# Population connectivity of southern flounder in the US South Atlantic revealed by otolith chemical analysis

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ABSTRACT: Reconstructing the movements of fish among different environments and incorporating patterns of spatial population structure contribute to improved accuracy in the assessment of marine fishery resources. The southern flounder *Paralichthys lethostigma* is a valuable flatfish throughout its range in the US South Atlantic and Gulf of Mexico, but stock conservation and management is hindered by critical knowledge gaps related to patterns of movement and the level of mixing among populations. Identifying stock structure and connectivity can be challenging in species with complex life histories, and otolith geochemical signatures have been effectively used as natural markers to estimate population connectivity in migratory fishes. With this approach, we inferred the degree of exchange of southern flounder among broad US South Atlantic regions by predicting the nursery origins of adults captured in North Carolina and South Carolina estuaries. Baseline nursery profiles were first established by analyzing stable isotopes ($\delta^{13}$C, $\delta^{18}$O) and trace elements (Mg:Ca, Mn:Ca, Sr:Ca, Ba:Ca) sampled from juvenile southern flounder otoliths. Quadratic discriminant analysis discriminated among state-scale nursery regions (North Carolina, South Carolina, and Florida) with 72% cross-validation accuracy. Adult southern flounder from the same cohort were subsequently classified to the atlas of nursery signatures obtained from the juvenile fish using a maximum likelihood mixed stock analysis. Results revealed a lack of nursery-state fidelity and the potential for broad-scale movement of post-migratory adults along the US South Atlantic coast, which will contribute to the definition of appropriate spatial scales for management.

KEY WORDS: Nursery origin · Stock structure · Migration · Stable isotopes · Laser ablation ICP-MS · *Paralichthys lethostigma*

## INTRODUCTION

Reconstructing the movements of fish among different environments and incorporating patterns of spatial population structure are necessary steps for comprehensive and accurate assessments of marine fishery resources (Goethel et al. 2011, Secor 2015). While fisheries management aims to preserve the sustainable productivity of a fish stock, these efforts are often hindered by the inherent difficulty both in identifying stocks and quantifying the degree of exchange among them (Begg et al. 1999). Complex

life histories, particularly those that include adult migration and larval dispersal, which each occur over broad spatial scales, make it especially challenging to isolate a geographically distinct stock (Begg & Waldman 1999, Stephenson 1999). Most contemporary studies of population connectivity have focused on movement in the context of early life history stages, modeling patterns of larval transport and retention that are driven largely by oceanographic processes. Recently, there has been renewed appreciation for the role of adult movements, motivated by an improved understanding of individual physiology and behavior (Nathan et al. 2008). This has provided a fuller understanding of connectivity and the spatial scale of fisheries productivity (Frisk et al. 2014). As such, determining the appropriate spatial scale of management requires consideration of the potential for movement at all life history stages of a species (Fogarty & Botsford 2007, Goethel et al. 2011).

For migratory fishes, multiple transitions among juvenile, adult, and spawning habitats can make quantifying the magnitude of mixing among populations complicated (Gillanders et al. 2003). Direct estimates of movement can be made through the application of artificial tags, using conventional non-transmitting tags combined with traditional mark–recapture designs to track movements among locations at broader spatial scales, or through the use of acoustic and/or satellite tags to reconstruct individual fish behavior with greater spatial resolution (McKenzie et al. 2012). However, conventional tagging studies are often limited by low recapture rates and reporting bias, and advanced acoustic/satellite tagging methods can be cost-prohibitive and are often logistically constrained in both spatial extent and sample size (reviewed in Elsdon & Gillanders 2003, Cadrin et al. 2013).

The use of natural tags, particularly otolith geochemical signatures, has emerged as a valuable method for indirectly inferring patterns of fish movement (reviewed in Elsdon et al. 2008). Otoliths (ear stones) grow by daily incremental deposits of calcium carbonate and are metabolically inert, so any chemical constituents incorporated from the ambient environment during the accretion process are permanently retained (Campana 1999). Thus, otoliths can provide a chronological record of the different environments that a fish has been exposed to throughout its lifetime. Determining patterns of residency and migration, as well as assessing stock structure and the degree of population mixing, only require that different water masses leave distinct chemical signatures in the otoliths of the fish residing in them

(Campana & Thorrold 2001). By comparing the water masses where fish resided as juveniles to the water masses where they were captured as adults, otolith geochemical signatures can be used as natural markers to study fish movement and population connectivity (Thorrold et al. 1998, Thorrold et al. 2001).

Southern flounder *Paralichthys lethostigma* inhabit estuarine and nearshore marine waters in the US South Atlantic and northern Gulf of Mexico, supporting commercial and recreational fisheries of economic and cultural importance (Ginsburg 1952, Gilbert 1986). At present, North Carolina is the only state in the US South Atlantic with a comprehensive management plan for southern flounder (NCDMF 2013). Southern flounder in North Carolina are currently categorized as depleted, and are managed as a unit stock, which assumes a closed population within state waters with no meaningful contribution of recruits or migrating adults from other regions (Takade-Heumacher & Batsavage 2009, NCDMF 2013).

The life history of southern flounder suggests the strong potential for mixing among geographic regions at spatial scales broader than state boundaries. Adult southern flounder emigrate from estuaries during fall, prior to winter spawning in continental shelf waters. Although the precise location of spawning is presently unknown, spawning activity is presumed to peak between December and February based on ichthyoplankton ingress surveys conducted in the northern portion of the species' range (Taylor et al. 2010). Pelagic larvae ingress through inlets, after spending 30 to 60 d in the water column, and recruit to estuarine habitats during late winter and early spring (Ginsburg 1952, Wenner et al. 1990, Burke et al. 1991). After settlement, juvenile southern flounder remain in estuaries until maturation, typically 2 to 3 yr of age (Midway & Scharf 2012), before initiating spawning-related migrations to oceanic waters. Following offshore spawning, adults may return to inshore waters, although the relative distribution of post-spawning individuals between estuarine and nearshore oceanic habitats remains unclear (Monaghan 1996, Watterson & Alexander 2004), and some adults may remain offshore permanently (Midway et al. in press). Conventional tag-return studies have revealed limited movement by southern flounder during estuarine residence, but also the potential for long-range directional movement during and after spawning migrations (Monaghan 1996, Craig et al. 2015). In addition, a lack of otolith morphometric and genetic differentiation across the US South Atlantic provides evidence of basin-wide mixing (Midway et al. 2014, Wang et al. 2015). The considerable uncer-

tainty regarding the oceanic movements and migratory patterns of adult southern flounder limits efforts to define stock boundaries that are appropriately aligned with the spatial scale of important ecological processes.

Here, we used an otolith geochemical approach, combining stable isotopes and trace elements to infer the degree of exchange among southern flounder subpopulations in the US South Atlantic. Trace elemental signatures in southern flounder otoliths have been used successfully in previous studies to examine migration timing, exposure to low salinity waters, and general patterns of estuarine habitat use (Taylor et al. 2008, Lowe et al. 2011, Farmer et al. 2013, Nims & Walther 2014), indicating their potential utility to inform population connectivity over broader spatial scales. First, we examined natural otolith tags in premigration juvenile southern flounder to determine whether geochemical profiles were distinct among geographically separate estuarine nurseries along the US South Atlantic coast. Subsequently, we used those juvenile otolith geochemical signatures as a baseline nursery atlas to retrospectively classify postmigration adult southern flounder to nurseries of origin, allowing for estimates of connectivity among US South Atlantic southern flounder following offshore migration.

