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A Comparison of Otolith Geochemistry and Stable Isotope Markers to Track Fish Movement: Describing Estuarine Ingress by Larval and Post-larval Halibut

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Abstract Estuarine recruitment of fishes is a potential bottleneck in the life cycle of many coastal species. We investigated patterns of size-at-ingress for larval and post-larval California halibut entering the Punta Banda Estuary (PBE), Mexico, using both otolith geochemistry and carbon stable isotope ratios (SIR). Juvenile halibut (n=126; 38–163 mm standard length [SL]) were collected from inside PBE and the adjacent exposed coast during the fall of 2003, and otoliths (geochemistry) and muscle tissues (SIR) were analyzed to reconstruct the environmental histories of individuals. Based on geochemical analyses, nearly all fish collected from PBE were characterized by a non-estuarine signature (e.g., low Mn and Ba) in the otolith growth bands deposited when fish were <30 mm SL. Although fish collected from the coast retained that signature throughout their lives, fish collected within PBE showed elevated concentrations of Mn and Ba in the otolith growth bands deposited once halibut were 30–70 mm SL, thereby recording ingress. Carbon SIR of juvenile halibut prey also differed between the estuary and coast. Muscle $\delta^{13}C$ values of halibut captured along the coast were consistent (ca.−15‰), while those captured in the estuary were variable and generally more enriched in ^{13}C (−16‰ to −11‰). Both natural tagging approaches agreed that most halibut (~75 %) enter PBE long after settlement (>>8–12 mm SL), although size-at-ingress estimates were significantly larger (mean difference = 27 mm; $p<0.001$) when derived via carbon SIR than with otolith geochemistry. Potential explanations for the differences in size-at-ingress estimates involve the magnitude of isotopic and trace element gradients at this ocean–estuary boundary, the temporal resolution of environmental tags stored within otoliths and soft tissues, and the size-at-capture or somatic growth rate of juvenile halibut. We conclude by discussing the relative merits of otolith geochemistry and SIR as natural tags, and by considering the implications of secondary dispersal into estuaries by post-larval fish.

Keywords California halibut · Larval ingress · Otolith geochemistry · Post-larval dispersal · Recruitment · Stable isotopes

Introduction

Many marine species have complex life histories in which transitions between life stages coincide with shifts in habitat use (Able 2005). For instance, many fishes and invertebrates have bipartite life histories in which larvae occupy water-column (often pelagic) habitat prior to transformation in to settled juveniles and adults that live in benthic (often coastal) environments. Because of their susceptibility to physical transport, expected ontogenetic migrations, and importance in overall population dynamics, larval and juvenile stages have been the focus of much attention for quantifying the rates, scales and consequences of exchange among marine habitats, populations or communities (Miller et al. 1991; Gillanders et al. 2003; Pineda et al. 2008).

Estuaries are known to provide critical nursery habitat for many ecologically and economically valuable species (Secor and Rooker 2005). Within the broad community of 'estuarine-dependent' fishes and mobile invertebrates, a large subset of species occupy the continental shelf or

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margin during the adult stage, where reproductive activity occurs (Able 2005). For these species, the movement of individuals during early life stages connect estuarine and ocean ecosystems (Gillanders et al. 2003). In some species, larvae recruit to estuaries prior to or coincident with metamorphosis (e.g., Burke et al. 1991; Tanaka et al. 1989). Other species, however, may settle along the exposed coast, and either reside there as juveniles (Woodland et al. 2012), or undergo a secondary dispersal phase as settled juveniles to estuarine habitat. Some species use both estuarine and coastal habitats throughout the juvenile stage (Able et al. 2006; Fodrie and Mendoza 2006). Thus, coastal settlement and secondary dispersal of juveniles to estuarine habitat may be a major pathway in which ingress occurs, although to our knowledge there are no direct measures of the relative importance of larval versus post-larvae ingress for any species. The timing of ingress is sometimes inferred indirectly via length–frequency analyses, although these estimates can be biased due to gear selectivity and size-specific mortality (Sogard 1997).

Measuring the connectivity between natal, nursery and adult habitats is greatly aided by the ability to track individuals. Unfortunately, early life history stages of fishes and invertebrates are particularly difficult to track due to their small size, high mortality, and patchy distribution over a range of scales (Levin 2006). In response to these difficulties, researchers have employed a suite of novel molecular (Kinlan and Gaines 2003), biophysical (Carson et al. 2011), natural (Herzka et al. 2002) and induced (Almany et al. 2007) tagging approaches to document the migratory paths of marine species across multiple spatial and temporal scales.

Geochemical tags stored within fish otoliths (teleost ear stones) have been employed with success to determine the migratory corridors and nursery origins of marine fishes recruiting to adult stocks (e.g., Gillanders and Kingsford 2000; Fodrie and Levin 2008). Otoliths grow as daily and annual rings that are deposited around a central core. As rings accrete, trace elements are deposited into successive growth bands in some relation to the ambient environment (Campana 1999). Provided that there are spatial gradients in environmental conditions (e.g., temperature, salinity, elemental concentration), otoliths can carry a permanent record of the environments or habitats experienced by individuals throughout their lives. Interestingly, despite the proliferation of studies employing otolith-based geochemical tags to identify the nursery origin(s) of egressing juveniles, the reverse process—the mode (larval, post-larval) and timing (size-at-ingress) of ingress—has not been examined using this approach.

Stable isotope ratio (SIR) analyses of soft tissues have been used successfully for tracking the estuarine ingress or emigration of larval and juvenile fishes and invertebrates (Fry 1981; Herzka et al. 2002). This approach takes advantage of differences in the isotopic composition of coastal and estuarine food webs (Michener and Lajtha 2007). Since over time the soft tissues of consumers integrate and reflect the isotopic composition of their foods (Fry and Arnold 1982), individuals that have recently migrated between estuarine and coastal habitat can be differentiated from residents based on their isotope signatures (Guelinckx et al. 2008). The size at which a dietary shift occurred can be estimated if the rate of isotopic turnover, which determines the period over which soft tissues integrate the isotopic composition of their foods, can be measured or estimated (Herzka 2005; Phillips and Eldridge 2005).

We explored the mode and timing of estuarine ingress by California halibut (*Paralichthys californicus*) to an estuary in Baja California using both of these tracking approaches that allow for the reconstruction of environmental histories experienced by individuals. We were simultaneously interested in ecological and methodological questions. First, we tested whether recently spawned halibut entered estuarine habitat prior to (larval) or following settlement (post-larval). For individuals that appeared to enter estuarine habitat following settlement, we attempted to estimate size-at-ingress. Second, we compared the size-at-ingress estimates derived from otolith geochemistry and muscle SIR. Based on the environmental gradients recorded in this system, we also discuss the utility and limitations of each approach.

Methods

Model Species

California halibut are common in the coastal waters along the west coast of North America. Along Baja California, spawning occurs in January–February and June–September in shallow coastal waters (<30 m). Following a ca. 30-day pelagic phase during which larvae are distributed across the shelf, transformed juveniles begin settling along exposed coasts and protected estuaries at 8–12 mm standard length (SL; Allen 1988).

