

Otolith Microchemistry Provides Information Complementary to Microsatellite DNA for a Migratory Fish

FREDERICK FEYRER*

*California Department of Water Resources, Aquatic Ecology Section,
901 P Street, P.O. Box 942836, Sacramento, California 94236, USA*

JAMES HOBBS

*Bodega Marine Laboratory, University of California–Davis,
Post Office Box 247, Bodega Bay, California 94923, USA*

MELINDA BAERWALD

*Genomic Variation Laboratory, Department of Animal Science, University of California–Davis,
1 Shields Avenue, Davis, California 95616, USA*

TED SOMMER

*California Department of Water Resources, Aquatic Ecology Section,
901 P Street, P.O. Box 942836, Sacramento, California 94236, USA*

QING-ZHU YIN

*Department of Geology and Interdisciplinary Center for Plasma Mass Spectrometry,
University of California–Davis, 1 Shields Avenue, Davis, California 95616, USA*

KEVIN CLARK

*California Department of Water Resources,
1416 Ninth Street, Room 252-35, P.O. Box 942386, Sacramento, California 94236, USA*

BERNIE MAY

*Genomic Variation Laboratory, Department of Animal Science, University of California–Davis,
1 Shields Avenue, Davis, California 95616, USA*

WILLIAM BENNETT

*Bodega Marine Laboratory, University of California–Davis,
Post Office Box 247, Bodega Bay, California 94923, USA*

Abstract.—We investigated the ability of otolith microchemistry to discriminate natal habitats of the splittail *Pogonichthys macrolepidotus*, a migratory cyprinid endemic to the San Francisco Estuary, California. Splittails are broadly distributed in the brackish and freshwater portions of the lower estuary and make long-distance upstream migrations during winter to rivers and floodplains for spawning. We found that the ratios of Sr : Ca and $^{87}\text{Sr} : ^{86}\text{Sr}$ in the otoliths (ascertained by laser ablation inductively coupled mass spectrometry) of age-0 fish collected from natal habitats significantly varied among four primary spawning rivers. Based on these two constituents, quadratic discriminant function analysis correctly classified 71% of the fish to their natal rivers. Recent work with microsatellite DNA indicates that splittails from these same rivers represent two genetically distinct populations.

Thus, integrating data obtained from otolith microchemistry and microsatellite DNA can provide complementary information on the natal origin and genetic structure of splittails at any life stage. This information will be valuable for studies of the population dynamics of mixed-stock samples collected from the estuary.

The distribution and movements of individuals and populations are critical components of research in ecology and population dynamics. Such information can lead to a better understanding of connectivity, philopatry, critical habitats, or specific life history events that may improve fitness, which ultimately will structure populations. This information, in turn, can be used to guide effective restoration and conservation strategies. Although many tools are presently available to physically track individual organisms, there is a limited ability to retrospectively obtain data across different life stages with conventional methods. A

* Corresponding author: ffeyrer@water.ca.gov

Received February 21, 2006; accepted November 20, 2006
Published online March 15, 2007

promising solution to this problem is the tandem application of multiple tools. For instance, genetic and biochemical techniques have been integrated in studies of migratory birds and butterflies (Clegg et al. 2003; Dockx et al. 2004; Kelly et al. 2005).

For fisheries scientists, otolith microchemistry and genetic markers represent two powerful tools to study population and community ecology. Recent advances in otolith microchemistry methodology have enabled scientists to elucidate fish migrations (Kennedy et al. 2002), stock structure (Campana et al. 2000), and natal habitats (Kennedy et al. 1997; Thorrold et al. 1998a, 1998b; Hobbs et al. 2005). Currently, population discrimination studies in fisheries science are heavily focused on microsatellites. This is, in part, because in contrast to other classes of DNA, they are abundant, highly variable, and dispersed throughout the genome, and data are readily obtained (O'Connell and Wright 1997). In some instances, in both marine and freshwater environments, otolith microchemistry has resolved uncertainties regarding natal habitats and stock structure that could not be addressed with genetic markers (Thorrold et al. 1998a; Campana et al. 2000; Milton and Chenery 2001). This resolution was possible because otolith microchemistry can be used to identify particular traits or events that have occurred within the time scale of an individual lifetime, whereas population divergence detected by genetic markers typically requires a longer time scale.

