Accurate Classification of Juvenile Weakfish
*Cynoscion regalis* to Estuarine Nursery Areas
Based on Chemical Signatures in Otoliths

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Accurate classification of juvenile weakfish 
*Cynoscion regalis* to estuarine nursery areas 
based on chemical signatures in otoliths

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ABSTRACT: We investigated the ability of trace element and isotopic signatures in otoliths to record the nursery areas of juvenile (young-of-the-year) weakfish *Cynoscion regalis* from the east coast of the USA. Juvenile *C. regalis* were captured with otter trawls at multiple sites in Doboy Sound (Georgia), Pamlico Sound (North Carolina), Chesapeake Bay (Virginia), Delaware Bay (Delaware) and Peconic Bay (New York), from July to September 1996. One sagittal otolith from each specimen was assayed for Mg/Ca, Mn/Ca, Sr/Ca and Ba/Ca ratios using inductively coupled plasma mass spectrometry (ICP-MS), while δ13C and δ18O values from the other sagittal otolith in the pair were determined using isotope ratio mass spectrometry (IR-MS). A multivariate analysis of variance determined that there were significant differences in trace element signatures among locations. Bootstrapped 95% confidence ellipses on canonical variates indicated that all 5 locations were significantly isolated in discriminant space. On the basis of these differences, linear discriminant function analysis (LDFA) and artificial neural network (ANN) models were used to classify individual fish to their natal estuary with an overall error rate of 37% for LDFA and 29.6% for ANN. Addition of δ13C and δ18O values to the LDFA and ANN models derived from the trace element data resulted in overall error around 10%. We therefore be able to use chemical signatures from the juvenile portion of adult *C. regalis* otoliths to accurately classify these fish to their natal estuary.

KEY WORDS: Estuarine nursery areas · Otolith chemistry · Trace elements · Stable isotopes · Neural networks

INTRODUCTION

Weakfish *Cynoscion regalis* landings along the Atlantic coast of the United States have recently shown a significant decline, with estimated commercial and recreational catches falling from $36 \times 10^6$ kg in 1980 to $3.6 \times 10^6$ kg in 1994. Efforts to manage this fishery are, however, handicapped by an inability to determine the spatial extent of weakfish stocks. The potential for presumptive stock mixing during over-wintering off the North Carolina coast has led to concerns that winter fisheries off North Carolina have been at least partly responsible for the decline of the weakfish catch in northern waters. Juvenile weakfish also represent a significant component of the South Atlantic shrimp trawl bycatch (Vaughan et al. 1991). At present there is no way of determining the effect these fisheries are having on adult populations in more northern waters.

Previous studies based on mark-recapture, morphology and life history differences have suggested there may be multiple stocks of weakfish along the mid-Atlantic coast (reviewed by Graves et al. 1992). However, allozyme (Crawford et al. 1988) and mtDNA (Graves et al. 1992) studies failed to support a multi-stock hypothesis. Weakfish spawn in estuarine and near-coastal waters, and larvae are believed to use selective tidal stream transport to remain within the...
estuary in which they were spawned (Rowe & Epifanio 1994). If larval drift is insignificant, and if spawning fish home to their natal area, weakfish should show evidence of stock separation along a geographic range that spans the entire U.S. Atlantic coast from Florida to Maine. However, the power of the genetic studies to test the null hypothesis of a single homogeneous stock was limited due to the low amount of overall genetic variation that was revealed. The mean nucleotide sequence diversity (the average amount of nucleotide differences found among individuals of a population) in the mtDNA analysis averaged 0.15%, one of the lowest values reported for any vertebrate (Graves et al. 1992). There is no way to determine, at this stage, if there is sufficient larval drift, or adult vagrancy, to prevent the formation of stock structure, or if the genetic techniques are not powerful enough to reveal what may be subtle genetic differences among adjacent populations.

