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# Lack of equivalence in the elemental and stable isotope chemistry within the sagittal otolith pair of the summer flounder, *Paralichthys dentatus*

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In fish that are not bilaterally symmetrical, the left and right sagittae are often not symmetrical, exhibiting divergent growth patterns and mass, and may have differences in chemical composition. We investigated this in the asymmetrical summer flounder *Paralichthys dentatus*, collected from different nursery habitats along the US east coast. Significant differences were detected in otolith mass, $\delta^{13}C$, $\delta^{18}O$, Li:Ca, Mg:Ca, and Sr:Ca, and overall chemical signatures. These results refute the hypothesis of left–right equivalence that is prevalent for bilaterally symmetrical fishes. We tested whether a specific side was better suited for classification. The best models differed between sagittae and resulted in different classification accuracies. The left otolith produced better classification accuracies. Simulated samples of randomized sets of left or right otoliths produced mean accuracies intermediate to classification and were often highly variable. We recommend that future otolith chemistry studies involving bilaterally asymmetrical species test the hypothesis of equivalence within the sagittae before randomly choosing an otolith for chemical analyses.

**Keywords:** otolith, Pleuronectiformes, sagittae, stable isotope, summer flounder, trace element.

## Introduction

Otolith chemistry is an expanding field of research that has been used to evaluate stock structure (Thresher, 1999; Thresher and Proctor, 2007), migratory patterns (Hamer *et al.*, 2006; Fairclough *et al.*, 2011), temperature and salinity histories (Secor *et al.*, 1995; Townsend *et al.*, 1995), and physiology (Wurster *et al.*, 2005; Solomon *et al.*, 2006), based on the use of the otolith as a natural tag (Campana, 1999). Because the otolith is metabolically inert, all incorporated environmental signatures are preserved (Campana, 1999). Thus, different elemental signatures may be used as markers to discern different environments, both spatially and temporally (Chesney *et al.*, 1998; Campana, 1999; Schaffler *et al.*, 2009).

In teleosts, there are three pairs of otoliths, with the sagittae being the most commonly used for otolith chemistry due to their larger size, with the notable exception of some Ostariophyseans, where sagittae are not used because the lapilli are larger (Long and Stewart, 2010). Sagittae, lapillae, and asteriscae do not carry matching elemental signatures (Meyer-Rochow *et al.*, 1992; Chesney *et al.*, 1998; Chittaro *et al.*, 2006; Smith and Jones, 2006), so the hypothesis of interest should dictate which pair of otoliths should be targeted (Smith and Jones, 2006). When otoliths grow at different rates, they emphasize different portions of the life history, albeit with sagittae reflecting lifetime habitat most closely (Smith and Jones, 2006). However, the researcher must still choose which otolith to use from the pair, and often randomly chooses with the assumption of equivalent chemistry between the paired otoliths (Thorrold *et al.*, 1997; Walther and Thorrold, 2006). This has been the case for both stable oxygen and carbon isotopes (Iacumin *et al.*, 1992; Thorrold *et al.*, 1997; Høie *et al.*, 2004) and trace element concentrations (Gauldie, 1996; Rooker *et al.*, 2001). These analysed species were bilaterally symmetrical with no differences in otolith mass and, perhaps as a consequence, no differences in elemental signatures between pairs. The generality of this phenomenon was further

investigated and confirmed in Atlantic cod (Campana *et al.*, 2000), striped bass (Secor *et al.*, 2001) and blackfin tuna (Arslan and Secor, 2008). However, there are instances where significant variation has been detected between the left and right otoliths of symmetrical fishes (Kalish, 1991; Outridge *et al.*, 2002). The question is further compounded if the species displays marked asymmetry, such as in many flatfishes (Nelson, 2006). Therefore, some researchers have chosen to systematically sample one member of the pair (Schaffler and Winkelman, 2008; Tanner *et al.*, 2012) and substitute the other member of the pair only in instances of loss or destruction of the targeted otolith (Campana, 1999; Loher *et al.*, 2008).

The summer flounder *Paralichthys dentatus* (Linnaeus, 1766) is an important target of commercial and recreational fisheries along the US east coast (Gutherz, 1967; Grimes *et al.*, 1989; Terceiro, 2011). The centre of abundance extends from Cape Cod in New England to Cape Hatteras in North Carolina, i.e. the Mid-Atlantic Bight (MAB) (Wilk *et al.*, 1980; Packer *et al.*, 1999), though other studies extend the area of abundance to South Carolina (Kraus and Musick, 2001). Summer flounder is a migratory species that moves between coastal and estuarine summer feeding grounds (Packer *et al.*, 1999) and continental shelf winter spawning grounds (Wilk *et al.*, 1980; Morse, 1981; Sackett *et al.*, 2007). Similar to many other offshore winter spawning species (Miller *et al.*, 1984; Warlen and Burke, 1990; Schaffler *et al.*, 2009), the larval period is protracted, extending between September and May; the larvae start to arrive into the coastal or estuarine systems that comprise their nursery habitats between November and April (Smith, 1973; Able *et al.*, 1990; Szedlmayer *et al.*, 1992; Kraus and Musick, 2001). The potential for high rates of mixing at the larval stage, combined with a lack of genetic differentiation among hypothesized subpopulations north and south of Cape Hatteras (Jones and Quattro, 1999), has led managers to consider the summer flounder as a single unit stock, extending from Cape Hatteras northwards to New England (Terceiro, 2011).