Since otolith microchemistry and microsatellite DNA could be used to identify unique and separate stock structure characteristics, their complementary application may be a powerful tool for scientists. In particular, such an application may be useful in studies of threatened or endangered species, taxa for which a thorough knowledge of population dynamics is needed for advanced research and management. However, there has apparently been limited integration of both techniques thus far in fisheries research. The studies conducted to date suggest that otolith microchemistry and genetic markers can provide corroborative information on population structure. Miller et al. (2005) found that microsatellite DNA and otolith microchemistry provided corroborative information on the population structure of black rockfish *Sebastes melanops*. In companion studies reported separately, Milton and Chenery (2001) and Salini et al. (2004) found similar spatial scales of population structure in the hilsa *Tenualosa ilisha* using otolith microchemistry and allozyme data, respectively. Patterson et al. (2004) found that otolith microchemistry produced results consistent with those from mitochondrial DNA and microsatellite studies on red drum *Sciaenops ocellatus* regarding the degree of mixing across large spatial

scales. These studies indicate that population structure determined from otolith microchemistry can sometimes act as a good proxy for genetic structure in broadly distributed fishes with multiple discrete spawning habitats. However, the interactions between population structure determined by genetics and otolith microchemistry have not been closely examined for fishes at smaller spatial scales. Situations where genetics and otolith microchemistry do not fully correspond may require a complementary application of these two approaches.

The splittail *Pogonichthys macrolepidotus* is a migratory cyprinid endemic to the San Francisco Estuary and its lower watershed in California's Central Valley (Moyle 2002). Recently removed from the U.S. Fish and Wildlife Service's (USFWS) list of threatened species, the splittail remains a high-profile species of special concern as designated by USFWS, California Department of Fish and Game, and other stakeholders because of uncertainties about long-term abundance trends. Adult splittails are broadly distributed in brackish habitats of upper San Francisco Estuary and make upstream migrations during winter and spring to freshwater rivers and floodplains for spawning (Moyle 2002; Feyrer et al. 2005). Based upon microsatellite DNA analyses of individual age-0 splittails collected from natal rivers, we previously identified two genetically distinct splittail populations present along the longitudinal axis of the estuary (Baerwald et al. 2006). The "downstream" population is represented by fish from the Petaluma and Napa rivers, while the "upstream" population is represented by fish from rivers of California's Central Valley (Baerwald et al. 2006). Since fish collected from individual rivers could not be genetically differentiated, this suggests that splittails do not always return to their natal river for spawning. However, it is believed that environmental conditions early in life, which can vary widely among rivers, control splittail abundance. Specifically, river flow may affect growth and survival of age-0 fish through the availability of inundated floodplains and river margins (Sommer et al. 1997). A tool capable of identifying natal origins of individuals collected from mixed samples in the estuary, where habitat and foraging locations may overlap during the nonbreeding season, would complement genetic information and facilitate advanced studies on the factors regulating abundance and population structure. Similar tools have been successfully developed in some areas of terrestrial ecology. For instance, Kelly et al. (2005) identified breeding origins of a neotropical bird in overwintering habitat by combining genetic differences between inland and coastal populations with the latitudinal gradients in hydrogen and sulfur isotopes.

