AN ABSTRACT OF THE DISSERTATION OF

Wade D. Smith for the degree of Doctor of Philosophy in Fisheries Science

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Title: Vertebral Elemental Markers in Elasmobranchs: Potential for Reconstructing Environmental History and Population Structure

Abstract approved:

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Differences in the chemical composition of calcified structures can be used to reveal natal origins, connectivity, metapopulation structure, and reconstruct the environmental history or movement patterns of many marine organisms. Sharks, skates, and rays (elasmobranchs) lack the calcified structures, known as otoliths, that are typically used for geochemical studies of dispersal and natal origin in fishes. If the incorporation of elements into shark and ray vertebrae is related to environmental conditions, the geochemical composition of cartilaginous vertebrae may also serve as natural tags and records of environmental history in elasmobranch populations. I used complementary laboratory and field studies to address several key assumptions regarding the incorporation of elements in elasmobranch vertebrae, providing the first detailed studies to assess relationships between water and vertebral chemical composition and the spatial and temporal variation of vertebral elemental signatures in

this subclass of fishes. To validate the uptake and incorporation of elements from water to vertebrae, I conducted two laboratory studies using round stingrays, *Urobatis halleri*, as a model species. First, I examined the effects of temperature (16°, 18°, 24° C) on vertebral elemental incorporation (Li/Ca, Mg/Ca, Mn/Ca, Zn/Ca, Sr/Ca, Ba/Ca) and found that temperature had strong, negative effects on the uptake (measured as a partition coefficient, $D_{Element}$) of magnesium and Ba and positively influenced manganese incorporation. Second, I tested the relationship between water and vertebral elemental composition by manipulating dissolved barium (Ba) concentrations (1x, 3x, 6x ambient concentrations) and found significant differences among rays from each treament. I also evaluated the influence of natural variation in somatic growth and vertebral precipitation rates on elemental incorporation. Finally, I examined the accuracy of classifying individuals to known environmental histories (temperature and barium treatments) using vertebral elemental composition. There were no significant relationships between elemental incorporation and somatic growth or vertebral precipitation rates for any elements with the exception of Zn. Relationships between somatic growth rate and D_{Zn} were, however, inconsistent and inconclusive. Elemental variation of vertebrae reliably distinguished *U. halleri* based on temperature (85%) and [Ba] (96%) history. These results support the assumption that vertebral elemental composition reflects the environmental conditions during deposition and validates the use of vertebral elemental signatures as natural markers in an elasmobranch.

To evaluate the utility of vertebral geochemistry as intrinsic markers of natal origin, I collected vertebrae of young-of-the-year scalloped hammerhead sharks (*Sphyrna lewini*) from artisanal fishery landings at six sites along the Pacific coast of Mexico and Costa Rica between 2007-2009. A total of 386 vertebrae were used to assess patterns of spatial and temporal variation in elemental composition using laser ablation-inductively coupled plasma mass spectrometry. A protracted pupping period was confirmed for *S. lewini*, with newborn pups being recorded from May through mid-October. Natal elemental signatures detected in the vertebrae of the sharks varied significantly among sites and could be used to identify source populations. All element-to-calcium ratios included in these analyses (Li/Ca, Mg/Ca, V/Ca, Cr/Ca, Mn/Ca, Rb/Ca, Sr/Ca, Ba/Ca, Pb/Ca) were useful for the discerning natal origins of sharks; however, Ba, Sr, Mn, and Mg ratios most consistently generated the greatest discriminatory power based on step-wise discriminant function analyses. Classification accuracy to putative nursery areas (natal signature) and location of capture (edge signature) based on step-wise discriminant function analysis ranged from low (30-60%) to high (80-100%) depending on the degree of spatial and temporal resolution by which the data were grouped for analysis (e.g. pooled across months, early season, late season). All classification accuracies exceeded chance expectations and assignment to putative nursery areas and sites of capture were accomplished with up to 100% accuracy in several models. I found significant intra-annual differences in natal elemental signatures within the three primary study sites, which likely contributed to the low assignment accuracies when data were analyzed

across months of collection. Significant differences in natal elemental signatures were also detected across years. However, pair-wise analyses revealed that site-specific inter-annual variation was driven by differences associated with samples collected in 2009. Natal elemental signatures were similar between 2007 and 2009, indicating some consistency in site-specific vertebral chemistry across years. These results confirmed that vertebral elemental signatures can be applied to distinguish individuals across small (5s km), moderate (100s km), and large spatial scales (>1000 km). The potential for intra-annual variation in natal signatures within a year-class highlights the importance of cohort-specific analyses and the development of a spatial atlas of natal vertebral elemental signatures for studies of natal origin and population connectivity.

The findings of my laboratory validation experiments and field study establish that geochemical analyses of vertebrae can provide reliable information on the spatial ecology and environmental history of shark and ray populations. The use of elemental markers offers a new approach for the study and conservation of this historically vulnerable group of fishes.

Vertebral Elemental Markers in Elasmobranchs: Potential for Reconstructing Environmental History and Population Structure

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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

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CONTRIBUTION OF AUTHORS

Dr. Selina Heppell was involved in project overview, assisted with lab work, and writing of Chapters 2 and 3. Dr. Jessica Miller was involved in experimental design, lab work, data analysis, and writing of Chapters 2 and 3. Dr. Fernando Márquez-Farías assisted with survey coordination and sample collection for Chapter 3.

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CHAPTER 1

INTRODUCTION

Shark and ray (elasmobranch) populations are now experiencing their highest rate of reduction through fishing activities than at any time in history (Clarke et al. 2006, Dulvy et al. 2008, Musick and Musick 2011). The increasing exploitation of this group is especially alarming because many of the life history traits typically associated with this group of fishes (including long life spans, slow growth, low fecundity, and late ages at maturity) severely restrict their ability to sustain fishing pressure or recover from over-exploitation (Hoenig and Gruber 1990, Smith et al. 1998). Many elasmobranchs form aggregations for food resources, mating, or birthing, thereby increasing their vulnerability to directed, localized fishing pressure (Springer 1967, Jacoby et al. 2012). Concerns about the resilience and persistence of elasmobranch populations are not new to fisheries science; Holden (1973, 1977) cautioned that this group of fishes offered limited opportunities for long-term exploitation and summarized the rapid rise and collapse of several historic shark fisheries.

Fisheries exploitation not only reduces population sizes but can alter community structure and function. As upper trophic level predators, sharks and rays have a substantial capacity to influence the abundance, composition, and behavior of prey species within a community. The removal of sharks and rays may have significant cascading effects on the biological diversity and structure of associated

communities and ecosystem connectivity (Stevens et al. 2000, Heithaus et al. 2007, Ferretti et al. 2010). In spite of this knowledge, conservation measures have not been enacted for the majority of the world's shark and ray populations and most fisheries for these species remain unregulated (Musick and Musick 2011). A general lack of detailed biological and fisheries information for exploited sharks and rays constrains efforts to develop appropriate conservation strategies.

Studies of sharks and rays are complicated due to their high mobility, broad spatial distributions, and use of diverse habitats. Individuals undertake large-scale (>1000 km) and small-scale (1-10 km) movements in response to reproductive and foraging needs, which influence population dynamics and community interactions across broad temporal and spatial scales (Papastamatiou et al. 2009, Speed et al. 2010). An improved understanding of elasmobranch dispersal pathways, shifts in habitat use, and migration would provide valuable insight into population structure, connectivity, and the evolution of life history patterns. Details on movement patterns and population structure are essential for devising effective conservation strategies, such as the delineation of appropriate spatial scales and priorities for conservation.

The analysis of natural elemental markers deposited in calcified structures provides a promising technique for identifying population connectivity, natal origins, and movement patterns (Campana 2000, Campana and Thorrold 2001). Elemental markers (also known as elemental signatures, fingerprints, or tags) have proven to be useful in ecological studies of bony fishes, providing insight into migratory patterns (Campana et al. 2007), population structure (Gillanders 2002), and dispersal (Miller

and Shanks 2004). This approach has been advanced by the chemical analysis of otoliths, inert calcified structures that form in the inner ear of fishes. Otoliths grow continuously over a lifetime and form daily and annual growth increments from which age can be determined (Campana 1999). Elements are assimilated from the surrounding environment as a byproduct of respiration and feeding and incorporated into otoliths as they grow (Campana and Thorrold 2001). Ratios and concentrations of elements are therefore continually incorporated into otoliths and may act as records of movements between physically or chemically distinct habitats or water masses. Because otoliths are metabolically inert, it is unlikely that the elemental composition is altered following deposition. Thus, all individuals within a population potentially carry chronological records of environmental history within their otoliths that may serve as intrinsic geochemical tags. Elemental analytical tools have recently been applied to assess natal origins, nursery ground contributions, and population structure for a diverse array of taxa, including mussels (Becker et al. 2007), embryonic crab (Carson et al. 2008), octopus (Doubleday et al. 2008), and squid (Zumholz et al. 2007). Analyses of naturally-occurring geochemical markers in highly mobile shark populations could provide a new and comparatively rapid approach for identifying movement patterns and population structure, enabling the identification and delineation of biological hotspots and spatial scales that are ecologically relevant to populations of concern. The validity of using these markers for sharks and rays, however, has not yet been evaluated.

Sharks and rays are cartilaginous fishes and lack otoliths which have been reliably used for studies of dispersal and population connectivity in bony fishes. Vertebrae of elasmobranchs also continue to grow throughout the life of the organism and develop alternating annual band pairs from which ages can be determined (Cailliet and Goldman 2004). Resorption or physiological reworking of elasmobranch vertebrae, as occurs in bone, would alter vertebral chemical composition and limit any potential utility as geochemical tags. However, the function and properties of elasmobranch cartilage, and vertebrae in particular, are fundamentally different from the cartilage or bone of other vertebrates (Clement 1992, Dean and Summers 2006). In most vertebrates, cartilage cells (chondrocytes) serve connective functions or are replaced by bone cells (osteocytes) to promote skeletal growth (Mayne and von der Mark, 1983). However, elasmobranch cartilage, unlike bone, lacks an internal blood supply and possesses a permanent mineralized rind that shows no evidence of remodeling or resorption (Doyle 1968, Mayne and von der Mark 1983, Clement 1992). Therefore, the elemental composition of elasmobranch vertebrae is likely to be stable and may have the potential to reveal site-specific markers and chemical clues from which environmental history could be reconstructed by referencing growth bands that correspond to a time interval of interest.

Several life history characteristics of elasmobranchs may enhance the potential for deposition of distinctive elemental markers within their vertebrae. Unlike most fishes, sharks and rays lack a larval period. Many live-bearing (viviparous) and egg-laying (oviparous) species rely on nursery areas for birthing and early development of

offspring (Heupel et al. 2007, Hoff 2010). Large, highly mobile sharks tend to give birth in shallow areas of estuaries, embayments, or nearshore coastal habitats that are not occupied by adults (Bass 1978, Simpfendorfer and Milward 1993). A growing number of studies have documented that females may demonstrate high levels of fidelity (philopatry) to natal sites, returning annually to the same areas to give birth (Keeney et al. 2003, Hueter et al. 2005, DiBattista et al. 2008). Newborn sharks and rays may remain within these areas for the first weeks, months, or years of their lives before moving onto secondary nurseries or adult habitats (Duncan and Holland 2006, Chapman et al. 2009). As a result, distinctive estuarine or region-specific elemental vertebral signatures may result from residence in different water masses. Because juveniles and adults tend to occupy different habitats, it may be possible to quantify the extent of connectivity and dispersal among populations and habitats, which may ultimately allow for the identification of the sites (or regions) that contribute the greatest proportions to overall population productivity (Beck et al. 2001, Heupel et al. 2007).

Before ecological questions regarding dispersal pathways and population connectivity of elasmobranchs may be tested using geochemical signatures, key assumptions regarding the chemical composition of cartilaginous vertebrae must be evaluated. My dissertation research was designed to evaluate the effects of temperature and water elemental composition on elemental incorporation in captive elasmobranchs and to explore the potential for geographically distinct elemental signatures in the vertebrae of sharks captured in the field. This investigation

represents the first attempt to examine factors regulating elemental incorporation in elasmobranchs in a controlled laboratory study and the first application of vertebral chemistry as a potential geospatial tag in an elasmobranch species. I applied a combination of experimental laboratory research and field collection to test the hypotheses that: (i) vertebral elemental signatures reflect the chemical composition of their ambient water mass; (ii) elemental incorporation is mediated by water temperature; and (iii) elemental signatures deposited within vertebrae can be used to distinguish natal origin of young-of-the-year sharks.

Laboratory Validation: Elemental Incorporation

In Chapter 2, I present the results of two controlled laboratory experiments. First, I determined the extent of discrimination and quantified the effects of temperature (three treatments) and growth rate on vertebral elemental composition of six element-to-calcium ratios (Li/Ca, Mg/Ca, Mn/Ca, Zn/Ca, Sr/Ca, Ba/Ca) using round stingrays, *Urobatis halleri*, as a model species. Second, I manipulated dissolved barium concentrations to determine if there was a relationship between water and vertebral barium-to-calcium ratios. I examined growth rate as a potential co-variate for the incorporation of specific elements and the resulting multi-elemental signatures. Finally, I assessed the accuracy of classifying individuals to known environmental histories (temperature and barium treatments) using vertebral elemental composition. This study is the first evaluation of trace and minor elemental incorporation into elasmobranch vertebrae, and serves as a key validation experiment for the

identification and potential variability of naturally occurring elements in wild populations.

Field Application: Natal Elemental Signatures

In Chapter 3, I evaluate spatial and temporal variation in vertebral elemental signatures of young-of-the-year scalloped hammerhead sharks (*Sphyrna lewini*). Samples were collected from three primary locations in Sinaloa, México and three distant locations between 2007-2009, spanning >3000 km of coastline. I determined the variation of natal signatures from each site to see if elemental composition could be used to accurately link individuals to their putative nursery grounds. I examined intra- and inter-annual variation in natal elemental signatures among sites. Results confirmed that vertebral elemental signatures can be applied to distinguish individual sharks across small (5s km), moderate (100s km), and large spatial scales (>1000 km), but variability among and within seasons will require consideration of local oceanographic and hydrologic characteristics when designing surveys, analyses of specific cohorts within sites and years, and expanded sampling of potential source populations to accurately assign natal origins and estimate connectivity.

Conclusions

In Chapter 4, I synthesize the primary findings of the laboratory experiments and field study. Additionally, I consider caveats, future directions, and applications of

this technique for improved conservation and management of elasmobranch populations.

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CHAPTER 2

ELEMENTAL MARKERS IN ELASMOBRANCHS: EFFECTS OF ENVIRONMENTAL HISTORY AND GROWTH ON VERTEBRAL CHEMISTRY

Wade D. Smith

ABSTRACT

Differences in the chemical composition of calcified skeletal structures (e.g. shells, otoliths) have proven useful for reconstructing the environmental history of many marine species. However, the extent to which ambient environmental conditions can be inferred from the elemental signatures within the vertebrae of elasmobranchs (sharks, skates, rays) has not been evaluated. To assess the relationship between water and vertebral elemental composition, we conducted two laboratory studies using round stingrays, *Urobatis halleri*, as a model species. First, we examined the effects of temperature (16°, 18°, 24° C) on vertebral elemental incorporation (Li/Ca, Mg/Ca, Mn/Ca, Zn/Ca, Sr/Ca, Ba/Ca). Second, we tested the relationship between water and subsequent vertebral elemental composition by manipulating dissolved barium concentrations (1x, 3x, 6x) in the tanks. We also evaluated the influence of natural variation in growth rate on elemental incorporation for both experiments. Finally, we examined the accuracy of classifying individuals to known environmental histories (temperature and barium treatments) using vertebral elemental composition. Temperature had strong, negative effects on the uptake of magnesium (D_{Mg}) and barium (D_{Ba}) and positively influenced manganese (D_{Mn}) incorporation. Temperature-

dependent responses were not observed for lithium and strontium. Vertebral Ba/Ca was positively correlated with ambient Ba/Ca. Partition coefficients (D_{Ba}) revealed increased discrimination of barium in response to increased dissolved barium concentrations. There were no significant relationships between elemental incorporation and somatic growth or vertebral precipitation rates for any elements except Zn. Relationships between somatic growth rate and D_{Zn} were, however, inconsistent and inconclusive. Geochemical variation of vertebrae reliably distinguished individual rays from each treatment based on temperature (85%) and Ba exposure (96%) history. These results support the assumption that vertebral elemental composition reflects the environmental conditions during deposition and validates the use of vertebral elemental signatures as natural markers in an elasmobranch. Vertebral elemental analysis is a promising tool for the study of elasmobranch population structure and habitat use that warrants further exploration.

INTRODUCTION

The trace and minor elemental composition of biomineralized structures can provide insight into the environmental conditions in which the elements were deposited. Elemental assays of coral skeletons and foraminifera tests, for example, have been commonly applied as surrogates of past climatic or oceanographic conditions (paleoproxies) [1, 2, 3]. Recently, considerable attention has been directed toward analyses of calcified structures, such as fish otoliths, to gain insight into contemporary ecological processes and inform management and conservation efforts

[4, 5, 6]. Elements are naturally acquired through respiratory and dietary pathways and assimilated into actively calcifying structures such as scales, shells, and otoliths [7, 8]. Ratios and concentrations of elements in these structures can reflect the physiochemical conditions of the ambient environment. If the calcified material is deposited in a temporally consistent pattern and is not subjected to resorption or reworking, elemental composition can provide permanent chronological records of the environmental conditions experienced over a lifetime.

The most widespread and expanding application of elemental markers in biomineralized structures has occurred using the otoliths of fishes [4, 5]. Otoliths are metabolically inert calcium carbonate structures (typically in the form of aragonite) that are used for balance and hearing in teleost fishes. Elements are incorporated into otoliths daily as new aragonite is crystallized onto an organic framework of proteins [8]. The elemental composition of other calcified structures, including vertebrae [9], scales [10], fin rays [11], and bone [12], have been evaluated as potential elemental markers in fishes. However, unlike otoliths, these calcium phosphate structures (in forms of hydroxyapatite) are metabolically active, subject to resorption and provide short-term and unstable geochemical records of environmental history [4].

The elemental composition of biogenic calcified structures is not a simple reflection of environmental conditions. A variety of physiological barriers and processes are encountered as elements are taken up from the water through the gills or intestine, transferred through the blood plasma, and eventually incorporated into biomineralized structures [8]. Physiological regulation of internal elemental

composition can result in active discrimination or preferential uptake of elements, thus modifying relationships with ambient environmental conditions. Trace metals such as manganese and zinc that are essential for metabolic and cellular transport processes are tightly regulated [13]. Conversely, physiological regulation of elements that do not play critical biological roles or generate toxic effects may be comparatively minimal [8, 14]. At the site of calcification, elemental incorporation can be inhibited or promoted by kinetic effects associated with mineralization. Elemental composition can be further modified by temperature, which has a profound influence on the rates of chemical and metabolic processes [15, 16]. Individual variation in growth rates, independent of temperature, can also influence elemental composition [17, 18]. Metabolic and kinetic effects on elemental incorporation, however, do not negate the utility of an element as a geochemical marker, providing the degree of regulation is constant or predictable.

Sharks, skates, and rays (elasmobranchs) are cartilaginous fishes that lack otoliths. Elasmobranch skeletons are composed of mineralized cartilage, an impure (non-stochiometric) form of carbonated calcium phosphate (hydroxyapatite) [19]. Like the otoliths of teleost fishes, elasmobranch vertebrae are deposited by the precipitation of elements onto a matrix of proteins and continue to grow throughout the life of the organism [19]. Vertebral growth bands are typically deposited seasonally, allowing individual ages to be determined. Resorption or physiological reworking of vertebrae, as has been observed in scale and bone hydroxyapatite, would alter the elemental composition and severely limit their utility as records of the

physiochemical environment. However, the function and properties of elasmobranch cartilage, and vertebrae in particular, are fundamentally different than those of other vertebrates [20, 21]. Whereas calcified cartilage is usually a transitional tissue that is ultimately replaced by bone, elasmobranch cartilage possesses a permanent mineralized rind that shows no direct evidence of remodeling or resorption [19, 22]. Doyle [23] confirmed that mineralization and growth of elasmobranch cartilage is accomplished through surface accretion that proceeds without altering the mineral or protein matrix. Therefore, the elemental composition of elasmobranch vertebrae is unlikely to be modified after deposition and could therefore provide permanent chronological records of the environmental conditions experienced by individuals.

Elemental and isotopic analyses of elasmobranch vertebrae to date have been predominately directed toward age validation [24, 25] and dietary studies [26, 27]. The potential use of vertebral elemental composition to delineate elasmobranch populations was first proposed by Edmonds et al. [28] following their analyses of jaw cartilage which revealed spatially explicit patterns of elemental variation. Age-related changes in vertebral chemistry have recently been examined to discern movement patterns in sharks [29, 30]. Although significant temporal and spatial variation in elemental composition have identified within elasmobranch vertebrae, interpretations of these differences are hindered by a lack of understanding as to how ambient vertebral chemistry relates to environmental conditions. Without an understanding of the factors that influence elemental incorporation and the extent of regulation, it is impossible to know if the elemental composition of a calcified structure presents a

reliable record of environmental history. Controlled laboratory validation studies provide a platform for quantifying abiotic and biotic effects on elemental incorporation and identifying those elements that are most likely to serve as useful indicators of the environment in which they were deposited. Incorrect assumptions about elemental relationships and an inadequate understanding of the mechanisms determining incorporation can lead to erroneous interpretations of field data.

Key assumptions regarding vertebral elemental incorporation in relation to the physical and chemical environment must be evaluated before broader ecological questions and hypotheses can be addressed using naturally occurring geochemical markers in elasmobranchs. We quantified the effects of temperature and growth rate on vertebral elemental incorporation through controlled laboratory studies using the round stingray, *Urobatis halleri*, as a model species. We manipulated environmental concentrations of barium (Ba) to determine the extent to which vertebral elemental ratios reflect the ambient environment. Finally, we tested the utility of these elemental markers to distinguish the environmental history experienced by individual rays using multivariate classification models. Our approach allowed us to test the hypotheses that: (*i*) elemental incorporation in vertebrae is mediated by water temperature; (*ii*) vertebral Ba to calcium ratios (Ba/Ca) reflects water Ba/Ca; (*iii*) growth rate does not significantly influence vertebral elemental composition; and (*iv*) vertebral elemental markers can distinguish individuals based on differences in environmental history. This investigation represents the first attempt to evaluate the utility of vertebral chemistry as potential records of environmental history in elasmobranchs.

MATERIALS AND METHODS

Ethics statement

This investigation was conducted with a permit from the California Department of Fish and Game (803099-01) and in strict accordance with guidelines established by the American Fisheries Society and National Institutes of Health for the use of fishes in research. Experimental protocol was approved by Oregon State University's Institutional Animal Care and Use Committee (3783).

Specimen collection

The round stingray, *Urobatis halleri*, is a benthic, live-bearing elasmobranch that occurs in estuaries and nearshore coastal soft bottom habitats from Panama to Eureka, California, USA [31]. The vertebrae of round rays are well-calcified and the annual deposition of a distinctive band pair (one opaque, one translucent growth band) has been validated, making reliable estimates of age and growth rates possible [32]. We selected *U. halleri* as a model elasmobranch species for vertebral elemental incorporation studies because of their record of hardiness in captivity, relatively small body size (to 31 cm disc width, DW), availability/occurrence in shallow coastal environments, and validated periodicity of vertebral growth band formation.

Juvenile *U. halleri* were collected by beach seine at Seal Beach, California (33° 44' N; 118° 06' W) on 6 February 2009 and transported to the Hatfield Marine Science Center (HMSC) in Newport, Oregon. A total of 108 rays were collected, consisting of 67 females and 41 males. Vertebral band counts performed at the end of

this study indicated that these rays were age 0 (i.e. young-of-the-year; n = 104) and age 1 (n = 4) at the time of capture.

Experimental design

We conducted two consecutive laboratory experiments using the same rays to evaluate the effects of: 1) temperature and 2) dissolved barium concentration on the incorporation of elements into the vertebrae of an elasmobranch. Following collection and transport, *U. halleri* were allowed to acclimate to lab conditions for five weeks. Total weight, DW, and sex were then recorded. Twelve rays were randomly assigned to each of nine 1,700 L independently re-circulating tanks containing a thin layer of sand substrate. Water from each tank circulated through individual wet-dry sumps containing biological filter media to reduce the build-up of potentially harmful nitrogenous waste products. The same combination of squid (*Doryteuthis opalescens*), herring (*Clupea pallasi*), or shrimp (*Pandalus jordani*) was provided daily. Remaining food and waste were removed daily. One-quarter to one-half volume water changes were completed approximately every 1-2 weeks to maintain water quality. Seawater was pumped from Yaquina Bay through HMSC's seawater system. Tanks were covered with clear plastic lids to reduce evaporation. A 12 hour light:dark photoperiod was established for both experiments. Temperature and salinity were recorded daily and water samples were collected weekly (Tables 1, 4).

