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Charles Darwin University

## Decoding fingerprints

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# Decoding fingerprints: elemental composition of vertebrae correlates to age-related habitat use in two morphologically similar sharks

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**ABSTRACT:** We compare vertebral microchemistry with previously described age-related movement patterns of bull sharks *Carcharhinus leucas* and pig-eye sharks *C. amboinensis* within coastal waters of north Australia. Laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) quantified the chemical signatures of nursery habitats within the vertebrae of juvenile and adult sharks. We examined evidence for adults returning to these habitats by applying LA-ICP-MS along a growth axis of their vertebrae. We transposed chemical signatures with growth increments in adult vertebrae to correlate with age estimates. Unique elemental signatures were identified in each of the freshwater nurseries, but we did not find them in adult vertebrae. Age-specific changes in vertebral microchemistry in mature female bull sharks correlate with periodic returns every 1 to 2 yr to less saline environments to pup. We were unable to discriminate among natal habitats of pig-eye sharks using elemental fingerprints, and age-specific changes in vertebral microchemistry were also absent. We conclude that changes in vertebral microchemistry correlate with known habitat use patterns of the bull and pig-eye sharks, showing the potential of vertebral microchemistry to discern movement patterns in sharks.

**KEY WORDS:** Vertebral microchemistry · LA-ICP-MS · Movement · Long-term · Resource partitioning · Carcharhinidae

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## INTRODUCTION

The persistence of sharks as apex predators in marine environments is threatened by over-exploitation and habitat change (Myers et al. 2007, Field et al. 2009). Their *K*-selected life history means that they have reduced resilience to rapid change and are likely to be slow to recover from population decline

(Schindler et al. 2002). Effective management and conservation of these vulnerable predators is hindered by poor knowledge of movement patterns (Speed et al. 2010). This information is essential because it can identify key habitats (e.g. pupping areas, nurseries) within distribution ranges and helps define the ecological role of species. Additionally, it can identify evolved behaviours such as natal and pupping site fidelity (sensu

Speed et al. 2010) that are critical to the maintenance of genetic diversity and replenishment of populations (Hueter et al. 2005).

Studies tracking shark movements and identifying patterns of habitat use in coastal regions typically involve tagging with standard (numerical), satellite or sonar tags (Speed et al. 2010). Such an approach is often logistically difficult and expensive because it first involves the capture, tagging and release (in good condition) of the shark. Furthermore, the animals must either be recaptured (standard tags), or tags must report to satellites or arrays of listening stations (sonar tags) for data acquisition (Voegeli et al. 2001, Simpfendorfer & Heupel 2004). Rates of recapture are usually low, while failure of expensive satellite tags to report is commonplace (Hays et al. 2007). Arrays of listening stations require considerable effort to deploy, download and maintain, which can limit the duration and spatial extent of a study using this approach. Despite these problems, studies using these techniques have mapped fine-scale (25 km) movements of different-age cohorts of sharks in shallow coastal waters (Simpfendorfer et al. 2005, Heupel & Simpfendorfer 2008, Yeiser et al. 2008, Heithaus et al. 2009, Ortega et al. 2009, Heupel et al. 2010), but the logistics, cost and limited life span of tags have restricted the number of target individuals and species and, in the case of sonar tags, the spatial extent of the sampling area.

To overcome the limitations associated with conventional tracking, natural chemical fingerprints are a developing tool to trace age-related movements of sharks among habitats throughout their lifetime. This method is similar to tracking fish movements based on otolith microchemistry. Environmental signatures ('fingerprints') stem from either pollution or natural leaching and weathering of elements from the Earth's crust into aquatic systems (Campana 1999, Gillanders 2005, Speed et al. 2010). As individuals living and growing in these environments osmoregulate, trace elements are absorbed across the skin and gills and can either be substituted for calcium or trapped within protein matrices of hard structures such as otoliths or vertebrae (Gillanders & Kingsford 2000, Dean & Summers 2006, Hale et al. 2006). In otoliths, elements are deposited in concentrations reflecting that of the aquatic environment and can be influenced by the physical properties of that medium (Gillanders & Kingsford 2000, Walther & Thorrold 2006, Brown & Severin 2009). For example, strontium (Sr) typically has high concentrations and is uniform in marine environments, while barium (Ba) shows the opposite pattern and is enriched in freshwater or during flood periods in the low salinity region of freshwater plumes (McCulloch et al. 2005, Crook & Gillanders 2006). Concentrations of elements in marine environments reflect proximity to freshwater

inputs or nutrient upwellings, with trace element:Ca ratios typically higher in less saline waters (Beamish et al. 2005, Kingsford et al. 2009). Correlating mineralisation with growth increments within these structures enables age-based interpretation of records that can be used to describe the periodicity of habitat use (Gillanders & Kingsford 2000, McCulloch et al. 2005).