The species is bilaterally asymmetrical as an adult because of the migration of the right eye to the left side of the head during metamorphosis (Keefe and Able, 1993; Martinez and Bolker, 2003). In flatfishes, even though the semicircular canals remain in their original symmetrical position and the vestibular systems conserve their morphology, the otoliths grow on top of each other because the fish is resting on its blind side (Leech, 1923; Platt, 1973). This can result in significant differences in mass between the otolith pairs (Sogard, 1991; Fischer and Thompson, 2004; Helling *et al.*, 2005), which may translate to differences in the incorporation of elemental signatures into the carbonate of the otoliths due to ontogenic shifts (Loher *et al.*, 2008). Loher *et al.* (2008) have conducted the only study of its kind in investigating the left–right differences in otolith chemistry in an asymmetrical species, the Pacific halibut, *Hippoglossus stenolepis*. They found significant differences in $\delta^{13}C$, $\delta^{18}O$, and Sr, but no differences in other trace elements (Loher *et al.*, 2008). This indicates that randomly selecting an otolith for geochemical analysis, or considering them interchangeable, could increase the variance observed in element and isotope concentrations, and ultimately reduce classification accuracies. The sampling plan is an integral part of any study designed to examine habitat-specific differences in otolith chemical signatures. Therefore, the question of equivalence in the otoliths with respect to this research aim takes on added importance.

We test the hypothesis of equivalence between the chemistries of left and right sagittal otoliths of summer flounder. This will be addressed by (i) testing for differences in otolith mass between the left and right sagittae, (ii) evaluating specific differences in $\delta^{13}C$ and $\delta^{18}O$ stable isotope ratios, and (iii) evaluation specific differences in trace elements Li, Mg, Mn, Rb, Sr, Y, Ba, and Pb between the left and right otoliths, as well as (iv) developing a simulation model to evaluate the classification success for either left or right otolith as compared with randomly selecting a left or right otolith. The results of this study provide important insights into the use of otolith chemistry as natural tags for species that are asymmetrical.

## Material and methods

### Field collections and otolith preparation

Scientists from US State natural resource agencies and universities collected juvenile summer flounder in fall 2011 from estuarine habitats along the US east coast. The areas sampled were Delaware Bay, Chesapeake Bay, Pamlico Sound, and the coastal inshore waters of South Carolina and Georgia (Figure 1). Fish were collected with bottom trawls operated in support of monitoring efforts. After capture, fish were frozen and transported to our lab (Center for Quantitative Fisheries Ecology at Old Dominion University), where total length ($L_T$) was measured to the nearest 1 mm and body mass ($M$) to the nearest 0.01 g for all individuals using an Ohaus CS200 balance (Ohaus Corporation, USA).

The sagittal otoliths were removed in a class-100 clean room using non-metallic acid-washed tools and cleaned on acid-washed glass slides to guard against contamination. We then placed the otoliths in Milli-Q (18 Megaohm) water and scraped all visible adhering tissue from the surface using glass probes, soaked them for 5 min in ultra-pure 30% hydrogen peroxide (VWR International, West Chester, Pennsylvania, USA) to dissolve any remaining tissue, triple-rinsed them with Milli-Q water, transferred them to acid-washed polycarbonate tubes, and left them to air-dry for 24 h under a Class 100 laminar-flow hood (Schaffler and Winkelman, 2008; Schaffler *et al.*, 2009). Subsequently, both sagittae were weighed to the nearest 0.01 mg ($M_O$) using a precision balance

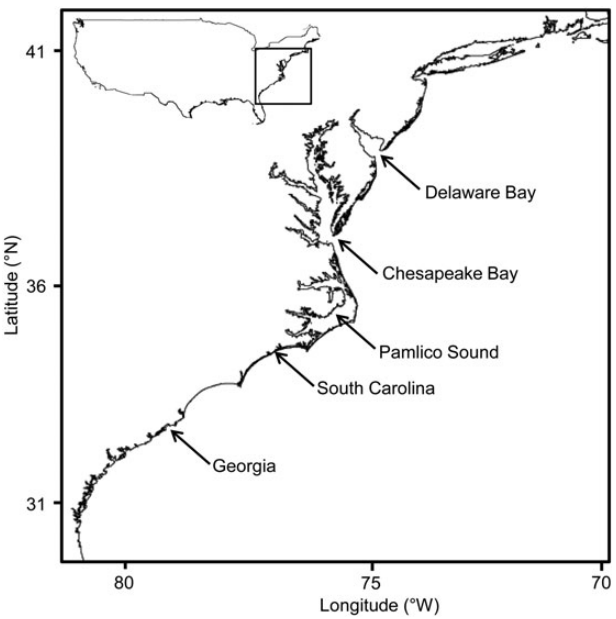


**Figure 1.** Areas sampled for summer flounder along the US east coast in 2011.

(Mettler Toledo AT261 DeltaRange®, Mettler-Toledo International Inc., Columbus, Ohio, USA).