## MATERIALS AND METHODS

### Sample collection

Sagittal otoliths for the baseline nursery atlas were collected between March and November 2012 from age-1 juvenile southern flounder that were part of the 2011 year class. Juveniles were collected from multiple estuarine regions along the US South Atlantic coast in 3 states: North Carolina (NC), South Carolina (SC), and Florida (FL) (Fig. 1, Table 1). Southern flounder ages were determined using otolith annulus counts. Juvenile otolith samples from NC and SC were obtained from archived collections associated with fishery-independent surveys conducted by the marine fishery management agency in each state. Within NC, samples originated from 3 regions: Pamlico Sound, the New River estuary, and the Cape Fear River estuary. Within SC, samples originated from 2 regions: northern SC (combining collection sites in Cherry Grove, Winyah Bay, and Cape Romain) and southern SC (combining collection sites in Charleston Harbor, Folly River, Kiawah River, and St. Helena Sound). Juvenile otolith sam-
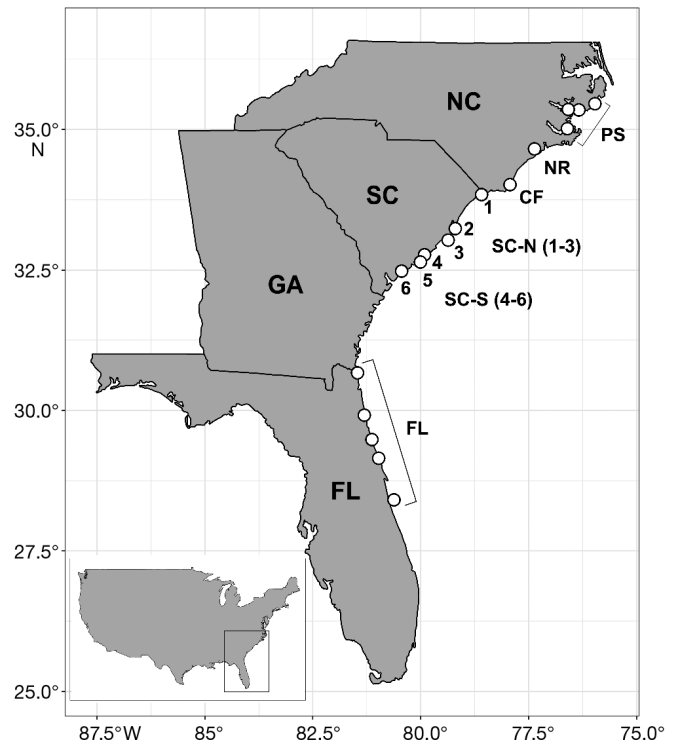


Fig. 1. State and regional-scale estuarine sampling for age-1 southern flounder along the US South Atlantic coast. North Carolina (NC; regions: PS: Pamlico Sound; NR: New River; CF: Cape Fear River), South Carolina (SC; regions: SC-N: South Carolina North; SC-S: South Carolina South), Georgia (GA; not sampled), and Florida (FL; all samples grouped, symbols represent approximate spatial range based on fish collection points). Within SC, individual collection sites are represented by numbers: (1) Cherry Grove, (2) Winyah Bay, (3) Cape Romain, (4) Charleston Harbor, (5) Folly and Kiawah Rivers, and (6) St. Helena Sound

ples from FL were sourced from licensed seafood dealers located along the northeast FL coast between Jacksonville and Titusville (approximately 28° 38′ N to 30° 40′ N). While all FL samples were confirmed to have been harvested in estuarine waters, otoliths

Table 1. Age, sample size (n), and mean total length (±1 SD) for southern flounder collected from 3 states across the US South Atlantic from 2012 to 2015. NC: North Carolina; SC: South Carolina; FL: Florida; ND: no data

| Collection | Age year | Year class | State | n | Total length (mm) |
|---|---|---|---|---|---|
| 2012 | 1 | 2011 | NC | 88 | 347.3 ± 51.3 |
| | | | SC | 77 | 290.5 ± 80.0 |
| | | | FL | 30 | 402.5 ± 38.5 |
| 2014 | 3 | 2011 | NC | 23 | 534.8 ± 62.3 |
| | | | SC | 17 | ND |
| 2015 | 4 | 2011 | NC | 7 | 539.6 ± 123.3 |

were pooled into a single FL state-scale group because exact capture locations of individual fish were not known. Southern flounder are present in Georgia estuaries, but preservation of otoliths during fishery-independent surveys was not standard practice at that time, so archived samples from the 2011 year class could not be obtained for this study.

Otoliths for adult classification were obtained from age-3 and age-4 southern flounder during 2014 and 2015, respectively; these fish were also in the 2011 year class. Adult southern flounder otoliths were obtained from archived collections associated with fishery-independent and fishery-dependent surveys in both NC and SC. NC adults were collected from estuarine and nearshore coastal waters in various locations across the state. With the exception of a single individual from Murrell's Inlet in northern SC, all SC adults were likely captured in Charleston Harbor (J. Tucker, SCDNR, pers. comm.). The exact capture locations of several SC adults are uncertain because most samples were obtained during recreational fishing tournaments. Poor catches of southern flounder during 2014 and 2015 in FL prevented us from obtaining otoliths of adult fish from FL waters. Archived otoliths from NC were stored in ethanol, while SC and FL otoliths were stored dry. Previous studies have shown that otolith elemental concentrations are not influenced by ethanol preservation (Milton & Chenery 1998, Hedges et al. 2004).

## Otolith preparation and analysis

Geochemical analyses for both juvenile and adult samples were conducted on material isolated from the region of the otolith corresponding to the first year of estuarine growth, between the core and first annulus, using southern flounder from the 2011 cohort. Otoliths for stable isotope analysis were first cleaned with 30% $H_2O_2$, then triple rinsed with $diH_2O$ and air dried. Otoliths were mounted on glass slides using thermoplastic adhesive, and cut using a low-speed saw to remove a 0.5 mm transverse section containing the core. The resulting sections were mounted on glass slides using thermoplastic adhesive, and a micromill sampling system (New Wave Research/Electro Scientific Industries) was used to isolate the region of the otolith corresponding to the first year of juvenile estuarine growth. Powdered otolith material was produced by milling between the core and the first annulus along a 600 µm drill path (Fig. 2) using a round carbide drill bit with a 300 µm head width (Brasseler), milling in successive drill depths (10 µm pass$^{-1}$) until a target depth of 300 µm was reached. Otolith powder was transferred to septum-capped glass vials for stable isotope analysis, during which samples were analyzed in random order to avoid systematic bias.

Otolith $\delta^{13}C$ and $\delta^{18}O$ were measured on a Delta V Plus isotope ratio mass spectrometer (Thermo Scientific) at the Isotope Ratio Mass Spectrometry Core Facility, Center for Marine Science, University of North Carolina Wilmington. Powdered samples were reacted with phosphoric acid under vacuum at 70°C, and the evolved carbon dioxide was measured for relative isotopic abundance. Carbonate standards NBS-18 and NBS-19 (National Institute of Standards and Technology, NIST) were measured to calibrate isotope ratio measurements; external precision was ± 0.09 ‰ (SD) for $\delta^{13}C$ and ± 0.14‰ for $\delta^{18}O$ based on repeated measures of the standards (9 of each standard per 60 otolith samples). Otolith isotopic compositions were reported in standard delta ($\delta$) notation as per mille (‰) variations relative to Vienna Pee Dee Belemnite.