Temperature experiment

Following acclimation and initial measurement, all specimens were injected with a 25 mg kg^{-1} dose of oxytetracycline [33] to provide a visual indicator within the vertebrae that coincided with the initiation of the experiment. Cooler water from Yaquina Bay was heated and three replicate treatments of 15°C, 18°C, and 24°C were established and maintained, corresponding with the mean winter, summer, and approximate maximum water temperatures at the site of collection [34]. The temperature experiment was conducted for eight months (April – December, 2009) to ensure that adequate vertebral deposition occurred for elemental analysis in all treatments. Three individuals died before the conclusion of the study and were excluded from analysis; praziquantel (Sigma-Aldrich) was subsequently administered to all tanks (10 mg/L) to eliminate parasitic flatworms during two weeks in July and August.

Barium manipulation experiment

The relationship between water and vertebral elemental composition was further evaluated through experimental manipulation of dissolved barium concentrations. Barium was selected because of its utility as a geospatial marker demonstrated in both field [35, 36] and laboratory studies [37, 38]. Upon conclusion of the temperature experiment, all specimens were weighed, measured, and injected with a 5 mg kg^{-1} dose of the fluorescent marker calcein to distinguish vertebral deposition between the experiments [39]. Water temperatures were gradually adjusted

to 19°C in all tanks over two weeks. Following this acclimation, Ba treatments were systematically assigned to each tank to ensure that one tank from each prior temperature treatment was represented within each Ba treatment. Three tanks were designated as controls that reflected ambient dissolved Ba/Ca ratios (1x). Three tanks were spiked with three times (3x) and three tanks were spiked with six times (6x) the estimated mean local Ba concentration of 4.50 μmol mol^{-1}, providing triplicate treatments of 1x, 3x, and 6x Ba concentrations. These values fall within the naturally occurring regional range for estuaries and coastal waters [38, 40]. Elevated Ba treatments were prepared by the addition of $BaCl_2$ (JT Baker) to ambient seawater. Diet, feeding, cleaning, and light regimes were maintained as previously described. Water changes were, however, made from an appropriate supply of 1x, 3x, or 6x Ba/Ca seawater sources. We excluded seven specimens from analysis (five from a single tank, 6x treatment) because of mortality that occurred prior to the completion of the study. All rays were sacrificed after 109 days (December, 2009 – April, 2010) with an injection of tricane methanesulfonate (Finquel, MS-222) in accordance with approved Institutional Animal Care and Use protocol. Rays were weighed and measured before vertebrae were excised and stored frozen for subsequent analysis.

Vertebral preparation and elemental analysis

Sample preparation for elemental analysis followed procedures typical to age and growth studies of elasmobranchs [e.g. 41] but incorporated processing methods associated with otolith chemistry studies [e.g. 42] to minimize contamination. Tissue

was removed from vertebrae with acid-washed non-metallic dissecting tools and individual centra were separated and dried in a Class 100 laminar flow bench. Vertebral centra were soaked for 10 minutes in ultrapure 30% hydrogen peroxide (ULTREX, J.T. Baker) to loosen remaining connective tissue, triple rinsed, and ultrasonically cleaned in Nanopure® (18 M Ohm, Barnstead International) water for 45 minutes. Samples were rinsed, dried, embedded in polyester casting resin infused with a spike of indium, and sectioned to a width of ~0.4 mm using a low speed diamond saw (Fig. 2.1a, 2.1b). Resulting thin-sections were mounted to acid-washed glass slides, polished with lapping film (3M^{TM}; 30, 12, 5, 3, 1 μm), and rinsed. Sectioned centra were randomly attached to acid-washed slides to prevent systematic bias. Sample slides were rinsed with ultrapure 1% nitric acid (HNO_3; ULTREX, J.T. Baker), cleaned ultrasonically for 15 minutes, triple rinsed, and dried in Class 100 conditions.

The elemental composition of *U. halleri* vertebrae was quantified using laser ablation-inductively coupled plasma mass spectrometry (LA-ICPMS). Analyses were conducted at Oregon State University's WM Keck Collaboratory for Plasma Spectrometry in Corvallis, Oregon using a VG PQ ExCell ICPMS with a DUV193 excimer laser (New Wave Research). The laser was set at a pulse rate of 5 Hz with an ablation spot size of 80 μm and translated across the sample at 5 μm s^{-1}. Laser transects were positioned within the corpus calcareum of the vertebral centrum to collect a time series of elemental composition that included both experimental periods (Fig. 2.1b). Transects were pre-ablated (100μm spot size, 2 Hz, 100 μm s^{-1}) to further

reduce potential sample contamination. We collected data on 17 elements: lithium, magnesium, calcium, titanium, vanadium, chromium, manganese, cobalt, copper, zinc, rubidium, strontium, zirconium, cadmium, barium, lanthanum, and lead. However, only magnesium (^{25}Mg), calcium (^{43}Ca), manganese (^{55}Mn), zinc (^{66}Zn), strontium (^{88}Sr), and barium (^{138}Ba) were consistently above detection limits. Lithium (^{7}Li) was often found in concentrations near and occasionally below detection limits but was included in analyses. Samples with measurements of Li that were not above background levels were dropped from further analysis (19% temperature experiment, 29% Ba manipulation experiment). Lead was incorporated into vertebrae at levels exceeding detection limits when specimens were living off Seal Beach, CA. However, Pb/Ca ratios were not consistently above detection limits ($\geq$4.19 μmol mol^{-1}) while rays were maintained at HMSC.

Data processing followed procedures described in Miller & Shanks [42]. To evaluate instrument drift and daily variation in instrument sensitivity, a National Institute of Standards and Technology (NIST) 612 glass standard was run with each sample slide. Background levels of analyte isotopes were measured and subtracted from values determined during vertebral ablation. Mean percent relative standard deviations (%RSD) of the NIST 612 standard were: Li = 5.2%, Mg = 12.6%, Ca = 3.3%, Mn = 4.5%, Zn = 8.3%, Sr = 3.7%, and Ba = 5.0% (n = 21). Time-resolved software (PlasmaLab®) allowed analyte counts to be integrated from specific positions along each vertebral transect. Regions for integration were determined using image analysis (Image ProExpress, Media Cybernetics®). We targeted areas that

corresponded with the mid-point of the temperature experiment and the final month of the Ba manipulation experiment for analysis to assure that adequate vertebral precipitation had occurred and to avoid sampling areas associated with the transition between experiments (Fig. 2.1c). Count data were normalized by ^{43}Ca to adjust for variability in instrument sensitivity and the amount of ablated material, then converted to elemental ratios based on measurements of the NIST 612 standard [43, 44]. Elemental ratios are presented in mmol mol^{-1} (Mg, Sr) or μmol mol^{-1} (Li, Mn, Zn, Ba).

Water collection and analysis

Dissolved elemental concentrations within and among treatments were evaluated by sampling the water from each tank weekly over the course of both experiments. Samples were collected in acid-washed plastic bottles, filtered with 0.2-μm syringe filters in a Class 100 laminar flow bench, acidified to <2 pH with ultrapure HNO_3 (ULTREX, J.T. Baker) and stored refrigerated at ~4°C until analysis. A subset of samples was analyzed to determine the concentrations of Li, Mg, Ca, Mn, Zn, Sr, and Ba during the temperature (n = 11 dates x 9 tanks) and Ba manipulation (n = 7 dates x 9 tanks) experiments. Water samples were selected to provide increased representation during the middle of the temperature experiment (August - October) and the latter half of the Ba manipulation experiment (February - March), the same time period from which vertebral elemental data were targeted.

Elemental concentrations were determined using a Leeman-Teledyne inductively coupled plasma optical emission spectrometer (ICP-OES) (Li at 670.8 nm, Mg at 279.1 nm, Ca at 317.9 nm, Mn at 259.4 nm, Zn at 206.2, Sr at 421.5 nm, and Ba at 493.4 nm). Filtered, acidified samples were diluted 100x for the determination of Mg, Ca, and Sr and 25x for Li, Mn, Zn, and Ba. Matrix-matched standards were created using SPEX Certiprep Group® certified reference materials (CRMs), NIST liquid standard (1643e), and a sodium chloride (NaCl) solution. Matrix-matched NIST standards and HNO_3 blanks were introduced throughout analysis to evaluate accuracy. Measured Li, Mg, Ca, Mn, Zn, Sr, and Ba concentrations were within 3%, 2%, 3%, 18%, 8%, 5%, and 3%, respectively, of certified values. A correction factor was applied to those elements that were ≥5% of known values (Mn, Zn and Sr). Repeated measurements of the same CRM calibration standard indicated that precision was within 1.2% for all elements (n = 7). Elemental concentrations are expressed as element to calcium ratios (Me/Ca) and presented in mmol mol^{-1} (Mg, Sr) or μmol mol^{-1} (Li, Mn, Zn, Ba).

Statistical analyses

Partition coefficients (D_{Me}, where the subscript typically indicates a trace metal (Me) of interest) characterize the relationship between the elemental composition of a solution with that of a solid, actively calcifying structure [45]. D_{Me} provide a standardized metric for comparing the effects of temperature, dissolved elemental concentration, and growth rates on elemental incorporation within and among species

and calcified structures. D_{Me} were calculated for each element by dividing a given element to calcium ratio (Me/Ca) measured from individual vertebrae by the mean Me/Ca ratio measured from the water of the corresponding tank [45].

Mean salinity, temperature, Me/Ca_{water}, and $Me/Ca_{vertebrae}$ were compared among treatments using parametric and non-parametric approaches. Data were screened for outliers and assessed for normality and homogeneity of variance using Shapiro-Wilk's and Levene's tests, respectively [46]. Temperature and salinity data did not meet the assumptions of normality following transformation and were examined using non-parametric Kruskal-Wallis analysis of variance by ranks [46]. Water (Me/Ca_{water}) and vertebral elemental ($Me/Ca_{vertebrae}$, D_{Me}) data required log_{10}-transformation to conform to the assumptions of parametric statistical analysis.

Data collected from the temperature and Ba manipulation experiments were analyzed separately using the same procedures. As a first step, one-way multivariate analysis of variance (MANOVA) was applied to test for differences in mean Me/Ca_{water} and $Me/Ca_{vertebrae}$ among treatments, where treatment (temperature or Ba concentration) was a fixed factor and elemental ratios (Li/Ca, Mg/Ca, Mn/Ca, Zn/Ca, Sr/Ca, Ba/Ca) were the response variables. When significant differences among treatments were identified, Tukey's Honestly Significant Difference (THSD) tests were conducted to determine which groups accounted for the observed differences [47]. Effects of temperature and Ba concentration on D_{Me} were evaluated in each experiment by ANOVA with tanks nested within treatments as random variables and

the corresponding temperature or Ba treatment as a fixed factor. All MANOVAs and ANOVAs were completed using JMP (Version 8.0) statistical software.

Effects of growth and precipitation rates

Somatic growth and vertebral precipitation rates were determined to evaluate their influence on elemental incorporation. Because rays were not individually marked, we assumed that sex-specific size ranks were maintained within each tank during the experiments and estimated individual growth rates from these ranks [48]. Somatic growth rates were calculated as the difference in body size (DW) between the start and end of each experiment divided by the number of months that the experiment was conducted, providing an estimate of growth in mm DW $month^{-1}$. Changes in centrum diameter during each experiment were similarly calculated by subtracting the vertebral diameter at the beginning of a study, as indicated by a fluorescent mark, from that measured at the end of an experiment using Image Pro Plus® (Media Cybernetics). Vertebral deposition/precipitation rates were expressed as mm DW $month^{-1}$. Mean monthly growth rates were estimated for all tanks (n = 9) and compared among treatments using ANOVA with treatment as a fixed factor. Regression analyses of D_{Me} against somatic growth and vertebral precipitation rates were performed within each treatment.

Classification

The ability to accurately classify individuals based on treatment/environmental history was evaluated with discriminant function analysis (DFA) of the vertebral

Me/Ca data (i.e. Mg, Mn, Sr, Ba) generated from our temperature and Ba manipulation experiments. Because Li/Ca measurements were not available for all samples (i.e. below detection limits), Li was not included in these anlayses. Group classification accuracy was assessed using a leave-one-out jack-knife procedure [49]. We assumed that prior probabilities of group membership were proportional to group sample sizes. A chance-corrected classification (Cohen's kappa, κ) was also calculated to determine if predicted group assignments exceeded that of randomly assigning individuals to groups in proportion to their sample sizes [50]. A κ of 0 indicates that no improvement over chance was provided by the DFA and a κ of 1 signifies that perfect agreement was achieved. SYSTAT (Version 12.0) was used for DFA.

RESULTS

Temperature experiment

Water temperatures differed significantly among treatments, as intended (Kruskal-Wallis, H = 69.96, $p < 0.001$; Table 2.1). Salinity varied during the experiment but remained equivalent among and within treatments (Kruskal-Wallis: H = 4.79, $p = 0.09$; Table 2.1). Additionally, water elemental ratios did not differ in response to temperature (MANOVA, Pillai's trace = 0.15, $p = 0.74$; Table 2.2). Of the six elemental ratios measured, Zn/Ca_{water} displayed the greatest variation (overall %CV = 47.5) and Sr/Ca_{water} the least (overall %CV = 1.1).

We observed significant and varied responses in vertebral elemental composition among temperature treatments (MANOVA, Pillai's trace = 10.80, $p < 0.001$; Table 2. 2). Vertebral Li/Ca and Sr/Ca did not vary among temperatures (Fig. 2.2a, 2.2e). Vertebral incorporation of Mg/Ca and Ba/Ca was significantly and negatively affected by temperature (Fig. 2.2b, 2.2f) whereas incorporation of Mn/Ca and Zn/Ca was significantly and positively related to temperature (Fig. 2.2c, 2.2d). The significant effect of temperature on both $Mg/Ca_{vertebrae}$ and $Mn/Ca_{vertebrae}$ was attributed to differences in the lowest temperature treatment. $Mg/Ca_{vertebrae}$ was elevated at 15° C (THSD, $p < 0.001$ for 15° v. 18° C and 15° v. 24° C), however, $Mn/Ca_{vertebrae}$ at 15° C was significantly less than those measured in *U. halleri* maintained 18° and 24° C (THSD, $p = 0.009$ for 15° v. 18° C and $p = 0.005$ for 15° v. 24° C). Mean $Zn/Ca_{vertebrae}$ was significantly greater at 24° C but did not differ between 15 and 18° C treatments (THSD, $p < 0.001$ for 15 v. 24° C, $p = 0.019$ for 18 v. 24° C). Significant variation in $Ba/Ca_{vertebrae}$ was evident across all treatments, with mean $Ba/Ca_{vertebrae}$ decreasing with increasing temperature (Fig. 2.2f; THSD, $p < 0.001$ for all pair-wise comparisons). Overall mean (± standard deviation, SD) $Ba/Ca_{vertebrae}$ was 0.97 ± 0.12, 0.71 ± 0.08, and 0.59 ± 0.09 μmol mol^{-1} for 15°, 18°, and 24°C treatments, respectively.

Varied responses to temperature were also observed among the partition coefficients calculated in this study (Table 2.3, Fig. 2.3). We detected no effect of temperature on Li incorporation. Although a significant temperature effect was associated with $Zn/Ca_{vertebrae}$, D_{Zn} indicated no evidence of temperature dependence.

D_{Sr} values showed a slight decrease with increasing temperatures, but the observed trend was statistically insignificant. Temperature had a significant, negative effect on D_{Mg} and D_{Ba} (Fig. 2.3b, 3f) and positively influenced D_{Mn} (Fig. 2.3c). Mean D_{Mg} declined with increasing temperature but the observed pattern was driven by differences between the 15° C and warmer treatments (THSD, $p < 0.001$ for 15° v. 18° C and 15° v. 24° C). A strong, negative effect of temperature on D_{Ba} was detected across treatments (THSD, $p < 0.001$ for all pair-wise comparisons). For D_{Ba}, treatment means (± SD) were 1.31 ± 0.18, 0.99 ± 0.12, and 0.81 ± 0.13 at 15°, 18°, and 24°C, respectively. The positive effect of temperature demonstrated by D_{Mn} was due to increased discrimination of Mn at 15° C (lower D_{Mn} values) compared with 18° and 24° C (THSD, $p = 0.017$ for 15° v. 18° C and $p = 0.001$ for 15° v. 24° C).

Ba manipulation experiment

Targeted Ba concentrations of 3x and 6x were successfully attained (Tables 4, 5). Mean Ba/Ca_{water} values differed significantly among treatments (Fig. 2.4b; THSD, $p < 0.01$ for all pair-wise comparisons). With the exception of Ba/Ca_{water}, dissolved elemental composition did not differ among treatments (MANOVA, Pillai's trace = 0.72, $p = 0.03$; Table 2.5). Salinity (Kruskal-Wallis, H = 5.71, p = 0.06) and temperature (Kruskal-Wallis, H = 1.18, $p = 0.56$) also did not differ among treatments (Table 2.4).

Ambient Ba concentration had a positive effect on $Ba/Ca_{vertebrae}$ (MANOVA, Pillai's trace = 0.95, $p < 0.001$; Table 2.5). Significant differences were found across

treatments (Fig. 2.4b; THSD, $p < 0.001$ for all pair-wise comparisons). Mean (± SD) vertebral Ba/Ca were 0.56 ± 0.11, 0.76 ± 0.05, and 0.99 ± 0.09 μmol mol^{-1} for 1x, 3x, and 6x treatments, respectively.

D_{Ba} decreased significantly with increasing dissolved Ba concentrations (Table 2.3, Fig. 2.5b). This negative relationship indicates that discrimination of Ba increases (less Ba is incorporated) in response to elevated environmental Ba concentrations. Mean D_{Ba} differed significantly among treatments (THSD, $p < 0.01$ for all pair-wise comparisons). Treatment means (± SD) of D_{Ba} were 0.81 ± 0.15 at 1x, 0.72 ± 0.05 at 3x, and 0.67 ± 0.05 at 6x.

Precipitation and growth rate effects

As anticipated, somatic growth (ANOVA, $F_{2,6} = 148.40$, $p < 0.001$) and vertebral precipitation (ANOVA, $F_{2,6} = 115.53$, < 0.001) rates were significantly affected by temperature. Mean growth rates increased with increasing temperatures, ranging between 1.8–6.2 mm DW $month^{-1}$ (Fig. 2.6a, Appendix A; S1). Vertebral deposition rates reflected a similar, positive response to temperature (Fig. 2.6b). No significant relationships were identifed between D_{Me} and somatic growth ($r \leq 0.30$, $p \geq 0.10$) or vertebral precipitation rates within temperature treatments ($r \leq 0.41$, $p \geq 0.12$) (Appendix B; S2), indicating that growth rates were not responsible for the variation in elemental composition observed among treatments.

Somatic growth (ANOVA, $F_{2,6} = 0.32$, $p = 0.80$) and vertebral precipitation rates (ANOVA, $F_{2,6} = 1.86$, $p = 0.23$; Fig. 2.6d) did not differ among Ba treatments.

Mean somatic growth rates ranged from 3.3–6.4 mm DW month^{-1} (Fig. 2.6c) and were consistently and significantly elevated in one tank within each treatment (ANOVA, $F \geq 9.4$, $p \leq 0.0006$ for all comparisons). Tanks with elevated mean growth rates in each treatment represented those that had experienced the least amount of temperature change (1° C) between the temperature and Ba manipulation experiments. Regression analyses indicated D_{Zn} was negatively correlated with somatic growth in two of the three Ba treatments (3x, 6x; Appendix B; S2). However, there was no detectable influence of vertebral precipitation rate on D_{Zn} or the other D_{Me} considered in this study (Appendix A; S1 and Appendix B; S2).

Classification

The multi-elemental composition of vertebrae successfully distinguished *U. halleri* based on their environmental (treatment) history (Table 2.6). Zinc was excluded from DFA because of the observed inconsistencies and potential to vary with growth rate. Therefore, DFAs were conducted using four elemental ratios (Mg/Ca, Mn/Ca, Sr/Ca, and Ba/Ca). For the temperature experiment (15°, 18°, 24°C), overall group classification success was 85%, which was significantly better than expected by chance. Classification of rays based on ambient Ba history (1x, 3x, 6x average local values), was accomplished with 96% success overall, which was also better than random chance. Ba/Ca ratios were the dominant variable used to predict group membership in both DFAs.

DISCUSSION

We demonstrated that the composition of certain minor and trace metal elements in elasmobranch vertebrae was related to the physical and chemical properties of the water. Vertebral incorporation of three of the six elements evaluated demonstrated significant temperature-dependent responses, revealing both positive (D_{Mn}) and negative relationships (D_{Mg} and D_{Ba}) with temperature. Vertebral Ba/Ca ratios in *U. halleri* were incorporated in proportion to Ba/Ca_{water}, supporting their application as a useful geochemical marker. Elemental incorporation of Li, Mg, Mn, Sr, and Ba did not appear to be mediated by somatic growth or vertebral precipitation rates, indicating that individual variation in growth rates are unlikely to be responsible for observed variation in vertebral elemental composition. Significant relationships between somatic growth rate and Zn incorporation were identified. However, correlations between D_{Zn} and somatic growth rate were inconsistent across treatments and between experiments, warranting further investigation. Using DFA, we reliably distinguished the environmental history of individual rays based on differences in vertebral elemental composition. These results indicate that vertebral elemental analysis is a promising tool for the study of elasmobranch populations.

Vertebral elemental composition and influences on incorporation

In the following, we consider the combined results of our temperature and Ba manipulation experiments individually for each element, compare these results with those reported from other calcified structures, consider pathways of uptake and

mechanisms of incorporation, and evaluate the utility of Li, Mg, Mn, Zn, Sr, and Ba as reliable indicators of environmental history and spatially-explicit geochemical tags.

Lithium

The Li/Ca composition of *U. halleri* vertebrae was occasionally below detection limits, highly variable, and not affected by temperature. In synthetic (nonbiogenic) hydroxyapatite, Li has been found to directly substitute for Ca and to increase proportionately with ambient dissolved concentrations [51]. The sparse experimental work on temperature effects on Li uptake in biogenic calcified structures provides mixed results. Negative temperature effects of Li/Ca incorporation into calcite and aragonite have been identified experimentally in some foraminifera, brachiopods, and coral [52, 53] whereas no effect has been observed in other foraminifera and coral species [54].

Otolith Li/Ca has been used as a geochemical marker in freshwater [55], diadromous [56], and marine teleosts [57], as well as an elasmobranch [30]. Fleishman et al. [58] determined that lithium (Li^+) concentrations in the blood plasma of elasmobranchs were 5-7 times lower than that of ambient seawater. This marked discrimination against Li^+ reflects the approach elasmobranchs evolved to maintain internal ionic and osmotic equilibrium (osmoregulation). Because concentrations of NaCl in the plasma of marine elasmobranchs are generally maintained below that of seawater, elasmobranchs experience an osmotic influx of NaCl that must be regulated [58]. Lithium, like Na^+, is a monovalent alkali metal that is unlikely to be

differentiated from Na^+ during osmoregulation [58]. In elasmobranchs, excess Na^+, chloride (Cl^-) and Li^+ are concentrated in the kidneys and renal gland and excreted with urine and other waste [59]. Discrimination of Li is reflected in the partition coefficients calculated from *U. halleri* vertebrae (overall D_{Li}: 0.85 ± 0.21 SD). However, elemental partitioning may be underestimated in our analyses because of the exclusion of samples that fell below instrument detection limits. If we include those samples as 0 values, the grand mean decreases (D_{Li}: 0.64 ± 0.39 SD).

Magnesium

The negative effects of temperature on Mg/Ca incorporation (and D_{Mg}) observed in *U. halleri* have also been reported in marine gastropods [60] and benthic foraminifera [61]. Among marine fishes, significant effects of temperature on otolith Mg/Ca ratios have not previously been reported. Experimental investigations of elemental incorporation into the otoliths of red drum (*Sciaenops ocellauts* [62]), spot (*Leiostomus xanthurus* [16]), gray snapper (*Lutjanus griseus* [63]), and Pacific cod (*Gadus macrocephalus* [40]) all concluded that otolith Mg/Ca was not affected by temperature. In contrast, Mg/Ca ratios in synthetic aragonite show a similar inverse relationship with temperature as observed within *U. halleri* vertebrae [64]. These results are likely due to underlying differences in the kinetics of mineralization associated with biogenic aragonite and hydroxyapatite and differences in ionic regulation between teleost fishes and elasmobranchs.

