generally make it the preferred approach. In addition to the catch location influence and whole life means used in this study, having access to the elemental profile time series gives the researcher access to maternal influence, birth location influence, or a given juvenile period data. Use of this method for stock structure analysis is therefore manageable, but deploying it for determination of individual migration or age and growth activities provides formidable challenges.

Microchemistry based stock structure analysis for sharks may complement existing genetics based techniques. Sharks tend to have lower mutation rates in the mtDNA than mammals, but the mtDNA still mutates at higher rates than nuclear DNA. As such, mtDNA is used more frequently in stock structure analyses. Over evolutionary time scales, nuclear DNA may be more useful (Heist 2004). Large mobile marine species will typically display little genetic population structure (Duncan et al. 2006). Mixing rates need only be 1% or even less between stocks to prevent genetic differentiation (Bentzen et al. 1996). It is important to note the difference between management units and evolutionarily significant units. Management units may be represented by shallow population

structure, with evolutionarily significant unit expressing a deeper structure. Genetics analysis is best for evolutionarily significant units, but may or may not detect management units (Avise 2004). These shallower management units may be where microchemical analysis of stock structure can detect separation. In terms of species preservation, trace element analysis in otoliths has proven invaluable in determining population connectivity and migration, which in turn helps in the design of marine reserves for conservation purposes (Nowlis and Friedlander 2004).

One advantage of ICPMS is that it has extremely low detection limits, often being sensitive to concentrations of parts per quadrillion or parts per trillion. This provides a wide range of elements to be analysed (Ludsin et al. 2006). In determining migratory patterns in teleosts, certain elements (eg. Sr, Ba) often show relationships with ambient concentrations, temperature or salinity. While others (eg. Na, K, S, P, and Cl) may not be useful as they are under heavy physiological regulation (Elsdon and Gillanders 2003). Careful selection of elements for inclusion in analyses is thus important.

4 Population Structure of Two Inshore Shark Species (*Sphyrna lewini* and *Rhizoprionodon acutus*) using laser ablation inductively coupled plasma mass spectrometry (LA-ICPMS) along the East Coast of Queensland Australia

4.1 Introduction

Sharks play an important role in the world's oceans as apex predators (Stevens et al. 2000a, Heithaus 2004); and recent declines in some populations have highlighted the need for improved management to ensure ongoing provision of ecosystem services and the maintenance of biodiversity (Heithaus et al. 2008). Sustainable management for sharks is especially important because many species have K-selected life history that result in low rates of population increase (Musick et al. 2000). The development of sustainable management systems depends on the availability of sound knowledge of several aspects of the biology and ecology of shark populations, including life history, population structure, change in abundance and susceptibility to fishing gear (Simpfendorfer and Donohue 1998). While the life history of sharks has been increasingly well studied (Carrier et al. 2004), stock structure has been poorly investigated even though knowledge of it dictates the spatial extent of management units. Improved knowledge of stock structure in shark populations will help improve the management of shark populations through identifying the appropriate spatial

scales at which actions are applied. In this study we define “stock” as a group of individuals that maintain spatial and temporal integrity by engaging in a distinct pattern of migration not shared by individuals of other contingents (Secor 1999).

A technique for examining stock structure that has become commonplace for use with teleost fishes is elemental analysis of their calcified structures using inductively coupled plasma mass spectrometry (ICPMS). Although usually applied to otoliths, several other structures of fish have been analysed using this technique, including scales, fin spines, eye lenses, etc. (Elsdon and Gillanders 2003). Despite the popularity of elemental analysis in teleosts, most shark population structure studies to date have been based on genetic markers (Keeney and Heist 2006, Ovenden et al. 2009), physical tagging (Stevens et al. 2000b), life history (Lenanton et al. 1990) or electronic tagging (Hunter et al. 2006). While effective within their scope, all techniques have limitations. Genetics can link fish to a population, but not to a specific geographic region (Ashford et al. 2005) because of the small amounts of migration between regions that can result in homology (Bentzen et al. 1996). Physical tagging on the other hand provides information for short time scales, but can be limited by the distribution of release and recapture effort and can be logistically challenging and costly (Ashford et al. 2005). Electronic tagging can provide data independent of recapture effort, but is expensive at the scale required to reliably identify stock structure (Sibert and Nielsen 2001). It is reasonable to expect ICPMS methods to bring a unique utility to stock structure analysis, while having their own specific shortcomings.

Queensland, Australia's East Coast Inshore Finfish Fishery (ECIFF) is the largest fishery in Queensland spanning the entire east coast. Of the 1800t of fish commercially harvested in 2006, elasmobranchs (sharks and rays) constituted approximately 35% (Harry et al. 2011b). Scalloped hammerhead sharks (*Sphyrna lewini*) and milk sharks (*Rhizoprionodon acutus*) represent two important species to this composition. At present, management of the fishery is based on quantitative population analysis, but does not account for population connectivity of the various species. Management techniques include seasonal closures, catch limits and gear restrictions, and are principally aimed at teleost fish. While reasonable knowledge exists regarding species volumes and composition of the shark fishery (Harry et al. 2011b), a need exists for studies of stock structure and population connectivity. This in turn can be utilised to establish more informed management structure.

Rhizoprionodon acutus is a fast growing productive species, having a continuous distribution from Indo-West Pacific region throughout the Indian Ocean, with isolated populations in the east Atlantic and Mediterranean Sea (Compagno et al. 2005). It is a coastal benthopelagic species that may range in maximum size from 1,000 mm to 1,500 mm (Musick et al. 2004), In the study area, they have recently been observed ranging to stretch total length of 859 mm (695 mm fork length - FL). At birth they are 300-400 mm (226-310 mm FL), and mature at approximately 700-800 mm (562-645 mm FL). Maturity is normally reached between one and two years of age, while full length is achieved at approximately

four years of age (Harry et al. 2010). They may occur in nursery areas, such as Cleveland Bay, Australia, at all times of year, but may emigrate as they approach maturity (Simpfendorfer and Milward 1993). Recent mitochondrial and nuclear genetic study indicates negligible stock structure across north eastern Australia (Ovenden et al. 2011). *Rhizoprionodon acutus* has an IUCN Red List status of "least concern" (IUCN 2001).

Sphyrna lewini is a circumtropical ranging species, using nearshore locations as nursery areas (Branstetter 1990, Castro 1993, Simpfendorfer and Milward 1993). Living to approximately 30 years (Piercy et al. 2007), they are a benthopelagic species that can grow to over 3,000 mm (Musick et al. 2004). However, in the study region of north eastern Australia, they have recently been determined to live to approximately 20 years while obtaining a stretch total length of 2,990 mm (2,219 mm FL). At birth, their length is 465-563 mm (346-421 mm FL). Some variation in reaching maturity exists between Queensland and northern NSW, where Queensland samples reached maturity at approximately 5.7 years at 1,300-1,500 mm (988-1142 mm FL). Samples from northern NSW matured at 8.9 years and length of 1,900-2,100 mm (1,449-1,604 mm FL) (Harry et al. 2011a). Being a larger and slow growing species, inshore areas may be used for extended periods as a nursery, displaying a large degree of site attachment (Knip et al. 2010). While juveniles tend to reuse core areas, they may be making occasional long distance excursions (Duncan and Holland 2006). Adults are more pelagic by nature (Klimley 1987), showing very little, if any genetic stock structure along coast lines in areas spanning thousands of kilometres (Nance et al. 2011, Ovenden et al. 2011). Adults have been known to display site fidelity at

locations 40 km apart (Hearn et al. 2010). However, transoceanic crossings for *S. lewini* appear uncommon (Kohler and Turner 2001, Duncan et al. 2006). *Sphyrna lewini*'s IUCN Red List status is "endangered" (IUCN 2001).

The purpose of this study was to deploy the laser ablation inductively coupled plasma mass spectrometry (LA-ICPMS) method as described in chapter 2 to determine fine scale stock structure of *S. lewini* and *R. acutus* along the east coast of Australia. In addition to their importance to the fishery, these species were chosen because they have differing life histories. *S. lewini* travels widely over its life time, while *R. acutus* is less known, but has been assumed to not move as far. Specifically, samples of both species were collected from six locations each several hundred kilometres apart for analysis of elemental composition and statistical comparison. The null hypothesis tested was that there is no difference in elemental composition of sharks between six regions along the east coast of Australia. This allowed inferences to be made regarding metapopulation structure and possible migration habits.

4.2 Materials and Methods

4.2.1 Sample Collection

Sharks were collected at inshore locations along the east coast of Australia by observers on commercial fishing vessels between September 2007 and June 2009. Collections occurred at six locations: Far North (14.0º S, 144.0º E), Cairns (16.7º S, 145.9º E), Townsville (19.3º S, 147.2° E), Mackay (21.0° S, 150.0° E),

Brisbane (27.0° S, 153.5° E), and Northern NSW (29.6° S, 153.5° E) (Figure 3.1). Attempts were made to minimise size variation of animals where possible, with *S. lewini* fork lengths ranging from 410 mm to 2,226 mm, and *R. acutus* ranging from 350 mm to 800mm (Figure 3.2). However, several adjacent locations of each species show little or no overlap in animal size. Sections of vertebral columns were cut from animals onboard vessels and frozen for future sample processing. Between 12 and 29 samples of each species at each location were collected, except NSW North, where no *R. acutus* were captured. Animal sex was only available for slightly more than half of total samples for both species. Of the samples recorded, approximately 2/3 were male. Since insufficient data was available on this, no consideration on sex was made during the analyses.

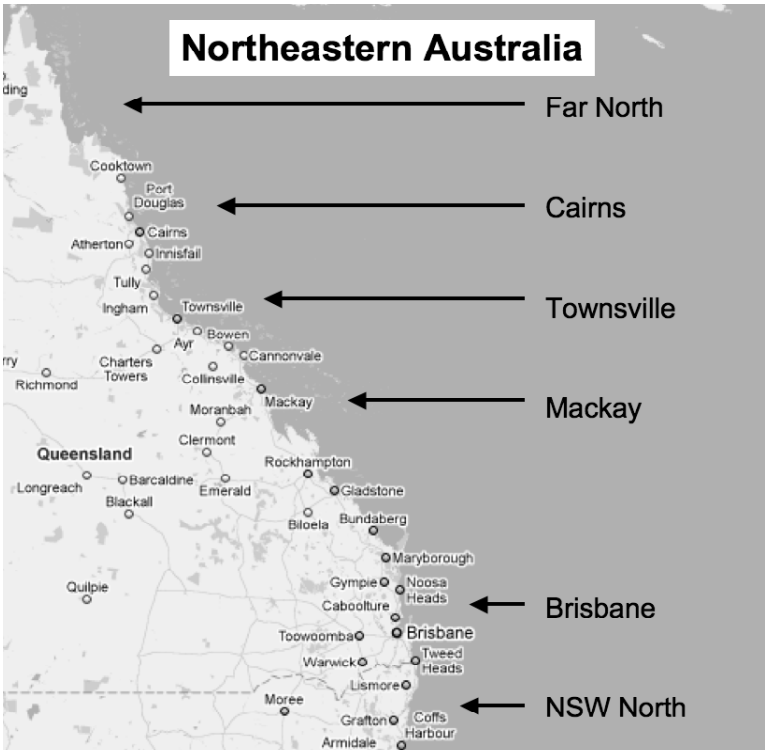


Figure 3.1 – Inshore sampling of northeastern Australian coast for both species. Note: no *R. acutus* samples were taken from northern NSW.

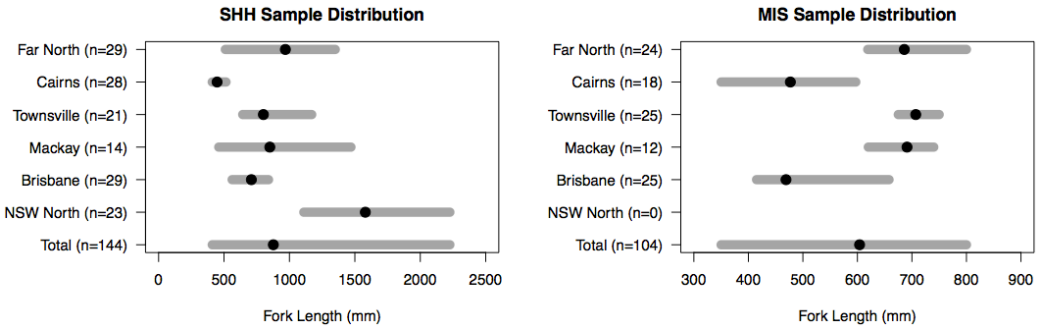


Figure 3.2 – Sample distribution for *S. lewini* (left) and *R. acutus* (right) samples, including location name, sample size, fork length range (grey bar), and mean (black dot).

4.2.2 Sample Preparation

Each section of vertebral column was defrosted and the neural arch and lateral processes removed prior to cleaning away as much organic tissue as possible. Individual centra were separated and soaked in 5% sodium hypochlorite solution for ~30 minutes. Samples were then dried for ~18 hours in a drying oven at 50°C. A section 500 microns thick was cut sagittally using a Buehler low speed Isomet diamond tipped rotary saw (Series 15HC wafering blade with tap water as coolant), then secured on a glass slide (25mm x 45mm) with clear polyester casting resin. The samples were lightly buffed for ~5 seconds with 3 micron lapping film while being rinsed in tap water. They were not polished due to potential for damaging the relatively soft sample material.

To analyse elemental composition of the vertebrae sections, a Coherent Geolas Pro 193 nm ArF Excimer laser unit was connected to a Varian 820-MS (Melbourne, Australia) Inductively Coupled Plasma Mass Spectrometer (ICPMS) via 3 metres of Tygon tubing (inner diameter 3.2mm). Vertebrae section slides were set on a chamber insert (55mm x 8mm height) and loaded into the circular sample chamber (55mm diameter x 15mm height). He was used as the carrier

gas, flowing at 235 ml/min. The instrument was optimised to the maximum sensitivity (238U signal > 2 million cps for NIST 610) while keeping the oxide ratio low (ThO/Th ~0.3%) and 238U/232Th ~ 1. Other instrumental parameters were: RF Power 1300 W, sampling depth 5mm, plasma flow 16.5 l/min, auxiliary flow 1.65 l/min, carrier gas flow 0.97 l/min, and dwell time 20 ms. The laser repetition rate was fixed at 10 Hz on energy density of 6 J/cm^2. A 31 µm laser beam was used and the scanning speed was set at 62 µm/s in a step repeat pattern. National Institute of Standards and Technology (NIST) 610 glass standard was processed at 60 to 120 minute intervals for purposes of correction of instrument drift during the data processing. Prior to measuring each transect, a cleaning run was made to remove surface contamination from the sample. The samples were processed in random order.

4.2.3 Sample Data Analysis

All data analysis was performed using R scripts. The output from LA-ICPMS was a time series for each element being analysed, with units of counts per second at the ICPMS detector. To process the raw data, several steps were undertaken. First, individual outlier points were identified as any point more than 40% above or below the ten point simple central moving average, ignoring the current point. Outlier values were replaced with the calculated simple central moving average value. Second, the entire time series was smoothed by a simple central moving average of width eleven points. Third, start and end points in the sample sequence were identified by visual plot inspection, determining at which point the signal moved substantially above the background signal or began dropping off

the sample signal at the end. Fourth, the background signal inherent in LA-ICPMS was removed by subtracting the simple mean of points 5 through 20 (before the sequence start) from the entire time series. Fifth, the birth ring sequence location was calculated and based on typical measured diameters for each species (*S. lewini* = 4.76mm, *R. acutus* = 2.84mm), laser scan speed, ICPMS detection measurement period and the physical geometry of each individual sample. Sixth, the catch location influence section was defined as the outer 0.2mm lateral distance from the centrum edge, accounting for LA-ICPMS scan parameters and centrum geometry. This outer section represents the final stage of the animal's life, the elemental composition of which would have been influenced by factors in that immediate geographic location. Seventh, the whole life mean was calculated as the simple mean of the values between the birth ring and sequence end. The mean elemental composition across the animal's life represents a weighted average of influencing factors across all geographic locations it has travelled. Eighth, the catch location influence mean was the mean of the values between the catch location influence point and sequence end. Finally, each of the elements investigated was divided by the corresponding Ca level as an internal standard.

To account for the inherent drift in ICPMS sensitivity over time NIST 610 standards were used for external calibration (Jarvis et al. 1992) of whole life and catch location influence mean ratio values. Each LA-ICPMS processing day began and ended with a pair of NIST 610 standard transects, with periodic pairs of standards run throughout the day at 60 – 120 minute intervals. Data from each standard sample was processed by taking the mean of each element for the

middle 60 points of the time series and calculating the ratio to corresponding Ca values. A linear interpolation model was created by using the mean of each pair of standard samples with run times as the independent variable, normalised to the first standard run of the first day of LA-ICPMS processing. An elemental ratio correction factor for each sample was calculated based on the actual sample run and date. Appropriate drift corrections for whole life mean and catch location influence mean for each sample were made by dividing the measured value by the corresponding correction factor.

It is important to note that resolving to actual elemental concentrations has not been performed and is not beneficial for this application. Also, no standard exists for shark vertebrae to make the calculation practical for laser ablation time series (laser ablation fractionation behaviour of soft vertebra samples may be different than that of hard NIST 610 standards). In the statistical analyses performed, it was important to know the relative variation between samples. The actual values are of little consequence if relative values between samples are accurate, regardless of whether samples are measured in parts per million, counts per second, or percentage of total weight. However, the sensitivity drift corrections based on the NIST standards are critically important to ensure consistency throughout and between the several days of processing.

All ratio data from the above preliminary processing was examined for normality and power transformed as appropriate. Principal components analysis (PCA) was used to provide visual exploratory analysis to determine obvious groupings

of samples based on location. Due to scale differences in concentrations of various elements, PCA's were based on correlations matrices (Quinn and Keough 2002). Groupings of categories were identified on the scores plot based on means of principal components 1 and 2, and a 95% confidence ellipsoid was plotted to visualise further the distribution characteristics. Hypothesis testing began with MANOVA. Pillai's trace was used as the MANOVA test statistic for its robustness (Quinn and Keough 2002). Post hoc univarite ANOVAs were run after each MANOVA to explore contribution of each response variable to overall variation between locations. Adjacent regions for each species were analysed using Hotelling's T^2 test for pair-wise comparisons. Five location comparisons for *S. lewini* and four for *R. acutus* required Bonferroni adjustment of $p_{slewini}$=.0100 and $p_{racutus}$=.0125 to achieve overall p=.05.

4.3 Results

Principal components analysis (PCA) for *S. lewini* whole life comparison showed detectable groupings within the region and separation between each region, but with heavy overlap (Figure 3.3, Table 3.1). The first two principal components explained 32% and 24% of overall variability. The largest variability corresponded to Mg, Mn, and Sr isotopes. Cairns had the most obvious grouping away from the overall means, associating heavily with Mg and Mn.

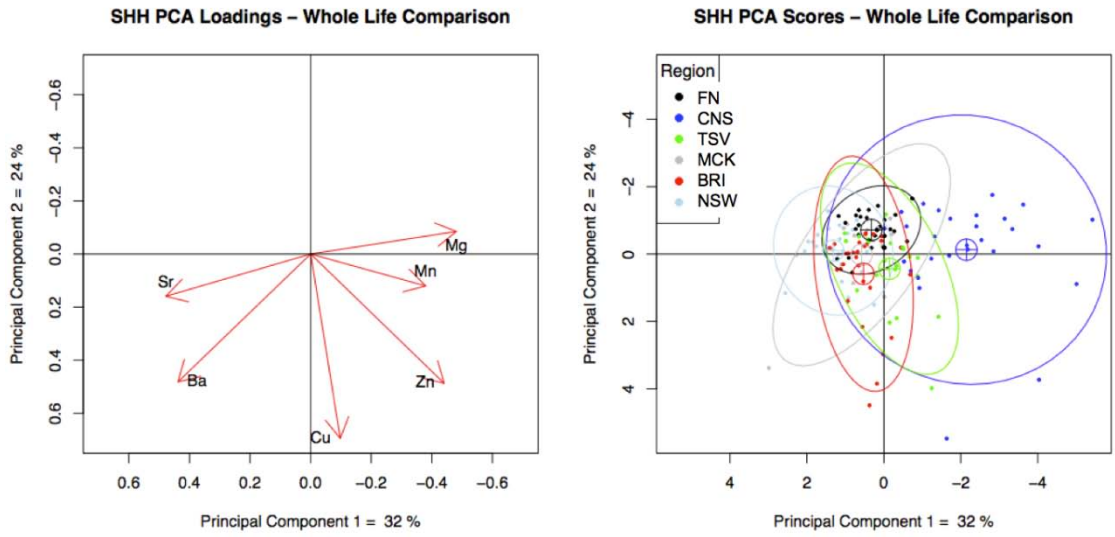


Figure 3.3 – Principal components analysis for *S. lewini* "whole life" LA-ICPMS. FN - Far North, CNS – Cairns, TSV – Townsville, MCK – Mackay, BRI – Brisbane, NSW – NSW North. Crosses represent mean for that region and ellipsoid represents 95% confidence interval.

Table 3.1 – Importance of components and loadings for PCA for *S. lewini* "whole life" analysis.

	PC 1	PC 2	PC 3	PC 4	PC 5	PC 6
Standard Deviation	1.39	1.21	0.97	0.85	0.71	0.67
Proportion of Variance	0.32	0.24	0.16	0.12	0.08	0.07
Cumulative Proportion	0.32	0.57	0.72	0.84	0.93	1.00

Loadings - Element	PC 1	PC 2	PC 3	PC 4	PC 5	PC 6
Mg	-0.48	0.08	0.19	0.81	0.26	0.04
Mn	-0.38	-0.12	-0.82	-0.07	0.08	0.41
Cu	-0.10	-0.69	0.22	0.14	-0.60	0.29
Zn	-0.44	-0.49	0.00	-0.26	0.26	-0.66
Sr	0.48	-0.16	-0.49	0.50	-0.18	-0.47
Ba	0.44	-0.48	0.09	0.06	0.68	0.31

PCA for *R. acutus* whole life comparison similarly showed separation between regions, with overlap (Figure 3.4, Table 3.2). The first two principal components explained slightly less at 26% and 20%, with Ba and Mg having the heaviest influence. Far North, Townsville, and Mackay have some association with Ba, while Cairns and Brisbane have some association with Mg.

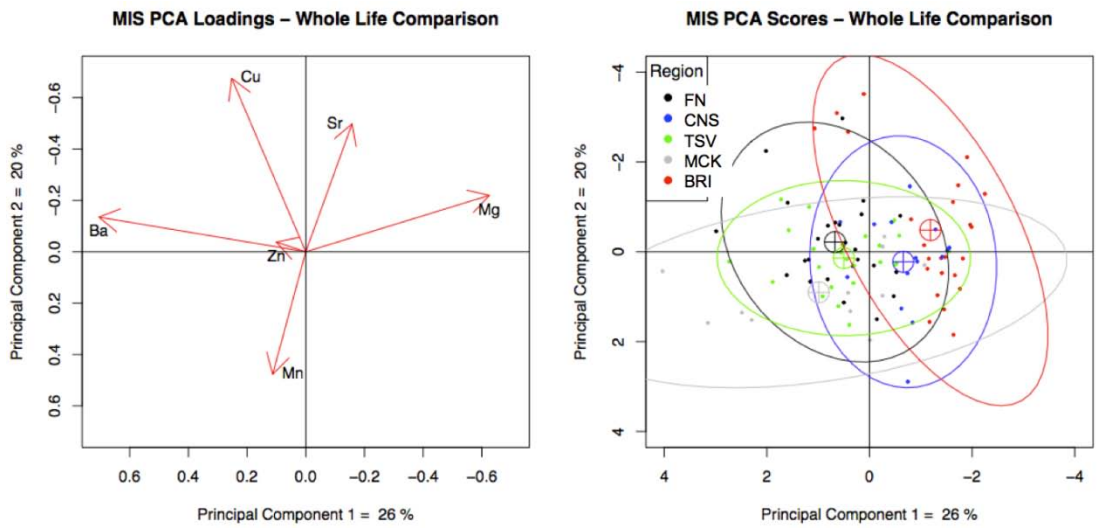


Figure 3.4 – Principal components analysis for *R. acutus* "whole life" LA-ICPMS. FN - Far North, CNS – Cairns, TVL – Townsville, MCK – Mackay, BRI – Brisbane. Crosses represent mean for that region and ellipsoid represents 95% confidence interval.

Table 3.2 – Importance of components and loadings for PCA for *R. acutus* "whole life" analysis.

	PC 1	PC 2	PC 3	PC 4	PC 5	PC 6
Standard Deviation	1.26	1.12	1.06	0.95	0.93	0.52
Proportion of Variance	0.27	0.21	0.19	0.15	0.14	0.05
Cumulative Proportion	0.27	0.48	0.66	0.81	0.95	1.00

Loadings - Element	PC 1	PC 2	PC 3	PC 4	PC 5	PC 6
Mg	-0.63	-0.22	0.26	-0.01	-0.42	0.56
Mn	0.11	0.48	0.59	0.35	-0.45	-0.30
Cu	0.25	-0.68	0.01	-0.21	-0.53	-0.39
Zn	0.10	-0.04	0.68	-0.64	0.34	0.03
Sr	-0.16	-0.50	0.34	0.60	0.46	-0.18
Ba	0.71	-0.14	0.12	0.24	-0.05	0.64

In considering the PCA for *S. lewini* catch location influence analysis, there is again some grouping between locations, but with heavy overlap (Figure 3.5, Table 3.3). The first two principal components explained 27% and 24% of overall variability. Contrasting to the whole life comparison, Cairns is not as clearly isolated from the other locations. Additionally, Mg has a negative association, where it had been strongly positive for the whole life analysis. Ba and Mg have

some association with Brisbane, while Cu and Zn are associated somewhat with Townsville.

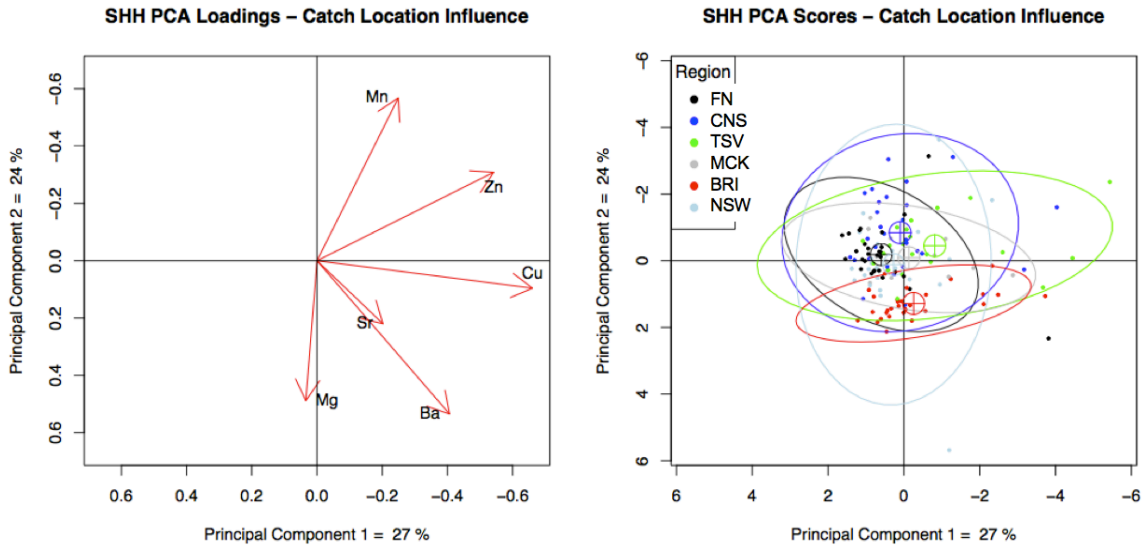


Figure 3.5 – Principal components analysis for *S. lewini* "catch location influence" LA-ICPMS. FN - Far North, CNS – Cairns, TVL – Townsville, MCK – Mackay, BRI – Brisbane. Crosses represent mean for that region and ellipsoid represents 95% confidence interval.

Table 3.3 – Importance of components and loadings for PCA for *S. lewini* "catch location influence" analysis.

	PC 1	PC 2	PC 3	PC 4	PC 5	PC 6
Standard Deviation	1.29	1.22	1.01	0.93	0.74	0.66
Proportion of Variance	0.28	0.25	0.17	0.14	0.09	0.07
Cumulative Proportion	0.28	0.52	0.69	0.84	0.93	1.00

Loadings - Element	PC 1	PC 2	PC 3	PC 4	PC 5	PC 6
Mg	0.03	0.49	-0.14	0.85	0.06	-0.14
Mn	-0.25	-0.57	0.33	0.33	0.62	-0.09
Cu	-0.66	0.10	-0.11	-0.16	-0.14	-0.71
Zn	-0.54	-0.31	-0.32	0.27	-0.40	0.53
Sr	-0.20	0.22	0.87	0.07	-0.36	0.13
Ba	-0.41	0.54	-0.04	-0.27	0.55	0.41

Clearer group separation occurred for the *R. acutus* catch location influence PCA (Figure 3.6, Table 3.4). However, the separation did not group geographically adjacent locations together. Far North, Townsville, and Mackay grouped

together, while Cairns and Brisbane were very distinct. Principal component 1 determines the primary separation between locations, with Mg, Sr, and Ba holding the greatest influence.

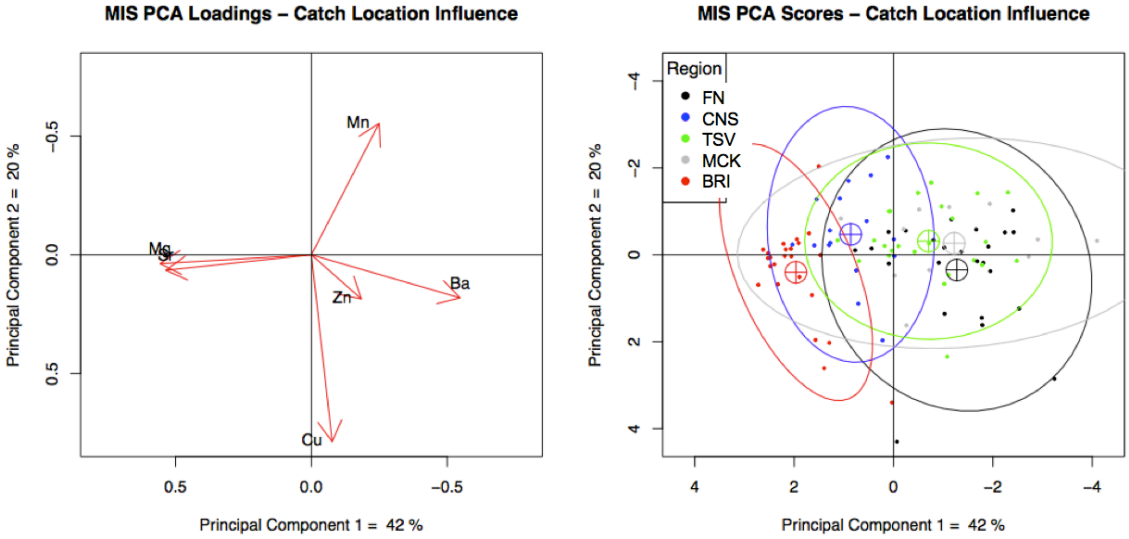


Figure 3.6 – Principal components analysis for *R. acutus* "catch location influence" LA-ICPMS. FN - Far North, CNS – Cairns, TVL – Townsville, MCK – Mackay, BRI – Brisbane. Crosses represent mean for that region and ellipsoid represents 95% confidence interval.

Table 3.4 – Importance of components and loadings for PCA for *R. acutus* "catch location influence" analysis.

	PC 1	PC 2	PC 3	PC 4	PC 5	PC 6
Standard Deviation	1.60	1.10	0.98	0.85	0.57	0.47
Proportion of Variance	0.43	0.20	0.16	0.12	0.05	0.04
Cumulative Proportion	0.43	0.63	0.79	0.91	0.96	1.00

Loadings - Element	PC 1	PC 2	PC 3	PC 4	PC 5	PC 6
Mg	0.56	0.04	-0.17	-0.20	0.26	0.74
Mn	-0.25	-0.55	-0.27	-0.74	0.06	-0.07
Cu	-0.08	0.79	0.03	-0.54	0.21	-0.20
Zn	-0.18	0.19	-0.93	0.26	0.01	-0.01
Sr	0.54	0.07	-0.16	-0.21	-0.77	-0.23
Ba	-0.55	0.18	0.11	-0.09	-0.54	0.60

The MANOVA hypothesis tests for both species and both analyses (whole life and catch location influence) showed high significance in testing for variation among groups (Table 3.5). Similarly the post-hoc ANOVAs for each element showed high significance among groups for most elements. One major exception is for Cu, which was likely due to contamination from the sectioning blade during preparation, as described in chapter 2. Zn showed high significance for both *S. lewini* post-hoc ANOVA's, but no significance for either of the *R. acutus* tests. Sr was highly significant for all tests except for *R. acutus* whole life analysis. It is logical to assume that the catch location influence analyses would show greater PCA groupings and hypothesis testing effect size than the whole life analyses since the composition should reflect only the very end of the animal's life. This was not the case. While there were some differences in effect sizes between the analyses, there were no consistent increases in effect sizes from whole life to catch location influence sequence analyses.

Table 3.5 – MANOVA and post-hoc ANOVA for both species and both analysis types (whole life or catch location influence).

Analysis	MANOVA	Mg	Mn	Cu	Zn	Sr	Ba
SHH Whole Life	Pillai=1.65 *** F(5,138)=11.3 p<.0001	F(5,138)=43.0 *** p<.0001	F(5,138)=26.1 *** p<.0001	F(5,138)=2.35 * p=.044	F(5,138)=7.13 *** p<.0001	F(5,138)=20.4 *** p<.0001	F(5,138)=14.6 *** p<.0001
MIS Whole Life	Pillai=1.14 *** F(4,94)=6.14 p<.001	F(4,94)=26.4 *** p<.0001	F(4,94)=16.4 *** p<.0001	F(4,94)=1.33 p=.266	F(4,94)=.297 p=.880	F(4,94)=1.62 p=.176	F(4,94)=10.7 *** p<.0001
SHH Catch Location Influence	Pillai=1.20 *** F(5,138)=7.18 p<.0001	F(5,138)=7.68 *** p<.0001	F(5,138)=17.8 *** p<.0001	F(5,138)=1.36 p=.242	F(5,138)=4.55 *** p=.0007	F(5,138)=6.12 *** p<.0001	F(5,138)=13.2 *** p<.001
MIS Catch Location Influence	Pillai=1.28 *** F(4,94)=7.21 p<.0001	F(4,94)=63.6 *** p<.0001	F(4,94)=10.9 *** p<.0001	F(4,94)=.863 p=.489	F(4,94)=2.45 p=.052	F(4,94)=24.0 *** p<.001	F(4,94)=20.5 *** p<.0001

While the above analyses appear to reject the simple hypothesis that there is no difference between sites, pair-wise comparisons address potential differences between adjacent sites. The Bonferroni adjusted Hotelling's T^2 comparisons for

both species' whole life sequences indicate highly significant separation between all adjacent locations except for Townsville – Mackay regions (Table 3.6). Both species exhibit similar trends in that the effect size is largest between Mackay / Brisbane, smallest between Cairns / Townsville, with Far North / Cairns between the other pairs. On inspection of these pair-wise comparisons in relation to animal sizes (Figure 3.2), there may be some issue with the difference of animals captured from each location. For example, both species have similar animal sizes from Townsville and Mackay, where the elemental composition difference is not significant. Cairns has animal sizes much smaller than either adjacent region, while all related Hotelling's tests show significance. However, this trend is not consistent. For *S. lewini*, Mackay and Brisbane have heavy overlap of animal sizes, but this pair has the largest effect size. Additionally, the Brisbane and NSW North have very substantial difference in animal sizes with no overlap, but the effect size is smaller than all but one of the other pair-wise comparisons.

Table 3.6 – Hotelling's T^2 pair-wise comparisons for whole life comparison. Bold entries are statistically significant.

Location Pair	*S. lewini*	*R. acutus*
Far North / Cairns	**T^2 = 18.4, p<.0001**	**T^2 = 15.9, p<.0001**
Cairns / Townsville	**T^2 = 11.8, p<.0001**	**T^2 = 11.8, p<.0001**
Townsville / Mackay	T^2 = 2.03, p=.09	T^2 = 1.22, p=.33
Mackay / Brisbane	**T^2 = 27.8, p<.0001**	**T^2 = 27.2, p<.0001**
Brisbane / NSW North	**T^2 = 4.71, p=.0009**	N/A

4.4 Discussion

The results obtained in this study provide additional insight into the mid-scale stock structure of both species for sites ranging in hundreds of kilometres apart. From an elemental profile viewpoint, they indicate several statistically significant isolated metapopulations, but with substantial overlap. Based on the size profile of *S. lewini* animals compared against life history parameters in the area (Harry et al. 2011a), most (but not all) animals in this study were juveniles. As such, conclusions made from these results should apply primarily to juveniles as treatment of nurseries. For *S. lewini*, the inferences of limited migration and structure are broadly aligned with expectations from other stock structure or migration determination methods. Using traditional tagging methods on the east coast of the United States, a total of 3,278 tagged *S. lewini* animals with a mean liberty time of 2.3 years (max 9.6 years), the average distance travelled between tagging and recapture was less than 100km (max of 1,600km) (Kohler and Turner 2001). Another traditional tag / recapture study in northern Australia of many shark species, including *S. lewini*, demonstrated some animals moving considerable distances (>1,000 km in some cases), mainly along shore. However, most sharks appeared to move very little, often staying within 50 km of initial tagging site (Stevens et al. 2000b). Within areas of regional residence, animals are known to move regularly between favoured locations 40 km away from each other (Hearn et al. 2010). In an early ultrasonic telemetry study in Kaneohe Bay, Hawaii, juvenile *S. lewini* were found to have activity ranges between 0.46 km^2 and 3.52 km^2 within the bay over a 12 day period (Holland et al. 1992). These studies indicate very limited home range for juvenile animals. Springer's (1967) general population model suggests that sharks come inshore

for pupping where the pups stay near nursery areas until they move offshore as adults. This is consistent with expectations for *S. lewini* in that most samples found on the present inshore study were juveniles.