We apply similar principles as a baseline approach to investigate whether changes in vertebral microchemistry correlate with known age-specific changes in habitat use of bull sharks *Carcharhinus leucas* and pig-eye sharks *C. amboinensis*. Both of these species are large apex predators (3400 and 2800 mm maximum total length in Australia, respectively) within shallow waters of tropical and subtropical coasts (Last & Stevens 2009). Pop-up satellite tags have confirmed the affinity of large bull sharks to shallow coastal environments and tracked a few individuals embarking on long-distance movements (1506 km) (Brunnschweiler et al. 2010, Carlson et al. 2010). Bull sharks use freshwater nurseries with juveniles remaining in these areas for approximately 4 yr (Thorburn & Rowland 2008, Heupel et al. 2010). Preliminary genetic assessment suggests female pupping site fidelity, although it is unclear how frequently females return to pup and whether males also return, in which case mating as well as pupping might occur in freshwater (Tillett et al. 2011b). Short-term tracking research (~1.5 yr) has suggested that habitat use is defined by maturity (Simpfendorfer et al. 2005, Heupel & Simpfendorfer 2008, Yeiser et al. 2008, Heupel et al. 2010). Conversely, adult pig-eye sharks do not show this pattern; rather, population genetic structure suggests restricted movement (Tillett et al. unpubl.). Acoustic tracking studies indicate age-based partitioning of habitat, with juveniles of this species occupying areas adjacent to creek and river mouths (Last & Stevens 2009, Knip et al. 2011).

We hypothesise that periodic chemical signatures of freshwater (indicated by low Sr:Ba ratios for bull sharks) or low-salinity environments (indicated by declines in element:Ca ratios for pig-eye sharks) will occur in vertebrae of adults, indicating returns to nurseries. These should be more evident in females because they return regularly to pup in freshwater or estuarine nurseries. If there is natal site fidelity occurring in these sharks, the chemical signatures from adult birth bands should be similar to those deposited in the vertebrae when they return to the same freshwater or estuarine nursery to pup. Second, we hypothesise that chemical signatures will change post-maturity coinciding with changes in habitat use. Third, Sr:Ba ratios should differ in young juvenile stages (inner 150 mm of the vertebra) of bull and pig-eye sharks because the former use freshwater nurseries while the

latter use nurseries in shallow coastal areas. As sharks grow and leave these nurseries, ratios should be increasingly similar between the species because of greater habitat overlap. Ultimately, post-maturity ratios should also be similar because both species are found in deeper coastal water (approximately 20 m) as adults.

## MATERIALS AND METHODS

**Vertebrae collection.** We removed 10 to 15 thoracic vertebrae from 88 pig-eye (39 adults, 49 juveniles) and 92 bull sharks (18 adults and 74 juveniles). Adult sharks were collected by scientific observers working with the Northern Shark Fishery operating along the Northern Territory coastline in 2009. In this fishery, long-lines must not exceed 15 nautical miles and have no more than 1000 snoods (hooks), and nets must be 1000 to 2500 m long with a square mesh size of 150 to 250 mm and a drop of 50 to 100 meshes. We obtained juvenile sharks from both commercial fisheries and our field work from 2002 to 2009. Fishery-independent studies were done using long-lines approximately 50 m long with 50 snoods (size 11/0) positioned 1 m apart, and nets approximately 50 m long with a square mesh size of 150 to 250 mm with a 16-mesh drop. Both

were weighted and deployed along the bottom in depths ranging from 5 to 15 m. We collected juvenile pig-eye sharks from inshore coastal waters around Broome, Western Australia, from the northeastern side of the Joseph Bonaparte Gulf to the Gulf of Carpentaria, Northern Territory, and from Townsville, Queensland (Fig. 1). We collected juvenile bull sharks from 6 different northern Australian river systems: the Liverpool, Roper, Towns, Fitzroy, Daly and East Alligator Rivers (Fig. 1). Sample sizes, sex ratio and capture dates from each location are supplied in Table S1 in the supplement at [www.int-res.com/articles/suppl/m434p133\\_supp.pdf](http://www.int-res.com/articles/suppl/m434p133_supp.pdf). We measured sex, total weight (TW) total length (TL) and fork length (FL) when possible. We inspected small individuals (<1 m TL) for the presence of umbilical scars as an indication of time since birth. Once thoracic vertebrae were removed, we stored them frozen or temporarily immersed them in 5% sodium hypochlorite solution before storing dry. Due to the morphological similarities between members of the genus *Carcharhinus*, we collected a small tissue fragment (~1 g) from each individual and genetically tested it to confirm species identification (Tillett et al. 2011b, Tillett et al. unpubl.).

