STABLE ISOTOPE ANALYSIS OF CENTRARCHID CONCENTRATION AREAS

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FINAL REPORT

Upper Colorado River Recovery Implementation Program
Project Number C-18/19

August 2007

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EXECUTIVE SUMMARY

This project addressed movement of nonnative fish into river reaches of critical habitat for endangered fishes from floodplain habitats. Nonnative fishes of the Family Centrarchidae, including largemouth bass *Micropterus salmoides*, bluegill *Lepomis macrochirus*, green sunfish *L. cyanellus*, and black crappie *Pomoxis nigromaculatus* are known to occur in floodplain ponds, backwaters, beaver ponds, washes and irrigation drainage ditches throughout the Grand Valley reach of the Colorado River. In riverine habitats, these species are most commonly associated with backwaters or side channels with slow-moving water. It is in these "low-velocity riverine habitats" that centrarchids are believed to pose a significant predatory threat to the young life stages of endangered and other native fishes. However, it has been uncertain to what extent the presence of centrarchid species in low-velocity riverine habitats is the result of escapement from off-channel ponds or from reproduction within the river itself. Determining origins and movements of nonnative fishes by conventional means has been impractical.

The primary objectives of this project were to determine whether the origins and movements of centrarchids in an 87-mile reach of the upper Colorado River and adjacent floodplain habitats could be identified using naturally occurring stable isotope and/or microchemical analyses, and determine the proportion of centrarchids in backwaters within the study area that originated from out-of-channel ponds versus in-channel habitats. Both of these objectives were achieved and the results of this research will provide managers with guidance to improve efficiency and effectiveness of nonnative fish control efforts. Two peer-reviewed scientific publications resulted from this project as well (Whitledge et al. 2006, Whitledge et al. 2007).

We focused our research seeking microchemical markers on two trace elements, strontium and selenium, and one stable isotope, hydrogen (deuterium, ^{2}H). While strontium had recently proven useful in studies of fish environmental history within freshwater systems, most of the previous work had been conducted on diadromous fishes. No literature existed on selenium or hydrogen isotopes as tracers in fish otoliths, although hydro-geochemical conditions in the Upper Colorado River basin create considerable spatial variability in both of these surface water constituents.

We found that while more research is needed to understand environment:otolith selenium dynamics, both of the other markers we examined worked very well. Deuterium proved to be an excellent naturally occurring marker for discriminating fishes originating from pond versus riverine habitats. Ponds and riverine habitats possessed distinct deuterium signatures (δD) with low temporal variation, and there was a strong and consistent relationship between δD signatures of the environment and the δD composition in otoliths. Strontium concentration (measured as Sr:Ca ratio) supplemented information garnered from δD because Sr can be analyzed with much higher resolution in the otolith, providing a time series of a fish's exposure to Sr, which we found to be highly correlated with salinity. The combination of δD and Sr:Ca yielded powerful insights into fish environmental histories. Successful development of these microchemical markers allowed us to determine origins and movements of centrarchids in the study area, and the proportion of centrarchids in backwaters that originated from out-of-channel ponds versus in-channel habitats.

Low-velocity backwater and beaver pond habitats were likely the primary source of three of the four species of centrarchids during this study, based the relative abundance of fish with riverine otolith core δD signatures and Sr:Ca ratios. Of those fish immigrating to riverine habitats from ponds, most came from ponds that were closely associated hydrologically with the Colorado River. To some degree these findings may be a result of timing of the study, which occurred during a prolonged drought. Drought may reduce connectivity between off channel ponds and the river and make conditions within the river itself more favorable for centrarchid recruitment. During wetter years the reverse may be true; our data on age at immigration from ponds suggested that ponds may be important sources of centrarchids to the river in some years.

Reevaluation of relative proportions of river-dwelling centrarchids with pond and riverine otolith signatures is recommended during and immediately following years of above average precipitation and river discharge. Such a follow-up study would be useful for assessing whether management of centrarchid abundance in critical habitat should always be focused within riverine habitats themselves or if additional emphasis should be placed on controlling centrarchid escapement from ponds to curtail immigration to riverine habitats during high-water years.

ACKNOWLEDGEMENTS

This study was funded by the Recovery Implementation Program for Endangered Fish Species in the Upper Colorado River Basin. The Recovery Program is a joint effort of the U.S. Fish and Wildlife Service, U.S. Bureau of Reclamation, Western Area Power Administration, states of Colorado, Utah, and Wyoming, Upper Basin water users, environmental organizations, the Colorado River Energy Distributors Association, and the National Park Service.

The following individuals and organizations provided valuable assistance during this project: U.S. Fish and Wildlife Service office in Grand Junction, CO (especially Bob Burdick, Doug Osmundson, and Rick Krueger); Kevin Bestgen, CSU Larval Fish Laboratory; Terry Whitledge, University of Alaska-Fairbanks; Alaska Stable Isotope Facility, Water and Environmental Research Center, University of Alaska-Fairbanks; Ian Ridley and Alan Koenig, USGS Mineral Resources Team, Denver, CO; Lori Martin, Kelli Rehder, Michael Carillo, Phil Schler, and Randy van Buren, Colorado Division of Wildlife; and the Great Lakes Institute for Environmental Research, University of Windsor, Windsor, Ontario, Canada. Chris Myrick, Ryan Fitzpatrick, Dan Gibson-Reinemer, Brendan McGinn, Britta Schilke, and Mike Gross also assisted with field sampling. We also thank the landowners in the Grand Valley that graciously allowed access to ponds on their property.

DISCLAIMER

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KEYWORDS

Provenance, nonnative fish, largemouth bass, black crappie, bluegill, green sunfish, smallmouth bass, fish control, Colorado River, otolith, microchemistry, hydrogen, δD, deuterium, strontium, selenium.

