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Otolith chemistry can discriminate signatures of hatchery-reared and wild spotted seatrout

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ABSTRACT

A central goal of stock enhancement is to increase species abundance and potential harvest by supplementing recruitment of wild populations. The efficacy of these efforts is predicated upon the survival of these hatchery-reared organisms after release, and determining their fate is essential to understanding the success of these programs. Along the northern Gulf of Mexico, spotted seatrout (*Cynoscion nebulosus*) represent the number one recreational fishery and in Texas are stocked in coastal bays and estuaries to enhance natural fish abundance. The natural chemical properties of fish otoliths represent one mechanism to track the fate of hatchery-reared fish in the wild and make inferences about their movement and survival. Establishing a baseline signature pattern to discriminate hatchery-reared from wild fish is an essential first step in this process. Spotted seatrout fingerlings were collected from three Texas bays and three Texas saltwater fish hatcheries. Sagittal otoliths were analyzed for stable isotopes ($\delta^{13}C$ and $\delta^{18}O$) and a suite of twelve trace elements. Multivariate and univariate analysis of variance tests indicated significant differences among natal origins and reduced the number of chemistry predictors to nine. Linear discriminant function analysis using jack-knife cross-validation successfully classified 92% of fish to their correct natal origin. These results show that otolith chemistry is a powerful tool for discriminating hatchery-reared from wild spotted seatrout; therefore, chemical signature patterns reflected in the otoliths establish a baseline for tracking the fate of hatchery-reared fish in the wild. This information can provide more quantifiable estimates of stock enhancement success to fishery managers by improving our understanding of the fate and contribution of hatchery-reared fish to wild populations.

1. Introduction

Stock enhancement programs are widely recognized as a useful fishery management tool to mitigate declines in wild fish populations occurring from overharvest, environmental stochasticity, and habitat degradation (Bell et al., 2006; Blaxter, 2000; Davis et al., 2005; Molony et al., 2003). The success of stock enhancement as a fishery management tool depends directly upon the survival of released hatchery-reared fingerlings (Blaxter, 2000; Leber et al., 1996). Thus, effective means of determining the fate of hatchery-released fish are necessary to evaluate the relative hatchery contribution and the success of stock enhancement programs.

One tool being used to investigate these hatchery contributions is otolith microchemistry, which takes advantage of the natural tag properties of fish otoliths (Campana, 1999). During otolith accretion, naturally occurring trace elements and stable isotopes in the ambient environment incorporate themselves into the metabolically inert aragonitic otolith matrix, and are permanently retained throughout the life of the fish (Campana, 1999). The natural tag properties of otoliths have been used to uncover many important life history questions including movement among habitat types (Chittaro et al., 2005; Gillanders and Kingsford, 1996), stock structure and spawning ground delineation (Newman et al., 2011; Rooker et al., 2001; Schaffler et al., 2009), and classification to natal origins (Comyns et al., 2008; Dorval et al., 2005; Gillanders and Kingsford, 2000; Rooker et al., 2010; Thorrold et al., 1998; Vasconcelos et al., 2007). Additionally, otolith chemistry has been used to identify hatchery-reared fish from wild fish for a number of species including red snapper (Gibson et al., 2010), walleye (Bickford and Hannigan, 2005), and various salmonids (Coghlan et al., 2007; Gibson-Reinemer et al., 2009; Zitek et al., 2010).

The spotted seatrout (*Cynoscion nebulosus*) is one of the most popular sportfish along the Gulf of Mexico (Gulf) coast and represents a valuable commodity for states bordering the Gulf

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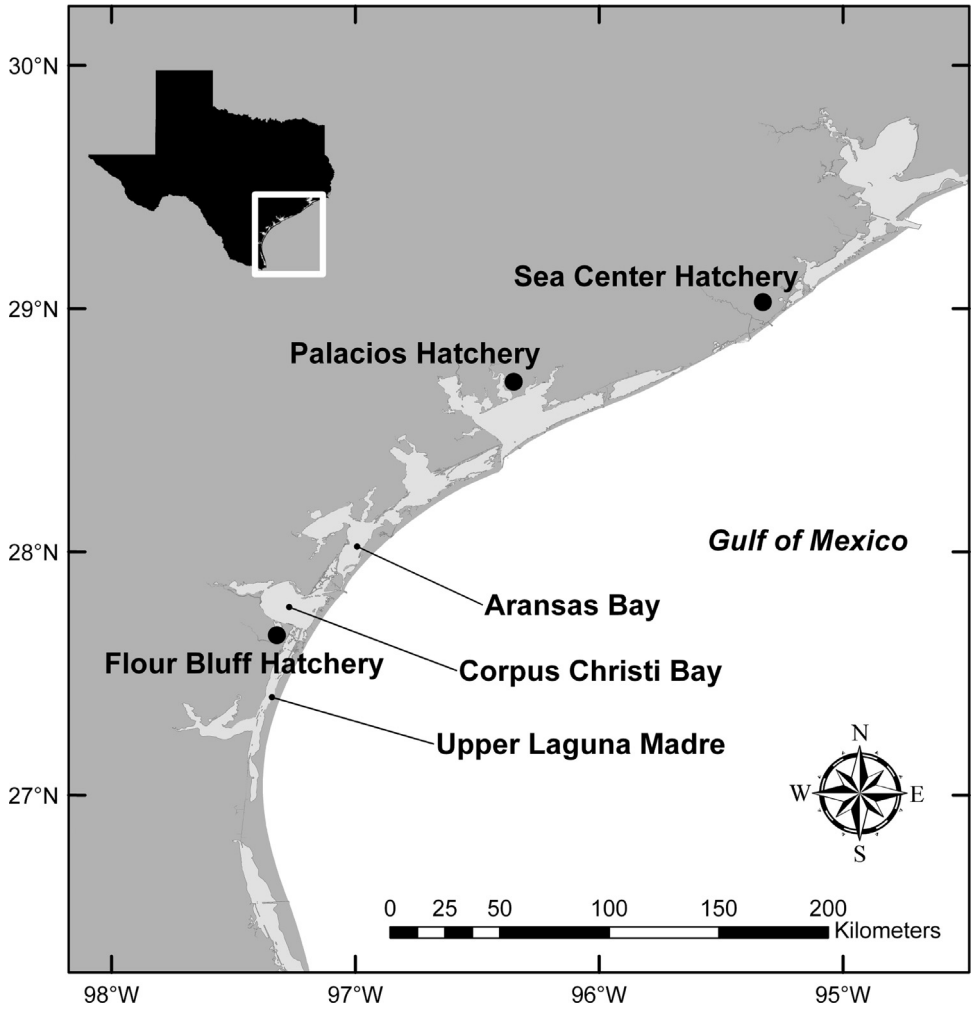

Fig. 1. The three south Texas bays and three Texas state saltwater fish hatcheries where samples were obtained. Wild specimens were collected in 3 to 4 seagrass patches around each bay. Hatchery-reared specimens were obtained from 3 to 4 grow-out ponds within each hatchery.

