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Natural variability and effects of cleaning and storage procedures on vertebral chemistry of the blacktip shark *Carcharhinus limbatus*

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Running head: VERTEBRAL CHEMISTRY VARIABILITY AND STABILITY

Using laser-ablation inductively coupled plasma mass spectrometry, key methodological assumptions regarding the degree of natural variability and influence of sample handling and storage of elasmobranch vertebral chemistry were assessed. Vertebral chemistry of juvenile blacktip sharks *Carcharhinus limbatus* was examined to identify whether differences existed among different regions of the vertebral column, between thoracic vertebrae of individual fish, or within individual vertebrae. Additionally, the effects of bleach exposure and storage in ethanol on vertebral chemistry were compared. No significant variation in vertebral chemistry was found among different regions of the vertebral column or between thoracic vertebrae, but significant differences among transect location within individual vertebrae were observed. The variation at all three levels appears comparable with published data on sagittal otoliths of bilaterally symmetrical teleost fishes. The experimental assessment of potential treatment effects indicated vertebral chemistry was not significantly affected by bleach or exposure to ethanol. Taken together, these results support the idea that vertebrae taken from the same region of the vertebral column can be treated as equivalent and at least certain elements remain robust to exposure to bleach and ethanol.

Key words: chemical equivalency; elasmobranch; LA-ICP-MS; post mortem contamination.

INTRODUCTION

The field of otolith chemistry has provided substantial insight into the ecology of bony fish species. Present as three pairs of calcium carbonate structures (sagittae, lapillae and asteriscae) within the vestibular canals of bony fishes, their metabolically inert nature, chronometric properties and capacity to incorporate elements derived from the environment have allowed researchers to estimate the timing of larval ingress (Hoover *et al*., 2012), identify life-history contingents (Secor *et al*., 2001) and study population connectivity (Thorrold *et al*., 2001; Almany *et al*., 2002). This myriad of applications, facilitated by constantly advancing analytical techniques, has driven researchers to explore the potential use of calcified structures to study other marine organisms (Edmonds *et al.*, 1996; Hand *et al.*, 2008; Tillett *et al.*, 2011).

The vertebrae of elasmobranchs are structures that hold particular promise as chemical chronographs. Although the mineral component of elasmobranch vertebrae [hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$)] differs from that of bony fish otoliths [calcium carbonate ($CaCO_3$)], elasmobranch vertebrae produce annual pairs of opaque and translucent bands similar to otolith growth zones and vertebral chemistry also appears to be partially influenced by the surrounding environment (Werry *et al.*, 2011; Scharer *et al.*, 2012; Smith *et al.*, 2013). Capitalizing on these characteristics, researchers have demonstrated the use of elasmobranch vertebrae to identify natal origin of neonate and young-of-the-year individuals (Lewis *et al*., 2016) and ontogenetic shifts in diet (Estrada

et al., 2006) and habitat (Werry *et al*., 2011). With the novel application of vertebral chemistry, however, comes the need to validate inherent methodological assumptions surrounding the degree of natural variability within and among individual vertebrae and the potential for *post mortem* contamination during sample preparation.

The need to understand the degree of natural variability of calcified structures stems from the fact that similar structures are presumed to be chemically equivalent. In bony fishes, the degree of variability between paired sagittal otoliths from a single individual appear to be negligible relative to the variability among fish (Arslan & Secor, 2008; Campana *et al*., 2000; Campana *et al*., 1994; Secor *et al*., 2001). Thus, individual otoliths within a pair are considered interchangeable. Exceptions to this assumed equivalence, however, do exist. For example, sagittae exhibit asymmetric growth patterns between sides of flatfishes, resulting in significant differences in mass that appear to drive a directional bias in otolith chemistry (Kajajian *et al*., 2014; Loher *et al*., 2008). Thus, treating sagittae of flatfish interchangeably can increase the variability in otolith chemical signatures and potentially affect conclusions drawn from quantitative analyses (Kajajian *et al*., 2014). Researchers have also observed differences in otolith chemistry among pairs of otoliths that again appear to be driven by differing patterns of growth (Smith & Jones, 2006). Sagittal and lapillar otoliths are relatively similar in size during the larval stage in bony fishes' development. Their growth patterns quickly diverge (David *et al*., 1994; Morioka & Machinandiarena, 2001), however, producing whole otolith signatures of