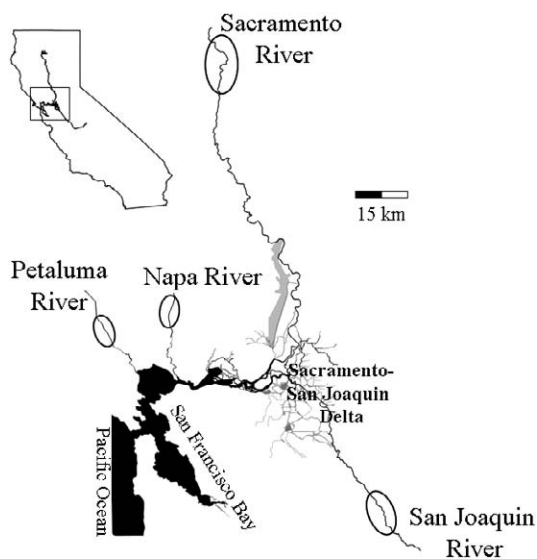


FIGURE 1.—Map of the study area in California, showing sampling regions where splittails were collected for an analysis of otolith microchemistry.

In this study, we completed otolith microchemistry analyses on individual age-0 splittails collected over two consecutive years from multiple natal rivers within each genetic population. We wanted to determine whether (1) otolith microchemistry varies geographically among rivers, (2) otolith microchemistry can be used to identify the splittail's natal rivers, and (3) data generated by otolith microchemistry and microsatellite DNA can provide complementary information on splittails.

Methods

Data collection.—Fish were collected from four primary spawning rivers in the San Francisco Estuary: the Petaluma, Napa, San Joaquin, and Sacramento rivers (Figure 1). The Petaluma and Napa rivers represent the “downstream” genetic population of splittails, while the Sacramento and San Joaquin rivers delineate the extreme northern and southern range limits, respectively, of the “upstream” population (Feyrer et al. 2005; Baerwald et al. 2006). Sample collection occurred during a 2-year (2002–2003) field study that examined distribution of age-0 splittails across the full range of the species (Feyrer et al. 2005). Samples were collected May–June and preserved in a 97% solution of ethanol. Individual fish were collected from at least two different sites on two different dates from each river, except that all fish were collected from a single site on a single day from the Sacramento River in 2003 and the San Joaquin River in 2002. It is

TABLE 1.—Mean standard length (SL), water temperature, and salinity at capture of age-0 splittail from four distinct natal rivers during May–June 2002 and 2003. Values in parentheses are SDs. Samples sizes are 10 fish per row.

| River | Year | SL (mm) | Temperature (°C) | Salinity (‰) |
|-------------|------|---------|------------------|--------------|
| Petaluma | 2002 | 35 (5) | 23 (1) | 13 (0) |
| | 2003 | 31 (6) | 23 (1) | 6 (1) |
| Napa | 2002 | 35 (9) | 27 (0) | 5 (3) |
| | 2003 | 34 (13) | 21 (4) | 0 (0) |
| San Joaquin | 2002 | 27 (2) | 23 (0) | 0 (0) |
| | 2003 | 29 (7) | 22 (1) | 0 (0) |
| Sacramento | 2002 | 21 (2) | 18 (1) | 0 (0) |
| | 2003 | 21 (4) | 19 (0) | 0 (0) |

possible that elements from fish tissue could have been mobilized by the ethanol preservation and contaminated the otoliths. However, all of our samples were handled in the same manner so any potential bias is likely to have been consistent across our samples. Further, Hedges et al. (2004) found that ethanol preservation did not affect Sr or Ba concentration in the otoliths of wild age-0 walleyes *Sander vitreus*. For this study, we selected 10 age-0 fish from each river each year that were as similar in size as possible (Table 1). Further, we selected fish collected from sampling sites within each river to ensure they had been produced and reared in their natal habitat until capture.

Lapilli otoliths were extracted from all fish with acid-washed utensils and rinsed briefly with a 10% solution of nitric acid to remove any attached organic tissue. They were then rinsed in deionized water and allowed to air dry. Dry otoliths were individually mounted on glass microscope slides in CrystalBond mounting media, polished to the core with 0.3- μ m lapping film, triple rinsed with a 5% solution of nitric acid, triple rinsed with milliQ water, and allowed to air dry in a class 100 laminar-flow hood.