**Preparation of vertebrae.** We defrosted frozen samples and excised excess tissue, neural and haemal arches to expose the centra. We separated individual

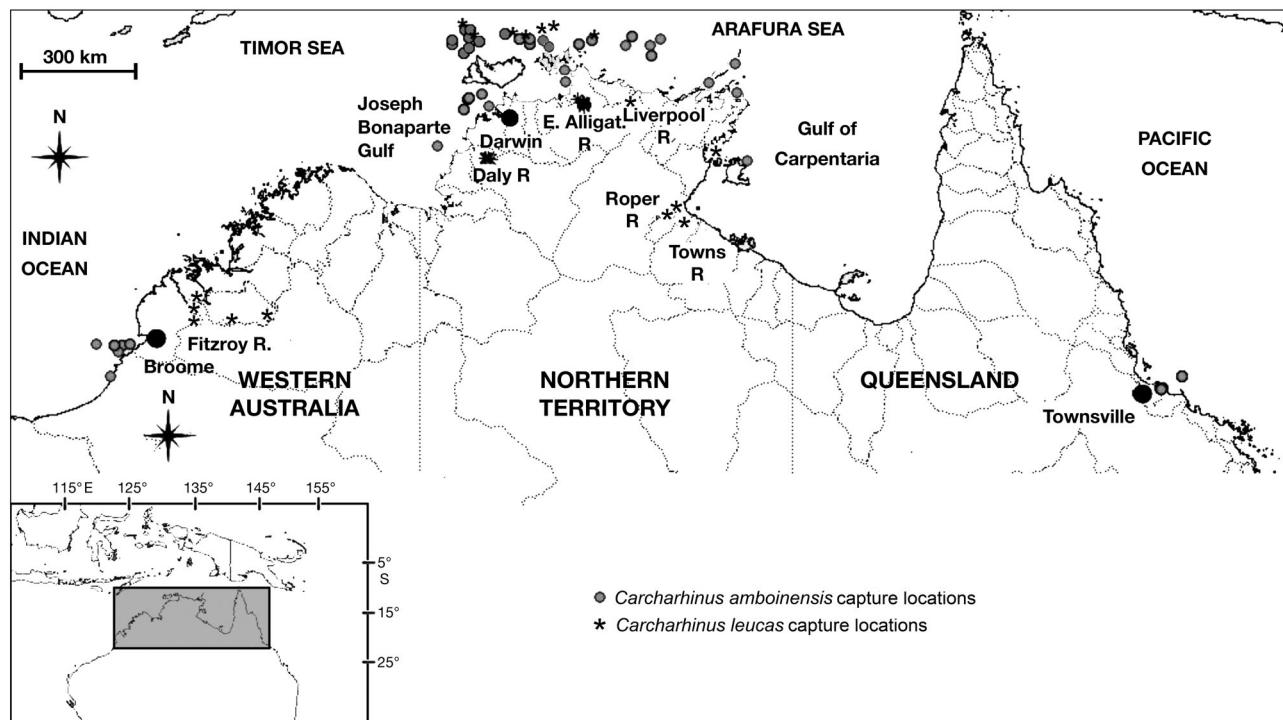


Fig. 1. Capture locations for pig-eye sharks *Carcharhinus amboinensis* (total n = 88; 39 adults, 49 juveniles) and bull sharks *Carcharhinus leucas* (total n = 92; 18 adults, 74 juveniles) across northern Australia

centra and removed any connective tissue with a scalpel blade or abrasive material. We subsequently washed centra in Milli-Q water and left polished centra to air-dry in a fume hood causing any remaining tissue to become brittle and peel away. We immersed remaining vertebrae (74 juvenile bull and 16 juvenile pig-eye sharks) in 5% sodium hypochlorite solution for approximately 1 min or until remaining flesh had been removed. Again, we excised neural and haemal arches to expose the centra. We weighed all cleaned vertebrae and embedded them whole in a 2-part casting-laminating epoxy resin (Barnes), through which we sectioned using a low-speed IsoMet diamond saw at approximately 240 rpm with a 250 g load weight. We ground sections on wet and dry paper until ~0.5 mm thick and rinsed again in Milli-Q water to remove potential contaminants (i.e. the outer edge potentially containing absorbed resin was removed). We mounted sections on glass slides using either Crystalbond™ 509 (ProSciTech, ) or with a temporary adhesive (Blu-Tack, Bostik) and took care not to contaminate vertebral regions to be ablated. We viewed sections under a Leica DM 400B compound microscope and marked the desired starting position for each analysis by a small incision in the surrounding resin.

**Ageing.** We viewed vertebral sections using the imaging software package OPTIMAS 6.5 (Media Cybernetics) and aged by counting growth bands (defined as one opaque and one translucent ring) visible along the corpus calcareum reading from the focus to the outer centrum edge (Fig. 2) (Campana 2001). We regarded the change of angle caused by differences in growth rate from intra-uterine to post-natal life history stages as the point of birth, or birth mark and recorded it as year zero (Cailliet & Goldman 2004). Annual growth band deposition has been confirmed for bull sharks (Branstetter & Stiles 1987, Neer et al. 2005) and pig-eye sharks (Tillett et al. 2011a).

**Preliminary study.** Initial analyses determined (1) the concentric manner in which the calcareous vertebral matrix was deposited and the corresponding 3-dimensional structure of the sagittal section, and (2) inter-vertebral variation in chemical composition of the matrix. We also tested to see whether temporary bleaching to remove excess tissue resulted in the leaching of elements from the vertebral matrix. For this analysis, we removed 4 thoracic vertebrae from 2 individuals of each species. After air drying, we removed excess connective tissue by trimming (2 vertebrae) or bleaching (2 vertebrae). We then set all 4 vertebrae in resin, sectioned and mounted them on slides as described above. We analysed chemical compositions to compare effects of treatments.