Catch rates of juvenile halibut (10–160 mm SL) are elevated by an order of magnitude in estuaries relative to other potential nurseries such as exposed coastal beaches (Allen 1988). For ocean-spawning species like halibut that disproportionately occupy coastal estuaries as juvenile habitat (termed facultative estuarine-dependent species; Able 2005), ingress through coastal inlets is generally described as occurring during the larval phase (Pearson 1929; Forward et al. 1999). Kramer (1990) conducted extensive bottom surveys within exposed coast and estuarine habitats of southern California, however, and hypothesized that in some years the majority of halibut first settle along the exposed coast, and then likely undergo a secondary dispersal phase as benthic

juveniles into estuaries. Specifically, Kramer (1990) noted that within estuaries, the 20–30 mm SL size class was often less abundant than the 40–50 mm SL size class, suggesting notable immigration of juvenile halibut ca. 30 mm in length. Simultaneously, densities of the 40-mm SL size class were dramatically lower than for the 20-mm SL size class along the exposed coast, potentially indicating emigration into nearby estuaries. While intriguing, these data are circumstantial and could have resulted from differences in gear selectivity among size classes and between habitats (Sogard 1997), as well as losses due to elevated predation along the exposed coast (Fodrie et al. 2009).

Study System

The Punta Banda Estuary (PBE) in Baja California, Mexico (Fig. 1), is a relatively unmodified system that has a main channel stretching 7.6 km, a median depth of 5 m and a simple L-shaped footprint (Ortiz et al. 2003). PBE is connected to the semi-enclosed Todos Santos Bay (TSB) by a single narrow inlet. Within TSB, a crescent-shaped coastal system located 100 km south of the United States (USA)–Mexico border, there is suitable habitat for juvenile halibut along an 8-km long beach with sandy, sub-tidal habitat. Within PBE and other estuarine systems of similar size (0.5–5 km^2), juvenile halibut densities are typically elevated toward the middle or inner regions of the embayment, relative to densities within the section of the estuary nearest the inlet (Fodrie and Mendoza 2006; López-Rasgado and Herzka 2009).

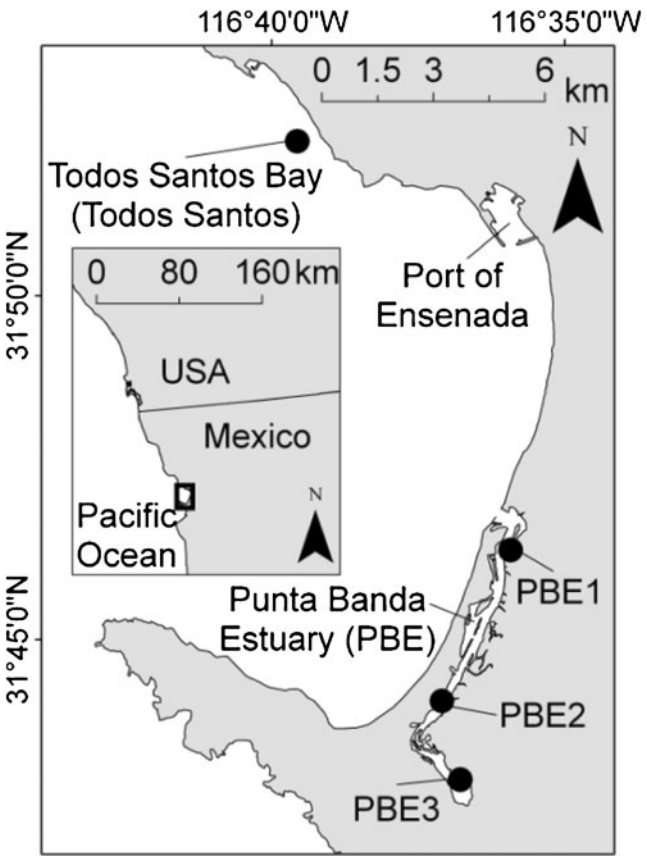

Fig. 1 Locations of juvenile halibut collections in the Punta Banda Estuary and Todos Santos Bay, Mexico (denoted by *filled circles*). Sites are labeled as: *PBE1* (outer estuary), *PBE2* (mid estuary), *PBE3* (inner estuary), and *Todos Santos* (coastal)

Specimen Collections

Juveniles were collected on October 2–3 and November 4–5, 2003, for analyses to determine the mode (larval, post-larval) and timing (size) of ingress into PBE for fishes spawned earlier that year. Overall, 126 juvenile halibut were collected from the outer (PBE1), middle (PBE2) and inner (PBE3) PBE (Fig. 1) to determine size-at-ingress based on either geochemical tags, stable isotope markers, or both. Eight juveniles were also collected from TSB to provided coastal reference specimens for geochemical and stable isotope analyses. Collections were made using an otter trawl (sampling methods described fully in Fodrie and Mendoza 2006), and halibut ranged between 38 and 163 mm SL, indicating that we collected individuals spawned throughout the summer and fall of 2003. We tested for differences in fish length among collection zones within PBE1 (95.8±4.3 mm SL; μ±1 SE), PBE2 (101.4±4.6 mm SL), and PBE3 (99.4±5.9 mm SL) and observed no significant difference (one-way analysis of variance [ANOVA]; $F_{3,122}$=0.202; p=0.818). Not all individuals could be analyzed using both natural tagging approaches due to limitations regarding the availability of instrument time (otolith geochemistry; fish were 105.5±5.0 mm SL) or fresh tissues (SIR; fish were 98.0±3.4 mm SL). Potential prey for halibut (mysids and small fishes; Plummer et al. 1983; Allen 1988) were also collected in PBE and TSB and analyzed for SIR to aid in the interpretation of the isotope data from halibut.

Otolith Geochemistry

Gradients in geochemical signatures have already been reported within PBE and TSB. Fodrie and Herzka (2008) collected and caged juvenile halibut within this system throughout 2002–2004, and reported increasing concentrations of Mn and Ba (and to a lesser degree, decreasing concentrations of Mg and Sr) in the otoliths of wild-caught and caged juveniles collected farther within PBE. Furthermore, all of PBE was enriched in Mn and Ba relative to TSB, the ability to correctly identify the collection location of individuals as estuarine or coastal was high (>90 % distinct).

Sagittal otoliths from the blind side of fish were dissected using sterile scalpels and ceramic forceps. Otoliths were sonicated for 5 min in 15 % H_2O_2 buffered with 0.05 mol l^{-1}

NaOH, 5 min in 3 % HNO_3^-, and rinsed with Milli-Q to remove attached organics. We mounted otoliths in crazy glue on petrographic slides, sanded them along the sagittal plane using 30-and 3-μm lapping paper, and polished them with a wet rock cloth. Mounted otoliths were given additional 5-min washes in 15 % H_2O_2, then 3 % HNO_3^-, and finally rinsed three times with Milli-Q before being dried and stored in a class-100 laminar flow hood. All containers, slides and forceps were rinsed with 3 % HNO_3^- before contact with otoliths.

