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Inter-laboratory comparison of Atlantic and Mediterranean bluefin tuna otolith microconstituents

D. H. Secor, S. E. Campana, V. S. Zdanowicz,
J. W. H. Lam, L. Yang, and J. R. Rooker

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Elemental analysis of juvenile (age 0+ and 1 year) Atlantic bluefin tuna *Thunnus thynnus* otoliths by isotope dilution and conventional inductively coupled plasma mass spectrometry (ID ICP-MS and ICP-MS) indicated that the concentrations of certain elements varied among putative nursery grounds in the Atlantic Ocean. Further, trace element fingerprints of age-1 tuna from each nursery – the western Atlantic and Mediterranean Sea – were distinct and varied sufficiently to distinguish individuals from different regions with moderate confidence. Overall correct classification rates for a simulated test set of age-1 tuna were 68% (using ICP-MS) and 81% (using ID ICP-MS), despite a small sample size (9 Mediterranean vs. 19 western Atlantic tunas). Although ID ICP-MS was the more accurate of the two ICP-MS technologies, inter-laboratory precision was moderately high (3–18%) for individual elemental concentrations (Li, Na, Mg, K, Ca, Mn, Sr, and Ba), and multi-variate elemental fingerprints were similarly ordinated between laboratories ($r=0.75$). Age-0 tuna samples were too small to permit statistical classification tests, but showed similar levels of elemental concentrations between laboratories. Our results indicate that it should be possible to assign nursery ground origin to adult bluefin tuna based on the elemental composition of their extracted otolith core.

Keywords: bluefin tuna, elemental fingerprint, migration, otolith microconstituents, stock structure.

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D. H. Secor: Chesapeake Biological Laboratory, University of Maryland Center for Environmental Science, Solomons, MD 20688, USA. S. E. Campana: Bedford Institute of Oceanography, Marine Fish Division, Dartmouth, NS, B2Y 4A2, Canada. V. S. Zdanowicz: National Marine Fisheries Service, James J. Howard Marine Sciences Laboratory, Highlands, New Jersey 07732, USA. Current address: US Customs Service Research Laboratory, 7501 Boston Boulevard, Suite 113, Springfield, VA 22153, USA. J. W. H. Lam and L. Yang: National Research Council Canada, Institute of National Measurement Standards, Ottawa, ON, K1A 0R6, Canada. J. R. Rooker: Texas A&M University, Department of Marine Biology, 5007 Avenue U, Galveston, TX 77551, USA.

Introduction

The northern bluefin tuna, *Thunnus thynnus*, occurring in the Atlantic Ocean, is a quintessential pelagic fish, epitomizing form, size, and energetics adaptive to highly migratory behaviour. Extremely high demand by the Japanese market for the fatty flesh of giant bluefin tuna (>130 kg) and increased commerce has contributed to declining abundances of Atlantic bluefin tuna caught in coastal waters adjacent to North America (NMFS, 1995). Abundance of adult Atlantic bluefin tuna declined ca. 80% during the period 1975–1994 (Magnuson *et al.*, 1994; NMFS, 1995), and has not since recovered (SCRS, 2000). Migratory behaviours of Atlantic bluefin tuna cause them to be harvested in jurisdictional waters of many countries, as well as in interjurisdictional regions. The high worldwide demand for giant Atlantic bluefin tuna and the fishes migratory behaviours dictate careful international management of this species.

Atlantic bluefin tuna occur throughout northern latitude Atlantic waters and historically ranged from

Brazilian to Norwegian waters. Despite pan-oceanic distribution, spawning habitats are hypothesized to be restricted to the Gulf of Mexico and the eastern Mediterranean Sea. In addition, tagging studies have revealed low rates (<5% on average) of transoceanic migrations by both juveniles and adults (NMFS, 1995). Despite disparate spawning regions and past evidence for low rates of transoceanic migrations, genetic studies, parasite markers, and microconstituent analysis of vertebrae all failed to provide compelling evidence against the null hypothesis of a single Atlantic bluefin stock (Magnuson *et al.*, 1994).

Otolith microconstituent analysis has been applied recently to study stock structure (Edmonds *et al.*, 1991, 1992, 1995; Campana *et al.*, 1999; Proctor *et al.*, 1995; Thresher, 1999) and migration rates (Secor and Piccoli, 1996; Secor *et al.*, 2001). The premise of this approach is that trace elements are incorporated into otoliths in direct proportion to their availability in surrounding water or food. Thus, larvae or young-of-the-year bluefin tuna exposed to either Gulf of Mexico or Mediterranean waters might be expected to incorporate different mixtures of elements into their otoliths. Few laboratory experiments have been conducted to verify the assumption that otoliths can record environmental histories, but such studies have supported this assumption for uptake of Sr and Ba (Secor *et al.*, 1995; Farrell and Campana, 1996; Bath *et al.*, 2000; Secor and Rooker, 2000). Physiological factors, temperature, and genetics may also affect uptake of specific elements into otoliths (Campana, 1999).