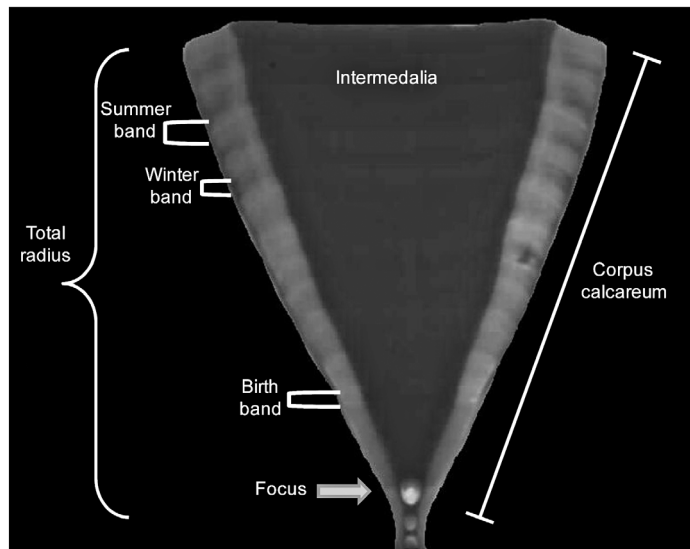


Fig. 2. *Carcharhinus leucas*. Sagittal section of bull shark vertebrae (aged 10 yr) displaying annual growth increments, and descriptions of vertebral attributes

**Laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS).** We analysed samples using a 213-nm laser (Nd:YAG, 5th harmonic; NewWave Research UP-213) coupled to an inductively coupled plasma mass spectrometer (Agilent 7500ce) connected to a Hitachi camera. Analyses were performed at Charles Darwin University, Darwin, Northern Territory, Australia. We optimised the LA-ICP-MS for maximum sensitivity by adjusting He and Ar flows and plasma power during ablation of the National Institute of Standards and Technology (NIST) 612 glass standard. We monitored oxide formation by the  $\text{ThO}^+:\text{Th}^+$  ratio which was typically <0.5%. We monitored instrumental drift by ablating the NIST 612 glass standard after ablating 7 shark samples. If variation between NIST 612 glass standard was greater than 5%, we retested the shark samples.

Prior to activation of the laser, we recorded elemental composition of the blank sample gas for 20 s. We made initial measurements of 13 elements ( $^7\text{Li}$ ,  $^{25}\text{Mg}$ ,  $^{27}\text{Al}$ ,  $^{31}\text{P}$ ,  $^{43}\text{Ca}$ ,  $^{55}\text{Mn}$ ,  $^{54}\text{Fe}$ ,  $^{63}\text{Cu}$ ,  $^{64}\text{Zn}$ ,  $^{86}\text{Sr}$ ,  $^{139}\text{La}$ ,  $^{137}\text{Ba}$  and  $^{238}\text{U}$ ) that vary in concentration in otoliths among different aquatic environments, but are not linked to diet (Milton & Chenery 2001, Cailliet & Goldman 2004, Kraus & Secor 2004, Martin & Thorrold 2005, Brown & Severin 2009). We can only assume that these same elements are equally unaffected by diet when isolated from vertebral tissues. From this list, we omitted from further analysis those elements that were not recorded consistently above detection limits (calculated as 3 times the standard deviation of the blank signal). Further analysis considered 6 elements ( $^7\text{Li}$ ,  $^{25}\text{Mg}$ ,  $^{55}\text{Mn}$ ,



$^{86}\text{Sr}$ ,  $^{64}\text{Zn}$  and  $^{137}\text{Ba}$ ) that were all well above detection limits calculated using the LA-ICP-MS software Glitter (Van Achterbergh et al. 2001) (0.006, 0.069, 0.070, 0.318, 0.061, 0.013  $\mu\text{g g}^{-1}$ , respectively).

For both species, we quantified elemental signatures of nursery areas by spot analyses of the later region of the first 6-mo period (typically translucent) of the birth band (see above, Fig. 2) of the sectioned vertebrae of juveniles (up to 850 mm TL, Last & Stevens 2009). We investigated age-related habitat use and the return to natal environments by ablating along the growth axis of the corpus calcareum (referred to as 'line scans') within the vertebrae initiating from the birth band, moving across annual growth increments towards the distal edge of the section (Fig. 2) on mature individuals (>2200 mm TL). We pre-ablated line scans to remove potential surface contaminants.