(Vanderkooy and Muller, 2003). Spotted seatrout comprises the number one recreational fishery along the Gulf coast in terms of recreational harvest, and in Texas alone is valued at $220 million (Vega et al., 2011). Fishery managers have enacted several fishing regulations for spotted seatrout to curb declining population sizes that have included bag and size limits; however, these top-down, more restrictive regulations are often unpopular with recreational anglers (Grimes, 1998; Travis et al., 1998). Enhancing populations through stock enhancement is another possible alternative for increasing spotted seatrout abundance that is typically popular with both fishery managers and recreational anglers. Three saltwater fish hatcheries managed by the Texas Parks & Wildlife Department have been operating active stock enhancement programs in the state for over 25 years for a number of recreationally popular sportfish, including spotted seatrout. These hatcheries combine to stock more than one million spotted seatrout fingerlings in bays and estuaries along the Texas coast annually and have cumulatively stocked over 50 million fingerlings since the inception of the spotted seatrout stock enhancement program in 1993 (Vega et al., 2011). With significant resources being spent on stock enhancement efforts, it is imperative to assess the relative contribution of these hatchery-reared fish to wild stocks.

Do hatchery and bay water chemistries impart different signature patterns in the otoliths of spotted seatrout fingerlings? If so, this technique could be used to retrace the natal origins of fish once they reach adulthood and serve as a baseline for future comparisons that will provide an estimate of long-term survival of hatchery-reared spotted seatrout in the wild. The necessary first step is to determine if discrimination of hatchery-reared from wild spotted seatrout is possible using otolith chemistry. Ultimately, this information can help estimate the relative contribution of hatchery-reared fish to wild populations and thereby provide a measure of success for stock enhancement efforts for spotted seatrout. Thus, the purpose of this study is to test the potential for using otolith microchemistry as a tool to discriminate hatchery-reared from wild spotted seatrout.

2. Methods

Wild spotted seatrout fingerlings were collected from three south Texas bays: Aransas Bay, Corpus Christi Bay, and the Upper Laguna Madre (Fig. 1) during October to November, 2007 and 2008 (Table 1). Wild fish were sampled at three sites per bay from seagrass habitats using a 6-m bag seine with 5-mm mesh wings and a 3-mm mesh bag and were stored on ice in the field until processing occurred in the laboratory. Hatchery-reared spotted seatrout fingerlings were obtained from three Texas Parks and Wildlife Department saltwater fish hatcheries (Fig. 1): CCA Marine Development Center located in Flour Bluff, TX; P.R. Bass Marine Fisheries Research Center located in Palacios, TX; and Sea Center Texas located in Lake Jackson, TX (hereafter, Flour Bluff

Table 1
Locations, origins, and sample sizes of spotted seatrout (*Cynoscion nebulosus*) specimens collected ($n = 211$). Group variable represents the unique combination of location plus year. Group is nested in Origin in downstream analyses.

Location	Year	Group	Origin	*n*
Aransas Bay	2007	AB07	Wild	18
Aransas Bay	2008	AB08	Wild	20
Corpus Christi Bay	2007	CC07	Wild	19
Corpus Christi Bay	2008	CC08	Wild	20
Upper Laguna Madre	2007	UL07	Wild	17
Upper Laguna Madre	2008	UL08	Wild	19
Flour Bluff Hatchery	2006	FB06	Hatchery	19
Flour Bluff Hatchery	2007	FB07	Hatchery	19
Flour Bluff Hatchery	2008	FB08	Hatchery	20
Palacios Hatchery	2008	PA08	Hatchery	20
Sea Center Texas	2008	SC08	Hatchery	20
Total				211

hatchery, Palacios hatchery, and Sea Center hatchery, respectively). Hatchery collections were sampled from an equal subset of 3–4 fingerling ponds to avoid individual pond bias. Specimens were obtained from the Flour Bluff hatchery for years 2006–2008 but only for 2008 from the other two hatcheries (Table 1). Palacios hatchery does not possess brooding facilities, only grow-out ponds; therefore, spotted seatrout larvae reared in the Flour Bluff facility are brought to Palacios hatchery 1.5 days post-hatch. Fish spend 28–30 days in ponds before being stocked into the wild. Hatchery fish for this experiment were collected 1–2 days prior to release into the wild. The water source for Flour Bluff Hatchery is the Upper Laguna Madre, Matagorda Bay supplies Palacios hatchery, and Galveston Bay supplies Sea Center hatchery. Hatchery fish were stored in 70% ethanol until measurements and otolith extractions occurred.

Spotted seatrout individuals were measured for SL (±0.1 mm), TL (±0.1 mm), and dry weight (±0.1 g). Cleaning and decontamination procedures followed published protocols (Rooker et al., 2001). All reagents used were ultrapure grade, and all instruments were cleaned by soaking in a dilute nitric acid (HNO_3) bath and triple rinsed with double deionized water ($DDIH_2O$). Left and right sagittal otoliths were removed using plastic forceps and placed in separate 1.5 mL polypropylene microcentrifuge tubes. Otoliths were soaked for several hours in $DDIH_2O$ followed by sonication for 5–10 min in 3% hydrogen peroxide (H_2O_2) to remove adhering residual tissue, transferred to a clean, pre-weighed (±0.1 mg) 1.5 mL microcentrifuge tube, and soaked for 1–2 min in dilute (0.001 N) nitric acid to remove any surface contamination. A final triple rinse with $DDIH_2O$ was performed and otoliths were allowed to dry overnight in a Class II biological safety flow hood (Fisher Hamilton). Microcentrifuge tubes containing otoliths were then re-weighed to obtain otolith weight to the nearest 0.1 mg.

Left sagittal otoliths were sent to the Environmental Isotope Laboratory, Department of Geosciences at the University of Arizona for stable isotopic analysis. Otolith samples were powdered using a mortar and pestle, and reacted with dehydrated phosphoric acid (H_2PO_4) under vacuum at 70 °C. An automated carbonate preparation device (KIEL-III) coupled to a gas-ratio mass spectrometer (Finnigan MAT 252) was used to measure $\delta^{13}C$ and $\delta^{18}O$ of carbonates. The isotope ratio measurement is calibrated based on repeated measurements of NBS-19 and NBS-18 used to define the Vienna Pee Dee Belemnite (VPDB) standard and precision is ±0.10‰ for $\delta^{18}O$ and ±0.08‰ for $\delta^{13}C$ (1 sigma).