Geochemical analyses of otoliths were performed at the Scripps Institution of Oceanography Unified Analytical Facility. We sampled growth rings using a New Wave UP 213-nm laser ablation unit. Otoliths were sampled by ablating 150-μm lines parallel to targeted growth bands at 0.5 mJ intensity, 15-μm s^{-1} scan speed and 20-μm spot size. The first ablation we made on each otolith was used to define geochemical fingerprints of estuarine (PBE 1, PBE 2 and PBE3) and coastal (TSB) habitats during the fall of 2003. These ablations were begun as close to the post-rostral apex as possible (i.e., last growth rings) and progressed ventrally along the post-rostral margin (PRM), thereby sampling the most recent growth increments.

Along the axis between the otolith core and the PRM, we also made a series of ablations to quantify the geochemical signatures present within otoliths representing every 10 mm of juvenile somatic growth. These ablations were spaced at every 165 μm along the posterior section of the otolith, with the first ablation in the series being set 165 μm posterior of the core (i.e., the core was not sampled). A constant gap distance between ablations was selected based on the linear relationship between otolith and somatic length across the range of halibut sizes considered in this study (R^2=0.84; p<0.001), and with respect to the proportion of otolith growth that occurs posterior to the core (48.0±0.1 %) representing each 10 mm of somatic growth. The number of ablations on each otolith depended on the length of the juvenile fish (sizes ranged from 38 to 143 mm SL; therefore, 3–14 ablations along the core–PRM axis). Across these ablations, the estimated size-at-ingress of each fish could be determined by examining where on each otolith estuarine-type signatures became evident (and using the tight linear relationship between otolith and somatic lengths to translate between the location of ablations on otoliths and fish size represented at each of those ablations). In all, the otoliths of 28 fish were examined in this manner.

Ablated material was transported using He gas (mixed with Ar) to a Thermoquest Finnigan Element 2 Inductively Coupled Plasma Mass Spectrometer. We sampled for the following isotopes to search for spatial discrimination: ^{26}Mg, ^{48}Ca, ^{55}Mn, ^{63}Cu, ^{88}Sr, ^{135}Ba, and ^{208}Pb (hereafter referred to by elemental abbreviation). Data processing to generate elemental concentrations standardized to calcium (X/Ca) followed Fodrie and Levin (2008). Detection limits for each element were as follows: 0.02 mmol mol^{-1} (Mg/Ca), <0.01 mmol mol^{-1} (Mn/Ca), 0.1 μmol mol^{-1} (Cu/Ca), 0.01 mmol mol^{-1} (Sr/Ca), <0.01 mmol mol^{-1} (Ba/Ca), and <0.001 mmol mol^{-1} (Pb/Ca). A glass standard spiked with trace elements (National Institute of Standards and Technology Standard Reference Material; NIST 612) was analyzed at the beginning and end of each day to account for machine drift.

Geochemical Data Analyses to Estimate Size at Ingress

Estuarine and non-estuarine geochemical signatures were generated from the PRM ablations on the otoliths of juvenile halibut. Element to calcium ratios (X/Ca) were analyzed using linear Discriminant Function Analysis (DFA). Using untransformed X/Ca PRM data as a reference set of signatures, fish were grouped as estuarine (collected from PBE1, PBE2 or PBE3) or non-estuarine (collected from TSB) residents. DFA was conducted stepwise by running the analysis on all element ratios and dropping the least significant variable as determined by the F-to-remove statistic. This procedure was repeated until all remaining element ratios scored F-to-remove values >2. Cross-validation of the DFA model was achieved by reclassifying each sample using the jackknife method to determine the probability of correct site assignment for PRM data. Since we were only interested in evaluating easily distinguishable estuarine and non-estuarine geochemical tags within halibut otoliths, and the DFA on PRM data demonstrated homogeneous within-group dispersions (along DFA Score 1), we did not employ additional statistical approaches on these data such as Random Forests, Artificial Neural Networks, or Markov Chain Monte Carlo methods (White et al. 2008; Mercier et al. 2011). Subsequently, we estimated size-at-ingress of each individual fish by evaluating core–margin transect data as 'unknowns' to determine when (at what size) estuarine signatures were recorded in otoliths.

Laboratory SIR Turnover Rate Experiment

To support the interpretation of isotope ratios from PBE recruits, we performed a dietary shift experiment to estimate the rate of isotopic turnover. Eggs were obtained from the Sea Laboratory (Redondo Beach, California, USA) and transported to the facilities of the Department of Aquaculture at the Centro de Investigación Científica y de Educación Superior de Ensenada. First-feeding larvae were fed rotifers (*Brachionus plicatilis*) until 19 days post-hatch and then switched to *Artemia* nuaplii enriched with highly unsaturated fatty acids (Zacarías-Soto and Lazo 2006). A few days prior to initiating the experiment, fifty halibut were placed in each of six 150-l tanks. The dietary switch experiment began at 86 days post-hatch (mean size = 25.3 mm SL). Dietary switches were

conducted at 15, 19 and 24 °C (n=2 tanks per treatment). This range of temperature was suitable for rearing California halibut larvae and representative of seasonal fluctuations in PBE and TSB.

To account for variations in growth and initial size when estimating rates of isotopic turnover, halibut were marked with 0.25×2 mm Coded Wire Tags (CWT; Northwest Marine Technologies, Inc.). Tags were injected into the dorsal muscle of the eyed-side after immersing fish in 1 g l^{-1} MS-222. Fish were measured and weight immediately after tagging. There was no mortality due to tagging.

At the start of the experiment juveniles were switched from the *Artemia* nauplii (δ^{13}C=−22.1±0.1; mean±SD, n=2) to frozen freshwater mysids (*Mysis relicta*) (δ^{13}C=−31.4±0.3; n=2). There was a 9.3‰ difference between the initial ($\delta_{initial}$) and final (δ_{final}) diets. Three fish per tank were sacrificed at the beginning of the experiment to measure $\delta_{initial}$. Fish were fed at 10 % of tank biomass per day, and three fish were sampled every 4 or 6 days for isotope analyses. All sampled individuals were measured, dried, weighed and frozen individually. Coded wire tags were removed by dissection and each individual identified. The rate of isotopic turnover was estimated by curve fitting an isotope turnover model to the laboratory data. The model partitions the contribution of growth (biomass gain) and metabolic turnover (catabolism and loss of body tissues) to isotopic turnover. The isotopic values of individual fish were plotted as a function of time and relative biomass gain (W_r=W_t/W_i), where W_t and W_i are wet weight at time t and the beginning of the experiment, respectively (Fry and Arnold 1982):