Several recent studies have suggested that otoliths may be an ideal natural tag for studies of fish population structure and for tracking individual migration paths (Campana et al. 1995, Thorrold et al. 1997a). Otoliths form through concentric additions of alternating protein and aragonite layers around a central nucleus. Because otoliths are acellular, once deposited, otolith material is neither resorbed nor metabolically reworked (Campana & Neilson 1985). The chemical composition of the otolith reflects, in turn, the physical and chemical characteristics of the ambient water, albeit not necessarily in a simplistic manner (Fowler et al. 1995, Thorrold et al. 1997a). In principle, then, water mass differences at the time and place of hatching will be reflected in chemical composition differences in the otolith nucleus. By corollary, migration among water masses at some age or date will be recorded by the chemical composition of the otolith in the appropriate otolith increments.

Most otolith chemistry studies have focused on quantifying elemental signatures of stock associations, with good success (e.g. Edmonds et al. 1989, 1991, 1992, Thresher et al. 1994, Campana et al. 1995). Significant information on environmental conditions may also reside, however, in carbon and oxygen stable isotope ratios within otoliths (e.g. Nelson et al. 1989, Patterson et al. 1993). For instance, Thorrold et al. (1997b) have shown that δ18O of juvenile Atlantic croaker Micropogonias undulatus is deposited in isotopic equilibrium with ambient water, and that the temperature fractionation relationship is similar to aragonite in hermatypic coral skeletons. On the basis of this temperature-dependent fractionation, Edmonds & Fletcher (1997) suggested that δ18O values in otoliths of adult pilchards Sardinops sagax may be a useful tool for stock identification because populations largely reside in waters with differing thermal regimes. While carbon isotopes are not necessarily reflective of the carbon isotopic composition of dissolved inorganic carbon in the environment, changes in otolith δ13C may record geographic differences in growth and condition (Thorrold et al. 1997b).

In this study, we investigated the ability of trace element and stable isotope signatures in otoliths to act as natural tags of estuarine nursery areas in juvenile (young-of-the-year) weakfish Cynoscion regalis. The observation that C. regalis larvae are able to maintain themselves within the estuary system in which they were spawned minimized the possibility of juveniles collected in an estuarine nursery area originating from elsewhere (Rowe & Epifanio 1994). The specific objectives of this study were to quantify geographic variation in the trace element and stable isotope chemistry of otoliths from juvenile C. regalis, and then to determine if these differences were sufficient to allow weakfish to be accurately classified to nursery areas based on these elemental and isotopic signatures.

**MATERIALS AND METHODS**

Juvenile weakfish were collected from 5 estuarine systems along the east coast of the United States, from Georgia to New York (Fig. 1). Within each location, fish were collected from several otter trawl sets at either 2 or 3 sites separated by at least 3 km (see Table 1), with the exception of Peconic Bay, where fish were collected from multiple tows and grouped into a single site. All samples were stored on ice in the field and subsequently frozen upon return to the lab. Surface and bottom temperature and salinity records were available from multiple sites at monthly intervals throughout the juvenile estuarine residency period for all locations except Pamlico Sound (Fig. 2).

In preparation for chemical analysis, sagittal otoliths were removed from the fish with acid-washed glass probes, placed in a drop of ultrapure (Milli-Q) water and cleaned of adhering tissue. Otoliths were then ultrasonically cleaned for 5 min, rinsed in ultrapure H2O2 to remove any remaining tissue, triple-rinsed in Milli-Q water, and air-dried under a Class 100 laminar flow hood. After drying for at least 24 h, otoliths were weighed to the nearest 10 µg and stored in acid-washed high density polyethylene vials. Blank vials were similarly prepared for blank corrections and to calculate limits of detection. One of the otoliths was then used to determine elemental concentrations, while the other otolith in the pair was used for stable isotope analysis.

**Otolith chemistry analyses.** Elemental concentrations in otoliths were determined by inductively cou-
Otolith solutions were assayed with a Finnigan-MAT SOLA quadrupole ICP mass spectrometer. A lab standard, consisting of *Micropogonias undulatus* otoliths ground to micron-sized particles (Campana et al. 1997), was also assayed periodically throughout the otolith analysis to assess measurement repeatability. All elemental data were subsequently converted to molar concentrations and expressed as ratios to Ca. Elements that substitute for Ca ions in the aragonite lattice may be expected to co-vary with elemental levels in the environment (Lea & Boyle 1993). Incorporation of these elements in the otolith is more accurately standardized to the number of Ca ions in the otolith rather than on total otolith weight.

