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RESEARCH ARTICLE

Effects of sample cleaning and storage on the elemental composition of shark vertebrae

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Rationale: Application of vertebral chemistry in elasmobranchs has the potential to progress our understanding of individual migration patterns and population dynamics. However, the influence of handling artifacts such as sample cleaning and storage on vertebral chemistry is unclear and requires experimental investigation.

Methods: Vertebrae centra from blacktip sharks (*Carcharhinus limbatus*) were cleaned with bleach (NaOCl) for 5 minutes (min), 1 hour (h) and 24 (h) in a cleaning experiment and stored frozen, in 70% ethanol, and 10% formalin treatments for 20 days in a storage experiment. Element concentrations (Li, Na, Mg, Mn, Cu, Zn, Sr, Ba, Pb) were quantified in the outer edges of vertebrae centra using laser ablation inductively coupled plasma mass spectrometry and the [element:Ca] molar ratios were compared among treatments and individual sharks.

Results: Bleach cleaning significantly increased [Na:Ca] and formalin storage decreased [Na:Ca] and [Mg:Ca], but ethanol storage did not affect any [element:Ca] ratios. Vertebrae edge [Sr:Ca], [Ba:Ca] and [Mn:Ca] varied among individual sharks, potentially reflecting different environments that they had previously inhabited.

Conclusions: This study shows how archiving methods for vertebrae cartilage can affect primary element:Ca compositions. We demonstrate greatest element:Ca stabilities for vertebrae with limited bleach exposure that are either stored in ethanol or frozen, supporting the use of comparably archived sample sets in future elemental studies.

1 | INTRODUCTION

Elemental time-series recorded in fish hard parts that grow throughout life have advanced the understanding of migration patterns, environmental histories and population dynamics of teleost fishes.[1,2] This technique relies on the assumption that elements are deposited into calcified structures in proportion to their availability in the ambient environment and are not altered post-mortem. Previous research has focused on calcified structures in teleost fish (i.e. otoliths, scales, spines), but recent work has expanded to calcified structures in elasmobranchs including vertebrae, jaws and teeth.[3] Shark vertebrae centra are widely used for determining age due to concentric growth, non-resorption of accreted material and seasonally influenced rates of mineralization.[4-6] In contrast to the highly calcified aragonite otoliths of teleost fishes, elasmobranch vertebral cartilage is composed of mineralized hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) surrounded by an extracellular matrix of proteins.[5] The appositional and non-resorbed mineralization of elasmobranch vertebrae makes these structures well suited for determining age and growth[6] and potentially for reconstructing previous environmental life histories using the elemental chemistry of vertebrae growth bands.

The trace elemental composition of vertebrae is increasingly used to reconstruct the environments previously encountered by elasmobranchs[7-13] and has been used to validate growth band deposition rate.[14-16] Despite the surge in recent studies that have utilized vertebral elemental chemistry, few have systematically investigated how cleaning[7,17] and storage[17] procedures may alter the primary elemental composition of hydroxyapatite structures. Such knowledge is crucial for the accurate interpretation of elasmobranch life history patterns established from historical collections.[18]

Vertebrae are routinely cleaned using bleach (sodium hypochlorite, NaOCl), but the duration of bleach exposure varies greatly among published studies. Storage methods also vary greatly, ranging from dry (frozen) to wet (ethanol or formalin). In order to assess whether primary compositions are retained in freshly processed or archived vertebrae, the effects of handling and storage protocols should therefore be experimentally tested, as previously validated for fish otoliths.[19-21] Towards this end, we applied a repeated measures

experimental design to determine how common cleaning and storage methods affect elemental chemistry in a ubiquitous coastal elasmobranch species – the blacktip shark (*Carcharhinus limbatus*).

2 | EXPERIMENTAL

2.1 | Vertebrae collection and experimental design

Two male and three female blacktip sharks, ranging in total length between 126 and 171 cm, were collected from commercial fisheries landings in Venice, LA, USA (29.2772°N, 89.3548°W) on February 10–11, 2017. For each shark, 12–15 vertebrae were isolated from the thoracic region below the dorsal fin and manually cleaned of adhering tissue using a filet knife. For storage experiments, three adjacent vertebrae per shark were placed in one of three treatments: dry (frozen), wet ethanol (70%), or wet formalin (10%). The order in which treatments were applied to groups of three vertebrae was randomized among individual sharks. The vertebrae remained in storage treatments for 20 days before being processed for elemental analysis. The storage experiments thus utilized 15 vertebrae per treatment (3 vertebrae × 5 sharks). An extra set of three vertebrae per shark was frozen in preparation for bleach cleaning experiments that were conducted later in the laboratory. For the latter, thawed vertebrae from each individual were submerged in ~30 mL of commercial Clorox bleach solution (8.25% sodium hypochlorite) in plastic containers for three different exposure periods: 5 min, 1 h, and 24 h. Frozen non-bleached vertebrae served as a control in the bleach cleaning experiment. Following bleach exposure, the vertebrae were rinsed with deionized water and air-dried.