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INTRODUCTION

Floodplain corridors bordering the main stem rivers in the Upper Colorado River Basin are considered an integral and necessary element in the recovery of the four endangered big river fish species. Four habitat types have been identified in the upper Colorado River basin in the area designated as critical habitat for these species: the main river channel, associated backwaters and beaver ponds (impounded backwaters) (these 3 habitats will hereafter be collectively referred to as "riverine" habitats), and floodplain ponds. All of these have been identified as critical habitat components in the life histories of the listed species, and generally important to the native fish community and ecological functions supporting the endangered fishes (Irving and Burdick 1995). Nonnative fish species are present throughout the Upper Basin (Martinez 2002, Trammel et al. 2002), and can adversely impact the recovery progress for endangered fishes through predation or competition at critical life stages or in critical locales (Tyus and Saunders 1996). Four species in the Family Centrarchidae (largemouth bass *Micropterus salmoides*, green sunfish *Lepomis cyanellus*, bluegill *Lepomis macrochirus* and black crappie *Pomoxis nigromaculatus)* are considered to be the most problematic (Osmundson 2003).

Control of nonnative fishes has been a recovery program goal since at least 1996, but control efforts have met with limited success, partly because the predominant source of nonnative fish in the Colorado River is unknown. The large number of potential sources and the inability to determine specific habitats where nonnative fishes are reproducing and recruiting have been vexing problems. Managers' work would be greatly facilitated by knowledge of the origins and movement patterns (provenance) of nonnative fishes, which could provide insights into the most promising and efficient management strategies to control them (Osmundson 2003, Martinez and Nibbelink 2004). However, it has not been possible to study nonnative fish provenance by conventional means because physical sampling and mark-recapture techniques are inadequate given the scale of the problem.

The advent of stable isotopic and microchemical analyses of otoliths has provided a new avenue for the study of fish provenance by exploiting natural markers that reflect a fish's environmental history throughout its lifetime (Campana and Thorrold

2001). Much of the previous work using otolith microchemistry for studies of fish environmental history has focused on trace element concentrations and isotopic ratios (e.g., strontium:calcium (Sr:Ca), barium:calcium (Ba:Ca), or strontium isotope ($^{87}Sr/^{86}Sr$) ratios) in estuarine and anadromous fishes (e.g., Thorrold et al. 1998; Limburg 2001; Secor et al. 2001; Kennedy et al. 2002) because large differences in salt and freshwater chemical composition are reflected and easily detected in otoliths (Graustein 1989; Campana 1999). However, otolith microchemistry and isotopic analysis have also recently proven useful in studies of fish origins and environmental history within freshwater systems, exploiting elements or isotopes (primarily Sr) exhibiting high spatial variability (e.g., Kennedy et al. 1997; Wells et al. 2003; Brazner et al. 2004). Otolith selenium concentration or selenium:calcium (Se:Ca) ratios had not previously been used as natural markers of fish environmental history. However, small amounts of selenium have been detected in otoliths of a few marine fish (Campana 1999), probably in association with otolith proteins. We hypothesized that selenium concentrations (and Se:Ca ratios) might be elevated in otoliths from fishes collected at sites in the Grand Valley that receive irrigation drainwater, which can be enriched in selenium leached from the soil. Stable hydrogen isotope ratio ($^{2}H/^{1}H$ or D/H, expressed as δD) was another potential environmental marker that had not been applied in any published studies of fish provenance. However, previous application of δD in research on origins of other migratory organisms, substantial spatial variation in water δD signatures, and presence of hydrogen in fish tissues and otolith proteins collectively suggested that δD had strong potential to serve as a natural marker of fish environmental history. Thus, a variety of stable isotopes and trace elements provided potential naturally occurring markers to track origins and movements of nonnative fishes in the upper Colorado River basin (e.g., Martinez et al. 2001).

In this study we evaluated the utility of several elemental and isotopic markers, some of which had never been tested previously, by performing assays on samples of water, fish otoliths and fish muscle tissue collected from a range of habitat types. We then used the most informative markers to identify sources of nonnative fish found within riverine reaches of critical habitat in the Grand Valley, Colorado. This report documents our research, some of which was recently published in the scientific

literature (Whitledge et al. 2006, 2007), and our resultant conclusions and recommendations for managers seeking to protect and recover the upper Colorado River basin's native fish assemblage.

OBJECTIVES

1. Determine whether the origins and movements (collectively termed provenance) of centrarchids in the study area can be identified using stable isotope and/or microchemical analyses.

2. Determine the proportion of centrarchids in backwaters within the study area that originated from out-of-channel ponds versus in-channel habitats.

3. If feasible, pinpoint "hotspots" where centrarchids present in connected backwaters have originated by narrowing the list of possible sources (e.g. from "off-channel ponds" to specific ponds or groups of ponds).

METHODS

Study area

The principal study area for this research encompassed a 140-km reach of the upper Colorado River and adjacent floodplain habitats in west-central Colorado from the town of Rifle (N 39° 31.73' W 107° 46.87') downstream to the mouth of Horsethief Canyon (N 39° 10.21' W 108° 48.87'). Horsethief Canyon is approximately 29 river km west of the Gunnison River confluence at Grand Junction, CO. Mean annual discharges upstream and downstream of the Gunnison River confluence are about 82 m^3/s and 170 m^3/s, respectively, with peak flow occurring during late May and June. A substantial portion of the river is diverted to canals in the Grand Valley (extending from ~24 river km upstream of Grand Junction to the lower limit of our study area) for agricultural and municipal use during spring, summer, and fall. Habitat in the study reach is composed primarily of runs and riffles with cobble and gravel substrate. The riverbanks and adjacent floodplain are composed of silt and sand with some segments

modified by levees or rip-rap. Ephemeral, backwater habitats with low-velocity water flow are common downstream of islands or at the mouths of secondary channels in braided reaches. Backwaters are small (< 0.3 ha) and shallow (maximum depth < 2 m) with silt and sand substrate and few or no aquatic macrophytes, although woody debris may be present. Many backwaters and side channels have been impounded by beavers *Castor canadensis*.

More than 400 ponds occur in the 100-year floodplain of the Colorado River within the study reach; most of these (~350) are within the Grand Valley. Ponds sampled for this study had surface areas < 4 ha and maximum depths < 5 m. Pond substrates consist of sand and silt with varying quantities of aquatic plants (mainly *Chara* and *Potamogeton*) depending on depth, slope, and water clarity. Some ponds are isolated from the Colorado River; others are permanently or periodically connected to the river by irrigation ditches or overland flow during spates. Salinity in many Grand Valley ponds and ditches frequently exceeds 1 ‰ (Butler and Osmundson 2000); salinities as high as 26 ‰ were measured in conjunction with this study. Elevated salinities are a consequence of irrigation water leaching minerals from Mancos shale, a marine formation that underlies the entire Grand Valley, coupled with high evaporation rates (Butler and Osmundson 2000). Irrigation water also leaches Sr, an element that commonly substitutes for Ca in otoliths, from Mancos shale; Sr concentrations are elevated in surface waters receiving irrigation water that has percolated through soils derived from Mancos shale and are further increased by evapotranspiration (Gerner et al. 2006). Leaching of selenium from Mancos shale as a result of irrigation activities has also led to elevated selenium concentrations in water and biota at several locations within the Grand Valley (Butler and Osmundson 2000).