Traditional tagging or telemetry techniques are powerful in tracking individuals' general movements, but are not necessarily conducive to comparing populations between sites with statistical methods. Existing stock structure analyses of *S. lewini* may be found with molecular evidence. Molecular methods are best used for determining structure over evolutionary time scales (Avise 2004). However, mixing rates need only be 1% or even less between stocks to prevent genetic differentiation (Bentzen et al. 1996). In a global *S. lewini* molecular based study, very little stock separation was found along coasts, while there was some level of structure detected across ocean basins (Duncan et al. 2006). In the present study's Australian location, the sites are inshore and hundreds of kilometres along the coast from each other. Coupling general site fidelity (or at least limited home range) with some animals occasionally travelling longer distances along the coast supports both the lack of coastal genetic structure and these microchemistry results. The microchemistry method addresses environmental influences on the animal from conception throughout life. It is logical that the technique will reveal shallower stock structure than molecular methods that reflect lineage up to the point of conception.

While substantial work has been performed on *S. lewini* stock structure and migration using other methods, very little has been done for *R. acutus*. However,

some activity has occurred to understand movement of other species in the genus *Rhizoprionodon*. For example, *R. terranovae* were studied in Florida, United States, using acoustic telemetry tracking (Carlson et al. 2008). It was found that juveniles had small home ranges, averaging 1.29 km^2. However, some animals disappeared from the study area for extended periods, returning after up to 1,352 days. Despite their small size, it appeared they use a series of coastal bays and estuaries as opposed to a discrete habitat. One individual was recaptured 169 km away from study site after 35 days at liberty (Carlson et al. 2008). A revised general model for smaller shark species suggests that despite limited site attachment, animals that spend their entire life-cycle inshore are less reliant on a specific habitat (Knip et al. 2010). Although the home range size and distance travelled may be less than that of juvenile *S. lewini*, it appears the same general behaviour of showing some site fidelity with occasional longer distance movement occurring for *R. acutus*. Again, this is generally consistent with the results of the present study. Based on life history data (Harry et al. 2010), the sampled animals in the present study represented approximately equal mix of juveniles and adults. This may indicate these results do not necessarily apply as a nursery area study, but a broad based behaviour of the species.

In simply trying to infer stock separation between regions, it is not necessary to determine how environmental variables, such as temperature, ambient elemental concentrations, diet availability or salinity, influence the chemical composition of the animals involved. It is only necessary to compare the multivariate elemental fingerprints of animal groups from the different regions (Elsdon and Gillanders 2003). The fact that the exploratory PCA showed some grouping with heavy

overlap between regions, and statistical hypothesis tests indicated significant separation between most regions may suggest that these species display general site fidelity, but with limited regional migration. However, one must be cautious in interpreting results such as these. Little can be inferred from differences in elemental fingerprints, other than that the stocks are different. If there is no difference in elemental fingerprints, such as between Townsville and Mackay in this study, one can not infer the stocks are the same (Campana et al. 2000). Since it is not known exactly what factors affect the trace elements in the vertebrae, it is not possible to make inferences about lack of difference between regions. It may be that the stocks freely migrate between the similar regions, or possibly that populations are isolated, but with similar influencing factors of each region.

While the present study suggests that several metapopulations exist along the northeastern Australian coast for both *S. lewini* and *R. acutus*, there are a number of uncertain factors in this microchemistry method. Factors affecting elemental variation in the animals include water temperature, salinity, diet availability or ambient concentrations (Bergenius et al. 2005). The migration behaviour we wish to make inferences about may be directly affected by these same factors. Water temperature may have direct effect on distribution and migration of sharks (Grubbs et al. 2007). Diets may vary with geographic location (McElroy et al. 2006) and could be due to local availability of prey (Bethea et al. 2006). Young *C. leucas* will change location based on salinity levels in a river estuary (Heupel and Simpfendorfer 2008). Although neither of the species in the present study would be expected to inhabit rivers, the salinity decreases from

river plumes during the wet season may affect animal movements (Knip et al. 2011).

Several trends in elemental profile were expected, but not observed in these results. Based on the assumption of temperature influencing elemental composition, it was expected that the PCA's would show a clear trend between proximal locations. For example, if Far North scores to show on far left, then adjacent locations of Cairns, Townsville, etc. generally would sequence to the right, with NSW North placed at the far right. However, no clear north to south pattern occurred in the PCA groupings. This may indicate that ambient concentrations have greater influence than temperature.

Another trend expected was some consistency between which elements were representative of each location. All four PCA's (*S. lewini*, *R. acutus*, each for whole life and catch location influence) indicated differing elemental profiles. It may be expected that different species reflect physiological and environmental variables differently (Elsdon and Gillanders 2003), but the differences in PCA loadings between whole life and catch location influence analyses is more difficult to explain. If considering a single sample, this could be explained by animal movement late in life. However, the statistically significant stock separation between locations makes this unlikely for entire sample location groups. Further analysis may clarify this phenomenon, but possible causes could be that the LA-ICPMS scan speed was too high (insufficient resolution) or ontogenetic changes in trace element apposition rate in cartilage. However, in otolith studies to

reconstruct salinity profiles, ontogeny showed no influence on key elements (Elsdon and Gillanders 2005). Further, in developing this method for shark vertebrae, specific testing was made to eliminate size as a contributing factor, albeit with different species and lower size variation (chapter 2).

An animal's microchemistry profile is affected by environmental ambient concentrations of various elements, but it is not a direct proxy for the environment (Campana and Thorrold 2001). As an inshore study with much of the area located in the tropics, it would be expected that run-off from the wet season will affect ambient concentrations of related elements. Detailed water analysis of elemental concentrations in study locations was beyond the scope of this study, as were the animals' specific physiological reaction related to these concentrations. Elemental concentration variation in sharks may be related simply to terrestrial runoff influencing ambient water concentrations and affecting the animals' uptake of related elements. As a simplistic example, if comparison is made for bedrock types of the underlying drainage basins for rivers near each of the study locations (Furnas 2003), an interesting pattern can be found corresponding to the Hotelling's T^2 pair-wise comparisons in the present study (Table 3.3). It can be seen that there are large changes in bedrock type for rivers feeding each adjacent pair of locations, except for Townsville and Mackay. This may be a coincidental similarity or may be a causal factor. The purpose here is to suggest that there are too many possible variables affecting an animal's elemental fingerprint to explain any causal effects. The conclusion to be drawn from these results are simply that the stocks from the different locations are significantly different from a statistical standpoint, with the exception of Townsville

/ Mackay. This reinforces what is expected from more traditional movement studies and does not contradict what has been found in prior molecular studies.

Table 3.3 – Bedrock type near each study location.

Region	River	Igneous % of Bedrock	Sedimentary % of Bedrock
Far North	Normanby	10%	90%
Cairns	Barron	38%	62%
Townsville	Ross	100%	0%
Mackay	Pioneer	98%	2%
Brisbane	Mary	20%	80%

Several additional areas can be addressed to increase the utility of this microchemistry method. In order to begin to understand what factors affect the animal's elemental profile, one must start with knowledge of where the animal has been. This may be from various tracking techniques, such as tag / recapture, acoustic telemetry, or preferably GPS tracking. Once the vertebrae and geographic history of many samples are acquired, it can be coupled with external environmental profiles. These would include items such as water chemical analysis, prey distribution models, or temperature profiles. Temporal effects must be considered to account for seasonal and annual variation in run-off or temperature. Analyses of this nature would be highly complex and require a very large sample size. It would also require much lower LA-ICPMS scan rate in order to substantially increase the resolution of transect data. However, if it is desired to infer more than "the stocks are different", this step must be taken.

This novel method for determining stock structure in sharks is useful to provide intermediate scale detail on the order of hundreds of kilometres between sites, with some limitations. Genetics based methods provide greater level of precision in analysis, but require greater scale for large wide travelling species. Acoustic or traditional tagging provides fine scale detail, but would require massive infrastructure to cover a larger scale. Caution is warranted in utilising ICPMS methods independently as there are many uncontrollable and unmeasurable variables that can influence results. It is unfeasible, with current knowledge, to reconstruct details of a specific animal's movement history from its elemental profile alone. While there is significant inshore population structure in both *S. lewini* and *R. acutus* along the Queensland and northern New South Wales coast, there is some regional migration. This should be sufficient to provide reasonable connectivity between populations as well as some capability of replenishment of depleted neighbouring populations. However, this connectivity appears limited so it would be prudent to manage the fishery on a regional basis to limit localised stock depletion.

5 General conclusions and recommendations for future research

5.1 How vertebral microchemistry fits with other stock structure methods

This project initially focused on developing a microchemistry approach to determining stock structure of sharks from their vertebrae, then followed with determination of medium scale stock structure of two species. Several other approaches exist for stock structure identification, each with their relative advantages and disadvantages.

As discussed in the literature review section, genetics methods are commonly used for stock structure analyses. A sister study based on genetic analysis occurred simultaneous to this microchemistry project, using a superset of samples of those used here. Lack of stock structure was found for both *R. acutus* and *S. lewini* along the entire study range (Ovenden et al. 2011). This was not unexpected, given the mobility of these species and relative proximity between study sites. With as little as 1% population mixing required to yield homogenous populations (Bentzen et al. 1996), genetics methods for these species should be more effective at larger (eg. global) scale. Genetics provides the benefit of having exact sequences to analyse, once the appropriate nuclear and/or mitochondrial markers are identified. The sequences are specifically defined, so genetics can provide a more precise analysis of stock separation.

This contrasts to the microchemistry approach that is largely based on statistical separation, often with indicated overlap. However, the microchemistry approach appears more effective at detecting shallower structure. Both methods are similar in sampling requirements, but the genetics has an advantage that it can be non-lethal. Preparation and laboratory processing are very different for each method, but similar in overall complexity. Since the genetic methods are more mature, software for data processing is readily available. For the SOL-ICPMS method, software is in place to determine adjusted elemental concentrations, so only a multivariate statistical package is required for analysis. However, LA-ICPMS will require substantial processing of the data to prepare it for the statistical analysis (see appendix). Packaged software exists for part of this activity, but not all of it. Software needs to be written for initial data reduction or the researcher needs to execute a very labour intensive exercise.

Another approach to determining stock structure is the traditional tag / recapture method. While it is beneficial to have two specific known locations and times for each animal, it does little to determine specific stock structure. It is very difficult to construct stock separation algorithms from a series of locations and times. However, inferences may be made from observing repeated and consistent behaviour between animals. A major challenge here is the heterogeneity of tag / recaptures and difficulty of simultaneous large-scale marking in the whole distribution area of a species (Jacobsen and Hansen 2005). To its advantage, tag / recapture techniques can provide accurate and very specific information about the migratory behaviour of animals being studied, but little about stock continuity or separation. Conversely, microchemistry techniques can not provide

specifics on animal movements, but can only infer population level heterogeneity. From a fishery management and conservation standpoint, both of these information types in conjunction with each other will prove invaluable. Tag / recapture techniques are logistically challenging and time consuming in that few animals may actually be recaptured and those that are, may take years for recovery.

Acoustic telemetry or GPS tagging can be very expensive to deploy and time consuming to maintain sufficient infrastructure. From an analysis perspective, there are similarities with traditional tag and recapture techniques in that movement of specific animals are identified, but with the benefit of having intermediate or continuous location data over a period of time. GPS tagging has the obvious shortcoming that the animals must surface periodically to transmit data. This type of tagging can prove very useful for understanding general migration or habitat utilisation, but is difficult to determine stock separation characteristics. It is difficult to manage a large scale study with acoustic telemetry, but drawing samples of geographically disparate locations for ICPMS analysis is comparatively easy.

Life history information was also utilised in a sister project to this stock structure study (Harry et al. 2010, Harry et al. 2011a). This generally involves considering various life history parameters, such as age at maturity, size at maturity, reproductive characteristics, or age growth curves analysed by location. Variation in life history characteristics may suggest separation of stocks at the

relevant locations. While this appears a very different approach to the microchemistry analysis, there is a strong similarity. Both life history characteristics and elemental composition may be affected by some of the same environmental variables. Age, growth and mortality figures may be influenced by environmental issues such as temperature or diet availability (Begg et al. 2005), which may also affect vertebral microchemistry profiles. Both methods are also be similar in that lack of differentiation between sites (elemental composition or life history parameters) does not necessarily indicate they are the same population. It may also indicate that the environmental conditions are similar or the animals simply evolved similarly. The sampling approach could be very similar, but the ICPMS methods will require greater use of technology.

It is possible to view a very high level summary of the various methods in terms of their relative strengths and weaknesses (Table 4.1). While the ratings are somewhat subjective and could be debated, it provides some guidance to the applicability of each method. For example, in comparing SOL-ICPMS and LA-ICPMS, both have the same field costs to acquire vertebrae. The SOL-ICPMS lab cost is lower than LA-ICPMS and the data (and related analysis) is less complex, but sacrifices historical information about the individual. Both should be supplemented with additional methods as confirmation of results. Genetics methods have greater capabilities as a standalone method, but only provide interesting results at a very wide scale. Acoustic telemetry has high cost in the field, provides limited information about stock structure, but very detailed information about the individuals being tracked.

Table 4.1 – Summary table summarising applicability of various shark stock structure methods. Field cost, lab cost, data complexity, and need for additional method are rated High, Med, or Low. Fine stock structure, wide stock structure and individual historical information rated as Good, Med, or Poor.

	Field Cost	Lab Cost	Data Complexity	Fine Stock Structure	Wide Stock Structure	Individual Historical Information	Need For Addl. Method
SOL-ICPMS	Med	Med	Low	Med	Good	Poor	High
LA-ICPMS	Med	High	High	Med	Good	Med	High
Genetics	Low	Med	Med	Poor	Good	Poor	Low
Tag/Recapture	High	Low	Low	Med	Poor	Good	Low
Life History	Med	Low	Med	Poor	Good	Poor	Med
Acoustic Telemetry	High	Low	Med	Med	Poor	Good	Low
GPS Tag	Med	Low	Low	Poor	Med	Good	Low

5.2 Challenges in method development - challenges in using the method

Substantial technical challenges had to be overcome throughout this project, both for SOL and LA-ICPMS methods. All of this led to large amounts of data analysis required to gain insight into the anomalies. The purpose here is not to detail all the challenges involved, but to provide a few examples and identify the lessons learned.

The SOL-ICPMS is relatively simple from the laboratory equipment standpoint in that no requirement for laser ablation exists. However, in this method the double blind sampling technique initially yielded inconsistent results outside the

anticipated ICPMS precision. Substantial effort was required in analysis and discussions to isolate errors in the ICPMS equipment prior to re-processing all samples.

Similarly, the laser ablation technique had several setbacks. After four days of processing LA-ICPMS samples for the main stock structure study of both species, equipment sensitivity changed, affecting all elemental ratios (details omitted for brevity). Due to the nature of the data, mathematical correction was not possible. It was not until the data analysis stage that the anomaly was detected, so months of time were lost. The issue was very difficult to articulate and prove fault to the equipment. Eventually the problem was specifically identified, but the root cause was never determined. As such, it was required to take the whole set of samples through the LA-ICPMS laboratory for re-processing.

The purpose of the above examples is to make the point that although the ICPMS equipment related challenges occurred during development of this method, there is nothing preventing the same issues reoccurring in a more routine utilisation of this method. In a method development project, substantial time to resolve these issues can be spent. However, in executing a stock structure analysis using this method, one should not expect to be confronted by these kinds of issues. Many researchers simply take ICPMS results at face value, without necessarily having a detailed understanding of what the values actually represent (Dr. Yi Hu, personal communication). Anyone utilising this method should have a solid

grounding in I.T., data analysis, and problem solving skills, as well as at least a very basic knowledge of the physics and chemistry behind ICPMS principles. Time should be spent in studying the data, applying tests of reasonableness for their intended application.

5.3 Extending the ICPMS method

While the method development should be considered a success, within the constraints and limitations described, various areas exist for refinement of the method for stock structure analysis or extension of the ICPMS to new areas. This final section details specific recommendations for future research that would be based directly on the method described in the present study.

5.3.1 Refining existing method

The method development section described the simple linear interpolation algorithm used for the LA-ICPMS drift calculation, based on repeatedly measuring NIST610 transects. While NIST610 was the standard identified as the closest to the apatite of the shark vertebrae, shortcomings exist with this strategy. All elements that were analysed were very low concentrations in the NIST 610 standard (with exception of Ca), varying between 305 and 492 µg/g (Pearce et al. 1997). The shark vertebrae had P as a major element, with relatively high concentrations of Mg and Mn. The drift correction algorithm assumed a linear relationship, which may or may not be a valid assumption and should be tested. For example, a given element may drift 10% for the NIST standard from 500 cps

(counts per second) to 550 cps. If a measurement of the shark vertebra was 20,000 cps, then the drift calculation would adjust it up that same 10%, to 22,000 cps. This is a major extrapolation of the algorithm. Ideally, a new standard would be developed that more closely resembles the shark vertebrae composition, particularly for P. Failing that, estimation of non-linear effects could be made by running the same vertebra sample just after each NIST 610 standard to model the effects. It may be possible to revise the overall data processing algorithms to improve precision in drift calculations. Another possibility to improve precision is exploring use of multiple internal standards to reduce variability (De Ridder et al. 2002). They were able to reduce measurement standard deviation by 30% by using two internal standards instead of just one.

In testing consistency of the SOL-ICPMS method, multiple solution samples were created from multiple vertebrae from multiple positions on the vertebral column. For LA-ICPMS, four different scans were taken either directly parallel with each other or from different legs of the corpus calcareum. These samples were always taken from the same animal and proved to be consistent within the expected variation of 5% - 8% for ICPMS technology (Durrant and Ward 2005). While this is beneficial to ensure consistency within the animal, it does not necessarily demonstrate consistency between animals with the same history. To achieve this, a controlled experiment similar in concept to that of Elsdon and Gillanders (Elsdon and Gillanders 2004), where a tank experiment could allow for control of environmental variables such as ambient concentrations and temperature. Understanding the exact history of samples being processed would allow specific conclusions to be drawn about a species' microchemical reaction to various

conditions. This understanding would assist in determining a set of rules to make inferences about an individual's specific migration history. Further, it may provide more detail to interpret how / why various stock structure patterns have developed.

Operating a manipulative experiment of this nature with sharks would likely prove logistically challenging, expensive and frustrating. As a compromise approach to this method, it would be substantially simpler to analyse vertebrae from multiple unborn pups from a pregnant female. While this does not allow for variable control or historical knowledge of ambient concentration or temperature, it ensures that samples taken have identical histories. Comparing this to simply drawing samples from the same location, it is not possible to know that movement histories of various animals would be identical. If LA-ICPMS were used, the time series sequences of the pups should also be compared to the mother for similarities. It would not be expected that the pups' sequences show direct equivalence to the outer edge sequence of the mother since the uterine environment would be under physiological regulation of the mother as well as being affected by the outer environment. While it would be expected that embryos of similar size will have had similar environmental and nourishment histories, this should allow confirmation of whether different animals of a given species will respond similarly in forming the chemical profile on their vertebrae. The only other factor that may affect composition of the vertebrae is physiological regulatory variation between fetuses due to genetic differences related to multiple paternity issues. This could easily be tested through nuclear genetics assays. From a statistical analysis standpoint, it would also be required to analyse several

sibling groups of a given species. If a sibling group of fetuses has similar microchemical profiles, but are different as a group than other sibling groups, then it could be inferred that animals of the species respond similarly to environmental influences. If statistical separation between sibling groups from mothers caught in the same area was not detected, it may indicate that external environmental factors have greater affect on the animal's composition than physiological factors.

For the LA-ICPMS method, limited lab time was available to optimise the scan parameters. In just a few hours of laboratory time, the primary parameters as defined in the methods development section were determined. These were largely determined based on the experience of the lab manager (Dr. Yi Hu) and the time available to process the estimated numbers of samples. For widespread application of this method, further research in to the optimum parameters is warranted. If primarily whole life analysis is required, and samples are relatively large, then it may be possible to substantially increase the scan speed to save processing time, without negative effect on accuracy. If 50 samples per day were processed at the 62um/s scan speed, each taking five minutes combined for cleaning and processing run, the total scan time for the day would be 250 minutes (4.2 hours). The remaining time is spent primarily on focusing the laser and changing slides in the ablation chamber. If a lower resolution scan lacks impact on the subsequent statistical analyses, the scan speed might possibly be increased two-fold or four-fold. It is also possible that reconfiguring the scanning software to improve the sample zoom capabilities could greatly increase the time efficiency related to the focusing process. For a larger project of 1,000 or more

samples, a 10% - 50% increase in LA-ICPMS processing efficiency could reduce project costs (laboratory and labour) by thousands of dollars.

Alternative to improving efficiency of the scanning process, it may be desirable to improve resolution, precision, and accuracy of the time series output of an LA-ICPMS scan. This may be to draw more detail from the end of life, or catch location influence, section of the transect. Higher resolution scans may also be required if LA-ICPMS were to be used for age and growth applications. As can be seen from the scan samples in the LA-ICPMS method development section, there is some natural latency in the signal that causes a low pass filtering effect. This is most obviously seen in the tail-off time at the end of the transect. This is caused by sample vapour being retained for a very short period in the ablation chamber or the 3 metre transport tube. Using a smaller chamber and/or shorter transport tube would decrease that filtering effect, which in turn increases accuracy of the signal at a given point in the time series. Alternatively, decreasing the scan speed would also lessen the filtering effect. Given that it may not be practical to decrease the chamber size or transport tube length, the scan speed may be the primary parameter to be modified to increase the resolution. Obviously this will also increase the time required per sample, thus decreasing the number of samples run per day. Many other parameters exist that can be modified, such as laser power, laser frequency, laser diameter, ICPMS sample frequency, etc. However, most of these parameters are set to ensure the correct amount of ablated sample material reaches the ICPMS sensor for the given sensitivity settings. The ICPMS sample frequency will affect the time series resolution from a data processing standpoint, but it is believed the

scan speed of the laser across the sample is more likely to improve the overall useable resolution in the processing.

In the method development section, there was a clear difference between the “whole life” and “catch location influence” analyses. The catch location influence demonstrated greater separation between location, as would be expected. However, upon analysing greater sample sizes and closer geographic locations, there was not a significant difference between the two analyses. Several possibilities exist to explain this, and multiple approaches could be pursued to try and isolate the issue. The first possibility is the scans may lack sufficient resolution, as described above. In this case the filtering effect of the relatively quick scan speed may be averaging out sufficient detail in the time series sequences. Second, the definition of catch location influence may need revision from using the final 0.2mm lateral distance from the vertebra edge. It is expected that this would not be a factor unless there is some physiological inconsistency between animals related to layering of new cartilaginous material onto the vertebrae. The third possibility is that it may be possible the assumption of chondrichthyans not resorbing cartilage, as has been assumed (Officer et al. 1995), is false. If resorption were to readily occur, this could explain a lack of variation between a whole life and catch location influence analysis. However, depositing of banding patterns and calcein marks remaining in place would then need to be explained. Isolation of this phenomenon would require combined LA-ICPMS analysis and data analysis (further discussion of resorption possibilities is addressed below). A similar overall approach to the stock structure determining method described herein would be required, but ablating samples at multiple

scan speeds and defining varying sequence lengths for the catch location influence. The various stock structure analyses could then be used to determine the best scan and data processing parameters. Alternatively, it may yield clues as to why the catch location influence analysis did not yield results stronger than the whole life in the main stock structure project.

In the methods development section, a range of animal sizes were compared to each other. Since it is necessary that all animals of this analysis were from the same location, there was an inherently limited size range of available samples. It would be prudent to test for a size effect more rigorously, by using a site with many samples and a very large evenly distributed size range. With the samples available in the present study, this was not possible. A robust analysis should include animal sizes ranging from neonate through to large adult. Since the historical movements of each animal is unknown, it would be necessary to compare the catch location influence portion of the sequences. Ideally, sixty to eighty samples spanning the size range of a given species could be captured from a single region. This would allow for three or four size classes to be defined and tested for variation. If no variation between size groups are identified, this would confirm what the present method development activity found, but with a more robust sample distribution. If variation exists, along with a trend (eg. the larger the animal, the more strontium gets deposited on their vertebra), then the method may require revision to use size as a covariate (MANCOVA instead of MANOVA).

Samples in the present study were captured across more than two years. Many of these would have been young-of-the-year or even neonates, meaning different samples would have had no overlapping life span. Being inshore species, it is logical that ambient concentrations of elements are affected by run-off in the variable wet seasons. Different animals would have lived through wet seasons or different years. Additionally, animals were captured at various times of year. For a five year old, this may not be significant. However if an animal is eighteen months of age and caught in March, it would have lived through two wet seasons (with related extra run-off) and one dry season, meaning 2/3 of its life was in waters with extra run-off and in summer temperatures. If an eighteen month old animal was caught in November, then the opposite would be true of having 2/3 of its life in dry season with winter temperatures. The present study did not yield sufficient sample time distribution to analyse these effects. In order to determine temporal and seasonal effects, sampling of a given species in a given region should occur in all seasons and across several years. Water and prey sampling should occur at the same time as well as recording of water temperatures. In addition to analysing the catch location influence section of the vertebrae, the water and prey samples can be analysed in a solution ICPMS to determine ambient concentrations of related elements. This sampling should include minimal range of sizes in order to eliminate as many possible sources of variation from the analysis. If clear trends are identified with the animals' elemental composition and a given environmental parameter (ambient concentration, temperature, etc.), then it may be possible to begin constructing algorithms to identify individual migration patterns based on the whole LA-ICPMS time series sequence. For example, if a strong positive relationship is identified between Sr

levels and water temperature, but not with ambient concentrations, this could be used in conjunction with ongoing hydrodynamic data along the coast. If the animal showed elevated Sr levels earlier in the sequence (ie. earlier in life), then it might be inferred it migrated up north for that period of its life. Without a detailed understanding of the relationships between elemental composition and all related contributing variables, it is impossible to make reasonable inferences about specific animal movements.

Another possible approach to determining direct relationships between environmental parameters and elemental profiles would be to study a known amphidromous species such as bull shark (*Carcharhinus leucas*). In teleosts, studying anadromous species is a common application due to salinity effects on otolith composition (Coutant and Chen 1993, Kimura et al. 2000, Bacon et al. 2004, Courtemanche et al. 2005, Arai and Hirata 2006, Vasconcelos et al. 2007). The minimum interesting analysis for sharks would be to run high resolution scans, looking for positive or negative spikes in any of the elements. If these spikes are of much greater magnitude than seen in other species, they may be related to salinity variation related to temporary migration up estuaries. Improvements in this analysis could be made if it were possible to recapture an animal that has been tracked acoustically through estuarine waters to have a known history of when it was present in reduced salinity conditions. Running a matching algorithm of various elemental profiles versus a timeline of known reduced salinity presence events may reveal a direct relationship that could be used to determine general estuarial utilisation habits of this species. It may also assist in understanding relationship between elemental composition and salinity

for other species as well. This could be of benefit in tropical situations where salinity levels might vary with the run-off from the wet seasons. However, caution is warranted in that a relationship between salinity in one species may not translate across to a similar relationship with other species (Elsdon and Gillanders 2005).

If the above analyses are executed successfully, and relationships between environmental variables and vertebral microchemistry are established, it may be feasible to progress this concept further. If vertebrae samples from wide ranging animals such as a great white (*Carcharodon carcharias*) or tiger shark (*Galeocerdo cuvier*) that have been tagged with GPS transmitters could be recovered, a wealth of analysis could ensue. It is acknowledged that this would be a difficult and expensive activity at best. Even with some relationship understanding between elemental composition and ambient concentrations or temperature, this would be a highly complex analysis. It would involve pattern matching with multiple response variables and multiple explanatory variables using a technology that only has 10% accuracy. Even if it were possible to draw a solid relationship between, say, temperature and a given element, this would still not lead to specific locations. For example, Adelaide may be at the same temperature as Auckland at a given point in time. Additionally, a robust database would be required incorporating temperature and elemental concentration values covering the water bodies and time frames involved. This author is highly sceptical that this method could be developed successfully. It is only discussed here because this utopian capability has frequently been requested by various researchers when discussing LA-ICPMS.

The microchemistry method appears to have identified “shallower” population structure than the genetics method could detect along the Queensland coast for the species being studied. In order to further understand the utility of each these methods in comparison to each other, it would be useful to study a species with both methods in a situation where deep genetic structure can be determined. For example, if vertebrae were available from Duncan (2006), LA-ICPMS could be utilised to compare and contrast against the global genetics study. That study clearly delineated between Atlantic and Indo-Pacific populations, suggesting limited trans-oceanic migrations. While the microchemistry method was able to detect subtle microchemical differences along the Queensland coast, it may be that much clearer and obvious separation exists between the globally disparate locations. Alternatively, it may be that all environmental factors are nearly identical at locations across the globe from each other, possibly causing lack of separation in elemental composition where separation exists in genetics studies. Regardless, this would assist in either validating or disproving the method for use on large scales. If ambient concentrations (in a multivariate sense) of various elements vary a reasonable amount at large scales, it would be expected to show not only statistical separation on a MANOVA, but also clearly distinguishable groups on a PCA.

5.3.2 Additional applications

The purpose of the present study was purely to analyse vertebrae to determine stock structure. Although not specifically considered here, many vertebrae

samples appeared to show amplitude patterns that may represent growth rings, similar to those found by Hale (2006). With the LA-ICPMS parameters as set in the present study, these growth ring patterns were identified in approximately 10% of samples. Using techniques to increase scan resolution as described above, it may be possible to obtain more consistent growth ring identification. Due to the ease of ageing sharks by vertebrae inspection under a microscope and the time/cost involved with LA-ICPMS, it is unlikely to use this method purely for ageing samples. However, if relationships are identified between various elements and any specific environmental variables, the amplitudes of growth rings may be used to infer seasonal migration or diet changes.

It has been a fundamental assumption throughout this study that no cartilage resorption occurs in sharks. Prior to widespread utilisation of this method, it would be prudent to test the assumption. This could be tested if a litter of pups could be captured and kept in captivity. Depending on sample sizes available, animals could be sacrificed for their vertebrae after various amounts of time. The animals that have been kept longer will obviously have larger vertebrae centra, with correspondingly longer time series associated with LA-ICPMS. The portion of the sequence inside the birth ring could be compared directly to corresponding time series sequences from pups that had been sacrificed earlier. If no resorption occurs, as is expected, the corresponding sections of time series would be very similar between the various aged sibling animals. However, if resorption does occur, the elemental composition inside the birth ring of older animals will be different from the siblings sacrificed at birth. If resorption were to

occur in sharks, it would have consequences for not only this stock structure method, but in our general understanding of shark developmental biology.

The final recommendation to extend LA-ICPMS research would be to improve and standardise the software involved with processing the data. Although the fundamental data structures and algorithms of the “R” code written for this project are sound, well documented, parameter driven, and use generally acceptable software development methods, it would be somewhat difficult for the code to be re-used by other researchers on other projects (see appendix). If there were interest in widespread use of LA-ICPMS to be used in the method developed by this research, it would be possible to commercialise the software or, at a minimum, create a well documented open source “R” library for general availability. If an “R” library were desired, expanded use of the input parameter files would be required. For example, the software developed for this project had many items, such as sequence definitions (moving average spread, outlier definition, etc.) set as externally identified parameters in an input file. Other items, such as the number of locations, types of analyses run, and elements analysed, are coded directly into the software. Restructuring the code to accommodate more generic use would be preferred overall to having each researcher write analysis software starting from nothing.

5.4 Conclusion

Vertebral microchemistry is a powerful method to help understand stock structure of shark species, but it is best when utilised as one portion of a comprehensive

suite of tests that include genetics, life history analysis, and traditional tag / recapture techniques. Critical advantages exist for each method, so they can all be used similar to puzzle pieces to construct a complete story for a given species. Furthermore, the ICPMS techniques developed in this research can be used as a foundation to enhance the method as well as developing new applications. The method is not without its technical challenges, where the data reduction requirements for raw LA-ICPMS data can be immense. As the method matures, openly available software should assist in the data analysis.

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7 Appendix – "R" Script for Data Processing