Otoliths for trace elemental analysis were pre-cleaned as outlined above for stable isotope analysis, then acid-washed with 1% $HNO_3$, triple rinsed with ultrapure water (Milli-Q; EMD Millipore Corpora-
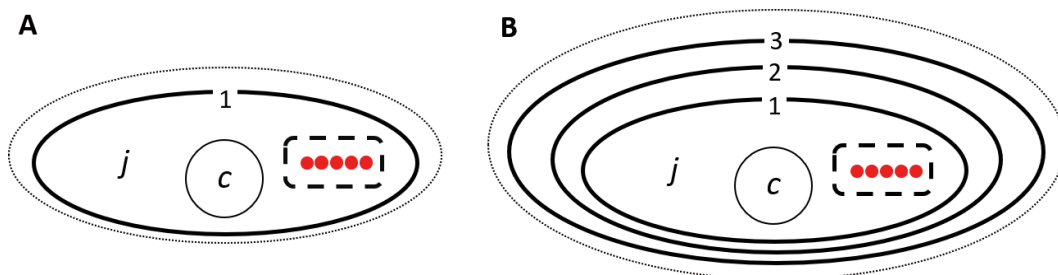
Fig. 2. Regions of southern flounder otolith sections sampled. Dashed rectangles: micromill drilling region for stable isotope analysis; solid red circles: laser ablation spots for trace element analysis in (A) age-1 and (B) age-3+ southern flounder. Regardless of fish age, all material was sampled within the juvenile (*j*) portion of the otolith, representing the first year of estuarine growth. Numbered bold lines represent annuli, and the otolith core is represented by the open circle (*c*). Although presented together in this schematic for visualization, stable isotope and trace element sampling were conducted on separate otolith sections

tion), and air-dried in polypropylene vials. All laboratory materials for trace elemental analysis preparation were acid-washed in 6 M $HNO_3$ prior to use. Cleaned otoliths were embedded in epoxy resin and a 1 mm transverse section including the core was removed using a low-speed saw. Embedded otolith sections were mounted on glass slides with thermoplastic adhesive, ground on one side with P800 and P2500 grit CarbiMet abrasive paper (Buehler) until the core was exposed, then polished manually with 0.3 µm Micro-Polish II alumina powder and microcloth (Buehler) until the surface was free of superficial blemishes. Polished sections were randomized, mounted on petrographic slides with thermoplastic adhesive, then sonicated in ultrapure water and air-dried.

Otolith elemental composition was measured using an XSERIES 2 inductively coupled plasma mass spectrometer (Thermo Fisher Scientific) coupled with a NWR213 laser ablation system (New Wave Research/Electro Scientific Industries) at Texas A&M University at Galveston, TX. We quantified $^7Li$, $^{24}Mg$, $^{55}Mn$, $^{59}Co$, $^{65}Cu$, $^{66}Zn$, $^{88}Sr$, and $^{137}Ba$, with $^{44}Ca$ measured as an internal standard. Five adjacent 50 µm spots were ablated along a transect (~400 µm) between the core and first annulus of each otolith, sampling a region comparable to the material sampled for stable isotope analysis (Fig. 2). Laser repetition rate was 20 Hz with a 12 s dwell time and 80% laser energy (~0.5 mJ). Each spot was pre-ablated prior to quantification to remove surface contamination. To calibrate the measurements and control for instrument drift and sensitivity, a glass reference standard (NIST 614) was analyzed 3 times prior to and once after every 5 to 10 ablation spots (1 to 2 otolith samples). External precisions were 8.3% for Li:Ca, 2.0% for Mg:Ca, 3.5% for Mn:Ca, 14.0% for Co:Ca, 13.9% for Cu:Ca, 9.9% for Zn:Ca, 2.2% for Sr:Ca, and 4.2% for Ba:Ca based on relative standard deviations of the reference material. Limits of detection (LOD) for each element were calculated as 3 standard deviations above the mean concentrations of the carrier gas blanks analyzed prior to each ablation. Elemental concentrations are expressed as µmol $mol^{-1}$ ratios relative to calcium (element:Ca). Estimates of LOD were 9.95 µmol $mol^{-1}$ for Li:Ca, 23.0 µmol $mol^{-1}$ for Mg:Ca, 1.79 µmol $mol^{-1}$ for Mn:Ca, 25.17 µmol $mol^{-1}$ for Co:Ca, 5.90 µmol $mol^{-1}$ for Cu:Ca, 6.12 µmol $mol^{-1}$ for Zn:Ca, 1.87 µmol $mol^{-1}$ for Sr:Ca, and 0.03 µmol $mol^{-1}$ for Ba:Ca. All Mg:Ca, Mn:Ca, Sr:Ca, and Ba:Ca sample measurements were above LOD; sample measurements for the remaining 4 elements consistently fell below LOD and were removed from further analysis.

## Otolith sampling for quality control analysis

Approximately 12% of juvenile otolith samples (n = 24) were subject to repeat stable isotope analysis beginning from the milling stage of preparation to ensure replicability. Due to availability, the otolith side quantified (left or right) varied, as did the precise location of sectioning along the transverse plane (centered or adjacent). When both otoliths in a pair were available, the left otolith was used for stable isotope analysis and the right otolith was used for trace element analysis (NC juvenile samples). When only one otolith from an individual was available, priority was given to the more intensive trace element preparation. In such instances, trace element preparation was first conducted on the centered transverse section, then a second transverse section directly adjacent and parallel to the original section was taken from the otolith for stable isotope analysis (SC and FL: right; NC adult: left). Although off-center, this adjacent transverse section still contains the region of juvenile estuarine growth within the first annulus that was targeted in all sampling efforts. NC adult paired otoliths (n = 21) were tested for differences in stable isotope quantification between right and left otoliths as well as between centered transverse sections and adjacent transverse sections. Due to limitations on instrument time, examination of these differences for trace elements was not feasible.

## Statistical analysis

Otolith signatures in our analyses consisted of 2 stable isotopes ($\delta^{13}C$, $\delta^{18}O$) and 4 trace elements (Mg:Ca, Mn:Ca, Sr:Ca, Ba:Ca). Of the 5 ablations quantified for trace elements for each otolith, the ablations with the highest and lowest concentrations for each trace element were excluded and the mean value of the remaining 3 ablations was used for all further analyses. Removal of the highest and lowest ablation concentrations for each trace element did not result in any appreciable bias, as comparisons with analyses retaining all 5 ablations resulted in no changes to quadratic discriminant analysis classification accuracy both overall and by state. Data for each stable isotope and trace element were visually assessed for univariate normality and log transformed as necessary (Mn:Ca, Ba:Ca), although most of the geochemical variables still exhibited mild departures from normality.

We assessed broad-scale discrimination among nursery sites by testing among states (NC, SC, FL) along the US South Atlantic coast. Analyses were conducted on a state scale because separation at the state level is most relevant from a management perspective. We also conducted analyses on a regional scale (estuaries within states) to evaluate finer ecological processes contributing to southern flounder connectivity (e.g. natal homing). Regional-scale results were less conclusive, and thus are largely not presented in full here, with the exception of several analyses specified below. First, multivariate analysis of variance (MANOVA) was used to test for differences in the combined otolith geochemical signature among nursery states. We used Pillai's trace test, which is considered to be the most robust of the MANOVA test statistics when distributional assumptions are violated (Quinn & Keough 2002). Analysis of variance (ANOVA) was then performed to test for differences in individual stable isotopes and trace elements among the 3 US South Atlantic states represented. Visual inspection of ANOVA model residuals revealed that departures from normality and homogeneity of variance were modest, so we proceeded with parametric analyses. Data were standardized (mean-centered, unit variance) prior to multivariate analyses.