Overview of approach and methodology

This project proceeded in two stages. First, we evaluated a variety of potential naturally occurring chemical markers and tested their ability to trace the origins of known-provenance fish. Second, after successfully developing suitable markers and associated analytical methods, we used the methodology to determine the locations and

habitat types that were contributing the majority of nonnative fishes to the Colorado River.

Development of markers – Se:Ca

Whole sagittal otoliths from thirteen fish were selected for analysis of Se:Ca ratios. Otoliths were removed using non-metallic forceps, rinsed with distilled water, and stored dry in polyethylene vials until preparation for analysis. Fishes sampled included 5 green sunfish (*Lepomis cyanellus*) from 30 Road (a high-selenium location, Butler and Osmundson 2000) near Grand Junction, 2 smallmouth bass (*Micropterus dolomieu*) collected in the Colorado River below the Gunnison River confluence (some selenium present), 3 smallmouth bass collected in the Colorado River upstream of the Grand Valley (very little selenium present), and 3 kokanee (*Oncorhynchus nerka*) from Blue Mesa Reservoir (another low-selenium site).

Whole otoliths for Se:Ca analysis were sent to the Center for Trace Analysis at the University of Southern Mississippi. Samples were washed in ultrapure 1% HNO_3 for 5 min, rinsed with ultrapure water, dissolved in ultrapure 1% HNO_3 and diluted to a Ca concentration of ~4 mM before analysis. Samples were analyzed using a ThermoFinnigan Element2 sector field ICPMS utilizing a teflon spray chamber and a 100μL/min microflow nebulizer. Isotopes measured included ^{43}Ca, ^{77}Se, ^{82}Se, and ^{115}In (internal standard). All isotopes were analyzed in both medium and high resolution. Isotopic counts were converted to Se:Ca ratios. The detection limit for Se:Ca was 0.4 μmol/mol (A. Shiller, University of Southern Mississippi, personal communication).

Development of markers - δD

To determine whether the provenance of centrarchids in the study area could be reliably identified using stable isotope and/or microchemical analyses we first needed to ascertain if ponds and “riverine” habitats possessed distinct chemical signatures and that these signatures exhibited low temporal variation. Then, we evaluated whether or not there were strong, consistent relationships between the environmental chemistry of each habitat type and the elemental and isotopic composition of otoliths taken from fish inhabiting each locale. To determine the generality of these relationships we also

collected water samples and fish of a variety of species from locations outside the Upper Colorado River basin.

Eleven species of fishes representing six families (Centrarchidae, Catostomidae, Esocidae, Percidae, Pleuronectidae, Salmonidae) were collected from 11 locations (Table 1) during 2004 by angling, electrofishing, seining, or gill netting. These locations were selected to encompass a broad range of water δD signatures and because fishes living in these locations were known or strongly suspected to have spent all or nearly all of their lives within that same water body. Sampling locations included isolated water bodies in which fishes were known to be naturally reproduced (College Lake, Dixon Reservoir, Bounds Pond, Audubon Pond) and sites where fish were hatched or stocked at age-0 and could not have originated from another source or subsequently traveled elsewhere (Research Hatchery, Highline Reservoir, Blue Mesa Reservoir). Fish may have entered two of our sampling locations from the Colorado River (Government Highline Canal, Horsethief Pond). However, the intake to Horsethief Pond is screened and would only permit passage of larval fishes, while Colorado River and Government Highline Canal δD signatures are indistinguishable (the canal is fed by Colorado River water). We cannot rule out the possibility that smallmouth bass collected from Lake Powell may have entered the lake from tributaries, but smallmouth bass reproduce in the lake and are not stocked. Pacific halibut collected near the mouth of Cook Inlet may have spent time in the adjacent Gulf of Alaska.

Five to 23 individuals were collected from each location along with a 20 ml water sample. At seven of the locations, one or two additional water samples were collected during subsequent seasons. Water samples were stored in scintillation vials containing minimal air space and sealed with Parafilm® to curtail evaporative loss and fractionation (Kendall and McDonnell 1998). Total length of each fish was measured to the nearest mm and sagittal otoliths were removed. Otoliths were blotted to remove organic residue, rinsed with distilled water, and air-dried. Dorsal muscle plugs were removed from fishes collected at seven of the 11 sampling locations, frozen on the date of capture, and stored at -10 °C. Muscle tissue samples were dried at 60 °C for 72 h; muscle and otolith samples were ground to a flour-like consistency with a mortar and pestle.

Water, otolith, and muscle samples were analyzed for hydrogen isotopic composition using a high temperature conversion elemental analyzer (TC/EA) interfaced with a Thermo Finnigan Delta Plus XL® isotope ratio mass spectrometer (Thermo Electron Corp.)* in the Water and Environmental Research Center at the University of Alaska-Fairbanks (Fairbanks, AK). Hydrogen isotope ratios are reported in standard δ notation, defined as the per mil deviation between isotope ratios of a sample and standard (Vienna Standard Mean Ocean Water (VSMOW), Krabbenhoft et al. 1994):

$$\delta D\ (‰) = [R_{sample} / R_{standard}) - 1] \ X \ 1000$$

where R represents $^{2}H/^{1}H$. Mean coefficient of variation among replicate measurements was 0.7% for water samples and 0.9% for solid samples.