Magnesium partition coefficients were expressed across a narrow range (D_{Mg} = 0.32-0.43) and exhibited the strongest discrimination (lowest D_{Me}) among the six elements considered in this investigation (e.g. mean D_{Mg} = 0.38, 0.39, 0.39 for 1x, 3x, 6x Ba treatments at 19° C; %CV = 5.6). Magnesium is an essential micronutrient that supports cellular metabolism, immune system function, and skeletal growth, among other physiological processes. In synthetic hydroxyapatites, Mg ions have been found to substitute for and compete with Ca and inhibit mineralization rates [65, 66]. Therefore, internal concentrations of Mg are likely subjected to a high degree of physiological regulation that would be reflected in a comparatively consistent pattern of incorporation, as was observed in *U. halleri*.

Manganese

Temperature influences on Mn incorporation into biomineralized structures have generally been found to be insignificant [63, 67] or negative [38, 68], but $Mn/Ca_{vertebrae}$ and D_{Mn} were positively affected by temperature in *U. halleri*. The effect was not expressed across temperatures but was driven by significantly lower incorporation of Mn/Ca within the coldest (15° C) treatment. Manganese is an essential micronutrient that is an important cofactor for many enzymes and supports metabolism, protein production, cellular signaling processes, and the activation of reproductive hormones. Though Mn/Ca uptake is proportional to Mn/Ca_{water} in synthetic hydroxyapatite [69], osmotic regulation of Mn ions would alter this direct relationship in biogenic hydroxyapatites. Furthermore, diet represents the primary

pathway of Mn uptake in elasmobranchs and other vertebrates [70, 71]. In a comparative study of radionuclide accumulation in a teleost and elasmobranch, Pentreath [72] concluded that uptake of Mn radioisotopes solely from water was insufficient to explain internal concentrations of the radionuclide. More recently, Mathews & Fisher [71] experimentally determined that >90% of the Mn accumulated in the soft tissue of lesser spotted dogfish (*Scyliorinus canicula*) was derived from dietary sources. The contribution of dietary Mn in addition to uptake from the environment at the gills offers an explanation for the elevated values of $Mn/Ca_{vertebrae}$ in comparison to water Mn/Ca in our experiment (Fig. 2.2c).

Considerable variation in D_{Mn} has been reported within and among species. D_{Mn} ranged between 0.10–1.90 in juvenile black bream (*Acanthopagrus butcheri* [73]), 0.018–1.02 in grey snapper [63], and 7.67–32.83 in a field-based study of spotted seatrout (*Cynoscion nebulosus* [74]). Strasser et al. [75] identified ontogenetic differences in D_{Mn} between larval (mean ± SD: 1.86 ± 0.19) and juvenile softshell clams (mean ± SD: 0.88 ± 0.13), *Mya arenaria*. Our estimates of D_{Mn} typically exceeded 1.0 (temperature experiment: 0.9–1.60; Ba manipulation experiment: 1.36–1.69) but fell within the broad range reported among these other investigations. Laboratory studies intended to assess the factors controlling Mn incorporation into otoliths have found no evidence of a relationship between Mn/Ca_{water} and $Mn/Ca_{otolith}$ (see review by Miller [38]). However, Limburg et al. [76] hypothesized that cyclical variation of $Mn/Ca_{otolith}$ ratios were associated with migrations into deep water hypoxic zones that are characterized by elevated Mn

concentrations, providing historic records of hypoxia intensity. Further research on the mechanisms of Mn incorporation is needed to clarify the utility of this element as as a geospatial tag or indicator environmental history.

Zinc

Our analyses of Zn incorporation into *U. halleri* vertebrae revealed a positive influence of temperature on $Zn/Ca_{vertebrae}$, no significant effect of temperature on D_{Zn}, and significant influences of somatic growth rates on D_{Zn}, providing a somewhat convoluted perspective on the factors influencing Zn incorporation. Few studies have attempted to experimentally validate Zn incorporation into biogenic calcified structures [65, 66, 77]. Zn is fundamental to a diverse array of physiological processes, including growth, neurotransmission, and cell signaling. It plays a vital role in protein production, structure, and maintenance [78]. Though branchial uptake of Zn is not inconsequential, diet represents the primary source of Zn intake in both elasmobranch and teleost fishes [69, 70]. The dietary contribution of Zn in the elasmobranch *S. canicula* (>80% [71]) is similar to those experimentally estimated for other fishes [79, 80]. Given our use of standardized diets, the observed inconsistencies may be the result of the high variability in Zn/Ca_{water} values (Tables 2.1, 2.4). Alternatively, variation in $Zn/Ca_{vertebrae}$ may be influenced by somatic growth rate and kinetic effects.

In synthetic hydroxyapatites, Zn substitution for Ca is minimal and the majority of Zn is incorporated through inclusion into interstitial spaces [81]. Elements

incorporated into interstitial spaces can be representative of environmental conditions [8, 15], but the pathways and mechanisms of Zn incorporation may differ in biogenic hydroxyapatite. Miller et al. [81] determined that the majority (40-60%) of Zn contained in Atlantic cod (*Gadus morhua*) otoliths was associated with the protein matrix rather than the mineralized aragonite structure or interstitial spaces. Given the critical role of Zn identified in more than 300 fish proteins [13], it is likely that much of the Zn contained in the biogenic hydroxyapatite of elasmobranch vertebrae is bound within the protein matrix as well. Because it is prevalent in the protein structure of otoliths and is assimilated primarily through dietary sources, Miller et al. [81] concluded that Zn is unlikely to be a reliable proxy of ambient environmental conditions. Zn has been reported to be useful for distinguishing shark populations [28] and movements of sharks between habitats [30]. Based on our results and a review of available literature, we do not anticipate vertebral Zn/Ca ratios and D_{Zn} to be commonly representative of ambient conditions.

Strontium

Strontium is one of the most commonly studied elemental markers in biogenic calcified structures. Unlike several of the elements previously considered in this study, a physiological role for Sr has not been identified in fishes [14]. Sr is primarily derived via branchial uptake in fishes and Sr/Ca ratios of otoliths are typically representative of ambient concentrations [83, 84, 85]. In synthetic hydroxyapatite and aragonite, Sr is known to compete with and substitute for Ca [86]. Temperature-

dependent responses in Sr/Ca incorporation have provided reliable indicators of temperature history in corals [87] and fishes [37], but Sr incorporation was not influenced by temperature in *U. halleri*. Temperature-independent patterns of Sr/Ca incorporation were also reported in juvenile *L. xanthurus* scales [88], common cuttlefish statoliths (*Sepia officinalis* [36]), and the otoliths of European eels (*Anguilla anguilla* [89]).

Barium

The strong, negative effect of temperature on Ba incorporation in *U. halleri* is similar to the pattern observed by Balter & Lécuyer [90] in laboratory studies of synthetic hydroxyapatite. Decreases in Ba/Ca ratios with increasing ambient water temperature have also been found in laboratory studies with synthetic aragonite [64], cephalopods [36], larval gastropods [67, 91], juvenile clams [92], and larval fish [39]. However, positive or no effect of temperature on Ba incorporation into otoliths has been much more commonly observed [38]. Studies of Ba incorporation into the hydroxyapatite of fish scales, bone, and teeth have also revealed either positive [12] or no relationship to temperature [88]. Given the inconsistency in temperature effects on Ba incorporation reported within the literature, it is likely that species-specific variation in this temperature response is widespread.

The significant positive relationship between vertebrae and water Ba/Ca supports the utility of Ba/Ca ratios as geospatial markers. However, our observation of a negative temperature effect on Ba/Ca incorporation indicates there are likely to be

interactive effects of temperature and water concentration on vertebral Ba incorporation. These effects could confound interpretations of field data, particularly in study areas with sharp gradients in both temperature and Ba/Ca_{water}. For example, vertebral Ba/Ca of *U. halleri* at 15.4° C and exposed to Ba/Ca_{water} of 6.3 μmol mol^{-1} ($Ba/Ca_{vertebrae}$ = 0.96) would be indistinguishable from *U. halleri* that had resided in water averaging 19.7° C with a mean Ba/Ca_{water} concentration of 31.7 μmol mol^{-1} ($Ba/Ca_{vertebrae}$ = 0.94). This finding highlights the importance of experimental validation studies, the utility of measuring multiple elemental markers, the value of temperature data from study areas, and need for caution when interpreting patterns from field studies.

Effects of growth and precipitation rates

Variation in growth rates can alter physiological and kinetic processes that directly modify patterns and rates of elemental discrimination and incorporation [45, 68]. For example, growth-mediated effects on elemental incorporation could produce significant variability among individuals with inherently different growth rates that occupy the same water mass. Indeed, growth rates have been found to influence the composition of some calcified structures [7, 16, 18]. In this study, we found no significant relationships between somatic growth or vertebral precipitation rates and D_{Me} for any elements except Zn in *U. halleri* (Appendix A; S1, Appendix B; S2), which supports the premise that growth rates do not generally alter vertebral elemental composition.

In contrast to other elements, D_{Zn}, independent of temperature, was significantly but inconsistently correlated with somatic growth. The effect of growth on D_{Zn} was restricted to the 3x and 6x Ba treatments (Appendix A; S1, Appendix B; S2). Vertebral precipitation rates (μm radius $month^{-1}$), however, were not significantly correlated with D_{Zn}. Zn/Ca_{water} values displayed the greatest variance among the six elemental ratios measured in this study (%CV = 47.8). Dissolved Zn concentrations can be highly variable, elevating during periods of increased river discharge and runoff [93]. Water changes that occurred during high flow events could have influenced Zn/Ca_{water}, $Zn/Ca_{vertebrae}$, and D_{Zn} in some tanks. Trace levels of Zn in seawater are also highly prone to contamination [94]. Given broad environmental variation and the potential for contamination, our sampling frequency may not have been sufficient to adequately characterize the uptake and partitioning of Zn in *U. halleri*. Further research is needed to clarify the relationships between Zn/Ca_{water}, $Zn/Ca_{vertebrae}$, and somatic growth rates, if this element is to be used as a reliable marker of habitat use or natal origins.

Ecological applications and future directions

Group classification of *U. halleri* based on environmental history within the controlled laboratory studies was highly successful (Table 2.6). Our results indicate that geochemical variation in elasmobranch vertebrae can reliably distinguish individuals based on differences in their environmental history or habitat – assuming differences among those habitats or time periods exist. Studies of vertebral elemental

chemistry could identify natal origins, biological hotspots, movement patterns, habitat use, and population structure of elasmobranchs, and generate critical information for spatially explicit conservation measures and advance future research in shark and ray population dynamics.

The significant temperature effects and the likelihood for interaction between temperature and ambient concentration on Ba incorporation observed in this study emphasize the importance of considering multiple elemental markers when making spatial and temporal inferences regarding environmental history. Measurements of vertebral bulk or compound-specific stable isotopic composition [95, 96], mapping of environmental chemical composition/isoscapes [97, 98], or molecular analyses [97] used in conjunction with minor and trace elemental assays should provide greater resolution than would be obtained from a single method alone. We anticipate that studies integrating complementary intrinsic markers will generate corroborative and more robust conclusions based on field data.

Our results prompt questions regarding the periodicity of growth band/increment deposition within elasmobranch vertebrae. Increments are deposited daily within fish otoliths and bivalve shells, a phenomenon that has not yet been found in elasmobranchs [4, 24]. Yet, microscopic examination of elasmobranch vertebrae typically reveals other increments and checks within the pair of annual growth bands [24, 100]. Are growth bands deposited at finer temporal scales within elasmobranch vertebrae? The ability to reconstruct environmental history and assay elemental markers with more refined

temporal resolution would enhance the utility of this tool in studies of elasmobranch populations.

Elemental composition of elasmobranch vertebrae may not provide a useful record of environmental history for all species. Vertebral elemental composition could differ among species due to species-specific environmental tolerances [101, 102] or extent of vertebral calcification [24, 103]. Additional laboratory or field-based experiments should be pursued to gain insight into the potential differences in elemental incorporation among species. Our validation study advances the use of vertebral elemental composition for the study of elasmobranch populations and provides a framework for interpreting the results of future field investigations.

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Table 2.1. Temperature experiment. Mean experimental conditions by tank and treatment. The summary includes the number of round rays (*Urobatis halleri*) per tank (n), their mean size in disc width (DW) at the onset of the study, water temperature (°C), salinity (parts per thousand, ‰), and dissolved element to calcium (Ca) ratios (Me/Ca) for lithium (Li), magnesium (Mg), manganese (Mn), zinc (Zn), strontium (Sr), and barium (Ba). Values in parenthesis are ± standard deviation. Untransformed Me/Ca concentrations are presented below but $\log_{10}$ transformation of these data was necessary to meet the assumptions for parametric statistical analysis.

Tank	Treament (°C)	n	Mean DW (mm)	Temperature (°C)	Salinity (‰)	Li/Ca_{water} ($\mu mol\ mol^{-1}$)	Mg/Ca_{water} ($mmol\ mol^{-1}$)	Mn/Ca_{water} ($\mu mol\ mol^{-1}$)	Zn/Ca_{water} ($\mu mol\ mol^{-1}$)	Sr/Ca_{water} ($mmol\ mol^{-1}$)	Ba/Ca_{water} ($\mu mol\ mol^{-1}$)
3	15	12	115.8 (17.4)	15.4 (1.2)	31.6 (1.6)	11.57 (0.26)	4987.52 (20.84)	13.64 (0.76)	18.88 (7.91)	8.77 (0.09)	6.31 (1.52)
6	15	12	117.5 (16.2)	15.0 (1.2)	31.6 (1.6)	11.54 (0.34)	4963.24 (60.88)	13.45 (0.52)	19.44 (9.55)	8.74 (0.12)	5.35 (1.48)
8	15	11	118.0 (20.5)	15.5 (1.3)	31.7 (1.6)	11.43 (0.34)	4984.04 (15.34)	13.45 (0.28)	27.42 (17.20)	8.76 (0.09)	5.14 (1.65)
1	18	11	100.0 (18.0)	18.8 (0.9)	32.3 (1.2)	11.63 (0.27)	4991.62 (18.84)	13.50 (0.24)	24.37 (10.57)	8.79 (0.07)	5.05 (0.95)
4	18	10	121.3 (20.8)	18.5 (0.8)	32.2 (1.2)	11.70 (0.21)	4990.09 (12.31)	13.76 (0.30)	20.51 (7.78)	8.79 (0.07)	5.22 (1.07)
7	18	12	115.9 (16.6)	18.6 (0.7)	32.4 (1.2)	11.64 (0.47)	4997.84 (18.48)	13.64 (0.54)	22.23 (13.27)	8.81 (0.08)	5.39 (1.24)
2	24	12	116.4 (19.6)	23.9 (0.9)	32.6 (1.4)	11.43 (0.64)	4983.72 (31.65)	14.26 (1.08)	24.10 (6.13)	8.78 (0.14)	5.37 (1.52)
5	24	12	119.7 (18.3)	24.3 (1.2)	33.0 (1.9)	11.42 (0.54)	4987.46 (17.37)	13.40 (1.28)	24.85 (13.90)	8.78 (0.11)	5.02 (1.30)
9	24	13	115.6 (14.2)	24.1 (0.8)	33.2 (1.4)	11.44 (0.33)	4983.24 (13.06)	13.68 (0.79)	28.18 (10.71)	8.76 (0.08)	5.63 (1.28)

Table 2.2. *Urobatis halleri*; temperature experiment. Univariate results from multivariate analysis of variance tests to evaluate the effect of temperature (Temp; 15°C, 18°C, and 24°C) on dissolved element to calcium ratios (Me/Ca) in water and vertebral Me/Ca among treatments. Significant p-values are indicated by bold font. Data were $\log_{10}$ transformed prior to analysis.

Source	Me/Ca	Effect	DF	MSE	F	p
Water	Li	Temp	2	< 0.001	3.32	0.107
		(Tank)	6	< 0.001		
	Mg	Temp	2	< 0.001	1.76	0.250
		(Tank)	6	< 0.001		
	Mn	Temp	2	< 0.001	0.27	0.774
		(Tank)	6	0.001		
	Zn	Temp	2	0.005	1.71	0.258
		(Tank)	6	0.003		
	Sr	Temp	2	< 0.001	2.98	0.126
		(Tank)	6	< 0.001		
	Ba	Temp	2	0.001	0.46	0.652
		(Tank)	6	0.001		
Vertebrae	Li	Temp	2	0.015	1.56	0.284
		(Tank)	6	0.010		
	Mg	Temp	2	0.002	49.81	**< 0.001**
		(Tank)	6	< 0.001		
	Mn	Temp	2	0.019	8.87	**0.016**
		(Tank)	6	0.002		
	Zn	Temp	2	0.028	12.97	**0.007**
		(Tank)	6	0.002		
	Sr	Temp	2	< 0.001	3.15	0.102
		(Tank)	6	< 0.001		
	Ba	Temp	2	0.118	91.78	**< 0.001**
		(Tank)	6	0.001		

Table 2.3. *Urobatis halleri*; temperature and barium manipulation experiments. Results of nested analysis of variance to evaluate the effect of temperature (Temp; 15°C, 18°C, and 24°C) and barium ([Ba]; 1x, 3x, and 6x average ambient values) treatments on mean partition coefficients (D_{Me}). Significant p-values are indicated by bold font. Data were $\log_{10}$ transformed prior to analysis.

Experiment	D_{Me}	Effect	DF	MSE	F	p
Temperature	D_{Li}	Temp	2	0.017	2.32	0.180
		(Tank)	6	0.007		
	D_{Mg}	Temp	2	0.001	47.86	**< 0.001**
		(Tank)	6	< 0.001		
	D_{Mn}	Temp	2	0.016	5.90	**0.038**
		(Tank)	6	0.003		
	D_{Zn}	Temp	2	< 0.001	0.17	0.850
		(Tank)	6	0.004		
	D_{Sr}	Temp	2	< 0.001	3.09	0.119
		(Tank)	6	< 0.001		
	D_{Ba}	Temp	2	0.191	31.77	**< 0.001**
		(Tank)	6	0.006		
Barium manipulation	D_{Li}	[Ba]	2	0.003	1.50	0.297
		(Tank)	6	0.002		
	D_{Mg}	[Ba]	2	< 0.001	1.69	0.261
		(Tank)	6	< 0.001		
	D_{Mn}	[Ba]	2	0.002	1.96	0.221
		(Tank)	6	0.001		
	D_{Zn}	[Ba]	2	0.137	0.61	0.575
		(Tank)	6	0.023		
	D_{Sr}	[Ba]	2	< 0.001	0.27	0.769
		(Tank)	6	0.001		
	D_{Ba}	[Ba]	2	0.015	20.44	**0.002**
		(Tank)	6	0.001		

Table 2.4. Barium manipulation experiment. Mean experimental conditions by tank and treatment. Treatments reflect ambient (1x) barium concentrations and targeted concentrations of three (3x) and six (6x) times the mean ambient value. The summary includes the number of round rays (*Urobatis halleri*) per tank (n), their mean size in disc width (DW) at the onset of the barium manipulation experiment, water temperature (°C), salinity (parts per thousand, ‰), and dissolved element to calcium (Ca) ratios (Me/Ca) for lithium (Li), magnesium (Mg), manganese (Mn), zinc (Zn), strontium (Sr), and barium (Ba). Values in parenthesis are ± standard deviation. Untransformed Me/Ca concentrations are presented below but $\log_{10}$ transformation of these data was necessary to meet the assumptions for parametric statistical analysis.

Tank	Treament	n	Mean DW (mm)	Temperature (°C)	Salinity (‰)	Li/Ca_{water} ($\mu mol\ mol^{-1}$)	Mg/Ca_{water} ($mmol\ mol^{-1}$)	Mn/Ca_{water} ($\mu mol\ mol^{-1}$)	Zn/Ca_{water} ($\mu mol\ mol^{-1}$)	Sr/Ca_{water} ($mmol\ mol^{-1}$)	Ba/Ca_{water} ($\mu mol\ mol^{-1}$)
2	1x	12	162.1 (14.0)	19.8 (0.2)	30.0 (0.7)	11.59 (0.28)	4987.78 (8.79)	14.18 (0.74)	44.54 (13.22)	8.76 (0.05)	4.68 (0.52)
6	1x	12	131.8 (15.5)	19.6 (0.4)	30.7 (0.6)	11.87 (0.42)	4984.19 (10.96)	13.77 (0.57)	24.34 (6.56)	8.76 (0.07)	4.85 (0.60)
7	1x	12	143.5 (12.6)	19.5 (0.2)	29.9 (0.7)	12.06 (0.64)	5000.91 (32.94)	13.24 (0.67)	27.08 (9.37)	8.80 (0.12)	4.96 (0.50)
3	3x	11	132.3 (15.9)	19.8 (0.4)	29.5 (1.0)	11.74 (0.35)	4995.85 (26.14)	13.95 (0.78)	23.12 (4.72)	8.78 (0.13)	10.21 (7.83)
4	3x	10	146.6 (15.4)	19.5 (0.4)	29.8 (0.9)	11.73 (0.30)	4985.15 (16.09)	14.54 (1.28)	25.08 (11.05)	8.76 (0.09)	16.94 (14.11)
9	3x	12	165.5 (7.7)	19.4 (0.3)	29.8 (1.0)	11.55 (0.37)	4986.28 (16.71)	14.67 (0.56)	48.99 (20.95)	8.76 (0.07)	14.15 (8.30)
1	6x	11	148.2 (9.5)	19.4 (0.3)	29.8 (0.7)	11.89 (0.79)	5005.43 (39.14)	14.86 (1.37)	47.85 (22.22)	8.74 (0.07)	31.99 (21.58)
5	6x	7	167.4 (9.5)	19.8 (0.2)	29.7 (0.8)	11.81 (0.25)	4992.87 (18.94)	14.80 (1.91)	35.90 (21.38)	8.78 (0.11)	44.22 (3.87)
8	6x	11	136.5 (17.9)	19.7 (0.4)	29.8 (1.0)	11.73 (0.22)	4993.88 (34.20)	13.89 (0.59)	36.10 (15.18)	8.79 (0.12)	31.73 (19.23)

Table 2.5. *Urobatis halleri*; barium manipulation experiment. Univariate results from multivariate analysis of variance tests to evaluate the effect of barium concentration ([Ba]; 1x, 3x, and 6x local ambient values) on dissolved element to calcium ratios (Me/Ca) in water and vertebral Me/Ca among treatments. Significant p-values are indicated by bold font. Data were $\log_{10}$ transformed prior to analysis.

Source	Me/Ca	Effect	DF	MSE	F	p
Water	Li	[Ba]	2	< 0.001	1.49	0.299
		(Tank)	6	< 0.001		
	Mg	[Ba]	2	< 0.001	0.70	0.534
		(Tank)	6	< 0.001		
	Mn	[Ba]	2	0.001	5.08	0.051
		(Tank)	6	< 0.001		
	Zn	[Ba]	2	0.016	0.91	0.451
		(Tank)	6	0.017		
	Sr	[Ba]	2	< 0.001	1.08	0.398
		(Tank)	6	< 0.001		
	Ba	[Ba]	2	0.424	28.19	**< 0.001**
		(Tank)	6	0.015		
Vertebrae	Li	[Ba]	2	0.004	1.14	0.382
		(Tank)	6	0.004		
	Mg	[Ba]	2	< 0.001	0.92	0.447
		(Tank)	6	< 0.001		
	Mn	[Ba]	2	< 0.001	0.16	0.858
		(Tank)	6	0.002		
	Zn	[Ba]	2	0.004	0.83	0.480
		(Tank)	6	0.002		
	Sr	[Ba]	2	< 0.001	0.19	0.829
		(Tank)	6	< 0.001		
	Ba	[Ba]	2	0.145	99.59	**< 0.001**
		(Tank)	6	0.001		

Table 2.6. *Urobatis halleri*. Group classification success determined from discriminant function analysis of vertebral elemental composition of magnesium, manganese, strontium, and barium (expressed as element to calcium ratios). Jack-knife classification success among groups based on known, controlled temperature and dissolved barium concentration histories. Overall percent classification success of groups and chance-corrected classification (κ) ± approximate standard error (ASE) represent independent measures of group classification performance.