Scientific evidence has been insufficient to support stock structure assumptions in the management of Atlantic bluefin tuna. Here, we conducted a multi-laboratory test for elemental fingerprint differences between juvenile Atlantic bluefin tuna collected in western and eastern Atlantic nursery regions. The objectives of the study were: (1) evaluate precision in otolith composition measures between two laboratories – one utilizing the more definitive approach (isotope dilution ICP-MS=ID ICP-MS) and the other utilizing more conventional ICP-MS technology; (2) test for differences in otolith chemistry between samples of juvenile bluefin tuna collected in the western Atlantic and Mediterranean Sea nurseries; and (3) determine whether two independent laboratories can determine similar levels of discrimination between the two samples using replicate otolith material (right or left sagittal otoliths).

Methods

Samples of age-1 (60–72 cm fork length [FL]) and age-0 (young-of-the-year; 31–39 cm FL) bluefin tuna were collected in fall 1998 in the Mediterranean Sea and along the Atlantic Coast of North America. Mediterranean age-0 specimens (n=15) were obtained from local catches from the Balearic Sea (Tarragona, Spain). Age-1 juveniles (n=9) were purchased at the fish market in Madrid, Spain; vendors there reported that the fish were captured from the Bay of Biscay. North American age-0 specimens (n=2) were captured by hook-and-line off Cape Hattaras (North Carolina, USA); age-1 juveniles (n=30) were angled in coastal waters off New Jersey and Rhode Island, USA. Otoliths were dissected shortly after harvest or purchase, adhering vestibular tissue was removed, and otoliths were stored dry in plastic vials.

Chemistry

In the laboratory, sagittal otoliths were carefully decontaminated. All reagents used were ultrapure grade and all implements and containers were cleaned with dilute nitric acid (HNO_3) and rinsed with 18 megohm doubly deionized water ($DDIH_2O$). First, otoliths were soaked in $DDIH_2O$ to hydrate any remaining biological residue adhering to the surface of the sample; this residue was removed using fine tipped forceps. Next the otoliths were soaked in 3% hydrogen peroxide for 5 min to dissolve any remaining biological residue. They were then immersed for 5 min in 1% nitric acid to remove surface contamination, and then flooded with $DDIH_2O$ for 5 min to remove the acid. Finally, they were dried under a Class 100 laminar flow hood, weighed to the nearest 0.01 mg, and stored in plastic vials.

We used immersion in 1% nitric acid as a rigorous method of decontamination (Campana *et al.*, 2000; Secor *et al.*, 2001). Acid immersion resulted in mass loss of 4–5%, independent of otolith mass or fish size. A study on handling and cleaning effects on Atlantic tunas (*Thunnus* spp.) (Rooker *et al.*, 2001a) showed that the decontamination procedure was effective in removing Mg, Mn, and Ba in deliberately contaminated otoliths (only these three elements were tested), without affecting the original composition of the otolith. For cod otoliths, Campana *et al.* (2000) showed that otolith Li, Sr, and Ba concentrations were unaffected by various decontamination procedures, but Mg concentrations were significantly higher in unwashed otoliths in comparison to acid-treated ones.

Elemental concentrations were determined using inductively coupled plasma mass spectrometers at laboratories in US (ICP-MS) and Canada (ID ICP-MS) according to established protocols at either laboratory (see Campana *et al.*, 2000; Secor *et al.*, 2001). Some noteworthy differences were (1) the use of isotope dilution standardization (^{6}Li, ^{25}Mg, ^{86}Sr, and ^{137}Ba used as ''isotope spikes'') at the Canadian laboratory, a more definitive method (Campana *et al.*, 2000); and (2) the use of atomic absorption spectrophotometry rather than

ICP-MS for measurement of Na and K at the US laboratory (Secor *et al.*, 2001).

Double blind experiment

Otoliths of individual fish were randomized within strata of age and site to reduce sequence effects. One otolith of each pair of sagittae (right or left) was randomly assigned to either the US laboratory (Lab 1: conventional ICP-MS) or the Canadian laboratory (Lab 2: ID ICP-MS), and sent to the two laboratories for independent analysis. Prior to running samples, the two laboratories conducted a preliminary study of bluefin tuna otolith material (10 pairs of sagittae from age-1 tuna) to identify elements that could be measured reliably and to provide a preliminary evaluation of precision. This was deemed prudent due to the limited availability of samples of otoliths from juvenile Atlantic bluefin tuna used for this study. Absolute paired differences for this preliminary data set indicated high precision (<94%) for Mg, K, Sr, and Ba.

Data from the test runs was initially screened for sequence effects, fish size (ontogenetic) effects, normality and presence of outliers. With the exception of Ca, no significant sequence effect was observed for either laboratory. Lab 1 and Lab 2, respectively showed a 5% increase ($p=0.05$) and 8% decrease ($p=0.001$) in Ca over the 55 sample run. Because samples were randomized, this minor sequence effect for Ca did not affect tests of central hypotheses. Significant fish length effects were observed for all elements, but these effects were due to differences between age-0 and age-1 juveniles. The direction and magnitude of ontogenetic effects were similar between the laboratories. In considering age-1 juveniles only, size effects were evaluated for each laboratory using analysis of covariance with site as the treatment factor and fork-length as the covariate. No significant effects due to the covariate FL were observed among elements and laboratories ($p>0.3$). Because analyses were restricted to this single age-class, elemental concentrations were not adjusted for length effects as has been done for similar analyses (Campana *et al.*, 2000).