$$\delta_t = \delta_{final} + (\delta_{initial} - \delta_{final}) * W_r^{\,c}$$

δ_t is the isotopic composition of individuals sampled throughout the experiment, $\delta_{initial}$ and δ_{final} are the isotopic composition of fish while equilibrated onto the *Artemia* and freshwater mysid diets, respectively (hereafter $\delta_{Artemia}$ and δ_{mysids}). The value of c, the coefficient of metabolic turnover, partitions the contribution of growth and metabolic turnover to isotopic turnover. Values of $c < -1$ indicate that growth and metabolic turnover drive isotopic turnover, while $c = -1$ indicates growth is the only detectable process. $\delta_{Artemia}$ was computed as the average isotope ratio of all fishes sampled at the beginning of the experiment (−20.5±0.2‰). The value of δ_{mysids} was calculated using the isotopic composition of the mysid feed corrected for trophic discrimination (TD). The carbon TD factor was calculated as the difference between the isotope ratio of the *Artemia* nauplii and that of fish at the beginning of the experiment (TD = 1.6‰; δ_{mysids} = −29.8±0.3‰, n=2). $\delta_{Artemia}$ and δ_{mysids} were fixed during the least-squares nonlinear curve-fitting procedure. We assumed that the TD value between mysids and fish tissue was the same as that calculated for *Artemia*.

SIR Analyses

California halibut collected from PBE, TSB or laboratory experiments were thawed and white muscle was dissected from the dorsal area. For teleost potential prey, we also used muscle tissue. Mysids were processed whole. All samples were rinsed thoroughly in distilled water and dried before processing. Ground samples (500–1,200 μg) of halibut or potential prey were placed in tin capsules and sent to the Stable Isotope Facility of the University of California, Davis for Carbon SIR analysis. The C/N ratios of muscle samples were 3.2±0.1, indicating a low lipid content that did not require lipid extraction or mathematical normalization (Post et al. 2007). Carbon isotope ratios were reported as δ values:

$$\delta_{sample}(‰) = \left(R_{sample} - R_{standard}\right)/R_{standard} \times 1,000$$

where δ_{sample} is the isotopic ratio of the sample and R is the ratio of the heavy to light isotopes (^{13}C/^{12}C) in the sample relative to the SIR standard Vienna Pee Dee Belemnite (V-PDB). During analyses, standards were routinely interspersed with study samples and instrument precision was found to be 0.2‰.

SIR Data Analysis to Estimate Size at Ingress

Carbon SIR of individual halibut collected in PBE and TSB were plotted as a function of SL to evaluate whether an isotopic shift from a coastal to estuarine signature was observed. To back-calculate size at ingress for individual halibut (n=109), Fry and Arnold's (1982) isotope turnover model was solved for weight at ingress (W_i) using field-based estimates of the isotopic composition of potential recruits ($\delta_{initial}$) and halibut equilibrated to food sources in PBE (δ_{final}). The value of c used in the model was obtained in the laboratory experiment. Wet weight (g) was converted to SL (mm) using a relationship derived from measurements on the fishes collected in PBE (SL = $(W_i/2.56\times10^5)^{0.34}$; R^2=0.92).

Dual Approaches to Determine Larval Ingress

We employed multiple statistical tests to compare the effects of tracking approach (geochemical tagging versus SIR) and collection location (PBE1, PBE2 and PBE3) on the size-at-ingress estimates for juvenile California halibut into the PBE. First, we used a two-way ANOVA on estimated size-at-ingress data with 'tracking approach' and 'PBE zone' as fixed factors. Data from all 126 fish collected and analyzed from PBE were included in this analysis. Only modest violations of data homoscedasticity were observed (all p>0.04), and therefore no data transformations were conducted prior to this ANOVA. For 11 individuals that we obtained geochemical and SIR data, we also used a

paired *t*-test to determine the effects of tracking approach (but not collection area within the estuary) on estimated size-at-ingress. Again, no transformations were required prior to conducting this analysis. Additionally, we used simple linear regression to compare the size-at-ingress estimates from the 11 fish for which both approaches were used.

Results

Size-at-Ingress Estimates Based on Otolith Geochemistry

PBE and TSB imparted distinct geochemical tags within juvenile halibut otoliths during the fall of 2003. Mg, Mn, Ba and Pb were included in the final DFA model generated from PRM ablations. Classification success between PBE and TSB was 92 %. Both Mn and Ba were elevated in otoliths of fishes collected from PBE, while Mg and Pb were generally more enriched in the otoliths of fishes from TSB (Fig. 2a).

For juvenile fishes collected in PBE and TSB, there were clear differences in the patterns of geochemical markers and ontogenetic migrations recorded within otoliths. For instance, juveniles collected from TSB had low Mn/Ca ratios throughout successive otolith bands, indicative of continuous residency along the exposed coast. For juveniles collected within PBE, Mn/Ca ratios were initially low, but over time became more enriched in the more-recently deposited otolith bands (Fig. 2b). The timing and magnitude of the shift varied among collection zones: fish from PBE1 demonstrated elevated Mn/Ca ratios (by a factor of ca. 2 over fish from TSB) once fish reached ca. 70 mm SL; fish from PBE2 showed elevated Mn/Ca ratios beginning at 40–50 mm SL (by a factor of ca. 3); and fish collected from PBE3 were characterized by elevated Mn/Ca ratios starting at ca. 30 mm SL, eventually reaching Mn/Ca ratios more than 5-fold higher than observed for fish collected in TSB (Fig. 2b).

Along the primary (sole) DFA axis generated from PRM data (Mg, Mn, Ba and Pb), the most negative DFA scores were indicative of non-estuarine residency, while more positive values (still potentially 'negative' on a raw integer scale) were indicative of fish occupying estuarine habitat (Fig. 2a,c). From the ablations sampling the early growth bands on individual halibut, nearly all fish collected in PBE (75 %) and TSB (100 %) demonstrated strongly negative DFA scores, indicative of non-estuarine habitat utilization (Fig. 2c). Only seven fish (one from PBE1, two from PBE2, four from PBE3) collected from inside PBE were defined by estuarine-type signatures in their earliest growth bands. For the remaining 21 fish collected in the estuary and analyzed for otolith geochemistry, we observed transitions across otoliths indicative of habitat shifts. At ca. 30 mm SL, the