Least-squares linear regression was applied to relate both mean otolith and mean muscle δD values to corresponding mean water δD values from our sampling locations. Bonferroni joint confidence intervals were used to test whether regression models had a y-intercept of zero and a slope of one. Assessment of possible effects of fish size and species on relationships between water and both otolith and muscle δD values was also of interest. However, regressions indicated that water-otolith and water-fish muscle differences in δD were a function of water δD signature. Therefore, otolith and muscle δD values for each fish were standardized to the mean water δD value of all sampling locations combined (-85.8 ‰) using regression equations described above. Standardized differences between mean water and fish (otolith and muscle) δD values were then regressed on fish total length (mm) to assess the influence of fish size on relationships between water and fish δD values. Standardized differences between mean water and fish (otolith and muscle) δD values were also compared among species using analysis of variance (ANOVA) followed by the Bonferroni multiple comparison method for separation of means.

Determining provenance

Water samples for stable hydrogen isotope analysis were collected from 27 floodplain ponds, 19 backwaters not impounded by beavers, five beaver-impounded backwaters, and 13 Colorado River main channel locations. Floodplain ponds were selected based on accessibility and included five sites upstream of the Grand Valley (one every 10-20 km) and at least one site every 4-8 river km within the Grand Valley. Ponds were also chosen to encompass the full range of river-pond connectivity (isolated, ditch-connected, and periodically connected ponds). River main channel sampling sites were adjacent to pond sampling locations. Water samples were obtained from backwaters sampled for fish and six additional unimpounded backwater and beaver-impounded backwater habitats. Samples were collected during November 2003, April 2004, and July 2004 to enable assessment of seasonal changes in water stable hydrogen isotopic composition. Water samples were stored in scintillation vials containing minimal air space and sealed with Parafilm® to curtail evaporative loss and fractionation (Kendall and Caldwell 1998). Conductivity (μS/cm) and salinity (‰) were measured in conjunction with each water sample using a portable meter.

Centrarchids (n=282; 141 green sunfish, 94 largemouth bass, 32 bluegill, and 15 black crappie) were collected from 18 backwaters (both beaver-impounded and unimpounded) in the Grand Valley during 2004 by electrofishing. Fish sampling locations were chosen to include backwaters with and without tributaries or inflowing ditches and locations above and below the Gunnison River confluence. Backwaters sampled for fishes were dispersed along the 53-km reach of the Colorado River within the Grand Valley; mean distance between backwater sampling sites was 2.8 river km. An additional 86 centrarchids (46 green sunfish, 25 largemouth bass, 11 bluegill, and 4 black crappie) were collected in the river main channel throughout the 140-km study reach. Total length of each fish was measured to the nearest mm. Fish were placed on ice immediately after capture and stored frozen until otolith removal.

In the lab, sagittal otoliths were removed from fishes using non-metallic forceps, rinsed with distilled water, and stored dry in polyethylene vials until preparation for analyses. One otolith was analyzed for stable hydrogen isotopic composition; protein was the source of hydrogen analyzed in otoliths. Otoliths < 2.5 mg used for hydrogen

isotope analysis were analyzed whole; otoliths > 2.5 mg were ground to obtain a 2-2.5 mg core sample centered on the otolith nucleus using a Dremel® rotary tool. Resolution of stable hydrogen isotope analysis using this procedure corresponded to about the first year of a fish's life based on mean otolith mass (± SE) for late age-0 (bluegill 1.7 ± 0.2 mg; green sunfish 1.8 ± 0.3 mg; largemouth bass 1.5 ± 0.1 mg) and age-1 (bluegill 3.5 ± 0.2 mg; green sunfish 3.9 ± 0.4 mg; largemouth bass 3.1 ± 0.4 mg) fishes from our study area aged with otolith annuli counts. The second otolith was embedded in Epo-fix® epoxy, sectioned in a transverse plane using an ISOMET® low-speed saw, and polished to reveal annuli. Age was estimated for each fish by counting otolith annuli. Otolith thin sections were prepared for analysis under a class 100 laminar flow hood and handled only with non-metallic, acid-washed forceps. Thin sections were mounted on acid-washed glass slides using double-sided tape, ultrasonically cleaned for 5 min in ultrapure water, and dried for 24 h under the laminar flow hood. Mounted and cleaned thin sections were stored in acid-washed polypropylene Petri dishes in a sealed container until analysis.

Water and otolith core samples were analyzed for stable hydrogen isotopic composition using a high temperature conversion elemental analyzer (TC/EA) interfaced with a Thermo Finnigan Delta Plus XL® isotope ratio mass spectrometer. Hydrogen isotope ratios are reported in standard δ notation. Mean coefficient of variation among replicate measurements was 0.7% for water samples (n=2-3 replicates per sample) and 0.9% for solid samples (n=2 replicates per sample).

Otolith thin sections were analyzed for ^{88}Sr and ^{44}Ca using a Perkin Elmer ELAN 6000 inductively coupled plasma mass spectrometer (ICPMS) coupled with a CETAC Technologies LSX-500 laser ablation system. A transect was ablated with the laser on each otolith thin section extending from the otolith nucleus to its edge along the longest axis (beam diameter = 25 μm, scan rate = 10 μm/s, laser pulse rate = 10 Hz, laser energy level = 9 mJ, wavelength = 266 nm). A standard developed by the USGS (MACS-1, $CaCO_3$ matrix) was analyzed every 12-15 samples to adjust for possible instrument drift. Each sample analysis was preceded by a gas blank measurement. Isotopic counts were converted to elemental concentrations (ppm) after correction for gas blank, matrix, and drift effects. Strontium concentrations were normalized to Ca

concentration based on the consideration of calcium as a pseudointernal standard (Bickford and Hannigan 2005; Ludsin et al. 2006); data are reported as Sr:Ca ratios (mmol/mol) for consistency with published otolith microchemistry literature and reflect differences in Sr concentration among samples. Mean limit of detection for ^{88}Sr was 0.09 ppm; otolith ^{88}Sr concentrations ranged from 494 to 6,952 ppm. Analytical precision for Sr:Ca was 3 % or better. Isotopic intensities from a blank epoxy sample did not exceed background levels for ^{88}Sr or ^{44}Ca.

Data analysis and determination of centrarchid origins

Differences in median water δD values among habitats (floodplain ponds, backwaters, beaver-impounded backwaters, and river main channel) were assessed using Kruskal-Wallis analysis of variance by ranks. This nonparametric procedure was used because water δD values in some habitats were not normally distributed (Shapiro-Wilkes test, $P < 0.001$) and could not be made so by simple transformations. Possible influences of conductivity, floodplain pond surface area (ha), and mean floodplain pond depth (m) on water δD were assessed using Spearman rank correlation coefficients.