7.1 Main Script for Laser Ablation ICPMS

```
#  Analysis for Laser Ablation ICP-MS

# notify user that processing has started

 cat('\nCommenced Processing\n')

# clear everything out of memory before starting

rm(list=ls())

# for MAC at home
file.location=('/Users/Ronbeau/JCU/R Statistics/Laser Ablation 5/')
# for PC at uni
#file.location=('G:\\JCU\\R Statistics\\Laser Ablation 4\\')
#file.location=('F:\\JCU\\R Statistics\\Laser Ablation 4\\')

# open and read sample information
index=read.csv(paste(file.location,'Sample Index.csv',sep=''))
```

```
# open and read input parameters and logicals
input.matrix=read.csv(paste(file.location,'Input Parameters.csv',sep=''))
input.logical=read.csv(paste(file.location,'Input Logicals.csv',sep=''))

# request input to vary input parameters

# input.matrix=edit(input.matrix)  temporarily blocked out ability to change defaults
# input.logical=edit(input.logical)

#  call up functions that have been written externally
source(paste(file.location,'ronbo_function_definitions.R',sep=''))

library(MASS)
library(Hmisc)
library(ICSNP) # for Hotellings T2
library(car)   # for data.ellipse

# suppress warnings printed by R
options(warn=-1)

# manipulate input parameters to input$ data.frame format
input=data.frame(t(input.matrix$parameter.value))
names(input)=input.matrix$parameter.input.variable

# manipulate input logicals to input.log$ data.frame format
input.log=data.frame(t(input.logical$value.t.f))
names(input.log)=input.logical$logical.input.variable

# define graphics output as screen (MAC quartz device) or pdf

if (input.log$pdf.graphics.output==FALSE) {
  quartz()
  ask=TRUE
}
if (input.log$pdf.graphics.output==TRUE) {
  pdf(paste(file.location,'Graphics Output.pdf',sep=''),width=12,height=6,
          onefile=T,family='Helvetica',title='Laser Ablation Data Analysis')
  ask=FALSE
}

# initiate vector of isotopes we're working with

isotope=c('Mg','P','Ca','Mn','Cu','Zn','Sr','Ba')
isotope.no.ca=c('Mg','P','Mn','Cu','Zn','Sr','Ba')

# initiate vector of isotopes, without P

isotope.no.p=c('Mg','Ca','Mn','Cu','Zn','Sr','Ba')
isotope.no.ca.no.p=c('Mg','Mn','Cu','Zn','Sr','Ba')

# run format pre-processor to convert raw data from laser ablation lab to data.frame

if (input.log$pre.process.format==TRUE) {

# notify user that processing has started

  cat('\nShuffling Raw Data\n')

# shuffle and stack data

  for (i in 1:8) {

# print file number to screen so you can track progress

          cat(paste('\nProcessing Raw File ',i,'\n'))

# read in raw data file

          raw.data=as.matrix(read.csv(paste(file.location,
                  'April Raw Data/Raw File ',i,'.csv',
                  sep=''),header=FALSE,col.names=c('scan','time','mg','p','ca','mn','cu','zn','sr','ba')))

# call up reformatting function to add day and run number columns to the matrix
```

```
        reshuffled.raw.data=la.data.reformat(raw.data,index)

# rename raw.data into variable

#               assign(paste('reshuffled.raw.data.jan.',i,sep=''),reshuffled.raw.data)

# save object for future work, without having to run pre-processing
        save(reshuffled.raw.data,file=paste(file.location,
                'April Raw Data/Raw_Data_',i,'.rda',sep=''),ascii=F)

 }

# stop processing from here

 stop('Done Shuffling Data')

}


#################################################################################
#       PRE-PROCESSING FOR FORMAT MANIPULATION FINISHES HERE
#################################################################################

# pre-processing of format must have already been run and saved previously
debug=1
if (input.log$pre.process.format==FALSE&input.log$pre.process.ts==TRUE) {

# print notice of loading shuffled data

 cat(paste('\nLoading Previously Shuffled Data\n'))

# load in first data file

 load(file=paste(file.location,
                'April Raw Data/Raw_Data_1.rda',sep=''))

# create new master table called raw.data.formatted (sorry about the names so close to reshuffled.raw.data)

 raw.data.formatted=reshuffled.raw.data
debug=2
# now loop through for the rest of the Jan 2009 data files

 for (i in 2:8){

        load(file=paste(file.location,
                'April Raw Data/Raw_Data_',i,'.rda',sep=''))

# append to existing raw.data.formatted

        raw.data.formatted=rbind(raw.data.formatted,reshuffled.raw.data)

 }
debug=3
debug=4


# don't ask me why, but all columns are "factors"!!!, we now need to convert to numeric

 raw.data.formatted=data.frame(matrix(as.numeric(raw.data.formatted),byrow=F,ncol=ncol(raw.data.formatted),
        dimnames=dimnames(raw.data.formatted)))

}


debug=5
#
#
#################################################################################
#       SEQUENCE DEFINITION PLOTS DONE HERE, PROGRAMME BREAKS AFTER PLOTS
#       FOR DATA ENTRY OF START AND END POINTS
#################################################################################
#

if (input.log$sequence.plot==TRUE) {
```

```
# print notice of plotting time series

 cat(paste('\nPlotting Ca Time Series\n'))


 for (i in 1:nrow(index)){

# create temporary subset of raw data based on run date and run number

        temp.raw.data=subset(raw.data.formatted,unique.id==index$unique.id[i])

        cat(i,' ')

# round to even 20, for scaling of plot

        xmax=ceiling(length(temp.raw.data$ca)/20)*20

        plot.ts(temp.raw.data$ca,main=paste('Sequence Definition Plot - Run Number ',index$run[i],' - Raw
 Data'),xlim=c(0,xmax))

        for (l in 1:(xmax/20)) {

                abline('v'=l*20,col='magenta',lwd=1,lty='dotted')

        }

        abline('v'=index$seq.start[i],col='green',lwd=1.5)
        abline('v'=index$seq.end[i],col='red',lwd=1.5)

 }

# since this is being run to determine sequence definition, rest of programme will likely end in error, so
# close the pdf connection if appropriate

 if (input.log$pdf.graphics.output==TRUE) {
        dev.off()
 }
}


#
#
##############################################################################################
#       PRE-PROCESSING FOR TIME SERIES BEGINS HERE
##############################################################################################
#


# run data pre-processor and save object as file for future use

if (input.log$pre.process.ts==TRUE) {

 cat('\nPre Processing Time Series\n')

# create data variable as a "list", just to initiate the variable
 data=c(1:3)
 data[1]=as.list(1)
 angle.lateral.outer.rad=1
 mean.sol=1

# eliminate 'NA' values by replacing them with 99999999, eventually to be picked up as outlier
# note: P will have several of these in a row based on April 2010 analysis. Eliminate from stats analysis

 raw.data.formatted$mg=ifelse(is.na(raw.data.formatted$mg),99999999,raw.data.formatted$mg)
 raw.data.formatted$p=ifelse(is.na(raw.data.formatted$p),99999999,raw.data.formatted$p)
 raw.data.formatted$ca=ifelse(is.na(raw.data.formatted$ca),99999999,raw.data.formatted$ca)
 raw.data.formatted$mn=ifelse(is.na(raw.data.formatted$mn),99999999,raw.data.formatted$mn)
 raw.data.formatted$cu=ifelse(is.na(raw.data.formatted$cu),99999999,raw.data.formatted$cu)
 raw.data.formatted$zn=ifelse(is.na(raw.data.formatted$zn),99999999,raw.data.formatted$zn)
 raw.data.formatted$sr=ifelse(is.na(raw.data.formatted$sr),99999999,raw.data.formatted$sr)
 raw.data.formatted$ba=ifelse(is.na(raw.data.formatted$ba),99999999,raw.data.formatted$ba)

# combine index and raw.data into appropriate data structure list of data
# the following large loop is executed once for each LAICPMS sample run
```

```
 for (i in 1:nrow(index)){

# print the run number to the screen

        cat(paste(' ',index$unique.id[i]))


# create temporary subset of raw data based on run number

        temp.raw.data=subset(raw.data.formatted,unique.id==as.character(index$unique.id[i]))

debug=1
# filter for outliers

        mg24.outlier.filt=outlier.filter(index$unique.id[i],'mg24',temp.raw.data$mg,
                input$outlier.spread,input$outlier.filter)
        p31.outlier.filt=outlier.filter(index$unique.id[i],'p31',temp.raw.data$p,
                input$outlier.spread,input$outlier.filter)
        ca44.outlier.filt=outlier.filter(index$unique.id[i],'ca44',temp.raw.data$ca,
                input$outlier.spread,input$outlier.filter)
        mn55.outlier.filt=outlier.filter(index$unique.id[i],'mn55',temp.raw.data$mn,
                input$outlier.spread,input$outlier.filter)
        cu65.outlier.filt=outlier.filter(index$unique.id[i],'cu65',temp.raw.data$cu,
                input$outlier.spread,input$outlier.filter)
        zn66.outlier.filt=outlier.filter(index$unique.id[i],'zn66',temp.raw.data$zn,
                input$outlier.spread,input$outlier.filter)
        sr88.outlier.filt=outlier.filter(index$unique.id[i],'sr88',temp.raw.data$sr,
                input$outlier.spread,input$outlier.filter)
        ba137.outlier.filt=outlier.filter(index$unique.id[i],'ba137',temp.raw.data$ba,
                input$outlier.spread,input$outlier.filter)

debug=2
# combine outlier.list data to deliver single overall outlier.list

        outlier.summary=rbind(mg24.outlier.filt[[2]],p31.outlier.filt[[2]],
                ca44.outlier.filt[[2]],mn55.outlier.filt[[2]],cu65.outlier.filt[[2]],
                zn66.outlier.filt[[2]],sr88.outlier.filt[[2]],ba137.outlier.filt[[2]])

# smooth each series by running through centralised simple moving average filter
debug=3

        smooth.mg24=smooth.series(mg24.outlier.filt[[1]],input$ma.spread)
        smooth.p31=smooth.series(p31.outlier.filt[[1]],input$ma.spread)
        smooth.ca44=smooth.series(ca44.outlier.filt[[1]],input$ma.spread)
        smooth.mn55=smooth.series(mn55.outlier.filt[[1]],input$ma.spread)
        smooth.cu65=smooth.series(cu65.outlier.filt[[1]],input$ma.spread)
        smooth.zn66=smooth.series(zn66.outlier.filt[[1]],input$ma.spread)
        smooth.sr88=smooth.series(sr88.outlier.filt[[1]],input$ma.spread)
        smooth.ba137=smooth.series(ba137.outlier.filt[[1]],input$ma.spread)

# calculate the background signal of each series based on points 5-20
debug=4
        back.sig.mg24=mean(smooth.mg24[5:20])
        back.sig.p31=mean(smooth.p31[5:20])
        back.sig.ca44=mean(smooth.ca44[5:20])
        back.sig.mn55=mean(smooth.mn55[5:20])
        back.sig.cu65=mean(smooth.cu65[5:20])
        back.sig.zn66=mean(smooth.zn66[5:20])
        back.sig.sr88=mean(smooth.sr88[5:20])
        back.sig.ba137=mean(smooth.ba137[5:20])

# combine data to be inserted into the main data set
debug=5
        background.signal=c(back.sig.mg24,back.sig.p31,back.sig.ca44,back.sig.mn55,
                back.sig.cu65,back.sig.zn66,back.sig.sr88,back.sig.ba137)
        names(background.signal)=isotope

# adjust the smoothed signal to remove the background signal
debug=6

        no.back.mg24=smooth.mg24-back.sig.mg24
        no.back.p31=smooth.p31-back.sig.p31
        no.back.ca44=smooth.ca44-back.sig.ca44
        no.back.mn55=smooth.mn55-back.sig.mn55
        no.back.cu65=smooth.cu65-back.sig.cu65
```

```
	no.back.zn66=smooth.zn66-back.sig.zn66
	no.back.sr88=smooth.sr88-back.sig.sr88
	no.back.ba137=smooth.ba137-back.sig.ba137
debug=7


# calculate sequence start and end from when ca44 crosses thresholds defined in
# input$parameters
# NIST standards need to use different thresholds due to lower abundances



# calculate start and end sequence

	start.point=index$seq.start[i]
	end.point=index$seq.end[i]
	length=end.point-start.point
	sequence=data.frame(start.point,end.point,length)


	if (index$profile[i]=='standard') {


# re-define correction sequence as 30 points in the middle to eliminate start and end tapers
# note that this modifies the original input sequences on the 'sample index' file

		sequence$start.point=sequence$start.point+round(
			(sequence$end.point-sequence$start.point)/2)-(input$standard.width/2)
		sequence$end.point=sequence$start.point+round(
			(sequence$end.point-sequence$start.point)/2)+(input$standard.width/2)

# calculate correction factors here for machine drift of ICPMS.  This will be required later for correction.
debug=8

		mean.corr.mg24=mean(no.back.mg24[sequence$start.point:sequence$end.point])
		mean.corr.p31=mean(no.back.p31[sequence$start.point:sequence$end.point])
		mean.corr.ca44=mean(no.back.ca44[sequence$start.point:sequence$end.point])
		mean.corr.mn55=mean(no.back.mn55[sequence$start.point:sequence$end.point])
		mean.corr.cu65=mean(no.back.cu65[sequence$start.point:sequence$end.point])
		mean.corr.zn66=mean(no.back.zn66[sequence$start.point:sequence$end.point])
		mean.corr.sr88=mean(no.back.sr88[sequence$start.point:sequence$end.point])
		mean.corr.ba137=mean(no.back.ba137[sequence$start.point:sequence$end.point])

# combine data for inclusion of main data set, all ratio to ca

		correction.signal=c(mean.corr.mg24,mean.corr.p31,mean.corr.mn55,mean.corr.cu65,
			mean.corr.zn66,mean.corr.sr88,mean.corr.ba137)/mean.corr.ca44

		names(correction.signal)=isotope.no.ca

# also save the raw data before ratios

		correction.signal.raw=c(mean.corr.mg24,mean.corr.p31,mean.corr.ca44,mean.corr.mn55,mean.corr.cu65,
			mean.corr.zn66,mean.corr.sr88,mean.corr.ba137)

		names(correction.signal.raw)=isotope


# since this is a standard, define dummy variables for later inclusion in 'data' list

		mean.catch=0
		mean.catch.ca44.ratio=0
		mean.sol.ca44.ratio=0
		mean.sol=0

	}

	else {

# run this stuff for non-standards

# calculate the angle between lateral and outer radii (in radians)

debug=9
		angle.lateral.outer.rad=asin((index$ventral.width[i]/2)/index$outer.radius[i])
```

```
# calculate the equivalent lateral step distances (point/micron),
# adjusting for the angle

                if (index$profile[i]=='edge') {
                        lateral.dist.step.equiv=input$laser.scan.speed*input$icpms.meas.period*
                                cos(angle.lateral.outer.rad)
                }
                else {
                        lateral.dist.step.equiv=input$laser.scan.speed*input$icpms.meas.period
                }
debug=10
# define start and end sequence

#                       sequence=define.sequence(no.back.ca44,input$start.hurdle,input$start.delta,
#                               input$end.hurdle,input$end.delta)

# calculate approximate number of points skipped due to not being able to ablate the
# absolute centre of the vertebrae

                skipped.start.points=input$start.distance/lateral.dist.step.equiv

# define birth ring diameter based on index

                birth.diam=switch(as.character(index$species[i]),'SHH'=input$shh.birth.diam,
                        'ABT'=input$abt.birth.diam,'MIS'=input$mis.birth.diam,'PIG'=NA,'GHH'=NA)
debug=11
# calculate the birth ring location (shh and abt only) based on lateral.dist.step.equiv
# and input$parameters

                if ((index$species[i]=='SHH')||(index$species[i]=='ABT')||(index$species[i]=='MIS')) {
                        sequence$birth.point=start.point+as.integer(((birth.diam/2)/
                                (lateral.dist.step.equiv))-skipped.start.points)
                }
                else {
                        sequence$birth.point=sequence$start.point
                }

debug=12

# calculate the mean values of each element from birth ring to death, for comparison
# to solution based ICPMS results needs to be corrected for drift, which is done later
# all ratio to Ca

                mean.sol.mg24=as.integer(mean(no.back.mg24[sequence$birth.point:sequence$end.point]))
                mean.sol.p31=as.integer(mean(no.back.p31[sequence$birth.point:sequence$end.point]))
                mean.sol.ca44=as.integer(mean(no.back.ca44[sequence$birth.point:sequence$end.point]))
                mean.sol.mn55=as.integer(mean(no.back.mn55[sequence$birth.point:sequence$end.point]))
                mean.sol.cu65=as.integer(mean(no.back.cu65[sequence$birth.point:sequence$end.point]))
                mean.sol.zn66=as.integer(mean(no.back.zn66[sequence$birth.point:sequence$end.point]))
                mean.sol.sr88=as.integer(mean(no.back.sr88[sequence$birth.point:sequence$end.point]))
                mean.sol.ba137=as.integer(mean(no.back.ba137[sequence$birth.point:sequence$end.point]))

                mean.sol=c(mean.sol.mg24,mean.sol.p31,mean.sol.ca44,mean.sol.mn55,mean.sol.cu65,
                        mean.sol.zn66,mean.sol.sr88,mean.sol.ba137)
                names(mean.sol)=isotope

                mean.sol.ca44.ratio=c(mean.sol.mg24,mean.sol.p31,mean.sol.mn55,mean.sol.cu65,
                        mean.sol.zn66,mean.sol.sr88,mean.sol.ba137)/mean.sol.ca44
                names(mean.sol.ca44.ratio)=isotope.no.ca
debug=13
# calculate catch location effect begin point.

                sequence$catch.loc.point=as.integer(sequence$end.point-(input$catch.loc.influence/
                        lateral.dist.step.equiv))

# calculate mean values of each element from catch influence point to death
# needs to be corrected for drift, which is done later
debug=14
                mean.catch.mg24=as.integer(mean(no.back.mg24[sequence$catch.loc.point:sequence$end.point]))
                mean.catch.p31=as.integer(mean(no.back.p31[sequence$catch.loc.point:sequence$end.point]))
                mean.catch.ca44=as.integer(mean(no.back.ca44[sequence$catch.loc.point:sequence$end.point]))
                mean.catch.mn55=as.integer(mean(no.back.mn55[sequence$catch.loc.point:sequence$end.point]))
                mean.catch.cu65=as.integer(mean(no.back.cu65[sequence$catch.loc.point:sequence$end.point]))
                mean.catch.zn66=as.integer(mean(no.back.zn66[sequence$catch.loc.point:sequence$end.point]))
```

```
                mean.catch.sr88=as.integer(mean(no.back.sr88[sequence$catch.loc.point:sequence$end.point]))
                mean.catch.ba137=as.integer(mean(no.back.ba137[sequence$catch.loc.point:sequence$end.point]))

                mean.catch=c(mean.catch.mg24,mean.catch.p31,mean.catch.ca44,mean.catch.mn55,
                        mean.catch.cu65,mean.catch.zn66,mean.catch.sr88,mean.catch.ba137)
                names(mean.catch)=isotope

                mean.catch.ca44.ratio=c(mean.catch.mg24,mean.catch.p31,mean.catch.mn55,mean.catch.cu65,
                        mean.catch.zn66,mean.catch.sr88,mean.catch.ba137)/mean.catch.ca44
                names(mean.catch.ca44.ratio)=isotope.no.ca

# if it's not a "standard", set correction.signal to NA

                correction.signal=c(NA,NA,NA,NA,NA,NA,NA)

        }


# calculate the ratio of element:ca for the sequence length (time series for each element)
# needs to be corrected for drift, which is done below
debug=15
                mg24.ca44.ratio=no.back.mg24[sequence$start.point:sequence$end.point]/
                        no.back.ca44[sequence$start.point:sequence$end.point]
                p31.ca44.ratio=no.back.p31[sequence$start.point:sequence$end.point]/
                        no.back.ca44[sequence$start.point:sequence$end.point]
                mn55.ca44.ratio=no.back.mn55[sequence$start.point:sequence$end.point]/
                        no.back.ca44[sequence$start.point:sequence$end.point]
                cu65.ca44.ratio=no.back.cu65[sequence$start.point:sequence$end.point]/
                        no.back.ca44[sequence$start.point:sequence$end.point]
                zn66.ca44.ratio=no.back.zn66[sequence$start.point:sequence$end.point]/
                        no.back.ca44[sequence$start.point:sequence$end.point]
                sr88.ca44.ratio=no.back.sr88[sequence$start.point:sequence$end.point]/
                        no.back.ca44[sequence$start.point:sequence$end.point]
                ba137.ca44.ratio=no.back.ba137[sequence$start.point:sequence$end.point]/
                        no.back.ca44[sequence$start.point:sequence$end.point]

debug=16
# create entry for list object that includes all sample
# indexing info and raw time series data

        temp.list=list(
                unique.id=index$unique.id[i],
                run=index$run[i],
                run.file=index$run.file[i],
                run.date=index$run.date[i],
                run.day=index$run.day[i],
                run.hour=index$run.hour[i],
                run.minute=index$run.minute[i],
                run.time=index$run.time[i],
                abs.time=index$abs.time[i],
                primary=index$primary[i],
                species=index$species[i],
                location=index$location[i],
                fl=index$fl[i],
                sex=index$sex[i],
                fish.num=factor(index$fish.num[i]),
                slide=index$slide[i],
                profile=index$profile[i],
#                       scan.speed=index$scan.speed[i],
#                       scan.freq=index$scan.freq[i],
                outer.radius=index$outer.radius[i],
#                       centre.radius=index$centre.radius[i],
                ventral.width=index$ventral.width[i],
                comment=as.character(index$comment[i]),
                background.signal=background.signal,
                correction.signal=correction.signal,
                sequence=sequence,
#                       mean.sol=data.frame(t(mean.sol)),
                mean.sol=mean.sol,
                mean.sol.ca44.ratio=data.frame(t(mean.sol.ca44.ratio)),
                mean.catch=data.frame(t(mean.catch)),
                mean.catch.ca44.ratio=data.frame(t(mean.catch.ca44.ratio)),
                outlier.summary=outlier.summary,
#                       point=ts(temp.raw.data$point),
                time=ts(temp.raw.data$time),
```

```
                mg24=ts(temp.raw.data$mg),
                p31=ts(temp.raw.data$p),
                ca44=ts(temp.raw.data$ca),
                mn55=ts(temp.raw.data$mn),
                cu65=ts(temp.raw.data$cu),
                zn66=ts(temp.raw.data$zn),
                sr88=ts(temp.raw.data$sr),
                ba137=ts(temp.raw.data$ba),
                mg24.out.filt=ts(mg24.outlier.filt[[1]]),
                p31.out.filt=ts(p31.outlier.filt[[1]]),
                ca44.out.filt=ts(ca44.outlier.filt[[1]]),
                mn55.out.filt=ts(mn55.outlier.filt[[1]]),
                cu65.out.filt=ts(cu65.outlier.filt[[1]]),
                zn66.out.filt=ts(zn66.outlier.filt[[1]]),
                sr88.out.filt=ts(sr88.outlier.filt[[1]]),
                ba137.out.filt=ts(ba137.outlier.filt[[1]]),
                mg24.smooth=ts(smooth.mg24),
                p31.smooth=ts(smooth.p31),
                ca44.smooth=ts(smooth.ca44),
                mn55.smooth=ts(smooth.mn55),
                cu65.smooth=ts(smooth.cu65),
                zn66.smooth=ts(smooth.zn66),
                sr88.smooth=ts(smooth.sr88),
                ba137.smooth=ts(smooth.ba137),
                mg24.no.back.sig=ts(no.back.mg24),
                p31.no.back.sig=ts(no.back.p31),
                ca44.no.back.sig=ts(no.back.ca44),
                mn55.no.back.sig=ts(no.back.mn55),
                cu65.no.back.sig=ts(no.back.cu65),
                zn66.no.back.sig=ts(no.back.zn66),
                sr88.no.back.sig=ts(no.back.sr88),
                ba137.no.back.sig=ts(no.back.ba137),
                mg24.ca44.ratio=ts(mg24.ca44.ratio),
                p31.ca44.ratio=ts(p31.ca44.ratio),
                mn55.ca44.ratio=ts(mn55.ca44.ratio),
                cu65.ca44.ratio=ts(cu65.ca44.ratio),
                zn66.ca44.ratio=ts(zn66.ca44.ratio),
                sr88.ca44.ratio=ts(sr88.ca44.ratio),
                ba137.ca44.ratio=ts(ba137.ca44.ratio),
                number=index$number[i],
#                       rep.group=index$rep.group[i],
                drift.group=index$drift.group[i],
                same.scan.group=index$same.scan.group[i],
                correction.signal.raw=correction.signal.raw,
                run.month=index$run.month[i],
                abl.yield.group=index$abl.yield.group[i]
        )
debug=17
# add data into main data object.  Samples can be accessed via format similar
# to data[[1]]$ca44.smooth

        data[[i]]=temp.list

 }   # end of main loop

# notify user that data is being saved

 cat('\nData Is Being Saved For Future Processing\n')

# save object for future work, without having to run pre-processing

 save(data,file=paste(file.location,'Data_Object_Pre_Drift.rda',sep=''),ascii=F)

}   # end of pre-processing

##############################################################################################
#       TIME SERIES PRE-PROCESSING FINISHES HERE
##############################################################################################

# pre-processing must have already been run and saved previously

if (input.log$pre.process.ts==FALSE&input.log$pre.process.drift==TRUE) {

# notify user that data is being saved
```

```
 cat('\nReading Data Object From Time Series Pre-Processing\n')

 load(file=paste(file.location,'Data_Object_Pre_Drift.rda',sep=''))
}
debug=29

################################################################################
#       DRIFT CORRECTION PRE-PROCESSING BEGINS HERE
################################################################################

# determine if we are to do drift corrections

if (input.log$pre.process.drift==TRUE) {

# notify user that correction processing has begun

 cat('\nProcessing Drift Corrections\n')

# set flag for ICPMS machine drift correction calculations

 first.correction.entry='TRUE'

# calculate correction factors for ICPMS machine drift
# start with subset of main data for "standard", using only NIST610 standard
# 'drift group' represents groups of standard runs that will be averaged together for correction

cat('\n      - building correction table\n')


 for (m in 1:nrow(index)) {

        if (index$species[m]=='NIST610'&index$drift.group[m]!=0){

# draw out data to be used in correction process

                correction.factor.temp=data.frame(run=data[[m]]$unique.id,species=data[[m]]$species,
                        slide=data[[m]]$drift.group,profile=data[[m]]$profile,element=isotope.no.ca,
                        run.day=data[[m]]$run.day,run.time=data[[m]]$run.time,
                                data[[m]]$correction.signal,0,0)

# define names for data.frame columns

                names(correction.factor.temp)=c('unique.id','species','drift.group','profile','element','run.day',
                        'run.time','value','norm.value','slide.mean.norm.value')

# this is required to initiate data.frame for first run through

                if (first.correction.entry=='TRUE'){
                        correction.factor=correction.factor.temp
                        first.correction.entry='FALSE'
                }
                else {
                        correction.factor=rbind(correction.factor,correction.factor.temp)
                }
        }
 }

debug=18
# rescale the correction factors to the first entry for each of the 8 elements
# samples selected are chosen so NIST610 mean on the start of earliest date is 1.0
# nist610-1a=[1:7], nist610-1b=[8:14]

 correction.factor$norm.value=correction.factor$value/((correction.factor$value[1:7]+
        correction.factor$value[8:14])/2)

# we need to have a column for absolute time (minutes) for the interpolation routine

 correction.factor$abs.time=(correction.factor$run.day-90)*24*60+correction.factor$run.time

# calculate mean value per element per drift group - couldn't figure how to do it without loop
# this adds another column onto the correction.factor data frame
# the term "slide" was used because I originally set it to have one pair of standards per slide

 for (q in 1:nrow(correction.factor)){
```

```
        correction.factor$slide.mean.norm.value[q]=mean(subset(correction.factor,
                element==element[q]&drift.group==drift.group[q])$norm.value)
 }
debug=19

# fit a simple linear interpolation function around data on each
# of the 7 elements
# note: correction data already in ratio to Ca

# run loop for correction

cat('\n     - building correction models\n')


 correction.model.mg=approxfun(correction.factor$abs.time
        [correction.factor$element=='Mg'],correction.factor$slide.mean.norm.value[
        correction.factor$element=='Mg'],rule=2)

#       correction.model.p=approxfun(correction.factor$abs.time
#               [correction.factor$element=='P'],
#               correction.factor$slide.mean.norm.value[correction.factor$element=='P'],rule=2)

 correction.model.mn=approxfun(correction.factor$abs.time
        [correction.factor$element=='Mn'],
        correction.factor$slide.mean.norm.value[correction.factor$element=='Mn'],rule=2)

 correction.model.cu=approxfun(correction.factor$abs.time
        [correction.factor$element=='Cu'],
        correction.factor$slide.mean.norm.value[correction.factor$element=='Cu'],rule=2)

 correction.model.zn=approxfun(correction.factor$abs.time
        [correction.factor$element=='Zn'],
        correction.factor$slide.mean.norm.value[correction.factor$element=='Zn'],rule=2)

 correction.model.sr=approxfun(correction.factor$abs.time
        [correction.factor$element=='Sr'],
        correction.factor$slide.mean.norm.value[correction.factor$element=='Sr'],rule=2)

 correction.model.ba=approxfun(correction.factor$abs.time
        [correction.factor$element=='Ba'],
        correction.factor$slide.mean.norm.value[correction.factor$element=='Ba'],rule=2)

# define a correction vector for all values that are not 'standard'
debug=20


 correction.vector.mg=correction.model.mg(index$abs.time[index$profile!='standard'])

#       correction.vector.p=correction.model.p(index$abs.time[index$profile!='standard'])

 correction.vector.mn=correction.model.mn(index$abs.time[index$profile!='standard'])

 correction.vector.cu=correction.model.cu(index$abs.time[index$profile!='standard'])

 correction.vector.zn=correction.model.zn(index$abs.time[index$profile!='standard'])

 correction.vector.sr=correction.model.sr(index$abs.time[index$profile!='standard'])

 correction.vector.ba=correction.model.ba(index$abs.time[index$profile!='standard'])


# now that we have the ICPMS drift correction models, we need to make all adjustments
# to each time series
debug=23

cat('\n     - making correction adjustments\n')

 for (s in 1:nrow(index)) {

        data[[s]]$mg24.corrected=as.ts(data[[s]]$mg24.ca44.ratio/
                correction.model.mg(data[[s]]$abs.time))
#               data[[s]]$p31.corrected=as.ts(data[[s]]$p31.ca44.ratio/
#                       correction.model.p(data[[s]]$abs.time))
        data[[s]]$mn55.corrected=as.ts(data[[s]]$mn55.ca44.ratio/
                correction.model.mn(data[[s]]$abs.time))
```

```
        data[[s]]$cu65.corrected=as.ts(data[[s]]$cu65.ca44.ratio/
                correction.model.cu(data[[s]]$abs.time))
        data[[s]]$zn66.corrected=as.ts(data[[s]]$zn66.ca44.ratio/
                correction.model.zn(data[[s]]$abs.time))
        data[[s]]$sr88.corrected=as.ts(data[[s]]$sr88.ca44.ratio/
                correction.model.sr(data[[s]]$abs.time))
        data[[s]]$ba137.corrected=as.ts(data[[s]]$ba137.ca44.ratio/
                correction.model.ba(data[[s]]$abs.time))

# now rescale the data to enable review of relative variation
debug=24

        data[[s]]$mg24.rescaled=as.ts(data[[s]]$mg24.corrected/data[[s]]$mg24.corrected[1])
#               data[[s]]$p31.rescaled=as.ts(data[[s]]$p31.corrected/data[[s]]$p31.corrected[1])
        data[[s]]$mn55.rescaled=as.ts(data[[s]]$mn55.corrected/data[[s]]$mn55.corrected[1])
        data[[s]]$cu65.rescaled=as.ts(data[[s]]$cu65.corrected/data[[s]]$cu65.corrected[1])
        data[[s]]$zn66.rescaled=as.ts(data[[s]]$zn66.corrected/data[[s]]$zn66.corrected[1])
        data[[s]]$sr88.rescaled=as.ts(data[[s]]$sr88.corrected/data[[s]]$sr88.corrected[1])
        data[[s]]$ba137.rescaled=as.ts(data[[s]]$ba137.corrected/data[[s]]$ba137.corrected[1])

# draw out solution and catch influence data for correction

        sol.ratio=data[[s]]$mean.sol.ca44.ratio
        catch.ratio=data[[s]]$mean.catch.ca44.ratio
        run.time=data[[s]]$abs.time

# prepare solution based and catch location influence data for stats analysis,
# only if NOT a standard
debug=25

        if(data[[s]]$profile!='standard'){

# calculate mean values of each element for solution based comparison

                sol.ratio.corrected.mg=sol.ratio$Mg/correction.model.mg(run.time)
#                       sol.ratio.corrected.p=sol.ratio$P/correction.model.p(run.time)
                sol.ratio.corrected.mn=sol.ratio$Mn/correction.model.mn(run.time)
                sol.ratio.corrected.cu=sol.ratio$Cu/correction.model.cu(run.time)
                sol.ratio.corrected.zn=sol.ratio$Zn/correction.model.zn(run.time)
                sol.ratio.corrected.sr=sol.ratio$Sr/correction.model.sr(run.time)
                sol.ratio.corrected.ba=sol.ratio$Ba/correction.model.ba(run.time)

# calculate mean values of each element for catch location influence
debug=26

                catch.ratio.corrected.mg=catch.ratio$Mg/correction.model.mg(run.time)
#                       catch.ratio.corrected.p=catch.ratio$P/correction.model.p(run.time)
                catch.ratio.corrected.mn=catch.ratio$Mn/correction.model.mn(run.time)
                catch.ratio.corrected.cu=catch.ratio$Cu/correction.model.cu(run.time)
                catch.ratio.corrected.zn=catch.ratio$Zn/correction.model.zn(run.time)
                catch.ratio.corrected.sr=catch.ratio$Sr/correction.model.sr(run.time)
                catch.ratio.corrected.ba=catch.ratio$Ba/correction.model.ba(run.time)

# now combine the elements for future stats analysis

#                       data[[s]]$sol.ratio.corrected=c(sol.ratio.corrected.mg,
#                       sol.ratio.corrected.p,sol.ratio.corrected.mn,sol.ratio.corrected.cu,
#                       sol.ratio.corrected.zn,sol.ratio.corrected.sr,sol.ratio.corrected.ba)

                data[[s]]$sol.ratio.corrected=c(sol.ratio.corrected.mg,
                sol.ratio.corrected.mn,sol.ratio.corrected.cu,
                sol.ratio.corrected.zn,sol.ratio.corrected.sr,sol.ratio.corrected.ba)

#                       names(data[[s]]$sol.ratio.corrected)=isotope.no.ca
                names(data[[s]]$sol.ratio.corrected)=isotope.no.ca.no.p

#                       data[[s]]$catch.ratio.corrected=c(catch.ratio.corrected.mg,
#                       catch.ratio.corrected.p,catch.ratio.corrected.mn,catch.ratio.corrected.cu,
#                       catch.ratio.corrected.zn,catch.ratio.corrected.sr,catch.ratio.corrected.ba)

                data[[s]]$catch.ratio.corrected=c(catch.ratio.corrected.mg,
                catch.ratio.corrected.mn,catch.ratio.corrected.cu,
                catch.ratio.corrected.zn,catch.ratio.corrected.sr,catch.ratio.corrected.ba)

#                       names(data[[s]]$catch.ratio.corrected)=isotope.no.ca
```

```
                names(data[[s]]$catch.ratio.corrected)=isotope.no.ca.no.p
debug=27

        }

 }
debug=28

# save object for future work, without having to run pre-processing

#         save(data,file=paste(file.location,'Data_Object_Post_Drift.rda',sep=''),ascii=F)
#         save(correction.factor,file=paste(file.location,'Correction_Factor.rda',sep=''),ascii=F)
  save.image(file=paste(file.location,'Data_Object_Post_Drift.rda',sep=''))

}

#
#
#================================================================================
#XXXXXXXXXX  Drift Correction Pre-Processing Finishes Here XXXXXXXXXXXXXXXXXXXXX
#================================================================================
#


# pre-processing must have already been run and saved previously

if (input.log$pre.process.ts==FALSE&input.log$pre.process.drift==FALSE) {

# notify user that data is being saved

  cat('\nReading Data Object From Drift Correction Pre-Processing\n')

  load(file=paste(file.location,'Data_Object_Post_Drift.rda',sep=''))

# re-open and read sample information
  index=read.csv(paste(file.location,'Sample Index.csv',sep=''))

# re-call up functions that have been written externally
  source(paste(file.location,'ronbo_function_definitions.R',sep=''))


# reload input logicals and parameters so we don't need to re run pre-processing each time we change it

  input.logical=read.csv(paste(file.location,'Input Logicals.csv',sep=''))
  input.matrix=read.csv(paste(file.location,'Input Parameters.csv',sep=''))

# manipulate input logicals to input.log$ data.frame format

  input.log=data.frame(t(input.logical$value.t.f))
  names(input.log)=input.logical$logical.input.variable

# manipulate input parameters to input$ data.frame format
  input=data.frame(t(input.matrix$parameter.value))
  names(input)=input.matrix$parameter.input.variable


}


#
#
#================================================================================
#XXXXXXXXXX  Plotting Begins Here XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
#================================================================================
#
#
#
# plot data to examine outliers, smoothed data, background signal, start time,
# and finish time on a single plot for each element and each sample
# i=sample run number, j+raw.offset=raw data, j+outlier.offset=outlier filtered, J+smooth.offset=smoothed data

# these have been set to variables because the number of items in the 'data' list has varied throughout development
# and this is relatively easy to change

#raw.offset=26
```

```
raw.offset=29
outlier.offset=raw.offset+8
smooth.offset=raw.offset+16

# notify user that plotting has begun

cat('\nPlotting Has Begun\n')


# run plots for each sample in index

for (i in 1:nrow(index)) {

# create temporary time series for plotting

 if (input.log$raw.data.plot==TRUE) {

# print progress to screen

        cat(paste(' ',i))


# set legend parameters
debug=30

        var=c('Raw Data','Smooth Data','Backgr Sig','Outliers','Seq Start','Birth Ring',
                'Catch Loc Infl','Seq End','Sol Base Mean','Catch Loc Mean')
        colour=c('black','green','blue','red','green','orange','grey','red','magenta','purple')
        line.type=c(1,1,2,1,2,2,2,2,2,1)
        line.width=c(2,1,2,2,3,3,3,3,2,3)

# draw legend

        plot(0,0,pch='',xlab='',ylab='',lab=c(1,1,1),tcl=0)
        legend('top',legend=var,col=colour,lty=line.type,lwd=line.width,horiz=FALSE)


# run plots for each of the 8 elements in each sample

        for (j in 1:8){

                raw.data.temp=as.ts(unlist(data[[i]][j+raw.offset]))
                outlier.temp=as.ts(unlist(data[[i]][j+outlier.offset]))
                smooth.temp=as.ts(unlist(data[[i]][j+smooth.offset]))

                raw.data.plot(data[[i]]$run,data[[i]]$species,data[[i]]$profile,isotope[j],
                        raw.data.temp,outlier.temp,smooth.temp,data[[i]]$background.signal[j],
                        data[[i]]$sequence$start.point,data[[i]]$sequence$end.point,
                        data[[i]]$sequence$birth.point,data[[i]]$sequence$catch.loc.point,
                        as.integer(data[[i]]$mean.sol[j]+data[[i]]$background.signal[j]),
                        as.integer(data[[i]]$mean.catch[j]+data[[i]]$background.signal[j]))
        }
 }
debug=31

# plot all non-normalised elemental ratios

 if (input.log$rescaled.ratio.plot==TRUE) {

# define color vector for plotting and legend

        colour7=c('black','green','blue','orange','magenta','grey','red')

#               ts.plot(data[[i]]$mg24.rescaled,data[[i]]$p31.rescaled,data[[i]]$mn55.rescaled,
#                       data[[i]]$cu65.rescaled,data[[i]]$zn66.rescaled,data[[i]]$sr88.rescaled,
#                       data[[i]]$ba137.rescaled,main=paste('Normalised Ratio to Ca44 - ',data[[i]]$run,
#                       ' - ',data[[i]]$species),xlab='Point Number',col=colour7)
        ts.plot(data[[i]]$mg24.rescaled,data[[i]]$mn55.rescaled,
                data[[i]]$cu65.rescaled,data[[i]]$zn66.rescaled,data[[i]]$sr88.rescaled,
                data[[i]]$ba137.rescaled,main=paste('Normalised Ratio to Ca44 - ',data[[i]]$run,
                ' - ',data[[i]]$species),xlab='Point Number',col=colour7)
        abline('v'=(data[[i]]$sequence$birth.point-data[[i]]$sequence$start.point+1),col='orange',
                lwd=3,lty=2)
        abline('v'=(data[[i]]$sequence$catch.loc.point-data[[i]]$sequence$start.point+1),col='gray',
                lwd=3,lty=2)
```

```
        abline('v'=(data[[i]]$sequence$start.point-data[[i]]$sequence$start.point+1),col='green',
                lwd=3,lty=2)
        abline('v'=(data[[i]]$sequence$end.point-data[[i]]$sequence$start.point+1),col='red',
                lwd=3,lty=2)
        legend('bottom',legend=isotope.no.ca.no.p,col=colour7,lty=1,lwd=3,horiz=T)
 }
}
debug=32

#
#
#=============================================================================
#XXXXXXXXXX  Run Plot for ICPMS machine correction   XXXXXXXXXXXXXX
#=============================================================================
#
#

if (input.log$drift.plot==TRUE){

# put all curves for each date on same plot
# can not use time series plot because run.time is irregular

# determine vector of unique dates from the sample index

 unique.date=unique(index$run.day)

# run loop to create drift plots for each unique date

 for (d in 1:length(unique.date)) {

# set 'date' based on 'd' in loop and unique.date

        date=unique.date[d]

        ymax=max(correction.factor$slide.mean.norm.value[correction.factor$run.day==date])

        ymin=min(correction.factor$slide.mean.norm.value[correction.factor$run.day==date])

# create offset for hours to bring x axis labels to be for that date only, and not from the beginning
# of the first date

        hour.offset=(date-unique.date[1])*24

        plot(correction.factor$run.time[correction.factor$run.day==date&
                correction.factor$element=='Mg']/60,correction.factor$slide.mean.norm.value
                [correction.factor$run.day==date&correction.factor$element=='Mg'],pch='',
                ylim=c(ymin,ymax),xlab='Time in hours (24 hour clock) on day of LA-ICPMS run',
                ylab='Relative concentration',main=paste('LA-ICPMS Machine Drift for all elements on day ',
                date,'\nBased on NIST610 Standard'))

debug=33

        lines(correction.factor$run.time[correction.factor$run.day==date&
                correction.factor$element=='Mg']/60,correction.factor$slide.mean.norm.value
                [correction.factor$run.day==date&correction.factor$element=='Mg'],lty=1,
                lwd=1.5,col='black')

#               lines(correction.factor$run.time[correction.factor$run.day==date&
#                       correction.factor$element=='P']/60,correction.factor$slide.mean.norm.value
#                       [correction.factor$run.day==date&correction.factor$element=='P'],lty=5,
#                       lwd=1,col='red')

        lines(correction.factor$run.time[correction.factor$run.day==date&
                correction.factor$element=='Mn']/60,correction.factor$slide.mean.norm.value
                [correction.factor$run.day==date&correction.factor$element=='Mn'],lty=4,
                lwd=1.5,col='blue')

        lines(correction.factor$run.time[correction.factor$run.day==date&
                correction.factor$element=='Cu']/60,correction.factor$slide.mean.norm.value
                [correction.factor$run.day==date&correction.factor$element=='Cu'],lty=3,
                lwd=2,col='magenta')

        lines(correction.factor$run.time[correction.factor$run.day==date&
                correction.factor$element=='Zn']/60,correction.factor$slide.mean.norm.value
                [correction.factor$run.day==date&correction.factor$element=='Zn'],lty=2,
```

```
                lwd=2,col='green')

        lines(correction.factor$run.time[correction.factor$run.day==date&
                correction.factor$element=='Sr']/60,correction.factor$slide.mean.norm.value
                [correction.factor$run.day==date&correction.factor$element=='Sr'],lty=1,
                lwd=3,col='light blue')

        lines(correction.factor$run.time[correction.factor$run.day==date&
                correction.factor$element=='Ba']/60,correction.factor$slide.mean.norm.value
                [correction.factor$run.day==date&correction.factor$element=='Ba'],lty=1,
                lwd=2,col='chocolate')

#               legend('bottomleft',c('Mg','P','Mn','Cu','Zn','Sr','Ba'),col=c('black','red','blue',
#                       'magenta','green','light blue','chocolate'),lty=c(1,5,4,3,2,1,1),
#                       lwd=c(1.5,1,1.5,2,2,3,2))

        legend('bottomleft',c('Mg','Mn','Cu','Zn','Sr','Ba'),col=c('black','red','blue',
                'magenta','green','light blue'),lty=c(1,5,4,3,2,1),
                lwd=c(1.5,1,1.5,2,2,3))


debug=34

# create vector of points for each element, for this given date

        points.mg=subset(correction.vector.mg,index$run.day[index$profile!='standard']==date)
#               points.p=subset(correction.vector.p,index$run.day[index$profile!='standard']==date)
        points.mn=subset(correction.vector.mn,index$run.day[index$profile!='standard']==date)
        points.cu=subset(correction.vector.cu,index$run.day[index$profile!='standard']==date)
        points.zn=subset(correction.vector.zn,index$run.day[index$profile!='standard']==date)
        points.sr=subset(correction.vector.sr,index$run.day[index$profile!='standard']==date)
        points.ba=subset(correction.vector.ba,index$run.day[index$profile!='standard']==date)

# run plots for correction

        correction.plot(date,'Mg',points.mg)
#               correction.plot(date,'P',points.p)
        correction.plot(date,'Mn',points.mn)
        correction.plot(date,'Cu',points.cu)
        correction.plot(date,'Zn',points.zn)
        correction.plot(date,'Sr',points.sr)
        correction.plot(date,'Ba',points.ba)

 }
}

debug=35

#
#
#================================================================================
#XXXXXXXXXX  Beginning of PCA, MANOVA, and DFA analyses   XXXXXXXXXXXXXX
#================================================================================
#
#

# notify user that plotting has begun

cat('\nStatistical Analyses Have Begun\n')


# write text output to file

sink(paste(file.location,'200901 LAICPMS Analysis Results.txt'))

# create header in output file

cat('Five Region 2009 LA-ICPMS Data Analysis\n')
cat('\nPrint Date:   ',date(),'\n\n\n')


# run PCA to compare directly to solution based results
# note that the 'sol' or 'solution' variables are left over from when we considered
# this comparing to solution based results.  The terminology has subsequently changed
# to 'whole life', but I have not changed all variable names.
```

```
debug=36
if (input.log$solution.pca==TRUE) {

# establish data frame for pca analysis

#         sol.pca=data.frame(0,'','',0,0,0,0,0,0,0,0)
#         names(sol.pca)=c('run','species','location','seq.length',isotope.no.ca)
 sol.pca=data.frame(0,'','',0,0,0,0,0,0,0)
 names(sol.pca)=c('run','species','location','seq.length',isotope.no.ca.no.p)

# run through the index and draw out all samples identified as part of primary study

 for (j in 1:nrow(index)) {

         if (index$primary[j]==TRUE) {

# draw out appropriate data from main data set for pca analysis

                 sol.pca.temp=data.frame(data[[j]]$run,data[[j]]$species,as.character(data[[j]]$location),
                         data[[j]]$sequence$length,t(data[[j]]$sol.ratio.corrected),check.names=F)

#                        names(sol.pca.temp)=c('run','species','location','seq.length',isotope.no.ca)
                 names(sol.pca.temp)=c('run','species','location','seq.length',isotope.no.ca.no.p)

# bind data from this record together with previous records

                 sol.pca=rbind(sol.pca,sol.pca.temp)
         }
 }
debug=37
# create subset of data for response matrix in pca

#         sol.pca.response=data.frame(sol.pca$Mg,sol.pca$P,sol.pca$Mn,sol.pca$Cu,sol.pca$Zn,
#                 sol.pca$Sr,sol.pca$Ba)
 sol.pca.response=data.frame(sol.pca$Mg,sol.pca$Mn,sol.pca$Cu,sol.pca$Zn,
         sol.pca$Sr,sol.pca$Ba)

#         names(sol.pca.response)=isotope.no.ca
 names(sol.pca.response)=isotope.no.ca.no.p

# transform Mn, Cu, and Zn for MIS      (this affects multiple pca/manovas for MIS)

#         sol.pca.response$Mn[sol.pca$species=='MIS']=sol.pca.response$Mn[sol.pca$species=='MIS']^.5
#         sol.pca.response$Cu[sol.pca$species=='MIS']=sol.pca.response$Cu[sol.pca$species=='MIS']^.5
#         sol.pca.response$Zn[sol.pca$species=='MIS']=sol.pca.response$Zn[sol.pca$species=='MIS']^.2


# run pca for the three species

#         pca.abt=princomp(sol.pca.response,cor=T,scores=T,subset=rep(sol.pca$species=='ABT'))
 pca.shh=princomp(sol.pca.response,cor=T,scores=T,subset=rep(sol.pca$species=='SHH'))
 pca.mis=princomp(sol.pca.response,cor=T,scores=T,subset=rep(sol.pca$species=='MIS'))

# run pca for three species at each location - region 1 and region 6

#
         pca.abt.region1=princomp(sol.pca.response,cor=T,scores=T,subset=rep(sol.pca$species=='ABT'&sol.pca$locatio
 n=='1'))
#
         pca.abt.region6=princomp(sol.pca.response,cor=T,scores=T,subset=rep(sol.pca$species=='ABT'&sol.pca$locatio
 n=='6'))
#
         pca.shh.region1=princomp(sol.pca.response,cor=T,scores=T,subset=rep(sol.pca$species=='SHH'&sol.pca$locati
 on=='1'))
#
         pca.shh.region6=princomp(sol.pca.response,cor=T,scores=T,subset=rep(sol.pca$species=='SHH'&sol.pca$locati
 on=='6'))
#
         pca.mis.region1=princomp(sol.pca.response,cor=T,scores=T,subset=rep(sol.pca$species=='MIS'&sol.pca$locatio
 n=='1'))
#
         pca.mis.region6=princomp(sol.pca.response,cor=T,scores=T,subset=rep(sol.pca$species=='MIS'&sol.pca$locatio
 n=='6'))

# set screen for biplots to be 2 plot areas wide
```

```
debug=38
 par(mfrow=c(1,2),new=F,ask=ask)

# simplify location in vector for eventual use as markers in bi-plots, all future reference must now be N and S, not 1 and 6

#        sol.pca$location=gsub('1','N',sol.pca$location)
#        sol.pca$location=gsub('6','S',sol.pca$location)

# define markers for scores plots

#        marker.abt=as.character(subset(sol.pca$location,sol.pca$species=='ABT'))
 marker.shh=as.character(subset(sol.pca$location,sol.pca$species=='SHH'))
 marker.mis=as.character(subset(sol.pca$location,sol.pca$species=='MIS'))

# determine means for pc1, pc2 and pc3 for each location

# create ellipses around conf.int confidence interval for plotting

 conf.int=.95


# for LA-5, I'm using coloured dots instead of region number characters as scores markers
# so change to colours - also changing ronbo.pca.biplot for "markers" to be colours instead of pch

 marker.col.shh=gsub('1','black',marker.shh)
 marker.col.shh=gsub('2','blue',marker.col.shh)
 marker.col.shh=gsub('3','green',marker.col.shh)
 marker.col.shh=gsub('4','grey',marker.col.shh)
 marker.col.shh=gsub('6','red',marker.col.shh)
 marker.col.shh=gsub('8','lightblue',marker.col.shh)

 marker.col.mis=gsub('1','black',marker.mis)
 marker.col.mis=gsub('2','blue', marker.col.mis)
 marker.col.mis=gsub('3','green', marker.col.mis)
 marker.col.mis=gsub('4','grey', marker.col.mis)
 marker.col.mis=gsub('6','red', marker.col.mis)

# define legend.vals for the ronbo.pca.biplot legend on the scores

 legend.vals.shh=c('1','2','3','4','6','8')
 legend.vals.mis=c('1','2','3','4','6')

# define legend.cols for the ronbo.pca.biplot legend on the scores

 legend.cols.shh=c('black','blue','green','grey','red','lightblue')
 legend.cols.mis=c('black','blue','green','grey','red')


# create biplots for ABT and SHH using Ronbo's fancy biplot routine

 ronbo.pca.biplot(pca=pca.shh,markers=marker.col.shh,title.loadings="SHH PCA Loadings - Whole Life Comparison",
        title.scores="SHH PCA Scores - Whole Life Comparison",input.log$user.label,ask,legend.vals=legend.vals.shh,
        legend.cols=legend.cols.shh)

 ronbo.pca.biplot(pca=pca.mis,markers=marker.col.mis,title.loadings="MIS PCA Loadings - Whole Life Comparison",