Limits of detection (LOD: 3σ + mean blank value from 21 analyses of 7 blank vials) for each of the elements were as follows: B (4.54 μg g⁻¹), Mg (3.99 μg g⁻¹), Ca (253 μg g⁻¹), Mn (0.87 μg g⁻¹), Zn (6.12 μg g⁻¹), Sr (4.28 μg g⁻¹), and Ba (0.08 μg g⁻¹). LODs were well below observed values for Mg, Ca, Mn, Sr and Ba. However, B and Zn counts were on occasion below detection limits. Estimates of precision (%RSD) based on repeated analyses of our lab standard (21 analyses of 7 digests) were as follows: B/Ca (80.7%), Mg/Ca (8.9%), Mn/Ca (2.0%), Zn/Ca (29.4%), Sr/Ca (4.8%) and Ba/Ca (7.2%). We eliminated B and Zn from statistical analyses as levels of...
both elements were below the LOD in at least some of the samples, and because of the poor precision in repeated analyses of the lab standard.

Otoliths for carbon and oxygen isotope ratio analysis were processed by an automated carbonate device (common acid bath at 90°C) attached to a Finnigan-MAT 251 gas ratio mass spectrometer. A number of otoliths were too large to be analyzed whole, so instead these otoliths were ground and a 1 mg sample was then taken from the resulting otolith powder for stable isotope analysis. Data were corrected for the usual isobaric interferences, using the method of Craig (1957) modified for a triple collector mass spectrometer, and are expressed relative to PDB (PeeDee Belemnite). External precision (calculated from replicate analyses of an internal laboratory calcite standard) was 0.02‰ for $\delta^{13}$C and 0.03‰ for $\delta^{18}$O.

**Data analysis.** Statistical analyses of chemical signatures were carried out using parametric and non-parametric multivariate approaches. To test for the statistical significance of among-location differences in otolith chemistry while accounting for spatial variation within locations, we implemented a mixed-model MANOVA (multivariate analysis of variance) design with collection sites (a random term) nested within locations (a fixed term). Assumptions of multivariate normality and equal variance-covariance matrices were assessed by examining each of the univariate variables for normal distribution of errors and homogeneity of variances using residual analysis (Winer 1971). All variables met the assumptions for univariate ANOVA, and we therefore assumed that the multivariate tests would also be valid. The MANOVA was followed by a canonical discriminant analysis (CDA) to visualize differences among locations. A posteriori contrasts among locations were performed by calculating 95% confidence ellipses on class means using a bootstrap approach (Efron & Gong 1983). Scores on each of the canonical variates were resampled 1000 times with replacement for the 5 locations. When confidence intervals were asymmetric around class means, the largest of the 2 intervals was used to produce a conservative and symmetric confidence ellipse.

Linear discriminant function analysis (LDFA) was used to determine if chemical signatures in otoliths accurately record natal estuaries of juvenile *Cynoscion regalis*. The cross-validation algorithm in the SAS Institute Inc. (1990) DISCRIM procedure, which uses a jackknife technique, was used to determine classification accuracy. Each sample is removed sequentially from the data set, the discriminant function is estimated from the remaining samples, and then the function is used to classify the absent observation.

We also assessed the ability of artificial neural networks (ANN) to accurately classify fish to their natal estuary. Feed-forward neural networks are becoming increasingly popular in pattern recognition and classification applications in a number of scientific disciplines, including geology (Malmgren & Nordlund 1996), population genetics (Cornuet et al. 1996), and ecology (Culverhouse et al. 1992). The neural network that we used consisted of an input matrix (the multivariate elemental data set) that passes to a single hidden layer after a tan-sigmoid transformation with a weight and bias term. The activity from the hidden neural layer is then passed through a simple linear transformation, with another weight and bias term, to the output layer consisting of a matrix that specifies the location to which an individual sample is to be classified (Fig. 3). The neural network is trained to classify individual samples by iteratively adjusting the weights and biases that connect each of the layers to minimize the model variance. After the network is adequately trained, it can then be used to classify unknown samples. In this instance, we used back-propagation to train the network, employing both momentum and adaptive learning rate to increase the speed and reliability of the back-propagation procedure (Demuth & Beale 1994). All data were first standardized by subtracting the grand mean of the variable from each observation, and then dividing by the standard deviation. It is important to note that, unlike linear discriminant analysis, neural networks do not have a single statistical solution. Therefore, all analyses were run with 5 different initial starting values, and results are means of the 5 model runs. Classification success was determined with the same cross-validation procedure used in the LDFA.