Trace element concentrations in otoliths were determined by laser ablation inductively coupled plasma mass spectrometry (LA-ICPMS; Agilent Technologies 7200A ICP-MS, coupled with a Nd:YAG 213-nm laser, New Ware Research UP213) at the University of California–Davis (UCD) Interdisciplinary Center for Plasma Mass Spectrometry. Barnett-Johnson et al. (2005) recently validated that Sr isotopes obtained by LA-ICP-MS are accurate and precise, and provide results identical to thermal ionization mass spectrometry. We ablated three 20- μ m spots near otolith cores for 30 s; the laser was operated at 5 Hz. We did not formally randomize how otolith samples entered the LA-ICP-MS. However, the samples were run such that consecutive samples typically alternated among rivers. We assayed for Sr, Mg, and Ba, summarized as molar ratios to Ca. We conducted a

pilot study to examine variability of within-otolith spot assays. Five fish per site per year were selected for three separate spot assays to be performed on a single otolith. We used a fixed-effects analysis of variance (ANOVA) with assay nested within otolith and calculated variance components to compare within-versus among-otolith variation in $\log(x + 1)$ -transformed molar ratios of each element. Extraction of variance components indicated that within-otolith variation in molar ratios of each element was minor ($\leq 1\%$) compared with among-otolith (fish) variation. Thus, we concluded a single assay could be conducted on all remaining otoliths, and we used average values for otoliths with three assays for data analyses. All element concentrations were standardized with National Institute of Standards and Technology (NIST) 612 glass standards. Overall laser stability, as evaluated by examining the relative standard deviation of target elements in the NIST 612 glass standards, was as follows: 1.06 for Sr : Ca, 0.90 for Ba : Ca, and 2.14 for Mg : Ca.

Strontium isotopic compositions (molar ratios of $^{87}\text{Sr} : ^{86}\text{Sr}$) in otoliths were determined with a multicollector LA-ICP mass spectrometer (Nu Plasma HR, Nu Instrument, Inc.) interfaced with a Nd : YAG 213-nm laser (New Wave Research UP213) following the methods of Hobbs et al. (2005). In most instances, we used the alternate otolith for this analysis. When only one otolith was available due to breakage, we ran both analyses on the single available otolith. Transects across the entire otolith surface were ablated using a laser beam size of 60 μm (width), 100% laser power, and 10-Hz repetition rate. Transect lengths were typically 50–80 μm . Based on these parameters, we estimated that crater depths were approximately 200–500 μm (Jones and Chen 2003). Typical ^{88}Sr signals of 2–6 V were obtained during analyses. Helium was used as a carrier gas in the LA cell to maximize sensitivity and minimize sample deposition at the ablation site. It was also mixed with additional argon after the laser sample cell but before sending the aerosols to the plasma source. Gas blank and background signals were monitored until ^{84}Kr and ^{86}Kr decayed and stabilized after the sample change (i.e., exposing sample cell to the air) and were measured for 30 s. The laser was then turned on typically for 30–60 s. Background signals were subtracted from the measured signals automatically. The $^{86}\text{Sr} : ^{88}\text{Sr}$ ratio of 0.1194 was used to correct for instrumental fractionation in accordance with exponential law. Peak intensities for ^{88}Sr , ^{87}Sr , ^{86}Sr , ^{85}Rb , and ^{84}Sr were measured simultaneously. Peak ^{85}Rb was monitored to correct for any ^{87}Rb interference on ^{87}Sr , which was negligible. The NIST Sr isotope standard SRM 987 was measured consistently during

the course of this study, giving an $^{87}\text{Sr} : ^{86}\text{Sr}$ ratio equal to 0.710253 ± 9.0 , compared with the certified value of 0.710258. In-house carbonate standard (a deepwater coral, which should give average modern seawater Sr isotope ratio) used as a LA standard gives an $^{87}\text{Sr} : ^{86}\text{Sr}$ ratio equal to 0.709177 ± 8.0 , which is precisely the known seawater value. For statistical analyses, we used data from ablation sites collected only near the core of the otoliths to be consistent with the trace element data. Typically, this was 20–80 μm (average = 45 μm) from the otolith core. This represents approximately 5–15 d of life after hatch.