Quadratic discriminant analysis (QDA) was conducted to test the ability of the multivariate geochemical signatures to accurately classify individual juvenile southern flounder to their known nurseries (states) of origin. QDA is robust to departures from normality and does not have the assumption of homogeneity of variance–covariance matrices (McGarigal et al. 2000). Classification accuracy was assessed with jackknife (leave-one-out) cross-validation, using uniform priors for group membership. We evaluated QDA classification accuracies for all possible combinations of trace elements and stable isotopes and found that the optimized combination of geochemical variables ($\delta^{13}C$, $\delta^{18}O$, Sr:Ca, Ba:Ca) only slightly outperformed the QDA with all variables ($\delta^{13}C$, $\delta^{18}O$, Mg:Ca, Mn:Ca, Sr:Ca, Ba:Ca), which yielded more consistent results. Thus, our multivariate analyses proceeded with all geochemical variables included. In linear discriminant analysis (LDA), canonical variates (linear combinations of the original variables) can be used to visualize the results. QDA does not produce natural canonical variates, so we used sliced average variance estimation (SAVE) (Pardoe et al. 2007) to calculate reduced-dimension variates for graphical QDA representation using the 'dr' package in R (Weisberg

2002). QDA classification accuracy was also assessed for nurseries on a regional scale (estuaries within states), but classifications were less successful so only a concise description of regional-scale QDA results is included.

Adult (age 3+) southern flounder collected in NC and SC waters were retrospectively classified to nursery states of origin (NC, SC, or FL) using a mixed stock algorithm to derive maximum likelihood estimates (MLE) (Millar 1987), with the sample means and covariance matrices for the juvenile nursery signatures providing the baseline for assignment. MLE was conducted using the 'mixtools' package in R (Benaglia et al. 2009), with 1000 bootstrap replicates to assess the error associated with the estimated nursery proportions. For classifying unknown individuals when true mixture proportions are uneven, MLE outperforms QDA and is the preferred method for estimating mixed-stock proportions and group assignments (White & Ruttenberg 2007).

One limitation of this assignment method is that MLE can only classify adults to characterized reference locations, and individuals are assigned to a nursery source even if their otolith chemical signatures are quite distinct from all sampled baseline sources. To evaluate the proportion of adults that were unlikely to have originated from any of the sampled nurseries, we followed the procedure used by Standish et al. (2008) in a similar classification scenario. We estimated the cumulative probability that the combined Ba:Ca and $\delta^{18}O$ otolith signature for each adult was drawn from any of the 3 nursery states, given the bivariate normal distribution defined by the means and covariances for each nursery state. Ba:Ca and $\delta^{18}O$ values were used in this analysis because they showed strong discrimination among nursery states. Adults were identified as having likely originated from an unsampled nursery state if they had probabilities <5% for all sampled nurseries.

For quality control, we analyzed 2 types of test stable isotope measurements: repeat measurements of the same otolith to test replicability, and measurements of paired otoliths to compare right/left otoliths as well as centered/adjacent transverse section locations. Stable isotope measurements were compared with paired $t$-tests. To assess how these differences would impact our results, we also substituted the test stable isotope measurements in the geochemical signatures for QDA (repeat measurements) and MLE analyses (paired otoliths). All statistical analyses were conducted in R v.3.3.0 (R Core Team 2016).

## RESULTS

### Nursery atlas

Otolith stable isotope ratios ($\delta^{13}$C, $\delta^{18}$O) and elemental concentrations (Mg:Ca, Mn:Ca, Sr:Ca, Ba:Ca) varied among juvenile southern flounder (n = 195) on a state scale (Fig. 3), with potential latitudinal patterns observed for $\delta^{13}$C, $\delta^{18}$O, and Mn:Ca. These patterns were less clear when comparing otolith geochemical variables on a regional scale (see Fig. S1 in the Supplement at www.int-res.com/articles/suppl/ m596p165_supp.pdf). The combined geochemical signatures differed significantly among states (MANOVA: Pillai's trace = 0.673, $F_{12,376}$ = 15.91, p < 0.001). Most of the individual stable isotope ratios and elemental concentrations also differed significantly among states (ANOVA: p < 0.05, see Table S1 in the Supplement), with the exception of Mg:Ca, which did not demonstrate significant spatial variation (ANOVA: p = 0.075, see Table S1).

Based on the multivariate geochemical signatures, cross-validated QDA accurately classified 71.8% of juvenile southern flounder to the state from which
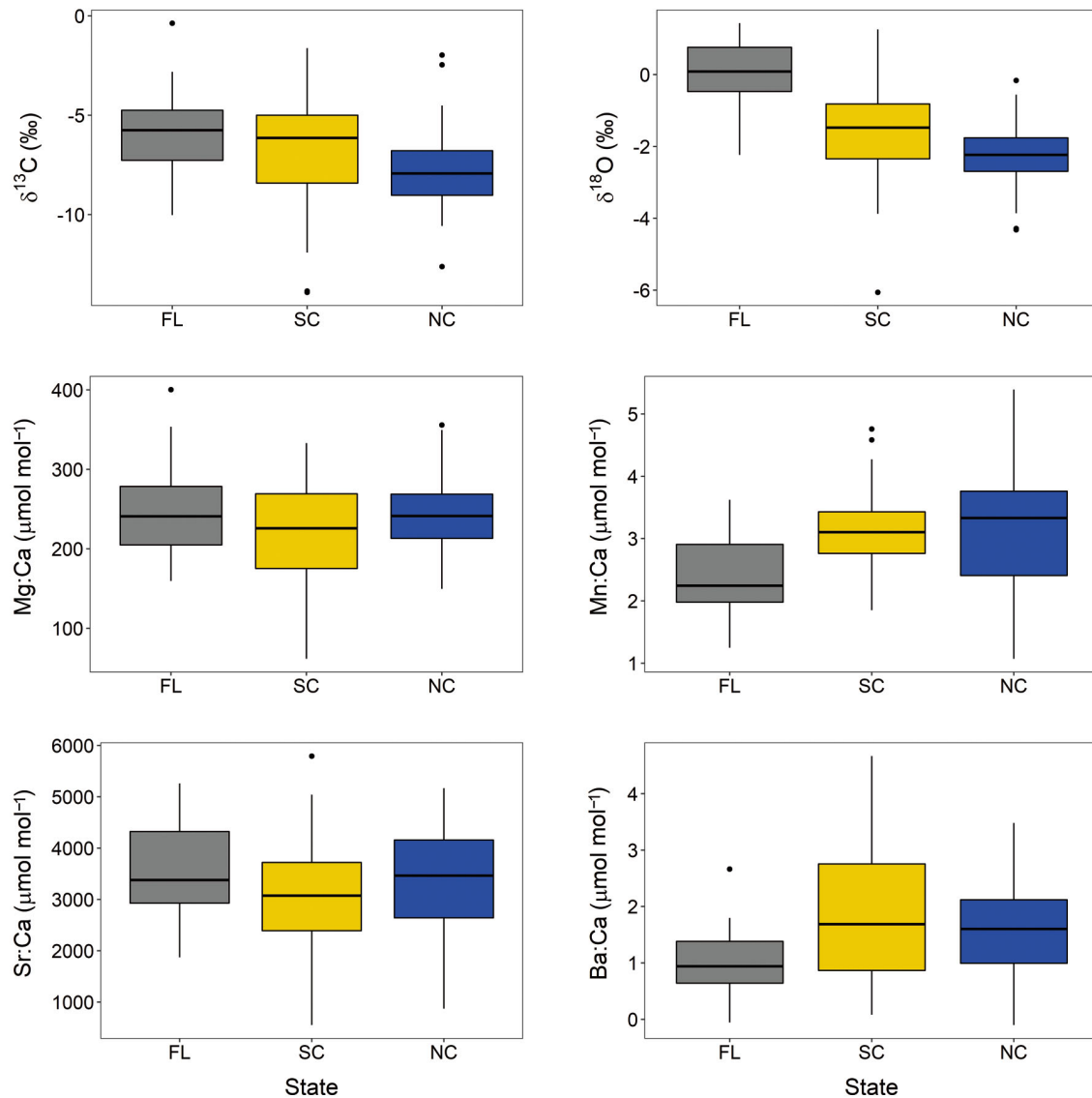