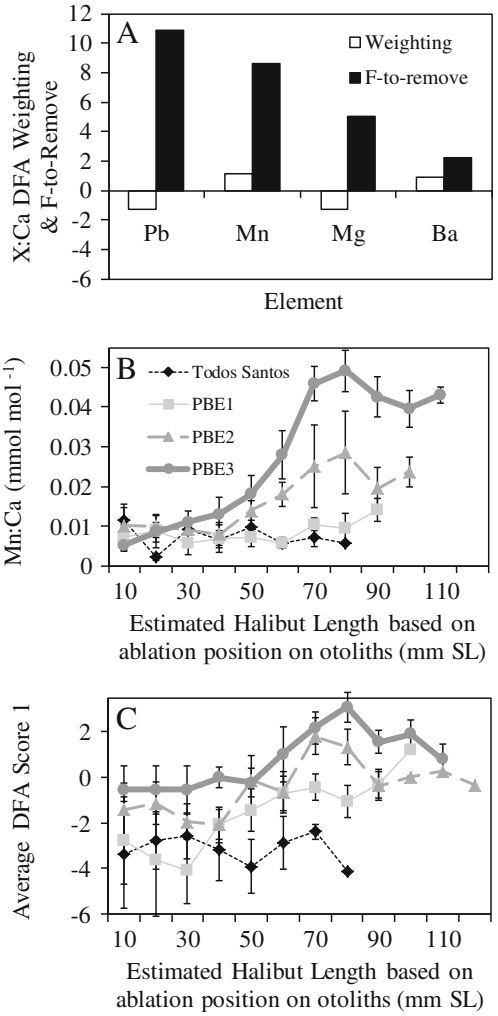

Fig. 2 Length-specific patterns in otolith geochemistry for juvenile halibut collected from PBE1, PBE2, PBE3 or TSB in the fall of 2003. **a** F-to-remove values and weighting for elements included in DFA algorithms to classify geochemical signatures as either estuarine or coastal based on data collected from the outer margin of fish otoliths. Positive values indicate the elements contributed toward estuarine DFA scores, while negative values indicate the elements contributed toward coastal DFA scores. **b** Mn/Ca concentrations (±1 SE) in the otoliths of fish, at every 10 mm SL, demonstrate the onset and intensity of estuarine signatures (i.e., elevated Mn/Ca values) for fish collected in PBE. **c** Average DFA scores of geochemical signatures (±1 SE) in the otoliths of fish representing either estuarine (higher scores) or coastal (lower scores) residency at every 10 mm SL somatic length (estimated from burn positions on individual otoliths)

environmental markers in the otoliths of juveniles from PBE3 changed so that they were identified as estuarine geochemical signatures (Fig. 2c), thereby recording ingress. Similarly, geochemical tags in the otoliths of fish from PBE2 and PBE1 were classified as estuarine signatures at ca. 40 and 70 mm SL, respectively (Fig. 2c). Once estuarine signatures were detected in an individual fish's otolith, DFA consistently identified all subsequent (relative to fish size) samples as characteristic of estuarine residency. Fishes from

TSB were found to have the most negative, non-estuarine signatures along their entire otoliths (Fig. 2c).

Laboratory-Based Isotope Turnover Rates

Halibut exhibited higher turnover rates with increasing temperature (Fig. 3a–c). Fish from the 15 °C, 19 °C and 24 °C treatments approximated isotopic equilibrium after about 50, 48 and 31 days, respectively. Curve-fitting Fry and Arnold's (1982) turnover model to our data yielded an estimate for the coefficient of metabolic decay (c) that did not differ significantly from −1 (one-tailed Student's t-test: p=0.35, 0.48 and 0.37 for the 15 °C, 19 °C and 24 °C treatments, respectively).

SIR of Potential Prey

The carbon isotope ratios of potential prey for juvenile halibut were more enriched in ^{13}C within PBE than in TSB. The mean±SD $\delta^{13}C$ values of small prey caught in the estuary were −12.2±0.9‰ for gobies (n=11), −9.8±1.8‰ for pipefish (a proxy for zooplanctivorous fishes; n=6) and −8.9±1.8 for mysid shrimp (n=11). No fishes were caught in TSB, and mysids had mean $\delta^{13}C$ values = −13.9±3.2‰ (n=4).

SIR as a Function of Size

The $\delta^{13}C$ values of fish from TSB were generally consistent (ca. −15‰; n=6), although two fish had isotope ratios enriched in ^{13}C by 1–2‰ (Fig. 4). Isotope ratios of fish collected in PBE were much more variable but typically more enriched in ^{13}C regardless of whether the fish were caught in PBE1, PBE2 or PBE3 (Fig. 5). The isotope ratios of the fish captured in the estuary did not have a normal distribution (Shapiro–Wilk p = 0.015), and we therefore applied non-parametric statistics to test for differences between sections of the estuary and between sampling months. There were no significant differences in $\delta^{13}C$ values of halibut collected in different sections of the estuary or months (Kruskal–Wallis test statistic = 2.817, p=0.589), and thus data were pooled. The relationship between $\delta^{13}C$ values and SL was significant (p=0.01) but very weak (R^2=0.08).

Estimates of Size at Ingress Based on SIR

To back calculate size-at-ingress, $\delta_{initial}$ was estimated as the average isotope ratio of halibut captured in TSB. The average $\delta^{13}C$ value of the most enriched 20th percentile was arbitrarily selected as the estuarine signature (δ_{final} = −11.2‰). To evaluate the sensitivity of size-at-ingress estimates to the value of

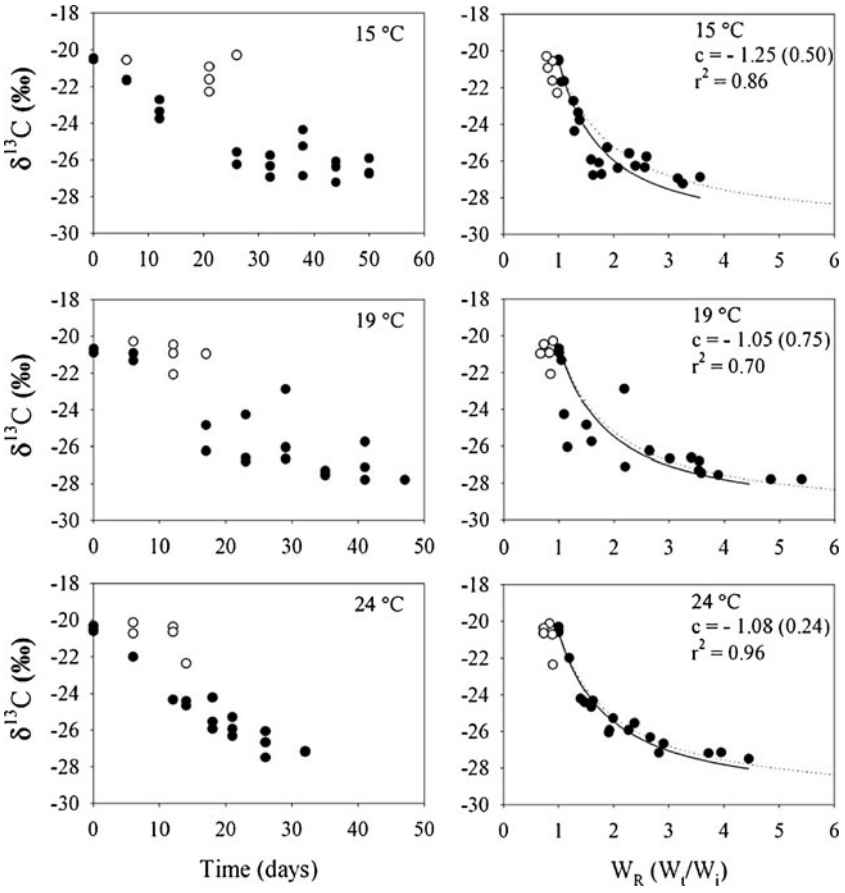

Fig. 3 Carbon isotope ratios of early juvenile California halibut switched to a diet of differing isotopic composition under controlled temperature conditions. Isotopic values are reported as a function of time (d) and relative wet weight gain in g (W_R = $weight_t/weight_{initial}$). *Open circles* represent fish that lost or did not gain weight during the experiment and were excluded from curve-fits. Fry and Arnold's (1982) isotope turnover model was fitted to the data (*black curve*). The pattern of isotopic turnover did not differ significantly from that predicted based on the dilution of the initial isotopic composition due to weight gain (*dotted curve*). The value of the coefficient of metabolic decay, c (SE), did not differ significantly from −1, which indicates that biomass gain drove isotopic turnover at the three temperatures