To determine the optimum number of neurons in the hidden layer, we generated a training data set from both the element/Ca ratios and the combined elemental and carbon and oxygen isotopic data. The data matrices were constructed by removing (by random selection) 12 samples from each location (approximately 20% of the total samples) as a test data set, while the remaining samples were used to train the neural network. We then tested the classification success of the ANN by running 10 independent trials for each model that had different numbers of neurons (3, 6, 9, 12, 18, 24, and 30) in the hidden layer. Error rates In all runs decreased exponentially with increasing numbers of training cycles ('epochs'). We chose 1000 epochs as a reasonable balance between the decrease in error rate associated with in-
Fig. 3. (a) Schematic representation of the architecture of a 2-layer back-propagation artificial neural network (ANN) in which the 4 element/Ca ratios in otolith samples enter the network, pass through a hidden layer with 7 neurons (note that the actual neural network used in this study has 18 hidden neurons), and then pass onto the output layer whose activity specifies the location to which an individual observation should be classified. (b) Schematic representation of the path of a single observation through the neural network, in which the initial input value \( i \) is multiplied by a weight \( \omega_{i1} \) and then passed through a sigmoidal transfer function before the addition of a bias term \( b_1 \). The resulting neural activity \( a \), is then multiplied by another weight term \( \omega_{a1} \), passed through a linear transfer function, and a second bias term is added \( b_{a1} \); the resulting activity of the second neuron is the model output \( o \).

Fig. 4. Change in error rate for a 2-layer back-propagation neural network, trained with a subset (approximately 80% of original observations) data matrix consisting of 4 element/Ca ratios \( \bullet \) and the combined elemental and isotopic data \( \otimes \) and then tested with the remaining 20% of the original observations, when the number of neurons in the hidden layer is increased from 3 to 30. Error rates are means ± SD from 10 independent runs of the neural network.

Fig. 5. Cynoscion regalis. Relationship between standard length and otolith weight for juvenile weakfish captured at Doboy Sound \( \bullet \), Pamlico Sound \( \bullet \), Chesapeake Bay \( \bullet \), Delaware Bay \( \otimes \), and Peconic Bay \( \bullet \).
Table 1. *Cynoscion regalis*. Summary information including collection site, collection date, number of fish analyzed (n), mean standard length (SL [mm], ±SE), and otolith weight (OW [mg], ±SE) from juvenile weakfish captured at various locations along the U.S. Atlantic coast between July and September of 1996.