Right sagittal otoliths were sent to the Research and Productivity Council, New Brunswick, Canada for trace element analysis using inductively coupled plasma mass spectrometry (ICP-MS). Prior to nitric acid digestion, all plastic beakers, sample tubes, and pipette tips were pre-cleaned by soaking in dilute trace-metal grade nitric acid. Samples were randomized to remove any bias related to calibration drift and analysis order. Otoliths were transferred into acid-cleaned 13 mL polypropylene tubes in sample racks. Each sample was placed into one of four groups on the basis of the sample weights provided (<2 mg, 2–4 mg, 4–10 mg, and >10 mg) for digestion purposes. Ultrapure nitric acid (30%) was added (0.5–2.5 mL, based upon sample weight) to each sample and specimens were allowed to react at room temperature for 1 h. Samples were heated to 80 °C in an oven for 20 min to complete the digestion. After cooling, an internal standard (Indium) for ICP-MS analysis was added and the solutions were diluted to volume (2–10 mL, depending upon sample weight). Samples were randomly ordered and analyzed by ICP-MS using a Thermo Series II ICP-MS instrument equipped with Thermo's "Xi" interface, designed for high sensitivity and tolerance to sample matrix. Indium was used as an internal standard for all elements (added to samples and standards during preparation). Limits of detection were based on external standardization. Blanks, rinses, reagent blanks, and matrix-matched standards were analyzed at regular intervals throughout the analytical run. Final values were normalized to a calcium concentration of 400,000 mg/kg (40%). A suite of twelve trace elements (Al, Ba, B, Mn, K, Rb, Na, Sr, Fe, Pb, Mg, and Zn) analyzed using ICP-MS were considered in this experiment.

Standard fish length (mm) and left otolith weight (mg) were compared among groups using analysis of variance (ANOVA; $\alpha = 0.05$) followed by a Tukey HSD post hoc test to determine pairwise differences between groups. Otolith weight was then regressed against each stable isotope and trace element and assessed with a Pearson's r correlation test to determine if response variables were influenced by fish weight and required weight-based correction (Campana, 2005). Multivariate analysis of variance (MANOVA) tests were used to determine overall model significance. Subsequent univariate ANOVA tests with Bonferroni correction for multiple tests for each trace element and stable isotope variable were performed to determine which variables were significantly different among groups and should be retained for discriminant function analysis. Additionally, a stepwise discriminant analysis was performed to confirm variable significance and inclusion by MANOVA and ANOVA analyses. Chemical signatures were normalized to 0 ± 1 (mean ± SD) to account for magnitude differences in scale between some predictor variables. Measurements characterized as outliers (>3 SD) were removed from the dataset and downstream analyses (Comyns et al., 2008; Fowler et al., 1995).

Linear discriminant function analysis (LDFA) was used to classify individuals to their natal origin. All observations from significant variables determined in ANOVA tests above were included in building the LDFA training dataset with prior probabilities of class membership reflecting class proportions from each group. Classification accuracy for the LDFA was validated using a jackknife (leave-one out) cross-validation procedure, whereby each observation was sequentially removed from the dataset, discriminant functions were estimated from the remaining observations, and the removed observation was then re-classified to a group by the discriminant functions. Three separate LDFAs were performed – one using only stable isotopes, one using only trace elements, and one combining the two – to determine relative classification success of these chemistries independently and combined. For the LDFA using all chemical predictors, linear discriminant function 1 was plotted against linear discriminant function 2 to visualize the separation of groups in multivariate space over two dimensions. To test whether grouping of individuals by LDFA occurred as a direct result from predictor variables or simply by random chance, simulation and permutation tests were performed (Manly, 2007; White and Ruttenberg, 2007). In each of 10,000 runs, the grouping variable in the data was randomized. Next, the same LDFA command was used on the randomized data as was used on the original data, and the rate of correct classification for each randomized run was

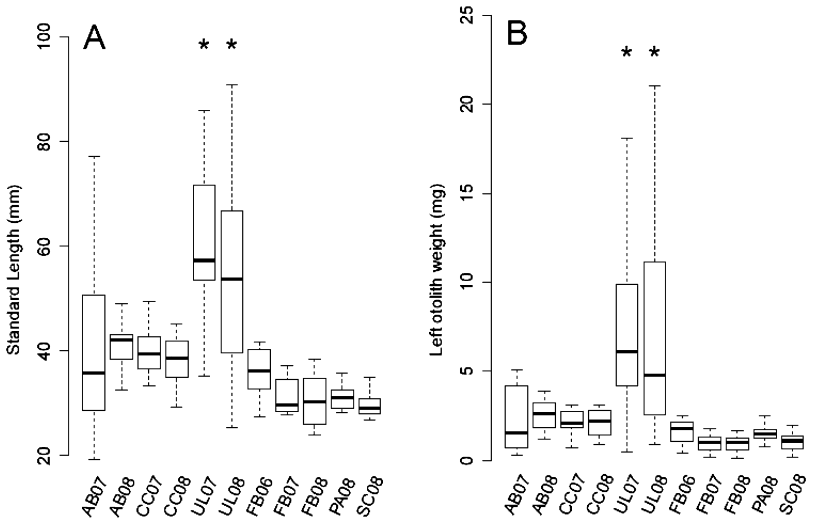

Fig. 2. (A) Standard fish length (mm) and (B) left otolith weight (mg). Thick horizontal lines represent the median for each group; the box is defined by the 25th and 75th percentiles (lower and upper quartiles); and the dotted line is 1.5× the difference between the median and lower or upper quartile. The eleven bay and hatchery groups (location/year combinations) are represented on the *x*-axis of each panel. The first six groups from left to right along the *x*-axis represent wild fish sources: AB = Aransas Bay, CC = Corpus Christi Bay, UL = Upper Laguna Madre. The last five groups represent hatchery fish sources: FB = Flour Bluff Hatchery, PA = Palacios Hatchery, SC = Sea Center Hatchery. Asterisk (*) denotes groups that are significantly different from all other groups as determined by Tukey-HSD post hoc tests.

compared with the rate of correct classification for the original data. Statistical analyses were performed using SAS version 9.3 (SAS, 2013) and R version 2.13.0 (R Development Core Team, 2013). Study site map (Fig. 1) generated using ArcMap version 9.3 (ESRI, 2013).

3. Results

An analysis was performed to determine if size differences existed among all the groups of wild and hatchery sampling locations. Juvenile spotted seatrout collected from all sampling locations ranged from 19 to 91 mm SL (mean ± SE). Standard fish length (mm) differed significantly among groups (ANOVA: $F = 20.493$; $df_{10,200}$; $p < 0.001$; $n = 211$), with Tukey-HSD post hoc tests revealing that fish from the Upper Laguna Madre in 2007 and 2008 were significantly larger than all other groups (Fig. 2A). Left otolith weight also differed among groups (ANOVA: $F = 15.593$; $df_{10,200}$; $p < 0.001$; $n = 211$), and similarly, the otoliths in Upper Laguna Madre fish from both years were significantly heavier than all other groups (Fig. 2B). Strong correlations existed between left otolith weight and standard length (Pearson's: $r = 0.93$, $p < 0.001$, $n = 211$), demonstrating that otolith weight could be used as a proxy for standard length. Regression of left otolith weight against chemical signatures showed weak but significant correlations for B, K, Na, Sr, and $\delta^{13}C$. To remove these correlations, for each variable we subtracted the within-group linear slope multiplied by the otolith weight from the initial otolith weight of each fish. Detrended data showed no residual correlations with otolith weight.