**Analysis.** Scanning electron microscopy (SEM) with X-ray Energy Dispersive Spectrometry (EDS) of 2 vertebral sections from each shark species confirmed constant percent composition of Ca (35 %) throughout the vertebrae, allowing use of this element as an internal reference standard (see Fig. S1 and Table S2 in the supplement) in LA-ICP-MS. This, combined with the reference silicate glass (NIST-612) as an external calibration standard within each analytical session, enabled the conversion of elemental count rates into concentrations (ppm) using the data-processing software Glitter (Van Achterbergh et al. 2001). NIST glasses have been used as a calibration standard for the analysis of carbonates and calcified teleost otoliths (Munksgaard et al. 2004, Hale et al. 2006, Steer et al. 2009). Glitter's data reduction accommodates gas blank corrections. The vertical homogeneity (signature does not change with depth through the section) of the sections enabled the user-selectable 'quantitation' window to quantify only the part of the analysis where the analyte signals were stable, thus eliminating potential contaminants ablated with surface material. We checked all analytical spectra for non-target signals represented by the rapid decline in  $^{43}\text{Ca}$  count rate. The variation in analyte concentrations of the NIST 612 standards remained within acceptable limits ( $\pm 5\%$ ) within each analytical session.

We compared the chemical signature for each nursery using Euclidean-distance similarity matrices of standardised elemental concentrations. We evaluated the evidence for differences between signatures using analysis of similarity (ANOSIM) which uses permutations to determine if the assigned groups are more similar in composition than samples from other groups (Chapman & Underwood 1999, Clarke & Gorley 2001). ANOSIM generates an *R*-statistic which indicates the magnitude of difference among groups and ranges from -1 to 1. A value of '0' indicates that there is no dif-

ference between groups and '1' that the groups differ completely. Statistical evidence for differences due to unique environmental signatures is determined by comparing the sample *R* grouped by nurseries with those produced by randomly assigning samples to groups. The portion of random arrangements with *R*-values greater than the sample gives the probability of observed patterns arising at random. Similarity percentages (SIMPER) then determined which elements differed between nursery signatures by calculating the average distance (based on Euclidean distance) within and between all nurseries estimating the percent contribution of each element to the overall distance (PRIMER-E). We screened data for the presence of outliers and conformity with multivariate normality, and removed those identified.

Analysis of line scans using Glitter provides an average composition of the selected scan (or portion of scan), but does not quantify spatial variation within the selected scan. Therefore, we transformed the analyte count data from each ICP-MS replicate into net count rates relative to net count rates for the internal standard (Ca) and transposed these ratios as a function of the ablated line scan distance using a customised Excel™ spreadsheet. We smoothed the data using a running median and average calculation of 8 ICP-MS replicates (equivalent to a scan distance of 35  $\mu\text{m}$ ) (Munksgaard et al. 2004). We correlated chemical signatures with growth increments to identify age-specific movement patterns. We looked for evidence of periodic movements of adults into nurseries shown by declines in Sr:Ba ratios (indicating time spent in marine/freshwater habitats for bull sharks) and reductions in element:Ca ratios (indicating time spent in offshore/onshore habitats for pig-eye sharks). Inter-specific Sr:Ba net counts per second (cps) ratios were also compared to infer different salinities of environments occupied within an individual's lifetime (McCulloch et al. 2005, Clarke et al. 2009). We used Glitter's 'quantitation' window to select birth bands and sections in the line scan analysis that might indicate return movements by adults into nursery environments. We compared similarity in multi-elemental composition between these returns and both juvenile signatures within adult vertebrae and nurseries identified from juvenile spot analysis using ANOSIM and principal component analysis (PCA).

## RESULTS

Elemental composition was consistent between spot analyses of same age sections of the corpus calcareum within vertebrae confirming the concentric growth of vertebrae (Global *R* = 0.095, *p* = 0.251; Global *R* =

0.067,  $p = 0.345$  for pig-eye and bull sharks, respectively). Similarly, elemental composition of spot analysis of the same age sections of the corpus calcareum was consistent between vertebrae, confirming intervertebral mineralisation was also constant (Global  $R = 0.058$ ,  $p = 0.222$ ; Global  $R = 0.086$ ,  $p = 0.191$  for pig-eye and bull sharks, respectively). Bleaching vertebrae to remove connective tissue did not cause leaching of elements from the vertebral matrix, supported by similar elemental composition of spot analysis between same-age sections of vertebrae treated differently to remove connective tissue (Global  $R = 0.039$ ,  $p = 0.296$ ; Global  $R = 0.009$ ,  $p = 0.378$  for pig-eye and bull sharks, respectively).

SIMPER of spot analyses on juvenile bull shark vertebrae quantified the percent contribution of each element to nursery signatures. The contribution of each element to these signatures differed between nurseries (Fig. 3). Barium ( $^{137}\text{Ba}$ ) was characteristic in the Liverpool River and almost absent from the East Alligator and Towns Rivers signatures. Lithium ( $^7\text{Li}$ ) and Strontium ( $^{86}\text{Sr}$ ) contributed similarity to signatures in all locations except the Liverpool River. ANOSIM confirmed differences (indicated by Euclidean distances) between nurseries were due to environmental signatures rather than randomly generated (Overall global  $R = 0.373$ ,  $p = 0.0001$ ; Table 1).