Classification of fishes as having floodplain pond or riverine δD signatures in their otolith cores was accomplished using a model that delimited expected otolith δD values for fishes from these two habitat types. To construct our source habitat classification model, the fifth percentile of floodplain pond water δD values (-116.5 ‰) and 95th percentile of riverine water δD values (-117.2 ‰) were identified. Expected values (± 2 SE) for otolith δD were calculated for each of the above water δD cutoff values using a regression model relating water and otolith δD developed with fishes of known environmental history (Whitledge et al. 2006). An upper 95% confidence limit of predicted riverine fish otolith δD and lower 95% confidence limit for predicted floodplain pond fish otolith δD served as thresholds in the model. Using this model, fish with otolith core δD values $\geq$ -128.8 ‰ were identified as having a floodplain pond signature during their first year of life, fish with otolith core δD values $\leq$ -134.2 ‰ possessed a riverine age-0 signature, and the origin of fish with intermediate otolith core δD values was uncertain. Variance associated with the regression model relating water and otolith δD (Whitledge et al. 2006) was responsible for the small region of overlap in predicted

ranges of otolith δD signatures expected for floodplain pond- and riverine-origin fish. The relationship between water and otolith δD values has previously been shown to be consistent among the species collected for this study (Whitledge et al. 2006).

Chi-square tests were applied to assess significance of differences in relative frequencies of centrarchids with floodplain pond, riverine, and uncertain otolith core δD signatures by species, fish age, and river reach (upstream versus within the Grand Valley, above versus below the Gunnison River confluence), and between individuals collected from main channel and backwater habitats. For fishes collected in backwaters, a chi-square test was used to evaluate differences in relative frequencies of individuals with pond, riverine, and uncertain otolith core δD signatures with respect to presence or absence of inflowing ditches or tributary washes. Alpha level (0.05) was divided by the number of chi-square tests to account for the possibility of encountering significant outcomes resulting from chance alone. Differences in median total length of fish with pond, riverine, and uncertain otolith core δD signatures were assessed for each species using Kruskal-Wallis analysis of variance by ranks. For fish with floodplain pond otolith core δD signatures, pond water δD was back-calculated using a regression model relating water and otolith δD developed with fishes of known environmental history (Whitledge et al. 2006).

Otolith Sr:Ca ratios complemented otolith δD analysis by identifying fish that previously resided in environments (some ponds, irrigation ditches) whose salinity exceeded that of riverine habitats. A threshold Sr:Ca ratio was used to distinguish periods of residence in high-salinity (salinity exceeding that of riverine habitats, high Sr:Ca) versus low-salinity (salinity not exceeding that of riverine habitats, low Sr:Ca) environments. This threshold Sr:Ca ratio was defined by an upper 95% confidence limit predicted for riverine-resident fish (2.09 mmol/mol, corresponds to a salinity of 1.7 ‰) using a relationship between otolith Sr:Ca ratio and environmental salinity (Figure 1) and the highest salinity value recorded in riverine habitats in conjunction with water sampling (1.2 ‰). The relationship between otolith Sr:Ca and salinity was developed using centrarchids collected from locations in which they were known to have lived solely within one water body (isolated ponds in which fish were naturally reproduced and no stocking occurred and stocked ponds with no opportunity for natural

immigration). Different species from the same location had statistically indistinguishable otolith Sr:Ca ratios (Kruskal-Wallis test, P=0.29); Sr:Ca varied by < 0.5 mmol/mol along laser-ablated transects from otolith core to edge for individual fish. The significant positive relationship between otolith Sr:Ca ratio and salinity of ponds in our study area is likely the result of higher Sr concentrations in waters with elevated salinities; concentrations of both Sr and major salinity-influencing ions are increased by evapotranspiration and influx of irrigation-derived water that has leached elements from Mancos shale underlying much of our study area (Gerner et al. 2006). Otolith Sr concentration reflects that of the water in which a fish lives (Howland et al. 2001; Zimmerman 2005); thus, our otolith Sr:Ca data are indicative of differences in Sr concentration among fish (and the environments in which they lived) because we treated Ca as an internal standard (Bickford and Hannigan 2005; Ludsin et al. 2006). Sr:Ca ratios for centrarchids of unknown history collected in riverine habitats were calculated based on integrations over entire laser transects when no evidence of fish movement from high-salinity to low-salinity environments was present (initial Sr:Ca $\leq$ 2.09 mmol/mol; Sr:Ca varied by < 0.5 mmol/mol from beginning to end of transect; Figure 2a). When evidence of fish emigration from high-salinity environments was present (initial Sr:Ca > 2.09 mmol/mol with at least one abrupt decline to a final Sr:Ca ratio < 2.09 mmol/mol; Figures 2b, 2c), Sr:Ca ratios were calculated separately for high-salinity and low-salinity portions of transects. Differences in median otolith core Sr:Ca ratios among fish with pond, riverine, and uncertain otolith core δD signatures and differences in median otolith core Sr:Ca ratios among species were both assessed using Kruskal-Wallis analysis of variance by ranks. Effect of fish age on otolith core Sr:Ca ratio was evaluated using Spearman rank correlation coefficients. Age at immigration was determined for individuals that showed evidence of movement from high-salinity to riverine environments by associating locations of abrupt declines in otolith Sr:Ca ratio along laser-ablated transects in relation to annuli.

RESULTS

Development of markers

Most otolith samples had Se:Ca ratios that were at or below the ICPMS detection limit (Table 2), including the green sunfish from 30 Road Pond (a high-selenium environment). Surprisingly, Se:Ca ratios of otoliths from the 3 kokanee collected at Blue Mesa Reservoir (a low-selenium environment) were significantly higher than those of otoliths from the other 10 fish analyzed.

Highly significant linear relationships were observed between both mean otolith and mean muscle δD values and mean water δD signatures at our sampling locations (Figure 3). All otolith and muscle samples were depleted in deuterium (^{2}H) with respect to corresponding water δD signatures. Bonferroni joint 95% confidence intervals indicated that slopes of regressions of mean otolith and mean muscle δD values on mean water δD signatures were significantly less than one and y-intercepts of these regression models were significantly less than zero. Slopes of regression relationships between otolith and water δD (0.50 ± 0.05 standard error (SE)) and muscle and water δD (0.49 ± 0.04 SE) were not significantly different from one another (heterogeneity of slopes test, $P > 0.1$), although mean muscle δD values were depleted in ^{2}H by an average of 31.43 ‰ (± 1.96 ‰ SE) with respect to corresponding mean otolith δD values.