We randomly selected a sample of 30 otolith pairs from all sampled areas. This broad range was selected to encompass as much variability in chemical signatures as possible. After cleaning, each sagitta was mounted on a glass slide using thermoplastic cement (CRYSTALBOND® 509) and a 0.7 mm transverse section was cut using a low-speed saw (Buehler®, Lake Bluff, Illinois, USA). The transverse section was then attached to a clean glass slide with thermoplastic cement and ~ 20 μm was ground from the surface of each otolith thin-section with 30 μm lapping film with an Aluminium oxide abrasive (Mark V Laboratory, East Granby, Connecticut, USA) to remove any contamination induced by the saw blades. Subsequently, each section was polished with 0.3 μm lapping film with an Aluminium oxide abrasive (Buehler®, Lake Bluff, Illinois, USA) until the surface was free from pits and scratches. After all sections had been prepared, the otolith thin-sections were attached to acid-washed petrographic slides in randomized order, sonicated in ultra-pure water (Milli-Q), and allowed to dry under a laminar-flow bench before chemical analysis.

## Otolith chemical analyses

The otolith thin-sections were analysed for trace element signatures with a thermo Finnegan Element 2 (Thermo-Fisher Scientific, Bremen, Germany) inductively-coupled plasma mass spectrometer (ICP-MS) coupled to a New Wave 193 nm excimer laser ablation system (Tanner *et al.*, 2012). The laser ablated material along a transect extending from the core to the dorsal edge of the otolith with a 25 μm spot diameter moving at a rate of 5 μm $s^{-1}$. The resulting trench depth was 30 μm (Jones and Chen, 2003). The ablated material was transported via a He-gas stream, mixed with a 5% $HNO_3$ aerosol, and the analyte transported to the ICP-MS via an Ar-carrier gas (Tanner *et al.*, 2012). For every sequence (i.e. block or petrographic slide containing 20 randomized samples) we measured four calibration standards prepared from stock single and multi-element standards to mimic concentrations typically observed in otoliths (Schaffler and Winkelman, 2008) at the beginning and end of each sequence as well as two certified otolith reference materials (Yoshinaga *et al.*, 2000; Sturgeon *et al.*, 2005) three times each and eight instrumental blanks of 5% $HNO_3$ interspersed throughout the block of 20 otoliths.

$^{7}Li$, $^{25}Mg$, $^{48}Ca$, $^{55}Mn$, $^{85}Rb$, $^{88}Sr$, $^{89}Y$, $^{138}Ba$, and $^{208}Pb$ were measured and the resultant counts per second converted to molar ratios by subtracting the blank value from each sample and then interpolating the data with the known-concentration calibration standards (Taylor, 2001). The concentrations were then converted into element to calcium molar ratios. Dissolved otolith certified reference materials (CRMs) (Yoshinaga *et al.*, 2000; Sturgeon *et al.*, 2005) were used to assess external precision as relative standard deviations (RSDs). Limits of detection (LODs) were calculated as the mean blank value plus 3 s.d. ($n = 56$), and presented as a percentage of the average sample intensity. If an excessive number of samples fell below the LOD for a certain element, that element was excluded from further analyses (Brazner *et al.*, 2004).

For stable isotope analyses, we used the two pieces of the otolith that remained after removing the thin-section that was used for trace element analysis. The thermoplastic cement was removed from the otolith pieces with dilute 1% $HNO_3$ and Milli-Q water. The otolith pieces were then dried overnight and crushed manually with a marble mortar and pestle into a homogeneous powder that was subsequently analysed with an automated Isoprime Micromass carbonate analyser at the University of Washington Stable Isotope Laboratory. Sagittal powder and limestone standards were both spiked with 100% phosphoric acid at 90°C with five standards every ten samples (Dorval *et al.*, 2007), and isotopic concentrations measured using the dual inlet method and coldfinger mode (Dorval *et al.*, 2005). The data produced were amended and reported in Peedee Belemnite (PDB). We also calculated the long-term precision of the machine by averaging the precisions of each sample.

## Statistical analyses

Prior to all analyses, the data were Box–Cox transformed to normalize them and homogenize the variances (Box and Cox, 1964). Prior to hypothesis testing, normality was assessed using the Shapiro–Wilk test. The null hypothesis of no difference in mass between the left and right otolith was tested with a paired *t*-test. Subsequently, the hypothesis of no difference in the chemistry (both trace element and stable isotope) was tested using Hotelling's $T^2$ test for pairwise multivariate data, followed by paired-sample *t*-tests to determine where differences occurred between the left and right otolith pairs.