Data analysis.—To address whether otolith microchemistry varied geographically among rivers, we used two-way multivariate ANOVA (MANOVA) with river and year as factors to examine the variability of element : Ca molar ratios and $^{87}\text{Sr} : ^{86}\text{Sr}$ in otoliths. Dependent variables were $\log(x + 1)$ -transformed to improve the fit of the data to assumptions regarding normality and homogeneity of variance, and significance was determined at less than or equal to 0.05. To evaluate whether otolith microchemistry could be used to identify natal rivers, we used the same data in a quadratic discriminant function analysis (DFA) to determine the discriminatory power of otolith microchemistry to identify splittail natal habitats. Multivariate Box *M* was used to test for homogeneity of variance–covariance matrices. Classification accuracy was based upon a traditional leave-one-out jackknife validation. We assessed whether data generated by splittail otolith microchemistry and microsatellite DNA provided complementary information by comparing our results with those of Baerwald et al. (2006) on splittail genetic structure obtained by microsatellite DNA (see Discussion).

Results

Seven otoliths (Napa River: 2, Petaluma River: 1, Sacramento River: 3, and San Joaquin River: 1) were damaged during the course of the study and could not be included in data analyses. Except for Mg : Ca, all element : Ca ratios and $^{87}\text{Sr} : ^{86}\text{Sr}$ varied significantly among rivers (multivariate $F = 10.5$, $P < 0.001$; Table 2; Figure 2). Additionally, Sr : Ca and Ba : Ca exhibited significant differences between years (Table 2; Figure 2). However, the river \times year interaction terms were not significant ($P > 0.10$), suggesting that the overall site differences remained stable across years. The most consistent pattern across rivers was exhibited by $^{87}\text{Sr} : ^{86}\text{Sr}$, which was lowest at the Sacramento River. The San Joaquin River had the highest values of Sr : Ca, although values for the Napa River in 2002 were also relatively high. The San Joaquin River had the lowest values of Ba : Ca.

TABLE 2.—Univariate results from the multivariate analysis of variance to test the effect of site, year, and site \times year on otolith elemental composition.

| Effect | Mean square | <i>F</i> | <i>P</i> |
|---------------------------------------------------|-------------|----------|----------|
| Mg/Ca | | | |
| Site | 0.00009 | 0.5 | 0.66 |
| Year | 0.004 | 15.5 | <0.001 |
| Site \times year | 0.00005 | 0.3 | 0.84 |
| Error | 0.0002 | | |
| Sr/Ca | | | |
| Site | 0.018 | 6.5 | 0.001 |
| Year | 0.016 | 5.6 | 0.021 |
| Site \times year | 0.006 | 2.1 | 0.11 |
| Error | 0.003 | | |
| Ba/Ca | | | |
| Site | 0.00001 | 3.8 | 0.014 |
| Year | 0.00005 | 15.5 | <0.001 |
| Site \times year | 0.000005 | 1.4 | 0.25 |
| Error | 0.000003 | | |
| $^{87}\text{Sr}/^{86}\text{Sr}$ | | | |
| Site | 0.000001 | 48.3 | <0.001 |
| Year | <0.000001 | 0.01 | 0.94 |
| Site \times year | <0.000001 | 1.2 | 0.32 |
| Error | <0.000001 | | |