Precision between laboratory measures of individual elements was evaluated between the paired otoliths (laboratory measures) by considering both absolute and relative differences. Differences in amplitude between laboratories were estimated as the arithmetic mean of absolute values of $(C_{ij1} - C_{ij2}) \times C_{ij2}^{-1}$, where C_{ij1} is the concentration for fish i, element j and Lab 1; and C_{ij2} is the concentration of the pair otolith for Lab 2. This estimate of average error uses the ID ICP-MS laboratory (Lab 2) as a reference value (i.e, how close is Lab 1 to Lab 2 value?). Relative differences between the rankings of individual fish between laboratories were evaluated using the correlation coefficient of pair-wise comparisons for the entire sample.

Due to our inability to obtain sufficient numbers of age-0 bluefin tuna from the western Atlantic nursery ($n=2$), tests of the effect of nursery and laboratory on otolith composition were limited to age-1 bluefin tuna. For this group, variance structure of elemental concentrations was examined for normality and outliers. Following removal of two records for Li (high values >0.4 ppm) and one value for Mg (high value of 52 ppm), variance assumptions were met for both laboratories.

The effects of site and laboratory on elemental fingerprints were tested using a multivariate repeated measures analysis. In this analysis, each subject (fish) was repeatedly measured by two laboratories (i.e. right and left otoliths analyzed by Lab 1 or Lab 2). Between-subject and within-subject effects corresponded to site and laboratory effects, respectively. The interaction between these two types of effects – site*laboratory – was also tested. Because multivariate responses (Li, Na, Mg, K, Ca, Mn, Sr, and Ba) had to be matched between laboratories, cases were omitted for those three subjects where outliers were removed to meet normality assumptions (see above). Pillai trace (V) was chosen as the test statistic since it is the most robust to violations of homogeneity of covariance (Wilkinson, 1998). Univariate t-tests were performed for each element. For each laboratory, discriminant analysis was used to develop elemental fingerprints and thereby classify juveniles from different nurseries. To simulate classification by discriminant function rules to an unknown test set, a jackknifed classification matrix was computed. Relative importance of individual elements in the discrimination function was assessed using the *F-to-remove* statistic (Wilkinson *et al.*, 1998). Covariance among elements and laboratories was evaluated using principal components analysis.

Results

Inter-laboratory precision

Precision was moderately high in paired elemental measures between laboratories, with Lab 1 deviating on average less than 20% from Lab 2 for Li (17.9%), Na (10.5%), Mg (10.3%), K (17.8%), Ca (8.0%), Mn (7.1%), Sr (11.6%), and Ba (3.2%). This low error rate indicated that laboratories could replicate measures for these elements despite substantial differences in instrumentation. In comparing differences in magnitude, Lab 2 (ID ICP-MS) showed higher mean levels of Li (mean difference=15%; paired t-test: $p=0.001$), Ca (mean difference=5%; $p=0.001$), and Sr (mean difference=11%; $p=0.001$); and lower levels of K (mean difference=9%; $p=0.001$). In light of its use in certifying reference materials, it seems probable that the ID ICP-MS more accurately measured elemental concentrations than the standard ICP-MS used by Lab 1. For K, atomic