Temperature Treatment (°C)	% Correctly Classified	Overall % Classification Success	κ (± ASE)	[Ba] Treatment	% Correctly Classified	Overall % Classification Success	κ (± ASE)
15	91	85	0.77 (0.09)	1x	100	96	0.94 (0.08)
18	86			3x	97		
24	76			6x	91		

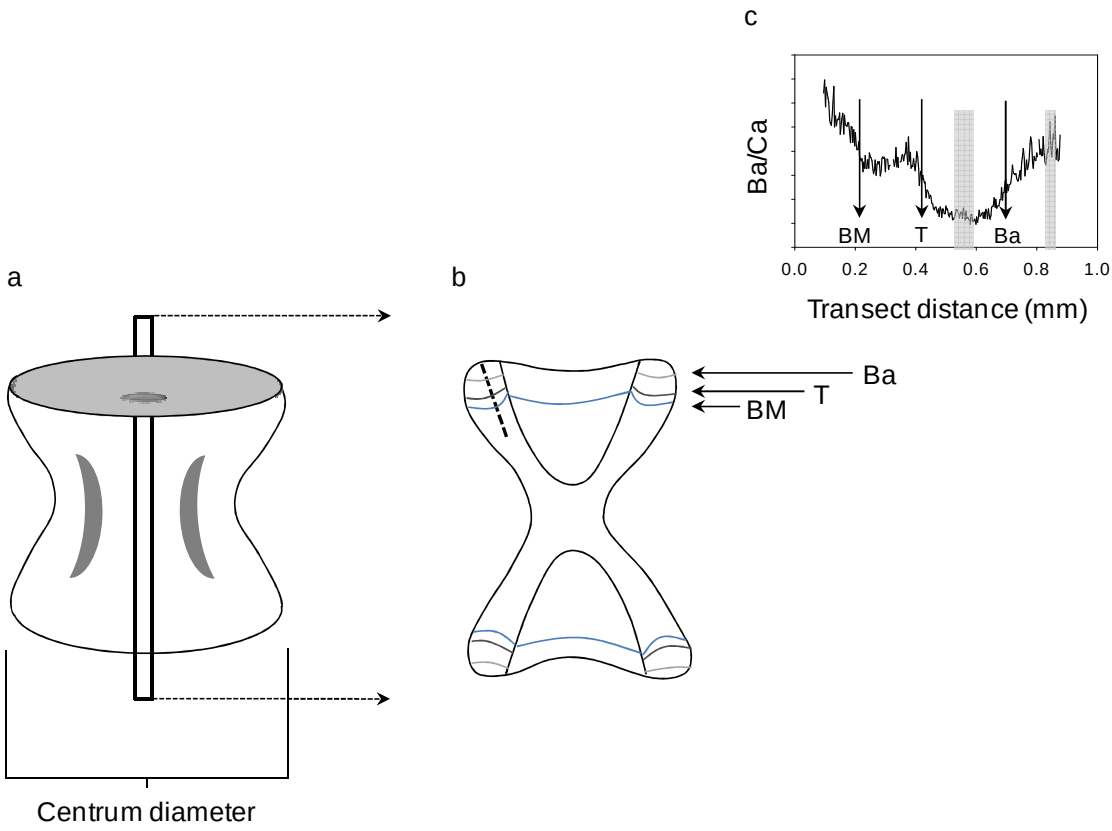

Figure 2.1. Depiction of a (a) whole and (b) thin-sectioned vertebral centra and (c) the barium to calcium ratio (Ba/Ca) profile collected along a representative laser transect. The birth mark (BM) provides an intrinsic reference and fluorescent markers injected into round rays (*Urobatis halleri*) at the beginning of the temperature and barium manipulation experiments provided visual references for identifying specific regions deposited during this study. T represents the beginning of the temperature experiment (indicated by an oxytetracycline mark). Ba represents the beginning of the barium manipulation experiment which was identifiable by a calcein mark. The dashed line in (b) exemplifies the transect pathway used for laser ablation. Grey vertical bars within (c) depict the regions integrated for analysis. The example in (c) characterizes the variation in vertebral Ba/Ca of a round ray that was maintained at 18° C and 6x ambient Ba concentration during the temperature and barium manipulation experiments, respectively.

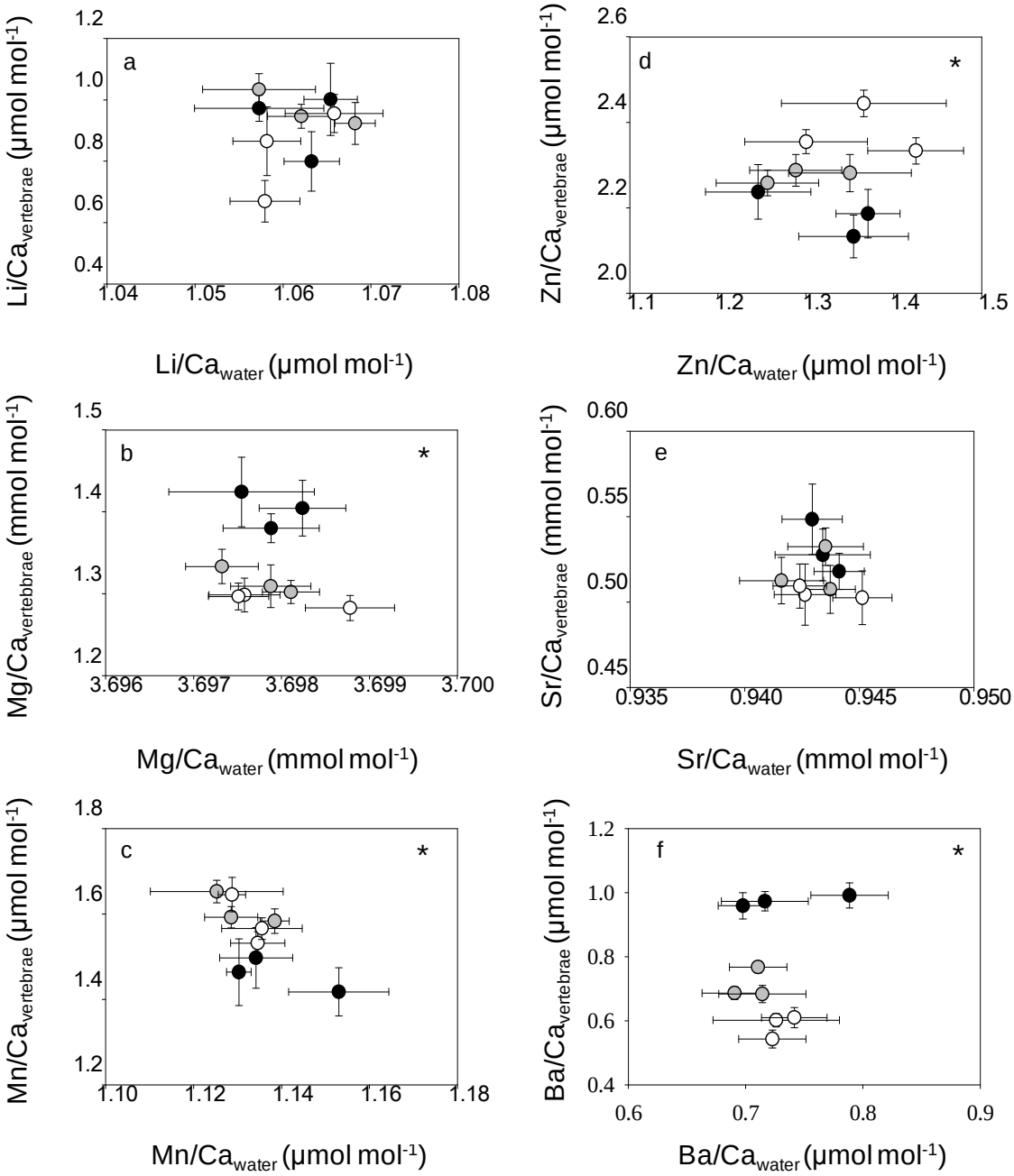

Figure 2.2. *Urobatis halleri*; temperature experiment. Mean ± standard error of element to calcium ratios (Me/Ca) for water and vertebral samples at 15° C (●), 18° C (◉), and 24° C (○) treatments. (a) lithium, (b) magnesium, (c) manganese, (d) zinc, (e) strontium, and (f) barium. Me/Ca ratios were $\log_{10}$-transformed. Significant temperature effects are indicated by (*).

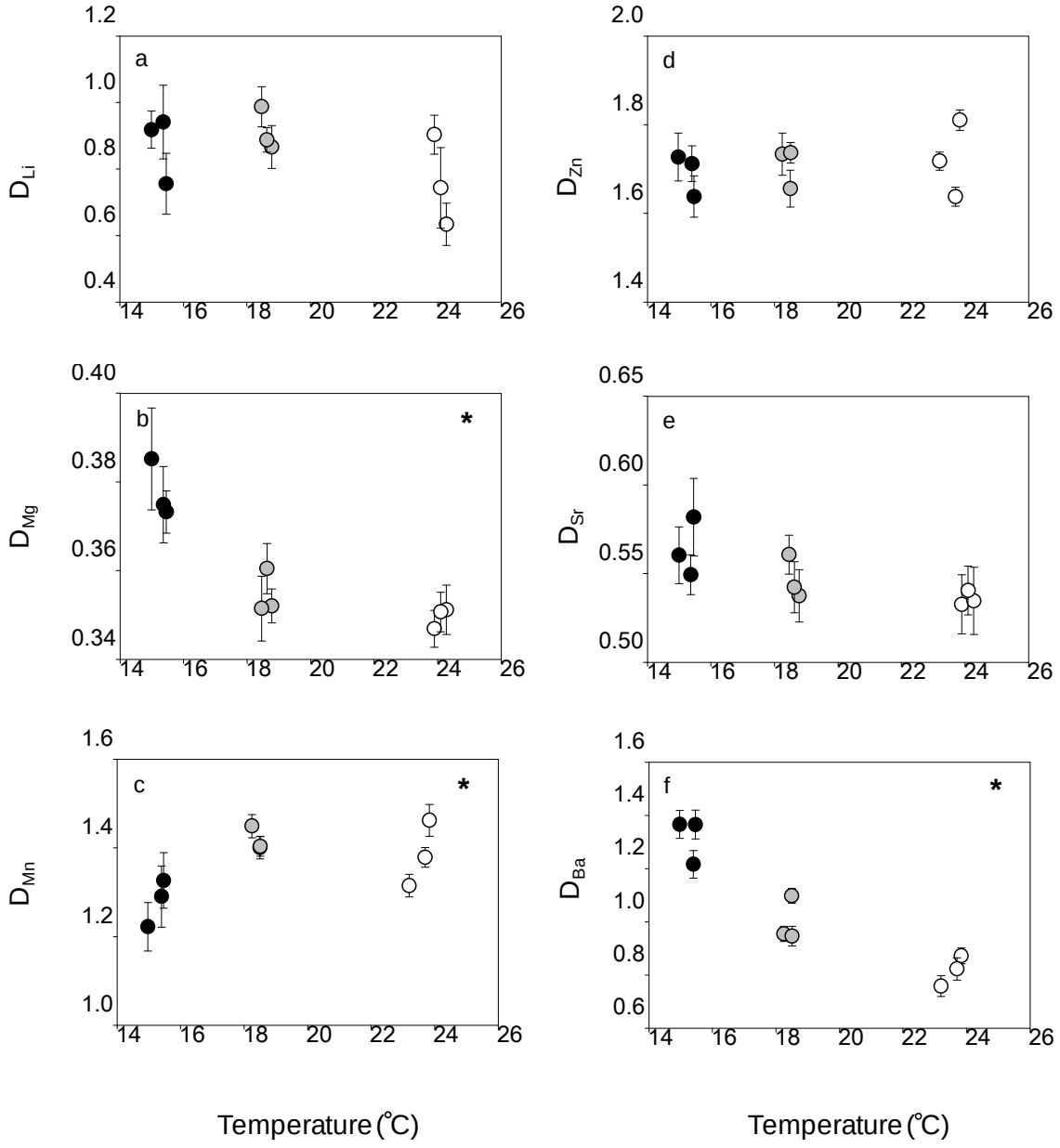

Figure 2.3. *Urobatis halleri*; temperature experiment. Mean ± standard error of partition coefficients (D_{Me}) for (a) lithium, (b) magnesium, (c) manganese, (d) zinc, (e) strontium, and (f) barium by treatment and mean tank temperature. Symbols represent 15° C (●), 18° C (◍), and 24° C (○) treatments. Significant responses of D_{Me} to temperature are indicated by (*).

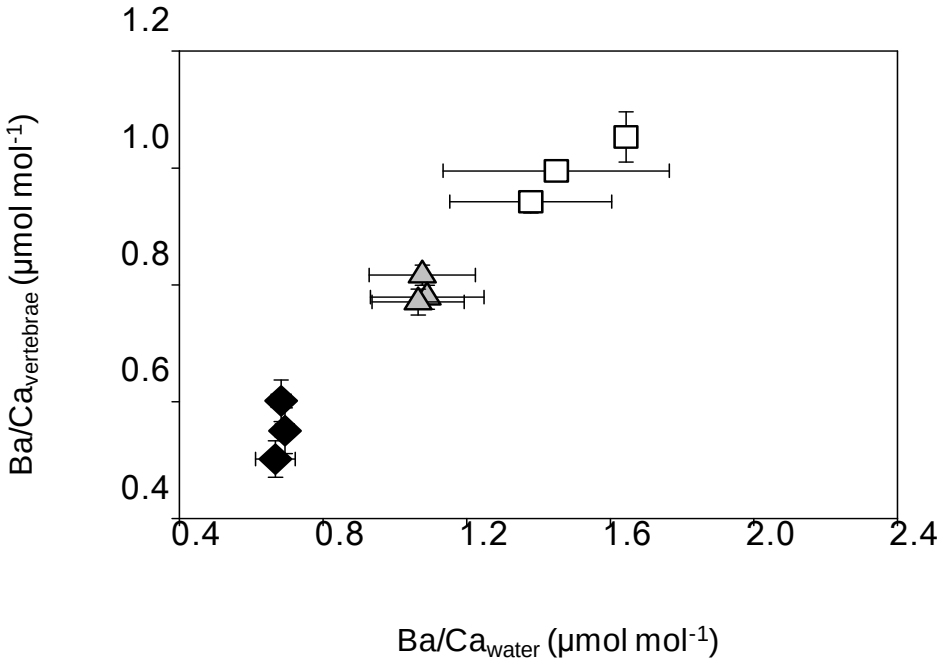

Figure 2.4. *Urobatis halleri*; barium manipulation experiment. Mean ± standard error of barium to calcium ratios for water and vertebral samples from 1x (◆), 3x (▲), and 6x (□) barium treatments. Element to calcium ratios were $\log_{10}$-transformed.

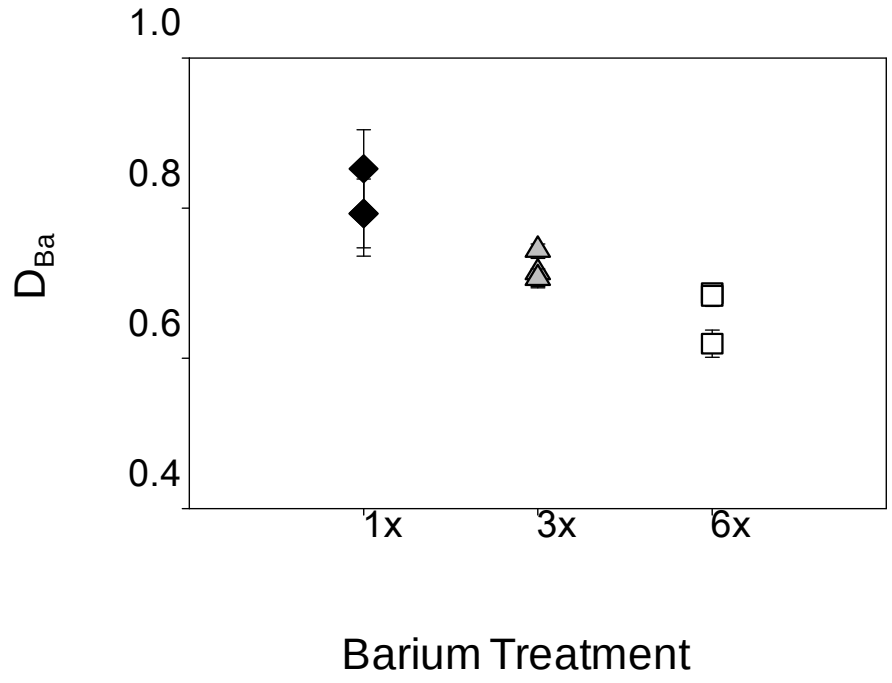

Figure 2.5. *Urobatis halleri*; barium manipulation experiment. Mean ± standard error of partition coefficients for barium (D_{Ba}) by barium treatment. Experimental barium concentrations were 1x (◆), 3x (▲), and 6x (□) that of average local values.

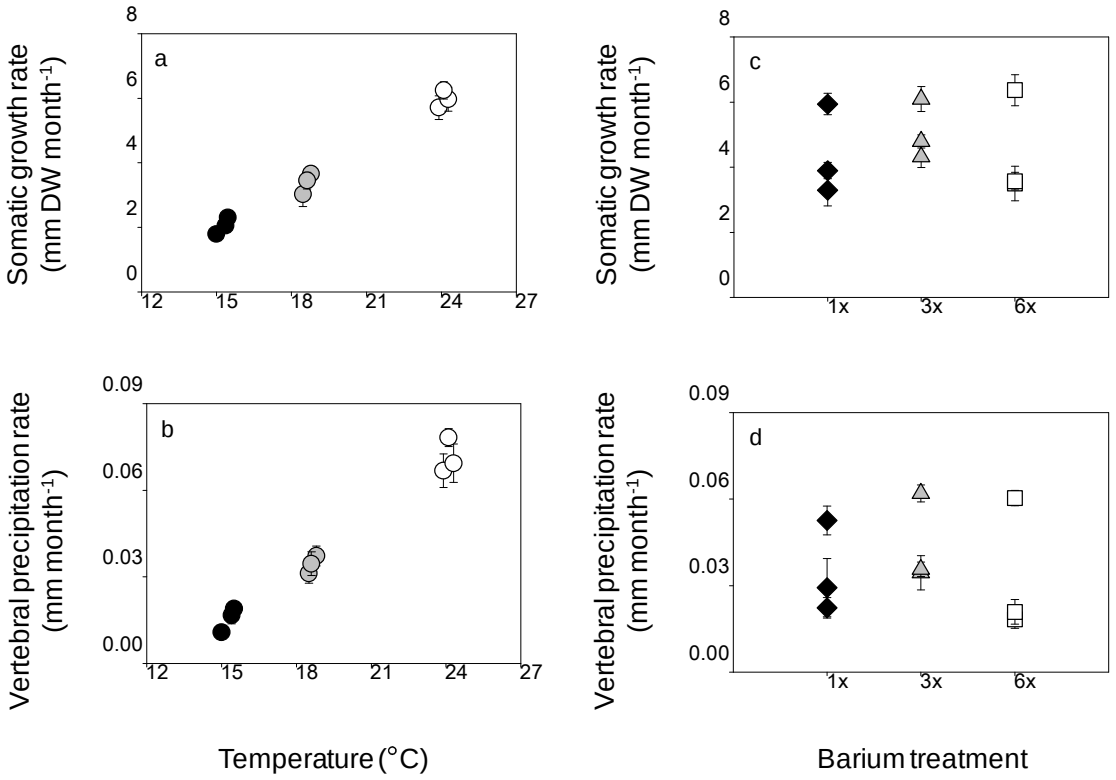

Figure 2.6. *Urobatis halleri*; temperature and barium manipulation experiments. Mean (a, c) somatic growth and (b, d) vertebral precipitation rates by tank, treatment, and experiment (± standard error). (a, b) Temperature treatments 15° C (●), 18° C (◉), and 24° C (○). (c, d) Experimental barium concentrations were 1x (●), 3x (▲), and 6x (□) that of average local values. Temperatures were equivalent among treatments (19° C) during the barium manipulation experiment.

CHAPTER 3

DISTINGUISHING NATAL ORIGINS AND CONNECTIVITY WITH VERTEBRAL ELEMENTAL SIGNATURES IN SHARKS

Wade D. Smith

ABSTRACT

Knowledge of movement patterns, habitat use and the spatial structure of populations is essential for effective management and conservation of wide ranging marine species. The chemical composition of calcified structures, such as otoliths and statoliths, has been used to distinguish natal origins, dispersal patterns, and population structure of many marine organisms. Because the use of discrete nursery areas is common among elasmobranchs, distinctive chemical markers may be incorporated into the vertebrae of individuals as they occupy these areas for the first months or years of their lives. The objective of this investigation is to determine if elemental signatures incorporated into the vertebrae of young-of-the-year elasmobranchs provide discrete markers of natal origin. Vertebrae were collected from artisanal fishery landings at six sites along the Pacific coast of Mexico and Costa Rica in 2007-2009 to assess patterns of spatial and temporal variation in elemental composition. The composition of vertebral elemental signatures (natal and edge) was measured using laser ablation-inductively coupled plasma mass spectrometry. A protracted pupping period was confirmed for *S. lewini*, with newborn pups present in fisheries samples from May through mid-October. Natal elemental signatures varied significantly

among putative nursery areas. All element-to-calcium ratios included in these analyses (Li/Ca, Mg/Ca, V/Ca, Cr/Ca, Mn/Ca, Rb/Ca, Sr/Ca, Ba/Ca, Pb/Ca) were useful for the discerning natal origins of sharks, however, Ba, Sr, Mn, and Mg ratios most consistently generated the greatest discriminatory power based on step-wise discriminant function analyses. All classification accuracies exceeded chance expectations and successfully discriminated among sites of capture, with overall success rates of 39-100%. Classification accuracy to putative nursery areas (natal signature) and location of capture (edge signature) improved from low to high when data were expressed with greater spatial and temporal resolution (e.g. early season, month of capture). Though significant differences in natal elemental signatures were detected across years, pair-wise analysis revealed that signatures were similar between 2007 and 2009, indicating some consistency in site-specific natal signatures. Our results confirmed that vertebral elemental markers can be used to distinguish individuals across small (5s km), moderate (100s km), and large spatial scales (>1000 km). The potential for intra-annual variation in natal signatures within a year-class, however, highlights the importance of cohort-specific analyses and the development of a spatial atlas of natal vertebral elemental signatures for studies of natal origin and population connectivity. Analyses of vertebral chemistry offer a promising new tool for the study of highly mobile shark populations.

INTRODUCTION

Movements of individuals define their interactions with the environment and within communities, thereby having profound consequences on individual fitness as well as population dynamics and distributions (Greenwood 1980, Sinclair 1988, Hastings and Botsford 2006). Tracing movement patterns, distinguishing natal origins of individuals, and quantifying the degree of exchange among subpopulations or habitats are fundamental aspects of applied ecology. Insight into dispersal pathways and population connectivity promotes the development of effective conservation and management practices at spatial and temporal scales that are relevant to a population (Fogarty and Botsford 2007, Clapham et al. 2008). Our ability to observe and identify movements of marine species, however, is complicated by the concealing nature of the environment, wide distribution of individuals, and complexity and vastness of potential habitat.

The majority of marine fishes possess a bipartite life cycle with a dispersive pelagic larval phase and relatively sedentary adult phase. In contrast, the life history of sharks and rays (elasmobranchs) is generally typified by a highly mobile adult stage and more restricted, localized movement as juveniles (Carrier et al. 2004). Studies of connectivity and movement patterns in shark and ray populations are frequently complicated because of their high mobility, broad spatial distributions, and tendency to segregate by size and sex (Speed et al. 2010, Jacoby et al. 2011). Individuals may undertake large- (>1000 km) and small-scale movements (1-10 km) in response to

reproductive and foraging needs, which influence population dynamics and community interactions across broad temporal and spatial scales (Kohler and Turner 2001, Heupel et al. 2012). Because many shark and ray populations are facing unprecedented population declines (Stevens et al. 2000, Ferretti et al. 2010) and have limited potential for rapid population recovery (Smith et al. 1998), new methods to improve our understanding of dispersal pathways, shifts in habitat use, and population connectivity in elasmobranchs could advance spatially-explicit management and conservation practices.