Bull sharks displayed large shifts in vertebral microchemistry with age (Fig. 4). Juvenile Sr:Ba net cps ratios differed among individuals and ranged from <100 to 300. Ratios either remained constant or steadily increased (to 300–700) until maturity (8 to 10 yr). Mature females ( $n = 14$ ) showed cyclic declines in Sr:Ba ratios (every 1 to 2 yr) (Fig. 4a), possibly indicating a periodic return to breeding grounds (marked by spots in Fig. 4a). Cyclic patterns were less distinct in male conspecifics ( $n = 2$ ) because declines in these individuals were predominately within the 5% variation attributable to instrument drift (Fig. 4b). Males also increased in Sr:Ba net cps ratios at younger ages (6 to 8 yr) than females (8 to 10 yr). Birth bands for adult bull sharks, irrespective of sex, did not group with any of the identified nurseries return periods. They were more similar to other adult birth bands than to any nursery defined from juvenile bull sharks (Table 2, Fig. S4 in the supplement).

In contrast to bull sharks, pig-eye nurseries could not be distinguished based on unique multi-elemental fingerprints (Global  $R = 0.109$ ,  $p = 0.084$ ; Table 3; Fig. 5). SIMPER analyses showed manganese ( $^{55}\text{Mn}$ ) as being most characteristic in Western Aus-

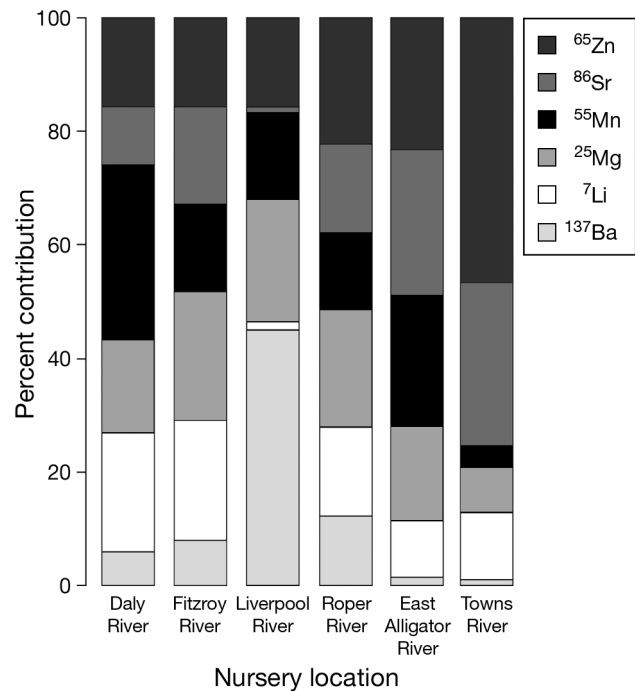


Fig. 3. *Carcharhinus leucas*. Similarity percentages (SIMPER) showing element contributions to bull shark nursery signatures. Total  $n = 74$  (Fitzroy River:  $n = 16$ , Daly River:  $n = 15$ , Liverpool River:  $n = 7$ , East Alligator River:  $n = 14$ , Roper River:  $n = 11$ , Towns River:  $n = 11$ )

tralia and least in the Northern Territory. Magnesium ( $^{25}\text{Mg}$ ) was also a major component of elemental signatures in Western Australia, making least contribution in north Queensland. Barium ( $^{137}\text{Ba}$ ) showed a complementary pattern, being most characteristic in north Queensland and least in Western Australia signatures.

Age-based differences in vertebral microchemistry were not evident in pig-eye sharks. Sr:Ba net cps ratios of juveniles were less variable than those of bull sharks and ranged between 200 and 450 (see Fig. S2 in the supplement). These values remained relatively constant with the onset of maturity. Any changes in Sr:Ba

Table 1. *Carcharhinus leucas*. Pairwise ANOSIM correlations among bull shark nurseries indicating whether differences are due to distinct environmental signatures or random factors.  $R$ -values ranging from ~0 (no difference) to 1 (highly different) are above the diagonal;  $p$ -values indicating the probability of differences arising purely at random are below the diagonal

	Fitzroy ( $n = 16$ )	Daly ( $n = 15$ )	Liverpool ( $n = 7$ )	East Alligator ( $n = 14$ )	Roper ( $n = 11$ )	Towns ( $n = 11$ )
Fitzroy	–	0.374	0.306	0.296	0.191	0.358
Daly	0.0001	–	0.61	0.475	0.252	0.389
Liverpool	0.0110	0.0001	–	0.529	0.58	0.495
East Alligator	0.0003	0.0001	0.0003	–	0.438	0.473
Roper	0.0090	0.0030	0.0002	0.0001	–	0.528
Towns	0.0007	0.0003	0.0007	0.0001	0.0001	–