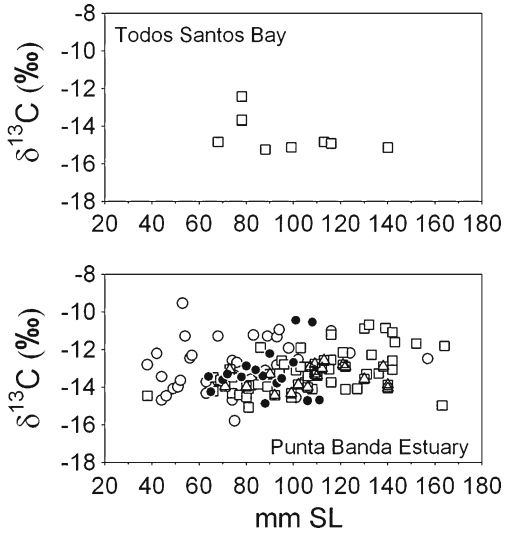

Fig. 4 $\delta^{13}C$ values of dorsal muscle tissue dissected from individual juvenile California halibut captured in TSB (*upper panel*) or PBE1, PBE2 and PBE3 (*lower panel*) in the fall of 2003. For fish caught in the estuary, *filled circles* indicate capture in the outer estuary (PBE1), *open circles* and squares represent collections from the mid estuary (PBE2) in October and November, respectively, and *open triangles* denote captures from the inner estuary (PBE3)

δ_{final}, calculations were also performed using δ_{final}±1.0‰ (−12.2 and −10.2‰; Fig. 6). Setting δ_{final} = −11.2‰ yielded a size-at-ingress estimates of 72±22 mm SL (mean±SD; n=100). Notably, fixing the value of δ_{final} at −10.2‰ and −12.2‰ yielded only moderately larger (75±23 mm SL; n=109) or smaller (67±21 mm SL; n=80) estimates of size at ingress, respectively.

Comparing Size-at-Ingress Estimates Using Dual Approaches

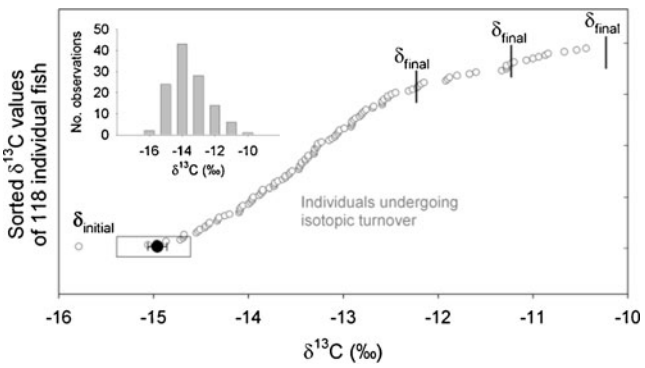

Fig. 5 $\delta^{13}C$ values of juvenile California halibut collected in PBE1, PBE2, PBE3 or TSB in the fall of 2003. Each *open circle* represents an individual fish (numbered along the *y*-axis), and isotopic values are plotted from the most depleted to the most enriched in ^{13}C. The isotopic composition of potential recruits ($\delta_{initial}$) and three possible values for halibut equilibrated onto estuarine foods (δ_{final}) are depicted. $\delta_{initial}$ and δ_{final} were used to back-calculate size-at-ingress for individuals considered to be undergoing isotopic turnover

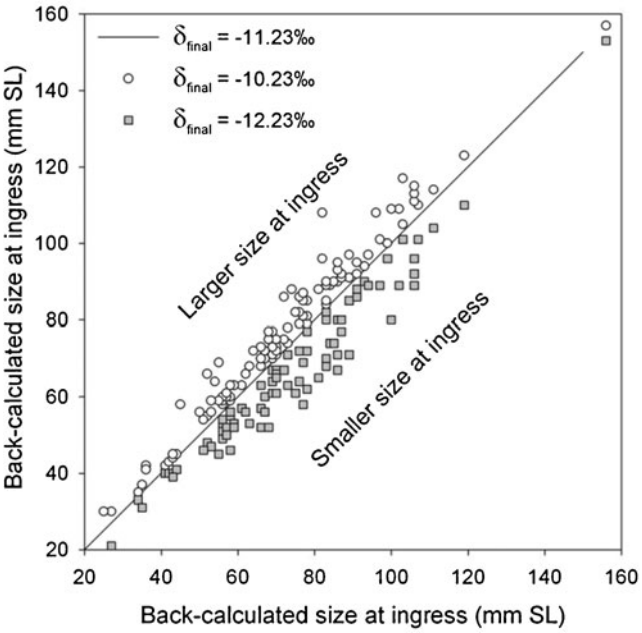

Fig. 6 Results of a sensitivity analysis used to examine the effect of using different equilibrium isotope values (δ_{final}) to back-calculate size at estuarine ingress of juvenile California halibut. Size-at-ingress was calculated using δ_{final} = −10.2‰, −11.2‰ and −12.2‰ (see text). Comparisons are performed against δ_{final} = −11.2‰ (the 1:1 line)

Using data from all 126 juvenile halibut, size-at-ingress estimates were interactively affected by the environmental marker we exploited (otolith geochemistry versus stable isotopes) and collection location of juvenile fish (PBE1, PBE2, PBE3; $F_{1,107}$=4.015; p=0.021). Notably, these approaches generated roughly similar size-at-ingress estimates for fish collected near the estuary mouth (PBE1: 60–70 mm), but markedly different size-at-ingress estimates for fish collected in PBE2 (mean difference: 22 mm) and PBE3 (mean difference: 50 mm) (Fig. 7a). Across all specimens, size-at-ingress estimates from stable isotope markers were larger than estimates generated via otolith geochemistry — ranging between 30 and 110 and between 10 and 80 mm SL, respectively (Fig. 7a–b). For the 11 fish analyzed using both tracking approaches, a paired t-test confirmed that stable isotope markers generated significantly larger size-at-ingress estimates than did geochemical tags (mean difference=27 mm; df=10; t=5.18; p<0.001). Regression analyses demonstrated that the differences in size-at-ingress estimates were most notable for individuals that may have entered PBE while very small (<40 mm SL) based on geochemical signatures (Fig. 7c).