To determine if otolith chemical signatures varied across locations and years, multivariate analysis of variance (MANOVA) and analysis of variance (ANOVA) was performed using a two-way model with origin (wild or hatchery) and group nested in origin as fixed factors. Fourteen total chemical predictors used in this study, including two stable isotopes and twelve trace elements, indicated that there were significant differences between hatchery and wild origins and among groups (Table 2). Subsequent univariate ANOVA tests for each chemical signature indicated specifically which variables differed among groups (Table 3). A Bonferroni correction ($\alpha' = \alpha/14 = 0.0035$) was applied to account for multiple, non-independent tests. Five trace element variables (Al, Fe, Pb, Mg, and Zn) did not differ significantly and were dropped from further analysis. Six of the original twelve trace element variables (Ba, Mn, K, Rb, Na, and Sr), and both stable isotopes ($\delta^{13}C$ and $\delta^{18}O$), differed significantly among groups and were retained for downstream analysis. Boron became marginally not significant following Bonferroni correction but showed a significant difference during stepwise discriminant analysis so was retained in the model. Stepwise discriminant analysis confirmed all other significant differences in variables from ANOVA tests.

Table 2
Multivariate analysis of variance (MANOVA) with Origin (hatchery or wild) and Group (unique bay or hatchery and year combination nested in Origin) as factors. All trace element (12) and stable isotope chemistry (2) variables included in the model.

	Pillai's trace	Num df	Den df	*F*	*p*
Origin	0.866	14	187	86.51	<.0001*
Group (Origin)	3.771	126	1755	10.05	<.0001*

* Significance at $p < 0.0001$.

Each of the nine significant chemical predictors was compared among the eleven hatchery and wild sampled groups. Trace element values were converted to a ratio of molar trace element concentration to molar calcium concentration (Table 4). Because chemical signatures differed from one another on the order of one magnitude, all nine predictor variables were normalized to 0 ± 1 (mean ± SD) prior to further analysis. Exploratory analysis identified nine values as outliers (±3 SD) and these observations were removed from the original dataset to reduce the total number of observations from 211 to 202. Comparisons of predictor variables in the normalized data revealed substantial differences among wild and hatchery groups with certain variables showing noticeable patterns (Fig. 3). Potassium (K) for wild groups was noticeably greater than hatchery groups, despite within-group variation of wild or hatchery groups being very small. Conversely, strontium (Sr) showed the opposite trend: wild groups showed noticeably less strontium in otoliths than hatchery groups. Stable isotopes $\delta^{13}C$ and $\delta^{18}O$ showed substantial year-to-year variation among wild groups. Wild fish from 2007 showed increasing $\delta^{13}C$ values moving south from Aransas Bay to Corpus Christi Bay to the Upper Laguna Madre, but exhibited little differentiation in $\delta^{13}C$ values for 2008. A slightly less pronounced but similar trend was seen in $\delta^{18}O$ values between the years 2007 and 2008 moving among the bays. Overall, temporal

Table 3
Univariate analysis of variance (ANOVA) tests with Origin (hatchery or wild) and Group (unique bay or hatchery and year combination nested in Origin) as factors for all trace element and stable isotope chemistry variables included in the MANOVA. Numbers in italics significant at $\alpha = 0.05$ but not significant with Bonferroni correction ($\alpha = 0.0035$) for multiple tests.

Chemical predictor	MS	df	*F*	*p*
Aluminum (Al)	1175.04	10	1.27	0.2516
Origin	57.39	1	0.06	0.8039
Group	1299.48	9	1.40	0.1899
Barium (Ba)	1803.54	10	61.62	<.0001*
Origin	4784.06	1	163.45	<.0001*
Group	1456.76	9	49.77	<.0001*
Boron (B)	1.14	10	2.54	*0.0067*
Origin	0.54	1	1.20	0.2741
Group	1.20	9	2.67	*0.0060*
Iron (Fe)	6252.00	10	1.24	0.2673
Origin	6839.68	1	1.36	0.2455
Group	6152.41	9	1.22	0.2841
Lead (Pb)	2.4e−06	10	0.90	0.5384
Origin	1.8e−05	1	0.01	0.9348
Group	2.7e−03	9	1.00	0.4454
Magnesium (Mg)	918.80	10	0.76	0.6632
Origin	3035.17	1	2.52	0.1137
Group	674.61	9	0.56	0.8279
Manganese (Mn)	1040.34	10	12.16	<.0001*
Origin	545.32	1	6.37	*0.0124*
Group	1089.44	9	12.73	<.0001*
Potassium (K)	1.1e05	10	39.64	<.0001*
Origin	6.1e05	1	213.74	<.0001*
Group	5.5e04	9	19.25	<.0001*
Rubidium (Rb)	3.4e−03	10	9.47	<.0001*
Origin	1.6e−02	1	43.90	<.0001*
Group	2.0e−03	9	5.53	<.0001*
Sodium (Na)	8.0e05	10	30.24	<.0001*
Origin	4.9e06	1	187.44	<.0001*
Group	3.3e05	9	12.65	<.0001*
Strontium (Sr)	1.5e06	10	59.56	<.0001*
Origin	9.0e06	1	350.60	<.0001*
Group	6.7e05	9	26.23	<.0001*
Zinc (Zn)	9.87	10	1.77	0.0673
Origin	17.12	1	3.08	0.0810
Group	9.07	9	1.63	0.1087
$\delta^{13}C$	62.71	10	77.74	<.0001*
Origin	95.26	1	118.10	<.0001*
Group	58.27	9	72.25	<.0001*
$\delta^{18}O$	21.32	10	258.88	<.0001*
Origin	24.23	1	294.23	<.0001*
Group	21.31	9	258.75	<.0001*

* Significance at $p < 0.0001$.

variability of wild fish over the two years appeared greater than the spatial variation exhibited among bays in either year.

Separation of sampling groups was visualized in multivariate space by plotting discriminant function 1 against discriminant function 2 for several combinations of wild and hatchery fish. In 2007, wild fish from the three bays were clearly distinguished from fish originating from Flour Bluff hatchery, the nearest hatchery to these three bays (Fig. 4A). Minimal overlap was observed among wild groups, but there was clear distinction between these wild groups and the single hatchery group. Similar patterns were observed for wild fish from 2008 and Flour Bluff hatchery fish from 2008 (Fig. 4B). Slightly more overlap among wild and hatchery groups occurred than in 2007 but there was still evident separation among 2008 groups. Discriminant function plots that included all hatchery-reared fish showed clear separation of each hatchery group spatially (Flour Bluff, Palacios, and Sea Center hatcheries in 2008) as well as temporally (Flour Bluff hatchery in 2006, 2007, and 2008; Fig. 4C). Very minimal overlap was evident among each of the five hatchery groups. Plotting all combinations of wild and hatchery fish together yielded considerably high group separation considering the inclusion of multiple years (Fig. 4D). The first two discriminant functions explained 75% of the cumulative variance

Table 4
Ratios of molar concentration of trace elements to molar calcium concentration, and $\delta^{13}C$ and $\delta^{18}O$ values (mean ± SD) among the eleven wild and hatchery reared fish groups.