Established methods for evaluating population structure and connectivity of marine organisms on ecological time scales focus largely on tagging and tracking individuals. These investigations are difficult to implement for wide ranging marine species and may be expensive, time-consuming, and limited in duration or area because of technological limitations (e.g. battery life) (Kohler and Turner 2001, Speed et al. 2010). Recapture success is typically low and though valuable insight has been gained from satellite tracking and biologging, these expensive tags remain prone to failure (Hays et al. 2007, Musyl et al. 2011) and restricted sample sizes.

The analysis of intrinsic elemental markers deposited in the hard (calcified) structures of fishes and other animals provides an alternative and comparatively rapid and inexpensive technique for identifying population structure and determining movement patterns on ecological time scales (Campana 2000, Campana and Thorrold 2001). Elemental markers (also known as elemental signatures or fingerprints) have

proven to be useful in ecological studies of bony fishes, providing insight into migratory patterns (Campana et al. 2007), population structure (Gillanders 2002), and dispersal (Miller and Shanks 2004). Naturally occurring elements are assimilated into actively calcifying structures as a byproduct of respiration and feeding (Campana and Thorrold 2001). A suite of physical and biological mechanisms can influence elemental incorporation into a calcified structure (Campana 1999). If the structure is metabolically inert and reflects, to some degree, the relative concentration of elements within the environment, then permanent geochemical records of environmental history may be preserved over the lifetime of an individual. Because the otoliths of bony fishes are metabolically inactive, grow throughout life, and are deposited in distinctive alternating bands from which ages can be determined, considerable attention has been directed toward extracting these chemical chronologies to identify intrinsic geospatial tags and trace movement and habitat use of teleost fishes (Elsdon et al. 2008). Elemental markers/tags confer an additional advantage over their extrinsic counterparts (floy tags, satellite tags) in that they are naturally occurring within all individuals within a population.

Sharks lack the calcified structures, known as otoliths, that are typically used for studies of dispersal and natal origin in bony fishes based on elemental markers. However, the vertebrae of sharks continue to grow throughout the lifetime of individuals and available data suggest that elements deposited into the vertebrae of sharks and rays are stable and not reworked (Clement 1992, Ashurst 2004).

Additionally, Smith et al. (2013) confirmed that at least some trace elements are detectable in elasmobranch vertebrae in relative proportion to their concentration in the environment. Tillet et al. (2011) found that vertebral elemental markers reflected patterns of age-specific habitat use in two shark species. Initial results from this emerging field indicate that the chemical composition of vertebrae can serve as valuable records of environmental history for sharks and rays over a lifetime. Analyses of naturally-occurring geochemical markers in highly mobile shark populations could provide a much needed alternative approach for identifying movement patterns and population structure, enabling the identification and delineation of movements and habitat use at spatial scales that are ecologically relevant to populations.

In this study, we investigated the utility of vertebral elemental signatures to discern the natal origins of young-of-the-year scalloped hammerhead sharks, *Sphyrna lewini*. As a first step toward assessing natal origins and connectivity in this population, we sought to determine if vertebral elemental composition differed among sharks within their putative nursery areas and the spatial and temporal scales over which this variation occurred. Our objectives were to: 1) examine patterns in natal vertebral chemistry across multiple spatial scales within years; 2) assess the extent of temporal variation in elemental signatures within and among individuals at the same sites within and among years; and 3) to determine if natal signatures can be used to accurately link individuals to their putative nursery grounds.

METHODS

Species selection and sample collection

Scalloped hammerhead sharks are a highly migratory species that occur in warm temperate and tropical estuarine, coastal, and pelagic marine habitats throughout the world (Compagno et al. 2005). Like many marine fishes, *S. lewini* use relatively shallow nearshore habitats as birthing and nursery areas (Clarke 1971, Simpfendorfer and Milward 1993). In addition to the resulting ontogenetic shifts in habitat use, *S. lewini* also exhibit spatial segregation by size and sex (Klimley 1987). Off the coast of Sinaloa, Mexico, juvenile hammerheads frequently aggregate at the mouths of rivers and near sand bars (Carvallo 1967). Tagging and acoustic telemetry studies have found that young-of-the-year scalloped hammerheads typically aggregate, display fidelity to core areas of activity, and may remain within nursery areas for a year or more (Holland et al. 1993, Duncan and Holland 2006). This pattern of restricted movement and extended residence within juvenile habitats suggests that *S. lewini* may be a good candidate for incorporating distinctive natal geochemical signatures. Additionally, young-of-the-year hammerhead sharks are a common component of small-scale fishery landings throughout much of the eastern Pacific (Peréz-Jiminéz et al. 2005, Bizzarro et al. 2009, Zanella et al. 2009), providing an existing framework for sample collection from a broad geographic area.

We opportunistically collected thoracic vertebrae of neonate and young-of-the-year scalloped hammerhead sharks from artisanal fishery landings along the Pacific

coast of Mexico and Costa Rica (Fig. 3.1a). More than 40 locations were surveyed during July-November, 2007-09 but a minimum number of samples ($n \geq 8$) was ultimately obtained from six sites, spanning >3000 km of coastline (Fig. 3.1a). Survey locations encompassed areas of contrasting geology and oceanographic circulation patterns (Castro et al. 2000, Tapia-Garcia et al. 2007), thus creating the potential for distinctive geochemical gradients to occur within the region of study. To evaluate intra-annual variability in elemental signatures within nursery areas, monthly surveys were undertaken at three locations in Sinaloa, Mexico between August-November, 2007-09 (Fig. 3.1b). Additional samples collected outside of our focal sampling period were provided by fishermen from June, 2008-09 and December, 2008. Samples (5-15 centra/individual) were stored frozen. Because *S. lewini* is a live-bearing species with placental connections to their embryos, the extent to which an individual's umbilical scar is healed can be used to approximate age (in weeks) and identify new-born sharks (Duncan and Holland 2006). Whenever possible, the location of capture, sex, total length (cm), total weight (kg), and status of the umbilical scar (opened/healed) were recorded from landed sharks. For the remaining sharks, size was estimated using the relationship between vertebral centrum diameter (CD, mm) and total length (TL, cm) determined from our samples:

$$TL = 9.26 * CD + 5.41 \ (n = 173; \ r^2 = 0.92)$$

Sample preparation

Vertebrae from young-of-the-year *S. lewini* were brought to Oregon State University for preparation and analysis. Cleaning and processing procedures followed those detailed in Smith et al. (2013). Individual centra were separated from vertebral segments, cleaned of tissue, and neural and haemal arches were removed using non-metallic, acid-washed (10% OmniTrace Ultra™ HNO_3, VWR™) dissecting tools. Centra were cleaned ultrasonically, dried, and embedded in polyester resin. The casting resin was infused with a spike of indium to provide a chemical reference of the centrum-resin interface for laser transects. Centra were thin-sectioned (~0.4 mm) using a low speed precision saw, mounted to acid-washed glass slides, and polished with lapping film (3M™; 30, 12, 5, 3, 1 μm). Polished centra were cleaned ultrasonically (45 min), triple rinsed, dried, and affixed to acid-washed petrographic slides (5-15/slide). Sample arrangements and groupings were randomized to prevent systematic bias. Nanopure® (18 M Ohm, Barnstead International) water was used during all cleaning stages. Sample preparation and drying procedures were completed in a Class 100 laminar flow work station. We viewed mounted vertebral sections under a dissecting scope and etched identifying marks in the resin adjacent to the birth mark to establish the transect position for elemental analysis (Fig. 3.2a). A distinctive change in angle of the intermedialia and a translucent band within the vertebrae are commonly associated with the transition from uterine to post-partum life history (Cailliet and Goldman 2004). Sample slides were then rinsed with ultrapure 1% nitric

acid, cleaned ultrasonically, triple rinsed, dried, and stored in plastic bags. Duplicate vertebral samples were prepared from 15 *S. lewini* to test the repeatability and consistency of elemental signatures within individuals.

Elemental analysis

We quantified the elemental composition of young-of-the-year *S. lewini* vertebrae using laser ablation-inductively coupled plasma mass spectrometry (LA-ICPMS). Analyses were completed at Oregon State University's WM Keck Collaboratory for Plasma Spectrometry using a VG PQ ExCell ICPMS coupled with a DUV193 excimer laser (New Wave Research). The laser was set with an ablation spot size of 80 μm at a pulse rate of 5 Hz and translated across the sample at 5 μm s^{-1}. Laser transects were positioned within the corpus calcareum and targeted the area of vertebral deposition immediately following the birth mark to characterize natal elemental signatures for all samples (Fig. 3.2b). Two additional laser transects were made within a subset of these samples to assess the elemental composition at different periods in the life history of individual sharks: 1) the area associated with pre-natal vertebral deposition (n = 47) and 2) the outer-most edge, which represented the vertebral elemental signature at the time of capture (n = 272). All transects were pre-ablated (100 μm spot size, 2 Hz, 100 μm s^{-1}) to further reduce potential sample contamination.

We collected data on 14 elements: lithium (Li), magnesium (Mg), calcium (Ca), titanium (Ti), vanadium (V), chromium (Cr), manganese (Mn), cobalt (Co), rubidium (Rb), strontium (Sr), cadmium (Cd), barium (Ba), lanthanum (La), and lead (Pb). Zinc (Zn) was purposely excluded as a potential marker due to evidence from lab validation studies that this element is more likely to reflect growth and physiological development than environmental conditions (Miller et al. 2006, Smith et al. 2013). To evaluate instrument drift and daily variation in instrument sensitivity, a National Institute of Standards and Technology (NIST) 612 glass standard was run at the beginning and end of analysis for each sample slide. Background levels of analytes were measured for 45 s prior to ablation and subtracted from those determined from counts obtained from the standard and vertebral samples. Estimates of precision (percent relative standard deviation, %RSD) for the NIST 612 standard and detection limits (DL, 3 x SD of the blank) are presented in Table 3.1. Analyte counts of vertebral transects were integrated and averaged using time-resolved software (PlasmaLab®). Cobalt (^{59}Co), cadmium (^{111}Cd), and lanthanum (^{139}La) counts were consistently below detection limits and were therefore excluded from analyses. Negative and subdetection level counts were occasionally obtained for V (n = 10, 3%) and Rb (n = 42, 11%). These samples, however, were retained for analyses and assigned equivalent replacement values (DL / $\sqrt{2}$) so as not to lose potentially relevant spatial or temporal information (Geffen et al. 2011). Measurements of titanium (^{47}Ti) were highly variable within the standard and among samples and were

therefore excluded from analyses. Thus, ^{7}Li, ^{25}Mg, ^{51}V, ^{52}Cr, ^{55}Mn, ^{85}Rb, ^{55}Sr, ^{138}Ba, and ^{208}Pb were ultimately examined in our study. Count data were normalized by ^{43}Ca to adjust for variability in instrument sensitivity and the amount of ablated material and converted to elemental ratios (e.g. Ba/Ca) based on measurements of the NIST 612 standard (Dove et al. 1996, Kent and Ungerer 2006). Elemental ratios are presented in mmol mol^{-1} (Mg, Sr) or μmol mol^{-1} (Li, V, Cr, Mn, Rb, Ba, Pb).

Statistical analyses

Elemental ratios were screened for outliers, assessed for normality, and tested for homogeneity of variances using univariate and multivariate techniques. Elemental ratio data were log transformed using the generalized procedure presented by McCune and Grace (2002). This approach can be particularly beneficial when working with low fractional values because it reduces the compression of data points that frequently result from transformation while maintaining the original order of magnitudes. Outliers were then identified and removed following visual inspection of frequency distributions and calculation of Euclidean distances. Those sample units whose mean distance to other sample units exceeded 3 SD of the grand mean of distances were excluded from analyses (McCune and Grace 2002). Transformation improved all distributional assumptions, however, univariate normality was not achieved for all elements (Shapiro-Wilk test) and multivariate distributions were negatively skewed in all three years (quantile-quantile plots). Variances were found to be equivalent for the

majority of elemental ratios in each year, with unequal variances detected among V/Ca and Mn/Ca in 2007, V/Ca in 2008, and Cr/Ca, Sr/Ca, and Pb/Ca in 2009 (Levene's test).

Individual variation

Growth rates and ontogenetic changes can influence elemental incorporation into calcified structures, potentially confounding efforts to discern spatial differences in elemental signatures (Campana 2005, Miller 2009). Therefore, we examined the relationships of each elemental ratio with TL to determine if differences in vertebral elemental composition were related to size. Correlations were evaluated by site, within month and year.

Initially, we characterized the consistency in vertebral elemental composition within individuals from a randomly selected subset of sharks (n = 15). For elemental signatures to serve as effective markers in calcified structures, elements should be deposited in a consistent fashion and elemental composition should reflect exposure/environmental history (Campana 2005, Elsdon et al. 2008). We therefore considered two hypotheses regarding elemental variation: 1) natal elemental ratios are equivalent between vertebrae within individual sharks; 2) elemental composition within the portion of vertebrae deposited while in-utero differs from post-partum elemental composition, because the environment of the mother is assumed to be

different from that of the free-living offspring (Table 3.2). These null hypotheses were both test using paired t-tests (Zar 1996).

Spatial variation

Variation in natal elemental signatures among sites was evaluated in three separate steps. First, one-way multivariate analysis of variance (MANOVA) was applied to test for differences in natal geochemistry among putative nursery areas using site as a fixed factor and elemental ratios as the response variables (Table 3.2). Because it is more robust in the case of small and unequal samples sizes and to departures from parametric assumptions, Pillai's trace was used as the test statistic (Tabachnick and Fidell 2007). Second, we assessed the ability of vertebral geochemical signatures to classify young-of-the-year *S. lewini* to their putative natal origins using forward step-wise discriminant function analysis (DFA). This multivariate technique was applied to evaluate the accuracy of group classification from elemental signatures and to identify those elements that contribute most to group separation (McGarigal et al. 2000). Step-wise selection removes variables that may be uninformative for distinguishing groups and seeks an optimal subset of discriminating variables. Thus, each DFA considered all nine elemental ratios but final classifications were based on a subset of these elements. Group classification accuracy was assessed using a leave-one-out jackknife procedure with the prior probabilities of group membership assumed to be uniform (White and Ruttenberg

2007). Both MANOVA and DFA are robust to departures from multivariate normality when the violation is the result of skewness rather than the influence of outliers (Tabchnick and Fidell 2007). Third, a chance-corrected classification (*Tau*) was calculated to determine if group assignments predicted by DFA exceeded that of randomly assigning individuals to groups (Klecka 1980). A maximum value for *Tau* of 1.0 signifies perfect agreement and a *Tau* of 0 indicates no improvement over chance. JMP (Version 8.0) was used for MANOVAs and DFAs were conducted using SYSTAT (Version 12.0).

Temporal Variation

Variation in vertebral elemental signatures within year and within site was evaluated with a blocked variation of the multi-response permutation procedure (MRPP) with data from our primary sample locations: Cospita, Mazatlán, and Tecapán (Table 3.2). MRPP calculates the average multivariate distance within *a priori* groupings and determines whether the average within-group distance is significantly smaller than those obtained from randomly assigning individuals to each group (McCune and Grace 2002, Mielke and Berry 2007). This non-parametric technique is not constrained by the distributional assumptions that are often difficult to satisfy with ecological and environmental data. We used blocked MRPP to test the null hypothesis of no difference in elemental composition between the natal signature and that of the outer-most vertebral edge within year and month of capture. Like parametric paired or

repeated measure tests, blocked MRPP is appropriate when samples are not independent (Mielke and Berry 2007). MRPP generates three measures for evaluating distances among groups: the test statistic (*T*), p-value, and a chance-corrected measure of within-group agreement (*A*). Separation between groups is characterized by *T*, with more negative values indicating greater separation between groups. Within-group homogeneity is summarized by *A* which provides a descriptor of effect size. If elemental signatures within a group were identical, *A* would be equal to 1.0. When heterogeneity within groups equals expectation by chance, then $A = 0$. We used Euclidean distance to determine average within-group distances and performed MRPP using PC-ORD. Test statistics were compared to a Pearson Type III distribution with mean, variance and skewness calculated from permuted datasets (McCune and Grace 2002).

If the chemical properties of vertebrae reflect, to some degree, the physical and chemical properties of the water mass they inhabit, then the elemental composition of vertebral edges should be indicative of ambient conditions at the time of capture. We therefore assessed changes in edge vertebral chemistry within each site across months as a measure of consistency in elemental composition within each year. Correlations (*r*) between the elemental composition of the outer-most vertebral edge and date of capture were evaluated for individual element/Ca ratios.

Inter-annual variation in natal geochemical signatures was examined using data from our primary sample locations (Table 3.2). First, a one-way MANOVA using

year as a fixed factor was conducted. Finally, where data were available, we assessed variation in elemental signatures within site and among years using standard MRPP (not blocked).

RESULTS

We sampled 1074 young-of-the-year *S. lewini* from artisanal fishery landings at seven locations during 2007-2009 (Fig. 3.1). We restricted our analyses to samples that represented discrete periods of capture within each month and were separated by a minimum of two weeks between months. LA-ICP-MS analyses were completed using the vertebrae of 440 sharks. Of these, 8 samples generated extremely low ^{43}Ca counts and were excluded from analysis. A total of 46 samples were identified as single or multi-elemental outliers and removed, reducing the total sample size in our study to 386 young-of-the-year *S. lewini* (Table 3.3).

Open umbilical wounds indicative of recent birth were present on some of the *S. lewini* collected between May and mid-October of each year (n = 121). Within our study area, therefore, parturition was protracted over at least a six month period. All sharks were captured in nearshore coastal habitats (non-estuarine) at depths of 15-35 m. Sharks ranged from 43.5-96.5 cm TL (Table 3.3; Appendix C). Elemental ratios were not significantly related to shark length ($r > 0.10$, $p > 0.06$).

Individual variation

The composition of natal elemental signatures did not differ between vertebrae within individuals (paired t-test; $t \geq 0.55$, $p \geq 0.14$, $n = 15$). This result provides support for the assumpation that elements are incorporated consistently within individuals and vertebrae.

Paired comparisons between vertebral regions that represented pre-natal and post-partum deposition revealed significant variation elemental composition. Ratios of V/Ca (paired t-test; $t = 3.37$, $p = 0.001$, $n = 47$), Mn/Ca (paired t-test; $t = 2.74$, $p = 0.013$, $n = 47$) and Sr/Ca ($t = 8.12$, $p < 0.001$, $n = 47$) were elevated within areas of the vertebrae that were deposited following birth when compared to the average elemental ratios measured within the pre-natal region of the same sample.

Spatial variation

Differences in natal elemental signatures were detected among the three putative nursery areas surveyed in 2007 (Fig. 3.3; MANOVA; Pillai's trace = 0.33, $F_{2,97} = 2.22$, $p = 0.006$). DFA of all 2007 data generated an overall jackknifed classification success of 54% (Table 3.4). Group assignment was most successful for the northernmost site, Cospita. Mn/Ca and Sr/Ca ratios accounted for 98% of the total variation among sites. Classification based on these discriminating variables achieved 37% fewer errors than would be expected by random assignment success (*Tau* = 0.37).

Spatial variation in natal elemental signatures was evident among the five locations examined in 2008 (Fig. 3.3; MANOVA; Pillai's trace = 0.74, $F_{4,117} = 2.71$, $p < 0.001$). Site-specific classification success was highly variable, ranging from 39-80% with the lowest and highest classification rates derived from Tecapán and Tárcoles, respectively (Table 3.4). Step-wise selection of variables indicated that optimal group separation could be achieved using four of the nine elements: Ba/Ca, V/Ca, Pb/Ca, and Mn/Ca ratios. These elemental ratios combined accounted for 100% of the observed dispersion among groups. Overall jackknifed classification success was 47% among 2008 samples, representing an improvement over random assignment to sites (*Tau* = 0.33).

We also found highly significant differences in natal elemental signatures among the five sites assessed in 2009 (Fig. 3.3; MANOVA; Pillai's trace = 0.84, $F_{3,118} = 2.71$, $p < 0.001$). DFA results for 2009 revealed moderate classification success of samples pooled across months (Table 3.4). An overall classification rate of 67% was calculated with site-specific values ranging from 50% (Tecapán) to 76% (Cospita). Sr/Ca ratios provided by far the largest discriminatory power with Mg/Ca and Cr/Ca ranking as the second and third most important variables for group assignments based on F-to-remove statistics. Group classification demonstrated an improvement over random assignment (*Tau* = 0.58).

If vertebral elemental composition is reflective of ambient environmental conditions then the outer-most edge of the vertebrae should be representative of the

most recent environmental conditions and provide a distinguishable site-specific signature where environmental differences exist among locations. Filtering of data reduced the overall sample size and available dates for analysis but revealed the potential for high classification success among putative coastal nursery areas (Table 3.5, Figs. 3.4, 3.5). Overall classification accuracy was high, ranging from 83-100% with predictions consistently exceeding chance expectations (*Tau* = 0.67–1.0). Annual site-specific edge classification ranged from 50-100%. *Sphyrna lewini* from the coast of Sinaloa (Cospita, Mazatlán, Tecapán) were consistently distinguished from the distant southern site of Puerto Madero with 100% accuracy (Figs. 3.4a, c, 3.5c). Sharks collected from Cospita and Mazatlán were classified to their respective sites of capture with high accuracy in all years. Ba/Ca, Sr/Ca, and Mg/Ca were also consistently identified as key discriminators when using the elemental composition of vertebral edge elemental. Li/Ca ratios were not included in all analyses because these data were not available for all of the 2007 samples. However, Li/Ca data were available from all sharks for analyses using vertebral edge chemistry and ranked as the primary contributor to group distinction in October 2008 and the second most important contributor in the September and October, 2009 groupings. The elemental signatures of vertebral edges were successful for discriminating among sites at scales of 10s, 100s, 1000 km.

Temporal variation

We observed significant intra-annual variation in natal elemental signatures within each of the primary study sites (Table 3.6). However, this variation was not consistent. In 2007, we did not detect significant differences across months (August September, October) within the site of Tecapán. Intra-annual differences in natal elemental signatures were, however, identified from Tecapán in the following year (August, November, December). Given the inconsistency of site-specific natal signatures, we re-examined classification accuracy by binning samples collected early (June-August) and those collected late (October-November) in each year into separate groups. Classification success was mixed and generally moderate when sharks were grouped into early and late season cohorts within each putative nursery area (Table 3.7). Among early season 2007 samples, classification accuracy ranged from 64-83% within sites, showing marked improvement in the ability to identify discrete natal signatures among sites using samples obtained earlier in the season. Mn/Ca ratios were the primary discriminator among sites in 2007 regardless of whether the data were pooled o r binned into early and late designations. Classification accuracy based on the late season 2008 designation was low (39%) in comparison to the initial results based on samples pooled across months (Table 3.4). However, assignment of sharks to the most distant sites, Puerto Madero and Tárcoles, in 2008 was high to moderate; 80% and 64%, respectively. Ba/Ca ratios ranked as the most useful identifier of groups in all three scenarios evaluated for 2008. In 2009, discrimination of natal

elemental signatures among the late grouping (71%) was more successful than that predicted for the early designation (63%). The contributions of elements to group discrimination differed among Early and Late designations within the 2009 data set. Mg/Ca and Sr/Ca were the most important variables contributing to group discrimination based on early natal signatures and Mn/Ca and Mg/Ca ranked as the principal variables used to discern groups within the late season designation. With the exception of 2008, classification performance improved from low to moderate levels when groups within each site were granted more specific temporal designations (i.e. month of capture rather than season of capture).

Similarity between natal elemental signatures and edge chemical composition generally decreased across months within a site, suggesting a change in vertebral chemistry over time (Appendix D). The duration over which natal and edge elemental signatures were similar varied among sites and years. Among sharks landed near Mazatlán in 2007, natal and outer edge vertebral signatures were equivalent through August (MRPP, $A = 0.12$, $p = 0.23$) and September (MRPP, $A = 0.02$, $p = 0.06$) but significantly differed in the following two months. Average within-group Euclidean distances of both natal and edge signatures are low, indicating that little variation in elemental composition exists within these groups. Site-specific elemental signatures, however, tend to become less similar as the year progresses (as measured by A).

Natal elemental signatures differed within sites and years (Table 3.5, Fig. 3.6). To determine which elements were associated with intra-annual variation, we

examined the relationships between each element-to-calcium ratio and the date of capture based on the elemental composition of vertebral edges (Appendix E). With the exception of Pb/Ca, element-to-calcium ratios displayed significant variation across months within at least one site and year. Mg/Ca, Sr/Ca, and Ba/Ca exhibited the most frequent variation. Mg/Ca, Sr/Ca, and Ba/Ca typically increased within vertebral edges with increasing dates of capture.