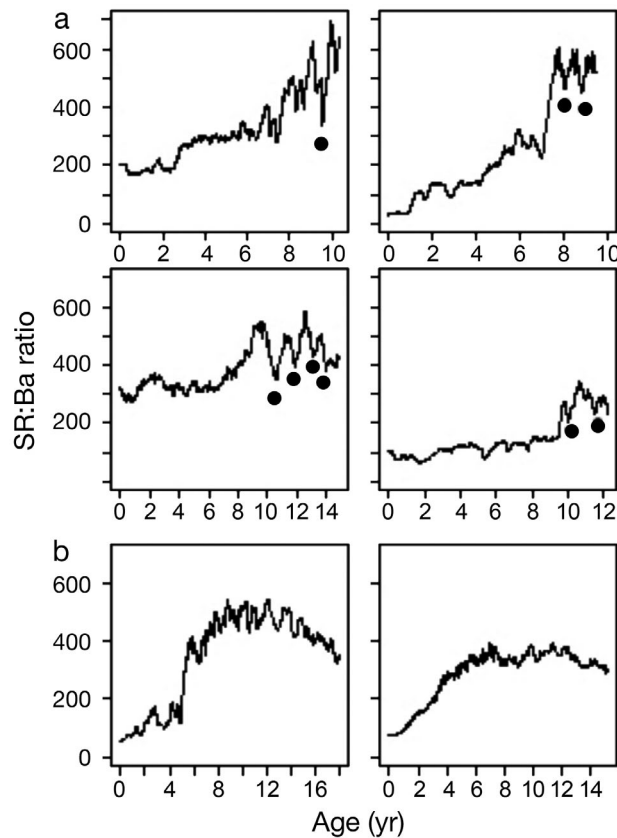


Fig. 4. *Carcharhinus leucas*. Typical plots of Sr:Ba ratio vs. age (yr) of individual (a) female and (b) male bull sharks. Dots in (a) indicate potential return of females to less saline waters

Table 2. *Carcharhinus leucas*. Average squared distance (standard deviation) between adult birth bands, females during postulated return periods to nurseries, and nurseries defined by juvenile bull sharks

	Adult birth bands	Returns
Fitzroy	17.20 (6.11)	22.52 (8.62)
Daly	17.44 (5.88)	23.25 (9.67)
Liverpool	19.86 (5.36)	26.34 (8.68)
East Alligator	14.68 (5.84)	18.23 (7.42)
Roper	12.79 (7.33)	18.85 (9.93)
Towns	9.32 (5.83)	12.11 (10.58)
Returns	5.89 (3.41)	–

net cps ratios were subtle, ranging between 100 and 200, and their frequency did not increase with age. Any declines in Sr:Ba ratios were commonly within the 5% variation attributable to instrument drift.

Similarly oscillations in element:Ca ratios were evident in some elements (e.g. 0.025 to 0.01 for  $^{55}\text{Mn}$ ), although predominately within the 5% variation attributable to instrument drift (see Fig. S3 in the supplement). Due to the absence of unique nursery fingerprints, and the lack of a distinctive nursery phase in

Table 3. *Carcharhinus amboinensis*. Pairwise ANOSIM correlations among pig-eye shark nurseries indicating whether differences are due to distinct environmental signatures or random variation.  $R$ -values ranging from  $\sim 0$  (no difference) to 1 (highly different) are above the diagonal;  $p$ -values indicating probability of differences arising purely at random are below the diagonal. WA: Western Australia, NT: Northern Territories, N QLD: north Queensland. Sample sizes for each nursery are given

	WA (n = 16)	NT (n = 27)	N QLD (n = 6)
WA	–	0.125	0.146
NT	0.070	–	0.078
N QLD	0.120	0.222	–

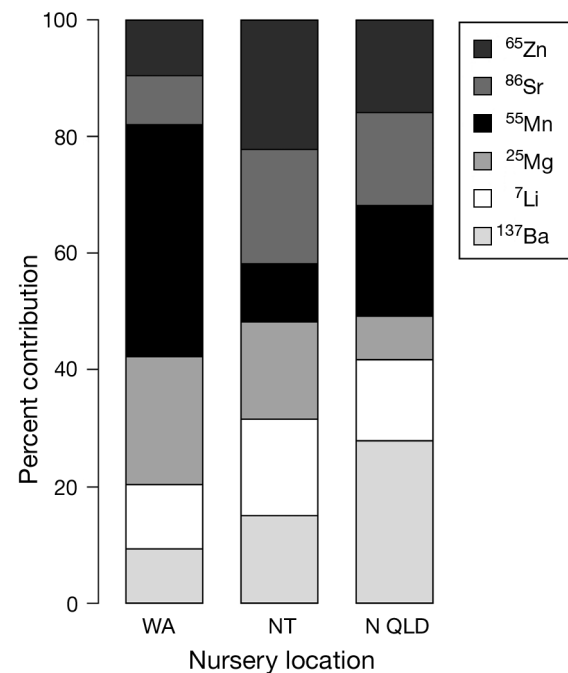


Fig. 5. *Carcharhinus amboinensis*. Similarity percentages (SIMPER) showing element contributions to pig-eye shark nursery signatures. Total  $n = 49$ ; Western Australia (WA)  $n = 16$ ; Northern Territories (NT)  $n = 27$ ; north Queensland (N QLD)  $n = 6$

element ratios, we did not attempt to identify chemical fingerprints of the juvenile phase within line scan analysis of adult pig-eye sharks.