        title.scores="MIS PCA Scores - Whole Life Comparison",input.log$user.label,ask,legend.vals=legend.vals.mis,
        legend.cols=legend.cols.mis)

debug=39
# now create response matrices for MANOVA.  the manova function does not allow subsetting, so separate matrix must
 be
# made for each manova call.  It can be based on the sol.pca.response though, so it won't be too cumbersome.

#        manova.response.abt=as.matrix(subset(sol.pca.response,sol.pca$species=="ABT"))
 manova.response.shh=as.matrix(subset(sol.pca.response,sol.pca$species=="SHH"))
 manova.response.mis=as.matrix(subset(sol.pca.response,sol.pca$species=="MIS"))

# create vector for manova with location as factor
debug=40
#               location.abt=as.factor(subset(sol.pca$location,sol.pca$species=='ABT'))
        location.shh=as.factor(subset(sol.pca$location,sol.pca$species=='SHH'))
        location.mis=as.factor(subset(sol.pca$location,sol.pca$species=='MIS'))

# now make manova and dfa calls using ronbo's routines - factor=location
debug=41
```

```
#         fit.abt.location=ronbo.manova(manova.response.abt,location.abt,title.data='Whole Life - ABT with location as
 factor',
#                   title.resid='ABT with location as factor: residuals',assumptions=input.log$assumptions.test)

#         lda.abt.location=ronbo.lda(manova.response.abt,location.abt,study.name='Whole Life - ABT - location as factor')

 fit.shh.location=ronbo.manova(manova.response.shh,location.shh,title.data='Whole Life - SHH with location as factor',
         title.resid='SHH with location as factor: residuals',assumptions=input.log$assumptions.test)

 lda.shh.location=ronbo.lda(manova.response.shh,location.shh,study.name='Whole Life - SHH - location as factor')

 fit.mis.location=ronbo.manova(manova.response.mis,location.mis,title.data='Whole Life - MIS with location as factor',
         title.resid='MIS with location as factor: residuals',assumptions=input.log$assumptions.test)

 lda.mis.location=ronbo.lda(manova.response.mis,location.mis,study.name='Whole Life - MIS - location as factor')

# now run series of pair-wise Hotellings T2 comparisons - region 1/2, 2/3, 3/4, etc

 hot.t2.shh.r1=subset(manova.response.shh,location.shh=='1')
 hot.t2.shh.r2=subset(manova.response.shh,location.shh=='2')
 hot.t2.shh.r3=subset(manova.response.shh,location.shh=='3')
 hot.t2.shh.r4=subset(manova.response.shh,location.shh=='4')
 hot.t2.shh.r6=subset(manova.response.shh,location.shh=='6')
 hot.t2.shh.r8=subset(manova.response.shh,location.shh=='8')

 hot.t2.mis.r1=subset(manova.response.mis,location.mis=='1')
 hot.t2.mis.r2=subset(manova.response.mis,location.mis =='2')
 hot.t2.mis.r3=subset(manova.response.mis,location.mis =='3')
 hot.t2.mis.r4=subset(manova.response.mis,location.mis =='4')
 hot.t2.mis.r6=subset(manova.response.mis,location.mis =='6')

# now run Hotellings T2 pair wise comparisons for each neighbouring region

 hotellings.shh.12=HotellingsT2(hot.t2.shh.r1,hot.t2.shh.r2)
 hotellings.shh.23=HotellingsT2(hot.t2.shh.r2,hot.t2.shh.r3)
 hotellings.shh.34=HotellingsT2(hot.t2.shh.r3,hot.t2.shh.r4)
 hotellings.shh.46=HotellingsT2(hot.t2.shh.r4,hot.t2.shh.r6)
 hotellings.shh.68=HotellingsT2(hot.t2.shh.r6,hot.t2.shh.r8)

 hotellings.mis.12=HotellingsT2(hot.t2.mis.r1,hot.t2.mis.r2)
 hotellings.mis.23=HotellingsT2(hot.t2.mis.r2,hot.t2.mis.r3)
 hotellings.mis.34=HotellingsT2(hot.t2.mis.r3,hot.t2.mis.r4)
 hotellings.mis.46=HotellingsT2(hot.t2.mis.r4,hot.t2.mis.r6)

# create summary tables for each species

 hotellings.shh=data.frame(matrix(c(
 hotellings.shh.12$statistic,hotellings.shh.12$p.value,
 hotellings.shh.23$statistic,hotellings.shh.23$p.value,
 hotellings.shh.34$statistic,hotellings.shh.34$p.value,
 hotellings.shh.46$statistic,hotellings.shh.46$p.value,
 hotellings.shh.68$statistic,hotellings.shh.68$p.value
 ),ncol=2,byrow=T,dimnames=list(c('R1-2','R2-3','R3-4','R4-6','R6-8'))))

 names(hotellings.shh)=c('t2.statistic','p.value')

 hotellings.mis=data.frame(matrix(c(
 hotellings.mis.12$statistic,hotellings.mis.12$p.value,
 hotellings.mis.23$statistic,hotellings.mis.23$p.value,
 hotellings.mis.34$statistic,hotellings.mis.34$p.value,
 hotellings.mis.46$statistic,hotellings.mis.46$p.value
 ),ncol=2,byrow=T,dimnames=list(c('R1-2','R2-3','R3-4','R4-6'))))

 names(hotellings.shh)=names(hotellings.mis)=c('t2.statistic','p.value')

 # print results to output text file

 cat('\n\nHotellings T2 Multivariate Pair-Wise Comparisons - Whole Life\nSHH\n')

 print(hotellings.shh)

 cat('\nMIS\n')

 print(hotellings.mis)
```

```
 cat('\n\n')


debug=42

 if (input.log$size.pca==TRUE) {

# re-plot the min length biplot, separating size & location instead of just location

# find median sequence lengths for each species,

#               median.abt=median(sol.pca$seq.length[sol.pca$species=='ABT'])
        median.shh=median(sol.pca$seq.length[sol.pca$species=='SHH'])
        median.mis=median(sol.pca$seq.length[sol.pca$species=='MIS'])

# find median sequence length for eachs species and location (region 1 and 6) combination

#               median.abt.region1=median(sol.pca$seq.length[sol.pca$species=='ABT'&sol.pca$location=='1'])
#               median.abt.region2=median(sol.pca$seq.length[sol.pca$species=='ABT'&sol.pca$location=='2'])
#               median.abt.region3=median(sol.pca$seq.length[sol.pca$species=='ABT'&sol.pca$location=='3'])
#               median.abt.region4=median(sol.pca$seq.length[sol.pca$species=='ABT'&sol.pca$location=='4'])
#               median.abt.region5=median(sol.pca$seq.length[sol.pca$species=='ABT'&sol.pca$location=='5'])
#               median.abt.region6=median(sol.pca$seq.length[sol.pca$species=='ABT'&sol.pca$location=='6'])

        median.shh.region1=median(sol.pca$seq.length[sol.pca$species=='SHH'&sol.pca$location=='1'])
        median.shh.region2=median(sol.pca$seq.length[sol.pca$species=='SHH'&sol.pca$location=='2'])
        median.shh.region3=median(sol.pca$seq.length[sol.pca$species=='SHH'&sol.pca$location=='3'])
        median.shh.region4=median(sol.pca$seq.length[sol.pca$species=='SHH'&sol.pca$location=='4'])
#               median.shh.region5=median(sol.pca$seq.length[sol.pca$species=='SHH'&sol.pca$location=='5'])
        median.shh.region6=median(sol.pca$seq.length[sol.pca$species=='SHH'&sol.pca$location=='6'])
        median.shh.region8=median(sol.pca$seq.length[sol.pca$species=='SHH'&sol.pca$location=='8'])

        median.mis.region1=median(sol.pca$seq.length[sol.pca$species=='MIS'&sol.pca$location=='1'])
        median.mis.region2=median(sol.pca$seq.length[sol.pca$species=='MIS'&sol.pca$location=='2'])
        median.mis.region3=median(sol.pca$seq.length[sol.pca$species=='MIS'&sol.pca$location=='3'])
        median.mis.region4=median(sol.pca$seq.length[sol.pca$species=='MIS'&sol.pca$location=='4'])
#               median.mis.region5=median(sol.pca$seq.length[sol.pca$species=='MIS'&sol.pca$location=='5'])
        median.mis.region6=median(sol.pca$seq.length[sol.pca$species=='MIS'&sol.pca$location=='6'])
debug=43
# find quantiles for three size class based on sequence length for each species

#               quant3.abt=quantile(sol.pca$seq.length[sol.pca$species=='ABT'],p=c(.33,.67))
        quant3.shh=quantile(sol.pca$seq.length[sol.pca$species=='SHH'],p=c(.33,.67))
        quant3.mis=quantile(sol.pca$seq.length[sol.pca$species=='MIS'],p=c(.33,.67))

# must eliminate first row of matrix for next step, also remove first row of response matrix to remove 0's for future manovas

        sol.pca=sol.pca[2:nrow(sol.pca),]
        sol.pca.response=sol.pca.response[2:nrow(sol.pca.response),]
debug=44
# define size.class, all as 'S' (small), with larger ones to be changed on the next step

        sol.pca$size.class.2='S'
        sol.pca$size.class.3='S'
        sol.pca$size.class.region1='S'
        sol.pca$size.class.region2='S'
        sol.pca$size.class.region3='S'
        sol.pca$size.class.region4='S'
#               sol.pca$size.class.region5='S'
        sol.pca$size.class.region6='S'
        sol.pca$size.class.region8='S'


        for (m in 1:nrow(sol.pca)) {

# define any values larger than median sequence length to be 'L' for 2 size class plots

                median=switch(as.character(sol.pca$species[m]),'ABT'=median.abt,'SHH'=median.shh,'MIS'=median.mis)
                if (median<sol.pca$seq.length[m]) sol.pca$size.class.2[m]='L'

# define any values larger than 33% [1] or 67% [2] to 'M' or 'L' as appropriate


        size.class.3.lo=switch(as.character(sol.pca$species[m]),'ABT'=quant3.abt[1],'SHH'=quant3.shh[1],'MIS'=quant3.mi
 s[1])
```

```
        size.class.3.hi=switch(as.character(sol.pca$species[m]),'ABT'=quant3.abt[2],'SHH'=quant3.shh[2],'MIS'=quant3.mi
 s[2])
                if (size.class.3.lo<sol.pca$seq.length[m]) sol.pca$size.class.3[m]='M'
                if (size.class.3.hi<sol.pca$seq.length[m]) sol.pca$size.class.3[m]='L'
debug=45
# define any values larger than median sequence length to be 'L' for species / location analysis

#
        median.region1=switch(as.character(sol.pca$species[m]),'ABT'=median.abt.region1,'SHH'=median.shh.region1,'M
 IS'=median.mis.region1)
#
        median.region2=switch(as.character(sol.pca$species[m]),'ABT'=median.abt.region2,'SHH'=median.shh.region2,'M
 IS'=median.mis.region2)
#
        median.region3=switch(as.character(sol.pca$species[m]),'ABT'=median.abt.region3,'SHH'=median.shh.region3,'M
 IS'=median.mis.region3)
#
        median.region4=switch(as.character(sol.pca$species[m]),'ABT'=median.abt.region4,'SHH'=median.shh.region4,'M
 IS'=median.mis.region4)
#
        median.region5=switch(as.character(sol.pca$species[m]),'ABT'=median.abt.region5,'SHH'=median.shh.region5,'M
 IS'=median.mis.region5)
#
        median.region6=switch(as.character(sol.pca$species[m]),'ABT'=median.abt.region6,'SHH'=median.shh.region6,'M
 IS'=median.mis.region6)

                median.region1=switch(as.character('SHH'=median.shh.region1,'MIS'=median.mis.region1))
                median.region2=switch(as.character('SHH'=median.shh.region2,'MIS'=median.mis.region2))
                median.region3=switch(as.character('SHH'=median.shh.region3,'MIS'=median.mis.region3))
                median.region4=switch(as.character('SHH'=median.shh.region4,'MIS'=median.mis.region4))
#                       median.region5=switch(as.character('SHH'=median.shh.region5,'MIS'=median.mis.region5))
                median.region6=switch(as.character('SHH'=median.shh.region6,'MIS'=median.mis.region6))
                median.region8=switch(as.character('SHH'=median.shh.region8))

                if (median.region1<sol.pca$seq.length[m]) sol.pca$size.class.region1[m]='L'
                if (median.region2<sol.pca$seq.length[m]) sol.pca$size.class.region2[m]='L'
                if (median.region3<sol.pca$seq.length[m]) sol.pca$size.class.region3[m]='L'
                if (median.region4<sol.pca$seq.length[m]) sol.pca$size.class.region4[m]='L'
#                       if (median.region5<sol.pca$seq.length[m]) sol.pca$size.class.region5[m]='L'
                if (median.region6<sol.pca$seq.length[m]) sol.pca$size.class.region6[m]='L'
                if (median.region8<sol.pca$seq.length[m]) sol.pca$size.class.region8[m]='L'

        }

# define markers for scores plots of 2 class size ('S' or 'L')

#               marker.abt.2=as.character(subset(sol.pca$size.class.2,sol.pca$species=='ABT'))
        marker.shh.2=as.character(subset(sol.pca$size.class.2,sol.pca$species=='SHH'))
        marker.mis.2=as.character(subset(sol.pca$size.class.2,sol.pca$species=='MIS'))

# define markers for scores plots of 3 class size ('S', 'M', or 'L')

#               marker.abt.3=as.character(subset(sol.pca$size.class.3,sol.pca$species=='ABT'))
        marker.shh.3=as.character(subset(sol.pca$size.class.3,sol.pca$species=='SHH'))
        marker.mis.3=as.character(subset(sol.pca$size.class.3,sol.pca$species=='MIS'))

# define markers for scores plots of 2 class size ('S' or 'L')


        marker.shh.region1=as.character(subset(sol.pca$size.class.region1,sol.pca$species=='SHH'&sol.pca$location=='
 1'))

        marker.shh.region2=as.character(subset(sol.pca$size.class.region2,sol.pca$species=='SHH'&sol.pca$location=='
 2'))

        marker.shh.region3=as.character(subset(sol.pca$size.class.region3,sol.pca$species=='SHH'&sol.pca$location=='
 3'))

        marker.shh.region4=as.character(subset(sol.pca$size.class.region4,sol.pca$species=='SHH'&sol.pca$location=='
 4'))
#
        marker.shh.region5=as.character(subset(sol.pca$size.class.region5,sol.pca$species=='SHH'&sol.pca$location=='
 5'))
```

```
        marker.shh.region6=as.character(subset(sol.pca$size.class.region6,sol.pca$species=='SHH'&sol.pca$location=='
 6'))

        marker.shh.region8=as.character(subset(sol.pca$size.class.region8,sol.pca$species=='SHH'&sol.pca$location=='
 8'))


        marker.mis.region1=as.character(subset(sol.pca$size.class.region1,sol.pca$species=='MIS'&sol.pca$location=='1'
 ))

        marker.mis.region2=as.character(subset(sol.pca$size.class.region2,sol.pca$species=='MIS'&sol.pca$location=='2'
 ))

        marker.mis.region3=as.character(subset(sol.pca$size.class.region3,sol.pca$species=='MIS'&sol.pca$location=='3'
 ))

        marker.mis.region4=as.character(subset(sol.pca$size.class.region4,sol.pca$species=='MIS'&sol.pca$location=='4'
 ))
#
        marker.mis.region5=as.character(subset(sol.pca$size.class.region5,sol.pca$species=='MIS'&sol.pca$location=='5'
 ))

        marker.mis.region6=as.character(subset(sol.pca$size.class.region6,sol.pca$species=='MIS'&sol.pca$location=='6'
 ))

debug=46
# create biplots for ABT, SHH, and MIS using Ronbo's fancy biplot routine - 2 size class

#               ronbo.pca.biplot(pca=pca.abt,markers=marker.abt.2,title.loadings=
#                       "ABT PCA Loadings - 2 Size Class - Whole Life Comparison",
#                       title.scores="ABT PCA Scores - 2 Size Class - Whole Life Comparison",input.log$user.label,ask)

        ronbo.pca.biplot(pca=pca.shh,markers=marker.shh.2,title.loadings=
                "SHH PCA Loadings - 2 Size Class - Whole Life Comparison",
                title.scores="SHH PCA Scores - 2 Size Class - Whole Life Comparison",input.log$user.label,ask)

        ronbo.pca.biplot(pca=pca.mis,markers=marker.mis.2,title.loadings=
                "MIS PCA Loadings - 2 Size Class - Whole Life Comparison",
                title.scores="MIS PCA Scores - 2 Size Class - Whole Life Comparison",input.log$user.label,ask)

# create biplots for ABT, SHH, and MIS using Ronbo's fancy biplot routine - 3 size class

#               ronbo.pca.biplot(pca=pca.abt,markers=marker.abt.3,title.loadings=
#                       "ABT PCA Loadings - 3 Size Class - Whole Life Comparison",
#                       title.scores="ABT PCA Scores - 3 Size Class - Whole Life
 Comparison",input.log$user.label,ask,ask)

        ronbo.pca.biplot(pca=pca.shh,markers=marker.shh.3,title.loadings=
                "SHH PCA Loadings - 3 Size Class - Whole Life Comparison",
                title.scores="SHH PCA Scores - 3 Size Class - Whole Life Comparison",input.log$user.label,ask)

        ronbo.pca.biplot(pca=pca.mis,markers=marker.mis.3,title.loadings=
                "MIS PCA Loadings - 3 Size Class - Whole Life Comparison",
                title.scores="MIS PCA Scores - 3 Size Class - Whole Life Comparison",input.log$user.label,ask)

# create biplots for each species and location using Ronbo's fancy biplot routine - 2 size class only

#               ronbo.pca.biplot(pca=pca.abt.region1,markers=marker.abt.region1,title.loadings=
#                       "ABT PCA Loadings - Region 1 - Whole Life Comparison",
#                       title.scores="ABT PCA Scores - Region 1 - Whole Life Comparison",input.log$user.label,ask)

        ronbo.pca.biplot(pca=pca.shh.region1,markers=marker.shh.region1,title.loadings=
                "SHH PCA Loadings - Region 1 - Whole Life Comparison",
                title.scores="SHH PCA Scores - Region 1 - Whole Life Comparison",input.log$user.label,ask)

        ronbo.pca.biplot(pca=pca.mis.region1,markers=marker.mis.region1,title.loadings=
                "MIS PCA Loadings - Region 1 - Whole Life Comparison",
                title.scores="MIS PCA Scores - Region 1 - Whole Life Comparison",input.log$user.label,ask)

#               ronbo.pca.biplot(pca=pca.abt.region6,markers=marker.abt.region6,title.loadings=
#                       "ABT PCA Loadings - Region 6 - Whole Life Comparison",
#                       title.scores="ABT PCA Scores - Region 6 - Whole Life Comparison",input.log$user.label,ask)

        ronbo.pca.biplot(pca=pca.shh.region6,markers=marker.shh.region6,title.loadings=
                "SHH PCA Loadings - Region 6 - Whole Life Comparison",
```

```
        title.scores="SHH PCA Scores - Region 6 - Whole Life Comparison",input.log$user.label,ask)

    ronbo.pca.biplot(pca=pca.mis.region6,markers=marker.mis.region6,title.loadings=
        "MIS PCA Loadings - Region 6 - Whole Life Comparison",
        title.scores="MIS PCA Scores - Region 6 - Whole Life Comparison",input.log$user.label,ask)


debug=47

# now create another manova response matrix, accounting for only region 1 (N)

#
    manova.response.abt.region1=as.matrix(subset(sol.pca.response,sol.pca$species=="ABT"&sol.pca$location=='1')
 )

    manova.response.shh.region1=as.matrix(subset(sol.pca.response,sol.pca$species=="SHH"&sol.pca$location=='1'
 ))

    manova.response.mis.region1=as.matrix(subset(sol.pca.response,sol.pca$species=="MIS"&sol.pca$location=='1')
 )

# now create another manova response matrix, accounting for only region 6 (S)

#
    manova.response.abt.region6=as.matrix(subset(sol.pca.response,sol.pca$species=="ABT"&sol.pca$location=='6')
 )

    manova.response.shh.region6=as.matrix(subset(sol.pca.response,sol.pca$species=="SHH"&sol.pca$location=='6'
 ))

    manova.response.mis.region6=as.matrix(subset(sol.pca.response,sol.pca$species=="MIS"&sol.pca$location=='6')
 )

# create vector for manova with size.class.2 as factor
debug=48
#       size.class.abt=as.factor(subset(sol.pca$size.class.2,sol.pca$species=='ABT'))
    size.class.shh=as.factor(subset(sol.pca$size.class.2,sol.pca$species=='SHH'))
    size.class.mis=as.factor(subset(sol.pca$size.class.2,sol.pca$species=='MIS'))

# create vector for manova with size.class.2 as factor for each location separately (N & S)
debug=49
#
    size.class.abt.region1=as.factor(subset(sol.pca$size.class.region1,sol.pca$species=='ABT'&sol.pca$location=='1')
 )

    size.class.shh.region1=as.factor(subset(sol.pca$size.class.region1,sol.pca$species=='SHH'&sol.pca$location=='1'
 ))

    size.class.mis.region1=as.factor(subset(sol.pca$size.class.region1,sol.pca$species=='MIS'&sol.pca$location=='1')
 )

#
    size.class.abt.region6=as.factor(subset(sol.pca$size.class.region6,sol.pca$species=='ABT'&sol.pca$location=='6')
 )

    size.class.shh.region6=as.factor(subset(sol.pca$size.class.region6,sol.pca$species=='SHH'&sol.pca$location=='6'
 ))

    size.class.mis.region6=as.factor(subset(sol.pca$size.class.region6,sol.pca$species=='MIS'&sol.pca$location=='6')
 )


debug=50
# now make manova and dfa calls using ronbo's routines - factor=size.class

#       fit.abt.size=ronbo.manova(manova.response.abt,size.class.abt,title.data='Whole Life - ABT with size as
 factor',
#           title.resid='ABT with size as factor: residuals',assumptions=input.log$assumptions.test)

#       lda.abt.size=ronbo.lda(manova.response.abt,size.class.abt,study.name='Whole Life - ABT - size.class as
 factor')

    fit.shh.size=ronbo.manova(manova.response.shh,size.class.shh,title.data='Whole Life - SHH with size as factor',
        title.resid='SHH with size as factor: residuals',assumptions=input.log$assumptions.test)
```

```
        lda.shh.size=ronbo.lda(manova.response.shh,size.class.shh,study.name='Whole Life - SHH - size.class as factor')

        fit.mis.size=ronbo.manova(manova.response.mis,size.class.mis,title.data='Whole Life - MIS with size as factor',
                title.resid='MIS with size as factor: residuals',assumptions=input.log$assumptions.test)

        lda.mis.size=ronbo.lda(manova.response.mis,size.class.mis,study.name='Whole Life - MIS - size.class as factor')

# now make manova and dfa calls using ronbo's routines - factor=size.class, done separately for each location - Region 1

#               fit.abt.size.region1=ronbo.manova(manova.response.abt.region1,size.class.abt.region1,
#                       title.data='Whole Life - ABT with size as factor - Region 1',
#                       title.resid='ABT with size as factor - Region 1: residuals',assumptions=input.log$assumptions.test)

#               lda.abt.size.region1=ronbo.lda(manova.response.abt.region1,size.class.abt.region1,
#                       study.name='Whole Life - ABT - size.class as factor - Region 1')

        fit.shh.size.region1=ronbo.manova(manova.response.shh.region1,size.class.shh.region1,
                title.data='Whole Life - SHH with size as factor - Region 1',
                title.resid='SHH with size as factor - Region 1: residuals',assumptions=input.log$assumptions.test)

        lda.shh.size.region1=ronbo.lda(manova.response.shh.region1,size.class.shh.region1,
                study.name='Whole Life - SHH - size.class as factor - Region 1')

        fit.mis.size.region1=ronbo.manova(manova.response.mis.region1,size.class.mis.region1,
                title.data='Whole Life - MIS with size as factor - Region 1',
                title.resid='MIS with size as factor - Region 1: residuals',assumptions=input.log$assumptions.test)

        lda.mis.size.region1=ronbo.lda(manova.response.mis.region1,size.class.mis.region1,
                study.name='Whole Life - MIS - size.class as factor - Region 1')

# now make manova and dfa calls using ronbo's routines - factor=size.class, done separately for each location - Region 6

#               fit.abt.size.region6=ronbo.manova(manova.response.abt.region6,size.class.abt.region6,
#                       title.data='Whole Life - ABT with size as factor - Region 6',
#                       title.resid='ABT with size as factor - Region 6: residuals',assumptions=input.log$assumptions.test)

#               lda.abt.size.region6=ronbo.lda(manova.response.abt.region6,size.class.abt.region6,
#                       study.name='Whole Life - ABT - size.class as factor - Region 6')

        fit.shh.size.region6=ronbo.manova(manova.response.shh.region6,size.class.shh.region6,
                title.data='Whole Life - SHH with size as factor - Region 6',
                title.resid='SHH with size as factor - Region 6: residuals',assumptions=input.log$assumptions.test)

        lda.shh.size.region6=ronbo.lda(manova.response.shh.region6,size.class.shh.region6,
                study.name='Whole Life - SHH - size.class as factor - Region 6')

        fit.mis.size.region6=ronbo.manova(manova.response.mis.region6,size.class.mis.region6,
                title.data='Whole Life - MIS with size as factor - Region 6',
                title.resid='MIS with size as factor - Region 6: residuals',assumptions=input.log$assumptions.test)

        lda.mis.size.region6=ronbo.lda(manova.response.mis.region6,size.class.mis.region6,
                study.name='Whole Life - MIS - size.class as factor - Region 6')

 }

}
debug=51



# do PCA, MANOVA, Hotellings T2, and DFA to catch location

if (input.log$catch.loc.analysis==TRUE) {

#       catch.pca=data.frame(0,'','',0,0,0,0,0,0,0,0,0)
#       names(catch.pca)=c('run','species','location','fl','seq.length',isotope.no.ca)
 catch.pca=data.frame(0,'','',0,0,0,0,0,0,0,0)
 names(catch.pca)=c('run','species','location','fl','seq.length',isotope.no.ca.no.p)

 for (j in 1:nrow(index)) {
        if (index$primary[j]==TRUE) {

# draw out appropriate data from main data set for pca analysis
```

```
                catch.pca.temp=data.frame(data[[j]]$run,data[[j]]$species,as.character(data[[j]]$location),
                        data[[j]]$fl,data[[j]]$sequence$length,t(data[[j]]$catch.ratio.corrected),check.names=F)

#                       names(catch.pca.temp)=c('run','species','location','fl','seq.length',isotope.no.ca)
                names(catch.pca.temp)=c('run','species','location','fl','seq.length',isotope.no.ca.no.p)

# bind data from this record together with previous records

                catch.pca=rbind(catch.pca,catch.pca.temp)
        }
 }
debug=52
# create subset of data for response matrix in pca

#       catch.pca.response=data.frame(catch.pca$Mg,catch.pca$P,catch.pca$Mn,catch.pca$Cu,
#               catch.pca$Zn,catch.pca$Sr,catch.pca$Ba)
 catch.pca.response=data.frame(catch.pca$Mg,catch.pca$Mn,catch.pca$Cu,
        catch.pca$Zn,catch.pca$Sr,catch.pca$Ba)

#       names(catch.pca.response)=isotope.no.ca
 names(catch.pca.response)=isotope.no.ca.no.p

# transform Mn, Cu, and Zn for MIS      (this affects multiple pca/manovas for MIS)

#       catch.pca.response$Mn[catch.pca$species=='MIS']=catch.pca.response$Mn[catch.pca$species=='MIS']^.5
#       catch.pca.response$Cu[catch.pca$species=='MIS']=catch.pca.response$Cu[catch.pca$species=='MIS']^.5
#       catch.pca.response$Zn[catch.pca$species=='MIS']=catch.pca.response$Zn[catch.pca$species=='MIS']^.2


# run pca for all three species

#       pca.catch.abt=princomp(catch.pca.response,cor=T,scores=T,
#               subset=rep(catch.pca$species=='ABT'))

 pca.catch.shh=princomp(catch.pca.response,cor=T,scores=T,
        subset=rep(catch.pca$species=='SHH'))

 pca.catch.mis=princomp(catch.pca.response,cor=T,scores=T,
        subset=rep(catch.pca$species=='MIS'))
debug=53
# set screen for biplots to be 2 plot areas wide

 par(mfrow=c(1,2),new=F,ask=ask)

# simplify location in vector for eventual use as markers in bi-plots

#       catch.pca$location=gsub('1','N',catch.pca$location)
#       catch.pca$location=gsub('6','S',catch.pca$location)

# define markers for scores plots

#       marker.abt=as.character(subset(catch.pca$location,catch.pca$species=='ABT'))
 marker.shh=as.character(subset(catch.pca$location,catch.pca$species=='SHH'))
 marker.mis=as.character(subset(catch.pca$location,catch.pca$species=='MIS'))
debug=54

# for LA-5, I'm using coloured dots instead of region number characters as scores markers
# so change to colours - also changing ronbo.pca.biplot for "markers" to be colours instead of pch

 marker.col.shh=gsub('1','black',marker.shh)
 marker.col.shh=gsub('2','blue',marker.col.shh)
 marker.col.shh=gsub('3','green',marker.col.shh)
 marker.col.shh=gsub('4','grey',marker.col.shh)
 marker.col.shh=gsub('6','red',marker.col.shh)
 marker.col.shh=gsub('8','lightblue',marker.col.shh)

 marker.col.mis=gsub('1','black',marker.mis)
 marker.col.mis=gsub('2','blue', marker.col.mis)
 marker.col.mis=gsub('3','green', marker.col.mis)
 marker.col.mis=gsub('4','grey', marker.col.mis)
 marker.col.mis=gsub('6','red', marker.col.mis)

# define legend.vals for the ronbo.pca.biplot legend on the scores

 legend.vals.shh=c('FN','CNS','TVL','MCK','BRI','NSW')
```

```
 legend.vals.mis=c('FN','CNS','TVL','MCK','BRI')

# define legend.cols for the ronbo.pca.biplot legend on the scores

 legend.cols.shh=c('black','blue','green','grey','red','lightblue')
 legend.cols.mis=c('black','blue','green','grey','red')


# create biplots for ABT and SHH using Ronbo's fancy biplot routine

#        ronbo.pca.biplot(pca=pca.catch.abt,markers=marker.abt,title.loadings=
#                "ABT PCA Loadings - Catch Location Influence",
#                title.scores="ABT PCA Scores - Catch Location Influence",input.log$user.label,ask)

 ronbo.pca.biplot(pca=pca.catch.shh,markers=marker.col.shh,title.loadings=
        "SHH PCA Loadings - Catch Location Influence",
        title.scores="SHH PCA Scores - Catch Location Influence",input.log$user.label,ask,legend.vals=legend.vals.shh,
        legend.cols=legend.cols.shh)

 ronbo.pca.biplot(pca=pca.catch.mis,markers=marker.col.mis,title.loadings=
        "MIS PCA Loadings - Catch Location Influence",
        title.scores="MIS PCA Scores - Catch Location Influence",input.log$user.label,ask,legend.vals=legend.vals.mis,
        legend.cols=legend.cols.mis)


# re-plot the min length biplot, separating size instead of location

# find mean sequence lengths for each species,

#        mean.abt=mean(catch.pca$seq.length[catch.pca$species=='ABT'])
 mean.shh=mean(catch.pca$seq.length[catch.pca$species=='SHH'])
 mean.mis=mean(catch.pca$seq.length[catch.pca$species=='MIS'])

# must eliminate first row of matrix for next step, also remove first row of response matrix to remove 0's for future manovas

 catch.pca=catch.pca[2:nrow(catch.pca),]
 catch.pca.response=catch.pca.response[2:nrow(catch.pca.response),]
debug=55

debug=57
# now create response matrices for MANOVA.  the manova function does not allow subsetting, so separate matrix must
 be
# made for each manova call.  It can be based on the sol.pca.response though, so it won't be too cumbersome.

#        manova.response.abt=as.matrix(subset(catch.pca.response,catch.pca$species=="ABT"))
 manova.response.shh=as.matrix(subset(catch.pca.response,catch.pca$species=="SHH"))
 manova.response.mis=as.matrix(subset(catch.pca.response,catch.pca$species=="MIS"))

# create vector for manova with location as factor

#        location.abt=as.factor(subset(catch.pca$location,catch.pca$species=='ABT'))
 location.shh=as.factor(subset(catch.pca$location,catch.pca$species=='SHH'))
 location.mis=as.factor(subset(catch.pca$location,catch.pca$species=='MIS'))
debug=58
# create vector for manova with size.class as factor

#        size.class.abt=as.factor(subset(catch.pca$size.class,catch.pca$species=='ABT'))
 size.class.shh=as.factor(subset(catch.pca$size.class,catch.pca$species=='SHH'))
 size.class.mis=as.factor(subset(catch.pca$size.class,catch.pca$species=='MIS'))

# now make manova calls using ronbo's routine - factor=location

#        fit.abt.location=ronbo.manova(manova.response.abt,location.abt,
#                title.data='Catch Location Influence - ABT - location as factor',
#                title.resid='ABT with location as factor: residuals',assumptions=input.log$assumptions.test)

#        lda.abt.location=ronbo.lda(manova.response.abt,location.abt,
#                study.name='Catch Location Influence - ABT - location as factor')

 fit.shh.location=ronbo.manova(manova.response.shh,location.shh,
        title.data='Catch Location Influence - SHH - location as factor',
        title.resid='SHH with location as factor: residuals',assumptions=input.log$assumptions.test)

 lda.shh.location=ronbo.lda(manova.response.shh,location.shh,
        study.name='Catch Location Influence - SHH - location as factor')
```

```
  fit.mis.location=ronbo.manova(manova.response.mis,location.mis,
          title.data='Catch Location Influence - MIS - location as factor',
          title.resid='MIS with location as factor: residuals',assumptions=input.log$assumptions.test)

  lda.mis.location=ronbo.lda(manova.response.mis,location.mis,
          study.name='Catch Location Influence - MIS - location as factor')
debug=59

# now run series of pair-wise Hotellings T2 comparisons - region 1/2, 2/3, 3/4, etc

  hot.t2.shh.r1=subset(manova.response.shh,location.shh=='1')
  hot.t2.shh.r2=subset(manova.response.shh,location.shh=='2')
  hot.t2.shh.r3=subset(manova.response.shh,location.shh=='3')
  hot.t2.shh.r4=subset(manova.response.shh,location.shh=='4')
  hot.t2.shh.r6=subset(manova.response.shh,location.shh=='6')
  hot.t2.shh.r8=subset(manova.response.shh,location.shh=='8')

  hot.t2.mis.r1=subset(manova.response.mis,location.mis=='1')
  hot.t2.mis.r2=subset(manova.response.mis,location.mis =='2')
  hot.t2.mis.r3=subset(manova.response.mis,location.mis =='3')
  hot.t2.mis.r4=subset(manova.response.mis,location.mis =='4')
  hot.t2.mis.r6=subset(manova.response.mis,location.mis =='6')

# now run Hotellings T2 pair wise comparisons for each neighbouring region

  hotellings.shh.12=HotellingsT2(hot.t2.shh.r1,hot.t2.shh.r2)
  hotellings.shh.23=HotellingsT2(hot.t2.shh.r2,hot.t2.shh.r3)
  hotellings.shh.34=HotellingsT2(hot.t2.shh.r3,hot.t2.shh.r4)
  hotellings.shh.46=HotellingsT2(hot.t2.shh.r4,hot.t2.shh.r6)
  hotellings.shh.68=HotellingsT2(hot.t2.shh.r6,hot.t2.shh.r8)

  hotellings.mis.12=HotellingsT2(hot.t2.mis.r1,hot.t2.mis.r2)
  hotellings.mis.23=HotellingsT2(hot.t2.mis.r2,hot.t2.mis.r3)
  hotellings.mis.34=HotellingsT2(hot.t2.mis.r3,hot.t2.mis.r4)
  hotellings.mis.46=HotellingsT2(hot.t2.mis.r4,hot.t2.mis.r6)

# create summary tables for each species

  hotellings.shh=data.frame(matrix(c(
  hotellings.shh.12$statistic,hotellings.shh.12$p.value,
  hotellings.shh.23$statistic,hotellings.shh.23$p.value,
  hotellings.shh.34$statistic,hotellings.shh.34$p.value,
  hotellings.shh.46$statistic,hotellings.shh.46$p.value,
  hotellings.shh.68$statistic,hotellings.shh.68$p.value
  ),ncol=2,byrow=T,dimnames=list(c('R1-2','R2-3','R3-4','R4-6','R6-8'))))

  names(hotellings.shh)=c('t2.statistic','p.value')

  hotellings.mis=data.frame(matrix(c(
  hotellings.mis.12$statistic,hotellings.mis.12$p.value,
  hotellings.mis.23$statistic,hotellings.mis.23$p.value,
  hotellings.mis.34$statistic,hotellings.mis.34$p.value,
  hotellings.mis.46$statistic,hotellings.mis.46$p.value
  ),ncol=2,byrow=T,dimnames=list(c('R1-2','R2-3','R3-4','R4-6'))))

  names(hotellings.shh)=names(hotellings.mis)=c('t2.statistic','p.value')

  # print results to output text file

  cat('\n\nHotellings T2 Multivariate Pair-Wise Comparisons - Catch Location Influence\nSHH\n')

  print(hotellings.shh)

  cat('\nMIS\n')

  print(hotellings.mis)

  cat('\n\n')

}

debug=60
#cv.analysis.matrix=na.omit(data.frame(cv.analysis.matrix[2:nrow(cv.analysis.matrix),]))
```

```
# test commands for reference
# cv=na.omit(data.frame(cv.mg$centre,cv.mg$edge))
# cv.mg=as.data.frame(tapply(cv.analysis.matrix$Mg,list(cv.analysis.matrix$fish.num,
#                     cv.analysis.matrix$profile),function(cv) std.dev(cv)/mean(cv)))

# create back to back histogram to gain insight on the size distribution by location of each species

if (input.log$sample.histogram==TRUE) {

# loop through one histogram for each species (other than NIST610)
debug=61
 for (i in 1:length(unique(index$species[index$species!='NIST610']))) {

# create variable of the current species being analysed

#                     i.species=switch(i,'ABT','SHH','MIS')
          i.species=switch(i,'SHH','MIS')
debug=62
# change from region codes to 'north' or 'south'

          index$location=gsub('1','North',index$location)
          index$location=gsub('6','South',index$location)


debug=63
          out=histbackback(split(index$outer.radius[index$species==i.species],
                    index$location[index$species==i.species]),probability=FALSE,
                    main=paste('Sample Size Distribution - ',i.species),
                    ylab='Outer Radius of Vertebrae (mm)',cex.lab=1.5,cex.axis=1.5,cex.main=1.5)
          barplot(-out$left,col='red',horiz=TRUE,space=0,add=TRUE,axes=FALSE)
          barplot(out$right,col='blue',horiz=TRUE,space=0,add=TRUE,axes=FALSE)

 }

}

# P not eliminated from the below analysis

if (input.log$jan.oct.compare==TRUE) {

# initialise a couple of data.frames for use in plotting

 nist.data=data.frame(matrix(c(0,0,0,0,0,0,0,0,0),ncol=9))
 names(nist.data)=c('run.date','run.month',isotope.no.ca)
 nist.data.temp=nist.data

 nist.data.raw=data.frame(matrix(c(0,0,0,0,0,0,0,0,0),ncol=10))
 names(nist.data.raw)=c('run.date','run.month',isotope)
 nist.data.raw.temp=nist.data.raw


# loop through one histogram for each species (other than NIST610)
debug=64
 for (i in 1:length(index$unique.id[index$species=='NIST610'])) {

# draw out appropriate data for plots

          nist.data[i,]=c(data[[index$unique.id[index$species=='NIST610'][i]]]$run.date,
                    data[[index$unique.id[index$species=='NIST610'][i]]]$run.month,
                    data[[index$unique.id[index$species=='NIST610'][i]]]$correction.signal)

debug=65

# now do the same for the raw data (not in ratio to Ca)

          nist.data.raw[i,]=c(data[[index$unique.id[index$species=='NIST610'][i]]]$run.date,
                    data[[index$unique.id[index$species=='NIST610'][i]]]$run.month,
                    data[[index$unique.id[index$species=='NIST610'][i]]]$correction.signal.raw)

 }

# initialise a couple more data.frames for use in plotting sample data
```

```
#         sample.data=data.frame(matrix(c(0,0,0,0,0,0,0,0,0),ncol=9))
#         names(sample.data)=c('run.date','run.month',isotope.no.ca)
 sample.data=data.frame(matrix(c(0,0,0,0,0,0,0,0),ncol=8))
 names(sample.data)=c('run.date','run.month',isotope.no.ca.no.p)
 sample.data.temp=sample.data

#         sample.data.raw=data.frame(matrix(c(0,0,0,0,0,0,0,0,0),ncol=10))
#         names(sample.data.raw)=c('run.date','run.month',isotope)
 sample.data.raw=data.frame(matrix(c(0,0,0,0,0,0,0,0,0),ncol=9))
 names(sample.data.raw)=c('run.date','run.month',isotope.no.p)
 sample.data.raw.temp=sample.data.raw


# loop through one histogram for each species (other than NIST610)
debug=65.1
 for (i in 1:length(index$unique.id[index$primary==TRUE])) {

# draw out appropriate data for plots

        sample.data[i,]=c(data[[index$unique.id[index$primary==TRUE][i]]]$run.date,
                data[[index$unique.id[index$primary==TRUE][i]]]$run.month,
                data[[index$unique.id[index$primary==TRUE][i]]]$mean.sol.ca44.ratio)

debug=65.2

# now do the same for the raw data (not in ratio to Ca)

        sample.data.raw[i,]=c(data[[index$unique.id[index$primary==TRUE][i]]]$run.date,
                data[[index$unique.id[index$primary==TRUE][i]]]$run.month,
                data[[index$unique.id[index$primary==TRUE][i]]]$mean.sol)

 }

# now add on a binary flag for whether run is before 7/8 Oct 2009 run date change

 nist.data$icpms.flag=floor(nist.data.raw$run.date/20091008)
 nist.data.raw$icpms.flag=floor(nist.data.raw$run.date/20091008)
 sample.data$icpms.flag=floor(sample.data.raw$run.date/20091008)
 sample.data.raw$icpms.flag=floor(sample.data.raw$run.date/20091008)


debug=66

# now start running all jan - oct comparison histograms - ratio data first

 jan.oct.hist(month.vec=nist.data$run.month,ca.ratio.flag=TRUE,data.vec=nist.data$Mg,element='Mg')
 jan.oct.hist(month.vec=nist.data$run.month,ca.ratio.flag=TRUE,data.vec=nist.data$P,element='P')
 jan.oct.hist(month.vec=nist.data$run.month,ca.ratio.flag=TRUE,data.vec=nist.data$Mn,element='Mn')
 jan.oct.hist(month.vec=nist.data$run.month,ca.ratio.flag=TRUE,data.vec=nist.data$Cu,element='Cu')
 jan.oct.hist(month.vec=nist.data$run.month,ca.ratio.flag=TRUE,data.vec=nist.data$Zn,element='Zn')
 jan.oct.hist(month.vec=nist.data$run.month,ca.ratio.flag=TRUE,data.vec=nist.data$Sr,element='Sr')
 jan.oct.hist(month.vec=nist.data$run.month,ca.ratio.flag=TRUE,data.vec=nist.data$Ba,element='Ba')

# now create histograms for raw data

 jan.oct.hist(month.vec=nist.data.raw$run.month,ca.ratio.flag=FALSE,data.vec=nist.data.raw$Mg,element='Mg')
 jan.oct.hist(month.vec=nist.data.raw$run.month,ca.ratio.flag=FALSE,data.vec=nist.data.raw$P,element='P')
 jan.oct.hist(month.vec=nist.data.raw$run.month,ca.ratio.flag=FALSE,data.vec=nist.data.raw$Ca,element='Ca')
 jan.oct.hist(month.vec=nist.data.raw$run.month,ca.ratio.flag=FALSE,data.vec=nist.data.raw$Mn,element='Mn')
 jan.oct.hist(month.vec=nist.data.raw$run.month,ca.ratio.flag=FALSE,data.vec=nist.data.raw$Cu,element='Cu')
 jan.oct.hist(month.vec=nist.data.raw$run.month,ca.ratio.flag=FALSE,data.vec=nist.data.raw$Zn,element='Zn')
 jan.oct.hist(month.vec=nist.data.raw$run.month,ca.ratio.flag=FALSE,data.vec=nist.data.raw$Sr,element='Sr')
 jan.oct.hist(month.vec=nist.data.raw$run.month,ca.ratio.flag=FALSE,data.vec=nist.data.raw$Ba,element='Ba')


# now let's look at the nist versus sample comparison of raw data, first draw out the full data, similar to above
# abl.yield.group looks at the 2 nearest vertebrae samples to each nist610 sample.  this way we can compare what's
# happening to ratios of sample versus standard as sensitivity drifts.  Specifically, this is looking for non-linear
# drift effects.

# initialise a couple of data.frames for use in plotting

 abl.yield.data=data.frame(matrix(c(0,0,0,0,0,0,0,0,0),ncol=10))
 names(abl.yield.data)=c('abl.yield.group','run.month',isotope)
 abl.yield.data.temp=abl.yield.data
```

```
 profile.vector=data.frame('')

# loop through one histogram for each species (other than NIST610)
debug=67
 for (i in 1:length(index$unique.id[index$abl.yield!=0])) {

# draw out appropriate data for plots, correction.signal.raw for standard, mean.sol for vertebrae sample

                if (data[[index$unique.id[index$abl.yield.group!=0][i]]]$profile=='standard') {

                abl.yield.data[i,]=c(data[[index$unique.id[index$abl.yield.group!=0][i]]]$abl.yield.group,
                        data[[index$unique.id[index$abl.yield.group!=0][i]]]$run.month,
                        data[[index$unique.id[index$abl.yield.group!=0][i]]]$correction.signal.raw)

        } else {  # assumes it is now 'edge' (vertebrae sample of any species)

                abl.yield.data[i,]=c(data[[index$unique.id[index$abl.yield.group!=0][i]]]$abl.yield.group,
                        data[[index$unique.id[index$abl.yield.group!=0][i]]]$run.month,
                        data[[index$unique.id[index$abl.yield.group!=0][i]]]$mean.sol)

        }

debug=68
# profile is character, so it must be pulled out separately

        profile.vector[i]=data[[index$unique.id[index$abl.yield.group!=0][i]]]$profile

 }

# now add profile to the data

 abl.yield.data$profile=t(profile.vector)
debug=69

# create table of means for each abl.yield.group x profile combination

 abl.yield.means.mg=as.data.frame(tapply(abl.yield.data$Mg,list(abl.yield.data$run.month,
        abl.yield.data$profile,abl.yield.data$abl.yield.group),mean))
 abl.yield.means.p=as.data.frame(tapply(abl.yield.data$P,list(abl.yield.data$run.month,
        abl.yield.data$profile,abl.yield.data$abl.yield.group),mean))
 abl.yield.means.ca=as.data.frame(tapply(abl.yield.data$Ca,list(abl.yield.data$run.month,
        abl.yield.data$profile,abl.yield.data$abl.yield.group),mean))
 abl.yield.means.mn=as.data.frame(tapply(abl.yield.data$Mn,list(abl.yield.data$run.month,
        abl.yield.data$profile,abl.yield.data$abl.yield.group),mean))
 abl.yield.means.cu=as.data.frame(tapply(abl.yield.data$Cu,list(abl.yield.data$run.month,
        abl.yield.data$profile,abl.yield.data$abl.yield.group),mean))
 abl.yield.means.zn=as.data.frame(tapply(abl.yield.data$Zn,list(abl.yield.data$run.month,
        abl.yield.data$profile,abl.yield.data$abl.yield.group),mean))
 abl.yield.means.sr=as.data.frame(tapply(abl.yield.data$Sr,list(abl.yield.data$run.month,
        abl.yield.data$profile,abl.yield.data$abl.yield.group),mean))
 abl.yield.means.ba=as.data.frame(tapply(abl.yield.data$Ba,list(abl.yield.data$run.month,
        abl.yield.data$profile,abl.yield.data$abl.yield.group),mean))

# draw plots of raw elemental values against each other

 plot.abl.yield(abl.yield.means.mg,'Mg')
 plot.abl.yield(abl.yield.means.p,'P')
 plot.abl.yield(abl.yield.means.ca,'Ca')
 plot.abl.yield(abl.yield.means.mn,'Mn')
 plot.abl.yield(abl.yield.means.cu,'Cu')
 plot.abl.yield(abl.yield.means.zn,'Zn')
 plot.abl.yield(abl.yield.means.sr,'Sr')
 plot.abl.yield(abl.yield.means.ba,'Ba')

# now plot each element v Ca for all NIST610 runs, separating between Jan and Oct runs

 raw.element.plot(nist.data.raw$Ca[nist.data.raw$run.month==1],nist.data.raw$Mg[nist.data.raw$run.month==1],

        nist.data.raw$Ca[nist.data.raw$run.month==10],nist.data.raw$Mg[nist.data.raw$run.month==10],'Ca','Mg','NIST61
0')

 raw.element.plot(nist.data.raw$Ca[nist.data.raw$run.month==1],nist.data.raw$P[nist.data.raw$run.month==1],
        nist.data.raw$Ca[nist.data.raw$run.month==10],nist.data.raw$P[nist.data.raw$run.month==10],'Ca','P','NIST610')

 raw.element.plot(nist.data.raw$Ca[nist.data.raw$run.month==1],nist.data.raw$Mn[nist.data.raw$run.month==1],
```

```
        nist.data.raw$Ca[nist.data.raw$run.month==10],nist.data.raw$Mn[nist.data.raw$run.month==10],'Ca','Mn','NIST61
0')

 raw.element.plot(nist.data.raw$Ca[nist.data.raw$run.month==1],nist.data.raw$Cu[nist.data.raw$run.month==1],

        nist.data.raw$Ca[nist.data.raw$run.month==10],nist.data.raw$Cu[nist.data.raw$run.month==10],'Ca','Cu','NIST61
0')

 raw.element.plot(nist.data.raw$Ca[nist.data.raw$run.month==1],nist.data.raw$Zn[nist.data.raw$run.month==1],

        nist.data.raw$Ca[nist.data.raw$run.month==10],nist.data.raw$Zn[nist.data.raw$run.month==10],'Ca','Zn','NIST610
')

 raw.element.plot(nist.data.raw$Ca[nist.data.raw$run.month==1],nist.data.raw$Sr[nist.data.raw$run.month==1],

        nist.data.raw$Ca[nist.data.raw$run.month==10],nist.data.raw$Sr[nist.data.raw$run.month==10],'Ca','Sr','NIST610'
)

 raw.element.plot(nist.data.raw$Ca[nist.data.raw$run.month==1],nist.data.raw$Ba[nist.data.raw$run.month==1],

        nist.data.raw$Ca[nist.data.raw$run.month==10],nist.data.raw$Ba[nist.data.raw$run.month==10],'Ca','Ba','NIST610
')

# now plot each element v Ca for all vertebrae sample "primary" runs, separating between Jan and Oct runs

 raw.element.plot(sample.data.raw$Ca[sample.data.raw$run.month==1],sample.data.raw$Mg[sample.data.raw$run.mont
h==1],
        sample.data.raw$Ca[sample.data.raw$run.month==10],sample.data.raw$Mg[sample.data.raw$run.month==10],
        'Ca','Mg','Vertebrae Sample')

 raw.element.plot(sample.data.raw$Ca[sample.data.raw$run.month==1],sample.data.raw$P[sample.data.raw$run.month=
=1],
        sample.data.raw$Ca[sample.data.raw$run.month==10],sample.data.raw$P[sample.data.raw$run.month==10],
        'Ca','P','Vertebrae Sample')

 raw.element.plot(sample.data.raw$Ca[sample.data.raw$run.month==1],sample.data.raw$Mn[sample.data.raw$run.mont
h==1],
        sample.data.raw$Ca[sample.data.raw$run.month==10],sample.data.raw$Mn[sample.data.raw$run.month==10],
        'Ca','Mn','Vertebrae Sample')

 raw.element.plot(sample.data.raw$Ca[sample.data.raw$run.month==1],sample.data.raw$Cu[sample.data.raw$run.mont
h==1],
        sample.data.raw$Ca[sample.data.raw$run.month==10],sample.data.raw$Cu[sample.data.raw$run.month==10],
        'Ca','Cu','Vertebrae Sample')

 raw.element.plot(sample.data.raw$Ca[sample.data.raw$run.month==1],sample.data.raw$Zn[sample.data.raw$run.month
==1],
        sample.data.raw$Ca[sample.data.raw$run.month==10],sample.data.raw$Zn[sample.data.raw$run.month==10],
        'Ca','Zn','Vertebrae Sample')

 raw.element.plot(sample.data.raw$Ca[sample.data.raw$run.month==1],sample.data.raw$Sr[sample.data.raw$run.month
==1],
        sample.data.raw$Ca[sample.data.raw$run.month==10],sample.data.raw$Sr[sample.data.raw$run.month==10],
        'Ca','Sr','Vertebrae Sample')

 raw.element.plot(sample.data.raw$Ca[sample.data.raw$run.month==1],sample.data.raw$Ba[sample.data.raw$run.month
==1],
        sample.data.raw$Ca[sample.data.raw$run.month==10],sample.data.raw$Ba[sample.data.raw$run.month==10],
        'Ca','Ba','Vertebrae Sample')

# create barplot of pre/post 7/8 Oct 2009 sample variations for raw data
# first need to create data.frame for barplot, row=nist, sample  and row=each element

 barplot.raw=as.data.frame(matrix(c(
        mean(nist.data.raw$Mg[nist.data.raw$icpms.flag==1])/mean(nist.data.raw$Mg[nist.data.raw$icpms.flag==0]),

        mean(sample.data.raw$Mg[sample.data.raw$icpms.flag==1])/mean(sample.data.raw$Mg[sample.data.raw$icpms.
 flag==0]),

        mean(nist.data.raw$P[nist.data.raw$icpms.flag==1])/mean(nist.data.raw$P[nist.data.raw$icpms.flag==0]),

        mean(sample.data.raw$P[sample.data.raw$icpms.flag==1])/mean(sample.data.raw$P[sample.data.raw$icpms.fla
 g==0]),
```

```
        mean(nist.data.raw$Ca[nist.data.raw$icpms.flag==1])/mean(nist.data.raw$Ca[nist.data.raw$icpms.flag==0]),

        mean(sample.data.raw$Ca[sample.data.raw$icpms.flag==1])/mean(sample.data.raw$Ca[sample.data.raw$icpms.
flag==0]),

        mean(nist.data.raw$Mn[nist.data.raw$icpms.flag==1])/mean(nist.data.raw$Mn[nist.data.raw$icpms.flag==0]),

        mean(sample.data.raw$Mn[sample.data.raw$icpms.flag==1])/mean(sample.data.raw$Mn[sample.data.raw$icpms.
flag==0]),

        mean(nist.data.raw$Cu[nist.data.raw$icpms.flag==1])/mean(nist.data.raw$Cu[nist.data.raw$icpms.flag==0]),

        mean(sample.data.raw$Cu[sample.data.raw$icpms.flag==1])/mean(sample.data.raw$Cu[sample.data.raw$icpms.
flag==0]),

        mean(nist.data.raw$Zn[nist.data.raw$icpms.flag==1])/mean(nist.data.raw$Zn[nist.data.raw$icpms.flag==0]),

        mean(sample.data.raw$Zn[sample.data.raw$icpms.flag==1])/mean(sample.data.raw$Zn[sample.data.raw$icpms.f
lag==0]),

        mean(nist.data.raw$Sr[nist.data.raw$icpms.flag==1])/mean(nist.data.raw$Sr[nist.data.raw$icpms.flag==0]),

        mean(sample.data.raw$Sr[sample.data.raw$icpms.flag==1])/mean(sample.data.raw$Sr[sample.data.raw$icpms.fl
ag==0]),

        mean(nist.data.raw$Ba[nist.data.raw$icpms.flag==1])/mean(nist.data.raw$Ba[nist.data.raw$icpms.flag==0]),

        mean(sample.data.raw$Ba[sample.data.raw$icpms.flag==1])/mean(sample.data.raw$Ba[sample.data.raw$icpms.f
lag==0])),

        byrow=FALSE,nrow=2),row.names=c('NIST','Samples'))

# rename data.frame columns

        names(barplot.raw)=isotope

# create the barplot

        barplot(as.matrix(barplot.raw),beside=TRUE,legend=rownames(barplot.raw),col=c('lightblue','pink'),
                main='Raw Element Intensity Variation Between NIST and Vertebrae Samples',
                xlab='Element',ylab='Ratio of Mean Values on 8 Oct or Later to Runs on 7 Oct or Earlier')

# now do the same thing for ratios

 barplot.ratio=as.data.frame(matrix(c(
        mean(nist.data$Mg[nist.data$icpms.flag==1])/mean(nist.data$Mg[nist.data$icpms.flag==0]),
        mean(sample.data$Mg[sample.data$icpms.flag==1])/mean(sample.data$Mg[sample.data$icpms.flag==0]),

        mean(nist.data$P[nist.data$icpms.flag==1])/mean(nist.data$P[nist.data$icpms.flag==0]),
        mean(sample.data$P[sample.data$icpms.flag==1])/mean(sample.data$P[sample.data$icpms.flag==0]),

        mean(nist.data$Mn[nist.data$icpms.flag==1])/mean(nist.data$Mn[nist.data$icpms.flag==0]),
        mean(sample.data$Mn[sample.data$icpms.flag==1])/mean(sample.data$Mn[sample.data$icpms.flag==0]),

        mean(nist.data$Cu[nist.data$icpms.flag==1])/mean(nist.data$Cu[nist.data$icpms.flag==0]),
        mean(sample.data$Cu[sample.data$icpms.flag==1])/mean(sample.data$Cu[sample.data$icpms.flag==0]),

        mean(nist.data$Zn[nist.data$icpms.flag==1])/mean(nist.data$Zn[nist.data$icpms.flag==0]),
        mean(sample.data$Zn[sample.data$icpms.flag==1])/mean(sample.data$Zn[sample.data$icpms.flag==0]),

        mean(nist.data$Sr[nist.data$icpms.flag==1])/mean(nist.data$Sr[nist.data$icpms.flag==0]),
        mean(sample.data$Sr[sample.data$icpms.flag==1])/mean(sample.data$Sr[sample.data$icpms.flag==0]),

        mean(nist.data$Ba[nist.data$icpms.flag==1])/mean(nist.data$Ba[nist.data$icpms.flag==0]),
        mean(sample.data$Ba[sample.data$icpms.flag==1])/mean(sample.data$Ba[sample.data$icpms.flag==0])),

        byrow=FALSE,nrow=2),row.names=c('NIST','Samples'))

# rename data.frame columns

        names(barplot.ratio)=isotope.no.ca

# create the barplot

        barplot(as.matrix(barplot.ratio),beside=TRUE,legend=rownames(barplot.ratio),col=c('lightblue','pink'),
```

```
                main='Ratio to Ca - Element Intensity Variation Between NIST and Vertebrae Samples',
                xlab='Element/Ca',ylab='Ratio of Mean Values on 8 Oct or Later to Runs on 7 Oct or Earlier')

}



debug=70

if (input.log$pdf.graphics.output==TRUE) {
  dev.off()
}

# re-enable warnings to screen
options(warn=1)

# close output text file
sink()

# save object for future work, without having to run pre-processing

#save(data,file=paste(file.location,'Data_Object_Analyses_Laser_Ablation_4.rda',sep=''),ascii=F)
```