2.2 | Vertebrae preparation and analysis

A low-speed Isomet saw equipped with a diamond tipped blade was used to cut 2-mm sagittal (longitudinal) sections from each vertebrae centrum, creating a 'bowtie' central section (Figure 1A). Deionized water was used as a blade lubricant during cutting. Halves of bowtie sections were glued in rows to petrographic slides using thermoplastic cement (Crystalbond) (Figure 1B). Treatments (frozen, ethanol, formalin, bleach) corresponding to adjacent vertebrae sections were randomized. The final mounted vertebrae were rinsed with ultrapure (18.2 MΩ cm) water, air-dried and stored in plastic bags until analysis.

The elemental concentrations in the marginal edge of the corpus calcareum of the vertebrae sections were measured on March 6–10, 2017, by laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) at the University of Texas at Austin (Austin, TX, USA) using an NWR193 excimer (193 nm wavelength, 4–6 ns pulse width) laser system (ESI, Portland, OR, USA) coupled to an 7500ce ICP-MS instrument (Agilent, Santa Clara, CA, USA). The laser system is equipped with a large format, two-volume laser cell, for direct sampling of the ablation plume with fast (<1 s) washout times to minimize spatial carryover, which accommodated all shark vertebrae mounts and standards in a single loading. The vertebrae were analyzed with spot analyses from the oldest marginal edge area (sampled in triplicate) (Figure 1C). The laser ablation parameters optimized from test ablations were 60% laser power, 10-Hz repetition rate, 60-s dwell interval, 100-μm spot, and a helium cell flow rate of 800 mL·min^{-1}. Prior to analysis, the samples and standards were pre-ablated at 60% power using a 125-μm spot with a 2-s dwell interval to eliminate potential surface contamination. Spot analyses were bracketed each hour by measurement of standards (USGS MAPS-4, MACS-3, and NIST 612; measured in triplicate for 60 s). The laser energy densities over the analytical sessions averaged 3.52 ± 0.12 J·cm^2. The ICP-MS instrument was operated at a radio-frequency (RF) power of 1600 W with an argon carrier gas flow rate of 800–850 mL·min^{-1}. The oxide production rates, as monitored by ThO·Th^{-1} on NIST 612, averaged 0.34 ± 0.10% over the analysis period. The quadrupole time-resolved method involved the measurement of 15 masses using integration times of 10 ms (^{23}Na, ^{24}Mg, ^{25}Mg, ^{43}Ca, ^{55}Mn, ^{88}Sr), 20 ms (^{7}Li, ^{63}Cu, ^{66}Zn, ^{68}Zn, ^{138}La-Ce-Ba, ^{208}Pb), and 25 ms (^{138}Ba, ^{139}La, and ^{140}Ce). The analytical sampling period of 0.2842 s, equivalent to a reading every 2.842 μm, corresponded to 90% measurement time. The time-resolved intensities were converted into concentration (ppm) equivalents using Iolite software (University of Melbourne, Hellstrom et al[22]) with ^{43}Ca as the internal standard and a Ca index value of 35 weight %.[3] The

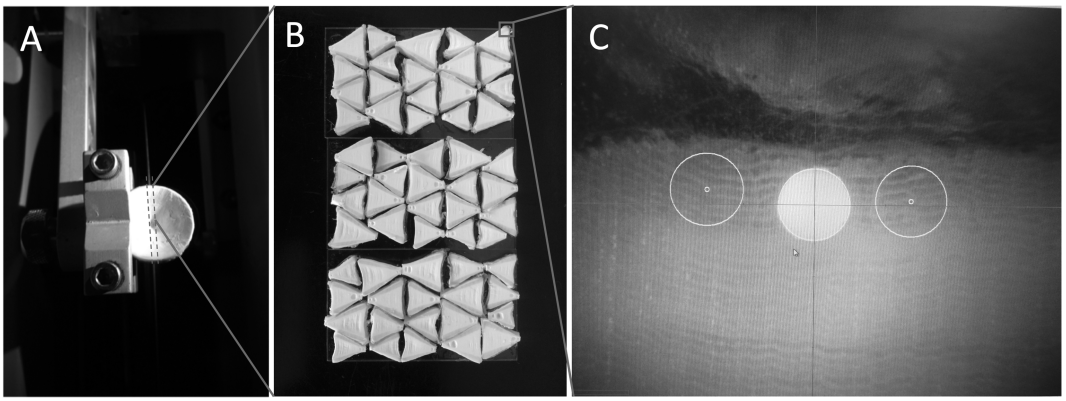

FIGURE 1 Schematic of the experimental design. Whole vertebrae centra were sectioned longitudinally using an Isomet saw (A); 2-mm 'bowtie' sections were cut in half and sections from each treatment/individual were randomly assorted and mounted onto glass slides (B); the outer marginal edge of the corpus calcareum (CC) was targeted with three triplicate spot ablations (100 μm diameter) per vertebrae section (C). Note the wavy horizontal growth increments visible on the vertebrae section surface ~17 μm thick that appear perpendicular to growth axis [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 1 Mean (± standard deviation) vertebral element concentrations in ppm and element:Ca molar ratios among experimental treatments