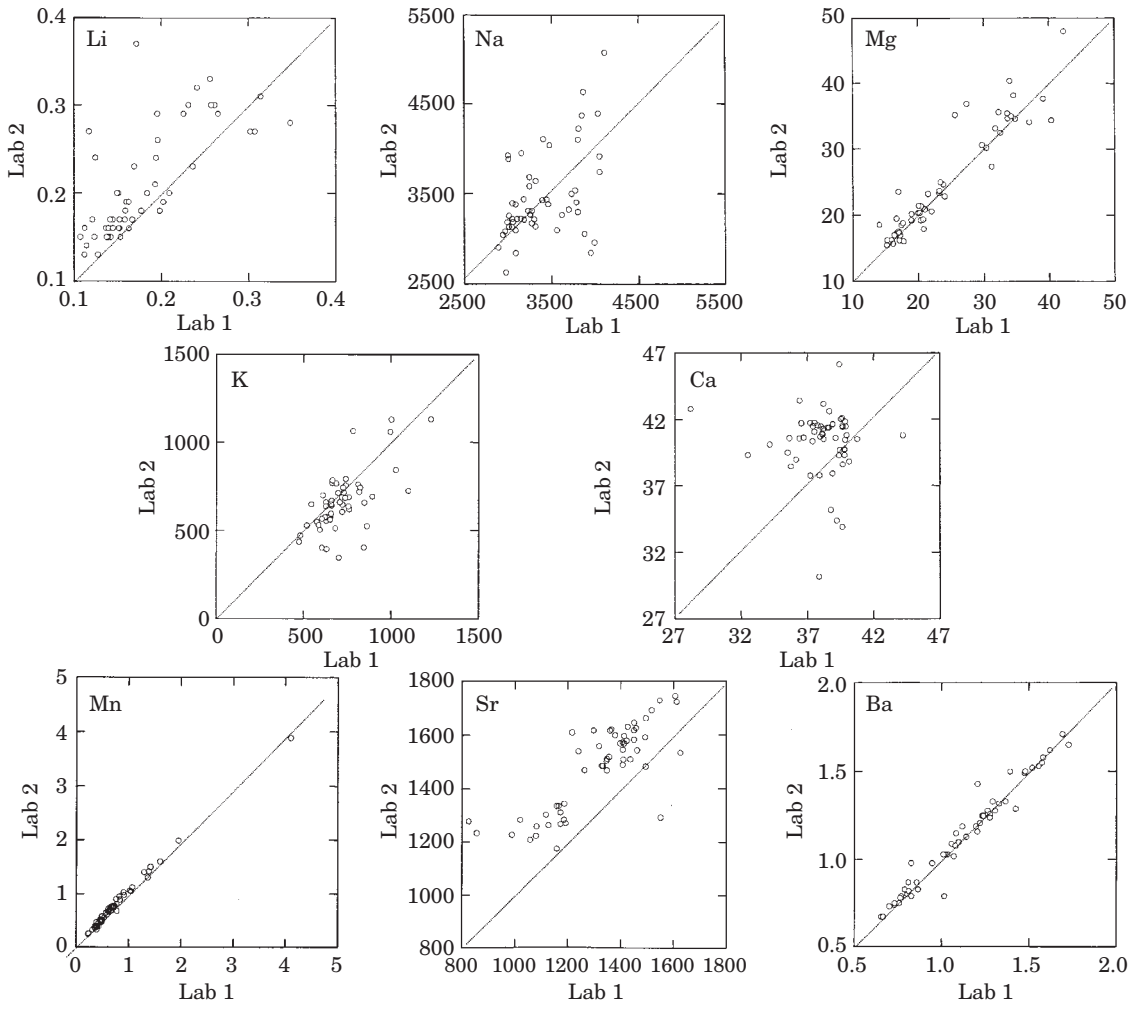

Figure 1. Bivariate comparisons of elemental concentrations between laboratories for otoliths of juvenile (age 0 and 1 year) Atlantic bluefin tuna. Concentrations are in ppm, except for Ca (%). Dotted lines of identity are presented for each plot.

absorption spectrophotometry was used by Lab 1, which may have contributed to the consistent difference between laboratories.

With the exception of Ca and Na, elemental concentrations showed high correspondence between laboratories. Regressions for elemental concentrations for samples pooled across ages and sites were significant and positive for Li ($r=0.71$), Na ($r=0.48$), Mg ($r=0.94$), K ($r=0.75$), Mn ($r=0.96$), Sr ($r=0.81$), and Ba ($r=0.96$) (Figure 1). Only Ca lacked significant covariance ($p=0.6$) between laboratories – this in part due to the low range of Ca values observed for either laboratory (coefficient of variation <6%). High correlation coefficients ($r>0.9$) for Mg, Mn and Ba indicated particularly high precision in measurement of these elements between laboratories.

Age-0 vs. age-1 juveniles

Significant ontogenetic effects were observed for most elements (Figure 2 vs. Figure 4). In comparison with age-1 juveniles, age-0 juveniles showed significantly elevated concentrations of Li, Na, Mg, K, and Mn; and depressed concentrations of Sr and Ba ($p<0.001$). Age did not significantly affect Ca concentration. This may be due to the low range of Ca values in comparison to other elements. The effect of age on elemental concentrations was statistically similar between laboratories (ANOVA test for the influence of the interaction between laboratory and age on elemental concentration; $p>0.1$).

Because the sample size for western Atlantic age-0 juveniles was only two, statistical tests were not