lapillae that are biased towards early life stage, while chemistry of sagittae more accurately reflecting the entire life history of an individual (Smith & Jones, 2006). The fact that deviations from presumed equivalency are most often attributed to differences in growth rate is of particular interest when considering equivalency among elasmobranch vertebrae. Significant axial variability in vertebral morphology and its effect on age estimates has been assessed in multiple species (Officer *et al.*, 1996; Piercy *et al.*, 2006; Natanson *et al.*, 2008). It is unknown, however, whether differences in vertebral morphology (Piercy *et al*., 2006; Natanson *et al*., 2008) among different regions of the vertebral column affect vertebral chemistry, though vertebral chemistry appears to be consistent within the thoracic region of the vertebral column (Tillett *et al.*, 2011; Smith *et al.*, 2016). Moreover, the degree of intra-vertebral variability has yet to be assessed, but has the potential to contribute process error to statistical analyses of vertebral chemistry.

The potential for *post mortem* contamination of vertebral chemistry is another concern that has received little attention to date. For example, several of the elements examined in bony-fish otoliths occur at trace (< mg l^{-1}) or ultra-trace (<μg l^{-1}) concentrations (Davies *et al*., 2011; Milton & Chenery, 1998; Proctor & Thresher, 1998; Smith *et al*., 2013), which can be easily influenced or contaminated during *post mortem* handling (Milton & Chenery, 1998; Hedges *et al*., 2004; Swan *et al*., 2006). Elements of interest, especially those that are incorporated into interstitial spaces (Izzo *et al.*, 2015) or associated with the

protein matrix (*e.g.* Mn, Cu and Zn; Miller *et al.*, 2006; Izzo *et al.*, 2015;), may be more prone to *post mortem* alteration. Thus, great care is typically taken to minimize the probability of contamination when samples are collected with the intent to quantify chemical constituents. Interest in analysing chemical constituents of archived elasmobranch vertebrae presents a unique issue, however, because archived samples historically have not been handled with the same degree of care. Elasmobranch researchers typically rely on diluted bleach to aid in tissue removal before storing vertebrae in isopropanol or ethanol prior to sectioning for age and growth analysis (Hale & Baremore, 2013). Although the use of bleach does not appear to affect vertebral chemistry significantly (McMillan *et al.*, 2017; Tillett *et al.*, 2011), the effects of a liquid preservative on vertebral chemistry is unknown but of concern given liquid preservatives can alter otolith chemistry (Milton & Chenery, 1998; Hedges *et al*., 2004). For example, Milton & Chenery (1998) and Hedges *et al*. (2004) noted that preservation of otoliths or whole fish in ethanol did not significantly alter chemical constituents that are likely to substitute for calcium in the carbonate matrix (*e.g.* Sr and Ba). Since whole otoliths are typically stored dry and not preserved in ethanol, it is unclear whether long-term exposure to a liquid preservative, such as isopropanol or ethanol, would alter vertebral chemistry.

The objectives of the present study were to assess both natural variability and potential *post mortem* contamination of juvenile blacktip shark *Carcharhinus limbatus* (Müller &

Henle 1839) vertebrae. Using laser-ablation inductively coupled plasma-mass spectrometry (LA-ICP-MS), natural variability in vertebral chemistry was assessed at three levels: among regions of the vertebral column; within the thoracic region of the vertebral column; within individual vertebrae. Whether exposure to bleach, long-term storage in 95% ethanol, or the application of both significantly affects elemental concentrations of *C.limbatus* vertebrae was also assessed.