<table>
<thead>
<tr>
<th>Location</th>
<th>Site</th>
<th>Date</th>
<th>n</th>
<th>Mean SL</th>
<th>Mean OW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doboy Sound, Georgia</td>
<td>South River</td>
<td>16 July 1996</td>
<td>23</td>
<td>69.8 ± 4.4</td>
<td>15.2 ± 1.9</td>
</tr>
<tr>
<td></td>
<td>Lower Duplin River</td>
<td>17 July 1996</td>
<td>17</td>
<td>80.0 ± 3.8</td>
<td>19.1 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>Upper Duplin River</td>
<td>17 July 1996</td>
<td>13</td>
<td>82.2 ± 4.1</td>
<td>21.2 ± 2.4</td>
</tr>
<tr>
<td></td>
<td>Combined</td>
<td></td>
<td>53</td>
<td>76.1 ± 2.6</td>
<td>17.9 ± 1.2</td>
</tr>
<tr>
<td>Pamlico Sound, North Carolina</td>
<td>Neuse River</td>
<td>1 August 1996</td>
<td>25</td>
<td>49.5 ± 3.4</td>
<td>6.3 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>Bluff Shoals</td>
<td>30 July 1996</td>
<td>29</td>
<td>77.1 ± 3.2</td>
<td>17.3 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>Combined</td>
<td></td>
<td>54</td>
<td>64.3 ± 3.0</td>
<td>12.2 ± 1.3</td>
</tr>
<tr>
<td>Chesapeake Bay, Virginia</td>
<td>James River</td>
<td>10 July 1996</td>
<td>18</td>
<td>60.0 ± 2.0</td>
<td>9.2 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>York River</td>
<td>10 July 1996</td>
<td>23</td>
<td>64.1 ± 2.5</td>
<td>8.5 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>Rappahannock River</td>
<td>10 July 1996</td>
<td>12</td>
<td>54.2 ± 3.0</td>
<td>7.5 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>Combined</td>
<td></td>
<td>53</td>
<td>60.5 ± 1.5</td>
<td>8.5 ± 0.4</td>
</tr>
<tr>
<td>Delaware Bay, Delaware</td>
<td>Mouth</td>
<td>20 September 1996</td>
<td>29</td>
<td>65.6 ± 5.3</td>
<td>12.9 ± 2.4</td>
</tr>
<tr>
<td></td>
<td>Lower Bay</td>
<td>20 September 1996</td>
<td>18</td>
<td>87.1 ± 3.7</td>
<td>25.7 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>Middle Bay</td>
<td>20 September 1996</td>
<td>11</td>
<td>91.7 ± 4.1</td>
<td>28.6 ± 2.4</td>
</tr>
<tr>
<td></td>
<td>Combined</td>
<td></td>
<td>58</td>
<td>77.2 ± 3.3</td>
<td>19.5 ± 1.6</td>
</tr>
<tr>
<td>Peconic Bay, New York</td>
<td></td>
<td>8 July 1996</td>
<td>56</td>
<td>67.8 ± 1.0</td>
<td>12.4 ± 0.4</td>
</tr>
</tbody>
</table>

Increasing epoch number and the computer time required to run the model for large numbers of epochs. All ANNs were performed using MATLAB's neural network toolbox (Demuth & Beale 1994).

**RESULTS**

Juvenile weakfish collected throughout the sampling locations ranged in size from approximately 30 to 120 mm SL (Table 1). There were, however, no significant differences in either standard length (ANOVA: df = 4, 7; MS = 4360; p = 0.3042) or otolith weight (ANOVA: df = 4, 7; MS = 1467; p = 0.1610) among locations. Similarly, fish from all the locations had a similar standard length/otolith weight relationship (Fig. 5). Correlation coefficients between otolith weight and chemical variables were calculated for each site, and these correlations were then averaged to give a mean correlation coefficient for each location (Fig. 6). Although mean coefficients ranged from 0.4 to −0.8, 23 of 30 coefficients were negative, indicating lower values for most of the elemental and isotopic variables with increasing otolith weight at most locations. Significant variability in the slopes of the relationship between otolith weight and each of individual elements and isotopes among locations meant, however, that we could not correct for the effect of otolith weight by using an analysis of covariance. It should be noted that variability in slopes among locations implies that there is little variation in trace element and isotopic signatures that can be attributed to a systematic effect of otolith weight on chemical signatures. We assumed, therefore, that any relationship between otolith chemistry and fish or otolith size (or growth rate) would not confound any differences in chemical signatures among locations.

**Trace element chemistry**

Elemental data from otoliths showed significant variations, both at sites within locations and among loca-

![Fig. 6. *Cynoscion regalis*. Mean correlation coefficients, averaged across sites, between chemical signatures and otolith weight from juvenile weakfish captured at Doboy Sound (○), Pamlico Sound (●), Chesapeake Bay (●), Delaware Bay (◇), and Peconic Bay (■).](image-url)
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Peconic Bay. The ANN provided similar results, although with somewhat better classification success rates. Fish from Delaware Bay proved most difficult to accurately classify for ANN (43% error rate), while Peconic Bay fish were again most easy to distinguish (12% error rate). The overall error rate of the LDFA analysis was 36.8%, while the ANN error rate was 29.6%.