Fig. 3. Age-1 southern flounder otolith stable isotope ratios and trace element concentrations by state. Horizontal lines: median value; lower and upper box edges: 25th and 75th percentiles, respectively; whiskers extend to the largest and smallest measured value within 1.5 × IQR (interquartile range; difference between 75th and 25th percentile); filled symbols: outliers beyond the range of 1.5 × IQR. Log-transformed values are presented for Mn:Ca and Ba:Ca to overcome considerable skewness in the raw data. FL: Florida; SC: South Carolina; NC: North Carolina
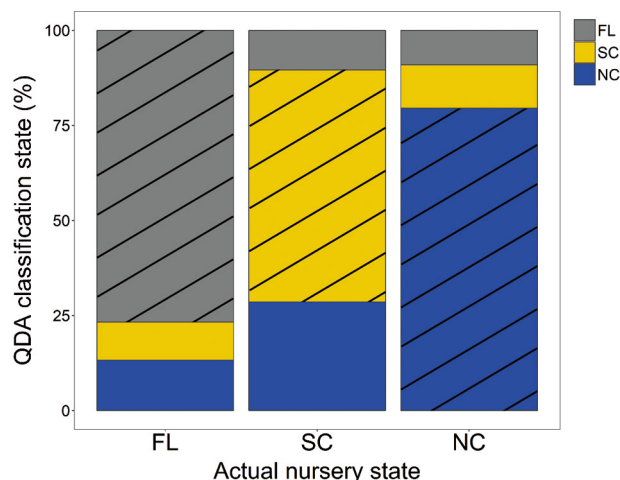
Fig. 4. Cross-validated classification accuracy by state for quadratic discriminant analysis (QDA) conducted on age-1 southern flounder otolith signatures. Known states of origin for juveniles are listed along the *x*-axis; the proportion of individuals (percentages) assigned to each state by QDA classification is indexed on the *y*-axis, as represented by colors. Diagonal patterns indicate correct assignment. FL: Florida; SC: South Carolina; NC: North Carolina
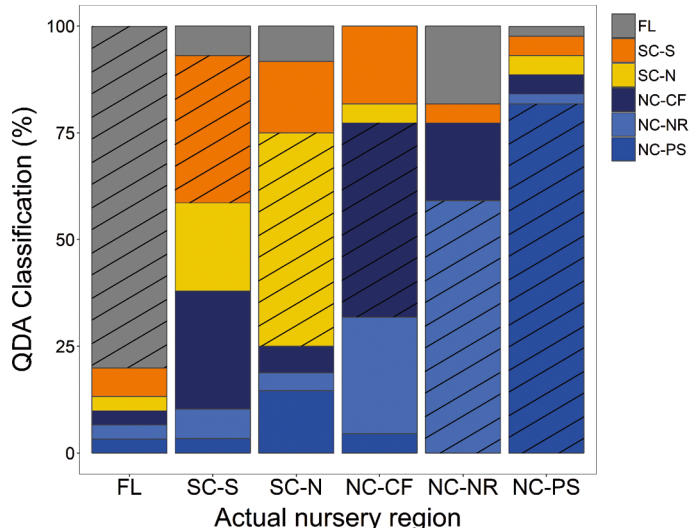


Fig. 5. Cross-validated classification accuracy by region (broad estuarine nurseries within states) for quadratic discriminant analysis (QDA) conducted on age-1 southern flounder otolith signatures. Known regions of origin for juveniles are listed on the *x*-axis; the proportion of individuals (percentages) assigned to each region by QDA classification is indexed on the *y*-axis, as represented by colors. Diagonal patterns indicate correct assignment. FL: Florida; SC-S: South Carolina south; SC-N: South Carolina north; NC-CF: North Carolina Cape Fear; NC-NR: North Carolina New River; NC-PS: North Carolina Pamlico Sound

they were collected. Classification accuracy was highest in NC (79.5%) and FL (76.7%), and lowest in SC (61.0%) (Fig. 4). Most of the misclassified juvenile signatures from SC were assigned to NC, the adjacent state. Regional-scale QDA was less successful, with cross-validated QDA accurately classifying 60.0% of juvenile southern flounder overall to the correct nursery region. Regional classification accuracy ranged from 34.5% (southern SC) to >80% (FL and Pamlico Sound, NC). Most regional classification errors still placed juvenile otolith signatures within the correct state (83.0, 62.3, and 80.0% correctly assigned to NC, SC, and FL, respectively), although individuals from SC regions were commonly misclassified to NC regions (Fig. 5). Because of low confidence in the regional nursery atlas to accurately discriminate among broad estuarine regions used as nurseries by southern flounder, regional-scale analyses were not pursued further. Only state-scale nursery analyses are presented hereafter.

We observed separation of nursery geochemical signatures among states along the first and second SAVE variates (estimates of canonical variates for QDA), although overlap in signatures was apparent, particularly between NC and SC (Fig. 6A). The first SAVE variate was influenced largely by differences in Ba:Ca concentrations (Table 2). The second and third SAVE variates were influenced most by Mn:Ca and $\delta^{18}O$, respectively (Table 2). Graphical visualiza-

tion of the second and third SAVE variates revealed a more well-defined distinction between northern (NC, SC) and southern (FL) otolith geochemical signatures, likely driven by differences in $\delta^{18}O$ among nursery habitats from states spanning a large latitudinal distance (Fig. 6B). Clear clustering of NC signatures and additional separation of SC can be visualized in plots of additional pairs of SAVE variates (see Fig. S2 in the Supplement).