MATERIALS AND METHODS

SAMPLE COLLECTION

Juvenile *C. limbatus* (n = 16) were collected opportunistically with gillnets or longlines in 2012 and 2013 as part of the Gulf of Mexico states' shark pupping and nursery survey (GULFSPAN) and fisheries independent sampling by Texas Parks and Wildlife. Four fish were euthanized in the field and transported to the lab whole to assess axial variability in vertebral chemistry. The vertebrae from the remaining 12 fish were removed in the field, transported on ice to the lab and stored frozen until processing.

SAMPLE PREPARATION AND LA-ICP-MS ANALYSIS

Details of experiments conducted to assess natural variability or storage medium effects on vertebral chemical signatures are described in subsequent sections. Here a

description of how vertebrae were prepared and analysed with laser ablation-inductively coupled plasma-mass spectrometry (LA-ICP-MS) is provided.

Cleaned vertebrae [Fig. 1(a)] were sectioned to a thickness of 0.5 mm with a Beuler Isomet low speed saw [www.bueler.com; Fig. 1(b)]. The radius of each sectioned vertebra was measured using a Nikon SMZ1500 dissecting microscope (www.nikon.com) and NIS-elements BR 4.00.07 imaging software (Nikon). Sections were then sonicated for 60 min in acid-leached polystyrene cells filled with ultrapure water (18 MΩ) and dried in a class 10 laminar-flow clean hood. Dried sections were subsequently mounted on glass microscope slides with double-sided tape and stored covered until trace metal analyses.

Analysis of vertebral chemistry occurred during two separate time periods. Analysis of vertebrae used to compare variability among body regions of the vertebral column took place in October 2014, while the vertebrae used to assess the variability within the thoracic region segment and effects of sample handling and storage were analysed in May 2015. On both occasions, the LA-ICP-MS configuration consisted of Nd: YAG NewWave 213 laser ablation system (ESI; www.esi.com) and an Agilent 7700 ICP-MS (www.agilent.com). Ablated material was swept from the ablation chamber with ultrapure He gas (flow rate of 0.7 l min^{-1}) and mixed with ultrapure Ar gas (flow rate of 1.1 l min^{-1}). Three parallel transects spaced 120 µm apart were run across the corpus calcareum of each vertebrae [Fig. 1(c)]. Transects were first pre-ablated (spot size of 100

μm, rep rate of 5 Hz and laser fluence of *c.* 0.4 mJ cm^{-2}) and then ablated with a laser spot size of 80 μm, using a repeat rate of 10 Hz and laser fluence of *c.* 4.0 mJ cm^{-2}. Transects were run on two certified reference materials (NIST 612 and MACS3) every 60 minutes to correct for instrument drift and convert signal intensity (counts s^{-1}) to units of concentration. Data were reduced using Trace_ElementIS in Iolite 2.5 (www.iolite-software.com) with ^{43}Ca as an internal standard (35% by mass). The focus was on eight elements that were assayed by monitoring the following isotopes: ^{7}Li, ^{24}Mg, ^{43}Ca, ^{44}Ca, ^{31}P, ^{55}Mn, ^{88}Sr, ^{137}Ba and ^{208}Pb. Concentrations of P were not included in the analysis, however, because per cent residual standard deviation (%R.S.D.) was above 10% and P is not certified in the MACS3. Concentrations of the remaining elements were normalized to ^{43}Ca and averaged over the postnatal portion of each transect [*i.e.* between the birthmark and distal edge; Fig. 1(c)]. Subsequent elemental ratios are expressed in units of μmol mol^{-1} (Li:Ca, Mn:Ca, Ba:Ca, Pb:Ca) or mmol mol^{-1} (Mg:Ca and Sr:Ca).