$\delta^{13}C$ and $\delta^{18}O$ values

Carbon isotope values in juvenile *Cynoscion regalis* otoliths ranged from approximately -9 to 0‰, while oxygen isotope values ranged from -8 to 0‰ (Fig. 9). There were considerable variations in both $\delta^{13}C$ and $\delta^{18}O$ among locations, as well as considerable variation at the site level in some, but not all, of the locations. For instance, $\delta^{13}C$ values from samples within Delaware Bay spanned nearly the entire range of values across all locations. Samples from Doboy Sound showed very little variation both within and among sites. Oxygen isotope values were also highly variable in the Delaware Bay samples, but showed significantly less variation within and among sites at all other locations.

When $\delta^{13}C$ and $\delta^{18}O$ values were added to the elemental data set, the resulting MANOVA found highly significant differences both at the site and location level (Table 2). The associated CDA analysis showed almost complete separation among locations (Fig. 10) when the first 3 canonical variates were plotted. The only locations not well separated on the CV1 and CV2 were Pamlico Sound and Doboy Sound. However, these locations were clearly distinguishable on CV3. Canonical coefficients revealed that CV1 was principally a contrast between $\delta^{18}O$ (positive values) and Mn/Ca (negative values), CV2 contrasted $\delta^{13}C$ and

Table 2. Results from MANOVA of element/Ca ratios, and $\delta^{13}C$ and $\delta^{18}O$ values, quantified in juvenile *Cynoscion regalis* otoliths from sites at locations along the U.S. Atlantic coast. All significance tests used Pillai's trace statistic. Note that the denominator for the Location $F$ approximation is the Site(Location) term, while the error term is the denominator in the Site(Location) $F$ approximation

<table>
<thead>
<tr>
<th>Source</th>
<th>Value</th>
<th>$F$</th>
<th>Num df</th>
<th>Den df</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dependent variables: Mg/Ca, Mn/Ca, Sr/Ca and Ba/Ca</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Location</td>
<td>2.225</td>
<td>2.19</td>
<td>16</td>
<td>28</td>
<td>0.0333</td>
</tr>
<tr>
<td>Site(Location)</td>
<td>6.784</td>
<td>9.12</td>
<td>26</td>
<td>1048</td>
<td>0.0001</td>
</tr>
<tr>
<td><strong>Dependent variables: Mg/Ca, Mn/Ca, Sr/Ca, Ba/Ca, $\delta^{13}C$ and $\delta^{18}O$</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Location</td>
<td>2.562</td>
<td>6.20</td>
<td>24</td>
<td>20</td>
<td>0.0001</td>
</tr>
<tr>
<td>Site(Location)</td>
<td>1.167</td>
<td>8.31</td>
<td>42</td>
<td>1446</td>
<td>0.0001</td>
</tr>
</tbody>
</table>
Mn/Ca ratios (positive values) with Mg/Ca ratios (negative values), and finally CV3 contrasted $\delta^{13}$C and Ba/Ca ratios (positive values) with Mg/Ca and Mn/Ca ratios (negative values). As a further check of the possible influence of differences in otolith weight among locations on the otolith chemical signatures, we calculated Pearson correlation coefficients between otolith weight (OW) and each of the 3 canonical variates. Coefficients for each of the 3 variates were low (CV1:OW = 0.18, CV2:OW = -0.17, CV3:OW = 0.24), suggesting that the effect of otolith weight on the resulting CDA analysis was negligible.

The increased separation among locations seen in the CDA plots was reflected in reduced error rates of both LDFA and ANN when the combined data matrix, consisting of 4 element/Ca ratios and $\delta^{13}$C and $\delta^{18}$O values, was used to classify samples according to natal estuary (Table 3). LDFA error rates were less than 20% for all locations. Delaware Bay fish proved most difficult to classify (18% error rate), while Peconic Bay fish were again classified most accurately (8% error rate). The ANN also showed better classification successes with the combined data set, with error rates ranging from 13% (Delaware Bay) to 2% (Pamlico Sound). Overall error rates were again lower with ANN (7% error rate) than LDFA (13% error rate).