## Classification of adults using nursery atlas

Geochemical signatures from adult southern flounder, sampled from the otolith region corresponding to the first year of juvenile estuarine residency (2011), were used to estimate the contributions of each state-scale nursery to older fish (age 3+) captured subsequently (2014 or 2015) in NC and SC (n = 47). MLE mixed stock predictions indicated extensive mixing within the US South Atlantic, with adults from NC and SC sourced to all characterized nursery states (Table 3, Fig. 7). Adults from NC were predicted to have originated from SC (36.7%) nearly as often having local NC nursery origins (40.0%). Adults from SC
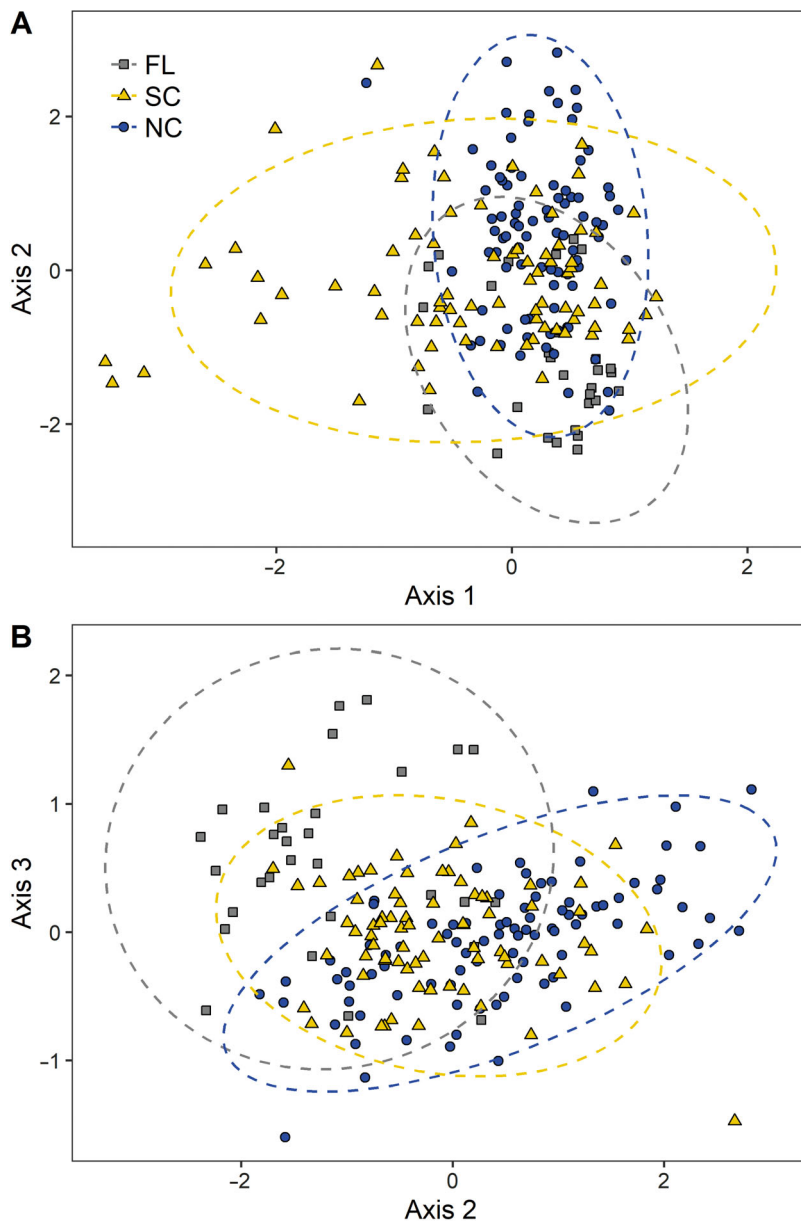
Fig. 6. Quadratic discriminant analysis (QDA) conducted on age-1 southern flounder otolith signatures among states, as visualized by the discriminant plot scores on 2 pairs of sliced average variance estimate (SAVE) variates: (A) Axis 1 and Axis 2; (B) Axis 2 and Axis 3. Ellipses: 95% confidence intervals. States are represented by different color/symbol combinations. FL: Florida; SC: South Carolina; NC: North Carolina

were estimated to be from a NC nursery source (47.1%) with greater frequency than from a local SC nursery source (23.5%). Further, 26.4% of NC and SC adults originated from FL nurseries.

Individual MLE classification probabilities were generally high, with all adults assigned to nursery states of origin with >70% probability, and all but 3 nursery assignments achieved >90% probability

(Fig. 7). Adult geochemical signatures plotted in nursery atlas ordination space fell almost entirely within the 95% confidence ellipses for the nursery signatures, with the exception of 2 individuals (Fig. 8). The presence of few stray adult signatures within the nursery atlas context suggests that potential bias due to unsampled nurseries may be limited (Chittaro et al. 2009). However, MLE can only classify adults to characterized sources, and all individuals (including those from unsampled sources) are assigned to a nursery state. Using Ba:Ca and $\delta^{18}O$ signatures, we estimated the proportion of adults that were unlikely to have come from either FL, SC, or NC, and we identified 11 adults (23%) with >95% probability of having originated from a source outside of our sampled nursery states.

## Quality control analysis

Assessments of repeated measurements and comparisons among paired sectioning locations of otolith $\delta^{13}C$ and $\delta^{18}O$ yielded mixed results. When evaluating juvenile otoliths subject to repeat stable isotope analysis, the mean difference between repeated $\delta^{13}C$ and $\delta^{18}O$ measurements was significantly greater than zero (Paired $t$-test: p < 0.05, see Table S2 in the Supplement). However, substituting the repeated stable isotope measurements in juvenile otolith signatures for the original values resulted in nearly identical QDA outcomes, with changes in <2% of cross-validated QDA classifications. For adult paired otoliths, $\delta^{13}C$ and $\delta^{18}O$ comparisons were made among 3 section types: left otolith adjacent sections (L-A), right otolith adjacent sections (R-A), and right otolith centered sections (R-C). Mean differences were not significantly different from zero for comparisons between $\delta^{13}C$ values from L-A compared with R-C or R-A, and $\delta^{18}O$ values from R-A compared with L-A or R-C (paired $t$-test: p > 0.05, see Table S2). Mean differences were significantly different from zero when comparing $\delta^{13}C$ values

Table 2. Eigenvalues and weights associated with each canonical variate for state-scale quadratic discriminant analysis conducted on otolith chemical signatures of age-1 southern flounder in the US South Atlantic, as estimated by sliced average variance estimation (SAVE). Eigenvalues and weights represent the discriminatory power of each SAVE variate and the contributing geochemical variables, respectively

|  | SAVE 1 | SAVE 2 | SAVE 3 | SAVE 4 | SAVE 5 | SAVE 6 |
|---|---|---|---|---|---|---|
| Eigenvalue | 0.620 | 0.370 | 0.295 | 0.239 | 0.177 | 0.031 |
| Geochemical variable weights |  |  |  |  |  |  |
| $\delta^{13}C$ | 0.021 | −0.387 | −0.431 | −0.787 | 0.488 | −0.116 |
| $\delta^{18}O$ | −0.222 | −0.459 | 0.728 | 0.178 | −0.043 | −0.038 |
| Mg:Ca | 0.076 | 0.113 | −0.102 | 0.443 | 0.548 | −0.618 |
| Mn:Ca | 0.138 | 0.734 | 0.480 | −0.356 | 0.262 | 0.294 |
| Sr:Ca | 0.434 | 0.181 | 0.115 | −0.133 | −0.545 | −0.415 |
| Ba:Ca | −0.859 | −0.233 | −0.173 | −0.087 | 0.308 | −0.587 |

Table 3. Predicted nursery origins of age-3+ southern flounder collected from North Carolina (NC) and South Carolina (SC) based on maximum likelihood estimation. Individual assignments and overall mixture proportions by section type are given as percentages. Estimates of error (±1 SE) based on bootstrap replicates are provided for mixture proportions. Predicted nursery origins (states) are also presented for mixture analyses conducted with stable isotope measurements from alternate test sections substituted for the original data (left-adjacent). FL: Florida

| Adult location | Predicted nursery origin | | |
|---|---|---|---|
|  | % NC | % SC | % FL |
| Summary of individual assignments |  |  |  |
| Overall | 42.6 | 31.9 | 25.5 |
| NC | 40.0 | 36.7 | 23.3 |
| SC | 47.1 | 23.5 | 29.4 |
| Mixture proportions by section type (overall) |  |  |  |
| Left-adjacent (L-A) | 41.3 ± 7.8 | 32.2 ± 7.0 | 26.4 ± 6.4 |
| Right-adjacent (R-A) | 37.9 ± 7.8 | 35.2 ± 7.5 | 26.9 ± 6.5 |
| Right-centered (R-C) | 47.1 ± 7.3 | 21.2 ± 5.5 | 31.7 ± 6.7 |

between R-A and R-C and $\delta^{18}O$ values between L-A and R-C (paired $t$-test: p < 0.05, see Table S2). When substituting the stable isotope measurements with the different section types in adult MLE mixed stock analysis, we observed discrepancies in 30% of the predicted nursery origin assignments among data sets containing the 3 section types. However, overall mixture proportions remained relatively stable and estimates of membership to each nursery state were within the bootstrapped confidence intervals among the section types (Table 3).