	Treatment						
		Bleach cleaning			Storage		
Element	Frozen control	5 min	1 h	24 h	ethanol	formalin	LOD (ppm)
[Li] (ppm)	1.26±0.211	1.206±0.241	1.195±0.336	1.013±0.177	1.206±0.171	1.089±0.196	0.059±0.014
[Li:Ca] (μmol·mol^{-1})	18.21±3.061	17.46±3.489	17.3±4.857	14.66±2.554	17.46±2.472	15.77±2.843	
[Na] (ppm)	17 410±2 797	18 213±3 066	26 528±6 224	27 710±4 035	15 325±2 696	8 828±1 244	5.043±1.391
[Na:Ca] (mmol·mol^{-1})	76.1±12.23	79.6±13.4	115.9±27.2	121.1±17.63	66.97±11.78	38.58±5.439	
[Mg] (ppm)	4 404±137	4 461±188	4 257±253	3 802±274	4 775±450	3 755±125	0.066±0.092
[Mg:Ca] (mmol·mol^{-1})	18.21±0.5683	18.44±0.7802	17.6±1.046	15.72±1.134	19.74±1.861	15.52±0.5164	
[Mn] (ppm)	28.08±6.56	30.01±8.35	27.37±6.62	30.7±3.87	29.44±5.38	29.94±5.69	0.053±0.017
[Mn:Ca] (μmol·mol^{-1})	51.35±12	54.89±15.26	50.05±12.1	56.14±7.077	53.83±9.83	54.75±10.41	
[Cu] (ppm)	1.253±0.310	0.84±0.117	1.351±1.005	1.329±1.077	2.52±2.065	3.64±3.413	0.105±0.027
[Cu:Ca] (μmol·mol^{-1})	1.981±0.4879	1.333±0.1831	2.135±1.589	2.102±1.702	3.984±3.264	5.754±5.398	
[Zn] (ppm)	48.56±6.60	48.42±6.83	51.62±16.94	45.45±4.61	53.69±6.16	58.81±9.49	0.058±0.025
[Zn:Ca] (μmol·mol^{-1})	74.61±10.14	74.4±10.49	79.31±26.03	69.84±7.091	82.49±9.46	90.37±14.58	
[Sr] (ppm)	1 587±102	1 596±96	1 618±99	1 650±80	1 616±89	1 569±121	0.0075±0.0067
[Sr:Ca] (mmol·mol^{-1})	1.82±0.1193	1.829±0.1092	1.856±0.1158	1.891±0.09266	1.853±0.1022	1.8±0.1386	
[Ba] (ppm)	14.63±3.12	14.42±2.19	15.86±3.32	15.43±2.05	16.32±4.75	21.61±5.37	0.0029±0.0033
[Ba:Ca] (μmol·mol^{-1})	10.7±2.285	10.55±1.606	11.6±2.429	11.29±1.501	11.94±3.478	15.81±3.931	
[Pb] (ppm)	1.602±0.517	1.533±0.225	1.777±0.437	1.199±0.184	1.662±0.524	1.761±0.342	0.0073±0.011
[Pb:Ca] (μmol·mol^{-1})	0.7767±0.2512	0.7433±0.109	0.862±0.2124	0.5807±0.08989	0.8058±0.2545	0.8543±0.1655	

LOD: limit of detection

TABLE 2 Repeated measures ANOVA comparing 8.25% bleach cleaning treatments (control, 5 min, 1 h, 24 h) on elemental composition of blacktip shark *Carcharhinus limbatus* vertebrae