Natal elemental signatures were not consistent within sharks collected from Cospita (MANOVA; Pillai's trace = 1.30, $F_{2,62} = 12.77$, $p < 0.001$), Mazatlán MANOVA; Pillai's trace = 1.14, $F_{2,66} = 10.01$, $p < 0.001$), or Tecapán (MANOVA; Pillai's trace = 1.01, $F_{2, 46} = 5.14$, $p < 0.001$) across consecutive years. Despite significant inter-annual variability in natal elemental signatures, pair-wise MRPP revealed similar monthly patterns in the natal signatures of sharks in 2007 and 2009 (Table 3.8). Vertebral multi-elemental signatures of sharks collected in 2008, however, differed significantly from the 2007 and 2009 natal signatures from the same sites.

DISCUSSION

The use of intrinsic elemental markers to distinguish among groups of fish that have occupied different environments requires that measureable, characteristic differences exist among sites (Campana 2005). Vertebral elemental composition showed significant variation among sites in each year of our study. Classification

accuracy to putative nursery areas (natal signature) and location of capture (edge signature) improved from low to high when data were analyzed with greater spatial and temporal resolution (Fig. 3.7). Our results indicate that vertebral elemental signatures can be applied to distinguish *S. lewini* across small (5s km), moderate (100s km), and large spatial scales (>1000 km) with moderate to high classification success.

Distinguishing groups using vertebral elemental signatures

The birthing period of *S. lewini* is reported to occur from late July–October in the eastern Pacific (Madrid et al. 1997). However, we observed neonates as early as May. Harry et al. (2011) documented births of *S. lewini* throughout the year in Australia. Similarly, Clarke (1971) documented neonates in Kaneohe Bay, Hawaii throughout the year but found a peak in parturition during the summer months. It is therefore possible that that the extent of the birthing period in our study area could be more protracted than we observed. Sharks pupped later in the year have the potential to be introduced into an environment that differs physically and chemically from that which was experienced by those born at the same location only a few months earlier. Although environmental conditions were not measured within these study sites (e.g. salinity, temperature), we would not expect the physical and chemical properties of this coastal environment to be static across a six month period. Concentrations of elements in seawater are influenced by many physical and biological processes including discharge from rivers, oceanographic circulation, local geology,

biogeochemical cycles, wind, and anthropogenic input of pollutants (Bruland and Lohan 2003). Indeed, in our study area the parturition period is punctuated by the region's maximum rainfall between July and September. Increased freshwater input into coastal areas during these months would alter salinity, temperature, and water chemistry (Elsdon and Gillanders 2006). Salinity, for example, can have significant positive effects on Sr incorporation into the otoliths of some marine fishes (Martin et al. 2004, Martin and Wuenschel 2006). Several elements, including Sr/Ca, were found to increase over the course of each year within our primary study sites (Fig. 3.6). Site-specific elemental variation in vertebral chemistry over time could be more explicitly examined by profile analysis based on laser ablation conducted from the birth mark across the entire length of the corpus calcareum (Elsdon et al. 2008, Smith et al. 2013). The inclusion of basic physical data (i.e. water temperature, salinity) would be highly informative additions to future studies of elemental markers in elasmobranch populations.

Within-site variation in natal signatures has been documented among other species with extended spawning/birthing periods. Cook (2011) also reported diminished classification accuracy for a marine fish (*Hypsypops rubicundus*) when data were pooled among sites across the entire three month spawning season. However, otolith elemental signatures were found to be effective indicators of natal origin when data were analyzed in two week bins (Cook 2011). Our assessment of vertebral edges (Tables 3.5, 3.7) suggests that monthly intervals provide the best

resolution for discerning natal sources in *S. lewini*. Among species with life history strategies that include a protracted birthing or spawning duration, sampling and analysis should occur on time scales that encompass the scale of variation within as well as among sites.

Behavioral and ecological processes can also diminish the potential to detect characteristic elemental signatures within a site. Tracking studies of the young-of-the year *S. lewini* within nursery grounds in Hawaii indicate extended residence times of 3-4 months before juveniles depart estuaries and move into oceanic habitat (Duncan and Holland 2006). However, movements of young-of-the-year *S. lewini* along a coastal area that is comprised of largely homogenous habitat may be quite different than that observed within a remote island archipelago. Furthermore, individuals born earlier in the season may expand their home ranges or disperse from natal sites, mixing with sharks that were born elsewhere. Heupel et al. (2003) found that tagged blacktip sharks (*Carcharhinus limbatus*) residing within nursery grounds in Florida estuaries responded to sharp drops in barometric pressure associated with tropical storms and hurricanes by leaving their nursery grounds and moving into deeper water. If a similar response occurs in scalloped hammerhead sharks, the potential for mixing among nursery grounds would increase during and after tropical storms or cyclones which affected the study area annually. Such movement patterns could produce a mixture of natal elemental signatures occurring among young-of-the-year within a site, particularly as the season progresses.

DFA assumes that all potential sources are represented within a data set (McGarigal et al. 2000, White et al. 2008). The presence of sharks derived from unknown but chemically distinctive natal sources within our study sites, therefore, could reduce classification accuracy. We did not attempt to sample all potential source populations and it may not be feasible to do so in studies of broadly distributed species or those with protracted spawning periods like the scalloped hammerhead. Because DFA has been commonly applied in studies of otolith chemistry, we adopted this classification technique to to better facilitate comparison with previous studies. However, a variety of alternative models are being increasingly used to estimate the proportion of individuals derived from different sources within a mixed population based on otolith chemistry. Maximum likelihood (White and Ruttenberg 2007), Markov Chain Monte Carlo (White et al. 2008), or Bayesian approaches (Munch and Clark 2008) address population mixture and should be applied in directed studies of natal origin, movement, or population structure using vertebral elemental signatures.

The protracted duration of parturition introduced confounding temporal and biological factors that did not make *S. lewini* an optimal model species for discerning natal origins from vertebral chemistry. Given these circumstances, it is perhaps noteworthy that overall classification success was was not lower than determined in this study when samples were combined with years. Elasmobranchs exhibit a wide array of reproductive strategies, including egg-laying, live birth, pulsed and protracted birthing durations. Extended parturition periods are not therefore representative of

elasmobranchs and are not necessarily typical of other sharks within the hammerhead family (Stevens and Lyle 1989). Given the elevated potential for mixing among natal sites and temporal shifts in site-specific natal signatures, it is perhaps surprising that classification success could be established at low, moderate, and high accuracies depending on the degree of temporal partitioning of the data. Other highly mobile elasmobranchs, including spiny dogfish (*Squalus suckleyi*; Tribuzio et al. 2005), blacktip sharks (*Carharhinus limbatus*; Castro 1996), and bat rays (*Myliobatis californica*; Martin and Cailliet 1988) exhibit much more discrete parturition periods (1-2 months) within estuaries, embayments, and coastal environments. Our approach may be particularly well-suited for inferring the natal origins of species with similarly discrete parturition periods.

Spatial variation

Our results confirm that spatial differences in vertebral geochemistry can provide intrinsic natural markers of natal origin in an elasmobranch. All classifications exceeded chance expectations. Classification accuracy, however, ranged from low to moderate depending on the degree of spatial and temporal refinement of our data set (Fig. 3.7). Group identification and assignment based on otolith elemental signatures among marine fishes are often highly variable in their success rates and highlight the variety of spatial scales at which significant geochemical variation can occur (Gillanders 2002, Bergenius et al. 2005, Miller 2007,

Ruttenberg et al. 2008). Miller and Shanks (2004) measured the otolith edge elemental signatures of black rockfish (*Sebastes melanops*) recruits over 120 km with classification successes averages of 67 to 81%. Similar, moderate classification rates were reported for bluefin tuna, *Thunnus thynnus* (Rooker et al. 2003). Site-specific classification accuracy based on Early and Late binning of our data produced overall successes of 39-73% (Table 3.4), falling within the range of classification success that has been observed for marine teleosts (Patterson et al. 2004, Brown 2006, Ruttenberg and Warner 2006). The degree of spatial and temporal refinement that was applied to our data set (e.g. all collection dates combined, Early vs. Late) had strong influence on classification success (Fig. 3.7). Though samples sizes were low, our analyses of vertebral edge chemistry based on precise locations and dates of capture generated the highest classification accuracies in our study.

Multivariate analyses revealed spatial variation in the vertebral elemental signatures of young-of-the-year *S. lewini* at large (1000s km), moderate (100s km), and relatively fine (5 to 10 km) spatial scales. Variation in vertebral elemental signatures at multiple spatial scales presents the opportunity to address a broad range of research questions. For example, Dorval et al. (2005) used otolith chemistry to discern habitat use of juvenile spotted seatrout (*Cynoscion nebulosus*) of specific seagrass beds at spatial scales of 15 km. Conversely, fine scales of variation in elemental signatures can inhibit the ability to trace patterns of connectivity and complicate interpretations of elemental data (Miller 2007, Ruttenberg et al. 2008). On

the scale of 10s-110 km, Fodrie and Levin (2008) successfully applied otolith chemistry to determine ontogenetic movements and the relative contributions of four coastal nursery habitats to adult California halibut (*Paralichthys californica*) populations. Our analyses of vertebral edge elemental signatures successfully discriminated between hammerhead sharks captured in the Gulf of Tehuantepec (P. Madero) and those from the entrance to the Gulf of California off Sinaloa, regions separated by >1300 km, with 100% accuracy (Table 3.7, Figs. 3.4, 3.5c). This strong geographic separation suggests differentiation between these water masses that could be useful to trace migration patterns over a lifetime or discern natal origins at a regional scale. Bluefin tuna (*Thunnus thynnus*) from distinct nursery areas separated by 100s-1000 km within the Mediterranean Sea were identified from otolith chemistry (Rooker et al. 2003). Spatially coarse, regional assessments based on vertebral elemental signatures may prove to be particularly informative for delineating the movements and population structure of highly mobile species.

All element-to-calcium ratios included in this study contributed to the assignment of natal origins of scalloped hammerhead sharks. The elements most consistently identified as principal discriminators among groups through step-wise DFA were Ba/Ca, Mn/Ca, and Sr/Ca. These elemental ratios have generally proven to be the most useful discriminators in studies of population structure and natal origin using fish otoliths (Thresher 1999, Campana 1999) and squid statoliths (Warner et al. 2009). Validation studies in fish and an elasmobranch support the assumption that

Ba/Ca is incorporated into otoliths (Martin et al. 2004) and vertebrae (Smith et al. 2013) in proportion to concentrations in the water. Ba incorporation into vertebrae, however, can also be affected by temperature Smith et al. (2013). Considerations of the interactive effects of ambient elemental concentrations and physical environmental conditions (i.e. salinity, temperature) on elemental incorporation are key to consider in reconstructions of environmental history. Further validation studies of elemental incorporation into elasmobranch vertebrae are needed to clarify relationships between elemental markers and the physiochemical environment.

The opportunistic use of fishery-derived specimens unfortunately did not allow sampling of sharks from the exact same location within a site over time. As a result, our samples were derived from general fishing areas associated with a landing site rather than discrete locations (i.e. reef, river mouth). Though fishing behavior at all locations changed across seasons and with markets, fishing effort out of Tecapán covered a broader spatial scale (~70 linear km) than was recorded from Cospita or Mazatlán during our surveys (Fig. 3.1b). Classification success in our study was also consistently lowest within the site of Tecapán. The potential for sampling unaccounted source groups or mixing of groups was likely greater among sharks sampled from this fishery. Alternatively, environmental conditions at this location may have not been sufficiently distinctive from Mazatlán or Cospita to generate a reliable degree of elemental variation among sites.

Temporal variation

Temporal differences in elemental signatures can obscure patterns of spatial variation. Although temporal stability of elemental signatures (and by extension the environment) within a site would improve and simplify the identification of geochemical markers in calcified structures, it is an unrealistic expectation in the dynamic marine environment. We detected both intra- and inter-annual variation in natal elemental signatures within our study sites. However, vertebral geochemistry was not found to differ significantly between 2007 and 2009 (Table 3.5). The extent of temporal variation in our study may have been exaggerated by relatively low sample sizes and unequal representation among months and sites.

Relatively few studies have examined geochemical variation on intra-annual scales, but short-term variation in natal elemental signatures has been documented on the order of weeks, months, and season (Hamer et al. 2003, Cook 2011, Tanner et al. 2012). Annual variation in elemental signatures has been more frequently documented and even where inter-annual variation has been shown to be minimal, improvements in group classification accuracy were reported when cohorts were examined by each year separately (Brown 2006). Because many species, including *S. lewini*, exhibit extended spawning/birthing/recruitment periods, care should be given to identify and collect distinct "chemical cohorts" that occur within a year-class in addition to evaluating inter-annual variation among year-classes (Gillanders 2002). This approach would generate a reference collection of vertebral elemental signatures

from which the most appropriate spatial and temporal scale could be identified for analyses (Gillanders 2002, Warner et al. 2005). The scale of temporal variation within a site could initially be evaluated by collecting and analyzing water samples. Mapping water chemistry data should provide a useful reference for the range of temporal variation within a site from which a robust and inclusive sampling program could be developed and potentially offer insights into relationships between water masses and the chemistry of calcified structures (Elsdon et al. 2008).

During 2008, logistic constraints limited restricted our early sampling efforts (August, September), greatly skewing the representation of samples toward later months (October, November). Because few neonate hammerheads were observed within the fishery by mid-October of each year, samples during these later dates have a higher potential to reflect mixed and unknown groups, thereby increasing the likelihood of misclassification in DFA. Classification accuracy within the 2008 dataset was consistently the poorest among the three years surveyed.

Recommendations and future directions

We suggest three methodological considerations that warrant evaluation and may improve the discriminatory power of assignments to sites of natal origin. First, given the potential for individual variation in growth and temporal shifts in site-specific elemental signatures, the spot size of 80 μm used for laser transects in this study may have been insufficient to adequately capture similar periods of deposition

among all young-of-the-year sharks. Second, to reduce expenses and maximize the number of samples analyzed in a day, we conducted horizontal transects near the birth mark and vertebral edge (Fig. 3.2). However, elemental profiles collected along the entire length of the corpus calcaerum (e.g. Smith et al. 2013) may provide greater flexibility and opportunities for analysis because the resulting data encompass the entire life history of an individual rather than a brief time interval. This approach requires a greater investment of time while running samples and when processing elemental data because the resulting elemental profiles are related to specific vertebral positions with the aid of measurement and image analysis. Finally, greater consistency among natal signatures within sites may have been achieved using a smoothing function when processing elemental count data. Smoothing approaches can eliminate noise from transects that occur at too fine of a scale to contribute to more generalized site-specific elemental signatures (Sinclair et al. 1998, Tillett et al. 2011).

Trace elements have often been used in combination with stable isotopic analysis as intrinsic tracers of movement and habitat use in aquatic environments (Best and Schell 1996, Graham et al. 2010). Walther and Thorrold (2008), for example, developed an atlas of elemental signatures that included strontium ($^{87}Sr/^{86}Sr$) and stable oxygen ($\delta^{18}O$) isotopes to reliably distinguish the natal origins of American shad (*Alosa sapidissima*). Stable carbon ($\delta^{13}C$) and oxygen isotopes have also been used to infer natal origins and population structure in marine fishes (Ashford and Jones 2007, Rooker et al. 2008). Analyses of stable carbon and nitrogen isotope ratios

within the vertebrae of elasmobranchs have primarily been used to identify ontogenetic dietary shifts (MacNeil et al. 2005, Estrada et al. 2006, Kerr et al. 2006). The extension of stable isotope studies beyond strictly trophic inquiries has only recently been pursued for elasmobranchs (Werry et al. 2011, Carlisle et al. 2012). We encourage the combined anlaysis of trace elements and stable isotopes in vertebrae as a method to generate more robust details on the spatial dynamics and environmental history of shark and ray populations.

How much time is required for ambient environmental conditions to be recorded in the vertebrae of an elasmobranch? Smith et al. (2013) confirmed that vertebral chemical composition, of at least some elements, is indicative of environmental history in round rays, *Urobatis halleri*. However, numerous factors, including temperature and water chemistry, influence elemental incorporation and composition of calcified structures (Campana 1999, Smith et al. 2013). Daily growth increments, evident in otoliths during the early life history of fishes, do not appear to be present in elasmobranch vertebrae (Campana and Thorrold 2001, Cailliet and Goldman 2004) and restrict the chronological record from which environmental history can be inferred. Changes in vertebral chemistry in response to external changes in the ambient environment were reported to occur within weeks in captive bull sharks (*Carcharnius leucas*; Werry et al. 2010). However, rapid somatic growth and associated rates of vertebral mineralization during the early juvenile stage is likely to generate records of environmental history on a finer temporal scale than that of

weeks. Elemental incorporation studies directed toward understanding response times associated with elemental uptake are needed to inform and guide future research.

The caveats and conclusions of our investigation highlight the need for assessing relevant spatial and temporal scales of variation in vertebral elemental signatures and the importance of cohort-specific analyses in studies of natal origin. These considerations and conclusions are not unique to studies of elasmobranch vertebral geochemistry (Gillanders 2002, Ruttenberg et al. 2008, Elsdon et al. 2008). As studies of vertebral elemental composition in elasmobranchs are extended from exploratory research to directed investigations of spatial ecology, researchers have the benefit of guidance from decades of work on otolith chemistry. Our findings, when considered in combination with two recent publications on vertebral chemistry (Tillett et al. 2011, Smith et al. 2013) indicate that studies of elemental composition are a promising new tool that can be applied independently or in conjunction with genetic (Miller et al. 2005), stable isotopic (Thorrold et al. 1998), or essential amino acid (McMahon et al. 2011) techniques to advance and improve our understanding of connectivity, movement, and habitat use in shark and ray populations.

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Table 3.1. Estimates of precision for laser ablation inductively coupled plasma mass spectrometry and limits of detection for specific isotopes. Values for %RSD (percent relative standard deviation) are dimensionless and were derived from measurements of a National Institute of Standards and Technology (NIST) glass standard (612). Units for detection limits are presented as mmol mol^{-1} (Mg, Sr) and μmol mol^{-1} for all other elements.

Isotope	NIST %RSD	Limits of Detection
^{7}Li	5.9	0.037
^{25}Mg	13.8	0.125
^{47}Ti	17.5	0.011
^{51}V	5.7	0.005
^{52}Cr	5.1	0.004
^{55}Mn	5.1	0.008
^{59}Co	5.3	0.012
^{85}Rb	6.2	0.008
^{88}Sr	4.4	0.018
^{90}Zr	7.7	0.027
^{111}Cd	14.4	0.013
^{138}Ba	6.0	0.002
^{139}La	6.0	0.007
^{208}Pb	8.5	0.004

Table 3.2. Summary of the primary hypotheses, scale of inquiry, and analytical approaches applied in this investigation. Region assayed refers to the location along the vertebral centra from which element-to-calcium ratio data were determined. MANOVA = multivariate analysis of variance, DFA step-wise discrminant function analysis, MRPP = multi-response permutation procedure, P = parametric statistical method, NP = nonparametric statistical method. MRPP analyses included standard and blocked designs.

Hypothesis	Scale of inquiry	Region assayed	Method	Procedure
Consistency in natal signatures within individuals	Individual	Natal	Paired-t	P
Differences in pre- and post-partum elemental composition	Individual	Natal, Pre-natal	Paired-t	P
Natal signatures differ among sites	Spatial	Natal	MANOVA	P
			DFA	P
Characteristic site-specific elemental signatures	Spatial	Edge	MRPP	NP
			DFA	P
Stability in elemental signatures within sites within year	Intra-annual	Natal, Edge	MANOVA	P
			MRPP	NP
Stability in elemental signatures within sites among years	Inter-annual	Natal	MANOVA	P
			MRPP	NP

Table 3.3. Summary of survey and collection efforts by location and date. Sites are presented in descending order from the northern- to the southern-most. Distance from previous site indicates an approximate linear distance. Months from which samples were collected and incorporated into analyses are identified with an "x". n represents the number of individuals from a site that were included in analyses. The average total length (TL) ± standard deviation (cm) of young-of-the-year scalloped hammerhead sharks (*Sphyrna lewini*) included in analyses are presented by site.

		Distance from	Month of collection									
Year	Location	previous site (km)	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	n	Mean TL (cm)
2007	Cospita					x	x	x	x		33	65.6 ± 10.6
	Mazatlán	120				x	x	x	x		38	60.6 ± 10.3
	Tecapán	66				x	x	x			27	64.4 ± 8.1
2008	Cospita								x		15	65.0 ± 9.2
	Mazatlán	120		x	x	x		x	x		47	58.6 ± 8.7
	Tecapán	66				x		x	x	x	33	74.3 ± 11.9
	P. Madero	1643							x		10	61.5 ± 5.1
	Tárcoles	1268							x		11	74.0 ± 6.7
2009	Cospita					x	x	x	x		49	66.8 ± 9.8
	Mazatlán	120				x		x			10	62.4 ± 10.9
	Tecapán	66		x		x					24	55.0 ± 3.5
	San Blas	88	x								7	52.3 ± 2.9
	P. Madero	1302					x	x			24	53.8 ± 3.7

Table 3.4. Cross-validated classification accuracy (%) of step-wise discriminant function analysis to putative natal origins by year. Samples were pooled across months within each year. Tau is a measure of improvement in classification accuracy over chance (1.0 = no errors in prediction, 0 no improvement over random chance).

Year	Overall % Classificaiton Success	Tau	Site	n	% Correctly Classified
2007	54	0.37	Cospita	33	70
			Mazatlán	38	61
			Tecapán	27	26
2008	47	0.33	Cospita	15	47
			Mazatlán	47	43
			Tecapán	33	39
			P. Madero	10	67
			Tárcoles	11	80
2009	67	0.58	Cospita	49	76
			Mazatlán	10	60
			Tecapán	24	50
			San Blas	7	57
			P. Madero	24	71

Table 3.5. Multi-response permutation procedure tests of difference in natal elemental signatures by month and year. Pair-wise comparisons of significance were identified using Bonferroni-corrected p-values. P-values presented in bold identify significant differences in multi-elemental composition within a site between years.

Location	Month	n	Years	T	p	A
Cospita	Aug	22	2007 vs. 2009	0.29	0.524	-0.01
	Nov	42	2007 vs. 2008	-9.37	**<0.001**	0.16
			2007 vs. 2009	-2.38	0.030	0.04
			2008 vs. 2009	-8.36	**<0.001**	0.14
Mazatlán	Aug	35	2007 vs. 2008	-9.06	**<0.001**	0.22
			2007 vs. 2009	-2.30	0.036	0.06
			2008 vs. 2009	-9.17	**<0.001**	0.21
	Oct	34	2007 vs. 2008	-6.99	**<0.001**	0.09
			2007 vs. 2009	-2.94	0.080	0.01
			2008 vs. 2009	-7.44	**<0.001**	0.11
Tecapán	Aug	27	2007 vs. 2008	-5.07	**0.002**	0.15
			2007 vs. 2009	0.92	0.924	-0.02
			2008 vs. 2009	-5.00	**0.002**	0.11

Table 3.6. Results of one-way multivariate analysis of variance (MANOVA) to examine intra-annual variation in natal elemental signatures within site and year. P-values presented in bold identify significant temporal differences in multi-elemental composition.

Site	Year	Factor	Months Analyzed	Pillai's Trace	F	df	p
Cospita	2007	Month	Aug, Sep, Nov	0.952	2.269	2, 6	**0.018**
	2009	Month	Aug, Sep, Oct, Nov	0.816	1.702	3, 7	**0.027**
Mazatlán	2007	Month	Aug, Sep, Oct, Nov	1.192	1.746	3, 6	**0.044**
	2008	Month	Jun, Jul, Aug, Oct, Nov	1.258	1.937	4, 7	**0.003**
	2009	Month	Aug, Oct	1.401	4.059	1, 7	**0.039**
Tecapán	2007	Month	Aug, Sep, Oct	0.689	1.183	2, 6	0.326
	2008	Month	Aug, Nov, Dec	1.058	2.745	2, 7	**0.003**
	2009	Month	Jun, Aug	1.645	2.559	1, 7	0.056

Table 3.7. Cross-validated classification accuracy (%) of step-wise discriminant function analysis to putative natal origins based on Early (July-August) and Late (October-November) season groupings by year and site. Tau is a measure of improvement in classification accuracy over chance (1.0 = no errors in prediction, 0 no improvement over random chance).