If significant differences in the chemistries occurred between left and right otoliths, the possibility exists that one otolith from the pair would provide better discrimination among areas. Homogeneity of variances was tested using O'Brien's test, and Mardia's test was used to test for multivariate normality based on skewness and kurtosis (Khattree and Naik, 2000; Schaffler *et al.*, 2009). A multivariate analysis of variance (MANOVA) was performed to detect differences in the left or right otoliths between areas. Pillai's trace statistic was used because it is robust to small and unequal sample sizes (Scheiner, 2001), followed by univariate analyses of variance (ANOVA) to determine which elements showed differences. Discriminant function analysis (DFA) was used to examine differences in classification performance between the left and right otoliths by examining all possible combinations of the transformed data. Equality of variance–covariance matrices was tested using Bartlett's test (Schaffler *et al.*, 2009) to determine which type of discriminant function (linear or quadratic) to use (Khattree and Naik, 2000). Classification success and error rates on all the trials were determined by cross-validation using the jackknife (leave-one-out) technique, with equal prior probabilities (White and Ruttenberg, 2007; Schaffler and Winkelman, 2008; Schaffler *et al.*, 2009).

## Simulations

Due to any inherent asymmetry in the sagittal pair, it is possible that the choice of left, right, or randomly chosen otolith can impact studies that aim to use the unique chemical signatures to distinguish between habitats. To illustrate the impacts of selecting a random otolith on our ability to discriminate nursery regions of summer flounder, a simulation model in R (R development core team) was developed to randomly select either a left or right otolith from the 30 paired observations and run all possible combinations on this randomized data set. We repeated this 10 000 times and built a classification function for these randomized datasets to compare with the best models based upon the left or right otolith datasets. Statistical analyses were conducted in either R or SAS® software (SAS Institute Inc., Cary, North Carolina, USA), and all hypothesis tests were performed based on the criterion $\alpha = 0.05$.

# Results

Juvenile summer flounder $L_T$ ranged between 177 and 234 mm, with a mean of 202.7 ($\pm$ 18.32) mm, and weighed between 49.7 and

**Table 1.** Biological characteristics of summer flounder from five estuarine nursery areas.

| Area | *n* | $L_T$ (mean ± sd) | M (mean ± sd) | Left $M_o$ (mean ± sd) | Right $M_o$ (mean ± sd) |
|---|---|---|---|---|---|
| Delaware Bay | 5 | 209.2 ± 13.27 | 96.1 ± 21.03 | 8.79 ± 2.20 | 9.12 ± 2.28 |
| Chesapeake Bay | 4 | 221.0 ± 4.65 | 93.9 ± 10.34 | 8.76 ± 0.84 | 9.01 ± 0.96 |
| Pamlico Sound | 12 | 193.2 ± 12.48 | 67.8 ± 11.16 | 8.01 ± 0.95 | 8.29 ± 0.99 |
| South Carolina | 5 | 211.8 ± 21.16 | 90.2 ± 29.30 | 10.11 ± 1.59 | 10.17 ± 1.57 |
| Georgia | 4 | 193.5 ± 20.01 | 75.6 ± 29.67 | 9.22 ± 1.48 | 9.53 ± 1.52 |
| Total | 30 | 202.7 ± 18.32 | 80.4 ± 23.48 | 8.75 ± 1.59 | 9.00 ± 1.61 |

$n$ = sample size, $L_T$ = total length in mm, $M$ = total body mass in g, $M_O$ = otolith mass in mg.

**Table 2.** Results of Box–Cox transformations, normality tests, and paired *t*-tests for different physical and chemical attributes of left and right sagittae.

| Quantity | λ | S-W | *t* | *p* | Diff (mean ± se) |
|---|---|---|---|---|---|
| *Mo*, left vs. right otolith | 0.762 4 | 0.0888 | −2.667 | 0.008 6* | −0.091 7 ± 0.036 0 |
| $\delta^{13}C$ left vs. right otolith | 0.945 4 | 0.125 8 | 8.816 | <0.000 1* | 0.304 2 ± 0.034 3 |
| $\delta^{18}O$ left vs. right otolith | 1.098 4 | 0.080 1 | 2.515 | 0.017 7* | 0.058 7 ± 0.022 7 |
| $^{7}Li$:$^{48}Ca$ left vs. right otolith | 1.102 6 | 0.344 0 | 3.670 | 0.000 9* | 0.471 9 ± 0.127 4 |
| $^{25}Mg$:$^{48}Ca$ left vs. right otolith | −0.693 4 | 0.634 4 | 2.769 | 0.009 7* | 21.999 2 ± 9.370 4 |
| $^{55}Mn$:$^{48}Ca$ left vs. right otolith | 0.126 9 | 0.966 7 | 0.471 | 0.640 9 | 1.400 5 ± 0.900 3 |
| $^{85}Rb$:$^{48}Ca$ left vs. right otolith | −0.521 8 | 0.704 8 | 2.020 | 0.052 7 | 0.010 9 ± 0.006 3 |
| $^{88}Sr$:$^{48}Ca$ left vs. right otolith | 1.366 6 | 0.770 7 | 2.075 | 0.046 9* | 0.056 9 ± 0.026 9 |
| $^{89}Y$:$^{48}Ca$ left vs. right otolith | −0.380 7 | 0.317 9 | 0.881 | 0.385 8 | 0.000 5 ± 0.000 6 |
| $^{138}Ba$:$^{48}Ca$ left vs. right otolith | 0.116 7 | 0.472 3 | 1.241 | 0.224 4 | 0.507 1 ± 0.468 4 |