ASSESSMENT OF NATURAL VARIABILITY

The evaluation of natural variability focused on three distinct spatial scales: among different regions of the vertebral column; between thoracic vertebrae; within thoracic vertebrae. Differences among regions of the vertebral column were based on element:Ca of one cervical, thoracic and precaudal vertebrae from four *C. limbatus* sampled in 2012. Cervical vertebrae were removed from the vertebral column immediately posterior to the

chondrocranium, while thoracic vertebrae were removed from below the first dorsal fin and precaudal vertebrae were removed from below the second dorsal fin (Fig. 2). Intra and intervertebral variability of the thoracic vertebrae were assessed based on element:Ca estimated from two randomly selected vertebrae from 12 individuals. These vertebrae also served as controls in the sample handling and storage experiment (see below). All vertebrae used to assess natural variability were stored frozen and cleaned manually with a scalpel.

SAMPLE HANDLING AND STORAGE EXPERIMENT

The sample handling and storage experiment consisted of one control and three experimental treatments. Two thoracic vertebrae from 12 different *C. limbatus* were randomly assigned to each treatment. The control treatment, as described above, consisted of storing vertebrae frozen and manual cleaning with a scalpel. Experimental treatment 1 consisted of manual cleaning vertebrae with a scalpel then storing them in polyethylene containers filled with 95% ethanol (Fisher Science; cat. num. S25310A; www.thermofisher.com) for 3 months. Experimental treatment 2 consisted of soaking vertebrae in 5% bleach for 5 minutes to aid the removal of excess tissue, manual cleaning with a scalpel and storing frozen. Experimental treatment 3 consisted of soaking vertebrae in 5% bleach for 5 minutes, manual cleaning with a scalpel then in 95% ethanol 3 months.

DATA ANALYSIS

Prior to statistical analyses, plots of individual transects were scanned for point outliers. The few that were present (0.12% of data points) were replaced with the mean of the points on either side of the outlier. Point measurements of Pb that were below the limit of detection (LOD; Table I) were also identified at this time. Measurements of Pb below the LOD were replaced with the LOD unless a given transect contained a large percentage (> 20%) of values below LOD. Fish that fell into the latter category were excluded from analysis.

Split-plot linear mixed-effect models were computed with the nlme package in R (Pinheiro *et al*., 2015) to test whether significant differences in vertebral element:Ca existed among regions of the vertebral column, between thoracic vertebrae and within vertebrae. Within these models, shark number was a random blocking factor, while transect location was considered a fixed factor nested within vertebral region (for comparisons among regions of the vertebral column), vertebra (for comparisons between thoracic vertebrae), or treatment (for comparisons among sample handling and storage methods). Vertebral region, vertebra and treatment were all treated as fixed factors. Vertebral radius was included as a covariate in the test among vertebral regions because of significant variability in vertebral morphology (Piercy *et al*., 2006), which was confirmed using a repeated-measures ANOVA ($F_{2,\ 6} = 10.57$, $P < 0.01$). Thoracic

vertebrae do not vary significantly in vertebral radius within individuals ($F_{3, 69} = 1.49$, $P > 0.05$); thus, vertebral radius was not included as a covariate in models that only used thoracic vertebrae. A series of diagnostic plots were constructed to assess whether within group errors were normally distributed and that random effects were approximately normally distributed. Three outliers attributed to a single fish were identified at this time and this fish was excluded from the analysis of treatment effects on Pb:Ca. Any deviations from model assumptions that remained were rectified by applying a constant variance function at the level of shark number. When significant differences among regions of the vertebral column, vertebrae and transect location were present, Tukey's multiple comparison was computed to further explore differences. Significant treatment effects were investigated further with Dunnett's multiple comparison to identify whether differences existed between experimental groups and the control group. All multiple comparisons were computed in R with the multcomp package (Hothorn *et al*., 2008). The significance level for all statistical analyses was set at 0.05.