## DISCUSSION

Our results show the potential for chemical signatures within shark vertebrae to track the long-term (lifetime) movements of these animals within marine, estuarine and freshwater habitats. Unique elemental signatures between bull shark nurseries support the assumption that vertebral microchemistry reflects



environmental signatures in some sharks. Declines in Sr:Ba net cps ratios in mature female bull sharks are consistent with predicted changes in vertebral microchemistry reflecting periodic returns to freshwater nurseries assuming that Sr:Ba net cps ratios reflect salinity changes as observed in other estuarine species (McCulloch et al. 2005, Allen et al. 2009). The lack of this pattern in male conspecifics suggests this behaviour might reflect pupping rather than mating events. This is further supported by the occurrence of this behaviour in 1 to 2 yr cycles correlating with the estimated 10 to 11 mo gestation period and rest year in the reproductive cycle (Last & Stevens 2009).

Assuming vertebral signatures are not altered because of metabolic exchange in the vertebrae as the individual ages (Campana et al. 2002), none of the adults were born or resided in any of the nurseries identified from the analysis of the vertebrae of juveniles, and thus we could not confirm the existence of return to natal areas solely on the basis of evidence from chemical fingerprints. Unfortunately, comparisons between adult return events and their natal fingerprints lacked statistical power due to the limited numbers of return periods per individual. Furthermore, the relationship between body surface area and absorption of elements needs to be confirmed before robust conclusions between adult and juvenile life stages can be drawn.

Gradual declines in some element:Ca net cps ratios (e.g. 0.025 to 0.01 for  $^{55}\text{Mn}$ ) in pig-eye sharks are consistent with adult conspecifics moving into deeper water away from nutrient-rich freshwater, particularly in areas such as northern Australia lacking nutrient upwellings. The lack of unique nursery signatures in juveniles prevented the identification of natal return behaviour in this species using vertebral microchemistry. Oceanic waters across northern Australia might not be chemically distinct due to mixing by oceanic and wind driven currents. In teleost fish, marine habitats can only be discriminated if there are large changes in water chemistry such as salinity, temperature or water gradients that are often present on narrower or more steeply sloping shelf systems (Gillanders & Kingsford 2000, Kingsford et al. 2009, Steer et al. 2009). This might represent a limitation of vertebral microchemistry to discern movements in some shark species.

Age-specific variation in vertebral microchemistry also correlates with described changes in habitat use following the same assumptions. Sr:Ba net cps ratios were typically lowest in bull sharks around the time of birth, in accordance with neonates occupying freshwater environments. Ratios then increased at 2 to 4 yr of age as juveniles move into more saline waters, increasing net cps ratios until maturity. Adults showed the

highest ratios, consistent with the consistent use of marine habitat (Yeiser et al. 2008, Heupel et al. 2010). Similar patterns in element:Ca net cps ratios in pig-eye sharks were not evident, again suggesting either a limitation of vertebral microchemistry to map fine-scale movements in sharks or that the use of nurseries is less defined in this species.

Surprisingly, vertebral microchemistry was more similar among juveniles and different among adult life stages between species than predicted. As expected, Sr:Ba net cps ratios of certain neonate stages of bull sharks were distinctly lower than equivalent pig-eye shark age classes, but other individuals were almost identical. This suggests a degree of habitat overlap between species as juveniles and highlights the individual variability among bull sharks. Furthermore, the greater variation in Sr:Ba net cps ratios in adult bull sharks might reflect the recently defined broad-scale movement patterns into cooler eastern and western Australian waters and the absence of such behaviour in pig-eye sharks (Brunnschweiler et al. 2010, Carlson et al. 2010).

The incorporation of elements within juvenile bull shark vertebrae highlights the potential for vertebral microchemistry as a valuable tool for discriminating complex behaviour such as pupping or natal site fidelity, although clarification of how elements are conserved in shark vertebrae, lag and cumulation effects, and whether these signatures are stable through time, are needed (Cailliet & Radtke 1987, Welden et al. 1987). Results suggest chemical cues can guide female returns to nursery and as such, implies large consequences should water chemistry change due to altering freshwater flows, or drainage from surrounding industries.

Conclusively attributing specific behaviour to certain vertebral signatures is beyond the scope of this study, but because bull sharks inhabit a wide diversity of habitats with specific age-related patterns of habitat use, changes in vertebral microchemistry should correlate with this behaviour. Conversely, such habitat-shifting behaviour is not described in pig-eye sharks, leading to the expectation—and our observation—of relatively lower variation in vertebral microchemistry in this species. Current results highlight the potential for vertebral microchemistry to describe shark movements between chemically distinct environments; however, the power of this method will depend on clearer definition of the mineralisation process in shark vertebrae.

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