Standardized differences between mean water δD and both muscle and otolith δD values for individual fish were not significantly correlated with fish total length (Pearson correlation coefficients, $P > 0.1$, $n = 119$ otolith samples, $n = 80$ muscle samples). Fish total lengths ranged from 39 mm to 1092 mm. Standardized differences between mean water δD and both mean otolith and mean muscle δD were also not significantly different among the 11 species included in this study (ANOVA, $P > 0.1$, $n \geq 5$ for each species; $1- \beta > 0.9$ for otolith data, $1-\beta = 0.8$ for muscle data for the largest observed effect sizes).

Determining provenance

Floodplain pond water samples were enriched in ^{2}H compared to water collected in the three riverine habitats (Figure 4); ranges of floodplain pond and riverine water δD values did not overlap. Median water δD was greater for floodplain ponds compared to beaver-impounded backwaters, unimpounded backwaters, and the river main channel ($P<0.0001$). Differences in median water δD values among the three riverine habitats were not significant ($P=0.33$). Absence of overlap in ranges of floodplain pond and riverine water δD values occurred despite incorporation of seasonal variation within habitats. Water δD was positively correlated with conductivity ($r_s=0.69$, $P<0.0001$), but conductivity ranged from 759 to 37,000 µS/cm among locations where water δD was >-80 ‰. Floodplain pond water δD was not correlated with mean pond depth ($P=0.65$) or surface area ($P=0.92$).

Median otolith core δD was -125.6 ‰ (inter-quartile range -122.8 ‰ to -127.6 ‰) for fish classified as being of floodplain pond origin (Figure 5). Median otolith core δD was -138.7 ‰ (inter-quartile range -136.5 ‰ to -141.4 ‰) for fish with riverine otolith core δD signatures. Back-calculation of water δD from otolith core δD (Whitledge et al. 2006) revealed that 68 of the 82 fish (83%) with floodplain pond otolith core δD signatures emigrated from ponds with water δD values between -100 and -116 ‰ (Mean -104.0 ‰ ± 2.0 ‰ SE; range -29.7 to -115.8 ‰).

Significant differences in relative proportions of individuals with floodplain pond, uncertain, and riverine otolith core δD signatures were present among species ($P=0.0003$). Approximately 70% of largemouth bass and bluegill collected exhibited an otolith core δD signature expected for riverine-resident fish, with 19% possessing a floodplain pond δD signature in the otolith core, and 10-11% being of uncertain origin (Figure 6). Slightly more than half of the green sunfish examined displayed a riverine otolith core δD signature. In contrast, the majority of black crappie collected had a floodplain pond otolith core δD signature.

Sixty of the 82 fish (73%) with floodplain pond δD signatures in their otolith cores were collected below the Gunnison River confluence. Relative proportions of individuals with floodplain pond, uncertain, and riverine otolith core δD signatures were different above versus below the Gunnison River confluence for both largemouth bass

and bluegill ($P<0.001$); proportions of floodplain pond and uncertain provenance individuals were higher below the Gunnison River confluence than above for both species (Table 3). Relative proportions of individuals with floodplain pond, uncertain, and riverine otolith core δD signatures were not different above versus within the Grand Valley ($P>0.05$) or among individuals collected in river main channel versus backwater habitats ($P>0.05$) for any species. For fish collected in backwaters, presence or absence of direct inflowing ditches or tributary washes did not have an effect on the relative proportions of individuals with floodplain pond, uncertain, and riverine otolith core δD signatures ($P>0.05$).

Median length of fish with a floodplain pond otolith core δD signature was greater ($P<0.05$) than that of fish with a riverine otolith core δD signature for all species except bluegill, whose median lengths were not different ($P=0.55$) among individuals with floodplain pond and riverine otolith core δD signatures. Relative proportions of individuals with floodplain pond, uncertain, and riverine otolith core δD signatures differed among age classes for all species ($P<0.001$). The proportion of fish possessing floodplain pond otolith core δD signatures increased and the proportion of individuals exhibiting riverine otolith core δD signatures declined with increasing fish age (Figure 7).

Otolith thin sections from 212 centrarchids collected from Colorado River backwaters were analyzed for Sr:Ca ratio using LA-ICPMS. All individuals with riverine otolith core δD signatures (n=79) exhibited otolith core Sr:Ca ratios below the upper 95% confidence limit expected for a riverine-resident fish (Figure 8). Eight fish whose origins were uncertain based on otolith core δD analysis exhibited elevated otolith core Sr:Ca ratios characteristic of residence in high-salinity ponds, resolving uncertainty regarding the source of these individuals based on δD analysis alone. Fish with floodplain pond δD signatures in their otolith cores (n=50) exhibited a wide range of otolith core Sr:Ca ratios. Median otolith core Sr:Ca ratios were higher for fish with floodplain pond (median Sr:Ca=1.51 mmol/mol, corresponding salinity=0.9 ‰) and uncertain (median Sr:Ca=1.42 mmol/mol, corresponding salinity=0.8 ‰) otolith core δD signatures compared to fish with riverine (median Sr:Ca=1.17 mmol/mol, corresponding salinity=0.3 ‰) otolith core δD signatures ($P<0.0001$). Otolith core Sr:Ca ratio was not associated with fish age for all individuals combined ($P=0.55$) or for fish with floodplain

pond δD signatures in their otolith cores (P=0.48). Median otolith core Sr:Ca ratio was higher for black crappie compared to the other three species (P<0.05; Table 4). Maximum estimated salinity corresponding to otolith core Sr:Ca ratios was highest for black crappie, intermediate for green sunfish and bluegill, and lowest for largemouth bass.

Twenty-two fish exhibited evidence of emigration from high-salinity habitats to the Colorado River based on changes in otolith Sr:Ca ratios along laser-ablated transects. Seventeen (77%) of these individuals were collected below the Gunnison River confluence. Four of the 22 fish immigrated to riverine habitats at age 0, eight immigrated at age 1, five moved from floodplain pond to riverine habitats at age 2, and five moved to riverine habitats at age 3. All five fish that showed evidence of immigration to riverine habitats at age 3 were black crappie.