Element	Factor	SS	df	MS	F	*p*-value
Li:Ca	Treatment	36.15	3	12.05	5.307	0.0459
	Individual	179.4	4	44.85	19.75	**<0.0001**
	Residual	27.24	12	2.27		
Na:Ca	Treatment	8368	3	2789	13.87	**0.0053**
	Individual	3106	4	776.4	3.86	**0.0306**
	Residual	2414	12	201.2		
Mg:Ca	Treatment	22.83	3	7.61	7.294	**0.0109**
	Individual	0.7277	4	0.1819	0.1744	0.9473
	Residual	12.52	12	1.043		
Mn:Ca	Treatment	124	3	41.33	0.7098	0.4675
	Individual	1595	4	398.7	6.848	**0.0041**
	Residual	698.7	12	58.23		
Cu:Ca	Treatment	2.116	3	0.7054	0.4389	0.5993
	Individual	3.49	4	0.8724	0.5428	0.7076
	Residual	19.29	12	1.607		
Zn:Ca	Treatment	224.2	3	74.73	0.4974	0.5275
	Individual	1961	4	490.3	3.263	0.0498
	Residual	1803	12	150.3		
Sr:Ca	Treatment	0.01506	3	0.005021	1.797	0.2407
	Individual	0.1591	4	0.03977	14.24	**0.0002**
	Residual	0.03352	12	0.002794		
Ba:Ca	Treatment	3.666	3	1.222	2.666	0.126
	Individual	58.32	4	14.58	31.8	**<0.0001**
	Residual	5.501	12	0.4584		
Pb:Ca	Treatment	0.2089	3	0.06964	7.226	**0.0378**
	Individual	0.3971	4	0.09927	10.3	**0.0007**
	Residual	0.1157	12	0.009638		
Ca CPS	Treatment	82836353500	3	27612117833	10.664	**0.004**
	Individual	6060865304	4	1515216326	0.5839	0.6803
	Residual	31141410065	12	2595117505		

Significant *p*-values (<0.05) in **bold.** SS = sum of squares; df = degrees of freedom; MS = mean square

concentrations were expressed in ppm and as molar ratios to calcium, except for ^{43}Ca, which was expressed as raw counts per second (CPS). The analyte baseline intensities were determined from 30-s gas blank intervals measured while the laser was off. USGS MAPS-4 was used as the primary reference standard. The analyte recoveries for secondary standards MACS-3 and NIST 612, respectively, averaged 106 ± 1.1% and 112 ± 0.1% (N = 18) versus GeoRem preferred values compiled in the GeoReM geochemical database for reference materials and isotopic standards.[23] Excluding Cu, Zn, La and Pb, these recoveries are 100 ± 0.04% and 106 ± 0.05%. The relative standard deviations (RSDs) of repeated measures of NIST 612 standards were: Li = 2.6%, Na = 5.7%, Mg = 1.1%, Mn = 1%, Cu = 5.4%, Zn = 3.1%, Sr = 1.9%, Ba = 1.8%, and Pb = 2.4%.

2.3 | Statistical analysis

A one-way repeated-measures analysis of variance (ANOVA) was used to partition variation in vertebrae element:Ca ratios among treatments nested within individual sharks to test the null hypothesis of no significant difference in element:Ca ratios among treatments. For the cleaning experiment, frozen non-bleached vertebrae were used as the control group. The D'Agostino-Pearson omnibus normality test was used to test if values come from a Gaussian distribution and a Brown-Forsythe test explored if the variance was equal among the treatment factors. Only [Cu:Ca] and [Pb:Ca] from the storage experiment did not exhibit normality and were thus log-transformed. Sphericity (equal variability of differences) was not assumed and a Geisser-Greenhouse epsilon hat correction was employed. A Holm-Sidak multiple comparisons test with adjusted p-values was used to examine significant differences among all treatments if the ANOVA revealed significant treatment effects. All statistical testing was completed in PRISM 7 (GraphPad Software).

3 | RESULTS

3.1 | Cleaning experiment

Exposing vertebrae to bleach significantly affected [Na:Ca], [Mg:Ca], [Pb:Ca] and Ca CPS; however, the effects varied among elements and by the duration of bleach exposure time (Tables 1 and 2; Figure 2). The vertebral [Na:Ca] significantly increased by 40 and 45 mmol·mol^{-1} (9 118 and 10 300 ppm) after 1 h and 24 h, respectively, compared with the control (Tables 1 and 2; Figure 2B). The vertebral [Mg:Ca] was significantly affected by the bleach treatment; however, no multiple comparisons were statistically significant (Figure 2C). The calcium signal significantly decreased by 178,000 CPS between 5 min and 24 h bleach exposure (Figure 2J). The [Pb:Ca] significantly