$M_O$ = otolith mass in mg, λ = lambda of Box–Cox transformation, S-W = *p*-value of Shapiro–Wilk's normality tests, $t$ = pairwise *t*-test statistic, $p$ = *p*-value of the *t*-test (* = significant difference at $\alpha = 0.05$), Diff = differences in raw elemental ratios between the left and right otoliths, se = standard error.

131.5 g, with a mean of 80.4 (± 23.48) g (Table 1). Otoliths chosen for ICP-MS ranged between 6.48 and 12.34 mg, with an overall mean of 8.88 (± 1.62) mg. Using all otoliths, there was a significant difference in otolith mass ($M_O$) between the left and right sagittal otoliths ($p = 0.0087$; Table 2). The right otolith is heavier than the left otolith in summer flounder.

## Differences in otolith chemistry

Relative standard deviations for both otolith certified reference materials were generally <5% (Table 3). In two cases the RSDs were slightly higher, but both were <10%, indicating very precise and repeatable measurements across all analyses. Likewise, blanks were very low for all measured elemental concentrations (<10% of the average sample intensity) except for Pb, and consequently >75% of all sample Pb measurements were below the detection limit. Therefore Pb was removed from further analyses. Average precision for the stable isotopes was 0.13‰ for $\delta^{13}C$ and 0.10‰ for $\delta^{18}O$.

After the application of Box–Cox transformations to the raw data, all elemental differences were normally distributed (Table 2). Hotelling's $T^2$ applied on the differences between the left–right chemical signatures showed a significant overall difference within the sagittal pair (Hotelling's $T^2 = 131.494$, d.f. = 9, $p < 0.0001$). Pairwise *t*-tests performed on each of the analysed chemical signatures showed significant differences in the Li:Ca, Mg:Ca, and Sr:Ca molar ratios, as well as $\delta^{13}C$ and $\delta^{18}O$ stable isotope ratios (Table 2). The mean difference for $\delta^{13}C$ was almost 10 s.e. between left and right, while that of Li was almost 4 s.d. No significant differences were detected in Mn, Rb, Y, and Ba.

## Discriminant function analyses

The decision to use a linear or quadratic discriminant function to classify members of each dataset was based on the results of Bartlett's test. We successfully transformed all available data and tested for the necessary assumptions that were mostly satisfied with Box–Cox transformations for both left and right otoliths across areas (Table 4). Estimated lambda values for the variables in the left and right otoliths were similar, indicating a similar initial distribution. Despite transformation, homogeneity of variances was not met for $\delta^{18}O$ with either the left or right otolith. All other variances were homogeneous. Mardia's test showed that the assumptions of multivariate normality were not met by both the left (skewness = 174.9, $p = 0.2843$; kurtosis = −2.09, $p = 0.0364$) and right otoliths (skewness = 153.7, $p = 0.7257$; kurtosis = −2.10, $p = 0.0359$) due to slight deviations in kurtosis. A linear discriminant function was used because Bartlett's test showed no deviation from homogeneity of the variance–covariance matrix in both the left ($\chi^2_{180} = 59.68$, $p > 0.9999$) and the right sagittae ($\chi^2_{180} = 59.62$, $p > 0.9999$).

**Table 3.** Relative standard deviations (RSD) of the two certified reference materials (JPN: Yoshinaga *et al.*, 2000; NRC: Sturgeon *et al.*, 2005), limits of detection (LODs) as percentage mean intensities, and percentage of samples with elemental concentrations greater than the respective LODs for the left and right otoliths.

| | RSD | | | % > LOD | |
|---|---|---|---|---|---|
| Element | JPN | NRC | % intensity | Left | Right |
| Li | 3.52 | 6.41 | 2.59 | 100.0 | 100.0 |
| Mg | 3.05 | 3.33 | 1.38 | 100.0 | 100.0 |
| Ca | 3.39 | 3.49 | 0.05 | 100.0 | 100.0 |
| Mn | 4.33 | 8.82 | 7.32 | 100.0 | 100.0 |
| Rb | 3.18 | 3.60 | 4.52 | 100.0 | 100.0 |
| Sr | 3.46 | 3.56 | 0.05 | 100.0 | 100.0 |
| Y | 4.51 | 4.31 | 2.25 | 100.0 | 100.0 |
| Ba | 3.49 | 3.83 | 0.20 | 100.0 | 100.0 |
| Pb | 3.07 | 3.35 | 66.34 | 16.7 | 23.3 |