Our final DFA model included Sr : Ca and $^{87}\text{Sr} : ^{86}\text{Sr}$ as prediction variables. We excluded Mg : Ca because it was not significantly different among sites and Ba : Ca because it was not a significant variable in DFA models. The final model met the assumption of equal variance–covariance matrices (Box *M* = 38.6; df = 9; *P* < 0.001). Overall leave-one-out, cross validated classification accuracy was 71%. Individual river classification accuracy was as follows: Petaluma River, 79%; Napa River, 50%; San Joaquin River, 69%; and Sacramento River, 89%. Misclassifications were not typically to the nearest neighboring river. The two misclassifications for the Sacramento River were assigned to the Napa River. Of the misclassifications for the San Joaquin River, one was assigned to the Sacramento River and the other five were assigned to the Napa River. The four misclassifications for the Petaluma River were spread equally between the Napa and San Joaquin rivers. The misclassifications for the Napa River were most widespread: four were assigned to the Petaluma and San Joaquin rivers, and one was assigned to the Sacramento River.

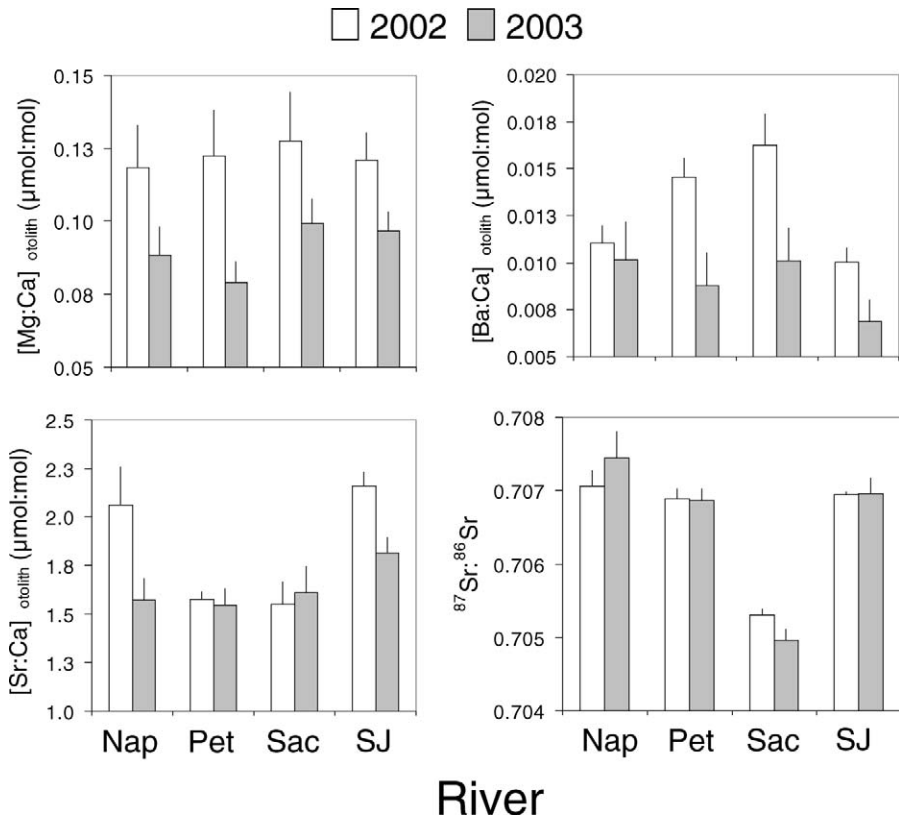


FIGURE 2.—Mean values (+SEs) of otolith constituents for age-0 splittails collected in 2002 and 2003 from the Napa (Nap), Petaluma (Pet), Sacramento (Sac), and San Joaquin (SJ) rivers, California.