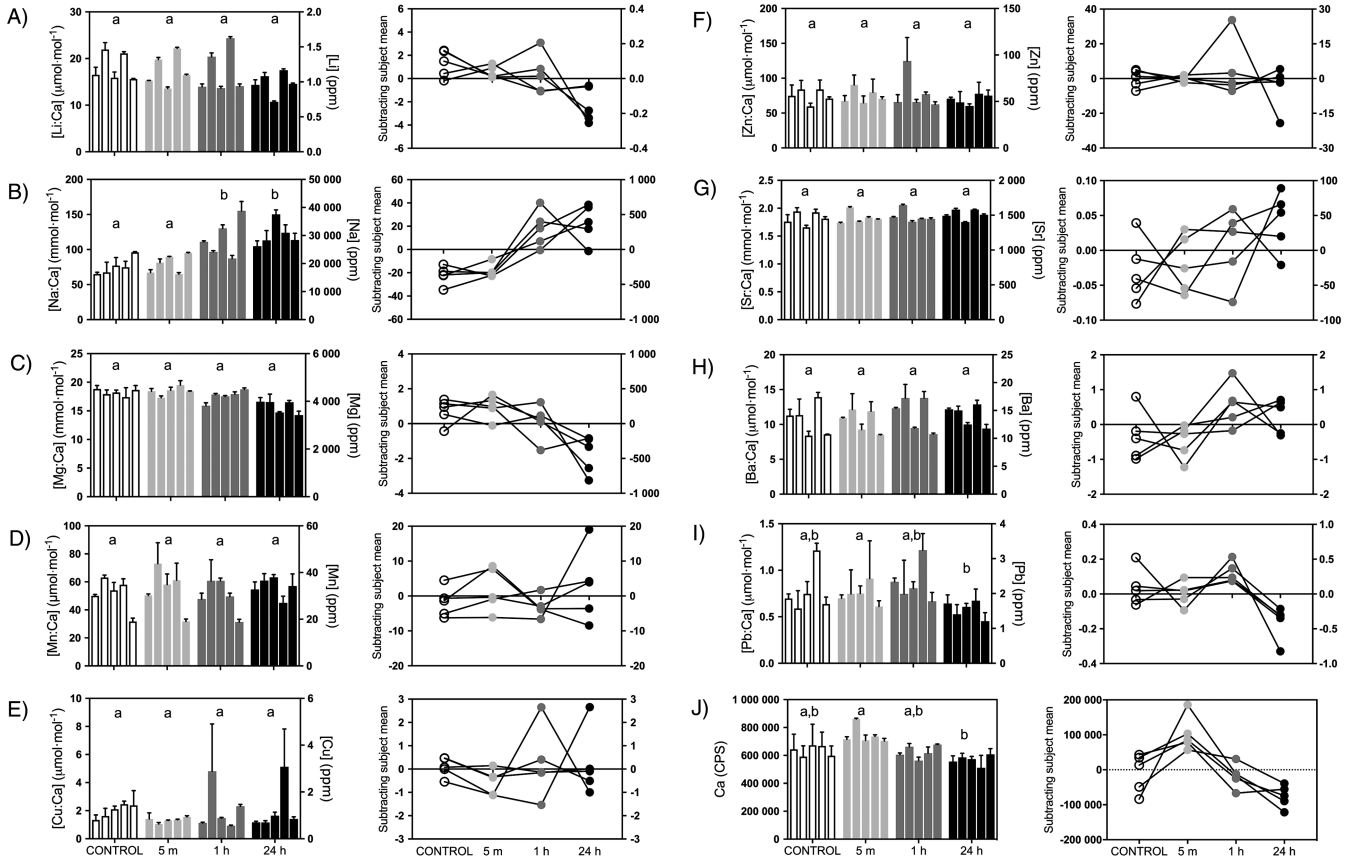

FIGURE 2 Elemental concentration of blacktip shark *Carcharhinus limbatus* (n = 5, individual bars) vertebrae (error bars: ± 1 SD, 3 replicate ablations per vertebrae) subject to control no bleach (white) 8.25% bleach cleaning for 5 min (light grey), 1 h (grey), or 24 h (black) in left panels; molar element:Ca ratios on left y-axis; element concentration on right y-axis. Grand total mean concentration (all treatments combined) subtracted from individual subject treatment means in right panels with lines connecting repeated measures on individual sharks. Lower case letters above bars indicate significant (Holm-Sidak adjusted p <0.05) multiple comparison tests

decreased by 0.16 μmol·mol^{-1} (0.3 ppm) between 5 min and 24 h bleach exposure, and was also significantly different among individual sharks (Table 2; Figure 2I). Individual sharks also differed significantly in [Li:Ca], [Na:Ca], [Mn:Ca], [Sr:Ca] and [Ba:Ca] (Table 2; Figures 2A, 2G, and 2H). [Cu:Ca] and [Zn:Ca] did not differ among bleach cleaning treatments and were consistent among individual sharks (Table 2).

3.2 | Storage experiment

Vertebral elemental compositions were not affected by storage in ethanol, but formalin storage affected the concentrations of several elements (Table 3; Figure 3). [Na:Ca] and [Mg:Ca] decreased by 37.5 mmol·mol^{-1} (8 583 ppm) and 2.7 mmol·mol^{-1} (649 ppm), respectively, in formalin treatments compared with in frozen treatments (Tables 1 and 3; Figures 3B and 3C). [Li:Ca] decreased by 2.4 μmol·mol^{-1} (0.17 ppm) and [Zn:Ca] increased by 15.8 μmol·mol^{-1} (10.3 ppm) in formalin compared with in frozen treatments, and significant differences among individual sharks were also detected (Tables 1 and 3, Figures 3A and 3F). [Sr:Ca] and [Mn:Ca] were significantly different among individual sharks, but not among storage treatments (Table 3; Figures 3D and 3G). There were no significant differences in log([Cu:Ca]), [Ba:Ca], log([Pb:Ca]) or Ca CPS among storage treatments or among individual sharks (Table 3, Figures 3E, 3H, 3I, and 3J).