DISCUSSION

Development of markers

Significant differences in otolith Se:Ca ratios were detected among fishes from our four sampling locations, although our simple hypothesis that otolith Se:Ca would be elevated in fishes residing in high-selenium environments was not supported. Four of 5 green sunfish from 30 Road Pond (a high-selenium environment) had otolith Se:Ca ratios that were at or below detection limits despite the fact that green sunfish tend to readily accumulate selenium in muscle and other tissues (B. Osmundson, USF&WS, personal communication). The reason for elevated Se:Ca ratios in Blue Mesa kokanee is unknown, although species-specific differences in selenium metabolism or in concentration of otolith proteins associated with selenium may be at least partly responsible. While the large differences between Se:Ca ratios of kokanee collected at Blue Mesa and other fishes analyzed in this pilot study suggest that Se:Ca may yet have potential as an environmental marker for fishes, the lack of correspondence between otolith Se:Ca and environmental selenium level indicates that relationships between these variables are not straightforward. A recent study (Palace et al. 2007),

the first published work on selenium in otoliths, confirms our suspicion that this trace element has merit as a marker and further research is warranted.

Significant linear relationships between both mean fish otolith and mean muscle δD values and mean water δD signature with coefficients of determination > 0.97 suggest that water stable hydrogen isotopic composition has a strong influence on fish δD and that stable hydrogen isotopes have great potential to serve as a new natural marker of fish environmental history when fish move among locations with distinct δD signatures. Water δD varies substantially among non-oceanic surface waters and between surface and groundwaters, often at spatial scales conducive to tracking movements of fishes among water bodies (Seal and Shanks 1998; Coplen and Kendall 2000). Although δD of surface waters may change seasonally (Krabbenhoft et al. 1994), differences in δD among connected aquatic environments can persist when spatial variation in water δD exceeds temporal variability within environments (Coplen and Kendall 2000; Whitledge, unpublished data). We expect that δD values of discrete otolith growth bands will reflect water δD signatures at the time of their deposition, as do isotopic compositions of other elements (e.g., $\delta^{18}O$, $^{87}Sr/^{86}Sr$) that have been successfully applied in studies of fish provenance (Gao and Beamish 1999; Kennedy et al. 2002) and δD values of migratory bird flight feathers (Hobson 1999). The 1-2 mg sample size requirement for stable hydrogen isotope analysis of otoliths by bulk analysis using isotope-ratio mass spectrometry may limit the resolution of δD as a natural marker of fish environmental history; advancement of microsampling techniques for δD analysis such as ion microprobe technology (Weber et al. 2002) would enhance the utility of δD as an environmental tracer for fishes. Application of muscle δD to issues of fish environmental history will require knowledge of metabolic turnover rates to establish time frames over which isotopic assays will integrate, as with isotopes of other elements (Hesslein et al. 1993). Analysis of multiple tissues with different turnover rates will likely prove useful for tracking movements of individuals, as has been the case for other isotopes (Hobson 1999).

Strong correlations between both otolith and muscle δD and δD of waters inhabited by fishes were evident despite lack of correction of otolith and muscle samples for hydrogen exchange. Non-carbon bonded hydrogen (e.g., O-H and N-H) in

organic matter may potentially exchange with ambient water, including laboratory water vapor that can vary temporally and geographically (Schimmelmann 1991; Cormie et al. 1994). For otolith protein (otolin), about 39% of the hydrogen is potentially exchangeable based on its amino acid composition, which is quite similar among fish species (Degens et al. 1969). Estimated proportions of non-exchangeable and potentially exchangeable hydrogen in otolin are similar to those in bird feather keratin (40% potentially exchangeable hydrogen; Hobson 1999); otolin strongly resembles keratin in amino acid composition (Degens et al. 1969). Although 40% of hydrogen in feather and whale baleen keratin is theoretically exchangeable, only about 15% effectively exchanges with ambient water vapor (Chamberlain et al. 1997; Wassenaar and Hobson 2003), suggesting that readily exchangeable hydrogen in otolin may be less than the 39% that is theoretically exchangeable. Mean proportion of exchangeable hydrogen was 19.3% (± 0.5% standard deviation) for quail muscle tissue (Wassenaar and Hobson 2000); proportion of exchangeable hydrogen in fish muscle tissue is unknown. Slopes of regressions of otolith and muscle δD on water δD may be indicative of the proportion of non-exchangeable hydrogen (acquired from the environment) in our samples if the ambient laboratory water vapor possessed a constant δD value. Highly significant relationships between both otolith and muscle δD and δD of waters inhabited by fishes strongly suggest that our otolith and muscle samples had equilibrated with ambient laboratory water vapor δD that did not change substantially during the time period of our analyses (Hobson 1999). We note that the 95% confidence interval for the slope of the regression of otolith δD on water δD includes 0.6, a figure very close to the predicted 61% non-exchangeable hydrogen in otolin. Additional evidence of constant laboratory water vapor δD is provided by mean δD values for four batches of otoliths collected on 30 March 2004 from one sampling location (Government Highline Canal) and analyzed during different months (February, August, and October 2004; March 2005). Mean δD values for these batches of samples were not significantly different (ANOVA, $P = 0.47$), indicating that any hydrogen exchange with ambient laboratory water vapor was not differentially affecting δD values of samples analyzed on different dates (i.e., any temporal variation in water vapor and exchangeable hydrogen δD was insufficient to cause differences among samples

analyzed during this study). Differences in δD among otolith or muscle samples therefore reflected differences in δD of non-exchangeable hydrogen. Stable isotopic composition of non-exchangeable hydrogen in metabolically inert otoliths will provide a permanent record of δD signatures from environments occupied by fish. Although our δD data are internally comparable, future applications of δD assays of fish otoliths and tissues should report results for non-exchangeable hydrogen only, using methods recently developed for bird feathers and other complex organic materials (Wassenaar and Hobson 2000, 2003). Reporting δD data in this manner will facilitate comparison of results among laboratories (Hobson 1999).