7.2 Input Parameters and Logicals

parameter.input.variable	parameter.value	expected units	comment
outlier.filter	0.4	0-1 decimal	Variation from EWMA to be considered outlier
outlier.spread	5	integer >0	Number of points on each side to average for outlier calculation
ma.spread	5	integer>0	Number of points on each side to use for central simple moving average
start.hurdle	110000	integer >0	Threshold to define sequence start for ca44 to rise above
start.delta	1	integer	Shift forward from crossing threshold to seqence start
end.hurdle	110000	>start.hurdle	Threshold to define sequence end for ca44 to drop below
end.delta	15	integer	Shift backward from crossing threshold to sequence start
standard.hurdle	10000	integer>0	Threshold to define sequence start for standards
standard.width	60	integer>0	Length of sequence for standard calculations
catch.loc.influence	200	decimal >0	Distance along outer radius representing catch location (microns)
start.distance	400	decimal >0	Distance along outer radius from centre to scan start (microns)
abt.birth.diam	3970	decimal >0	ABT birth ring diameter (microns) - NOT radius
shh.birth.diam	4760	decimal >0	SHH birth ring diameter (microns) - NOT radius
mis.birth.diam	2840	decimal >0	MIS birth ring diameter (microns) - NOT radius (estimated)
icpms.meas.period	0.2323	decimal >0	Time period (1/freq) for ICPMS measurement (not LA)
laser.scan.speed	62	decimal>0	Laser ablation scan speed (micron/s)
conf.int	0.9		confidence interval for pca biplot ellipse and ellipsoid

logical.input.variable	value.t.f	units	comment
pdf.graphics.output	T	logical T-F	Should graphics go to pdf instead of screen
pre.process.format	F	logical T-F	Run pre-processor to format raw data into data.frame
sequence.plot	F	logical T-F	Run plots of Ca to manually define sequence start and end
pre.process.ts	F	logical T-F	Run pre-processor to do original time series data manipulation
pre.process.drift	F	logical T-F	Run drift correction algorithms
solution.pca	T	logical T-F	Recreate solution based pca analysis (inside birth ring eliminated)
size.pca	F	logical T-F	Run size based PCA analyses for each location (whole life)
catch.loc.analysis	T	logical T-F	Should catch location analysis be performed
sample.histogram	F	logical T-F	Should histogram plot comparing sample size/location be made
same.scan.analysis	F	logical T-F	Should same scan pca analyses be performed
raw.data.plot	F	logical T-F	Should raw data pre-processing results be plotted
rescaled.ratio.plot	F	logical T-F	Should drift rescaled ratio data results be plotted
drift.plot	F	logical T-F	Should plot to view drift from standards be shown
user.label	F	logical T-F	Should user define labels manually in biplots
assumptions.test	F	logical T-F	Should assumptions test be used for MANOVA
cv.analysis.flag	F	logical T-F	Should the coefficient of variation analysis be run
jan.oct.compare	F	logical T-F	Should comparison for Jan runs to Oct runs be made
gobi.dump	T	logical T-F	should data be dumped to csv file for gobi analysis