Table 3. Results of linear discriminant function (LDFA) and artificial neural network (ANN) analyses for classifying juvenile Cynoscion regalis to natal estuary based on trace element and isotopic signatures in otoliths. Samples came from Doboy Sound (DS), Pamlico Sound (PA), Chesapeake Bay (CB), Delaware Bay (DE) and Peconic Bay (GP). Bold values indicate percent of individuals successfully classified to their natal estuary ($n$ = number of samples analyzed)

<table>
<thead>
<tr>
<th>From location:</th>
<th>DS</th>
<th>PA</th>
<th>CB</th>
<th>DE</th>
<th>GP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cross-validation results (%) with Mg/Ca, Mn/Ca, Sr/Ca and Ba/Ca as dependent variables</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DS (n = 53)</td>
<td>LDFA 56.6</td>
<td>38.8</td>
<td>76.6</td>
<td>32.1</td>
<td>0</td>
</tr>
<tr>
<td>ANN 73.6</td>
<td>5.6</td>
<td>7.6</td>
<td>13.2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>PA (n = 54)</td>
<td>LDFA 3.7</td>
<td>50.0</td>
<td>13.0</td>
<td>25.9</td>
<td>7.4</td>
</tr>
<tr>
<td>ANN 5.6</td>
<td>64.4</td>
<td>3.7</td>
<td>11.1</td>
<td>14.8</td>
<td></td>
</tr>
<tr>
<td>CB (n = 53)</td>
<td>LDFA 7.6</td>
<td>9.4</td>
<td>66.0</td>
<td>9.4</td>
<td>7.6</td>
</tr>
<tr>
<td>ANN 13.2</td>
<td>5.7</td>
<td>69.8</td>
<td>5.7</td>
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<tr>
<td>DE (n = 58)</td>
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<td>58.6</td>
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<td>56.9</td>
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<tr>
<td>Cross-validation results (%) with Mg/Ca, Mn/Ca, Sr/Ca, Ba/Ca, $\delta^{13}$C and $\delta^{18}$O as dependent variables</td>
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<tr>
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<td>6.2</td>
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<tr>
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<tr>
<td>GP (n = 51)</td>
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Fig. 10. *Cynoscion regalis*. (a) Canonical variates 1 and 2 and (b) canonical variates 1 and 3, summarizing variations in trace element signatures combined with δ13C and δ18O values from juvenile weakfish otoliths collected from Doboy Sound (*), Pamlico Sound (*), Chesapeake Bay (**), Delaware Bay (*) and Peconic Bay (*). Different symbol shapes indicate different sites within a location. Shaded areas represent bootstrapped 95% confidence ellipses around location means for each variate.

**Chesapeake Bay rivers**

The highly significant differences in chemical signatures among sites at some locations suggested that otolith chemistry may vary over smaller spatial scales as well as among locations. To examine this in more detail, we used samples collected from 3 rivers that flow into the western shore of Chesapeake Bay—the James, York and Rappahannock Rivers. While sample sizes were smaller than the among location comparisons, CDA plots still showed that there was considerable separation among the rivers using the combined elemental and isotopic data (Fig. 11). Indeed, only 2 fish from the Rappahannock River overlapped in discriminant space with fish from the York River; the remaining 52 samples separated clearly into the specific river in which they were captured.

**DISCUSSION**

The elemental and isotopic composition of juvenile *Cynoscion regalis* otoliths varied considerably both within and among the estuarine systems we sampled. The most important finding in the present study was that chemical signatures in otoliths from each of the estuaries were distinct enough to be used as a natural mark of nursery area, despite statistically significant variations at sites within each location. By combining analyses of trace elements with carbon and oxygen isotope values, we were able to generate more powerful classification algorithms than if we had used the trace element data alone. It remains to be seen if the enhanced accuracy of the carbon and oxygen stable isotopes comes at the expense of higher error rates due to inter-annual variations within the different estuarine systems.