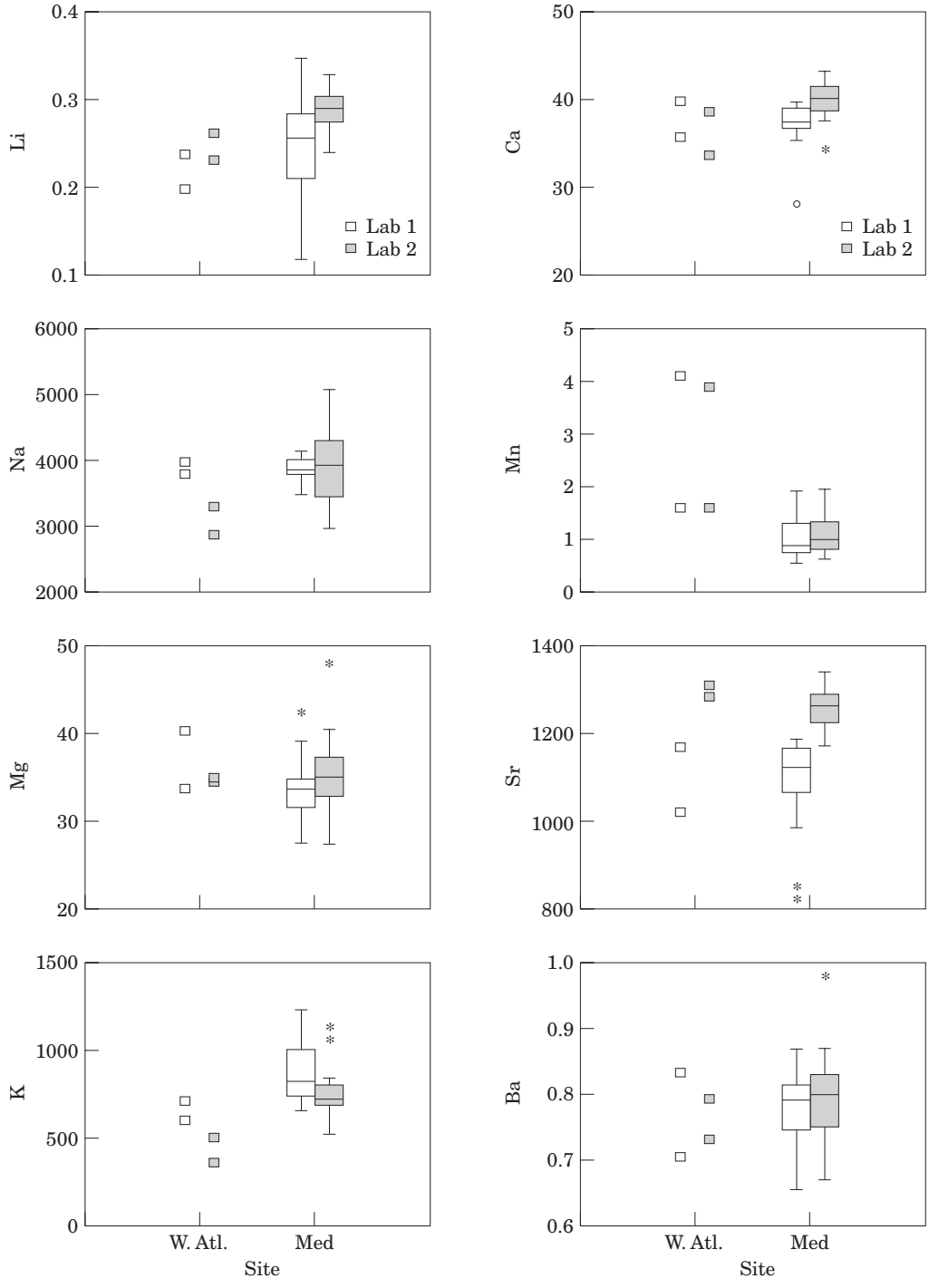

Figure 2. Data points and box whisker plots of elemental concentrations of otoliths from age-0 Atlantic bluefin tuna between western Atlantic and Mediterranean nurseries. Data points and box plots are further separated by laboratory. Data points, rather than box plots, are given for the western Atlantic due to small sample size (n=2). Concentrations are in ppm, except for Ca (%).

performed to evaluate differences between sites or between laboratories. Still, distribution plots suggested that alkali metals [Li (Lab 2), Na (Lab 2), and K (Lab 1,2)] were lower in western Atlantic age-0 juveniles than in the Mediterranean sample (Figure 2).

Table 1. Classification matrix on reference set and simulated test set for age-1 Atlantic bluefin tuna. Subjects (cases) in row categories are classified into columns. Classifications were based upon linear discriminant analysis. Samples sizes were 28 and 9 for western Atlantic and Mediterranean groups, respectively.

	Test (Jack-knifed) Set Classification Matrix					
	W. Atlantic		Mediterranean		%Correct	
Site	Lab 1	Lab 2	Lab 1	Lab 2	Lab 1	Lab 2
W. Atlantic	21	25	8	4	72%	86%
Mediterranean	4	3	5	5	56%	63%
Total	25	28	13	9	68%	81%

Lab 1=conventional ICP-MS; Lab 2=ID ICP-MS.

Discrimination of age-1 juveniles

Elemental fingerprints for 1-yr-old juveniles varied significantly between sites (MANOVA: $p=0.0008$) and laboratories (MANOVA: $p=0.004$). Site and laboratory did not interact in their effect on the response vector ($p=0.6$). Thus, elemental fingerprints were significantly different between the Mediterranean and western Atlantic juveniles, regardless of differences contributed by laboratory protocols. Classification rates for discriminant analysis performed separately for each laboratory were 68% and 81% for Lab 1 (ICP-MS) and Lab 2 (ID ICP-MS), respectively. Higher discrimination by Lab 2 is probably attributable to the use of a more accurate and precise instrument, but the difference in classification accuracy was relatively small (13%) and this small difference was not detectable in the MANOVA (no significant interaction between laboratory and site). Highest misclassification for both laboratories occurred for the Mediterranean group and is probably due to its low sample size ($n=9$). For instance, each fish that is incorrectly classified into or out of the Mediterranean category contributes an 11% error rate, so the potential for substantial sampling error is high.

The most influential elements in the classification functions for Lab 1 were Mg, Li, Ba, and Na; for Lab 2 they were Mg, Mn, Sr, and Na. Subject canonical scores showed that three of the Mediterranean fish overlapped with the western Atlantic sample between laboratories, which generally agreed with classification rates of 56% and 63% for this group (Table 1). Subject scores between laboratories were strongly correlated ($r=0.75$; $p=0.0001$) and showed similar rankings between laboratories (Figure 3). For both laboratories, univariate contrasts showed significant site differences for Li ($p<0.003$) and Mg ($p<0.001$). The direction and magnitude of differences between sites was similar for each element (Figure 4). For instance, in comparison to western Atlantic juveniles, Mg was 23% and 29% higher in Mediterranean fish for Lab 1 and Lab 2, respectively. Similarly, K was 11% and 16% higher in Mediterranean fish for Lab 1 and Lab 2, respectively. In comparisons between laboratories, Lab 1 showed lower levels of Li, Ca, and Sr, but higher levels of K than Lab 2.