Discussion

Our ability to classify natal habitats of splittails (71% overall accuracy) compares reasonably well with other studies in freshwater environments. A study on ciscoes *Coregonus artedii* in Lake Superior achieved only 45% overall success rate (Bronte et al. 1996). However, studies on yellow perch *Perca flavescens* in Lake Superior accurately classified fish to presumed natal habitats at 76–86% (Brazner et al. 2004a, 2004b). Wells et al. (2003) were able to accurately classify habitats of westslope cutthroat trout *Oncorhynchus clarkii lewisi* in the Coeur d'Alene River, Idaho, at 100%. Our results compare quite well with these previous single-year studies, considering that our work was conducted over 2 years in a highly variable environment. Studies on freshwater natal habitats of anadromous species (taxa with life history traits generally similar to those of the splittail) have documented results similar to ours (e.g., Thorrold et al. 1998a; Milton and Chenery 2001; Tomás et al. 2005).

A number of interacting factors probably contributed to the relatively unique otolith microchemistry among sites and our ability to accurately classify natal rivers. Ambient water chemistry is believed to be a primary factor contributing to otolith chemistry (Campana 1999). Wells et al. (2003) found that the trace element concentration in the otoliths of westslope cutthroat trout was related to that of the water. We do not have trace element : Ca or Sr isotope data for our study rivers during the study period, and thus we cannot directly test the relationship between the chemical composition of the water and the otoliths. However, the underlying geology of the system is varied (Mount 1995), which is likely to promote water chemistry variation among rivers. The Sacramento and San Joaquin rivers drain the northern (younger volcanic basin) and southern (older granitic basin) regions of California's Central Valley, respectively. Ingram and Weber (1999) demonstrated $^{87}\text{Sr} : ^{86}\text{Sr}$ in water of Central Valley rivers (including the Sacramento and San Joaquin rivers) varied according to underlying geology and was relatively stable. The Napa and Petaluma rivers drain the southern interior region of California's Coastal Range, which is dominated by volcanic and sedimentary rock (Mount 1995).

In addition to geological characteristics, the four rivers also differ in flow, water temperature, and salinity profiles due to variation in watershed characteristics and proximity to marine habitats (Feyrer et al. 2006). During our study period, flow patterns matched historical trends in that they were highest in the Sacramento River, intermediate in the San Joaquin River, and lowest in the Napa and Petaluma rivers. Mean daily temperatures

during the lives of the fish we collected (obtained from continuous recorders we deployed for another study) ranged from 12–15°C in March to 20–23°C in June, and on average were slightly warmer in 2002. Salinities measured at the time of fish sampling (Table 1) were relatively high for the Petaluma River (means = 13‰ in 2002 and 6‰ in 2003) and Napa River (5‰ in 2002 and 0‰ in 2003), while the Sacramento and San Joaquin rivers are freshwater. Bath Martin et al. (2004) found that Sr : Ca in the otoliths of spot *Leiostomus xanthurus* was positively related to water temperature and salinity. Although this relationship has not been validated for splittails, we found that Sr : Ca in splittail otoliths was highest in the warmest rivers (San Joaquin and Napa rivers) and also highest in the year with the overall highest water temperatures (2002). There was no apparent relationship between Sr : Ca and salinity in our data.

Another possible factor leading to differences in otolith chemistry is variation in fish growth rates. In a related study (Feyrer, Sommer, and Hobbs, unpublished), we found the only differences in growth rate among locations were that fish from the Sacramento River appeared to grow slower than fish from the San Joaquin, Petaluma, and Napa rivers. This related study included most of the fish examined for otolith microchemistry as well as many other individuals, providing a comprehensive assessment of the growth of age-0 splittails. Our results, therefore, do not follow the model that that slower-growing fish tend to incorporate more Sr in their otoliths than faster-growing fish (Campana 1999). However, this relationship would be best tested under highly controlled laboratory conditions. This is because the aforementioned differences in river temperature, salinity, and presumed Sr concentrations may have overwhelmed this phenomenon for the splittails we studied, if this phenomenon even exists.