Group	Ba (μmol mol^{-1})	B (μmol mol^{-1})	Mn (μmol mol^{-1})	K (mmol mol^{-1})	Rb (μmol mol^{-1})	Na (mmol mol^{-1})	Sr (mmol mol^{-1})	$\delta^{13}C$ (‰)	$\delta^{18}O$ (‰)
Aransas Bay' 07	19.03 ± 5.22	17.11 ± 13.47	47.83 ± 10.19	1.75 ± 0.22	0.10 ± 0.01	13.47 ± 0.59	2.05 ± 0.10	−6.66 ± 0.64	−1.97 ± 0.34
Aransas Bay' 08	33.21 ± 6.97	12.44 ± 3.48	52.97 ± 15.60	1.77 ± 0.11	0.08 ± 0.01	13.41 ± 0.40	2.12 ± 0.13	−2.61 ± 0.78	0.11 ± 0.47
Corpus Christi Bay' 07	16.71 ± 3.06	13.53 ± 5.39	55.59 ± 8.54	1.77 ± 0.10	0.09 ± 0.01	13.37 ± 0.44	2.18 ± 0.12	−5.09 ± 0.50	−1.74 ± 0.10
Corpus Christi Bay' 08	13.72 ± 2.07	14.41 ± 5.02	44.62 ± 14.49	1.81 ± 0.17	0.10 ± 0.06	13.76 ± 0.48	2.14 ± 0.13	−2.82 ± 0.47	−0.30 ± 0.18
Upper Laguna Madre' 07	18.42 ± 5.18	11.18 ± 8.63	30.21 ± 7.63	1.63 ± 0.22	0.07 ± 0.02	12.63 ± 1.19	2.34 ± 0.21	−3.09 ± 0.81	−1.26 ± 0.08
Upper Laguna Madre' 08	14.16 ± 2.28	11.35 ± 4.04	33.03 ± 7.28	1.80 ± 0.13	0.07 ± 0.01	12.19 ± 1.61	2.34 ± 0.11	−2.54 ± 0.54	0.56 ± 0.37
Flour Bluff Hatchery' 06	12.01 ± 1.18	18.05 ± 3.72	17.94 ± 3.22	1.36 ± 0.13	0.06 ± 0.01	11.19 ± 0.49	2.72 ± 0.16	−7.30 ± 1.60	1.29 ± 0.08
Flour Bluff Hatchery' 07	12.87 ± 2.67	16.52 ± 5.19	56.29 ± 36.73	1.49 ± 0.11	0.06 ± 0.01	12.81 ± 0.55	2.67 ± 0.35	−3.78 ± 0.95	−0.50 ± 0.43
Flour Bluff Hatchery' 08	16.50 ± 6.18	20.97 ± 9.41	35.64 ± 8.40	1.55 ± 0.13	0.08 ± 0.03	12.94 ± 1.33	3.08 ± 0.18	−4.29 ± 0.58	0.33 ± 0.29
Palacios Hatchery' 08	15.49 ± 1.97	14.57 ± 5.02	54.88 ± 30.11	1.41 ± 0.15	0.06 ± 0.01	11.36 ± 0.79	2.40 ± 0.19	−8.10 ± 0.90	0.04 ± 0.25
Sea Center Hatchery' 08	4.31 ± 0.52	13.82 ± 2.95	26.03 ± 3.94	1.15 ± 0.04	0.06 ± 0.01	12.30 ± 0.23	2.85 ± 0.12	−4.98 ± 0.51	−1.62 ± 0.17

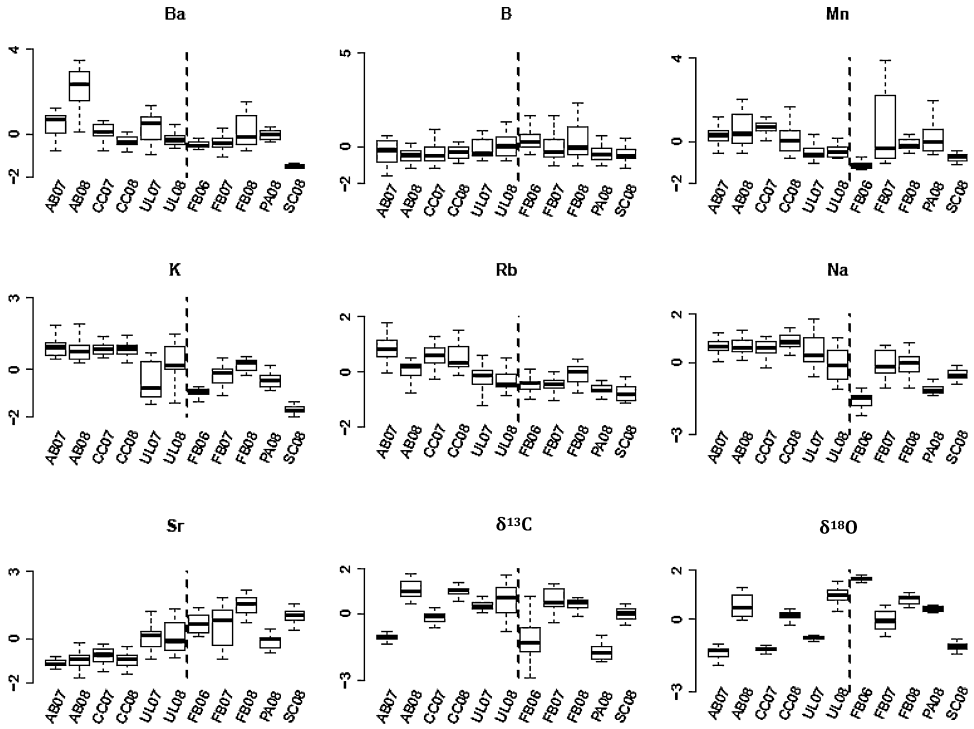

Fig. 3. Boxplots of nine chemical signatures determined to be significant from univariate ANOVA tests. Thick horizontal lines represent the median for each group; the box is defined by the 25th and 75th percentiles (lower and upper quartiles); and the dotted line is 1.5× the difference between the median and lower or upper quartile. All variables normalized to 0 ± 1 (mean ± SD) to account for magnitude differences in scale. The eleven bay and hatchery groups (location/year combinations) are represented on the *x*-axis of each panel. Vertical dashed line in each panel divides wild fish from hatchery fish groups. The first six groups from left to right along the *x*-axis represent wild fish sources: AB = Aransas Bay, CC = Corpus Christi Bay, UL = Upper Laguna Madre. The last five groups represent hatchery fish sources: FB = Flour Bluff Hatchery, PA = Palacios Hatchery, SC = Sea Center Hatchery.

in the LDFA (Table 5). The third and fourth discriminant functions explained an additional 15% and 4% of the variance for a cumulative variance of 91% and 95%, respectively, for the first four discriminant function variables (Table 5).