4 | DISCUSSION

The elemental chemistries of shark vertebrae were significantly affected by cleaning with bleach over relatively long periods (24 h) compared with brief (5 min) exposures, which increased [Na:Ca] and decreased [Pb:Ca] and Ca. Vertebrae storage in formalin decreased [Na:Ca] and [Mg:Ca] compared with frozen treatments, but ethanol storage did not affect any elemental ratios. Of great significance, we find that several elemental ratios commonly used to trace environmental temperature or salinity variations ([Sr:Ca], [Ba:Ca], [Mn:Ca]) were unaffected by cleaning or storage treatments. For elasmobranch ecological tracer studies, it is of obvious importance that the vertebrae retain their primary compositions post-mortem. Vertebrae should therefore have short bleach exposure times (<1 h) and not be stored in formalin.

4.1 | Bleach cleaning

The concentrated bleach solution used in this experiment (8.25% NaOCl) acts as a strong oxidizing agent that can break down molecular bonds in proteins. The increase in vertebrae [Na:Ca] following bleach exposure may result from Na ions substituting for weakly bound ions within the hydroxyapatite structure (e.g. Mg, Li). Microscopic examination of vertebrae section surfaces (Figure 3C) revealed opaque and translucent increments ~17 μm in diameter

TABLE 3 Repeated-measures ANOVA comparing storage treatments (frozen, 70% ethanol, 10% formalin) on elemental composition of blacktip shark *Carcharhinus limbatus* vertebrae

Element	Factor	SS	df	MS	F	*p* value
[Li:Ca]	Treatment	15.69	2	7.846	11.71	**0.0082**
	Individual	88.89	4	22.22	33.16	**<0.0001**
	Residual	5.361	8	0.6701		
[Na:Ca]	Treatment	3828	2	1914	13.22	**0.0045**
	Individual	113.7	4	28.44	0.1965	0.9334
	Residual	1158	8	144.7		
[Mg:Ca]	Treatment	45.47	2	22.74	20.33	**0.0057**
	Individual	7.263	4	1.816	1.624	0.2591
	Residual	8.947	8	1.118		
[Mn:Ca]	Treatment	30.95	2	15.47	2.139	0.1959
	Individual	1338	4	334.5	46.24	**<0.0001**
	Residual	57.87	8	7.233		
Log[Cu:Ca]	Treatment	0.1503	2	0.0752	1.063	0.3788
	Individual	0.442	4	0.1105	1.562	0.2736
	Residual	0.5658	8	0.0707		
[Zn:Ca]	Treatment	620.4	2	310.2	8.061	**0.0137**
	Individual	1312	4	328.1	8.525	**0.0055**
	Residual	307.9	8	38.48		
[Sr:Ca]	Treatment	0.007159	2	0.003579	4.198	0.0625
	Individual	0.1676	4	0.0419	49.14	**<0.0001**
	Residual	0.00682	8	0.0008525		
[Ba:Ca]	Treatment	71.15	2	35.58	4.945	0.0848
	Individual	73.52	4	18.38	2.555	0.1206
	Residual	57.55	8	7.194		
Log[Pb:Ca]	Treatment	0.0071	2	0.00355	0.465	0.6231
	Individual	0.111	4	0.0277	3.637	0.0568
	Residual	0.06107	8	0.0076		
Ca CPS	Treatment	868352309	2	434176154	0.06068	0.88
	Individual	1940835646	4	485208912	0.06781	0.9899
	Residual	57241712403	8	7155214050		

Significant *p*-values (<0.05) in **bold.** SS = sum of squares; df = degrees of freedom; MS = mean square