Despite uncertainty regarding the specific mechanisms, otolith microchemistry variation among sites appears to be stable enough to permit accurate classification of splittail natal habitats. Similar to our results, many studies have identified interyear variation in otolith microchemistry (e.g., Thorrold et al. 1998a; Gillanders 2002; Patterson et al. 2004). In some instances, interyear variation had the potential to confound spatial comparisons (Gillanders 2002). Our results suggest that interannual differences are not likely to affect natal habitat discrimination of young splittails. Nonsignificant site \times year interaction terms in the MANOVA model observed across years with different environmental conditions suggest that site differences were relatively stable. San Francisco Estuary and its watershed are subject to extreme interannual environmental variation in the form of

droughts and wet periods within the 7–10-year life span of splittails. Thus, the temporal extent to which these interactions remain nonsignificant will require study.

Two limitations of our study are that we did not have information on water chemistry (previously discussed) and that we did not examine the otoliths from all potential natal sites. Although it is possible for splittails to spawn in virtually any freshwater region of the system, the only major spawning river that we did not assess was the Cosumnes River (Moyle et al. 2004; Feyrer et al. 2005). Substantial production of splittails can sometimes occur in the Cosumnes River in wet years, when the river's floodplain inundates for extended periods of time (Crain et al. 2004). The Cosumnes River is immediately adjacent to, and is a tributary of, the Mokelumne River. Ingram and Weber (1999) found that $^{87}\text{Sr} : ^{86}\text{Sr}$ values in waters of the Mokelumne and lower San Joaquin rivers were indistinguishable, but both were distinct from that of the Sacramento River. Therefore, it may not be possible to differentiate splittails from the Cosumnes River and those from the San Joaquin River.

In this study, we were fortunate to have data on the genetic structure, as determined by microsatellite DNA (Baerwald et al. 2006), for comparison with our results on otolith microchemistry. Whereas some previous studies have found that these two types of data have provided corroborative information on population structure (Milton and Chenery 2001; Patterson et al. 2004; Miller et al. 2005), our study indicates that otolith microchemistry and genetic information can provide unique and complementary information on the population structure of fishes. For splittails, otolith microchemistry can elucidate natal habitats, but this information does not directly corroborate genetic structure (Baerwald et al. 2006). For instance, otolith microchemistry alone could have never determined that fish from the Central Valley rivers, or from the Napa and Petaluma rivers, respectively, represent distinct interbreeding populations. However, otolith microchemistry can be used to track movement patterns of fish from particular rivers, which is something that genetic markers are incapable of doing. This information is very valuable because genetic markers cannot determine where fish from particular rivers choose to forage during the nonbreeding season. Given that the Petaluma and Napa River populations are probably considerably smaller than the Central Valley populations, it would be useful to ascertain where fish from these rivers live and forage during the nonbreeding season to monitor these areas for conservation management purposes. Thus, integrating data obtained from otolith microchemistry and microsatellite DNA can provide complementary information on the natal

origin and genetic structure of splittails at any life stage. Such integration provides a foundation for advanced studies in population and community ecology. For instance, it will now be possible to quantify movements and philopatry within and among genetic populations, the effect of environmental conditions in spawning rivers on abundance, and overall connectivity among rivers and populations. This information, in turn, can be used to develop strong science-based management strategies for special-status fish species such as the splittail.

Acknowledgments

Our research was supported by the San Francisco Bay–Delta Science Consortium, Interagency Ecological Program, and California Department of Water Resources. Funding by the National Science Foundation and access to facilities at the UCD Interdisciplinary Center for Plasma Mass Spectrometry are sincerely acknowledged. We thank Michelle Gras for assistance with trace element LA-ICP-MS analysis. Thorough reviews by R. Barnett-Johnson and two anonymous reviewers significantly improved the manuscript. This work represents UCD Interdisciplinary Center for Plasma Mass Spectrometry contribution number 0011.

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