Reproducibility of vertebral chemistry at each spatial scale and between treatments was further evaluated by calculating the average per cent error ($\%\bar{\epsilon}$, Beamish & Fournier, 1981): $\%\bar{\epsilon} = (\sum_{j=1}^{n} \%\bar{\epsilon}_j)n^{-1}$, where $\%\bar{\epsilon}_j = 100(\sum_{i=1}^{R} \frac{|x_{ij} - \bar{x}_j|}{\bar{x}_j})R^{-1}$ and where $\%\bar{\epsilon}_j$ is the average per cent error for the jth shark, x_{ij} is the ith element:Ca estimate on the jth shark, $\bar{x}_j$ is the mean element:Ca estimate for the jth shark, R is the number of element:Ca estimates for each shark, which was the same for each shark and n is the number of sharks in the

sample. Estimates of ϵ were calculated with the FSA package in R 3.2.1 (Ogle, 2015). All estimates of ϵ among regions of the vertebral column and between thoracic vertebrae were based on comparisons of equivalent transects (*i.e.* transect 1 *v.* transect 1, transect 2 *v.* transect 2 and transect 3 *v.* transect 3). Estimates of ϵ within individual vertebrae were based on comparisons among all three transect locations (*i.e.* transect 1 *v.* transect 2, transect 1 *v.* transect 3 and transect 2 *v.* transect 3).

RESULTS

The majority of the samples contained concentrations of individual elements well above LOD. Samples run in May 2015, however, did contain four *C. limbatus* that were removed because Pb was not consistently above the LOD. Per cent R.S.D. varied between the NIST 612 and MACS-3, but remained in the ranges of 3.7–9.9% and 5.6–10.1%, respectively (Table I).

ASSESSMENT OF NATURAL VARIABILITY

Among regions of the vertebral column, no significant differences were detected in vertebral chemistry (Table II). Vertebral chemistry was also not significantly different between thoracic vertebrae (Table II and Fig. 3). Because differences between thoracic

vertebrae for both Li:Ca and Pb:Ca were near the *a priori* threshold for statistical significance, however, multiple comparison tests were computed to identify which transect locations differed between vertebrae. Results indicated that only element:Ca ratios from the interior-most transects were not significantly different between thoracic vertebrae across the range of elements examined (Table III).

Despite the lack of statistically significant differences in chemistry among vertebrae from different regions of the vertebral column, there were some significant differences among transects within vertebrae (Table II). Among regions of the vertebral column, Sr:Ca and Pb:Ca exhibited significant disordinal interactions between region and transect location, which suggests that observed intra-vertebral variability was not consistent among regions of the vertebral column (Fig. 4).

Within thoracic vertebrae, there were significant differences among transects for Mg:Ca, Mn:Ca, Sr:Ca, Ba:Ca and Pb:Ca, including significant ordinal interactions for Mg:Ca, Ba:Ca and Pb:Ca (Table II). The latter three elemental ratios were also the only elements for which all multiple comparisons were significant within each vertebra. Additionally, unlike the inconsistent patterns of intra-vertebral variability among body segments, intra-vertebral variability of thoracic vertebrae was more consistent (Fig. 5). Similar patterns of intra-vertebral variability were observed for Mg:Ca, Sr:Ca and Ba:Ca, which exhibited a decline in concentration with increasing distance from the intermedialia. The opposite was true for Li:Ca, although differences were not significant.

Concentrations of Pb:Ca tended to be higher on the interior and edge of the corpus calcareum while no clear pattern was observed for Mn:Ca.

When comparing estimates of $\%\bar{\epsilon}$ (*i.e.,* reproducibility) at different spatial scales, the greatest error was observed between vertebrae collected from different regions of the vertebral column (Fig. 6). Average per cent error ($\%\bar{\epsilon}$) between thoracic vertebrae and within an individual vertebra was lower, often d 5% for Li:Ca, Mg:Ca, Mn:Ca, Sr:Ca and Ba:Ca.

SAMPLE HANDLING AND STORAGE EXPERIMENT

The results of the sample handling and storage experiment indicated only Li:Ca and Mn:Ca were significantly different among treatments (Table II). Results of *post hoc* comparisons, however, indicated there were no differences between the control vertebrae and those exposed to experimental treatments ($P > 0.05$). There was significant intra-vertebral variability for all element:Ca ratios but the same general trends existed across treatments (Fig. 7). For the six elements examined, there was no significant interaction between treatment and transect location. Average per cent error between vertebrae exposed to the same treatment also was similar (*e.g.* $\%\bar{\epsilon} < 9\%$), thus further supporting the inference that handling and storage treatments had minimal effects on vertebral chemistry.