Unique elemental signatures within both left and right sagittae were found between the areas sampled. There were significant differences in the multivariate elemental signatures between sampled areas in both left (Pillai's trace = 2.89, $F_{36,80} = 5.76$, $p = <0.0001$) and right sagittae (Pillai's trace = 2.76, $F_{36,80} = 4.97$, $P = <0.0001$). In the left otolith, $\delta^{13}C$, $\delta^{18}O$, Mn, Rb, Y, and Ba were significantly different between areas, whereas in the right otolith, $\delta^{13}C$, $\delta^{18}O$, Mg, Mn, Rb, Sr, Y, and Ba were significantly different (Table 4).

**Table 4.** Results of Box–Cox transformations, variance and normality tests, and univariate analyses of variance (ANOVA) for left and right sagittae separately.

| Quantity | λ | O'Brien | S-W | ANOVA | |
|---|---|---|---|---|---|
| | | | | $F_{4,25}$ | *p* |
| **Left otolith** | | | | | |
| $\delta^{13}C$ | 0.806 | 0.4232 | 0.7060 | 20.10 | <0.0001* |
| $\delta^{18}O$ | 0.710 | 0.0074* | 0.5789 | 51.78 | <0.0001* |
| $^{7}Li:^{48}Ca$ | 1.109 | 0.5974 | 0.5184 | 2.74 | 0.0509 |
| $^{25}Mg:^{48}Ca$ | −0.924 | 0.2930 | 0.6096 | 1.60 | 0.2060 |
| $^{55}Mn:^{48}Ca$ | 0.198 | 0.7900 | 0.2990 | 5.00 | 0.0042* |
| $^{85}Rb:^{48}Ca$ | −0.826 | 0.4756 | 0.7369 | 11.30 | <0.0001* |
| $^{88}Sr:^{48}Ca$ | 0.248 | 0.5900 | 0.9888 | 1.90 | 0.1410 |
| $^{89}Y:^{48}Ca$ | −0.425 | 0.3991 | 0.5928 | 4.72 | 0.0056* |
| $^{138}Ba:^{48}Ca$ | 0.063 | 0.6148 | 0.0752 | 5.16 | 0.0036* |
| **Right otolith** | | | | | |
| $\delta^{13}C$ | 0.944 | 0.1010 | 0.6375 | 14.75 | <0.0001* |
| $\delta^{18}O$ | 0.761 | 0.0120* | 0.5350 | 50.66 | <0.0001* |
| $^{7}Li:^{48}Ca$ | 1.737 | 0.3104 | 0.1532 | 1.18 | 0.3445 |
| $^{25}Mg:^{48}Ca$ | −0.268 | 0.4860 | 0.2271 | 7.48 | 0.0004* |
| $^{55}Mn:^{48}Ca$ | 0.036 | 0.3337 | 0.7579 | 3.18 | 0.0304* |
| $^{85}Rb:^{48}Ca$ | −0.180 | 0.6969 | 0.2994 | 19.23 | <0.0001* |
| $^{88}Sr:^{48}Ca$ | 1.485 | 0.6768 | 0.6332 | 3.76 | 0.0158* |
| $^{89}Y:^{48}Ca$ | −0.082 | 0.4990 | 0.5466 | 8.29 | 0.0002* |
| $^{138}Ba:^{48}Ca$ | 0.183 | 0.6884 | 0.5612 | 5.45 | 0.0027* |

λ = lambda of Box–Cox transformation, O'Brien = *p*-value of O'Brien's test for homogeneity of variances, S-W = *p*-value of normality tests, $F_{4,25}$ = ANOVA's *F*-statistic, *p* = *p*-value of the test. *denotes any significant differences in all the tests performed.

The models that produced the highest classification accuracy for 2-variable through 7-variable combinations were different between the left and right otoliths (Table 5), and in general indicated that the left otolith produced slightly higher classification accuracies. The model with the highest classification and fewest elements was a 4-variable model in the left otolith (highlighted in Table 5). This 4-variable model, based on $\delta^{13}C$, $\delta^{18}O$, Mn, and Ba, presented the highest classification accuracy at 90%, as opposed to other models that had lesser accuracies with fewer variables or similar accuracies with more variables. Conversely in the right otolith, the model with the highest classification and fewest elements was a 5-variable model. This model was based on $\delta^{13}C$, $\delta^{18}O$, Rb, Sr, and Ba, resulting in 90% correct classification. Again, other models produced lower accuracies with fewer variables or similar accuracy with more variables.