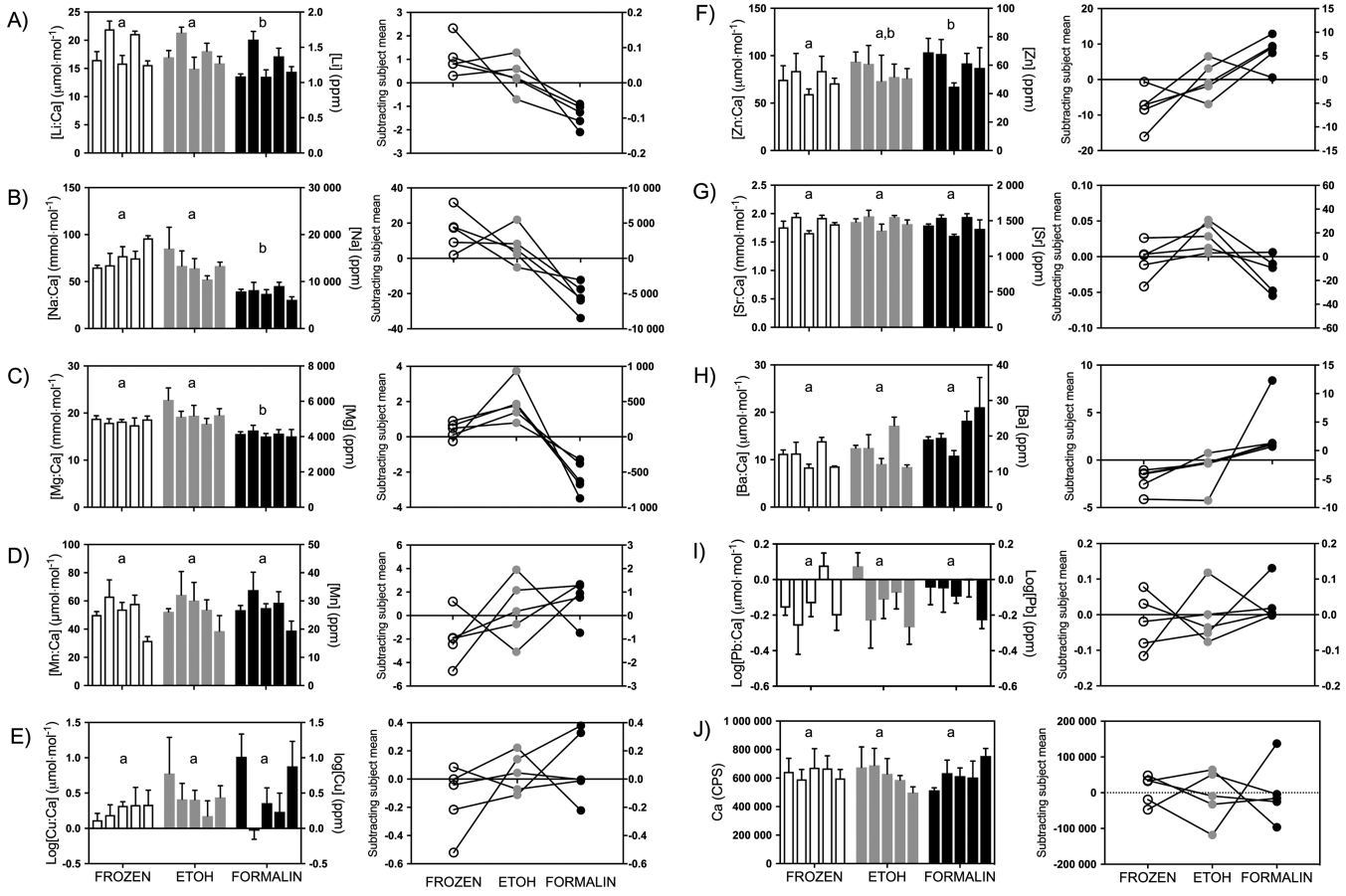

FIGURE 3 Elemental concentration of blacktip shark *Carcharhinus limbatus* (n = 5, individual bars) vertebrae (error bars: ± 1 SD, 3 replicate ablations per vertebrae) subject to storage treatments frozen (white), 70% ethanol (ETOH: grey), or 10% formalin (black) in left panels; molar element:Ca ratios on left y-axis; element concentration on right y-axis. Grand total mean concentration (all treatments combined) subtracted from individual subject treatment means in right panels with lines connecting repeated measures on individual sharks. Lower case letters above bars indicate significant (Holm-Sidak adjusted p <0.05) multiple comparison tests

perpendicular to the growth axis, which may represent the canalicular passageways to transport liquids and dissolved elements.[5] Previous otolith studies have suggested that Na and Mg ions are weakly absorbed within the aragonite-proteinaceous matrix and not structurally bound within the aragonite crystal lattice.[20] Na and Mg concentrations in Terubok, tropical shad *Tenualosa toli*, otoliths were higher in samples immediately removed and stored dry, than in samples stored frozen or in 70% ethanol.[21] McMillan et al[3] (see their Supplemental Materials, Table S4) found that vertebrae [Na:Ca] significantly increased with bleach exposure (40 vs 120 min), whereas [Mg:Ca] decreased, and [Mn:Ca] increased only after 2 h. Lewis[17] investigated how exposure to bleach for 5 min and then ethanol storage for 3 months affected the chemistry of blacktip shark vertebrae and found a significant difference in [Mn:Ca] (p = 0.04) due to the handling and storage treatment, but no change in [Li:Ca], [Mg:Ca], [Sr:Ca], [Ba:Ca] or [Pb:Ca]. However, Lewis[17] did not quantify [Na:Ca], and sonicated the experimental vertebrae in ultrapure water for 60 min before analysis, which may have equalized the concentrations of the more labile elements ([Na:Ca] and [Mg:Ca]), or perhaps the short bleach treatment of 5 min was not long enough to induce elemental changes as we found. In addition, Lewis[17] utilized laser transects and not spot analyses, so averaging values over the entire transect may have masked potential bleach effects near the edge of the vertebrae. Tillett et al[7] found no elemental variations between vertebrae pairs exposed and unexposed to bleach, but only four specimens were considered and bleach exposure procedures were not documented.