Year/ Period	Overall % Classificaiton Success	Tau	Site	n	% Correctly classified	Year/ Period	Overall % Classificaiton Success	Tau	Site	n	% Correctly Classified
2007	73	0.62	Cospita	11	64	2007	63	0.48	Cospita	17	71
Early			Mazatlán	13	83	Late			Mazatlán	22	59
			Tecapán	11	70				Tecapán	10	60
2008	70	0.39	Mazatlán	21	72	2008	39	0.24	Cospita	15	27
Early			Tecapán	12	63	Late			Mazatlán	23	13
									Tecapán	25	39
									P. Madero	10	80
									Tárcoles	11	64
2009	63	0.51	Cospita	31	72	2009	71	0.49	Cospita	21	71
Early			Mazatlán	10	60	Late			Mazatlán	7	43
			Tecapán	24	58				P. Madero	15	85
			San Blas	7	45						
			P. Madero	12	71						

Table 3.8. Cross-validated classification accuracy (%) of step-wise discriminant function analysis to collection locations based on the elemental signatures of vertebral edges and discrete temporal and spatial group designations within each site. Samples were drawn from a select subset of sharks that were captured on the same date, at known locations. Tau is a measure of improvement in classification accuracy over chance (1.0 = no errors in prediction, 0 no improvement over random chance). Note that sample availability allowed us to compare the elemental signatures of vertebral edges between sharks captured at two different locations within the broader Mazatlán fishery in October, 2008 (Mazatlán A, Mazatlán B).

Year	Month	Overall % Classificaiton Success	Tau	Site	n	% Correctly Classified
2007	August	94	0.89	Cospita	11	91
				Mazatlán	6	100
2008	October	83	0.67	Mazatlán A[1]	7	86
				Mazatlán B[1]	11	82
	November	86	0.68	Cospita	15	93
				Tecapán	6	50
				P. Madero	7	100
2009	August	72	0.72	Cospita	11	64
				Mazatlán	6	83
				Tecapán	8	75
	September	100	1.00	Tecapán	10	100
				P. Madero	13	100
	October	89	0.84	Cospita	7	71
				Mazatlán	5	100
				P. Madero	7	100

[1] Elemental variation between two groups captured at different locations within the fishery off Mazatlán. Mazatlán A (Marmol) is approximately 7 km north of the site designated as Mazatlán B.

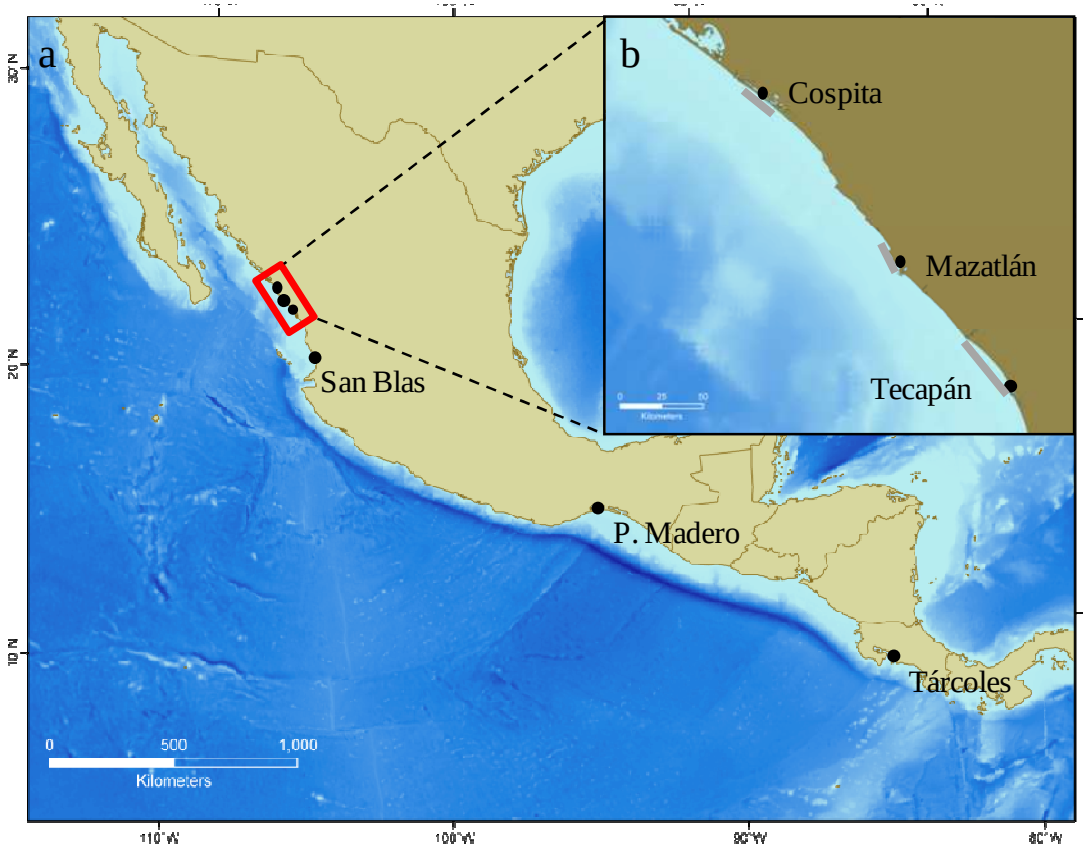

Figure 3.1. Collection sites for young-of-the-year scalloped hammerhead sharks, *Sphryna lewini*, 2007-2009. (a) The region contained in the rectangle indicates the three sampling locations from which regular monthly sampling was conducted. (b) Areas of fishing activity associated with the three primary landing sites surveyed in this study (grey bars).

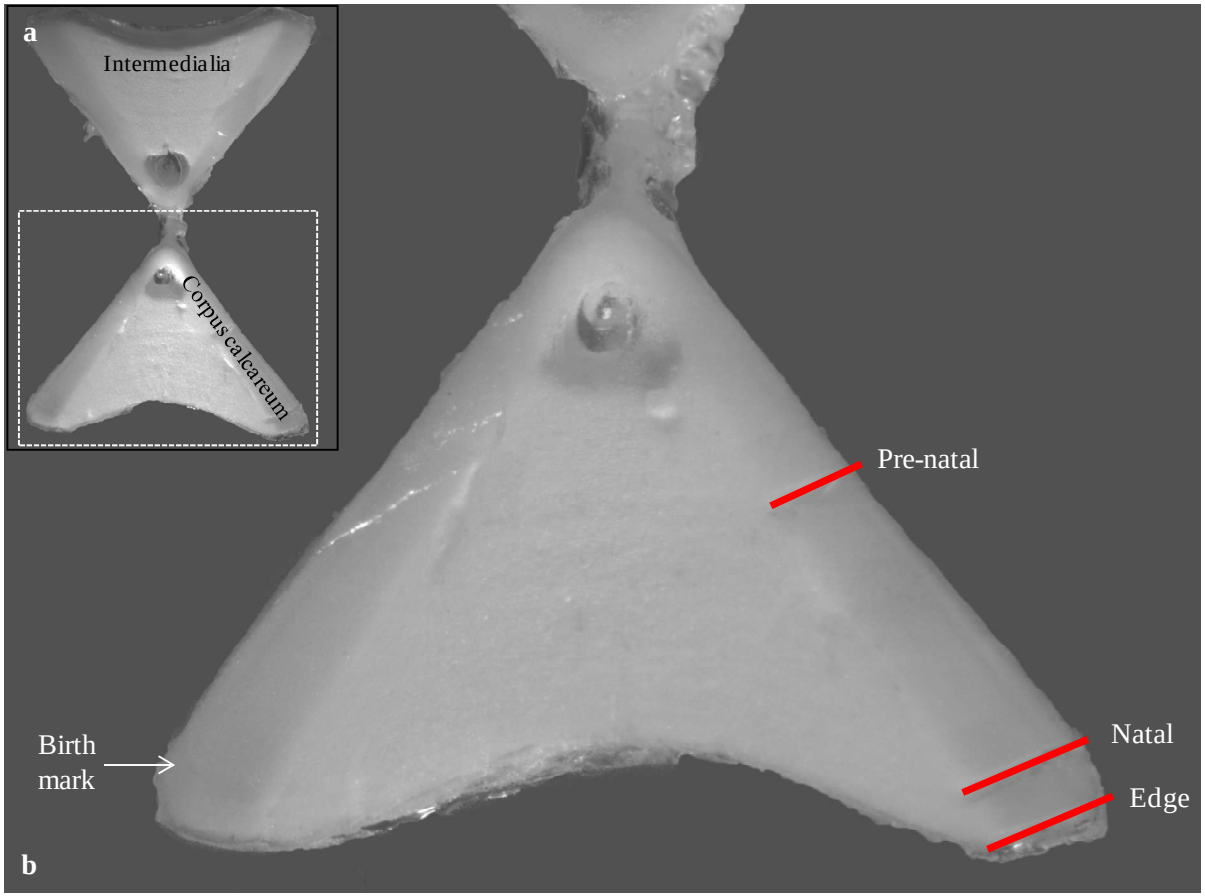

Figure 3.2. Sagittal section of young-of-the-year scappoped hammerhead (*Sphryna lewini*) vertebrae. (a) Examples of a whole, thin-sectiononed vertebra and (b) the regions selected for laser ablation in this study.

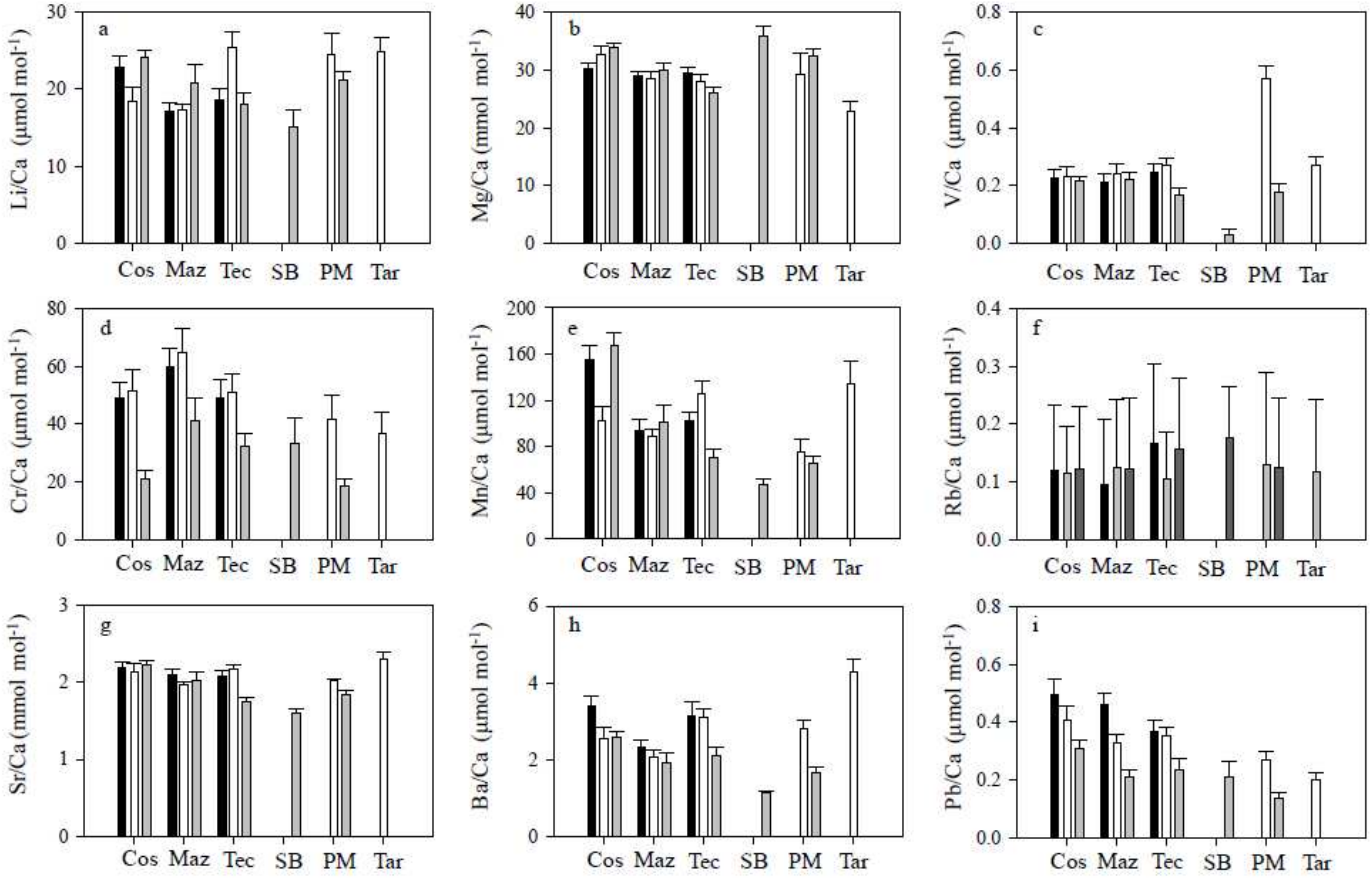

Figure 3.3. Average (± SE) element/calcium ratios of (a) lithium, (b) magnesium, (c) vanadium, (d) chromium, (e) manganese, (f) rubidium, (g) strontium, (h) barium, and (i) lead observed within sites by year. Sites are arranged from north to south and include the primary study locations of Cospita (Cos), Mazatlán (Maz), and Tecapán (Tec) as well as San Blas (SB), Puerto Madero (PM), and Tárcoles (Tar). Black bars = 2007, white = 2008, grey = 2009. Note that measurements of Li were only available from a subset of samples in 2007.

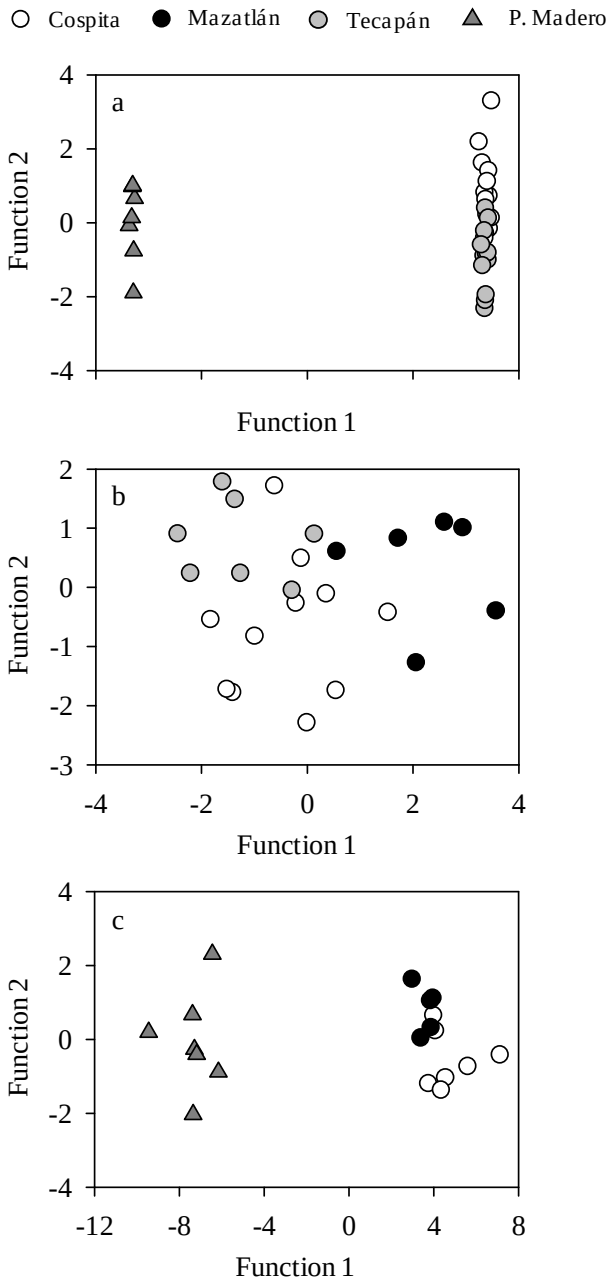

Figure 3.4. Canonical function plots of step-wise discriminant function analyses based on the elemental signatures of vertebral edges and discrete temporal and spatial group designations by site, month, and year of capture. a) Cospita, Tecapán, and Puerto Madero (P. Madero), November, 2008; b) Cospita, Mazatlán, and Tecapán, August, 2009; and c) Cospita, Mazatlán, and Puerto Madero, October, 2009.

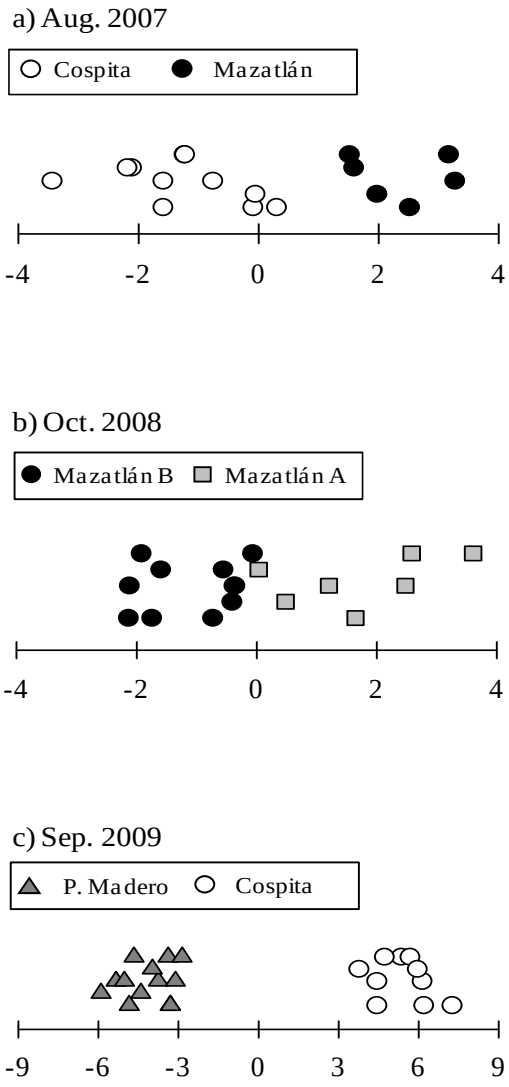

Figure 3.5. Jittered dot density plots of canonical function 1 resulting from step-wise discriminant function analyses of vertebral edge elemental signatures and discrete temporal and spatial group designations by site, month, and year of capture. a) Cospita, Mazatlán, August, 2007; b) two sites nested within Mazatlán, Mazatlán A, Mazatlán B, October, 2008; and c) Cospita, Puerto Madero (P. Madero), September, 2009.

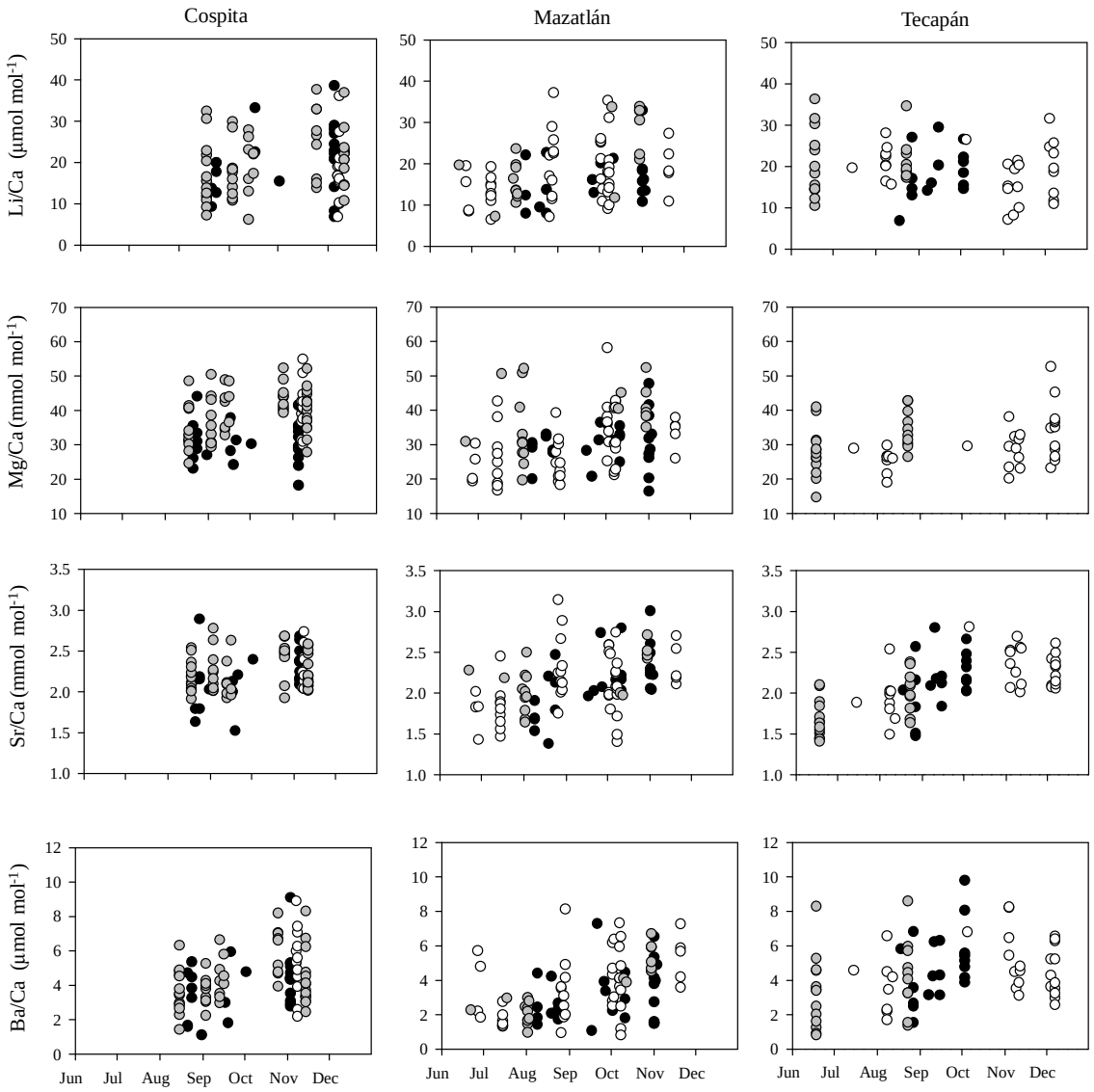

Figure 3.6. Intra-annual variation in selected element/calcium ratios by site. Lithium (Li/Ca), magnesium (Mg/Ca), strontium (Sr/Ca), and barium (Sr/Ba) displayed significant increases across months within years. Elemental ratios determined from vertebral edges of specimens captured within each month. Shaded circles represent the year of collection: black = 2007, white = 2008, and grey = 2009.

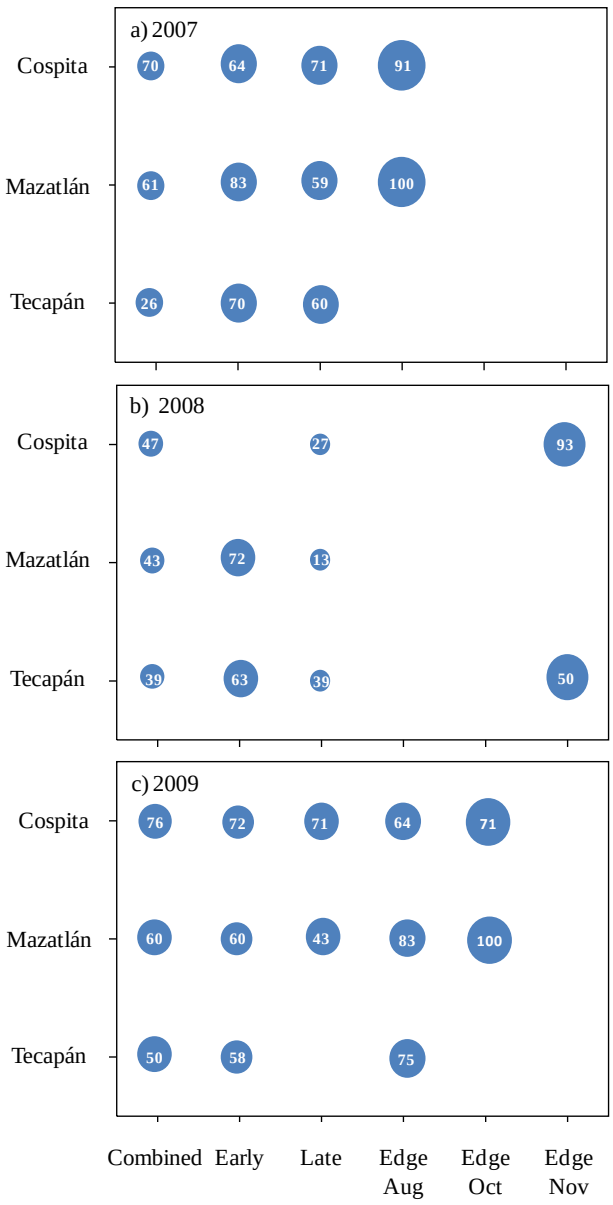

Figure 3.7. Cross-validated classification accuracy (%) of step-wise discriminant function analysis to putative natal origins/sites of capture by site, year, and category of analysis. Analyses of natal elemental signatures are presented as Combined, Early, and Late. Combined indicates analyses in which natal elemental signatures were pooled across months of collection. Early (July-August) and Late (October-November) designates analyses that were restricted to generalized dates of collection within each year. Vertebral edge elemental signatures were based on discrete temporal and spatial group designations by site, month, and year of capture. Circle size represents overall classification success. Values presented within the circles are site-specific classification accuracies as determined from discriminant function analysis.