Using the combination of stable isotopes and trace element chemistries, LDFA performed over all wild and hatchery groups (Fig. 4D) correctly predicted true group assignment with accuracies between 82 and 100% for an overall accuracy of 95% and jack-knife cross-validation accuracies between 72 and 100% for an overall accuracy of 92% (Table 6A). The lowest jack-knife cross-validation classification accuracy occurred for wild fish from Upper Laguna Madre 2008, with four out of 18 fish misclassified as individuals from Corpus Christi Bay 2007 and one misclassified as Flour Bluff hatchery 2008. This was the only case where a wild fish was misclassified as a hatchery fish or vice versa. The highest classification accuracy of 100% was shared among Corpus Christi Bay, Flour Bluff hatchery, Palacios hatchery, and Sea Center hatchery in 2008. Using only stable isotopes δ^{13}C and δ^{18}O in the LDFA yielded classification accuracies between 25 and 100% for an overall average accuracy of 66% and a jack-knife cross-validation accuracy of 64% (Table 6B). Using only trace element chemistries fared just slightly better in LDFA yielding classification accuracies between 61 and 100% for an overall average of 80% and a jack-knife cross-validation accuracy of 75% (Table 6C). Misclassifications were much more prevalent when using only stable isotopes or only trace elements and, unlike when combining the two chemistries, frequently misclassified wild fish as hatchery fish and hatchery fish as wild fish.

To ensure that group classification success was due to our linear discriminant model and not achieved solely based on chance, simulation and permutation analyses were performed. Simulation runs testing that grouping occurred solely by random chance showed a maximum rate of correct classification of 17% and the permutation runs using our actual data produced a slightly higher maximum rate of correct classification of 36% (Table 7). These methods both

Table 5
Standardized coefficients of linear discriminant (LD) functions using the combination of trace elements and stable isotopes for each variable in LDFA. Proportion of explained variance and cumulative variance appears below each linear discriminant function.

Variable	LD 1	LD 2	LD 3	LD 4	LD 5	LD 6	LD 7	LD 8	LD 9
Ba	0.188	−1.937	0.357	−1.211	0.841	−0.355	−0.023	−0.210	−0.093
B	0.179	−0.368	−0.146	0.471	0.297	−0.162	0.185	−0.922	0.773
Mn	0.137	0.340	−0.086	−0.059	0.487	1.037	−0.826	0.134	0.369
K	−0.274	−0.280	−0.555	1.753	0.593	0.904	1.456	−0.566	−0.620
Rb	0.518	−0.804	0.166	0.152	−0.198	−1.406	−2.357	−0.164	−0.344
Na	−0.475	0.576	−1.016	0.808	0.315	−0.813	0.511	1.805	0.900
Sr	−0.596	1.807	−0.420	0.689	1.684	−0.182	0.056	0.245	−0.262
δ^{13}C	−0.119	0.523	−1.783	−0.909	−0.554	0.228	−0.372	−0.755	−0.281
δ^{18}O	4.082	0.238	−0.426	0.344	−0.330	−0.219	−0.317	0.781	0.322
Proportion	0.487	0.267	0.153	0.043	0.028	0.017	0.002	0.001	0.001
Cumulative	0.487	0.754	0.907	0.950	0.979	0.996	0.998	0.999	1.000

Table 6
Jack-knife (leave-one out) cross-validation classification success matrices. (A) Trace elements and stable isotopes combined in LDFA, (B) stable isotopes only, and (C) trace elements only.

	AB07	AB08	CC07	CC08	UL07	UL08	FB06	FB07	FB08	PA08	SC08	% Correct
A. Trace elements and stable isotopes combined												
Aransas Bay' 07	14	0	3	0	0	0	0	0	0	0	0	82%
Aransas Bay' 08	0	19	0	1	0	0	0	0	0	0	0	95%
Corpus Christi Bay' 07	0	0	18	0	1	0	0	0	0	0	0	95%
Corpus Christi Bay' 08	0	0	0	19	0	0	0	0	0	0	0	100%
Upper Laguna Madre' 07	0	0	1	1	15	0	0	0	0	0	0	88%
Upper Laguna Madre' 08	0	0	0	4	0	13	0	0	1	0	0	72%
Flour Bluff Hatchery' 06	0	0	0	0	0	1	17	0	0	0	0	94%
Flour Bluff Hatchery' 07	0	0	0	0	0	0	0	15	4	0	0	79%
Flour Bluff Hatchery' 08	0	0	0	0	0	0	0	0	17	0	0	100%
Palacios Hatchery' 08	0	0	0	0	0	0	0	0	0	18	0	100%
Sea Center Hatchery' 08	0	0	0	0	0	0	0	0	0	0	20	100%
											Total	92%
B. Stable isotopes only												
Aransas Bay' 07	17	0	0	0	0	0	0	0	0	0	0	100%
Aransas Bay' 08	0	5	0	5	0	6	0	4	0	0	0	25%
Corpus Christi Bay' 07	1	0	11	0	0	0	0	0	0	0	7	58%
Corpus Christi Bay' 08	0	2	0	14	0	0	0	3	0	0	0	74%
Upper Laguna Madre' 07	0	0	0	0	16	0	0	0	0	0	1	94%
Upper Laguna Madre' 08	0	6	0	2	0	5	1	0	4	0	0	28%
Flour Bluff Hatchery' 06	0	0	0	0	0	2	16	0	0	0	0	89%
Flour Bluff Hatchery' 07	0	5	0	3	4	0	0	7	0	0	0	37%
Flour Bluff Hatchery' 08	0	0	0	0	0	10	0	0	7	0	0	41%
Palacios Hatchery' 08	0	0	0	0	0	0	0	0	0	18	0	100%
Sea Center Hatchery' 08	0	0	6	0	1	0	0	0	0	0	13	65%
											Total	64%
C. Trace elements only												
Aransas Bay' 07	11	0	2	2	1	0	0	0	0	1	0	65%
Aransas Bay' 08	4	16	0	0	0	0	0	0	0	0	0	80%
Corpus Christi Bay' 07	1	0	13	4	1	0	0	0	0	0	0	68%
Corpus Christi Bay' 08	0	0	7	11	0	1	0	0	0	0	0	58%
Upper Laguna Madre' 07	1	0	1	2	10	2	1	0	0	0	0	59%
Upper Laguna Madre' 08	0	0	2	3	1	10	0	2	0	0	0	56%
Flour Bluff Hatchery' 06	0	0	0	0	0	0	18	0	0	0	0	100%
Flour Bluff Hatchery' 07	0	0	1	0	0	2	0	11	5	0	0	58%
Flour Bluff Hatchery' 08	0	0	0	0	0	0	0	2	15	0	0	88%
Palacios Hatchery' 08	0	0	0	0	0	0	1	0	0	17	0	94%
Sea Center Hatchery' 08	0	0	0	0	0	0	0	0	0	0	20	100%
											Total	75%

Table 7
Simulation method and permutation method using actual data showing classification success based solely on chance. Compare with overall total classification success of linear discriminant function analysis with jack-knife cross-validation results (92%).

	Simulation method	Permutation method
Min	0.032	0.000
2.5%	0.055	0.020
Median	0.091	0.084
97.5%	0.141	0.203
Max	0.168	0.356

produced correct classification rates much lower than our LDFA with cross-validation result of 92% (Table 6A). Because there were 10,000 permutations used, the null hypothesis that our rate of correct classification was due to chance is rejected ($p < 0.0001$). Thus, chance alone cannot explain the high rates of classification success produced from LDFA.