7.3 Function Definition Script

```
#===============================================================================
#XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
#===============================================================================
#
#
# This function creates a biplot on for a previously run pca analysis
#
#
# Inputs:
#       pca - data.frame output from princomp() (eg. pca=princomp(resp_matrix,...))
#       markers - character string for scores plot (default = pca$scores row names)
#       title.loadings - character string for title (default = "PCA Biplot Loadings")
#       title.scores - character string for title (default = "PCA Biplot Scores")
#       self.label - logical allowing user to place vector labels on plot with mouse
#                   (default=FALSE) - used to make publication quality
#                   assumes you know which vector to mark (use previous plot)
#                       ask - logical to ask if you should prompt between plots or not
#        legend.vals - values to be shown for legend
#        legend.cols - colours for legend
#        conf.int - confidence interval to draw ellipse
#
# Outputs:
#       pca biplot on current graphics device
#
# Requirements for use:
#       - "scores" must have been set to TRUE in princomp command (default)
#       - recommend having 2 plot areas active - eg. "par(mfrow=c(1,2))"
#
#
# This function was created because I didn't like default "biplot()" function:
#       - separates scores and loadings into 2 separate plots
#       - variance component calculated and placed on the axes
#       - min/max limits for scaling are calculated
#       - plots are forced to be square and zero centred
#       - algorithm to place loading vector labels in desireable place
#       - include your own score markers for scores plot
#       - allows user to define vector marker locations with mouse click
#
#

ronbo.pca.biplot=function(pca,markers=dimnames(pca$scores)[[1]],title.loadings="PCA Biplot Loadings",
 title.scores="PCA Biplot Scores",self.label=FALSE,ask=FALSE,legend.vals,legend.cols,conf.int=.95){

# set screen parameters to 2 per page

par(mfrow=c(1,2),new=F,ask=ask)

# calculate variance components

varcomp=as.integer(100*pca$sdev^2/sum(pca$sdev^2))

# create data for plotting

loadings.x=pca$loadings[,1]
loadings.y=pca$loadings[,2]
loadings.max=max(abs(c(loadings.x,loadings.y)))
loadings.lim=c(loadings.max,-loadings.max)
scores.x=pca$scores[,1]
scores.y=pca$scores[,2]
scores.max=max(abs(c(scores.x,scores.y)))
scores.lim=c(scores.max,-scores.max)

# create plots for loadings data

plot(loadings.x,loadings.y,pch=" ",axes=T,xlim=loadings.lim,ylim=loadings.lim,xlab=paste("Principal Component 1 = ",
 varcomp[1],"%"),ylab=paste("Principal Component 2 = ",varcomp[2],"%"),main=title.loadings)

#draw arrows for laoding vectors

arrows(rep(0,length(loadings.x)),rep(0,length(loadings.y)),loadings.x,loadings.y,cod=2,col="red")
```

```
# draw zero axes

abline(v=0)
abline(h=0)

# calculate preferred marker position based on Ronbo's special algorithm

marker.position=rep(0,length(loadings.x)) #define marker.position vector
for(i in 1:length(loadings.x)){ # determine which half of which quadrant point is in
  if (abs(loadings.x[i])>=abs(loadings.y[i])&&loadings.y[i]<0) marker.position[i]=1
  if (abs(loadings.x[i])<abs(loadings.y[i])&&loadings.x[i]<0) marker.position[i]=2
  if (abs(loadings.x[i])>=abs(loadings.y[i])&&loadings.y[i]>=0) marker.position[i]=3
  if (abs(loadings.x[i])<abs(loadings.y[i])&&loadings.x[i]>=0) marker.position[i]=4}

# draw labels for loading vectors using label placement algorithm

if (self.label==FALSE) text(loadings.x,loadings.y,dimnames(pca$loadings)[[1]],pos=marker.position)

# draw labels for loading vectors using locator()
# to use this interactive tool, simply point to where you want the vector labels to be placed and click
# this must be done in order (first Ba, then Cu,....), so you may need to look at plot on screen first
# and then re-run it to know which vector is which.
#
# if you his return on the console while locator() is awaiting input, it will give you an error
#

if (self.label==TRUE) text(locator(length(loadings.x)),dimnames(pca$loadings)[[1]],adj=0)

# now plot scores (each sample)

#plot(scores.x,scores.y,xlim=scores.lim,ylim=scores.lim,xlab=paste("Principal Component 1xxx = ",varcomp[1],"%"),
#          ylab=paste("Principal Component 2 = ",varcomp[2],"%"),main=title.scores,pch=markers,cex=2)
plot(scores.x,scores.y,xlim=scores.lim,ylim=scores.lim,xlab=paste("Principal Component 1 = ",varcomp[1],"%"),
  ylab=paste("Principal Component 2 = ",varcomp[2],"%"),main=title.scores,col=markers,pch=20,cex=.6)

# draw zero axes

abline(v=0)
abline(h=0)

# add legend to the scores plot

legend('topleft',pch=20,pt.cex=1.5,col=legend.cols,cex=1.1,legend=legend.vals,title='Region')

# run loop to draw confidence interval ellipses

for (i in 1:length(legend.vals)){

e.x=subset(scores.x,markers==legend.cols[i])
e.y=subset(scores.y,markers==legend.cols[i])

# draw data.ellipse

data.ellipse(e.x,e.y,levels=conf.int,col=legend.cols[i],plot.points=F,add=T,center.pch=10,center.cex=3,lwd=1)

}

}

#============================================================================
#XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
#============================================================================
#
# simple function to return standard deviation
#

std.dev=function(x) sqrt(var(x))

#============================================================================
#XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
#============================================================================
#
# function to standardise COLUMNS of a matrix  (X-mean(X))/std.dev(X)
#
```

```
ronbo.matrix.column.standardise=function(x){
x.std=x
for (i in 1:ncol(x)) x.std[,i]=(x[,i]-mean(x[,i]))/std.dev(x[,i])
x.std}

#==============================================================================
#XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
#==============================================================================
#
# function to perform discriminant function analysis and write outputs
#
#
#  Inputs:
#         response   - i x j matrix holding i cases of j response variables
#         grouping   - i length vector holding grouping variable
#                    (may work as ixk if k variables, but this is not tested)
#         study.name - character string for output header
#                    (defaults to "Discriminant Function Analysis")
#
#  Outputs:
#         lda.results - data.frame holding dfa analysis information
#         classification and percent correct data printed to file
#


ronbo.lda=function(response,grouping,study.name="Discriminant Function Analysis"){

 # standardise matrix

 std.response=ronbo.matrix.column.standardise(response)

 # create input data frame with both response and grouping
 lda.input=data.frame(std.response,grouping,row.names=c(1:nrow(std.response)))

 # run lda with grouping as a function of everything else in response matrix
 lda.results=lda(grouping~.,lda.input)

 # create classification table
 classification.table=table(grouping,predict(lda.results)$class)

 # calculate success rate of classification
 percent.correct=100*sum(diag(classification.table))/sum(classification.table)

 # print all related data
 cat('\n\nDiscriminant Function Analysis Results\n\n')
 print(lda.results)
 cat('\n\nClassification Table for ',study.name,'\n')
 print(classification.table)
 cat('\n\nPercent Correctly Classified = ',percent.correct,' %\n\n')
 lda.results}

#==============================================================================
#XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
#==============================================================================
#
# function to perform simple single factor MANOVA's and write outputs
#
#
#  Inputs:
#         response    - i x j matrix holding i cases of j response variables
#         grouping    - i length vector holding grouping variable
#                     (may work as ixk if k variables, but this is not tested)
#         title.data  - title string for data histograms and boxplots
#         title.resid - title string for q-q residuals plots
#         assumptions - logical to see if assumptions tests should be run
#         ask         - logical to determine if prompting should occur for graphics
#
#  Outputs:
#         fit.manova - data.frame holding dfa analysis information
#         manova table printed to current text file output
#         qq and data histogram plots on current graphics device
#         summary ANOVA tables printed to text file output
#
#  Notes:
#         - max of 8 response variables as plots come out in 2 x 4 format
```

```
#          - model is set for single factor only
#

ronbo.manova=function(response,grouping,title.data='MANOVA response data',title.resid='MANOVA residuals',
  assumptions=TRUE,ask=TRUE) {

# Perform MANOVA (can not use "subset" in manova(), so vectors must be created)


fit=manova(response~grouping)

sum.fit=summary(fit,test="Pillai")

# print outputs to file

cat('\n\n=================================================================\n\n\n')
cat(title.data,' MANOVA Table\n\n')
print(sum.fit)

# print summary ANOVA's to file - one for each response variable

cat('\n\n=================================================================\n\n\n')
cat(title.data,' Summary ANOVA Tables\n\n')
print(summary.aov(fit))

# test if assumptions flag is set to TRUE.  If not, don't run tests

if (assumptions[1]==TRUE){

# various plots for MANOVA assumptions testing

resfit=residuals(fit)

# normality - histograms

par(mfrow=c(2,4),new=F,ask=ask)
for (i in 1:length(response[1,])) hist(response[,i],xlab=dimnames(response)[[2]][i],main=title.data)
par(mfrow=c(2,4),new=F,ask=ask)
for (i in 1:length(response[1,])) boxplot(response[,i],xlab=dimnames(response)[[2]][i],main=title.data)

# residuals histograms and q-q plots

par(mfrow=c(2,4),new=F,ask=ask)
for (i in 1:length(resfit[1,])) hist(resfit[,i],xlab=dimnames(resfit)[[2]][i],main=title.resid)
par(mfrow=c(2,4),new=F,ask=ask)
for (i in 1:length(resfit[1,])) {
  qqnorm(resfit[,i],xlab=dimnames(resfit)[[2]][i],main=title.resid)
  qqline(resfit[,i])}
}
fit}



#============================================================================
#XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
#============================================================================
#
#
#          Outlier filter
#                    Takes a series of data and eliminate outliers based on predefined
#                    rules which are inputted by the user
#
#          Inputs
#                    - run     - run name (character)
#                    - element          - element (subset of run) (character)
#                    - input            - input series (ts)
#                    - spread           - how many points before and after for mean calculation
#                    - filt       - how far above local mean does it need to be to be outlier
#
#          Outputs (combined as list object)
#                    - [[1]] output - output series (ts) with outliers replaced by local mean
#                    - [[2]] outlier.list - data frame of outlier points being replaced
#
#          Requirements / assumptions
#                    - all outliers are ABOVE local mean (as is the case with icpms)
```

```
#                   - there are no outliers in first or last few points of the series
#                   - outliers are not eliminated, but replaced with local mean value
#
#
#
# create outlier filter function

outlier.filter=function(run,element,input,spread,filt) {

# define outlier counter for saving to outlier.list for future review

 cnt=0

# create output and output.list variables

 output=1
 outlier.list=list(' ',' ',0,0,0)
 names(outlier.list)=c('run','series','point','old.val','new.val')

# smooth initial and final few points where moving average can't work,
# assumes no outliers in the very beginning and very end of the series

 output[1:spread]=input[1:spread]
 output[(length(input)-spread):length(input)]=input[(length(input)-spread):length(input)]

# loop through series, calculating local means and averaging any outliers
# save appropriate data to outlier.list

 for (j in (1+spread):(length(input)-spread)-1){

        if (input[j]>((1+filt)*(mean(input[(j-spread):(j-1)])+mean(input[(j+1):
                (j+spread)])/2))) {

                cnt=cnt+1
                outlier.list$run[cnt]=as.character(run)
                outlier.list$series[cnt]=element
                outlier.list$point[cnt]=j
                outlier.list$old.val[cnt]=input[j]
                output[j]=as.integer(mean(input[(j-spread):(j-1)])+
                        mean(input[(j+1):(j+spread)])/2)
                outlier.list$new.val[cnt]=output[j]
        }

        else {

# if no outlier, then output=input

                output[j]=input[j]
        }
 }

# combine output series and outlier.list as list for output

result=list(output,as.data.frame(outlier.list))}

#==============================================================================
#XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
#==============================================================================
#
#
#       Smoothing Function
#                   Takes a series of data and and smooths it based on predefined parameters
#                   using centralised simple moving average function
#
#       Inputs
#                   - input   - input series (ts)
#                   - spread          - how many points before and after for mean calculation
#
#       Output
#                   - output          - output series (ts) smoothed by moving average
#
#       Requirements / assumptions
#                   - earliest and latest points averaged only on one side
#
#
```

```
# define function

smooth.series=function(input,spread) {

# create output variable
 output=1

# calculate moving average of points away from edge of series

 for (i in (spread+1):(length(input)-spread-1)) {
        output[i]=mean(input[(i-spread):(i+spread)])
 }

# calculate the simple average of the first few series points

 for (i in 1:spread) {
        output[i]=mean(input[1:spread])
 }

# calculate the simple average of the last few series points

 for (i in (length(input)-spread):length(input)) {
        output[i]=mean(input[(length(input)-spread):length(input)])
 }

result=output}


#================================================================================
#XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
#================================================================================
#
#
#
#       Plotting of all raw data information
#               Plots raw data series, and adds information such as outliers, background signal, smoothed curve, start
 and end
#               of sequence
#
#       Inputs
#               - run                   - name of run for title (character)
#               - species               - name of species for title (character)
#               - profile       - type of profile for this sample, 'edge', 'standard', 'centre', etc. (character)
#               - element               - element being plotted for title (character)
#               - raw.data              - input series (ts) of raw and unfiltered data
#               - outlier       - input series (ts) of raw data with outliers filtered out
#               - smoothed              - input series (ts) smoothed data to plot over the raw data
#               - background    - background value (numeric) drawn from smoothed data
#               - start.point   - start of actual sequence of sample laser ablation
#               - end.point             - end of actual sequence of sample laser ablation
#               - birth.point   - point where birth ring occurs based on previous measurements
#               - catch.point   - point beyond which is considered influenced by catch point
#               - post.birth.mean - mean signals between birth and death
#               - catch.mean    - mean signal between catch influence point and death
#
#       Output
#               - plot to the current device
#
#       Requirements
#               - time series must not be in a list for plotting (don't know why)
#

raw.data.plot=function(run,species,profile,element,raw.data,outlier,smoothed,background,start.point,end.point,birth.point,
 catch.point,post.birth.mean,catch.mean){

 if (profile=='standard') {

        ts.plot(raw.data,outlier,smoothed,line=background,line=0,col=c('red','black','green','blue','black'),
                xlab='Point Number',ylab='Hits Per Second',
        lty=c(1,1,1,2,1),lwd=c(2,2,1,2,2),main=paste('Raw Data Manipulation Plot - ',run,' - ',species,' - ',
                element,' - mean conc. (post-birth)',post.birth.mean),ylim=c(0,(1.4*max(smoothed))))

# draw various lines

#               abline('v'=birth.point,col='orange',lwd=3,lty=2)
```

```
		abline('v'=start.point,col='green',lwd=3,lty=2)
		abline('v'=end.point,col='red',lwd=3,lty=2)
#			abline('v'=catch.point,col='gray',lwd=3,lty=2)
#			lines(c(catch.point,end.point),c(catch.mean,catch.mean),col='pink',lwd=3,lty=1)
#			lines(c(birth.point,end.point),c(post.birth.mean,post.birth.mean),col='magenta',lwd=2,lty=2)

 }
 else{

# calculate max x and round up to nearest 20, for plotting
		xmax=ceiling(length(raw.data)/20)*20

		ts.plot(raw.data,outlier,smoothed,line=background,line=0,col=c('red','black','green','blue','black'),
			xlab='Point Number',ylab='Hits Per Second',lty=c(1,1,1,2,1),lwd=c(2,2,1,2,2),
			main=paste('Raw Data Manipulation Plot - ',run,' - ',species,' - ',
			element,' - mean conc. (post-birth)',post.birth.mean),ylim=c(0,(1.4*max(smoothed))),
			xlim=c(0,xmax))

# draw various lines

		abline('v'=birth.point,col='orange',lwd=3,lty=2)
		abline('v'=start.point,col='green',lwd=3,lty=2)
		abline('v'=end.point,col='red',lwd=3,lty=2)
		abline('v'=catch.point,col='gray',lwd=3,lty=2)
		lines(c(catch.point,end.point),c(catch.mean,catch.mean),col='pink',lwd=3,lty=1)
		lines(c(birth.point,end.point),c(post.birth.mean,post.birth.mean),col='magenta',lwd=2,lty=2)

		axis(1,tck=1,lty='dotted',lwd=1,xaxp=c(0,xmax,xmax/20),labels=FALSE,col='black')
		axis(2,tck=1,lty='dotted',lwd=1,col='black')
 }
}

#===============================================================================
#XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
#===============================================================================
#
#
#
#	Define the start and end of a sequence in the time series from the laser ablation
#
#	Inputs
#		- input			- name of run for title (character)
#		- start.hurdle	- threshold to cross for starting the sequence
#		- start.delta	- number of points to shift to right after start.hurdle is crossed
#		- end.hurdle	- threshold to cross to end the sequence
#		- end.delta		- number of points to shift to the left after end.hurdle is crossed
#
#	Output
#		- data frame of start point, end point, and length of sequence
#
#

define.sequence=function(input,start.hurdle,start.delta,end.hurdle,end.delta) {

 for (k in 1:length(input)) {
		if (input[k]>start.hurdle) {
			start.point=k+start.delta
			break
		}
 }

# to find end.point, start counting from back until value is above end.hurdle

 for (l in length(input):1) {
		if (input[l]>end.hurdle) {
			end.point=l-end.delta

# if laser ablation didn't get to end of sample before terminating, the sample is not complete

			if (l==length(input)) end.point=l
			break
		}
 }

 length=end.point-start.point
```

```
result=data.frame(cbind(start.point,end.point,length))}

#==========================================================================
#XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
#==========================================================================
#
#
#
#       Normalise time series
#
#       Inputs
#               - input             - name of run for title (character)
#               - start.hurdle      - threshold to cross for starting the sequence
#               - start.delta       - number of points to shift to right after start.hurdle is crossed
#               - end.hurdle        - threshold to cross to end the sequence
#               - end.delta         - number of points to shift to the left after end.hurdle is crossed
#
#       Output
#               - data frame of start point, end point, and length of sequence
#
ts.norm=function(input) {
 mean=mean(input)
 std.dev=sqrt(var(input))
 results=(input-mean)/std.dev}


#==========================================================================
#XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
#==========================================================================
#
#
#       correction.plot
#
#       Plot correction data for each date and element
#               - this routine assumes access to data from the main "Laser Ablation Method.r"
#
#       Explicit Inputs
#               - date                  - date value of runs (eg. 20080522)
#               - element               - element name for this plot (eg. Mg)
#               - correction.vector     - vector of correction values to be plotted
#
#       Output
#               - plot to current output device
#
#

correction.plot=function(date,element,correction.vector){

# calculate minimum and maximum time for x-axis

 xlim=c(min(correction.factor$run.time[correction.factor$run.day==date&
       correction.factor$element==element],index$run.time[index$run.day==date&
       index$profile!='standard']),max(correction.factor$run.time[correction.factor$run.day==date&
       correction.factor$element==element],index$run.time[index$run.day==date&
       index$profile!='standard']))/60

# calculate maximum drift from max level

 ymax=max(correction.factor$slide.mean.norm.value[correction.factor$run.day==date&
       correction.factor$element==element])

 ymin=min(correction.factor$slide.mean.norm.value[correction.factor$run.day==date&
       correction.factor$element==element])

 max.drift=round(100*(ymax-ymin)/ymax)

# plot correction.factor for each element

 plot(correction.factor$run.time[correction.factor$run.day==date&correction.factor$element==element]/60,
       correction.factor$norm.value[correction.factor$run.day==date&correction.factor$element==element],
       pch=3,cex=1.5,xlim=xlim,xlab='Time in hours (24 hour clock) on day of LA-ICPMS run',col='dark grey',
       ylab='Relative concentration',main=paste('LA-ICPMS Machine Drift for ',element,':Ca  on ',date,
       '\nBased on NIST610 Standard - Max Drift = ',max.drift,'%'))
```

```
  box(lwd=1.5)

  points(correction.factor$run.time[correction.factor$run.day==date&correction.factor$element==element]/60,
        correction.factor$slide.mean.norm.value[correction.factor$run.day==date&
        correction.factor$element==element],pch=21,cex=1.0,col='red')

  points(index$run.time[index$run.day==date&index$profile!='standard']/60,correction.vector,pch=5,cex=.6,
  col='blue')
}




#===============================================================================
#XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
#===============================================================================
#
#
#       same.scan.plot
#
#       Plot same scan repeats for each element on top of each other
#               - this routine assumes access to data from the main "Laser Ablation Method.r"
#               - total of four runs will be plotted
#
#       Explicit Inputs
#               - scan                  - character string for title, identifying which scan/sample in plots
#               - run1                  - number of first run, next runs assumed to be sequential
#
#       Output
#               - plot to current output device
#
#

same.scan.plot.4=function(scan,run1) {

  colours=c('black','red','blue','green')
  legend.value=c('Run 1','Run 2','Run 3','Run 4')

  ts.plot(data[[run1]]$mg24.corrected,data[[run1+1]]$mg24.corrected,data[[run1+2]]$mg24.corrected,data[[run1+3]]$mg24.
  corrected,main=
        paste('Multiple Rep of Same Scan Comparison - ',scan,' - Mg:Ca'),col=colours,xlab='Point Number')
  legend('bottom',legend=legend.value,col=colours,lty=1,lwd=3,horiz=T)

#
        ts.plot(data[[run1]]$p31.corrected,data[[run1+1]]$p31.corrected,data[[run1+2]]$p31.corrected,data[[run1+3]]$p31.c
  orrected,main=
#               paste('Multiple Rep of Same Scan Comparison - ',scan,' - P:Ca'),col=colours,xlab='Point Number')
#       legend('bottom',legend=legend.value,col=colours,lty=1,lwd=3,horiz=T)

  ts.plot(data[[run1]]$mn55.corrected,data[[run1+1]]$mn55.corrected,data[[run1+2]]$mn55.corrected,data[[run1+3]]$mn55.
  corrected,main=
        paste('Multiple Rep of Same Scan Comparison - ',scan,' - Mn:Ca'),col=colours,xlab='Point Number')
  legend('bottom',legend=legend.value,col=colours,lty=1,lwd=3,horiz=T)

  ts.plot(data[[run1]]$cu65.corrected,data[[run1+1]]$cu65.corrected,data[[run1+2]]$cu65.corrected,data[[run1+3]]$cu65.cor
  rected,main=
        paste('Multiple Rep of Same Scan Comparison - ',scan,' - Cu:Ca'),col=colours,xlab='Point Number')
  legend('bottom',legend=legend.value,col=colours,lty=1,lwd=3,horiz=T)

  ts.plot(data[[run1]]$zn66.corrected,data[[run1+1]]$zn66.corrected,data[[run1+2]]$zn66.corrected,data[[run1+3]]$zn66.cor
  rected,main=
        paste('Multiple Rep of Same Scan Comparison - ',scan,' - Zn:Ca'),col=colours,xlab='Point Number')
  legend('bottom',legend=legend.value,col=colours,lty=1,lwd=3,horiz=T)

  ts.plot(data[[run1]]$sr88.corrected,data[[run1+1]]$sr88.corrected,data[[run1+2]]$sr88.corrected,data[[run1+3]]$sr88.corre
  cted,main=
        paste('Multiple Rep of Same Scan Comparison - ',scan,' - Sr:Ca'),col=colours,xlab='Point Number')
  legend('bottom',legend=legend.value,col=colours,lty=1,lwd=3,horiz=T)

  ts.plot(data[[run1]]$ba137.corrected,data[[run1+1]]$ba137.corrected,data[[run1+2]]$ba137.corrected,data[[run1+3]]$ba13
  7.corrected,main=
        paste('Multiple Rep of Same Scan Comparison - ',scan,' - Ba:Ca'),col=colours,xlab='Point Number')
  legend('bottom',legend=legend.value,col=colours,lty=1,lwd=3,horiz=T)
```

```
}
#================================================================================
#XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
#================================================================================
#
#
#	parallel.scan.plot
#
#	Plot same scan repeats for each element on top of each other
#		- this routine assumes access to data from the main "Laser Ablation Method.r"
#		- total of four runs will be plotted
#
#	Explicit Inputs
#		- scan				- character string for title, identifying which scan/sample in plots
#		- run1				- number of first run, next runs assumed to be sequential
#
#	Output
#		- plot to current output device
#
#

parallel.scan.plot=function(run1) {

 colours=c('black','red','blue')
 legend.value=c('Run 1','Run 2','Run 3')

 ts.plot(data[[run1]]$mg24.corrected,data[[run1+1]]$mg24.corrected,data[[run1+2]]$mg24.corrected,main=
	paste('Parallel Scan Across Vertebrae - Mg:Ca'),col=colours,xlab='Point Number')
 legend('bottom',legend=legend.value,col=colours,lty=1,lwd=3,horiz=T)

#	ts.plot(data[[run1]]$p31.corrected,data[[run1+1]]$p31.corrected,data[[run1+2]]$p31.corrected,main=
#		paste('Parallel Scan Across Vertebrae - P:Ca'),col=colours,xlab='Point Number')
#	legend('bottom',legend=legend.value,col=colours,lty=1,lwd=3,horiz=T)

 ts.plot(data[[run1]]$mn55.corrected,data[[run1+1]]$mn55.corrected,data[[run1+2]]$mn55.corrected,main=
	paste('Parallel Scan Across Vertebrae - Mn:Ca'),col=colours,xlab='Point Number')
 legend('bottom',legend=legend.value,col=colours,lty=1,lwd=3,horiz=T)

 ts.plot(data[[run1]]$cu65.corrected,data[[run1+1]]$cu65.corrected,data[[run1+2]]$cu65.corrected,main=
	paste('Parallel Scan Across Vertebrae - Cu:Ca'),col=colours,xlab='Point Number')
 legend('bottom',legend=legend.value,col=colours,lty=1,lwd=3,horiz=T)

 ts.plot(data[[run1]]$zn66.corrected,data[[run1+1]]$zn66.corrected,data[[run1+2]]$zn66.corrected,main=
	paste('Parallel Scan Across Vertebrae - Zn:Ca'),col=colours,xlab='Point Number')
 legend('bottom',legend=legend.value,col=colours,lty=1,lwd=3,horiz=T)

 ts.plot(data[[run1]]$sr88.corrected,data[[run1+1]]$sr88.corrected,data[[run1+2]]$sr88.corrected,main=
	paste('Parallel Scan Across Vertebrae - Sr:Ca'),col=colours,xlab='Point Number')
 legend('bottom',legend=legend.value,col=colours,lty=1,lwd=3,horiz=T)

 ts.plot(data[[run1]]$ba137.corrected,data[[run1+1]]$ba137.corrected,data[[run1+2]]$ba137.corrected,main=
	paste('Parallel Scan Across Vertebrae - Ba:Ca'),col=colours,xlab='Point Number')
 legend('bottom',legend=legend.value,col=colours,lty=1,lwd=3,horiz=T)

}


#================================================================================
#XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
#================================================================================
#
#
#	Function to process raw data from laser ablation
#		Converts from spreadsheet having run number and date/time as header to
#		to insert day of year and run number in column in data frame
#
#	Inputs
#		- input	- input file, raw data from laser ablation software
#
#	Output
#		- output		- data.frame with day of year and run number inserted in first 2 columns
#
#	Requirements / assumptions
#		- format not changed from laser ablation software.
```

```
#       - ensure no scans exist in raw data that are not in the Index.  Unpredictable and bad things happen.
#
#
# define function

la.data.reformat=function(input.file,index) {

# initialise raw.data.mod to begin building it up.  First row will be ignored, so don't worry.

 raw.data.mod=matrix(c(0,0,0,0,0,0,0,0,0,0,0,0,0),ncol=13,dimnames=list(c(''),
        c('unique.id','run.day','run.num','scan','time','mg','p','ca','mn','cu','zn','sr','ba')))

# initialise delete record flag

 delete.record=0

# now loop through to get rid of headers and add a column with run number

 for (j in 1:nrow(raw.data)) {

# check to see if 'Processed Time/Date' is in first entry, this marks beginning of run header

        if ((raw.data[j,1]!='Processed Time/Date')&&(delete.record==0)) {

# stack new row onto matrix

                raw.data.mod=rbind(raw.data.mod,c(unique.id,day.of.year,run.num,raw.data[j,]))

        }
        else {

# if we are here, this means we are in a header,  if 'Processed Time/Date' is first entry, then it is first row of header

                if (raw.data[j,1]=='Processed Time/Date') {

# calculate day of year in 2009 the run was made by converting text based date to POSIX and taking day of year

                        day.of.year=as.numeric(format(strptime(raw.data[j,2],format='%a %b %d %H:%M:%S %Y'),'%j'))

# pick out run.num for the next set of data, it is 1 entry below 'Processed Time/Date' line

                        run.num=raw.data[j+1,1]

# now determine the unique.id from index, day.of.year, and run.num.

                        unique.id=index$unique.id[index$run.day==day.of.year&index$run==run.num]

# set delete.record counter to 2, meaning to skip the next 2 records

                        delete.record=2

# print run number to screen so you can track progress

                        print(paste('Processing Day of Year ',day.of.year,' Run Number ',run.num))

                }
                else {

# if we are here, this means we are in header, but not the first row of it,  simply decrement delete.record counter and go on

                        delete.record=delete.record-1

                }
        }
 }

# create new object eliminating leading row of 0's

 raw.data.mod.final=raw.data.mod[2:nrow(raw.data.mod),]


result=raw.data.mod.final}


#==========================================================================
```

```
#XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
#==============================================================================
#
#
#  This function creates a back to back histogram for comparing Jan and Oct NIST runs
#
#
#  Inputs:
#        month.vec - month vector showing which month runs were made in
#        ca.ratio.flag - flag to know whether numbers are raw or in ratio to Ca
#        data.vec - vector of either raw data or calcium ratio data
#                              element - character of which element is represented in data.vec above
#
#  Outputs:
#        back to back histogram comparing Jan and Oct runs
#
#  Requirements for use:
#        -
#
#
#  This function was created because I didn't like default "histbackback()" function:
#
#

jan.oct.hist=function(month.vec,ca.ratio.flag,data.vec,element){

# determine break points based on the whole dataset (both Jan and Oct data)

 breaks=hist(data.vec,breaks=8,plot=FALSE)$breaks

# create left and right side of histograms, based on break points for overall data set

 h.jan=hist(data.vec[month.vec==1],plot=FALSE,breaks=breaks)
 h.oct=hist(data.vec[month.vec==10],plot=FALSE,breaks=breaks)

# determine limits of histogram to ensure symmetry on x-axis

 xlim=1.1*c(-max(h.jan$counts,h.oct$counts),max(h.jan$counts,h.oct$counts))

# define y.label and main.label titles

 if (ca.ratio.flag==TRUE) {
        y.label=paste(element,' ratio to Ca',sep='')
        main.label=paste('NIST610 Comparison Between Jan and Oct Runs - ',element,' Ratio to Ca',sep='')
 }else{
        y.label=paste(element,' (counts per second)',sep='')
        main.label=paste('NIST610 Comparison Between Jan and Oct Runs - ',element,' Raw Count Data',sep='')
 }

# draw main barplot with titles and x axis labels - left side only

        barplot(-h.jan$counts,col='red',horiz=TRUE,space=0,add=FALSE,axes=TRUE,
                xlab='Jan            Oct',ylab=y.label,xlim=xlim,
                main=main.label)

# add right side of barplot

        barplot(h.oct$counts,col='blue',horiz=TRUE,space=0,add=TRUE,axes=FALSE)

# create labels for y axis

        y.axis.labels=as.character(breaks)

# create y axis manually

        axis(2,at=seq(1,length(breaks),by=1)-1,labels=y.axis.labels)
}
#==============================================================================
#XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
#==============================================================================
#
#
#  This function creates a ratio plot of standard v sample comparing Jan and Oct NIST runs
#
#
```

```
#  Inputs:
#        means.data - tapply table separating raw data of standard v sample v month
#                                    which is data.frame with $edge.10, $edge.1, $standard.10 and $standard.1
#                                                representing profile.month
#                           element - element character string being plotted
#
#
#  Outputs:
#        plot of standard v sample raw data with separate markers for jan and oct runs
#
#  Requirements for use:
#        -
#
#

plot.abl.yield=function(means.data,element){

# first calculate min and max values for x and y plots

 xlim=c(0,max(means.data$edge.10,means.data$edge.1))
 ylim=c(0,max(means.data$standard.10,means.data$standard.1))

# create main plot, using october runs

 plot(means.data$edge.10,means.data$standard.10,pch='o',col='blue',
          main=paste('Raw Data - NIST610 v Vertebra Sample - ',element,sep=''),
          ylab=paste('NIST610 Standard - ',element,' (counts per second)',sep=''),
          xlab=paste('Vertebrae Sample - ',element,' (counts per second)',sep=''))

 abline(lm(means.data$standard.10~means.data$edge.10),col='blue')

 points(means.data$edge.1,means.data$standard.1,pch='+',col='red')

 abline(lm(means.data$standard.1~means.data$edge.1),col='red')


 legend(x='topleft',c('January Runs','October Runs'),pch=c('+','o'),col=c('red','blue'))


}
#================================================================================
#XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
#================================================================================
#
#
#  This function creates an 'element.x' v 'element.y' plot comparing Jan and Oct NIST runs
#
#
#  Inputs:
#         vector.x.jan - vector of element.x values for jan
#                           vector.y.jan - vector of element.y values for jan
#         vector.x.oct - vector of element.x values for oct
#                           vector.y.oct - vector of element.y values for oct
#                           element.x - character string of element for x axis
#                           element.y - character string of element for y axis
#                           type - character string used in main header prefix
#
#
#  Outputs:
#        plot of element.y v element.x raw data with separate markers for jan and oct runs
#
#  Requirements for use:
#        -
#
#

raw.element.plot=function(vector.x.jan,vector.y.jan,vector.x.oct,vector.y.oct,element.x,element.y,type){

# first calculate min and max values for x and y plots

 xlim=c(0,max(vector.x.jan,vector.x.oct))
 ylim=c(0,max(vector.y.jan,vector.y.oct))

# create main plot, using october runs
```

```
  plot(vector.x.oct,vector.y.oct,pch='o',col='blue',
          main=paste(type,' Raw Data - ',element.y,' v ',element.x,sep=''),
          ylab=paste(element.y,' (counts per second)',sep=''),
          xlab=paste(element.x,' (counts per second)',sep=''))

  abline(lm(vector.y.oct~vector.x.oct),col='blue')

  points(vector.x.jan,vector.y.jan,pch='+',col='red')

  abline(lm(vector.y.jan~vector.x.jan),col='red')


  legend(x='topleft',c('January Runs','October Runs'),pch=c('+','o'),col=c('red','blue'))


}
```

7.4 Script for Daily Consistency Checks for LA-ICPMB

```
#  Analysis for Laser Ablation ICP-MS

# notify user that processing has started

  cat('\nCommenced Processing\n')

# clear everything out of memory before starting

rm(list=ls())

# for MAC at home
file.location=('/Users/Ronbeau/JCU/R Statistics/Laser Ablation 5/')

# open and read sample information
#index=read.csv(paste(file.location,'Sample Index.csv',sep=''))

# open and read input parameters and logicals
input.matrix=read.csv(paste(file.location,'Input Parameters.csv',sep=''))
input.logical=read.csv(paste(file.location,'Input Logicals.csv',sep=''))

# request input to vary input parameters

# input.matrix=edit(input.matrix)  temporarily blocked out ability to change defaults
# input.logical=edit(input.logical)

#  call up functions that have been written externally
source(paste(file.location,'ronbo_function_definitions.R',sep=''))

library(MASS)
library(Hmisc)

# suppress warnings printed by R
options(warn=-1)

# manipulate input parameters to input$ data.frame format
input=data.frame(t(input.matrix$parameter.value))
names(input)=input.matrix$parameter.input.variable

# manipulate input logicals to input.log$ data.frame format
input.log=data.frame(t(input.logical$value.t.f))
names(input.log)=input.logical$logical.input.variable

# define graphics output as screen (MAC quartz device) or pdf

if (input.log$pdf.graphics.output==FALSE) {
  quartz()
  ask=TRUE
}
if (input.log$pdf.graphics.output==TRUE) {
  pdf(paste(file.location,'Graphics Output.pdf',sep=''),width=12,height=6,
          onefile=T,family='Helvetica',title='Laser Ablation Data Analysis')
```

```
  ask=FALSE
}

# initiate vector of isotopes we're working with

isotope=c('Mg','P','Ca','Mn','Cu','Zn','Sr','Ba')
isotope.no.ca=c('Mg','P','Mn','Cu','Zn','Sr','Ba')

# run format pre-processor to convert raw data from laser ablation lab to data.frame

if (input.log$pre.process.format==TRUE) {

# notify user that processing has started

  cat('\nCommenced Processing\n')

# notify user that data shuffling has started

  cat('\nShuffling Raw Data\n')


# read in raw data file for the next day

  raw.data=as.matrix(read.csv(paste(file.location,
          'April Raw Data/SharkVertebrae20-04-10.csv',  ##################################################
          sep=''),header=FALSE,col.names=c('scan','time','mg','p','ca','mn','cu','zn','sr','ba')))

# call up reformatting function to add day and run number columns to the matrix

  reshuffled.raw.data=la.data.reformat(raw.data)

# save object for future work, without having to run pre-processing

  save(reshuffled.raw.data,file=paste(file.location,
          'April Raw Data/File_10.rda',sep=''),ascii=F)   ##############################################

# stop processing from here

#         stop('Done Shuffling Data')

}



##############################################################################################
#       PRE-PROCESSING FOR FORMAT MANIPULATION FINISHES HERE
##############################################################################################

# pre-processing of format must have already been run and saved previously
debug=1
if (input.log$pre.process.ts==TRUE) {

# print notice of loading shuffled data

  cat(paste('\nLoading Previously Shuffled Data\n'))

# load in first data file

  load(file=paste(file.location,
                  'April Raw Data/File_1.rda',sep=''))

# don't ask me why, but all columns are "factors"!!!, we now need to convert to numeric

  reshuffled.raw.data=data.frame(matrix(as.numeric(reshuffled.raw.data),byrow=F,ncol=ncol(reshuffled.raw.data),
          dimnames=dimnames(reshuffled.raw.data)))

# for testing purposes, ensure that raw.data.formatted$run.num is unique by adding multiple of 1000 to each subsequent
  file

  reshuffled.raw.data$run.num=reshuffled.raw.data$run.num+1000

# create new master table called raw.data.formatted (sorry about the names so close to reshuffled.raw.data)

  raw.data.formatted=reshuffled.raw.data
```

```
debug=2
# now loop through for the rest of the data files

 for (i in 2:10){      ##########################################################################################

        load(file=paste(file.location,
                'April Raw Data/File_',i,'.rda',sep=''))

# don't ask me why, but all columns are "factors"!!!, we now need to convert to numeric

        reshuffled.raw.data=data.frame(matrix(as.numeric(reshuffled.raw.data),byrow=F,ncol=ncol(reshuffled.raw.data),
                dimnames=dimnames(reshuffled.raw.data)))

# for testing purposes, ensure that raw.data.formatted$run.num is unique by adding multiple of 1000 to each subsequent
 file

        reshuffled.raw.data$run.num=reshuffled.raw.data$run.num+1000*i

# since the 2nd day was broken by the machine failing, separate plots accordingly

        if (i==2) reshuffled.raw.data$run.day=98.1
        if (i==3) reshuffled.raw.data$run.day=98.2
        if (i==4) reshuffled.raw.data$run.day=98.3

# append to existing raw.data.formatted

        raw.data.formatted=rbind(raw.data.formatted,reshuffled.raw.data)

 }
debug=3


# don't ask me why, but all columns are "factors"!!!, we now need to convert to numeric

#       raw.data.formatted=data.frame(matrix(as.numeric(raw.data.formatted),byrow=F,ncol=ncol(raw.data.formatted),
#               dimnames=dimnames(raw.data.formatted)))

}


debug=5
#
# print notice of summarising data
 cat(paste('\nSummarising Data\n'))



# now summarise data to take mean of largest values [10:50] of each element
# this should eliminate outliers and all of the non-signal values

# first create summary data.frame

sum.dat=data.frame(matrix(0,ncol=10,nrow=length(unique(raw.data.formatted$run.num))))

names(sum.dat)=c('run.day','run.num','mg','p','ca','mn','cu','zn','sr','ba')

# draw out data for run.day and run.num (there are probably easier ways to do this, but this method ensure
# it comes out in the same order as the elemental tapply's)

sum.dat$run.day=tapply(raw.data.formatted$run.day,raw.data.formatted$run.num,mean)
sum.dat$run.num=tapply(raw.data.formatted$run.num,raw.data.formatted$run.num,mean)

# now draw out data for each element

sum.dat$mg=tapply(raw.data.formatted$mg,raw.data.formatted$run.num,function(sm)
 mean(sort(sm,decreasing=T)[10:50]))
sum.dat$p=tapply(raw.data.formatted$p,raw.data.formatted$run.num,function(sm) mean(sort(sm,decreasing=T)[10:50]))
sum.dat$ca=tapply(raw.data.formatted$ca,raw.data.formatted$run.num,function(sm)
 mean(sort(sm,decreasing=T)[10:50]))
sum.dat$mn=tapply(raw.data.formatted$mn,raw.data.formatted$run.num,function(sm)
 mean(sort(sm,decreasing=T)[10:50]))
sum.dat$cu=tapply(raw.data.formatted$cu,raw.data.formatted$run.num,function(sm)
 mean(sort(sm,decreasing=T)[10:50]))
```

```
sum.dat$zn=tapply(raw.data.formatted$zn,raw.data.formatted$run.num,function(sm)
  mean(sort(sm,decreasing=T)[10:50]))
sum.dat$sr=tapply(raw.data.formatted$sr,raw.data.formatted$run.num,function(sm) mean(sort(sm,decreasing=T)[10:50]))
sum.dat$ba=tapply(raw.data.formatted$ba,raw.data.formatted$run.num,function(sm)
  mean(sort(sm,decreasing=T)[10:50]))


# print notice of plotting

  cat(paste('\nCreating Plots\n'))



# now create plots of each element versus Ca, using different colour for each run.day
# combine samples and NIST, but it should be easily identifiable

daily.check.plot(sum.dat$ca,sum.dat$mg,sum.dat$run.day,'Mg')
daily.check.plot(sum.dat$ca,sum.dat$p,sum.dat$run.day,'P')
daily.check.plot(sum.dat$ca,sum.dat$mn,sum.dat$run.day,'Mn')
daily.check.plot(sum.dat$ca,sum.dat$cu,sum.dat$run.day,'Cu')
daily.check.plot(sum.dat$ca,sum.dat$zn,sum.dat$run.day,'Zn')
daily.check.plot(sum.dat$ca,sum.dat$sr,sum.dat$run.day,'Sr')
daily.check.plot(sum.dat$ca,sum.dat$ba,sum.dat$run.day,'Ba')


# tidy things up after running

if (input.log$pdf.graphics.output==TRUE) {
  dev.off()
}

# re-enable warnings to screen
options(warn=1)



stop('FINISHED PROCESSING')
```