CHAPTER 4

CONCLUSIONS

Population connectivity is fundamental to the persistence and maintenance of species with patchy distributions (Hastings and Botsford 2006, Kritzer and Sale 2006). Studies of connectivity in marine ecosystems have recently been invigorated by advances in elemental analysis of fish otoliths (Thorrold et al. 2007). The use of intrinsic chemical markers to trace dispersal and exchange of individuals among discrete habitats or subpopulations invites a metapopulation perspective of ecology and conservation of threatened species, which may be essential for their long-term persistence. In marine ecosystems, the concept of a metapopulation has evolved to be more generally viewed as a system of discrete local populations that are connected, to some degree, by the exchange of individuals through dispersal processes (Sale et al. 2006). This exchange or connectivity may have a significant influence on population demographics, as many of the dispersal events for marine species are directed ontogenetic shifts where habitat needs are life-stage specific. Habitat-specific survival rates, source and sink dynamics, and the extent of self-recruitment represent important areas of directed geochemical research for marine organisms with larval early life history stages (Levin 2006, Cowen et al. 2007).

Despite a tendency for broad-scale movements, both within and across life history stages, shark and ray populations are often highly structured and segregated among discrete habitats. However, metapopulation dynamics of sharks and rays have

rarely been considered or discussed. The tendency for complex, spatially-segregated population structure connected via adult-mediated, rather than larval, dispersal lends itself to the metapopulation perspective. Intrigued by attempts to trace sources, patterns, and distances of larval dispersal using elemental markers in calcified structures, I began to consider the utility of chemical tools to studies of elasmobranch metapopulations. However, I was quickly confronted with basic but essential questions about elemental incorporation into cartilaginous vertebrae and the assumption that these calcium phosphate structures could even provide reliable records of environmental history. Thus, a proof of concept approach and test of primary assumptions was necessary before directed studies of connectivity could be supported. The results of this dissertation research represent the first inquiry into the potential application of multi-elemental analysis of vertebrae to studies of natal origin and population connectivity in elasmobranch fishes.

The identification of nursery areas for threatened shark populations has been a frequently recommended conservation measure (Branstetter 1990, Applegate et al. 1993, Heupel et al. 2007). Recent evidence suggests that adult females of many species may return to their site of natal origin (natal fidelity, philopatry) (Keeney et al. 2003, Hueter et al. 2005). If natal site fidelity is a widespread adaptation, specific nursery areas would essentially be "selected for" because they were successfully contributing reproductive adults to the population. Vertebral geochemistry could be applied to quantify the extent of connectivity and dispersal among populations and identify sites (or regions) that contribute the greatest proportions to overall population

productivity (Beck et al. 2001, Gillanders 2002, Dahlgren et al. 2006). Identifying and protecting nursery areas that contribute disproportionately to the adult segment of the population could have significant benefits for the conservation and recovery of elasmobranchs. While recognizing that protection of nursery grounds affects a limited portion of the population (Kinney and Simpfendorfer 2009), I view nursery areas as destination points that act as a nexus in spatially-segregated populations. As such, nursery areas represent more than depositories for offspring or sites for possible protection of early life history stages. The delineation of nursery grounds provides critical reference points from which population and ecosystem connectivity can be evaluated.

Effective management strategies for highly mobile shark and ray populations will require the recognition and consideration of multiple spatial and temporal scales. Effort should be directed toward not only identifying sites of natal fidelity but those of breeding and feeding fidelity as well. Identification of sites of fidelity or biological/diversity hotspots will require multi-national effort and evaluation on the scales of region and large marine ecosystem for those species that regularly traverse geopolitical boundaries (Myers and Worm 2005, Wallace et al. 2010, Lucifora et al. 2011). In many cases, these sites will benefit more than a single species (Speed et al. 2010). Recently, shark sanctuaries that include a range of habitats and protect different life history stages have been adopted by a number of nations, including Palau and Honduras. Spatial closures, such as marine protected areas, need not be the only spatially-explicit management technique considered. Pondella and Allen (2008), for

example, found that gear restrictions (gill net ban) may have been critical to the recovery of several over-exploited, long-lived species off the coast of California, including the tope shark, *Galeorhinus galeus*. Temporal restrictions on fishing effort could also be implemented in areas known for seasonal breeding or birthing aggregations.

Although the concept of connectivity is perhaps most commonly associated with the exchange of individuals among populations, in other fields of ecology the term is also applied to describe the exchange at evolutionary, community, and individual scales (Sheaves et al. 2009, Boström et al. 2011). Connectivity between habitats has a variety of consequences on community dynamics through trophic interactions and energy transfer. Additionally, connectivity can be evaluated at finer spatial and temporal scales to determine the use of habitat within a nursery area, for example, or identify ontogenetic movements between coastal and offshore habitats. Habitat use at these scales has been examined using telemetry (Papastamatiou et al. 2009, Chin et al. 2013) but elemental profile analyses of vertebrae could also be used to quantify age-specific habitat use among individuals to gain insight into connectivity on the scale of a seascape.

Discerning patterns of connectivity, habitat use, or natal origin from elemental markers is based on the assumption that elements of interest are incorporated into calcified structures in relation to their concentration in the water. Certain elements are thought to be incorporated into otoliths through substitution for calcium ions (Campana 1999), but environmental variables such as temperature and salinity can

influence elemental incorporation (De Vries et al. 2005, Martin and Wuenschel 2006). To determine the extent to which vertebral elemental ratios reflect the ambient environment, I manipulated water temperature and environmental concentrations of barium (Ba) in an experimental setting using round stingray (*Urobatis halleri*) as a model species (Chapter 2). I tested several hypotheses related to the validity of elemental signatures as a means to identify environmental history/habitats for elasmobranchs.

Like other laboratory validation studies of elemental incorporation to date, my results indicate that vertebral elemental composition reflects the physical and chemical environment, albeit with significant physiological regulation and element-specific temperature dependence. Despite the lack of a simplistic, direct relationship between vertebral and environmental chemistry, the combined laboratory experiments confirmed that changes in environments experienced by round rays were temporally matched by changes in vertebral chemistry. These results show great promise for application to studies of elasmobranch populations. I demonstrated that the composition of certain minor and trace metal elements in elasmobranch vertebrae was related to the physical and chemical properties of water. Vertebral incorporation of three (Mg, Mn, Ba) of the six elements evaluated demonstrated significant temperature-dependent responses, revealing the importance of abiotic factors, other than water chemistry, in regulating elemental incorporation. Vertebral Ba/Ca ratios in *U. halleri* were incorporated in proportion to Ba/Ca_{water}, though the relative uptake of Ba decreased with increasing environmental concentrations. These observations

confirm that elemental incorporation into elasmobranch vertebrae is a complex, multi-causal process that influences each element in different ways. These interactive effects do not negate the ability of elemental signatures to distinguish among individuals that have occupied different environments but rather provide a basis for interpreting patterns of variation in specific elements from field studies. For example, classifications to putative natal origins based on Ba/Ca ratios as the primary discriminating factor could be the result of differences in water temperature (same [Ba]) between sites or differences in Ba concentration (similar temperature) between the water masses.

Elemental incorporation of Li, Mg, Mn, Sr, and Ba did not appear to be mediated by somatic growth or vertebral precipitation rates, indicating that individual variation in somatic growth is unlikely to be responsible for observed variation in vertebral elemental composition, even during periods of rapid juvenile growth. Although some evidence of a possible growth rate effect on zinc incorporation was detected, this evidence was inconclusive and I found no correlations between growth or precipitation rates on the incorporation of any other elements in the study.

These experiments represent the first to assess factors influencing elemental incorporation into elasmobranch vertebrae and focused on the effects of temperature, growth/precipitation rates, and dissolved Ba concentrations on elemental incorporation of one species. Further studies on the influence of water chemistry, temperature, and salinity on elemental incorporation in other species are needed to advance the ecological application of elemental markers to elasmobranch populations. If

predictive relationships between the physical and chemical properties of water (i.e. elemental concentration, temperature, salinity) and vertebral chemistry are better understood, analyses of water from areas of interest could serve as a proxy for vertebral chemistry of some elements, expediting comparisons and evaluations of source populations. However, correlations between otolith chemistry and water chemistry have proven to be elusive (Warner et al. 2005), redirecting efforts toward the development of atlases of elemental signatures based on the species of interest rather than the environment (e.g. Rutteberg et al. 2006).

The elemental composition of scalloped hammerhead (*Sphyrna lewini*) vertebrae revealed significant spatial variation across small (5s km), moderate (100s km), and large spatial scales (>1000 km). Results of the field study confirmed that vertebral elemental signatures can distinguish sharks from different nursery areas. However, classification accuracy was constrained within and among years by the likelihood of intra-annual environmental variation (water chemistry, temperature, and salinity) within sites, possible mixing of individuals among sites, broad areas associated with fishery-derived samples within sites, and incomplete sampling of potential source populations. Classification accuracy to putative nursery areas (natal signature) and location of capture (edge signature) improved from low to high when data were expressed with greater spatial and temporal resolution. Though significant differences in natal elemental signatures were detected across years, pair-wise analysis revealed that signatures were consistent between 2007 and 2009, indicating some consistency in site-specific natal signatures. These observations confirm the

importance of cohort-specific analyses and the development of annual spatial atlases of natal vertebral elemental signatures for studies of natal origin and population connectivity.

The protracted duration of parturition introduced confounding temporal and biological factors that did not lend *S. lewini* as an optimal model species for distinguishing natal origins from vertebral chemistry. However, given the potential for environmental variation across months during the course of the pupping season and sampling efforts that were directed at broad fishing locations rather than discrete sites, the classification success determined in this study can be interpreted as quite successful. Notably, the caveats, challenges, and recommendations for future work based on the results of this study are not unique to the application of elemental analyses to an elasmobranch. Similar confounding factors and conclusions have frequently been reported in studies of marine teleost fishes (Gillanders 2002, Cook 2011). Future field studies should consider the life history of the species (including the birthing period and extent of movement), local hydrology, and local oceanographic patterns to develop effective survey designs.

Although spatial variation in elemental signatures can be useful without knowledge of incorporation mechanisms, controlled laboratory studies provide a valuable platform for interpreting observed patterns and assessing those elements that are most likely to serve as useful spatial indicators. Strontium, manganese, magnesium, lithium, and barium were most commonly identified as key discriminators among putative nursery areas in the field study. Additional elements, not considered

in these lab and field studies, may also be useful elemental markers, including: bromine (Br), molybdenum (Mo), and uranium (U).

The combined use of intrinsic (chemical and genetic) and external electronic tags/markers is likely the most effective way to study connectivity in wide-ranging marine species. Measurements of vertebral bulk or compound-specific stable isotopic composition, mapping of environmental chemical composition/isoscapes, or molecular analyses used in conjunction with studies of vertebral chemistry should provide greater resolution of movements population structure than would be obtained from a single method alone. Otoliths are paired structures and offer relatively few opportunities for multiple analyses because of limited sample material. However, many vertebrae are typically collected from sharks and rays, enhancing the opportunity for integrative analyses. New insights may therefore be gained from archived samples of vertebrae, extending the value of sample material beyond that of age and growth studies.

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APPENDICES

APPENDIX A. Temperature experiment: Influence of growth rate on elemental incorporation.

Supporting Information 1. Correlations (*r*) between partition coefficients (D_{Me}), somatic growth rates (mm disc width $month^{-1}$), and vertebral precipitation rates (μm radius $month^{-1}$) for the temperature (T) experiment. The number of round rays (n, *Urobatis halleri*) included in growth rate estimates, observed range of individual somatic growth, and vertebral precipitation rates are reported for each treatment. Significant p-values are indicated by bold font.

		Somatic growth rate				Precipitation rate	
Treatment	D_{Me}	*r*	p	Treatment	D_{Me}	*r*	p
T = 15 °C	Li	0.288	0.162	T = 15 °C	Li	0.041	0.970
n = 32	Mg	0.035	0.849	n = 28	Mg	0.408	0.213
Range: 0.5-3.3 mm $month^{-1}$	Mn	0.269	0.174	Range: 5.5-31.2 μm $month^{-1}$	Mn	0.017	0.960
	Zn	0.119	0.545		Zn	0.323	0.333
	Sr	0.296	0.127		Sr	0.297	0.324
	Ba	0.051	0.779		Ba	0.275	0.388
T = 18 °C	Li	0.289	0.181	T = 18 °C	Li	0.219	0.472
n = 33	Mg	0.298	0.103	n = 30	Mg	0.193	0.473
Range: 1.4-4.5 mm $month^{-1}$	Mn	0.173	0.369	Range: 12.8-49.6 μm $month^{-1}$	Mn	0.245	0.379
	Zn	0.280	0.128		Zn	0.320	0.226
	Sr	0.184	0.323		Sr	0.337	0.202
	Ba	0.029	0.889		Ba	0.283	0.307
T = 24 °C	Li	0.248	0.215	T = 24 °C	Li	0.023	0.919
n = 33	Mg	0.208	0.246	n = 30	Mg	0.117	0.552
Range: 3.6-8.0 mm $month^{-1}$	Mn	0.202	0.272	Range: 36.7-101.0 μm $month^{-1}$	Mn	0.037	0.855
	Zn	0.139	0.465		Zn	0.318	0.121
	Sr	0.193	0.282		Sr	0.089	0.653
	Ba	0.264	0.166		Ba	0.109	0.603

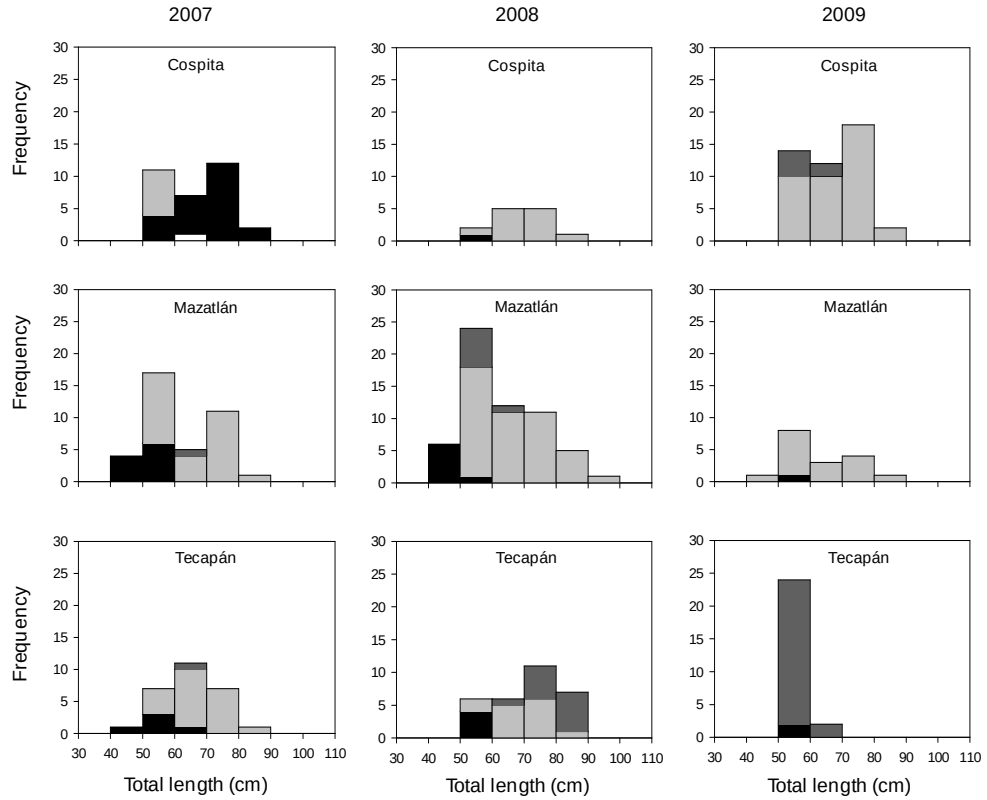
APPENDIX B. Barium experiment: Influence of growth rate on elemental incorporation.

Supporting Information 2. Correlations (*r*) between partition coefficients (D_{Me}), somatic growth rates (mm disc width $month^{-1}$), and vertebral precipitation rates (μm radius $month^{-1}$) for the barium manipulation ([Ba]) experiment. The number of round rays (n, *Urobatis halleri*) included in growth rate estimates, observed range of individual somatic growth, and vertebral precipitation rates are reported for each treatment. Significant p-values are indicated by bold font.

		Somatic growth rate				Precipitation rate	
Treatment	D_{Me}	*r*	p	Treatment	D_{Me}	*r*	p
[Ba] = 1x	Li	0.163	0.479	[Ba] = 1x	Li	0.421	0.105
n = 34	Mg	0.089	0.628	n = 30	Mg	0.006	0.979
Range: 1.8-7.8 mm $month^{-1}$	Mn	0.210	0.265	Range: 7.3-80.1 μm $month^{-1}$	Mn	0.258	0.259
	Zn	0.327	0.083		Zn	0.043	0.854
	Sr	0.175	0.338		Sr	0.076	0.723
	Ba	0.012	0.953		Ba	0.337	0.146
[Ba] = 3x	Li	0.101	0.622	[Ba] = 3x	Li	0.172	0.525
n = 34	Mg	0.003	0.960	n = 29	Mg	0.167	0.471
Range: 2.0-6.5 mm $month^{-1}$	Mn	0.209	0.260	Range: 7.4-69.7 μm $month^{-1}$	Mn	0.016	0.949
	Zn	0.462	**0.013**		Zn	0.103	0.684
	Sr	0.176	0.320		Sr	0.157	0.496
	Ba	0.087	0.635		Ba	0.050	0.835
[Ba] = 6x	Li	0.022	0.932	[Ba] = 6x	Li	0.039	0.900
n = 29	Mg	0.197	0.297	n = 28	Mg	0.173	0.454
Range: 2.0-7.3 mm $month^{-1}$	Mn	0.126	0.522	Range: 7.3-73.4 μm $month^{-1}$	Mn	0.228	0.334
	Zn	0.498	**0.010**		Zn	0.097	0.296
	Sr	0.086	0.665		Sr	0.253	0.296
	Ba	0.244	0.229		Ba	0.428	0.076

APPENDIX C. Size frequency distribution of scalloped hammerhead sharks (*Sphyrna lewini*).

Length frequency distributions (total length, cm) and status of the umbilical opening of young-of-the-year scalloped hammerhead sharks (*Sphyrna lewini*) included in our analyses by site and year of collection. Samples are presented for the three primary sampling locations in Sinaloa, Mexico. Black bars = open umbilical scars, light grey bars = closed umbilical scars, and dark grey bars = unknown umbilical scar status.



APPENDIX D. Intra-annual variation in elemental signatures.

Blocked multi-response permutation procedure (MRPP) tests of differences in the elemental composition of vertebral natal and edge signatures by year, site and month. Average Euclidean distances are presented as a measure of dispersion in multi-elemental signatures within each group. Smaller Euclidean distances indicate greater similarity within a group. P-values presented in bold identify significant differences in multi-elemental composition within a site between years.

				Average Within Group Differences				
Year	Location	Month	n	Natal	Edge	T	p	A
2007	Cospita	August	10	1.916	1.414	-4.23	**0.005**	0.20
		November	13	0.818	0.828	-7.75	**<0.001**	0.52
	Mazatlán	August	8	1.764	1.668	-2.90	**0.016**	0.13
		September	5	1.583	1.629	-3.08	**0.015**	0.42
		October	6	0.895	1.816	-3.84	**0.008**	0.53
		November	9	1.238	1.151	-5.85	**0.001**	0.59
	Tecapán	August	6	1.325	1.425	-2.36	**0.031**	0.19
		September	8	1.020	1.195	-4.09	**0.006**	0.38
		October	9	1.235	1.284	-5.80	**0.002**	0.56
2008	Cospita	November	14	0.618	2.233	-9.44	**<0.001**	0.81
	Mazatlán	June	6	0.791	7.493	-1.13	0.129	0.14
		July	8	5.138	7.140	-1.00	0.159	0.06
		August	11	1.403	4.666	-6.71	**0.001**	0.76
		October	17	1.396	3.902	-11.51	**<0.001**	0.80
		November	5	1.356	0.873	-2.45	**0.029**	0.92
	Tecapán	August	12	3.159	5.037	-3.87	**0.008**	0.43
		November	12	1.141	0.953	-8.12	**<0.001**	0.93
		December	12	1.080	3.174	-8.05	**<0.001**	0.85
2009	Cospita	August	6	2.311	1.721	-7.37	**<0.001**	0.70
		September	8	1.106	1.426	-13.07	**<0.001**	0.91
		October	7	1.028	2.024	-4.58	**0.004**	0.91
		November	14	0.971	0.902	-9.54	**<0.001**	0.93
	Mazatlán	August	11	4.639	4.830	-1.9179	0.054	0.17
		October	7	1.100	1.032	-4.58	**0.004**	0.92
	Tecapán	August	10	2.953	2.053	-6.00	**0.001**	0.74

APPENDIX E. Correlations of Element/Calcium Ratios by month and year.

Correlations (*r*) of element/calcium ratios across months by location and year. The number of sharks included in analyses is indicated by (*n*) and p-values in bold font identify element-to-calcium ratios that differed significantly across months of collection.

		Cospita			Mazatlán			Tecapán		
Year	Element/Ca	n	*r*	p	n	*r*	p	n	*r*	p
2007[1]	Li	26	0.082	0.208	28	0.245	**0.001**	19	0.185	0.527
	Mg		0.000	0.961		0.153	0.134		0.093	0.709
	V		0.018	0.517		0.280	**0.043**		0.214	0.380
	Cr		0.035	0.361		0.200	0.083		0.371	0.118
	Mn		0.073	0.182		0.154	0.133		0.169	0.490
	Rb		0.304	**0.004**		0.280	**0.035**		0.266	0.271
	Sr		0.176	**0.033**		0.436	**0.005**		0.389	0.100
	Ba		0.071	0.197		0.768	**<0.001**		0.462	0.054
	Pb		0.114	0.091		0.262	0.061		0.134	0.585
2008	Li	12	0.042	0.523	42	0.267	0.087	32	0.019	0.922
	Mg		0.039	0.537		0.367	0.307		0.426	**0.015**
	V		0.050	0.485		0.199	0.206		0.509	**0.003**
	Cr		0.093	0.363		0.262	0.093		0.162	0.376
	Mn		0.030	0.306		0.268	0.086		0.251	0.166
	Rb		0.123	0.264		0.309	0.051		0.094	0.616
	Sr		0.123	0.263		0.309	**0.046**		0.472	**0.006**
	Ba		0.148	0.216		0.495	**0.010**		0.350	**0.049**
	Pb		0.026	0.634		0.162	0.634		0.079	0.668
2009	Li	53	0.193	0.171	16	0.495	**0.001**	20	0.134	0.573
	Mg		0.338	**0.013**		0.392	0.134		0.548	**0.010**
	V		0.266	0.056		0.529	**0.043**		0.009	0.970
	Cr		0.228	0.099		0.447	0.083		0.478	**0.028**
	Mn		0.383	**0.005**		0.393	0.133		0.394	0.077
	Rb		0.186	0.191		0.530	**0.035**		0.281	0.244
	Sr		0.142	0.310		0.660	**0.005**		0.535	**0.013**
	Ba		0.363	**0.008**		0.876	**<0.001**		0.347	0.134
	Pb		0.104	0.469		0.512	0.061		0.324	0.164

[1] Li/Ca data were not available for all samples in 2007: Cospita = 22; Mazatlán = 21; Tecapán = 14.