4. Discussion

We were able to successfully discriminate hatchery-reared fish from wild fish using otolith microchemistry of both stable isotopes and trace element chemistry. Differences in otolith signatures were shown to exist among groups of wild and hatchery fish and among years. The LDFA correctly classified fish to their natal origins (either wild or hatchery) with high success rates (overall 92%), and was shown to be significantly higher than classifications based solely on chance. These findings are similar to previous studies attempting to discriminate other species of hatchery-reared fish from wild fish using otolith chemistry. For red snapper, hatchery-reared fish were distinguished from wild fish with 100% accuracy (Gibson et al., 2010), hatchery-reared brook and rainbow trout were distinguished from wild trout with 100% and 94% accuracy (Coghlan et al., 2007), and walleye were assigned to their natal hatchery with 92% accuracy (Bickford and Hannigan, 2005). Studies identifying natal origin of juvenile fishes showed true classification accuracies of 87% for juvenile weakfish (Thorrold et al., 1998), 93% for spotted seatrout (Comyns et al., 2008), and between 70 and 92% for five different estuarine species (Vasconcelos et al., 2007).

Among the suite of chemical predictor variables analyzed, stable isotopes $\delta^{13}C$ and $\delta^{18}O$ were two of the most influential discriminators for assigning group membership. The discriminatory power of these two isotopes in these environments is likely attributed to the dramatic salinity gradient that exists in bays along the Texas coastline. From north to south, bays transition from mesohaline positive estuaries toward hypersaline negative estuaries with some areas of the Upper Laguna Madre having reported salinity levels upwards of 70 ppt (Quammen and Onue, 1993). This gradient is driven by decreasing precipitation (i.e. freshwater inflow) coupled with increasing rates of evaporation from north to south. Increasing salinity has been positively correlated with increasing $\delta^{13}C$ and $\delta^{18}O$ in otoliths (Elsdon and Gillanders, 2002; Pruell et al., 2012). These two stable isotopes also successfully discriminated juvenile

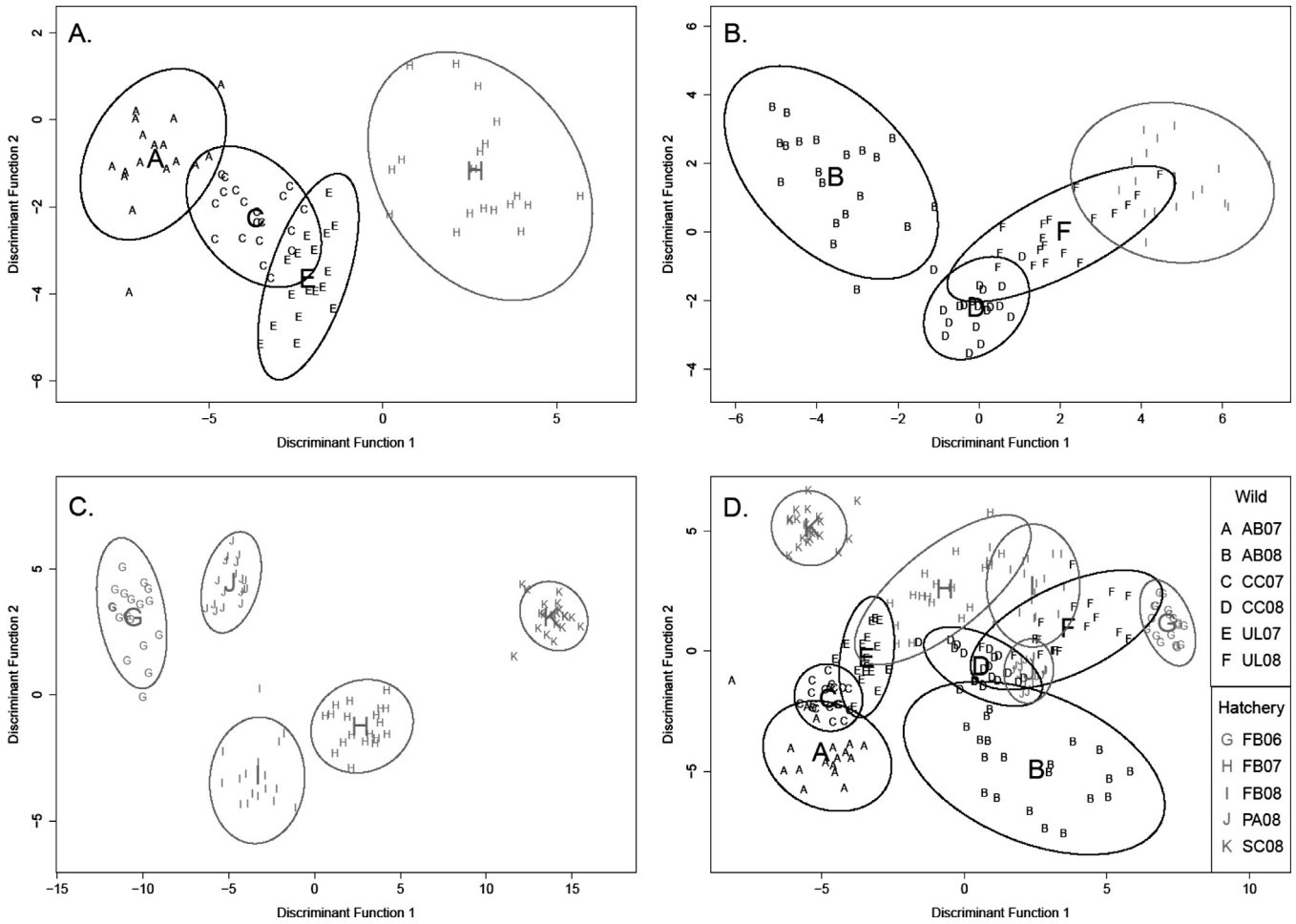

Fig. 4. Linear discriminant analysis plots for true classes of the first two discriminant functions for (A) 2007 wild and Flour Bluff hatchery samples, (B) 2008 wild and Flour Bluff hatchery samples, (C) hatchery-reared only, and (D) all group combinations. Small letters show individual observations per group. Large, bold letters represent group centroids (multivariate means). Ellipses represent 95% confidence interval. Black letters indicate spotted seatrout of wild origin; gray letters indicate spotted seatrout of hatchery origin. Wild fish sources: AB = Aransas Bay, CC = Corpus Christi Bay, UL = Upper Laguna Madre; Hatchery fish sources: FB = Flour Bluff Hatchery, PA = Palacios Hatchery, SC = Sea Center Hatchery.

red drum, another Sciaenid, along the Texas coastline (Rooker et al., 2010). Interestingly, we saw this pattern of increasing otolith $\delta^{13}C$ and $\delta^{18}O$ (although less pronounced) with increasing salinity moving south from Aransas Bay to Corpus Christi Bay to the Upper Laguna Madre in 2007 but not in 2008. One possible explanation for the year-to-year change is the dramatic difference in the amount of rainfall received between these two years. Rainfall was substantially greater in 2007 than in 2008 in South Texas (NCDC-NOAA, 2012). In 2007, sufficient rainfall may have promoted the formation of the north-south salinity gradient and created salinity differences among the three bays. The differences in stable isotopic signatures $\delta^{18}O$ and $\delta^{13}C$ among the bays reflect these differences in salinity. Conversely, in 2008, the lack of rainfall may have had a homogenizing effect on salinity and minimized the differences among the three bays, which created only marginal differences in stable isotopic $\delta^{18}O$ and $\delta^{13}C$ within the otoliths. Nonetheless, $\delta^{18}O$ and $\delta^{13}C$ showed the first and second greatest discriminatory power among all chemical predictors in this study, and linear discriminant analysis using only stable isotopes categorized fish to their natal origin with 64% classification success overall. Considering the increased costs and technical difficulties associated with processing trace element chemistries, knowing that stable isotopes will provide most of the discriminatory power in assigning group memberships can prevent unnecessary processing expenses in future studies. However, where studies require more precision or are performed over multiple years, the addition of trace element chemistry to stable isotopes can provide even greater discriminatory power for group assignment.