## DISCUSSION

Combined stable isotope ($\delta^{13}C$, $\delta^{18}O$) and multi-elemental (Mg:Ca, Mn:Ca, Sr:Ca, Ba:Ca) otolith signatures allowed for accurate discrimination among state-scale southern flounder nurseries in the US South Atlantic. By comparing the nursery otolith signatures of adult southern flounder to the baseline juvenile nursery atlas, we found evidence for extensive mixing of southern flounder adults among US South Atlantic states. This mixing presumably occurs following offshore spawning migrations, as flounder migrate back to estuaries. Broad-scale mixing of southern flounder in the US South Atlantic basin inferred from our results is consistent with recent investigations of stock structure using otolith morphometrics and population genetics, which found only weak evidence for population differentiation among states (Midway et al. 2014, Wang et al. 2015).
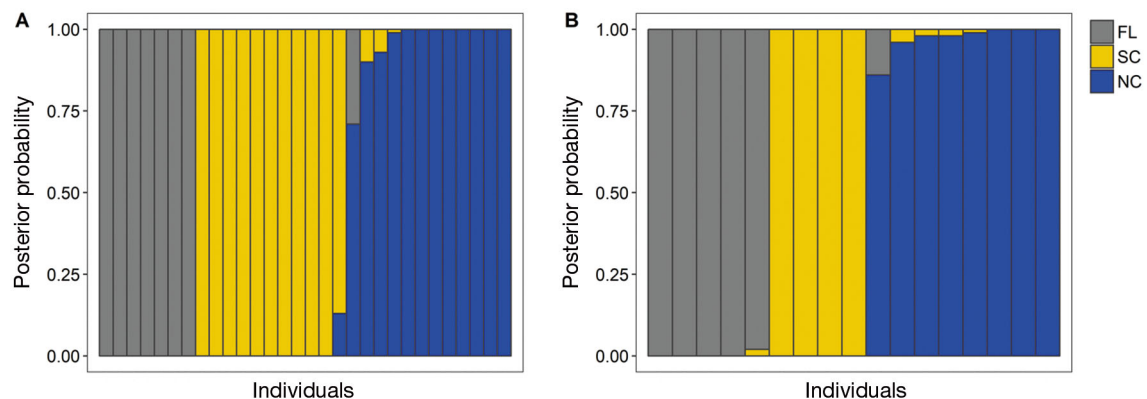


Fig. 7. Predicted state-scale nursery origins for age 3+ southern flounder captured in (A) North Carolina (NC) and (B) South Carolina (SC) estuaries using maximum likelihood estimation. Individuals are represented on the *x*-axis, and the proportion of each color indexed on the *y*-axis represents the probability of assignment to each nursery state. FL: Florida
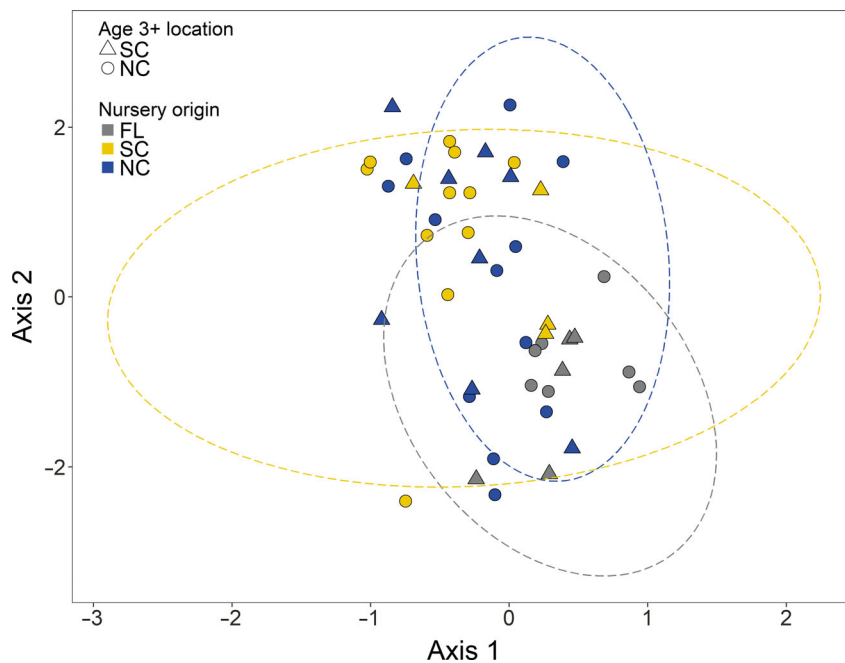
Fig. 8. Confidence ellipses (95%) for state-scale quadratic discriminant analysis (QDA) conducted on age-1 southern flounder otolith signatures, with discriminant scores plotted for adult (age 3+) southern flounder otolith signatures. Scores and ellipses are visualized on the first and second sliced average variance estimate (SAVE) variates, represented here as Axis 1 and Axis 2. Circles: North Carolina (NC) adults; triangles: South Carolina (SC) adults; colors represent predicted nursery origin. FL: Florida

Genetic differentiation is influenced by many factors, including population size, connectivity, and history, which can confound efforts to identify ecologically relevant levels of migration and connectivity that would be of interest to fisheries managers (Hauser & Carvalho 2008). Marine population connectivity, and thus reductions in genetic differentiation, can result from dispersal at any life history stage, including broad larval dispersal and even rare straying of adults. While our conclusions are based on the analysis of a single cohort of southern flounder (2011 year class), the mixing detected was widespread and re-affirmed prior evaluations of stock structure, so we anticipate that examination of other cohorts would yield similar patterns. Our findings provide additional evidence of large-scale oceanic movements by adult southern flounder with limited site fidelity during the post-migration period, which contributes to extensive mixing within the US South Atlantic basin.

Large-scale (50 to 500+ km) movements by southern flounder within the US South Atlantic basin have been observed previously during conventional tagging studies conducted in Georgia, South Carolina, and North Carolina, including numerous recaptures of tagged individuals outside of the tagging state

(Music & Pafford 1984, Wenner et al. 1990, Craig et al. 2015). Nearly all large-scale movements of tagged individuals were in a southerly direction, regardless of the release location. The size and timing of movements indicates that large-scale southward movements are likely associated with maturation and spawning behavior, and suggest that offshore spawning activities by southern flounder may be concentrated in the southern portion of the US Atlantic range (Craig et al. 2015).

Using otolith chemistry to infer connectivity, we detected both northward and southward movements of southern flounder adults between the first-year nursery period and mature adult stages (ages 3 and 4). Overall, slightly less than half of individuals were estimated to have originated from a nursery state located south of their adult capture location, an indication that northward movements are common. This finding contradicts observations from past conventional tagging studies, which documented nearly uniform directional movement to the south when fish had been recaptured at large distances (50 to 500+ km) from the release site. However, southern flounder tag-return studies have not been conducted in Florida waters, so it is possible that fish originating near the southern edge of the Atlantic range make regular northward movements that have gone undetected due simply to a lack of tagging effort in these regions. It is also important to note that some of the directed southward movements detected by conventional tagging are likely associated with pre-spawning migrations (based on season and time at large), while the northward movements indicated by otolith chemical signatures represent haphazard (non-homing) returns to estuarine waters following offshore spawning. Unfortunately, the assessment of population connectivity using otolith chemistry does not allow for precise estimates of the timing and seasonality of movements. While not commonly observed during previous conventional tagging studies, large-scale northward movements by southern flounder were noted on 2 occasions, including one in which a small (<350 mm total length [TL]), and presumably immature, individual moved rapidly (<50 d

at large) between Georgia and North Carolina, a distance over 500 km (Music & Pafford 1984). This observation suggests that large-scale movement behavior can occur which may not be directly associated with spawning.