Discussion

Our data represent a first attempt to directly track ingress for individual larval or post-larval fishes into estuarine nursery habitat using dual natural tagging approaches. Both approaches

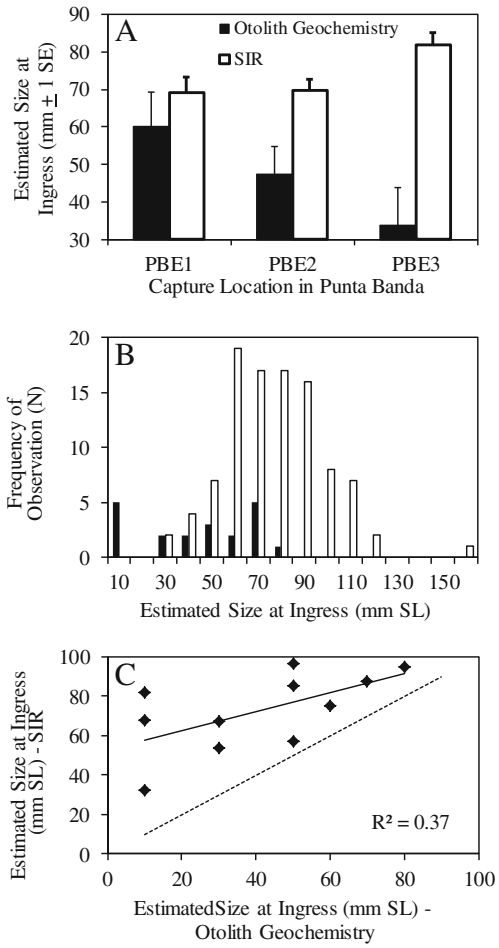

Fig. 7 Relationship in size-at-ingress estimates for juvenile halibut based on either otolith geochemistry or SIR signatures (based on δ_{final} = −11.23). **a** Mean (±1 SE) estimated size-at-ingress of juvenile halibut collected throughout the Punta Banda Estuary (*outer*, PBE1; *middle*, PBE2; *inner*, PBE3) based on geochemical and stable isotope markers. **b** Length-frequency distribution, in 10-mm bins, of size-at-ingress estimates for juvenile halibut in the Punta Banda Estuary (all zones) based on geochemical and stable isotope markers. **c** Relationship between geochemical- and stable-isotope-based size-at-ingress estimates for the 11 individual fish in which both approaches were employed. Also shown are the best-fit line, regression coefficient and 1-to-1 line

we employed indicated that the great majority of halibut first settled along the open coast (i.e., TSB, as indicated by low Mn/Ca and negative DFA 1 scores within the earliest growth bands) and then underwent a secondary dispersal phase across the ocean–estuary boundary. Specifically, geochemical tagging data demonstrated that only 25 % of individual halibut had estuarine signatures present in otolith growth bands deposited at the time those fish were 10 mm SL (5 of 20 juveniles). Since halibut settle at 8–12 mm SL (Allen 1988), those are the only fish that appear to have immigrated into PBE prior to the transition from planktonic larvae to benthic juveniles. The remaining 75 % of fish appeared to enter the estuary at between 30 and 80 mm SL as revealed by ontogenetic changes in Mg, Mn, Ba and Pb within otoliths. Alternatively, SIR analyses indicated that none of the fish we collected appeared to have entered PBE until at least 32 mm SL, with ingress continuing up until fish were >100 mm SL. These findings support previous work in southern California that hypothesized mostly post-larval ingress based on indirect evidence (length–frequency data; Kramer 1990).

Methodologically, a major goal of our study was to compare the patterns of ocean–estuary connectivity via larval and post-larval ingress as determined by dual natural tagging approaches. Although both tagging approaches agreed that the great majority of halibut enter PBE long after settlement (at 8–12 mm SL), size-at-ingress estimates were, on average, 27 mm larger when derived via carbon isotope ratios than with otolith geochemistry. Potential explanations for the differences in size-at-ingress estimates involve the magnitude of isotopic and trace element gradients at this ocean–estuary boundary, the temporal resolution of environmental tags stored within otoliths versus soft tissues, and the size-at-capture or somatic growth rate of juvenile halibut.

For either natural tagging approach, we observed distinct environmental gradients across the estuary–ocean boundary in the PBE–TSB system. Within otoliths, Mn, Pb, Mg and Ba contribute to distinct geochemical tags that allow for the reconstruction of the residency and movement of juvenile halibut (Fodrie and Herzka 2008). This is not surprising given the repeated demonstrations of distinct estuary and coastal signatures in other semi-arid regions (e.g., Gillanders and Kingsford 2000). Similarly, the $\delta^{13}C$ values of juveniles caught within PBE were, on average, more enriched in ^{13}C than those captured in TSB. This is consistent with the well-documented differences in the isotopic composition of the benthic primary producers that support estuarine food webs versus those from coastal systems in which production is driven by phytoplankton (France 1995; Kwak and Zedler 1997), and which is reflected in the isotopic composition of fishes that ingress from coastal to estuarine habitat as part of their life cycle (Herzka et al. 2002; Ciancio et al. 2008). It is also consistent with the presence of potential prey for halibut enriched in ^{13}C within the estuary.

However, the carbon isotope ratios of individual halibut caught within the estuary spanned a range of about 5‰, regardless of size, sampling month or capture location. The high variability in the $\delta^{13}C$ values of estuarine juveniles (not observed in otolith geochemistry) could be due to differences in individual feeding preferences or small-scale variations in the isotopic composition of available prey. Also, halibut of various sizes could be at different stages of isotopic turnover from the coastal to the estuarine signature (see below). Regardless of the underlying cause of the variability, the absence of a clearly identifiable 'estuarine' $\delta^{13}C$ value (i.e., δ_{final}) made it difficult to distinguish between individual halibut undergoing isotopic turnover from those that had reached isotopic equilibrium to estuarine prey.

We also recognize important differences in the time scales over which otoliths and SIR integrate local environmental signatures. Geochemical tags stored in otoliths are permanent 'snap-shots' that can be related to age, size or specific stages of the life cycle (Campana 1999). Based on the width of daily otolith bands and the laser settings we employed, ca. 2 weeks of environmental information were integrated in each sample we collected. Alternatively, the isotopic composition of soft tissues integrates the feeding niche of individuals over a period of time that is interactively determined by growth rate and metabolism. Unlike otolith-based tags, the ability to detect migration via SIR depends on collecting individuals during the transition period from coastal to estuarine isotopic signatures. The dietary shift experiment performed on early juvenile halibut indicated that isotopic turnover was rapid (30–50 days) at temperatures and growth rates representative of estuarine conditions. Similarly, experiments on winter flounder (*Pseudopleuronectes americanus*; Bosley et al. 2002), Japanese flounder (*Paralichthys olivaceus*; Tominaga et al. 2003) and summer flounder (*Paralichthys dentatus*; Buchheister and Latour 2010) all demonstrated that isotopic turnover was tightly linked to individual growth rate, and occurred quickly (from 2 to 60 days depending on fish size).