7.5 Solution Based ICPMS – Method Development

```
#
# This R script runs a series of statistical analyses related to
# the shark vertebrae solultion based microchemistry results executed
# by Ron Schroeder through the JCU AAC.  It requires a data file and
# a functions definition file as identified below.
#
# It pops up an edit window, which can be simply closed to accept the
# defaults, or edited to change the run parameters.
#
# quartz device (MAC) should be set to 2:1 aspect ratio (eg. 12" wide x 6" high)
#

#  call up functions that have been written externally
source("/Users/Ronbeau/JCU/R Statistics/2008 Minor Project/ronbo_function_definitions.R")
library(MASS)

# open data file
fulltable=read.csv("/Users/Ronbeau/JCU/R Statistics/2008 Minor Project/200803 ICPMS
  Results.csv",header=TRUE,nrows=69,blank.lines.skip=T)

# write text output to file
sink("/Users/Ronbeau/JCU/R Statistics/2008 Minor Project/200803 ICPMS Analysis Output.txt")

# create header in output file
cat('March 2008 Minor Project Data Analysis\n')
cat('\nPrint Date:  ',date(),'\n\n\n')


#  Collect input to be used in this analysis.  Except for column 3, we
#  are only concerned with the first cell of each column for the input.
```

```
#
#  Column 1 - first cell of first column determines what is to be tested
#  Change the cell to change what analyses to run
#
#     a - all analyses  (default)
#     m - only main geographic location analysis (both species)
#     s - only spine location anlaysis
#     d - only duplicates based analysis
#
#  Column 2 used for labels to identify parameter for first input
#
#  Column 3 determines which elements will be used (ratio to Ca)
#     Set unwanted elements to '' (blank).
#     DO NOT just delete the row, or it will not be read correctly.
#
#  Column 4 is to set "self.label" to TRUE or FALSE
#     This allows vector biplots to be labelled interactively
#     This would be used for final presentation quality plots, and you
#     must know ahead of time which vector is for which response element.
#
#  Column 5 - first element defines graphics output to 'screen' or 'pdf'
#
#  Column 6 - logical to define whether assumptions tests are performed
#


# set default input parameters

analyse=c('a',' ',' ','a','m','s','d')
choice.of.analyses=c(' ',' ',' ','all','main','spine','duplicates')
element=c('Ba','Cu','Mg','Mn','P','Sr','Zn')
self.label=as.logical(c(FALSE,' ',' ',' ',' ',TRUE,FALSE))
graphics.dev=c('screen',' ',' ',' ',' ',' ','screen','pdf')
assumptions.test=as.logical(c(FALSE,' ',' ',' ',' ',TRUE,FALSE))
input=data.frame(cbind(analyse,choice.of.analyses,element,self.label,graphics.dev,assumptions.test))

# request input to vary input parameters

input=edit(input)

# write input parameters to output file

cat('================================================================\n\n\n')
cat('Input Parameters Matrix\n\n')
print(input)

# define graphics outputs as screen (MAC quartz device) or pdf

if (input$graphics.dev[1]=='screen') quartz()
if (input$graphics.dev[1]=='pdf') pdf('/Users/Ronbeau/JCU/R Statistics/2008 Minor Project/200803 ICPMS Analysis
  Output.pdf',width=12,height=6,onefile=TRUE,family='Helvetica',title='March 2008 Minor Project Data Anlysis')

# little bit of error trapping here, just for some fun

if ((input$self.label[1]==TRUE)&&(input$graphics.dev[1]=='pdf')) {
  quartz()
  plot(c(-1,1),c(-1,1),main='YOU DUMB ARSE')
  text(0,.3,'You can not set self.label to true and plot to a pdf file')
  text(0,0,'self.label allows you to interctively mark where vectors labels go')
  text(0,-.3,'graphics.dev will be set to screen mode')
  input$graphics.dev[1]='screen'}

# define logical for future par(...ask) statements, to avoid waiting for 'return' when graphics going to pdf file

if (input$graphics.dev[1]=='pdf') ask='F' else ask='T'

# eliminate outliers as appropriate

fulltable=subset(fulltable,rep(run_num!="150640")) #very small shark with insufficient amount for full digestion
fulltable=subset(fulltable,rep(run_num!="211066")) #Ba level was extraordinarily high

# set up various vectors to be used from the full table

species=fulltable$species # SHH or ABT
location=fulltable$location # PCB or BGB
```

```
fl=fulltable$fl # fork length in mm
sex=fulltable$sex # M or F
samp_wt=fulltable$samp_wt # weighted digested in acid (g)
fish.num=fulltable$fish_num # unique fish number, often (but not always )same as tag number
spine_loc=fulltable$spine_loc # n, d1, or d2
tag=fulltable$tag # tag number (different from fish.num for spine location study)
run_num=fulltable$run_num # unique identifier for each sample sent through the ICPMS
study=fulltable$study # main (geographic loc.), loc_spine (spine loc.), duplicate (prep type)
dup_study=fulltable$dup_study # TRUE if we have multiple icpms runs from this particular fish
method=fulltable$method # orig, whole, powder, store, split, alt diss (see external notes)
ba=fulltable$ba # raw element concentrations from icpms
ca=fulltable$ca
cu=fulltable$cu
mg=fulltable$mg
mn=fulltable$mn
p=fulltable$p
sr=fulltable$sr
zn=fulltable$zn

# create ratio columns to calcium in vector
# BE CAREFUL - "Ba" - ratio to calcium, where "ba" is raw figure (note case sensitivity)

Ba=fulltable$ba/fulltable$ca
Cu=fulltable$cu/fulltable$ca
Mg=fulltable$mg/fulltable$ca
Mn=fulltable$mn/fulltable$ca
P=fulltable$p/fulltable$ca
Sr=fulltable$sr/fulltable$ca
Zn=fulltable$zn/fulltable$ca

# define response matrix with all elements but omitting elements as per input matrix

elem.temp=cbind(Ba,Cu,Mg,Mn,P,Sr,Zn)[,na.omit(input$element)]

# shorten location names for use as markers in pca scores plot
# there are cleaner ways to do this, but this is working and I can't be bothered changing

location=gsub("BGB","B",location)
location=gsub("PCB","P",location)
elem_response=matrix(elem.temp,nrow(elem.temp),ncol(elem.temp),dimnames=list(c(as.character(location)),c(dimnames
  (elem.temp)[[2]])))


#===============================================================================
#XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
#===============================================================================
#
# MAIN ANALYSIS LOOKING AT TWO GEOGRAPHIC LOCATIONS AND TWO SPECIES (ABT & SHH)
#
# first is principal components analysis of main study looking at two locations
#

# only run this section if 'a' all, or 'm' main has been selected in input

if (input$analyse[1]=='a' || input$analyse[1]=='m'){

#run analysis, correlations - ABT

pca.abt=princomp(elem_response,cor=T,scores=T,subset=rep(study:species=="main:ABT"))

# set screen for biplots to be 2 plots areas wide

par(mfrow=c(1,2),new=F,ask=ask)

# define markers for scores plots

marker.abt=as.character(subset(location,species=="ABT"))

#create biplots for ABT using Ronbo's special biplot routine

ronbo.pca.biplot(pca=pca.abt,markers=marker.abt,title.loadings="ABT Main Study Loadings",title.scores="ABT Main Study
  Scores",input$self.label[1])

# print summary pca data to text file
```

```
cat('=================================================================\n')
cat('XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX\n')
cat('=================================================================\n\n\n')
cat('ABT PCA Summary\n\n')
print(summary(pca.abt))

# prep data for MANOVA (can not use "subset" in manova() command, so vectors must be created)

elem_response.abt=subset(elem_response,species:study=="ABT:main")

location.abt=as.factor(subset(location,species:study=="ABT:main"))

# run manova fit, test assumptions, and output text and graphics data

fit.abt=ronbo.manova(elem_response.abt,location.abt,title.data='ABT Main Study Data',title.resid='ABT Main Study
 Residuals',assumption=input$assumptions.test,ask)

# Perform DFA Analysis for ABT using Ronbo's special dfa function

cat('\n\n=================================================================\n')
cat('ABT Discriminant Function Analysis\n\n')

lda.abt=ronbo.lda(elem_response.abt,location.abt,study.name='ABT Main Study')

#-----------------------------------------------------------------------------
# now do the same analyses for SHH

#run analysis, correlations - SHH

pca.shh=princomp(elem_response,cor=T,scores=T,subset=rep(study:species=="main:SHH"))

# set screen for biplots to be 2 plots areas wide

par(mfrow=c(1,2),new=F,ask=ask)

# define markers for scores plots

marker.shh=as.character(subset(location,species=="SHH"))

#create biplots for SHH

ronbo.pca.biplot(pca=pca.shh,markers=marker.shh,title.loadings="SHH Main Study Loadings",title.scores="SHH Main
 Study Scores",input$self.label[1])

# print summary pca data to text file

cat('=================================================================\n')
cat('XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX\n')
cat('=================================================================\n\n\n')
cat('SHH PCA Summary\n\n')
print(summary(pca.shh))

# prep data for MANOVA (can not use "subset" in manova(), so vectors must be created)

elem_response.shh=subset(elem_response,species:study=="SHH:main")

location.shh=as.factor(subset(location,species:study=="SHH:main"))

# run manova fit, test assumptions, and output text and graphics data

fit.shh=ronbo.manova(elem_response.shh,location.shh,title.data='SHH Main Study Data',title.resid='SHH Main Study
 Residuals',assumption=input$assumptions.test,ask)

# Perform DFA Analysis for SHH
cat('\n\n=================================================================\n\n\n')
cat('SHH Discriminant Function Analysis\n\n')

lda.shh=ronbo.lda(elem_response.shh,location.shh,study.name='SHH Main Study')
}

#===============================================================================
#XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
#===============================================================================
#
# ANALYSIS BASED ON THREE SPINE LOCATIONS (N, D1, D2) FROM 5 SHARKS (ALL ABT PCB)
```

```
#
#
#
#  start with pca and related biplots
#
#

# only run this section if 'a' all, or 's' spine has been selected in input

if (input$analyse[1]=='a' || input$analyse[1]=='s'){

# print header information in file

cat('=================================================================\n')
cat('XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX\n')
cat('=================================================================\n\n\n')
cat('Spine Location Analysis (neck, D1, D2)\n\n')

# transform Mn data by ^.2 for this analysis

elem_response.trnsf=subset(elem_response,study=='loc_spine')
elem_response.trnsf[,4]=elem_response.trnsf[,4]^.2

#pca.spine=princomp(elem_response,cor=T,scores=T,subset=rep(study=="loc_spine"))
pca.spine=princomp(elem_response.trnsf,cor=T,scores=T)

# create markers for scores biplot

marker.spine=as.character(subset(fish.num,study=="loc_spine"))

# create pca biplot for spine location analysis

par(mfrow=c(1,2),new=F,ask=ask)
ronbo.pca.biplot(pca=pca.spine,markers=marker.spine,title.loadings="Spine Location Analysis -
  Loadings",title.scores="Spine Location Analysis - Scores",input$self.label[1])

#-----------------------------------------------------------------------------

# Prep MANOVA data (can not use "subset" in manova(), so vectors must be created)

#elem_response.spine=subset(elem_response,study=="loc_spine")
elem_response.spine=elem_response.trnsf

# confusing part here where "spine_loc" is column name and "loc_spine" is entry in "study" column
# location.spine = n, d1, d2
# fish.num.spine = 1,2,3,4,5

location.spine=as.factor(subset(spine_loc,study=="loc_spine"))
fish.num.spine=as.factor(subset(fish.num,study=='loc_spine'))

# perform two factor manova based on spine location (n,d1,d2) and fish number (1,2,3...)
# not enough degrees of freedom to do interaction, so only look at primary effects

#fit.spine=manova(elem_response.spine~fish.num.spine+location.spine) this line commented out to eliminate fish.num
fit.spine=manova(elem_response.spine~location.spine)

# print outputs to file

cat('\n\n=================================================================\n\n\n')
cat('MANOVA Table - Factors: spine location \n\n')
print(summary(fit.spine),test='Pillai')

# print summary ANOVA's to file - one for each response variable

cat('\n\n=================================================================\n\n\n')
cat('Summary ANOVA Tables - Factors: spine location \n\n')
print(summary.aov(fit.spine))

# test if assumptions flag is set to TRUE.  If not, don't run tests

if (input$assumptions.test[1]==TRUE){

# various plots for MANOVA assumptions testing

resfit=residuals(fit.spine)
```

```
# normality - histograms

par(mfrow=c(2,4),new=F,ask=ask)
for (i in 1:length(elem_response.spine[1,]))
 hist(elem_response.spine[,i],xlab=dimnames(elem_response.spine)[[2]][i],main='Spine Location Data Histogram')
par(mfrow=c(2,4),new=F,ask=ask)
for (i in 1:length(elem_response.spine[1,]))
 boxplot(elem_response.spine[,i],xlab=dimnames(elem_response.spine)[[2]][i],main='Spine Location Data Histogram')

# residuals histograms and q-q plots

par(mfrow=c(2,4),new=F,ask=ask)
for (i in 1:length(resfit[1,])) hist(resfit[,i],xlab=dimnames(resfit)[[2]][i],main='Spine Location Residuals')
par(mfrow=c(2,4),new=F,ask=ask)
for (i in 1:length(resfit[1,])) {
 qqnorm(resfit[,i],xlab=dimnames(resfit)[[2]][i],main='Spine Location Residuals')
 qqline(resfit[,i])}
}


# Perform DFA Analysis for spine location, factor = spine location (n,d1,d2)

cat('\n\n=====================================================================\n\n\n')
cat('Spine Location Discriminant Function Analysis - Factor = Location\n\n')


lda.spine.location=ronbo.lda(elem_response.spine,location.spine,study.name='Spine Location Study - Spine Location
 DFA')


# perform DFA analysis for spine location, factor = fish number

cat('\n\n=====================================================================\n\n\n')
cat('Spine Location Discriminant Function Analysis - Factor = fish.num\n\n')

lda.spine.fish.num=ronbo.lda(elem_response.spine,fish.num.spine,study.name='Spine Location Study - Fish Number
 DFA')
}

#==================================================================================
#XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
#==================================================================================
#
# ONE WAY MANOVA COMPARING VARIATION FROM VARIOUS STAGES OF
# PREPARATION AND ICPMS PROCESSING
#
#
#
#  start with pca and related biplots
#
# first create prep.type vector for subsetting

# only run this section if 'a' all, or 'd' duplicates has been selected in input

if (input$analyse[1]=='a' || input$analyse[1]=='d'){

# print header information in file

cat('====================================================================\n')
cat('XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX\n')
cat('====================================================================\n\n\n')
cat('Duplication Analysis Across Five Individuals\n\n')

# recategorise method variable to 'physical', 'chemical', or 'original'

prep.type=method
prep.type=gsub('whole','physical',prep.type)
prep.type=gsub('powder','physical',prep.type)
prep.type=gsub('split','physical',prep.type)
prep.type=gsub('store','chemical',prep.type)
prep.type=gsub('alt diss','chemical',prep.type)
prep.type=gsub('orig','original',prep.type)

#run analysis, correlations
```

```
pca.prep=princomp(elem_response,cor=T,scores=T,subset=rep(dup_study=="TRUE"))

# set screen for biplots to be 2 plots areas wide

par(mfrow=c(1,2),new=F,ask=ask)

# define markers for scores plots

marker.prep=as.character(subset(tag,dup_study=="TRUE"))
marker.prep=gsub('211060','A',marker.prep)
marker.prep=gsub('211078','B',marker.prep)
marker.prep=gsub('234645','C',marker.prep)
marker.prep=gsub('234647','D',marker.prep)
marker.prep=gsub('4n','E',marker.prep)
marker.prep=gsub('4d2','E',marker.prep)
marker.prep=gsub('4d1','E',marker.prep)

#create biplots for both species

ronbo.pca.biplot(pca=pca.prep,markers=marker.prep,title.loadings="Preparation Type Study
  Loadings",title.scores="Preparation Type Study Scores",input$self.label[1])

# Perform MANOVA (can not use "subset" in manova(), so vectors must be created)

elem_response.prep=subset(elem_response,dup_study=="TRUE")

prep.type=as.factor(subset(prep.type,dup_study=="TRUE"))
prep.fish.num=as.factor(subset(fish.num,dup_study=="TRUE"))

#fit.prep=manova(elem_response.prep~prep.fish.num+prep.type+prep.fish.num:prep.type)
fit.prep=manova(elem_response.prep~prep.fish.num*prep.type)

sum.prep=summary(fit.prep,test="Pillai")

# now do a manova for just fish.num, without prep.type

fit.repeated.samples=manova(elem_response.prep~prep.fish.num)

sum.repeated.samples=summary(fit.repeated.samples,test='Pillai')


# print outputs to file
cat('================================================================\n\n\n')
cat('Variation Based on Repeated Samples (fish.num only) - MANOVA Table\n\n')
print(sum.repeated.samples)

# print summary anova outputs to file
cat('================================================================\n\n\n')
cat('Variation Based on Repeated Samples (fish.num only) - Summary ANOVA Tables\n\n')
print(summary.aov(fit.repeated.samples))

# print outputs to file
cat('================================================================\n\n\n')
cat('Variation Based on Preparation Type - MANOVA Table\n\n')
print(sum.prep)

# print summary anova outputs to file
cat('================================================================\n\n\n')
cat('Variation Based on Preparation Type - Summary ANOVA Tables\n\n')
print(summary.aov(fit.prep))

# test if assumptions flag is set to TRUE.  If not, don't run tests

if (input$assumptions.test[1]==TRUE){

# various plots for MANOVA assumptions testing

resfit=residuals(fit.repeated.samples)

# normality - histograms

par(mfrow=c(2,4),new=F,ask=ask)
for (i in 1:length(elem_response.prep[1,]))
  hist(elem_response.prep[,i],xlab=dimnames(elem_response.prep)[[2]][i],main='Replicate Samples Data Histogram')
```

```
par(mfrow=c(2,4),new=F,ask=ask)
for (i in 1:length(elem_response.prep[1,]))
  boxplot(elem_response.prep[,i],xlab=dimnames(elem_response.prep)[[2]][i],main='Replicate Samples Data Histogram')

# residuals histograms and q-q plots

par(mfrow=c(2,4),new=F,ask=ask)
for (i in 1:length(resfit[1,])) hist(resfit[,i],xlab=dimnames(resfit)[[2]][i],main='Replicate Samples Residuals')
par(mfrow=c(2,4),new=F,ask=ask)
for (i in 1:length(resfit[1,])) {
  qqnorm(resfit[,i],xlab=dimnames(resfit)[[2]][i],main='Replicate Samples Residuals')
  qqline(resfit[,i])}
}


# Perform DFA Analysis for Prep Type
cat('\n\n=================================================================\n\n\n')
cat('Prep Type Discriminant Function Analysis - Grouping = Prep type\n\n')

lda.prep=ronbo.lda(elem_response.prep,prep.type,study.name='Prep Type Variability Study - Grouping = prep.type')

# perform DFA for fish number

cat('\n\n=================================================================\n\n\n')
cat('Prep Type Discriminant Function Analysis - Grouping = Fish number\n\n')

lda.prep=ronbo.lda(elem_response.prep,prep.fish.num,study.name='Prep Type Variability Study - Grouping = Fish
  Number')

}

if (input$graphics.dev[1]=='pdf') dev.off()
#graphics.off()
sink()
```

7.6 Sample Index File

unique.id	run	file.name	primary	species	drift.group	bl.yield.group	ocation	fl	sex	fish.num	slide	run.date	un.month	run.day	run.hour	un.minute	run.time	abs.time	profile	outer.radius	entral.width	seq.start	seq.end
1	101	harkVertebrae07-04-10.csv	F	NIST610	1	1					na	20100407	4	97	10	15	615	10695	standard			167	266
2	102	harkVertebrae07-04-10.csv	F	NIST610	1	1					na	20100407	4	97	10	21	621	10701	standard			188	293
3	103	harkVertebrae07-04-10.csv	T	SHH	0	1	3	730	m	216083	SHH N	20100407	4	97	10	35	635	10715	edge	4.8	4.9	200	479
4	104	harkVertebrae07-04-10.csv	T	SHH	0	1	2	445	f	283666	SHH N	20100407	4	97	10	48	648	10728	edge	3	3.6	176	335
5	105	harkVertebrae07-04-10.csv	T	SHH	0	0	3	850	m	150620	SHH N	20100407	4	97	11	4	664	10744	edge	5.2	6.2	192	497
6	106	harkVertebrae07-04-10.csv	T	SHH	0	0	2	420	m	210911	SHH N	20100407	4	97	11	15	675	10755	edge	2.9	2.7	148	318
7	107	harkVertebrae07-04-10.csv	T	SHH	0	2	4	900	m	296676	SHH N	20100407	4	97	11	41	701	10781	edge	6.3	6.3	108	471
8	108	harkVertebrae07-04-10.csv	F	NIST610	2	2					na	20100407	4	97	11	47	707	10787	standard			122	231
9	109	harkVertebrae07-04-10.csv	F	NIST610	2	2					na	20100407	4	97	11	52	712	10792	standard			106	221
10	110	harkVertebrae07-04-10.csv	T	SHH	0	2	2	460	m	210934	SHH N	20100407	4	97	12	20	740	10820	edge	3.4	3.2	231	392
11	111	harkVertebrae07-04-10.csv	T	SHH	0	0	2	502	f	241982	SHH N	20100407	4	97	12	29	749	10829	edge	3.5	3.5	97	296
12	112	harkVertebrae07-04-10.csv	T	SHH	0	3	2	465	m	210832	SHH N	20100407	4	97	12	36	756	10836	edge	3.2	3.2	80	201
13	113	harkVertebrae07-04-10.csv	F	NIST610	3	3					na	20100407	4	97	13	35	815	10895	standard			156	267
14	114	harkVertebrae07-04-10.csv	F	NIST610	3	3					na	20100407	4	97	13	41	821	10901	standard			70	183
15	115	harkVertebrae07-04-10.csv	T	SHH	0	3	2	425	m	210935	SHH N	20100407	4	97	13	49	829	10909	edge	2.8	2.8	149	281
16	116	harkVertebrae07-04-10.csv	T	SHH	0	0	2	465	f	242063	SHH N	20100407	4	97	13	59	839	10919	edge	2.8	2.8	132	270
17	117	harkVertebrae07-04-10.csv	T	SHH	0	0	4	###	m	296660	SHH I	20100407	4	97	14	30	870	10950	edge	6.5	6.6	84	460
18	118	harkVertebrae07-04-10.csv	T	SHH	0	0	2	470	f	242005	SHH I	20100407	4	97	14	38	878	10958	edge	3.2	3.2	79	242
19	119	harkVertebrae07-04-10.csv	T	SHH	0	0	2	410	f	146583	SHH I	20100407	4	97	14	45	885	10965	edge	2.4	2.5	81	195
20	120	harkVertebrae07-04-10.csv	T	SHH	0	4	3	665	m	216027	SHH I	20100407	4	97	14	53	893	10973	edge	4.6	4.6	64	310
21	121	harkVertebrae07-04-10.csv	F	NIST610	4	4					na	20100407	4	97	14	57	897	10977	standard			90	201
22	122	harkVertebrae07-04-10.csv	F	NIST610	4	4					na	20100407	4	97	15	1	901	10981	standard			83	196
23	123	harkVertebrae07-04-10.csv	F	SHH	0	4	2	465	m	242039	SHH I	20100407	4	97	15	19	919	10999	edge	3.2	3.4	85	225
24	124	harkVertebrae07-04-10.csv	T	SHH	0	0	3	685	m	146568	SHH I	20100407	4	97	15	26	926	11006	edge	4.6	4.6	119	375
25	125	harkVertebrae07-04-10.csv	T	SHH	0	0	3	775	m	146569	SHH I	20100407	4	97	16	19	979	11059	edge	5.5	6.1	154	459
26	126	harkVertebrae07-04-10.csv	T	SHH	0	5	3	710	m	216070	SHH I	20100407	4	97	15	52	952	11032	edge	5.1	4.6	65	307
27	127	harkVertebrae07-04-10.csv	F	NIST610	5	5					na	20100407	4	97	16	38	998	11078	standard			67	183
28	128	harkVertebrae07-04-10.csv	F	NIST610	5	5					na	20100407	4	97	16	44	1004	11084	standard			98	216
29	129	harkVertebrae07-04-10.csv	T	MIS	0	5	2	440		312030	MIS M	20100407	4	97	16	54	1014	11094	edge	3.2	4	102	250
30	130	harkVertebrae07-04-10.csv	T	MIS	0	0	3	728		220523	MIS M	20100407	4	97	17	5	1025	11105	edge	5	5.8	132	400
31	131	harkVertebrae07-04-10.csv	T	MIS	0	6	4	620		296603	MIS M	20100407	4	97	17	13	1033	11113	edge	4.6	6.2	106	280
32	132	harkVertebrae07-04-10.csv	T	MIS	0	6	3	750		317091	MIS M	20100407	4	97	17	21	1041	11121	edge	5.5	7.5	120	451
33	133	harkVertebrae07-04-10.csv	F	NIST610	6	6					na	20100407	4	97	17	27	1047	11127	standard			89	207
34	134	harkVertebrae07-04-10.csv	F	NIST610	6	6					na	20100407	4	97	17	32	1052	11132	standard			146	263
35	250	harkVertebrae08-04-10.csv	F	NIST610	7	7					na	20100408	4	98	14	58	898	12418	standard			92	197
36	251	harkVertebrae08-04-10.csv	F	NIST610	7	7					na	20100408	4	98	15	1	901	12421	standard			93	194
37	252	harkVertebrae08-04-10.csv	T	MIS	0	7	3	700		316044	MIS M	20100408	4	98	15	19	919	12439	edge	4.9	5.9	120	408
38	253	harkVertebrae08-04-10.csv	T	MIS	0	7	4	680		296634	MIS M	20100408	4	98	15	41	941	12461	edge	4.9	6.6	106	399
39	254	harkVertebrae08-04-10.csv	T	MIS	0	0	4	665		296626	MIS M	20100408	4	98	15	49	949	12469	edge	5.2	6.4	110	358
40	255	harkVertebrae08-04-10.csv	T	MIS	0	0	3	695		317116	MIS M	20100408	4	98	16	0	960	12480	edge	5.1	7	143	470
41	256	harkVertebrae08-04-10.csv	T	MIS	0	8	3	720		317060	MIS M	20100408	4	98	16	22	982	12502	edge	5.2	6.1	98	373
42	257	harkVertebrae08-04-10.csv	F	NIST610	8	8					na	20100408	4	98	16	27	987	12507	standard			69	171
43	258	harkVertebrae08-04-10.csv	F	NIST610	8	8					na	20100408	4	98	16	30	990	12510	standard			86	186
44	259	harkVertebrae08-04-10.csv	T	SHH	0	8	2	434	f	283664	SHH P	20100408	4	98	16	47	1007	12527	edge	2.6	3	99	225
45	260	harkVertebrae08-04-10.csv	T	SHH	0	0	4	630	f	296674	SHH P	20100408	4	98	16	54	1014	12534	edge	4.2	4.6	102	300
46	261	harkVertebrae08-04-10.csv	T	SHH	0	9	2	430	m	210908	SHH P	20100408	4	98	17	3	1023	12543	edge	3	2.5	114	249
47	262	harkVertebrae08-04-10.csv	T	SHH	0	9	2	455	m	210923	SHH P	20100408	4	98	17	11	1031	12551	edge	3	3	85	244
48	263	harkVertebrae08-04-10.csv	F	NIST610	9	9					na	20100408	4	98	17	15	1035	12555	standard			81	180
49	264	harkVertebrae08-04-10.csv	F	NIST610	9	9					na	20100408	4	98	17	19	1039	12559	standard			100	200
50	301	harkVertebrae09-04-10.csv	F	NIST610	10	10					na	20100409	4	99	9	30	570	13530	standard			132	230
51	302	harkVertebrae09-04-10.csv	F	NIST610	10	10					na	20100409	4	99	9	34	574	13534	standard			82	179
52	303	harkVertebrae09-04-10.csv	T	SHH	0	10	2	485	f	134628	SHH P	20100409	4	99	9	46	586	13546	edge	3	3	99	250
53	304	harkVertebrae09-04-10.csv	T	SHH	0	10	2	460	f	242019	SHH P	20100409	4	99	9	54	594	13554	edge	3.2	3.1	121	301
54	305	harkVertebrae09-04-10.csv	T	SHH	0	0	3	750	m	134136	SHH P	20100409	4	99	10	20	620	13580	edge	5.4	5.6	127	328
55	306	harkVertebrae09-04-10.csv	T	SHH	0	11	3	680	m	216023	SHH P	20100409	4	99	10	29	629	13589	edge	4.9	5	115	387
56	309	harkVertebrae09-04-10.csv	F	NIST610	11	11					na	20100409	4	99	11	6	666	13626	standard			103	180
57	310	harkVertebrae09-04-10.csv	F	NIST610	11	11					na	20100409	4	99	11	12	672	13632	standard			99	184
58	311	harkVertebrae09-04-10.csv	T	MIS	0	11	6	615	f	118085	MIS B	20100409	4	99	11	20	680	13640	edge	4.5	5.8	89	333
59	312	harkVertebrae09-04-10.csv	T	MIS	0	0	6	601	m	165524	MIS B	20100409	4	99	11	27	687	13647	edge	4.4	5.4	114	300

unique.id	run	file.name	primary	species	drift.group	bl.yield.group	ocation	fl	sex	fish.num	slide	run.date	run.month	run.day	run.hour	run.minute	run.time	abs.time	profile	outer.radius	entral.width	seq.start	seq.end
60	313	harkVertebrae09-04-10.csv	T	MIS	0	0	1	680	m	211036	MIS B	20100409	4	99	11	35	695	13655	edge	5	6.5	153	419
61	314	harkVertebrae09-04-10.csv	T	MIS	0	0	6	437	f	165520	MIS B	20100409	4	99	11	41	701	13661	edge	2.9	4.3	100	228
62	315	harkVertebrae09-04-10.csv	F	NIST610	12	12					na	20100409	4	99	11	50	710	13670	standard			95	188
63	316	harkVertebrae09-04-10.csv	F	NIST610	12	12					na	20100409	4	99	12	2	722	13682	standard			22	118
64	317	harkVertebrae09-04-10.csv	T	MIS	0	12	1	685	m	210972	MIS B	20100409	4	99	11	46	706	13666	edge	5.2	5.2	265	566
65	318	harkVertebrae09-04-10.csv	T	MIS	0	12	1	675	m	211027	MIS B	20100409	4	99	12	11	731	13691	edge	5.5	7	104	375
66	319	harkVertebrae09-04-10.csv	T	MIS	0	0	6	418	f	165542	MIS B	20100409	4	99	12	18	738	13698	edge	3.3	4.5	102	259
67	320	harkVertebrae09-04-10.csv	T	MIS	0	13	1	660	m	211084	MIS B	20100409	4	99	12	26	746	13706	edge	5.9	8.4	114	420
68	321	harkVertebrae09-04-10.csv	F	NIST610	13	13					na	20100409	4	99	13	43	823	13783	standard			108	205
69	322	harkVertebrae09-04-10.csv	F	NIST610	13	13					na	20100409	4	99	13	48	828	13788	standard			144	245
70	323	harkVertebrae09-04-10.csv	T	SHH	0	13	1	1190	m	243594	SHH G	20100409	4	99	14	17	857	13817	edge	8.8	9	105	643
71	324	harkVertebrae09-04-10.csv	T	SHH	0	0	1	###	m	211058	SHH G	20100409	4	99	14	37	877	13837	edge	10.4	10.3	119	690
72	325	harkVertebrae09-04-10.csv	T	SHH	0	14	1	###	m	211060	SHH G	20100409	4	99	14	49	889	13849	edge	8.1	7.9	104	560
73	326	harkVertebrae09-04-10.csv	F	NIST610	14	14					na	20100409	4	99	15	0	900	13860	standard			100	199
74	327	harkVertebrae09-04-10.csv	F	NIST610	14	14					na	20100409	4	99	15	4	904	13864	standard			181	282
75	328	harkVertebrae09-04-10.csv	T	MIS	0	14	3	700		187667	MIS J	20100409	4	99	15	32	932	13892	edge	5	6.2	92	380
76	329	harkVertebrae09-04-10.csv	T	MIS	0	0	3	703		243527	MIS J	20100409	4	99	15	43	943	13903	edge	5.6	7.4	34	339
77	330	harkVertebrae09-04-10.csv	T	MIS	0	0	2	591		134606	MIS J	20100409	4	99	15	49	949	13909	edge	3.8	4.5	199	414
78	331	harkVertebrae09-04-10.csv	T	MIS	0	0	2	480		134621	MIS J	20100409	4	99	15	56	956	13916	edge	3	3.3	91	246
79	332	harkVertebrae09-04-10.csv	T	MIS	0	0	3	705		187647	MIS J	20100409	4	99	16	2	962	13922	edge	4.3	6.2	220	489
80	333	harkVertebrae09-04-10.csv	T	MIS	0	0	2	597		134601	MIS J	20100409	4	99	16	9	969	13929	edge	3.9	4.8	100	280
81	334	harkVertebrae09-04-10.csv	F	MIS	0	15	2	464		134610	MIS J	20100409	4	99	16	14	974	13934	edge	2.7	3.1	93	197
82	335	harkVertebrae09-04-10.csv	F	NIST610	15	15					na	20100409	4	99	16	18	978	13938	standard			86	191
83	336	harkVertebrae09-04-10.csv	F	NIST610	15	15					na	20100409	4	99	16	22	982	13942	standard			99	200
84	337	harkVertebrae09-04-10.csv	T	SHH	0	15	6			199357	SHH C	20100409	4	99	16	51	1011	13971	edge	4.3	4.7	105	322
85	338	harkVertebrae09-04-10.csv	T	SHH	0	0	1		m	142711	SHH C	20100409	4	99	16	59	1019	13979	edge	6.1	6.2	89	466
86	339	harkVertebrae09-04-10.csv	T	SHH	0	16	1	935	f	142709	SHH C	20100409	4	99	17	8	1028	13988	edge	6.3	6.7	97	462
87	340	harkVertebrae09-04-10.csv	T	SHH	0	16	6			199364	SHH C	20100409	4	99	17	20	1040	14000	edge	5.1	5.6	90	356
88	341	harkVertebrae09-04-10.csv	F	NIST610	16	16					na	20100409	4	99	17	24	1044	14004	standard			101	202
89	342	harkVertebrae09-04-10.csv	F	NIST610	16	16					na	20100409	4	99	17	28	1048	14008	standard			98	200
90	401	harkVertebrae12-04-10.csv	F	NIST610	17	17					na	20100412	4	102	9	34	574	17854	standard			86	197
91	402	harkVertebrae12-04-10.csv	F	NIST610	17	17					na	20100412	4	102	9	38	578	17858	standard			101	210
92	403	harkVertebrae12-04-10.csv	T	SHH	0	17	1	###	m	210996	SHH C	20100412	4	102	9	55	595	17875	edge	6.8	7	87	516
93	404	harkVertebrae12-04-10.csv	T	SHH	0	17	1	827	f	142712	SHH C	20100412	4	102	10	4	604	17884	edge	6	6.2	83	430
94	405	harkVertebrae12-04-10.csv	T	SHH	0	0	6		m	199373	SHH C	20100412	4	102	10	14	614	17894	edge	5.4	5.8	122	433
95	406	harkVertebrae12-04-10.csv	T	SHH	0	18	1	510	f	211008	SHH C	20100412	4	102	10	32	632	17912	edge	3.3	3.3	101	237
96	407	harkVertebrae12-04-10.csv	F	NIST610	18	18					na	20100412	4	102	11	4	664	17944	standard			97	199
97	408	harkVertebrae12-04-10.csv	F	NIST610	18	18					na	20100412	4	102	11	9	669	17949	standard			114	217
98	409	harkVertebrae12-04-10.csv	T	MIS	0	18	1		f	211082	MIS C	20100412	4	102	11	30	690	17970	edge	5.8	7.5	103	455
99	410	harkVertebrae12-04-10.csv	T	MIS	0	0	6	445	f	165535	MIS C	20100412	4	102	11	44	704	17984	edge	3	3.8	93	267
100	411	harkVertebrae12-04-10.csv	T	MIS	0	0	1		f	211081	MIS C	20100412	4	102	11	51	711	17991	edge	5.9	8.9	101	451
101	412	harkVertebrae12-04-10.csv	T	MIS	0	0	1	670	m	211062	MIS C	20100412	4	102	11	57	717	17997	edge	5.1	6.5	93	350
102	413	harkVertebrae12-04-10.csv	T	MIS	0	0	1		m	211064	MIS C	20100412	4	102	12	7	727	18007	edge	4.5	6.6	107	352
103	414	harkVertebrae12-04-10.csv	T	MIS	0	0	1	685	m	211085	MIS C	20100412	4	102	12	14	734	18014	edge	5.5	7	108	446
104	415	harkVertebrae12-04-10.csv	T	MIS	0	0	6	432	m	165533	MIS C	20100412	4	102	12	20	740	18020	edge	3.3	4	166	343
105	416	harkVertebrae12-04-10.csv	T	MIS	0	19	1	690	m	210971	MIS C	20100412	4	102	12	26	746	18026	edge	4.8	6.3	95	344
106	417	harkVertebrae12-04-10.csv	F	NIST610	19	19					na	20100412	4	102	12	34	754	18034	standard			86	190
107	418	harkVertebrae12-04-10.csv	F	NIST610	19	19					na	20100412	4	102	12	37	757	18037	standard			97	202
108	419	harkVertebrae12-04-10.csv	T	SHH	0	19	4	###	m	296655	SHH M	20100412	4	102	13	19	799	18079	edge	6.8	7.8	100	479
109	420	harkVertebrae12-04-10.csv	T	SHH	0	0	3	765	m	234627	SHH M	20100412	4	102	13	28	808	18088	edge	5.2	5.5	111	413
110	421	harkVertebrae12-04-10.csv	T	SHH	0	0	4	570	f	296605	SHH M	20100412	4	102	13	35	815	18095	edge	3.8	3.8	101	315
111	422	harkVertebrae12-04-10.csv	T	SHH	0	20	4	760	m	296638	SHH M	20100412	4	102	13	48	828	18108	edge	4.9	5.2	104	392
112	423	harkVertebrae12-04-10.csv	F	NIST610	20	20					na	20100412	4	102	13	52	832	18112	standard			76	186
113	424	harkVertebrae12-04-10.csv	F	NIST610	20	20					na	20100412	4	102	13	56	836	18116	standard			83	197
114	425	harkVertebrae12-04-10.csv	T	SHH	0	20	4	850	m	296633	SHH M	20100412	4	102	14	6	846	18126	edge	5.3	5.5	88	350
115	426	harkVertebrae12-04-10.csv	T	SHH	0	0	3	730	m	216067	SHH M	20100412	4	102	14	14	854	18134	edge	4.8	5.3	81	359
116	427	harkVertebrae12-04-10.csv	T	SHH	0	0	2	475	f	210926	SHH M	20100412	4	102	14	19	859	18139	edge	3	3.3	87	251
117	428	harkVertebrae12-04-10.csv	T	SHH	0	0	3	755	m	146572	SHH M	20100412	4	102	14	27	867	18147	edge	4.8	5.3	144	387
118	429	harkVertebrae12-04-10.csv	T	MIS	0	0	4	690		296654	MIS L	20100412	4	102	14	39	879	18159	edge	4.7	6	95	371