Overall, roughly one-third (34%) of southern flounder adults were captured in the same state where they were predicted to have spent their nursery period. Because movement was inferred by comparing adult capture location to otolith chemistry-classified juvenile nursery origin, information about the movements of individuals while 'at large' is not available. Therefore, we cannot distinguish with certainty whether these individuals returned to their home states following offshore spawning migrations or simply did not emigrate from their original nursery estuaries. We presumed that all adult southern flounder included in the present study had participated in offshore migrations to spawning areas. In North Carolina waters, the length at which 50% of female southern flounder are predicted to be mature is 408 ± 65.6 mm TL (mean ± SE), and 76% of individuals were mature at age 2 (Midway & Scharf 2012). Additionally, conventional tagging studies conducted in North Carolina found that southern flounder ≥450 mm TL were more than 3 times as likely to emigrate from estuarine systems than fish ≤375 mm TL (Craig et al. 2015). The sizes (>500 mm TL) and ages (3 and 4 years old) of adult southern flounder used in the present study (Table 1) support the notion that the vast majority of individuals were mature and did emigrate from their original nursery estuaries to spawn before subsequently returning to inshore waters, with some fraction of fish re-entering waters in the same state from which they emigrated. Whether adult southern flounder demonstrated natal homing behavior, by returning to the same estuary where they had spent their nursery period, could not be discriminated given the broad state-scale spatial resolution of our baseline nursery atlas.

Tests of otolith stable isotope signatures ($\delta^{13}C$, $\delta^{18}O$) between left and right otoliths revealed the potential for lack of chemical equivalence in southern flounder paired sagittae within individuals. Intra-fish variability in isotopic signatures have been found to be negligible in bilaterally symmetrical species (Iacumin et al. 1992, Thorrold et al. 1997, Høie et al. 2004, but see Kalish 1991), and this equivalence has also been established for trace elemental signatures (Rooker et al. 2001). Thus, left and right otoliths are often assumed to be interchangeable and are frequently chosen at random in studies of otolith chemistry (e.g. Thorrold et al. 1997, Walther & Thorrold 2008, Stanley et al. 2016). However, for bilaterally asymmetrical species, differences in otolith chemistry among paired sagittae have been recently demonstrated, although evidence for chemical asymmetry is equivocal depending on the geochemical variable measured and otolith region sampled. Loher et al. (2008) found significant differences in $\delta^{13}C$, $\delta^{18}O$, and Sr:Ca when examining left–right differences using whole otolith preparations in Pacific halibut *Hippoglossus stenolepis* but found no differences in other trace elements (including Mg:Ca, Mn:Ca, and Ba:Ca; elements measured in this study). Gao et al. (2015) found significant differences in $\delta^{13}C$ and $\delta^{18}O$ using micromill-isolated material from left and right Pacific halibut otolith cores, but not in material representing later life stages (5th and 8th annulus). Kajajian et al. (2014) examined sagittal otolith pair equivalence in summer flounder *Paralichthys dentatus* and found significant differences in Li:Ca, Mg:Ca, and Sr:Ca sampled with laser ablation transects, but no differences were detected in Mn:Ca and Ba:Ca, among other elements. Significant differences were also detected in $\delta^{13}C$ and $\delta^{18}O$ prepared from the whole otoliths remaining after trace element analysis. In contrast, Marriott et al. (2016) tested left and right whole otoliths of juvenile plaice *Pleuronectes platessa* and found no differences in all 10 elements sampled (including Mg:Ca, Mn:Ca, Sr:Ca, and Ba:Ca).

In the present study, we detected differences in $\delta^{13}C$ and $\delta^{18}O$ among some comparisons of left and right otoliths, as well as in some comparisons of otolith sectioning region, but changes in stable isotope ratios did not have a large influence on the overall mixture proportions for the nursery origins of adult southern flounder (Table 3). While we were unable to conduct similar tests for bilateral equivalence in trace elemental concentrations, the stability in Mn:Ca and Ba:Ca concentrations detected among paired otoliths in previous flatfish studies (Loher et al. 2008, Kajajian et al. 2014) provides us with additional confidence in the accuracy of the classification analyses, as Mn:Ca and Ba:Ca were large contributors to discriminatory power in the juvenile nursery atlas (Table 2). Ideally, otolith chemistry studies in flatfish should be conducted on a single consistent otolith (left or right) across all specimens, but this is not always feasible when sampling from archived otolith collections or when conducting multiple preparations that consume otolith material. Otherwise, testing the assumption of equivalence becomes necessary to understand the effect of otolith choice on analysis outcomes.

While otolith geochemical signatures were effective for discriminating among southern flounder nurseries among states, more overlap occurred between NC and SC, resulting in relatively lower reclassification accuracy (61.0%) for individuals collected from SC nurseries (Figs. 4 & 6). Overlap in otolith geochemical signatures may be due to fish experiencing similar chemical environments in the estuarine nurseries of states that are geographically adjacent. The first 3 axes of discrimination in the nursery atlas were driven by Ba:Ca, Mn:Ca, and $\delta^{18}$O, respectively (Table 2). These 3 variables illustrate clear separation when comparing otolith concentrations between FL and both NC and SC, but considerable overlap between the 2 northern states is present (Fig. 3).

Misclassified SC individuals in the baseline nursery atlas were most commonly assigned to NC, so it is possible that the subsequent mixed stock analysis to infer adult nursery origins is underestimating the proportion of adults from SC nurseries, while at the same time overestimating the proportion of adults from NC nurseries. Additionally, we identified up to 23% of adults that likely originated from unsampled nurseries based on Ba:Ca and $\delta^{18}$O signatures. Georgia was not represented in the baseline nursery atlas, and the presence of adult geochemical signatures from unsampled nursery sources suggests that some portion of the adults from NC and SC could have spent their juvenile period in Georgia estuaries. The contribution of Georgia estuaries to post-migratory adults in the 2 northern states would not be unexpected, given the level of coastal scale mixing identified here.

Given the degree of mixing among US South Atlantic states detected here, along with previously discussed conventional tagging evidence suggesting large-scale movement potential, the unit stock assumption for NC southern flounder appears to be violated. The classic approach to managing discrete fish stocks assumes that populations will respond to reductions in fishing pressure—an assumption that only holds if populations are essentially closed, with population dynamics mediated by internal processes such as growth, recruitment, and fishing and natural mortality (Frisk et al. 2014). With the frequent movement of adults among estuaries, southern flounder more closely resemble a metapopulation, an open subpopulation where immigration and emigration make nontrivial contributions to local population dynamics, and rates of exchange among groups must be taken into account in designing effective management strategies (Kritzer & Sale 2004). Further compli-

cating matters, the fishery for southern flounder occurs almost entirely within estuaries that can vary considerably in fish population demographics, harvest rates, and fleet behavior (Scharf et al. 2017). Along the US South Atlantic coast, this translates to a fishery consisting of multiple discrete spatial units, each with distinct fishery dynamics that can react to shifting environmental and economic conditions over short temporal scales. Not accounting for fine-scale variation in fish and fishery dynamics can lead to misalignment between the spatial scale of management strategies and patterns of exploitation (Ying et al. 2011, Wilson et al. 2012, Kerr et al. 2014, White 2015). However, understanding the interplay between fine-scale variation in fish demography and fishery dynamics and broad-scale patterns of mixing will be paramount in establishing effective management strategies and realizing optimal fishery yields for southern flounder in the US South Atlantic.

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