These studies indicate that in young flatfish the rate of isotopic turnover of muscle tissue is relatively fast, that it is somewhat proportional to the size at which a dietary shift takes place, and that it is driven primarily by dilution due to weight gain. If early juvenile California halibut can ingress to estuaries over a range of sizes, as our otolith data and previous studies based on abundance and size distributions indicate, then it seems likely that the variability we observed in the $\delta^{13}C$ values of halibut caught in the estuary was at least partially due to the capture of individuals at different stages of isotopic turnover. Part of the variability we observed could also be due short-term variations in the baseline isotopic composition of the food web within the estuary.

Given the variability of $\delta^{13}C$ observed for juvenile halibut within PBE, the difficulty in establishing a δ_{final}, and the unequivocal patterns of Mn, Ba, Pb and Mg signatures, we propose that geochemical tags provided a more accurate estimate of size-at-ingress for post-larval halibut in PBE. Notably, our otolith-based results agree closely with Kramer's (1990) expectation that estuarine immigration occurs at ca. 30 mm SL. We do note, however, that our results suggest that immigration continues until fish are much larger (70–100 mm SL), and that the size-at-ingress is related to the within-estuary residency of fish (i.e., PBE appeared to fill with recruiting fish at the 'upper end first'). Our results do not suggest that SIR analyses would not be equally or more valuable than otolith geochemistry for documenting ingress in other systems where environmental gradients are more distinct or the growth rate of fishes are slower (e.g., Herzka et al. 2002). Furthermore, despite some methodological uncertainties, both approaches indicated that post-settlement, secondary dispersal was the dominant mode of ingress into PBE, and therefore we consider the broader implications of that finding.

Since Pearson (1929) first noted the passage of larval Sciaenids through Texas inlets, ingress of fishes into estuaries has been appreciated as a potentially important bottleneck in the life histories of many ecologically and economically valuable species. Indeed, both fishes and invertebrates have developed behavioral adaptations such as selective tidal stream transport that allow them to interact with tidal- (Churchill et al. 1999), diel- (Forward et al. 1999) and wind-driven processes near coastal inlets to enhance passage into putative estuarine nurseries. The evolution of these behaviors, as well as complex sensory abilities to process chemical (Forward et al. 1999) and physical (Fuchs et al. 2010) cues that aid in migration toward and through inlets, provide strong support that larval ingress contributes toward regulation of overall population fitness. Still, our results lead to questions regarding the relative importance of post-settlement, secondary dispersal into coastal estuaries for ocean-spawned species.

The California halibut is among a large guild of fishes defined as facultative users of estuarine nurseries because juveniles occur in estuaries as well as along the exposed coast. Along the Mid-Atlantic Bight, long-term survey data have shown that ca. 50 % of species with nearshore, settled juveniles are found both in estuaries and on the exposed coast (Able 2005; Able et al. 2006; Woodland et al. 2012). In some instances (e.g., bluefish, *Pomatomus saltatrix*), seasonal plasticity is evident in estuarine versus exposed coast settlement and habitat utilization due to spatiotemporal gradients in temperature that ultimately define habitat 'suitability' (Taylor et al. 2007; Callihan et al. 2008). Thus, intra-annual and cohort-specific patterns of estuarine ingress are also important considerations for defining the nursery function of coastal environments. Still, for halibut and similar species, fitness benefits generally accrue for individuals that ultimately make their way into estuarine nurseries (Fodrie et al. 2009). Therefore, for these fishes, particularly species with 'hard-wired' life-history transitions following a set pelagic larval duration, settlement along the exposed coast and post-settlement ingress would increase the window of opportunity to locate the most productive nursery habitats.

By settling first along the exposed coast and entering estuaries as larger, settled (often cryptic, benthic) individuals, fishes may also be subject to significantly reduced threats from potential predators. Larvae of many species ingress only during nighttime flood tides due, in part, to the significant risk of predation for meroplankton upon entering estuaries (Forward et al. 1999). Coastal species that migrate offshore for spawning or that have larvae transported away from estuaries (or coral reefs) prior to estuarine recruitment presumably

evolved these strategies to minimize the risk of predation. Thus, delaying ingress until size or behavioral refuges are attained may provide fitness advantages for early juvenile fish during the transition from ocean to estuarine environments (Miller et al. 1991).

The relative importance of post-settlement ingress (versus larval ingress) should vary geographically as well as among years due to fluctuations in regional estuarine–ocean conditions or the magnitude of initial settlement pulses. Estuaries along the semi-arid coasts of Alto/Baja California, South Africa and parts of Australia are more saline (seasonally hypersaline), smaller and less contiguous than the larger, river-fed, estuarine systems of the eastern US or western European coasts (Zedler 1982). As a result, the environmental cues used by larvae to locate coastal inlets are diminished or absent along arid coastlines, and fishes may require more time and flexibility in locating estuarine habitats. Additionally, many estuaries in our study region are lagoonal in nature, and can become periodically closed (Fodrie and Mendoza 2006). Therefore, secondary, post-larval dispersal into estuarine habitats may represent a 'bet-hedging' strategy along arid coasts.

In contrast, well-studied congeners of California halibut found along the east coast of the US, the summer flounder (*P. dentatus*) and southern flounder (*P. lethostigma*), do not appear to settle along exposed coasts, and juveniles are classified as obligate estuarine users (Burke et al. 1991). The Japanese flounder (*P. olivaceus*) also has a life history strategy in which settlement occurs coincident with ingress into estuarine habitat (Tanaka et al. 1989). Thus, despite a shared evolutionary history, these species appear to have adapted unique early life-history strategies in response, to some degree, to the availability and characteristics of the coastal estuaries they encounter. Even along southern California, Kramer (1990) noted that from one year to the next, juvenile halibut could be abundant or mostly absent along exposed coast, perhaps in response to the strength of estuary plumes and estuary–ocean gradients along the coast.

Following from our limited sample size and geographic/temporal scope, certainly more work would be needed to rigorously quantify the overall importance of post-settlement ingress as a mechanism connecting ocean and estuarine environments across species (or even for California halibut), regions or years (e.g., climate cycles). More broadly, there is a general dearth of information on the functional role of putative nurseries within the coastal seascape and particularly across the estuary–ocean ecotone (few studies properly consider the 'offshore estuary'; Able 2005). This includes migratory patterns and processes among habitats/environments (Gillanders et al. 2003). Thus, the major advance we offer is to highlight for the first time via direct, individual-based markers that estuarine ingress can be driven largely by post-settlement, secondary dispersal (complimentary to indirect, survey based methods: Kramer 1990). For halibut, the exposed coast is most likely an important corridor habitat for juveniles even if it is not their primary nursery habitat. Furthermore, ours is among only a few studies to employ dual natural tagging approaches to track the movements of fishes. Quantitative differences between otolith geochemistry and stable isotope markers highlight the importance of local environmental gradients in designing and interpreting tracking studies based on natural tags.

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