## Discussion

The chemistry of left and right otoliths cannot be used interchangeably in the summer flounder and perhaps other bilaterally asymmetrical flatfishes. Numerous studies performed on asymmetrical species (different flatfishes) have considered the otoliths interchangeable (de Pontual *et al.*, 2003; Swearer *et al.*, 2003; Vasconcelos *et al.*, 2007; Reis-Santos *et al.*, 2008; Vasconcelos *et al.*, 2008; Leakey *et al.*, 2009), although these studies fail to test the hypothesis of equivalent chemistries between left and right otoliths. In Pacific halibut, left and right otolith chemistries were not equivalent (Loher *et al.*, 2008). Our results in summer flounder agree with those in Pacific halibut, finding the same $\delta^{13}C$, $\delta^{18}O$, and Sr:Ca differences between otoliths. Taken together, our results

**Table 5.** Model selection results showing the best classifications produced for left and right otoliths, as compared with the mean accuracies for the same models using a randomized data set of left–right mixtures.

| | | | Randomized dataset | |
|---|---|---|---|---|
| *n* | Elements | Accuracy | Mean | sd |
| **Left otolith** | | | | |
| 1 | O | 60 | 65 | 5.2 |
| 2 | O, Mg | 87 | 72 | 6.0 |
| 3 | C, O, Mn | 87 | 81 | 4.6 |
| 4 | C, O, Mn, Ba | 90 | 85 | 0.4 |
| 5 | C, O, Li, Mg, Ba | 87 | 83 | 0.5 |
| 6 | C, O, Li, Mg, Y, Ba | 90 | 86 | 0.5 |
| 7 | C, O, Li, Mg, Sr, Y, Ba | 90 | 84 | 0.5 |
| 8 | C, O, Li, Mg, Rb, Sr, Y, Ba | 83 | 81 | 0.6 |
| 9 | ALL | 77 | 82 | 0.6 |
| **Right otolith** | | | | |
| 1 | O | 63 | 65 | 5.2 |
| 2 | C, O | 73 | 81 | 4.7 |
| 3 | C, O, Ba | 83 | 84 | 4.0 |
| 4 | O, Rb, Sr, Ba | 87 | 80 | 0.6 |
| 5 | C, O, Rb, Sr, Ba | 90 | 84 | 0.5 |
| 6 | C, O, Li, Rb, Sr, Ba | 90 | 83 | 0.5 |
| 7 | O, Li, Mg, Rb, Sr, Y, Ba | 90 | 80 | 0.6 |
| 8 | C, O, Li, Mg, Rb, Sr, Y, Ba | 90 | 81 | 0.6 |
| 9 | ALL | 83 | 82 | 0.6 |

The boxed functions show the models that produced the highest accuracy with the fewest variables. *n* = number of elements used, sd = standard deviation. Accuracy and mean in percentage (%).

and those of Loher *et al.* (2008) indicate that processes acting on the otoliths of bilaterally asymmetrical fishes invalidate the assumption of equivalent chemistry between otolith pairs which has been demonstrated in bilaterally symmetrical fishes (Iacumin *et al.*, 1992; Gauldie, 1996; Thorrold *et al.*, 1997; Rooker *et al.*, 2001). Further, it appears that differences in otolith accretion rates (Helling *et al.*, 2005) may be responsible for differences observed in otolith chemistries between left and right sagittae.

There were significant mass differences between the left and right otoliths of the summer flounder. These mass differences likely reflect differential carbonate accretion rates that directionally skew the elemental signatures in a way similar to the differences between sagittae and lapillae (Meyer-Rochow *et al.*, 1992; Chesney *et al.*, 1998; Chittaro *et al.*, 2006; Smith and Jones, 2006), which have divergent growth patterns from the time of hatching onwards (Loher *et al.*, 2008). The Pacific halibut, which shows differences in both stable isotopes and trace elements within the sagittal pair, demonstrates this trend, where the left otolith is significantly larger than the right otolith (Loher *et al.*, 2008). In the case of the summer flounder, the right otolith is significantly heavier than the left otolith. It is noteworthy in these two flatfishes that the Pacific halibut is dextral, where the left eye migrates to the right side, as opposed to the summer flounder, which is sinistral; in both fishes, the blind-side otolith is the larger one. Loher *et al.* (2008) suggest that the smaller right otolith might reflect early life history to a greater extent than the left one in the Pacific halibut; this might also apply to the summer flounder, where the smaller left sagitta is more indicative of early life. Further investigations into the morphological or ontogenic drivers for this trend will likely result in a greater understanding of what the otolith chemistry of flatfishes tells us about their ecology.