Oxygen isotopes in fish otoliths are deposited approximately in equilibrium with the δ18O values of
Carbon isotope ratios of DIC in Delaware co-vary with independent of precipitation rate (Thorrold et al. fish are common throughout this range (Grecay 1990). Oxygen isotopes are generally considered to be de-
Finally, as analysis of whole otoliths invariably loses information on size-specific migration patterns, it is possible that larger juveniles are moving into waters of differing elemental or isotopic composition than smaller fish. This is an adequate explanation for the $\delta^{13}C$ and $\delta^{18}O$ data (see above) and the observation that some locations (e.g. Peconic Bay) consistently showed low and positive correlations between otolith weight and elemental signatures while other locations (e.g. Delaware Bay) consistently showed high, negative correlations between the same variables.

We were surprised to see such clear differences in otolith chemical signatures among the 3 rivers sampled in Chesapeake Bay. However, there were also significant differences in salinity regimes in these rivers during the late spring and summer of 1996 (Fig. 2). The proximity of the James River to the mouth of Chesapeake Bay meant that high salinities (up to 27%) were observed during periods of low freshwater input. However, during periods of high freshwater input, salinities in the lower James River approached 0%. The York River showed less salinity variation than the James River, although salinities as high as 20% were recorded in the lower reaches during May 1996, indicating significant exchange with Chesapeake Bay water. The Rappahannock River has a shallow sill at its mouth, which acts to restrict water exchange with Chesapeake Bay. Average salinity values in the lower Rappahannock during 1996 were, therefore, lower than either the James or York Rivers, and also showed the smallest range around the mean. We hypothesize that differences in dissolved metal levels among the rivers, as indicated by salinity, were sufficient to generate the observed differences in trace element signatures in the otoliths of juvenile weakfish. Although definitive data on the trace element composition of these waters is lacking, we plan on collecting this data in the near future to test the relationships between trace element chemistry and levels of dissolved metals present in the different water masses.

While a number of workers have clearly demonstrated the potential of otolith chemistry to distinguish among adjacent adult fish populations (e.g. Edmonds et al. 1989, Campagna et al. 1995), it has proved more difficult to demonstrate that chemical signatures may also be useful as a natural marker of juvenile nursery or natal spawning area of individual fish (Campana et al. 1994, Thresher et al. 1994, Proctor et al. 1995, Severin et al. 1995, Gillanders & Kingsford 1996). The results of this study, and results from similar work on juvenile Alosa sapidissima, argue that elemental and isotopic signatures in otoliths can indeed record the natal areas of individual fish with error rates of less than 10% (Thorrold et al. 1998). We also saw a reduction in error rates of between 5 and 7% with the use of ANN to classify unknown samples when compared to LDFA. Although this is the first time that ANN has been used in an otolith chemistry application, similar improvements in classification success rates were reported in a chemostratigraphy study that also compared ANN with LDFA (Malmgren & Nordlund 1996).

It should be noted that ANNs are well suited for handling genetic information as well. It would, therefore, be easy to merge data from DNA markers with otolith chemistry signatures into a single matrix for input into an ANN model, as can be done with maximum likelihood estimation procedures.

There are important implications for the interpretation of chemical signatures in otoliths if, as we believe, the signatures are largely determined by spatial variability in the physical environment. Significant differences in trace element and isotopic signatures among geographically separated groups do not imply reproductive isolation, as would such a finding based on genetic data. Rather, these data suggest that geographic isolation has persisted for a significant portion of an individual's life. Management strategies designed to conserve genetic diversity will need to continue to rely upon DNA analyses to delineate the spatial extent of genetic mixing. However, a significant problem with genetic markers is that ecologically inconsequential rates of genetic exchange, probably less than 1%, are sufficient to maintain Hardy-Weinberg equilibrium among populations. This appears to be a common situation in marine fishes, in which geographically isolated populations are genetically linked by even small amounts of larval dispersal or adult vagrancy (e.g. Graves et al. 1992, Gold et al. 1997). An environmentally induced natural tag such as trace element or isotopic signatures in otoliths will allow fish ecologists to quantify exchange rates among meta-populations in situations where these rates could not be quantified using genetic approaches.

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