DISCUSSION

The success of employing otolith chemistry to examine different questions about bony fish ecology has prompted exploratory research of other calcified structures with the hope of identifying structures of similar use. Regardless of the type of calcified structure, equivalent chemistry among potential structures within an individual is a fundamental assumption (Gauldie, 1996; Rooker *et al.*, 2001) and one that appears valid for thoracic vertebrae of *C. limbatus*. Others have recently reported similar results for bull shark *Carcharhinus leucas* (Valenciennes 1839), pigeye shark *Carcharhinus amboinensis* (Müller & Henle 1839) and scalloped hammerhead *Sphyrna lewini* (Griffith & Smith 1834) (Smith *et al.*, 2016; Tillett *et al.*, 2011). When compared with fish otoliths, estimates of precision among vertebrae were remarkably similar. In their assessment of base equivalency between right and left tuna otoliths, Rooker *et al*. (2001) reported APE of 2-8%. Moreover, vertebral chemistry among vertebrae from different regions of the vertebral column did not significantly differ and most elements exhibited $\%\bar{\epsilon}$ between 5 and 8%, indicating variability among regions of the vertebral column may also be comparable with paired otoliths.

Equivalent chemistry of most bony fish sagittae appears to be the result of selective pressure to maintain morphological symmetry of the vestibular system. Conversely, flatfish (Order: Pleuronectiformes) sagittae, which exhibit asymmetry in mass (Kajajian

et al., 2014; Loher *et al*., 2008; Morat *et al*., 2013) and morphology (Jackman *et al*., 2015), do not appear to be chemically equivalent (Jackman *et al*., 2015; Kajajian *et al*., 2014; Loher *et al*., 2008; Morat *et al*., 2013). With such a strong correlation between growth pattern and chemical equivalency, the observed equivalency among *C. limbatus* vertebrae from different regions of the vertebral column is somewhat of a surprise. Though low overall, vertebrae from different regions of the vertebral column resulted in the highest $\%\bar{\epsilon}$. Considering vertebrae were sectioned at the same thickness and significant differences in vertebral morphology were observed, it is possible a temporal mismatch was experienced in vertebral chemistry that increased $\%\bar{\epsilon}$. This may also have contributed to the significant interaction between region of the vertebral column and transect location for Sr:Ca and Pb:Ca. Given the fact that the degree of variation in vertebral morphology has an ontogenetic component (Natanson *et al.*, 2008), the amount of process error introduced during the sectioning process may vary depending on the species and life stages of interest. Also worth noting is that there are instances where calcified structures that exhibit differing growth rates are chemically equivalent. For example, Gulf menhaden *Brevoortia patronus* Goode 1878 reared under constant salinity regimes yielded lapillus and sagitta with chemically equivalent Sr values (Chesney *et al*., 1998). Perhaps the observed equivalency among body segments reflects environmental histories that were relatively stable. Although a more thorough evaluation is warranted

before generalizing all vertebrae within an individual are chemically equivalent, it does appear plausible under certain conditions.