4.2 | Formalin effects

Formalin works as a sample preservative by penetrating tissues and slowly binding aldehyde groups to proteins to form stable methylene crossed-linked bridges over several days.[24] In the present study, formalin effects occurred over 20-day storage intervals, but samples in historical collections may be stored over years to decades. Studies of the effects of formalin preservation on mammalian bone demonstrate that formalin leaches Ca, Mg and P ions, which can lower the fracture toughness of bone and make it brittle.[25] In our study, formalin exposure did not change shark vertebrae Ca compared with frozen or ethanol treatments but, similar to bleach exposure treatments, it did significantly decrease both Na and Mg concentrations in all specimens, suggesting that formalin leaches these ions from the hydroxyapatite matrix.[25,26] There was also a significant increase in $[Zn]^{2+}$ and decrease in $[Li]^{1+}$ due to formalin treatment, but there were also significant differences in these elements among individual sharks that preclude clear interpretation of the treatment differences. Element binding studies within otoliths have determined that 'non-essential'

alkaline metals (e.g. Sr, Ba) with ionic radii similar to that of Ca directly substitute for Ca during aragonite crystal formation.[27-29] Metals essential for physiological osmoregulation (e.g. Na, Mg) probably do not substitute for Ca within the crystal lattice, but may be loosely bound to protein or interstitial spaces of the mineralized crystal. The effect of formalin on biological tissues depends on the strength and mode of element binding in the tissue.[26] To our knowledge, no studies have examined element binding sites in mineral hydroxyapatite or in the protein fraction of elasmobranch vertebrae cartilage. Our vertebrae treatment studies indicate that Na and Mg are particularly susceptible to loss by formalin and bleach exposure. More controlled experimental work is needed to elucidate element incorporation dynamics and species-specific uptake and regulation of elements.

4.3 | Ecological ramifications

Only the formalin storage treatment and bleach cleaning for 1–24 h significantly altered element concentrations in shark vertebrae. Although shifts in element concentrations for [Na:Ca] and [Mg:Ca] were statistically significant, the shifts were relatively minor (30–40 $mmol{\cdot}mol^{-1}$ for [Na:Ca]; 2–3 $mmol{\cdot}mol^{-1}$ for [Mg:Ca]). Lewis et al[9] found differences in [Mg:Ca] ranging between 1 and 5 $mmol{\cdot}mol^{-1}$ among blacktips sampled in the Gulf of Mexico. Differences in [Mn:Ca] were of the order of 100 $\mu mol{\cdot}mol^{-1}$ among regions, while the [Ba:Ca] ranged by 5 $\mu mol{\cdot}mol^{-1}$, indicating that natural spatial variation in blacktip vertebrae chemistry is larger than the difference detected in this experiment. Importantly, elements that are typically incorporated into elasmobranch vertebrae in proportion to their availability in the environment, such as barium[12] and strontium,[16] were not affected by cleaning or storage treatments. In fact, the environmental tracers [Sr:Ca], [Ba:Ca] and [Mn:Ca] were the only elemental ratios that significantly differed among individual sharks. Since laser sampling targeted the distal margin of the vertebrae, the resolved elemental signatures should reflect ambient environmental conditions in the weeks or months prior to capture. The coastal Louisiana area, where all sharks in this study were collected, experiences dynamic temperature, salinity and dissolved oxygen variations associated with the influence of the Mississippi River plume. The significant difference among individuals in [Sr:Ca], [Ba:Ca] and [Mn:Ca] further supports these elements as environmental proxies in blacktip vertebrae.[9] However, for shark species that inhabit stable environments without highly variable water chemistries, storage and cleaning treatment effects on primary compositions may be more pronounced.

5 | CONCLUSIONS

Our investigations suggest that chemical alteration is least likely for (1) vertebrae samples subjected to less than 1 h of bleach exposure, and (2) vertebrae samples stored frozen or in ethanol. We also find that important elemental tracers ([Sr:Ca], [Ba:Ca], [Mn:Ca]) are likely to be preserved in samples stored in formalin. For properly prepared and archived specimens, elasmobranch vertebrae chemistry holds promise to advance our understanding of life histories and population dynamics, similar to knowledge gained for bony fish from elemental analysis of otoliths.

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