Specific differences were detected in the concentrations of $\delta^{13}C$, $\delta^{18}O$, Li:Ca, Mg:Ca, and Sr:Ca. The remaining elemental concentrations did not deviate significantly between sagittae. Summer flounder showed more deviations in the signatures than the Pacific halibut that had differences in $\delta^{13}C$, $\delta^{18}O$, and Sr:Ca (Loher *et al.*, 2008). In this regard, Sr:Ca might be a trace element that could potentially be used routinely in the detection of left–right differences in the sagittae. $\delta^{13}C$ and $\delta^{18}O$ also have a similar trend in the Pacific halibut and the summer flounder, with the potential to be used as markers. Loher *et al.* (2008) discussed the possible effect of low elemental concentration with respect to detection limits, contamination, or procedural bias as factors that might confound other left–right differences. In our study, we measured Rb and Y, which do not have certified values in the CRMs used (Yoshinaga *et al.*, 2000; Sturgeon *et al.*, 2005) and are not routinely used. Moreover, Rb has the potential for isobaric interferences due to the Ar-carrier gas used in the ICP-MS. However, the RSDs for both Rb and Y demonstrate that these elements are consistently measured with high precision (RSDs $< 5\%$); the ability to repeatedly obtain the same value from a CRM, regardless of the accuracy of the value, is of utility when evaluating a marker stock discriminator, especially since the obtained ratios are being investigated in relation to one another and not necessarily for absolute concentrations. However, excluding Rb and Y from the classification functions, due to the lack of certified values in CRMs and possible isobaric interferences, would not affect the left otolith-based functions, as neither element contributes towards the models with the highest classification success. In the right otolith, removing Rb and Y from the analyses resulted in no changes in classification success in the four- and five-variable models (C, O, Li, Ba: 87% and C, O, Li, Sr, Ba: 90%).

Loher *et al.* (2008) also hypothesized that the differences seen in the chemical signatures were not location specific, as they sampled from two locations, one for stable isotopes and the other for trace elements, using solution-based ICP-MS. Our study expanded on that idea by sampling from five different locations and incorporating random fish from the entire range in analyses of both stable isotopes and trace metals on the same fish. Our results show that there is an overarching asymmetry within the sagittal pair independent of the location sampled.

Moreover, Loher *et al.* (2008) hypothesize that there might be a greater asymmetry in the left–right signatures of flatfishes that use estuaries as nurseries due to larger variability in environmental and biotic factors, as in the case of the common sole (de Pontual *et al.*, 2003), English sole (Brown, 2006), and southern flounder (Lowe *et al.*, 2011). The life history of summer flounder might advocate a stronger divergence within the sagittae, stemming from the fact that summer flounder are spawned on the continental shelf but develop as juveniles in the estuarine nurseries along the US east coast (Smith, 1973; Able *et al.*, 1990; Szedlmayer *et al.*, 1992; Kraus and Musick, 2001). It is clear from our study and Loher *et al.* (2008) that the left and right sagittae of flatfishes are often not equivalent.

The discriminant function analyses show that exclusively choosing either the left or right otolith will alter the classification model used to discriminate populations as well as the observed classification success. As such, the model with the highest accuracy and fewest elements was a four-variable model using the left otolith. The left otolith produced better classification schemes than the right otolith, as seen with the optimal DFA. The elements in the most useful models differed between the left and right sagittae as well, highlighting the differences in chemical signatures useful for classification success and further underlining the asymmetry of this flounder. Thus, introducing an added source of variation (choice of left or right otolith) can have far-reaching consequences for studies aiming to discover useful classification functions and to understand connectivity and population structure in poorly studied organisms.

There are many examples of otolith chemistry studies performed on flatfish species; while some have specifically selected an otolith from the sagittal pair (Brown, 2006; Fodrie and Levin, 2008; Cuveliers *et al.*, 2010; Lowe *et al.*, 2011; Tanner *et al.*, 2011), others make no reference to selection, presumably as they have randomly selected an otolith (Toole *et al.*, 1993; de Pontual *et al.*, 2003; Swearer *et al.*, 2003; Vasconcelos *et al.*, 2007; Reis-Santos *et al.*, 2008; Vasconcelos *et al.*, 2008; Leakey *et al.*, 2009). Based upon our results, the left and right otoliths of flatfish are not interchangeable, and random selection has the potential to produce greater error and highly variable results. Not accounting for left–right variability in these cases could result in increased variability in the elemental signatures and reduced classification accuracy. Our results also have practical and logistic implications when applying a classification function based on only a left or right otolith. Many labs age recreationally and commercially important species, some of which have asymmetrical otoliths, in support of stock assessment work. This potentially means that, if randomly selected, up to half of all archived otoliths from asymmetrical species may not be available for chemical analyses. Moreover, if all available otoliths were to be used disregarding their orientation (i.e. left or right), estimates of population structure based on otolith chemistry could be strongly biased. For example, we have demonstrated that the accuracy of the classification function could be dramatically reduced, and the

researchers may conclude that little population structure exists when in fact structure was present but obscured by incorrectly sampling both left and right otoliths due to an inflated variance. Further, it is possible that incorrectly sampling from both left and right otoliths could generate spurious relationships that would lead researchers to falsely conclude population structure exists when in fact there is none. And though it is reasonable to expect otoliths to be interchangeable in symmetrical species (e.g. Campana *et al.*, 2000; Secor *et al.*, 2001; Arslan and Secor, 2008), we recommend testing the assumption of equivalence in otolith chemistry before sagittae are used interchangeably in asymmetrical species.

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