Elevated levels of otolith strontium were found in spotted seatrout from the Upper Laguna Madre compared to Aransas and Corpus Christi Bays, which likely reflects higher salinity levels associated with this bay system. Significant correlations between otolith strontium and salinity have also been observed in other studies with increases in salinity producing elevated levels of strontium in otoliths (Kraus and Secor, 2004; Secor et al., 1995; Secor and Rooker, 2000; Tzeng, 1996). We did not see substantial annual variation in otolith strontium as with stable isotopic signatures, suggesting that strontium is less susceptible to annual fluctuations in climate and may represent a more temporally stable marker in otoliths. This characteristic makes strontium useful in studies involving longer time scales or spanning multiple ontogenetic stages (de Pontual et al., 2003; Friedland et al., 1998; Kafemann et al., 2000; Kennedy et al., 2002; Walther and Thorrold, 2009). There were noticeably higher levels of strontium present in the otoliths of hatchery-reared fish compared to wild fish. As Flour Bluff hatchery obtains its seawater from the Upper Laguna Madre, we would expect to see comparable levels of strontium occurring in otoliths in wild fish from the Upper Laguna Madre and hatchery-reared fish from Flour Bluff hatchery. Instead, substantial differences in otolith strontium were seen between the

Flour Bluff hatchery and the Upper Laguna Madre in both 2007 and 2008. The observable difference indicates strontium uptake is either additionally regulated by physiological forces (Fowler et al., 1995; Kalish, 1989) or that the hatchery residency period imparts a unique water chemistry signature onto otoliths that is different from its bay source. In laboratory trials, the contribution of water source to otolith strontium levels was estimated at 83% for juvenile mummichog (Walther and Thorrold, 2006) indicating that water chemistry dominates the uptake of strontium into the otolith aragonite matrix for this marine species. It seems likely therefore, that the majority of otolith strontium for our hatchery-reared spotted seatrout is derived from the ambient water chemistry of hatchery ponds but is partially supplemented from additional sources such as food. Hatchery ponds may also experience higher rates of evaporation compared with nearby bays, thereby increasing salinity levels in hatchery ponds relative to their bay water source, and the higher levels of otolith strontium in hatchery fish compared to wild fish reflects this difference. Another possible explanation is that wild fish may be moving across wider salinity gradients within the estuaries compared to hatchery fish, which are restricted to higher salinity water derived directly from more marine sources. Unfortunately, we were unable to obtain nominal salinity values for the hatchery ponds or the bays during this study to compare salinities between these two environments. However, the hatchery-rearing process clearly imparts a unique signature through the uptake of trace elements that makes fish reared in these hatcheries distinguishable from nearby wild populations.

In addition to using the natural chemical variation of otoliths to discriminate hatchery signatures, managers are also using artificial manipulation methods during the hatchery rearing process. Such strategies may involve spiking concentrations of specific isotopes such as ^{137}Ba, producing a detectable and transgenerational marking in the otoliths. Successful detection of such artificial markings has been achieved through direct maternal injection (Thorrold et al., 2006), immersion of larvae or juveniles in enriched ponds (Munro et al., 2008), and through dietary transmission (Woodcock et al., 2013). Where natural chemistries are too variable to adequately distinguish hatchery-reared fish with confidence, artificial manipulation may provide the complementarity necessary to achieve high levels of classification success.

Substantial temporal and spatial variation was evident among wild fish otolith signatures and hatchery fish otolith signatures. The high classification accuracies using LDFA among hatchery-reared fish show that otolith signature patterns are distinct not only among different hatcheries separated spatially but are also dynamic on year-to-year time scales within the same hatchery (i.e. Flour Bluff hatchery 2006–2008). Temporal variability was also seen among groups of wild fish over the two years and was greater than the spatial variation exhibited among bays in either year. If we consider that greater variation among groups leads to more accurate classification success, we would also expect that less variation among groups creates greater overlap and less accurate classification success. Of the eleven misclassifications that occurred among wild groups using LDFA, only one fish was misclassified to the wrong year, and it was also misclassified to the wrong bay. The ten other misclassifications occurred among bays within a single year. This indicates that the temporal variability seen in otolith signatures was greater than the spatial variability over the period of our study. Substantial annual variation within the same bay or hatchery means that if adult assignment to natal origin is the goal, then investigators must maintain a bank of cohorts from each year as well as each location for accurate retrospective classification. Simply using one bay or hatchery as the benchmark for comparison, while ignoring the year, may be insufficient because of high temporal variability. The loss in resolution power may be compounded when using only stable isotopes for otolith signatures, as they have been shown to fluctuate even more than certain trace elements (i.e. strontium) over temporal scales (Walther and Thorrold, 2009). We recommend, therefore, that when studies span longer than one year, trace element chemistries are necessary in addition to stable isotopes to provide greater discriminatory power to achieve accurate classifications to natal origin. For single year studies, using only stable isotopic signatures may prove sufficient and would be preferred when minimizing costs is necessary.

Otolith chemistry can be a powerful tool to discriminate between hatchery-reared and wild spotted seatrout. The ability to discriminate these two groups from each other based on their unique otolith chemistry fingerprint provides the crucial key first step toward measuring the contribution of hatchery-reared fish to wild stocks. The next step would be to capture adult fish in the wild and, using otolith chemistry as a tool for assignment as demonstrated in this study, classify these individuals to wild or hatchery origin. This process would involve milling out the core of the otolith on captured adult fish, which represents the chemical signature recorded as a fingerling and matching that core signature with the baseline signatures in fingerlings from this study. From there it is possible to calculate the proportion of hatchery fish that have survived into adulthood. Given the temporal variability associated with these chemical fingerprints (especially in stable isotopes), we recommend hatcheries maintain an otolith bank of fish each year to serve as the baseline from which retrospective assignments can be made. This is easily accomplished by setting aside a number of fish each year to use for otolith chemistry assays. With significant capital funneled into stock enhancement programs each year, it is critical to understand the contribution of hatchery enhancement to wild stocks. This is reflected largely by the number of hatchery-reared fish that survive to adulthood and recruit into the directed fishery. By using the otolith chemistry techniques presented here fishery managers can better optimize hatchery programs to improve the efficacy of stock enhancement programs.

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