Principal component analysis of multivariate element vectors showed strong covariance between laboratories in elemental fingerprints (Figure 5). In particular, K, Mn, and Ba mirrored each other between laboratories. Among elements, covariance was seen for four groups: (1) Li, K, and Mg; (2) Ba; (3) the transition metal Mn; and (4) a group clustered towards the center that did not show strong correspondence with the first three eigenvectors: Na, Ca, and Sr. With the exception of Na, these groupings were similar between laboratories.

Discussion

Elemental analysis of juvenile Atlantic bluefin tuna otoliths indicated that the concentration of certain

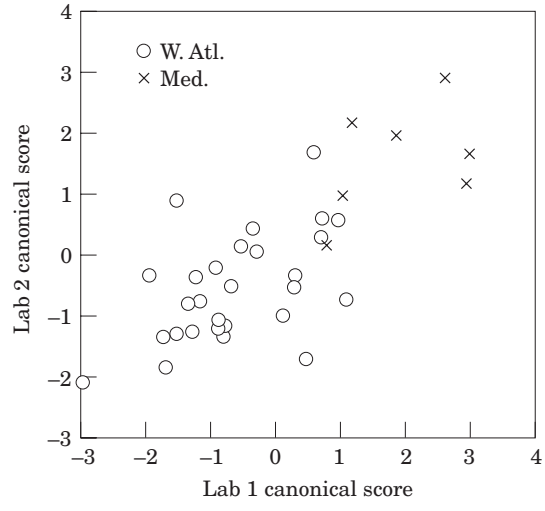

Figure 3. Bivariate comparison of canonical scores between laboratories for elemental fingerprints of age-1 Atlantic bluefin tuna.

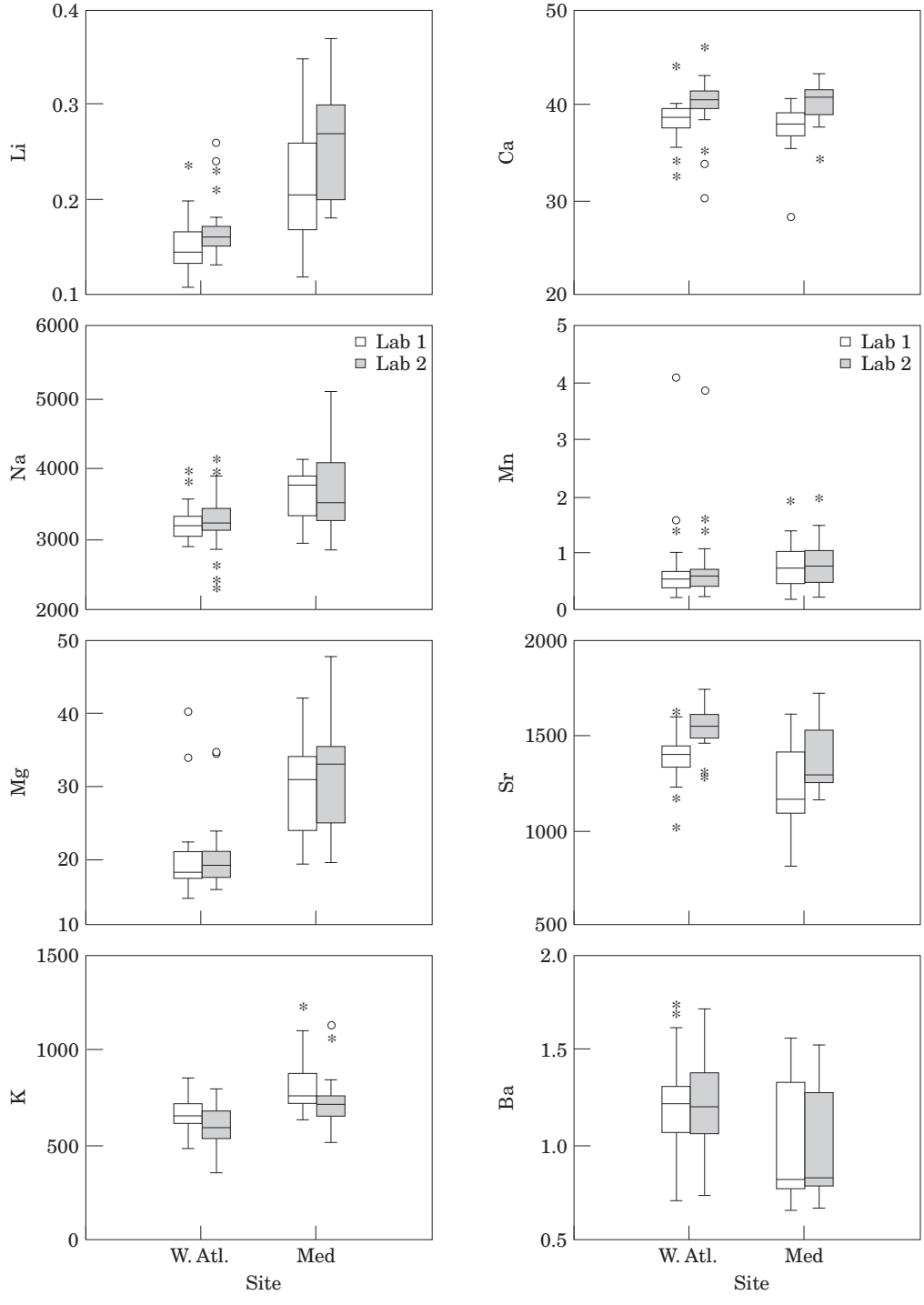

Figure 4. Box whisker plots of elemental concentrations of otoliths from age-1 Atlantic bluefin tuna between western Atlantic and Mediterranean nurseries. Box plots are further separated by laboratory. Concentrations are in ppm, except for Ca (%).