Somewhat surprising was the fact that significant variability was present within individual vertebrae. Tillett *et al*. (2011) did not find significant variability in chemical signatures within vertebrae of *C. leucas* or *C. amboinensis*. These differing results may reflect differences in statistical approaches (*i.e.,* non-parametric multivariate model *v.* univariate mixed-effects model) and sample sizes and their combined influence on statistical power. When it comes to detecting significant group differences for a particular dependent variable, a univariate approach can be considerably more powerful (Tabachnick & Fidell, 2007). Additionally, the split-plot design used in the current study has the advantage of higher precision at the sub-plot level (*i.e.* transect location) compared with the main plot level (*i.e.* vertebra; Hoshmand, 2006). Such fine-scale differences observed in vertebral chemistry have also been reported in otoliths (Gillanders, 2002; Hamer *et al*., 2003; Di Franco *et al*., 2014). Within otolith variability, however, is typically lower than observed variability between otoliths of different individuals (Gillanders, 2002; Hamer *et al*., 2003)**.** Hamer *et al*. (2003) reported that the per cent variation between otoliths was 58, 67 and 91% for Mn, Sr and Ba, respectively, whereas per cent variation within otoliths was 42, 33 and 9%, respectively. The same does not appear true for *C. limbatus* and the fact that estimated $\%\bar{\epsilon}$was consistently higher among transect locations within vertebrae versus between vertebrae suggests that

inconsistent transect or spot placement may introduce process error (*i.e.* natural variability) into statistical analyses of nursery sources or population connectivity that is avoidable (Kajajian *et al*., 2014).

Results of the sample handling and storage experiment suggest vertebral chemistry was not affected by the treatments tested here. The use of bleach to aid in tissue removal is a common practice and the results indicate that brief exposure to bleach did not have a significant effect on vertebral chemistry. This is consistent with the findings of Tillett *et al*. (2011) and McMillan *et al.*, (2017). Because bleach is not commonly used during otolith processing, there is little need to test for potential affects. The use of nitric acid as a means to decontaminate otoliths, however, has been assessed, along with distilled water and hydrogen peroxide. Although nitric acid appears to be an effective method for removing surficial contaminants, this method for decontamination also has the potential to reduce otolith mass (Rooker *et al*., 2001; Patterson *et al*., 2008; Davies *et al*., 2011). Exposure to 3% hydrogen peroxide, which is more comparable with the bleach treatment in the current study, does not appear to have much influence on otolith chemistry (Davies *et al*., 2011).

This was the first study to assess the combined effect of bleach and ethanol and the effect of ethanol alone on shark vertebral chemistry. Because otoliths are typically stored dry, studies focused on the influence of ethanol storage on otolith chemistry are not common, but do exist in the literature, specifically for early life stages. The effect of

ethanol storage on otolith Sr:Ca and Ba:Ca appears to be minimal (Hedges *et al*., 2004; Proctor & Thresher, 1998). Proctor & Thresher (1998) reported otolith Cl was significantly affected by preserving newly settled greenback flounder *Rhombosolea tapirina* Günther 1862 in 100% ethanol for 60 days, as was otolith Na, K, S and Cl of juvenile jackass morwong *Nemadactylus macropterus* (Forster 1801) exposed to the same treatment. The only study that evaluated the effects on ethanol storage on otoliths themselves was Milton & Chenery (1998)**,** in which otolith storage in analytical grade alcohol did not affect Li, Na, Mg, Mn, Co, Sr, or Ba concentrations . The present results for *C. limbatus* vertebrae are consistent with those of Milton & Chenery (1998), but it is not clear whether longer storage intervals would increase the likelihood of contamination.

The results reported here for *C. limbatus* indicate that natural variability among thoracic vertebrae is comparable with the degree of variation between sagittal otoliths and further supports the utility of vertebral chemistry as a tool to study elasmobranch ecology and population dynamics. Variability among regions of the vertebral column appeared low (%$\bar{\epsilon}$d 10%) and perhaps may be treated as equivalent under certain circumstances. Considering the small sample size and limited number of age classes, however, a more extensive evaluation is needed to determine under what conditions and for what species and life stages vertebrae from different body segments should be considered equivalent. No treatment effects for bleach, ethanol, or the combined use of bleach and ethanol were observed. Considering the degree of intra-vertebral variation within a given treatment

was similar to that of the control group, it appears the observed variability can be explained by natural variability or other forms of process error. What remains to be determined is whether longer-term exposure to either bleach or ethanol would produce similar results. Nevertheless, the present results provide preliminary support for using archived shark vertebrae stored in ethanol for chemical analysis.

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