unique.id	run	file.name	primary	species	drift.group	bl.yield.group	ocation	fl	sex	fish.num	slide	run.date	un.month	run.day	run.hour	un.minute	run.time	abs.time	profile	outer.radius	entral.width	seq.start	seq.end
119	430	harkVertebrae12-04-10.csv	T	MIS	0	0	2	515		312028	MIS L	20100412	4	102	14	46	886	18166	edge	3.3	4.5	95	270
120	431	harkVertebrae12-04-10.csv	F	MIS	0	0	5	720		142741	MIS L	20100412	4	102	14	53	893	18173	edge	4.4	5	131	376
121	432	harkVertebrae12-04-10.csv	T	MIS	0	0	2	350		242111	MIS L	20100412	4	102	15	4	904	18184	edge	1.8	2.3	97	210
122	433	harkVertebrae12-04-10.csv	T	MIS	0	0	3	700		317125	MIS L	20100412	4	102	15	11	911	18191	edge	5.2	6.4	82	372
123	434	harkVertebrae12-04-10.csv	T	MIS	0	0	4	700		296650	MIS L	20100412	4	102	15	18	918	18198	edge	5.1	6.6	92	396
124	435	harkVertebrae12-04-10.csv	T	MIS	0	21	3	710		317094	MIS L	20100412	4	102	15	25	925	18205	edge	5.4	6.4	144	406
125	436	harkVertebrae12-04-10.csv	F	NIST610	21	21					na	20100412	4	102	15	30	930	18210	standard			118	231
126	437	harkVertebrae12-04-10.csv	F	NIST610	21	21					na	20100412	4	102	15	33	933	18213	standard			95	208
127	438	harkVertebrae12-04-10.csv	T	MIS	0	21	2	538		242022	MIS L	20100412	4	102	15	39	939	18219	edge	3.5	4.7	135	337
128	439	harkVertebrae12-04-10.csv	T	SHH	0	0	8			999037	SHH T	20100412	4	102	16	48	1008	18288	edge	13.9	13.2	84	873
129	440	harkVertebrae12-04-10.csv	T	SHH	0	0	8			999039	SHH T	20100412	4	102	17	1	1021	18301	edge	12.2	13.8	86	755
130	441	harkVertebrae12-04-10.csv	T	SHH	0	22	8			999012	SHH T	20100412	4	102	17	14	1034	18314	edge	15.3	15.3	84	856
131	442	harkVertebrae12-04-10.csv	T	SHH	0	22	8			999038	SHH T	20100412	4	102	17	25	1045	18325	edge	13.1	14	80	835
132	443	harkVertebrae12-04-10.csv	F	NIST610	22	22					na	20100412	4	102	17	32	1052	18332	standard			43	158
133	444	harkVertebrae12-04-10.csv	F	NIST610	22	22					na	20100412	4	102	17	36	1056	18336	standard			115	230
134	501	harkVertebrae13-04-10.csv	F	NIST610	23	23					na	20100413	4	103	9	11	551	19271	standard			108	220
135	502	harkVertebrae13-04-10.csv	F	NIST610	23	23					na	20100413	4	103	9	19	559	19279	standard			91	203
136	503	harkVertebrae13-04-10.csv	T	SHH	0	23	8			999018	SHH Q	20100413	4	103	9	27	567	19287	edge	10.1	10.9	89	651
137	504	harkVertebrae13-04-10.csv	T	SHH	0	23	8			999046	SHH Q	20100413	4	103	9	52	592	19312	edge	14.1	14.9	92	930
138	505	harkVertebrae13-04-10.csv	T	SHH	0	0	8			999044	SHH Q	20100413	4	103	10	11	611	19331	edge	10.2	9	101	626
139	506	harkVertebrae13-04-10.csv	T	SHH	0	0	8			999043	SHH Q	20100413	4	103	10	21	621	19341	edge	8.9	8.7	92	551
140	507	harkVertebrae13-04-10.csv	T	SHH	0	24	8			999031	SHH Q	20100413	4	103	10	30	630	19350	edge	10.6	10.7	102	697
141	508	harkVertebrae13-04-10.csv	F	NIST610	24	24					na	20100413	4	103	10	36	636	19356	standard			115	235
142	509	harkVertebrae13-04-10.csv	F	NIST610	24	24					na	20100413	4	103	10	41	641	19361	standard			108	229
143	510	harkVertebrae13-04-10.csv	T	SHH	0	24	8			999006	SHH V	20100413	4	103	10	53	653	19373	edge	9.9	9	101	618
144	511	harkVertebrae13-04-10.csv	T	SHH	0	0	8			999042	SHH R	20100413	4	103	11	29	689	19409	edge	14.3	14.3	92	885
145	512	harkVertebrae13-04-10.csv	T	SHH	0	0	8			999027	SHH R	20100413	4	103	11	40	700	19420	edge	11.4	12	91	762
146	513	harkVertebrae13-04-10.csv	T	SHH	0	0	8			999011	SHH R	20100413	4	103	11	52	712	19432	edge	11.8	12.3	92	751
147	514	harkVertebrae13-04-10.csv	T	SHH	0	25	8			999025	SHH R	20100413	4	103	12	3	723	19443	edge	11.4	11.3	95	709
148	515	harkVertebrae13-04-10.csv	F	NIST610	25	25					na	20100413	4	103	12	10	730	19450	standard			111	228
149	516	harkVertebrae13-04-10.csv	F	NIST610	25	25					na	20100413	4	103	12	15	735	19455	standard			145	259
150	517	harkVertebrae13-04-10.csv	T	SHH	0	25	4	###	m	296671	SHH O	20100413	4	103	12	31	751	19471	edge	7	6.9	84	405
151	518	harkVertebrae13-04-10.csv	T	SHH	0	0	2	420	f	283663	SHH O	20100413	4	103	13	11	791	19511	edge	2.9	3.1	86	220
152	519	harkVertebrae13-04-10.csv	T	SHH	0	0	4	###	m	296659	SHH O	20100413	4	103	13	21	801	19521	edge	10.4	10.5	89	703
153	520	harkVertebrae13-04-10.csv	T	SHH	0	0	3	1170	m	317210	SHH O	20100413	4	103	13	30	810	19530	edge	7.9	7.7	101	550
154	521	harkVertebrae13-04-10.csv	T	SHH	0	26	4	###	m	296678	SHH O	20100413	4	103	13	43	823	19543	edge	9.3	9.4	84	532
155	522	harkVertebrae13-04-10.csv	F	NIST610	26	26					na	20100413	4	103	13	48	828	19548	standard			63	173
156	523	harkVertebrae13-04-10.csv	F	NIST610	26	26					na	20100413	4	103	13	52	832	19552	standard			87	202
157	524	harkVertebrae13-04-10.csv	T	MIS	0	26	1	675	m	210974	MIS F	20100413	4	103	14	4	844	19564	edge	5.5	6.4	79	350
158	525	harkVertebrae13-04-10.csv	T	MIS	0	0	6	438	m	165536	MIS F	20100413	4	103	14	10	850	19570	edge	3.4	4.3	87	254
159	526	harkVertebrae13-04-10.csv	T	MIS	0	0	6	420	m	165538	MIS F	20100413	4	103	14	15	855	19575	edge	3.4	4.3	99	264
160	527	harkVertebrae13-04-10.csv	T	MIS	0	0	6	595	f	165531	MIS F	20100413	4	103	14	20	860	19580	edge	4.2	5.7	86	359
161	528	harkVertebrae13-04-10.csv	T	MIS	0	0	6	419	f	165546	MIS F	20100413	4	103	14	26	866	19586	edge	3.2	4.3	87	251
162	529	harkVertebrae13-04-10.csv	T	SHH	0	0	1	850	m	210964	SHH D	20100413	4	103	14	36	876	19596	edge	4.9	5.6	96	392
163	530	harkVertebrae13-04-10.csv	T	SHH	0	0	6		f	199384	SHH D	20100413	4	103	14	42	882	19602	edge	5.3	5.6	85	373
164	531	harkVertebrae13-04-10.csv	T	SHH	0	0	1	825	m	210992	SHH D	20100413	4	103	14	48	888	19608	edge	6.3	6.5	110	437
165	532	harkVertebrae13-04-10.csv	T	SHH	0	0	6		m	199378	SHH D	20100413	4	103	14	54	894	19614	edge	5.1	5.4	90	371
166	533	harkVertebrae13-04-10.csv	T	SHH	0	0	1	780	f	210987	SHH D	20100413	4	103	15	10	910	19630	edge	5.8	6	100	368
167	534	harkVertebrae13-04-10.csv	T	SHH	0	27	1		m	142707	SHH D	20100413	4	103	15	20	920	19640	edge	6.4	6.4	90	409
168	535	harkVertebrae13-04-10.csv	F	NIST610	27	27					na	20100413	4	103	15	24	924	19644	standard			81	196
169	536	harkVertebrae13-04-10.csv	F	NIST610	27	27					na	20100413	4	103	15	28	928	19648	standard			98	213
170	537	harkVertebrae13-04-10.csv	T	SHH	0	27	6		f	199394	SHH D	20100413	4	103	15	42	942	19662	edge	5.2	5.2	93	306
171	538	harkVertebrae13-04-10.csv	T	SHH	0	0	6			199363	SHH D	20100413	4	103	15	49	949	19669	edge	4.8	4.8	168	459
172	539	harkVertebrae13-04-10.csv	T	MIS	0	0	6	597	f	216158	MIS G	20100413	4	103	16	0	960	19680	edge	4.8	6.3	99	362
173	540	harkVertebrae13-04-10.csv	T	MIS	0	0	6	415	f	165547	MIS G	20100413	4	103	16	6	966	19686	edge	3	4.1	85	274
174	541	harkVertebrae13-04-10.csv	T	MIS	0	0	6	430	f	118086	MIS G	20100413	4	103	16	12	972	19692	edge	2.9	3.7	84	219
175	542	harkVertebrae13-04-10.csv	T	MIS	0	0	1	680	m	211031	MIS G	20100413	4	103	16	18	978	19698	edge	5.3	7	91	372
176	543	harkVertebrae13-04-10.csv	T	MIS	0	0	1	680	m	210966	MIS G	20100413	4	103	16	24	984	19704	edge	5.1	6.6	89	381
177	544	harkVertebrae13-04-10.csv	F	MIS	0	0	3	705		187650	MIS H	20100413	4	103	16	36	996	19716	edge	4.9	6.3	90	349

unique.id	run	file.name	primary	species	drift.group	bl.yield.group	ocation	fl	sex	fish.num	slide	run.date	un.month	run.day	run.hour	un.minute	run.time	abs.time	profile	outer.radius	entral.width	seq.start	seq.end
178	545	harkVertebrae13-04-10.csv	T	MIS	0	0	3	698		130698	MIS H	20100413	4	103	16	41	1001	19721	edge	4.5	5.6	87	339
179	546	harkVertebrae13-04-10.csv	T	MIS	0	0	3	716		146536	MIS H	20100413	4	103	16	55	1015	19735	edge	4.7	5.6	96	370
180	547	harkVertebrae13-04-10.csv	T	MIS	0	28	3	720		242141	MIS H	20100413	4	103	17	8	1028	19748	edge	4.9	5.7	118	339
181	548	harkVertebrae13-04-10.csv	F	NIST610	28	28					na	20100413	4	103	17	13	1033	19753	standard			88	201
182	549	harkVertebrae13-04-10.csv	F	NIST610	28	28					na	20100413	4	103	17	16	1036	19756	standard			101	215
183	550	harkVertebrae13-04-10.csv	T	MIS	0	28	2	450		242044	MIS H	20100413	4	103	17	21	1041	19761	edge	3.2	3.9	94	271
184	551	harkVertebrae13-04-10.csv	T	MIS	0	29	3	719		220516	MIS H	20100413	4	103	17	27	1047	19767	edge	5.4	6.9	83	359
185	552	harkVertebrae13-04-10.csv	T	MIS	0	29	3	684		317127	MIS H	20100413	4	103	17	34	1054	19774	edge	5.2	7.1	94	422
186	553	harkVertebrae13-04-10.csv	F	NIST610	29	29					na	20100413	4	103	17	40	1060	19780	standard			83	197
187	554	harkVertebrae13-04-10.csv	F	NIST610	29	29					na	20100413	4	103	17	43	1063	19783	standard			83	191
188	601	harkVertebrae14-04-10.csv	F	NIST610	30	30					na	20100414	4	104	9	17	557	20717	standard			107	302
189	602	harkVertebrae14-04-10.csv	F	NIST610	30	30					na	20100414	4	104	9	22	562	20722	standard			84	230
190	603	harkVertebrae14-04-10.csv	T	MIS	0	30	6	460	m	165529	MIS D	20100414	4	104	9	31	571	20731	edge	3.3	4.9	98	284
191	604	harkVertebrae14-04-10.csv	T	MIS	0	30	6	433	f	118095	MIS D	20100414	4	104	9	37	577	20737	edge	3.5	4.7	89	265
192	605	harkVertebrae14-04-10.csv	T	MIS	0	0	1	690	m	211030	MIS D	20100414	4	104	9	42	582	20742	edge	5.6	7.6	101	424
193	606	harkVertebrae14-04-10.csv	T	MIS	0	0	1	680	m	210999	MIS D	20100414	4	104	9	49	589	20749	edge	6	7.9	89	371
194	607	harkVertebrae14-04-10.csv	T	MIS	0	0	1	720	m	210969	MIS D	20100414	4	104	9	56	596	20756	edge	5.2	7.6	89	399
195	608	harkVertebrae14-04-10.csv	T	MIS	0	0	1	680	m	211037	MIS D	20100414	4	104	10	3	603	20763	edge	4.7	6.3	85	380
196	609	harkVertebrae14-04-10.csv	T	MIS	0	0	6	440	m	165526	MIS D	20100414	4	104	10	8	608	20768	edge	3.4	4.2	101	261
197	610	harkVertebrae14-04-10.csv	T	MIS	0	0	1	670	m	210973	MIS D	20100414	4	104	10	15	615	20775	edge	5.1	7	115	418
198	611	harkVertebrae14-04-10.csv	T	MIS	0	0	4	695		296615	MIS N	20100414	4	104	10	26	626	20786	edge	5	6.4	87	379
199	612	harkVertebrae14-04-10.csv	T	MIS	0	0	2	455		242096	MIS N	20100414	4	104	10	36	636	20796	edge	3.2	3.3	86	200
200	613	harkVertebrae14-04-10.csv	T	MIS	0	31	4	690		296621	MIS N	20100414	4	104	10	57	657	20817	edge	4.8	6.4	111	376
201	614	harkVertebrae14-04-10.csv	F	NIST610	31	31					na	20100414	4	104	11	4	664	20824	standard			83	191
202	615	harkVertebrae14-04-10.csv	F	NIST610	31	31					na	20100414	4	104	11	7	667	20827	standard			81	186
203	616	harkVertebrae14-04-10.csv	T	MIS	0	31	3	719		220522	MIS N	20100414	4	104	11	13	673	20833	edge	5.2	6.4	88	342
204	617	harkVertebrae14-04-10.csv	T	MIS	0	0	2	395		210833	MIS N	20100414	4	104	11	19	679	20839	edge	2.5	2.8	121	260
205	618	harkVertebrae14-04-10.csv	T	MIS	0	0	2	474		312029	MIS N	20100414	4	104	11	25	685	20845	edge	3.3	4	88	261
206	619	harkVertebrae14-04-10.csv	T	MIS	0	0	2	445		242017	MIS N	20100414	4	104	11	30	690	20850	edge	3.2	4.2	83	232
207	620	harkVertebrae14-04-10.csv	T	MIS	0	0	4	740		296629	MIS N	20100414	4	104	11	38	698	20858	edge	5.1	6.4	92	397
208	621	harkVertebrae14-04-10.csv	T	SHH	0	0	1	###	m	309081	SHH A	20100414	4	104	12	9	729	20889	edge	10.6	10.6	81	728
209	622	harkVertebrae14-04-10.csv	T	SHH	0	0	1	###	m	243577	SHH A	20100414	4	104	12	18	738	20898	edge	7.8	8.3	80	532
210	623	harkVertebrae14-04-10.csv	T	SHH	0	32	1	###	m	243593	SHH A	20100414	4	104	12	27	747	20907	edge	9.8	9.2	81	598
211	624	harkVertebrae14-04-10.csv	F	NIST610	32	32					na	20100414	4	104	12	33	753	20913	standard			89	190
212	625	harkVertebrae14-04-10.csv	F	NIST610	32	32					na	20100414	4	104	12	37	757	20917	standard			90	191
213	626	harkVertebrae14-04-10.csv	T	SHH	0	32	1	###	m	309076	SHH A	20100414	4	104	13	13	793	20953	edge	9.4	10	87	641
214	627	harkVertebrae14-04-10.csv	T	SHH	0	0	1	1130	m	243592	SHH A	20100414	4	104	13	22	802	20962	edge	8.2	8.7	90	541
215	628	harkVertebrae14-04-10.csv	T	SHH	0	0	1	985	m	309077	SHH A	20100414	4	104	13	35	815	20975	edge	7.4	7.4	98	511
216	629	harkVertebrae14-04-10.csv	T	SHH	0	0	1	1130	m	210976	SHH A	20100414	4	104	13	45	825	20985	edge	8.9	8.9	93	568
217	630	harkVertebrae14-04-10.csv	T	SHH	0	33	1	###	m	210986	SHH A	20100414	4	104	13	54	834	20994	edge	9.6	10.1	83	627
218	631	harkVertebrae14-04-10.csv	F	NIST610	33	33					na	20100414	4	104	14	0	840	21000	standard			81	190
219	632	harkVertebrae14-04-10.csv	F	NIST610	33	33					na	20100414	4	104	14	3	843	21003	standard			82	189
220	633	harkVertebrae14-04-10.csv	T	SHH	0	33	1	868	m	142705	SHH E	20100414	4	104	14	13	853	21013	edge	7.2	6.7	86	413
221	634	harkVertebrae14-04-10.csv	T	SHH	0	0	1	810	m	211009	SHH E	20100414	4	104	14	19	859	21019	edge	6.2	5.8	124	433
222	635	harkVertebrae14-04-10.csv	T	SHH	0	0	6		m	199381	SHH E	20100414	4	104	14	26	866	21026	edge	5	5.5	83	365
223	636	harkVertebrae14-04-10.csv	T	SHH	0	0	6		f	199385	SHH E	20100414	4	104	14	32	872	21032	edge	5	5	99	375
224	637	harkVertebrae14-04-10.csv	T	SHH	0	0	1	835	m	243580	SHH E	20100414	4	104	14	41	881	21041	edge	6.6	6.4	91	449
225	638	harkVertebrae14-04-10.csv	T	SHH	0	0	6			199369	SHH E	20100414	4	104	14	47	887	21047	edge	4.8	5	83	354
226	639	harkVertebrae14-04-10.csv	T	SHH	0	0	6		f	199390	SHH E	20100414	4	104	14	53	893	21053	edge	5.7	6	84	363
227	640	harkVertebrae14-04-10.csv	T	SHH	0	0	6		f	199389	SHH E	20100414	4	104	15	1	901	21061	edge	3.6	3.4	80	290
228	641	harkVertebrae14-04-10.csv	T	MIS	0	0	4	690		296604	MIS I	20100414	4	104	15	12	912	21072	edge	5	6.7	92	389
229	642	harkVertebrae14-04-10.csv	T	MIS	0	0	3	675		150525	MIS I	20100414	4	104	15	17	917	21077	edge	5.1	6.1	89	329
230	643	harkVertebrae14-04-10.csv	T	MIS	0	0	2	440		134598	MIS I	20100414	4	104	15	22	922	21082	edge	2.7	3.2	85	221
231	644	harkVertebrae14-04-10.csv	T	MIS	0	0	2	484		283652	MIS I	20100414	4	104	15	27	927	21087	edge	3.1	3.9	99	255
232	645	harkVertebrae14-04-10.csv	T	MIS	0	0	3	713		243518	MIS I	20100414	4	104	15	33	933	21093	edge	5.5	6.3	91	344
233	646	harkVertebrae14-04-10.csv	T	MIS	0	0	3	679		150536	MIS I	20100414	4	104	15	39	939	21099	edge	4.9	5.8	85	321
234	647	harkVertebrae14-04-10.csv	T	MIS	0	34	2	510		134619	MIS I	20100414	4	104	15	45	945	21105	edge	3.1	3.4	120	279
235	648	harkVertebrae14-04-10.csv	F	NIST610	34	34					na	20100414	4	104	15	50	950	21110	standard			101	203
236	649	harkVertebrae14-04-10.csv	F	NIST610	34	34					na	20100414	4	104	15	55	955	21115	standard			85	185

unique.id	run	file.name	primary	species	drift.group	bl.yield.group	ocation	fl	sex	fish.num	slide	run.date	un.month	run.day	run.hour	un.minute	run.time	abs.time	profile	outer.radius	entral.width	seq.start	seq.end
237	650	harkVertebrae14-04-10.csv	T	MIS	0	34	3	702		243537	MIS I	20100414	4	104	16	0	960	21120	edge	5.6	7.5	89	395
238	651	harkVertebrae14-04-10.csv	T	SHH	0	0	1	835	f	243565	SHH F	20100414	4	104	16	11	971	21131	edge	6	6.7	87	460
239	652	harkVertebrae14-04-10.csv	T	SHH	0	0	6			199359	SHH F	20100414	4	104	16	24	984	21144	edge	5.1	6.3	99	402
240	653	harkVertebrae14-04-10.csv	T	SHH	0	0	6			199371	SHH F	20100414	4	104	16	30	990	21150	edge	5.3	5.5	88	367
241	654	harkVertebrae14-04-10.csv	T	SHH	0	0	1		m	142714	SHH F	20100414	4	104	16	40	1000	21160	edge	6.9	6.8	91	432
242	655	harkVertebrae14-04-10.csv	T	SHH	0	0	1	863	m	243573	SHH F	20100414	4	104	16	48	1008	21168	edge	6.6	7	99	411
243	656	harkVertebrae14-04-10.csv	T	SHH	0	0	6		m	199380	SHH F	20100414	4	104	16	57	1017	21177	edge	4.9	5.4	106	378
244	657	harkVertebrae14-04-10.csv	T	SHH	0	35	6			199358	SHH F	20100414	4	104	17	3	1023	21183	edge	6	6.2	163	490
245	658	harkVertebrae14-04-10.csv	T	SHH	0	35	1	835	m	243567	SHH F	20100414	4	104	17	10	1030	21190	edge	6.3	6.4	91	445
246	659	harkVertebrae14-04-10.csv	F	NIST610	35	35					na	20100414	4	104	17	16	1036	21196	standard			92	191
247	660	harkVertebrae14-04-10.csv	F	NIST610	35	35					na	20100414	4	104	17	20	1040	21200	standard			89	191
248	661	harkVertebrae14-04-10.csv	T	SHH	0	36	1	820	f	142719	SHH H	20100414	4	104	17	30	1050	21210	edge	6.2	6.5	138	506
249	662	harkVertebrae14-04-10.csv	T	SHH	0	36	6		f	199393	SHH H	20100414	4	104	17	38	1058	21218	edge	5.9	5.7	90	359
250	663	harkVertebrae14-04-10.csv	F	NIST610	36	36					na	20100414	4	104	17	44	1064	21224	standard			86	189
251	664	harkVertebrae14-04-10.csv	F	NIST610	36	36					na	20100414	4	104	17	47	1067	21227	standard			106	215
252	801	harkVertebrae19-04-10.csv	F	NIST610	37	37					na	20100419	4	109	10	57	657	28017	standard			93	213
253	802	harkVertebrae19-04-10.csv	F	NIST610	37	37					na	20100419	4	109	11	0	660	28020	standard			100	225
254	803	harkVertebrae19-04-10.csv	T	SHH	0	37	6		m	199375	SHH H	20100419	4	109	11	11	671	28031	edge	4.5	5	90	357
255	804	harkVertebrae19-04-10.csv	T	SHH	0	37	6			199361	SHH H	20100419	4	109	11	18	678	28038	edge	4.6	4.5	102	346
256	805	harkVertebrae19-04-10.csv	T	SHH	0	0	1	847	m	142710	SHH H	20100419	4	109	11	25	685	28045	edge	6.5	6.4	105	430
257	806	harkVertebrae19-04-10.csv	T	SHH	0	0	6		f	199386	SHH H	20100419	4	109	11	32	692	28052	edge	4.8	4.3	100	291
258	807	harkVertebrae19-04-10.csv	T	SHH	0	0	6			199370	SHH H	20100419	4	109	11	39	699	28059	edge	5.2	5.3	99	364
259	808	harkVertebrae19-04-10.csv	T	MIS	0	0	6	430	m	165514	MIS E	20100419	4	109	11	58	718	28078	edge	3.1	3.9	93	248
260	809	harkVertebrae19-04-10.csv	T	MIS	0	0	1	680	f	211010	MIS E	20100419	4	109	12	3	723	28083	edge	4.9	5.3	107	379
261	810	harkVertebrae19-04-10.csv	T	MIS	0	0	6	420	f	118094	MIS E	20100419	4	109	12	8	728	28088	edge	2.9	4.1	117	283
262	811	harkVertebrae19-04-10.csv	T	MIS	0	0	6	430	f	118091	MIS E	20100419	4	109	12	13	733	28093	edge	3.1	4.2	100	279
263	812	harkVertebrae19-04-10.csv	T	MIS	0	0	6	445	m	118090	MIS E	20100419	4	109	12	18	738	28098	edge	3.5	4.3	87	259
264	813	harkVertebrae19-04-10.csv	T	MIS	0	38	6	423	f	118080	MIS E	20100419	4	109	12	23	743	28103	edge	3.2	4.1	91	262
265	814	harkVertebrae19-04-10.csv	F	NIST610	38	38					na	20100419	4	109	12	27	747	28107	standard			85	210
266	815	harkVertebrae19-04-10.csv	F	NIST610	38	38					na	20100419	4	109	12	31	751	28111	standard			88	216
267	816	harkVertebrae19-04-10.csv	T	MIS	0	38	6	465	f	165534	MIS E	20100419	4	109	13	27	807	28167	edge	3.7	4.7	102	291
268	817	harkVertebrae19-04-10.csv	T	MIS	0	0	1	680	f	211022	MIS E	20100419	4	109	13	34	814	28174	edge	5.1	7.3	90	382
269	818	harkVertebrae19-04-10.csv	T	SHH	0	39	2	416	m	283658	SHH J	20100419	4	109	13	44	824	28184	edge	2.8	2.7	90	218
270	819	harkVertebrae19-04-10.csv	F	NIST610	39	39					na	20100419	4	109	13	48	828	28188	standard			90	220
271	820	harkVertebrae19-04-10.csv	F	NIST610	39	39					na	20100419	4	109	13	52	832	28192	standard			100	230
272	821	harkVertebrae19-04-10.csv	T	SHH	0	39	4	470	m	296689	SHH J	20100419	4	109	14	2	842	28202	edge	5.3	6.1	93	400
273	822	harkVertebrae19-04-10.csv	T	SHH	0	0	3	###	m	215964	SHH J	20100419	4	109	14	13	853	28213	edge	7	6.8	119	528
274	823	harkVertebrae19-04-10.csv	T	SHH	0	0	3	830	f	216054	SHH J	20100419	4	109	14	21	861	28221	edge	6.8	5.8	83	398
275	824	harkVertebrae19-04-10.csv	T	SHH	0	0	2	463	f	242060	SHH J	20100419	4	109	14	26	866	28226	edge	3.6	3.1	87	240
276	825	harkVertebrae19-04-10.csv	T	SHH	0	0	2	420	f	242006	SHH J	20100419	4	109	14	31	871	28231	edge	3.1	3	89	247
277	826	harkVertebrae19-04-10.csv	T	SHH	0	0	2	440	f	283665	SHH J	20100419	4	109	14	36	876	28236	edge	3	3	83	210
278	827	harkVertebrae19-04-10.csv	T	SHH	0	0	4	590	f	296607	SHH J	20100419	4	109	14	41	881	28241	edge	3.6	3.7	90	288
279	828	harkVertebrae19-04-10.csv	T	MIS	0	0	3	705		187648	MIS K	20100419	4	109	14	51	891	28251	edge	5.2	7	88	382
280	829	harkVertebrae19-04-10.csv	T	MIS	0	0	3	690		134115	MIS K	20100419	4	109	14	58	898	28258	edge	5.1	6.5	100	381
281	830	harkVertebrae19-04-10.csv	T	MIS	0	0	4	705		296631	MIS K	20100419	4	109	15	6	906	28266	edge	5.3	6.7	97	392
282	831	harkVertebrae19-04-10.csv	T	MIS	0	40	2	495		312016	MIS K	20100419	4	109	15	11	911	28271	edge	3.6	4.7	93	278
283	832	harkVertebrae19-04-10.csv	F	NIST610	40	40					na	20100419	4	109	15	15	915	28275	standard			93	225
284	833	harkVertebrae19-04-10.csv	F	NIST610	40	40					na	20100419	4	109	15	19	919	28279	standard			100	235
285	834	harkVertebrae19-04-10.csv	T	MIS	0	40	3	710		243525	MIS K	20100419	4	109	15	27	927	28287	edge	5.6	7.3	96	381
286	835	harkVertebrae19-04-10.csv	F	MIS	0	0	2	460		134608	MIS K	20100419	4	109	15	37	937	28297	edge	3	3.1	108	240
287	836	harkVertebrae19-04-10.csv	T	MIS	0	0	4	700		296632	MIS K	20100419	4	109	15	43	943	28303	edge	4.6	6.3	88	338
288	837	harkVertebrae19-04-10.csv	T	MIS	0	0	3	739		317130	MIS K	20100419	4	109	15	50	950	28310	edge	5.9	8	89	407
289	838	harkVertebrae19-04-10.csv	T	SHH	0	0	3	940	f	146580	SHH K	20100419	4	109	16	12	972	28332	edge	4.7	5.1	109	341
290	839	harkVertebrae19-04-10.csv	T	SHH	0	0	3	###	m	234642	SHH K	20100419	4	109	16	20	980	28340	edge	7.1	6.5	99	460
291	840	harkVertebrae19-04-10.csv	T	SHH	0	0	4	460	f	296690	SHH K	20100419	4	109	16	26	986	28346	edge	5	7	99	342
292	841	harkVertebrae19-04-10.csv	T	SHH	0	0	3	643	m	316026	SHH K	20100419	4	109	16	32	992	28352	edge	4.3	4.3	88	318
293	842	harkVertebrae19-04-10.csv	T	SHH	0	0	3	###	m	234680	SHH K	20100419	4	109	16	44	1004	28364	edge	7.2	7.3	115	525
294	843	harkVertebrae19-04-10.csv	T	SHH	0	41	3	725	m	234678	SHH K	20100419	4	109	16	50	1010	28370	edge	5.1	5.6	97	363
295	844	harkVertebrae19-04-10.csv	F	NIST610	41	41					na	20100419	4	109	16	58	1018	28378	standard			113	253

unique.id	run	file.name	primary	species	drift.group	bl.yield.group	ocation	fl	sex	fish.num	slide	run.date	un.month	run.day	un.hour	un.minute	run.time	abs.time	profile	outer.radius	entral.width	seq.start	seq.end
296	845	harkVertebrae19-04-10.csv	F	NIST610	41	41					na	20100419	4	109	17	5	1025	28385	standard			100	239
297	846	harkVertebrae19-04-10.csv	T	SHH	0	41	2	425	m	210929	SHH K	20100419	4	109	16	55	1015	28375	edge	3.3	2.9	97	259
298	847	harkVertebrae19-04-10.csv	T	SHH	0	42	6		f	199391	SHH B	20100419	4	109	17	15	1035	28395	edge	4.9	4.4	83	329
299	848	harkVertebrae19-04-10.csv	F	NIST610	42	42					na	20100419	4	109	17	20	1040	28400	standard			92	221
300	849	harkVertebrae19-04-10.csv	F	NIST610	42	42					na	20100419	4	109	17	23	1043	28403	standard			105	240
301	901	harkVertebrae20-04-10.csv	F	NIST610	43	43					na	20100420	4	110	9	19	559	29359	standard			89	216
302	902	harkVertebrae20-04-10.csv	F	NIST610	43	43					na	20100420	4	110	9	22	562	29362	standard			96	221
303	903	harkVertebrae20-04-10.csv	T	SHH	0	43	6		f	199392	SHH B	20100420	4	110	9	28	568	29368	edge	5.5	5.5	133	445
304	904	harkVertebrae20-04-10.csv	T	SHH	0	43	6		m	199379	SHH B	20100420	4	110	9	34	574	29374	edge	4	3.4	91	295
305	905	harkVertebrae20-04-10.csv	T	SHH	0	0	6		f	199388	SHH B	20100420	4	110	9	40	580	29380	edge	5	4.9	92	360
306	906	harkVertebrae20-04-10.csv	T	SHH	0	0	6			199367	SHH B	20100420	4	110	9	47	587	29387	edge	4.5	4.9	90	332
307	907	harkVertebrae20-04-10.csv	T	SHH	0	0	6		f	199383	SHH B	20100420	4	110	9	57	597	29397	edge	4.9	5.2	89	339
308	908	harkVertebrae20-04-10.csv	T	SHH	0	0	6		f	199387	SHH B	20100420	4	110	10	3	603	29403	edge	4.2	3.8	150	400
309	909	harkVertebrae20-04-10.csv	T	SHH	0	0	6		m	199377	SHH B	20100420	4	110	10	9	609	29409	edge	4.5	4.9	87	340
310	910	harkVertebrae20-04-10.csv	T	SHH	0	0	4	660	m	130681	SHH L	20100420	4	110	10	18	618	29418	edge	4.1	4.4	135	332
311	911	harkVertebrae20-04-10.csv	T	SHH	0	0	2	515	m	242050	SHH L	20100420	4	110	10	23	623	29423	edge	3.6	3.6	86	276
312	912	harkVertebrae20-04-10.csv	T	SHH	0	44	3	713	m	243505	SHH L	20100420	4	110	10	30	630	29430	edge	4.8	4.8	97	323
313	913	harkVertebrae20-04-10.csv	F	NIST610	44	44					na	20100420	4	110	10	38	638	29438	standard			90	207
314	914	harkVertebrae20-04-10.csv	F	NIST610	44	44					na	20100420	4	110	10	42	642	29442	standard			93	209
315	915	harkVertebrae20-04-10.csv	T	SHH	0	44	2	440	m	242003	SHH L	20100420	4	110	11	1	661	29461	edge	3.3	3.1	90	258
316	916	harkVertebrae20-04-10.csv	T	SHH	0	0	2	446	m	312075	SHH L	20100420	4	110	11	6	666	29466	edge	3.2	2.9	90	185
317	917	harkVertebrae20-04-10.csv	T	SHH	0	0	2	420	m	210927	SHH L	20100420	4	110	11	12	672	29472	edge	2.9	2.9	129	247
318	918	harkVertebrae20-04-10.csv	T	SHH	0	0	2	440	m	210905	SHH L	20100420	4	110	11	16	676	29476	edge	3.1	3.1	89	249
319	919	harkVertebrae20-04-10.csv	T	SHH	0	0	2	420	f	210910	SHH L	20100420	4	110	11	21	681	29481	edge	2.8	3.1	88	241
320	920	harkVertebrae20-04-10.csv	T	SHH	0	0	2	450	NA	241991	SHH L	20100420	4	110	11	27	687	29487	edge	3	2.9	120	279
321	921	harkVertebrae20-04-10.csv	T	SHH	0	0	3	660	m	216076	SHH L	20100420	4	110	11	34	694	29494	edge	4.7	4.8	90	326
322	922	harkVertebrae20-04-10.csv	T	MIS	0	0	1	630	m	211011	MIS A	20100420	4	110	11	44	704	29504	edge	4.9	6.7	106	391
323	923	harkVertebrae20-04-10.csv	T	MIS	0	0	1	680	m	211035	MIS A	20100420	4	110	11	52	712	29512	edge	5.7	7.3	88	418
324	924	harkVertebrae20-04-10.csv	F	MIS	0	0	6	440	f	118084	MIS A	20100420	4	110	11	56	716	29516	edge	3	4.3	80	241
325	925	harkVertebrae20-04-10.csv	T	MIS	0	45	1	685	m	211028	MIS A	20100420	4	110	12	2	722	29522	edge	4.5	7.5	77	283
326	926	harkVertebrae20-04-10.csv	F	NIST610	45	45					na	20100420	4	110	12	11	731	29531	standard			89	191
327	927	harkVertebrae20-04-10.csv	F	NIST610	45	45					na	20100420	4	110	12	15	735	29535	standard			88	184
328	928	harkVertebrae20-04-10.csv	T	MIS	0	45	6	658	m	165528	MIS A	20100420	4	110	12	24	744	29544	edge	4.3	5.8	126	389
329	929	harkVertebrae20-04-10.csv	T	MIS	0	0	6	425	m	165539	MIS A	20100420	4	110	12	29	749	29549	edge	2.8	3.8	89	231
330	930	harkVertebrae20-04-10.csv	T	MIS	0	0	1	680	m	211017	MIS A	20100420	4	110	12	34	754	29554	edge	5	5.9	98	364
331	931	harkVertebrae20-04-10.csv	T	SHH	0	0	8			999019	SHH V	20100420	4	110	12	58	778	29578	edge	16.4	16.2	100	1104
332	932	harkVertebrae20-04-10.csv	T	SHH	0	46	8			999014	SHH U	20100420	4	110	13	14	794	29594	edge	10.5	11.7	81	684
333	933	harkVertebrae20-04-10.csv	F	NIST610	0	46					na	20100420	4	110	13	22	802	29602	standard			101	180
334	934	harkVertebrae20-04-10.csv	F	NIST610	46	46					na	20100420	4	110	13	27	807	29607	standard			84	203
335	935	harkVertebrae20-04-10.csv	F	NIST610	46	46					na	20100420	4	110	13	33	813	29613	standard			99	220
336	936	harkVertebrae20-04-10.csv	T	SHH	0	47	8			999047	SHH U	20100420	4	110	15	37	937	29737	edge	12	11.8	99	810
337	937	harkVertebrae20-04-10.csv	F	NIST610	47	47					na	20100420	4	110	15	44	944	29744	standard			83	203
338	938	harkVertebrae20-04-10.csv	F	NIST610	47	47					na	20100420	4	110	15	49	949	29749	standard			96	216
339	939	harkVertebrae20-04-10.csv	T	SHH	0	47	8			999036	SHH U	20100420	4	110	15	58	958	29758	edge	12.1	12.8	100	813
340	940	harkVertebrae20-04-10.csv	T	SHH	0	0	8			999035	SHH S	20100420	4	110	16	13	973	29773	edge	13.7	13.6	100	810
341	941	harkVertebrae20-04-10.csv	T	SHH	0	0	8			999016	SHH S	20100420	4	110	16	33	993	29793	edge	16.9	18.3	99	1030
342	942	harkVertebrae20-04-10.csv	T	SHH	0	0	8			999015	SHH S	20100420	4	110	16	44	1004	29804	edge	13.1	14	106	920
343	943	harkVertebrae20-04-10.csv	T	SHH	0	48	8			999034	SHH S	20100420	4	110	16	53	1013	29813	edge	9.5	9	129	684
344	944	harkVertebrae20-04-10.csv	T	SHH	0	48	8			999033	SHH S	20100420	4	110	17	3	1023	29823	edge	9.7	9.3	83	661
345	945	harkVertebrae20-04-10.csv	F	NIST610	48	48					na	20100420	4	110	17	10	1030	29830	standard			133	250
346	946	harkVertebrae20-04-10.csv	F	NIST610	48	48					na	20100420	4	110	17	15	1035	29835	standard			84	210