elements varied between putative nursery regions in the Atlantic Ocean and Mediterranean Sea. Further, trace element fingerprints from each nursery were distinct and varied sufficiently to distinguish individuals from different regions with moderate confidence. Overall correct classification rates for the simulated test set were 68%

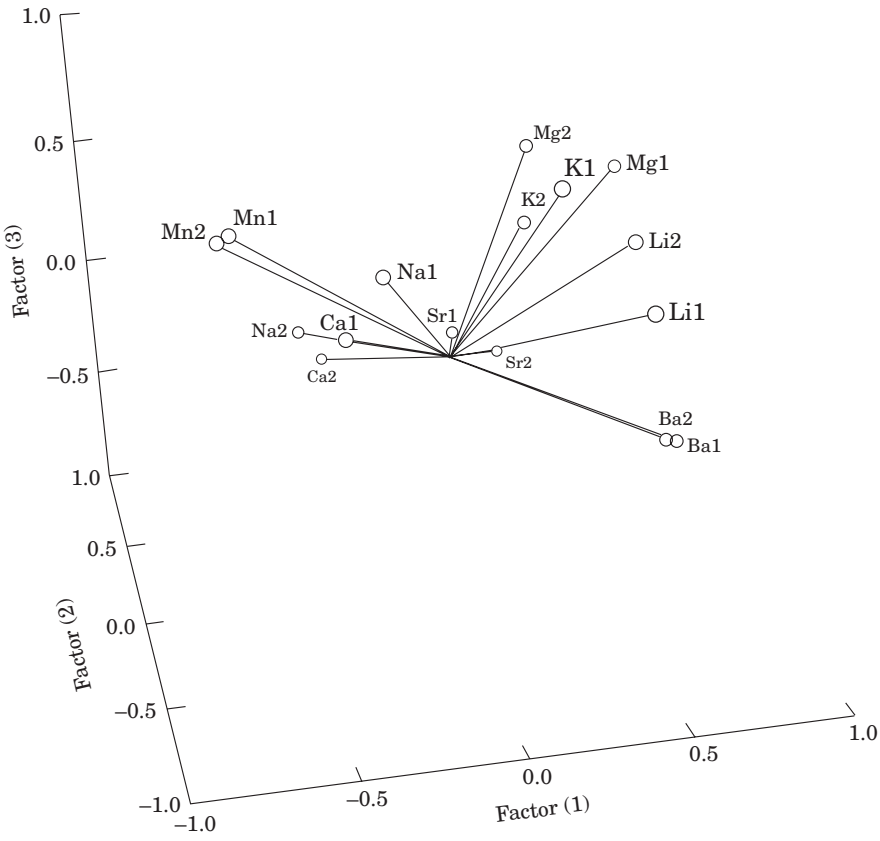

Figure 5. Principal component factor loadings for first three eigenvectors for combined elements measured in the otoliths of age-1 Atlantic bluefin tuna by two laboratories. Numbers 1 or 2 appended to each element corresponds to Lab 1 and Lab 2, respectively.

(using ICP-MS) and 81% (using ID ICP-MS), despite a small sample size (9 Mediterranean vs. 19 western Atlantic tunas). The relatively low classification rate of the Mediterranean sample (56% and 63%) may have been due to a small sample size and sampling error, or may reflect more variability associated with Mediterranean source fish. Rooker *et al.* (in press) reported a slightly higher level of discrimination (85%) between age-1 bluefin tuna collected in 1999 from the western Atlantic and Mediterranean. Li and Mg were similarly important discriminators in the classifications of 1998 (this study) and 1999 samples.

The significant, albeit modest, discrimination reported here and by Rooker *et al.* (in press) suggests an underlying cause related to exposure to different environmental conditions between the two principal nurseries. Although the relationship between exposure to certain elements in seawater and their uptake into otoliths is not well-understood (Campana, 1999; Thresher, 1999), the use of elemental fingerprints of juvenile Atlantic bluefin tuna as tracers of nursery origin was supported in these initial comparisons. We are continuing our sampling efforts to evaluate both temporal and spatial stability in elemental fingerprints, and must await additional years' sampling to statistically evaluate temporal and spatial stability of elemental fingerprints of age-1 juveniles. However, for more easily sampled age-0 bluefin tuna in the Mediterranean, Rooker *et al.* (in press) showed significant regional differences (Alboran, Ligurian, vs. Tyrrhenian Seas) in elemental concentrations. Also, interannual differences in elemental concentrations were observed in the Alboran Sea, but regional classification accuracies were not negatively affected. These regional and interannual differences in elemental fingerprints highlight the need for stratified designs in sampling juvenile bluefin tuna for future development of classification functions.

Two independent ICP-MS laboratories successfully replicated measurement of elemental fingerprints and their discrimination. The use of isotope dilution standardization (ID ICP-MS) by Lab 2 is the more definitive method and served to verify the more routine ICP-MS standardization procedures (external calibration) used by Lab 1. The higher level of discrimination by Lab 2 may have been due to higher recovery levels and greater precision and accuracy, but the lack

of interactive effects of laboratory and site in the multi-variate analysis suggests that any difference in discriminatory capabilities were minor. Precision for individual elements was moderately high, particularly in considering the regression of one laboratory's measures upon the other. The slope statistic is more relevant than intercept differences (i.e., mean differences in concentration) because correspondence in ranking of elemental concentrations among individuals will define elemental fingerprints. Here and elsewhere (Campana *et al.*, 2000; Secor *et al.*, 2001; Rooker *et al.*, 2001a), we have examined protocol and precision issues by making contrasts between right and left sagitta of the same individual. This method is advantageous because it accounts for unexplained sources of variation expected to occur between fishes, while using otolith pairs as replicates or as a means to apply a treatment effect. In this study, differences within otolith pairs could be statistically isolated and attributed to ICP-MS protocol differences between laboratories.

A previous inter-laboratory calibration study comparing probe-based assays was much broader in scope and identified large differences in elemental concentrations among instrument types and between laboratories within instrument type (Campana *et al.*, 1997). With the exception of Sr, precision levels were several fold lower than those reported here. Since that study, there has been growing convergence in the selection of ICP-MS as a method that can simultaneously measure a range of elements, and do so at moderately sensitive detection limits <1 ppm (Campana, 1999; Thresher, 1999). Results reported here support the reliability of ICP-MS measures, particularly for alkali and alkaline earth metals Li, Na, Mg, K, Ca, Sr, and Ba, and the transition metal Mn. Accuracy of this approach has also been recently demonstrated (Secor *et al.*, 2001; Rooker *et al.*, 2001a) through the use a certified otolith reference material (Yoshinaga *et al.*, 1999, 2000).

In past studies on bluefin tuna otolith composition, discrimination was reported among nursery regions for Atlantic and Pacific bluefin tuna (*Thynnus orientalis*) (Rooker *et al.*, 2001b, in press) and between Atlantic and Pacific bluefin tuna (Secor and Zdanowicz, 1998; Rooker *et al.*, in press). The Secor and Zdanowicz study included one transition metal – Ni – that was erroneously detected and measured, the result of polyatomic interference by calcium oxide. While omission of this element still resulted in significant discrimination between test groups (Secor unpubl. data), it is noteworthy that the set of elements used in stock discrimination applications has diminished in recent years and now includes very few transition metals (Thresher, 1999). The accuracy of transition metal measures should be viewed critically because they typically occur at very low concentrations ($\ll$1 ppm) and their measurement is susceptible to interferences and contamination in their measurement. Because transition metals may serve as particularly useful tracers of coastal water masses, we expect that innovations that permit their reliable measurement would substantially improve the ability to discriminate natal origins in bluefin tuna.

Beyond innovation to increase our ability to measure a greater number of elements, the ability to characterize nursery origin in adults will require a larger sample of age-0 juveniles from the western Atlantic. During the period 1997–2000, directed efforts by federal sampling agents, federal, state and academic scientists, and charter boat fishers have resulted in a sample of only two individuals, those reported in this study. Historically, small Atlantic bluefin tuna were common and targeted in purse seine fisheries in the North and Middle Atlantic Bight (Wagner, 1996). The correspondence between their apparent scarceness and declining trends in adult biomass and larval abundance in the Gulf of Mexico (SCRS, 2000) could indicate low recruitment. Still, because there has not been a targeted fishery for individuals <66 cm fork length since 1974, we remain cautiously optimistic that future sampling efforts could be successful. In addition, over 100 age-1 juveniles have been collected during this same period, suggesting some recruitment should be occurring. Here too caution dictates over-interpreting their relative abundance as local recruitment; age-1 Atlantic and Pacific bluefin tuna are known to migrate across entire ocean basins (Bayliff, 1994; Magnuson *et al.*, 1994; Polovina, 1996; Kitagawa *et al.*, 2000). If such migratory juveniles were included in our reference set, then discrimination between nursery zones would be substantially compromised.

Recent efforts to understand spawning migrations by adult Atlantic bluefin tuna through the use of electronic tags and satellite telemetry has uncovered an unexpected migration trajectory: large adults tagged in the fall in the North Atlantic Bight were recorded several months later – during spawning season – in the mid-Atlantic Ocean near the Azores (Lutcavage *et al.*, 1999). While spawning activity has not been documented outside the Gulf of Mexico/Florida Straits and Mediterranean Sea, another spawning region cannot be ruled out, particularly considering the spawning behaviour of Pacific bluefin tuna, which spawns in the ocean basin and in two marginal sea (Okiyama, 1974; Okiyama and Yamamoto, 1979). While a third spawning population would complicate the application of elemental fingerprints to stock discrimination, we expect that were age-0 samples available we could discriminate among three groups, as has been done for age-0 Pacific bluefin tuna (Rooker *et al.*, 2001b).

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