Depletion of δD values for fish otolith and muscle samples relative to water hydrogen isotopic signature is consistent with published studies that have demonstrated lower $^{2}H/^{1}H$ ratios in aquatic biota compared to the water they inhabit (Estep and Dabrowski 1980). Twenty to 30% of hydrogen in quail tissues is derived from drinking water, with the rest derived from the diet (Hobson et al. 1999); the relative contribution of water and diet to hydrogen in fishes is unknown. Consistently lower δD values for muscle samples compared to otoliths from fish collected at a given location may be related to the presence of lipids in muscle samples; lipids are typically depleted in ^{2}H compared to proteins (Smith and Epstein 1970; Estep and Hoering 1980). Further experimental research to refine our understanding of sources and behavior of stable hydrogen isotopes in fishes and other aquatic food web components is warranted.

Relationships between both fish muscle and otolith δD and water δD encompass a wide range of water δD signatures and appear to be consistent across distantly related fish species and a wide range of fish sizes. High r^2 values for linear regressions of fish otolith and muscle δD on water δD were observed despite inclusion of fishes from locations with diverse thermal regimes (Mean maximum water temperatures ranged from 10.1-27.4 °C (Whitledge, unpublished data); research hatchery fish were held at 12 ± 1 °C (Phil Schler, Colorado Division of Wildlife Research Hatchery, Box 96, Bellvue, CO 80512, personal communication)), suggesting that the relationship between water and fish δD values is not strongly affected by water temperature as is otolith $\delta^{18}O$. Thus, δD may provide a valuable alternative to otolith $\delta^{18}O$ analysis for discriminating among locations in which distinct thermal regimes prevent spatial differences in water

$\delta^{18}O$ from being expressed in otoliths. The discovery of highly significant relationships between water and fish δD provides a foundation for stable hydrogen isotope ratios to serve as a valuable additional tool in research directed at reconstructing fish environmental history.

Utility of otolith δD and Sr:Ca as environmental markers

This study represents the first application of otolith δD analysis to determine location of origin for individual fish and illustrates the utility of otolith δD as an environmental marker for fishes when clearly defined spatial differences in water δD exist. Whereas δD has been used to track movements of migratory terrestrial animals on a continental scale (Hobson 2005), here we showed that δD was capable of discriminating source locations for fishes on a much smaller scale (m to km). Water δD was enriched in ^{2}H in floodplain ponds compared to riverine habitats due to greater opportunity for evaporative fractionation (Kendall and Caldwell 1998) to be expressed in floodplain ponds as a result of their longer water residence time relative to the Colorado River. Differences in water δD among floodplain ponds reflected varying degrees of hydrologic isolation from the Colorado River, but not dissimilarity of pond morphology or conductivity. That conductivity was a relatively poor predictor of water δD values is likely due to the fact that surface water conductivity in our study area is a function of both evaporation and leaching of elements from Mancos Shale (Butler and von Guerard 1996), whereas water δD is primarily affected by evaporation. We expect that δD will likely be applicable as an environmental tracer for fishes in other locations, particularly in arid or semi-arid regions where differential evaporative fractionation has ample opportunity to create spatial variation in water δD.

Accurately assigning fish to a source location using otolith microchemistry or stable isotopic composition when individuals sampled differ in age or year of collection depends on the inter-annual stability of signatures among locations (Gillanders 2002). Water δD values for 15 samples collected from the Colorado River in our study area at 1-4 month intervals between December 1984 and June 1987 (Coplen and Kendall 2000) were within the range of water δD values for riverine habitats measured in this study, suggesting that the δD signature of riverine habitats in our study area is stable

among years. No data are available regarding inter-annual variation of water δD in Grand Valley floodplain ponds. However, overlap between Colorado River and floodplain pond δD signatures would only be expected to occur during periods when river discharge was sufficient to inundate ponds; many ditch-connected ponds are semi-isolated from the river by levees would not be inundated except during extreme floods. Relatively low variation in Sr:Ca ratios along laser-ablated transects from otolith core to edge (< 0.5 mmol/mol) for known origin, floodplain pond fish (age-0 to age-5) and fish collected from riverine habitats that possessed a riverine otolith core δD signature (age-1 to age-5) is indicative of inter-annual stability in water chemistry within habitats and demonstrates that differences in otolith Sr:Ca signatures of fish from riverine and high-salinity, floodplain pond habitats (up to 11 mmol/mol) can persist among years. These findings are consistent with previous research that demonstrated strong associations between water and otolith microchemistry (Wells et al. 2003) and inter-annual stability of Sr:Ca signatures in some freshwater environments (Zimmerman and Reeves 2002; Wells et al. 2003; Munro et al. 2005; Ludsin et al. 2006).

Centrarchid source habitats

The relative abundance of fish with riverine otolith core δD signatures and Sr:Ca ratios indicates that low-velocity backwater habitats are likely the primary source of three of the four species of centrarchids included in this study. All four species analyzed in this study are associated with low-velocity, river margin habitats (Dettmers et al. 2001; Barko and Herzog 2003) and construct nests in these areas (Pflieger 1997; Scott and Crossman 1998). Black crappie was the only species for which the majority of individuals collected showed evidence of having emigrated from floodplain ponds, which may be a consequence of their tendency to spawn in or near vegetation (Edwards et al. 1982; Pope and Willis 1997); macrophytes are common in Grand Valley floodplain ponds but are rare or absent in backwaters (Martinez et al. 2001). Black crappie recruitment in many backwaters may also be limited by high turbidity given that negative associations between age-0 *Pomoxis* spp. density and turbidity have been documented in other systems (Mitzner 1991). Our results indicate that efforts to control abundance of largemouth bass, bluegill, and green sunfish in critical habitat for native

threatened and endangered fishes should be concentrated in backwaters. Management of black crappie abundance in critical habitat would require an emphasis on restricting escapement from floodplain ponds; however, black crappie are the least numerous of the five centrarchids present in our study area.

Resolution of the approach used for otolith δD analysis corresponded to approximately the first year of a fish's life based on otolith size (mass) for known age centrarchids collected in our study area. Thus, the possibility exists that individuals that emigrated from floodplain ponds very early during age-0 may have been misclassified as being of riverine origin, because material indicative of riverine residence could dominate the otolith core δD signature under such a scenario. Largemouth bass, bluegill, and green sunfish exhibit parental care (Pflieger 1997), which would likely limit the extent of emigration from ponds by age-0 individuals of these species during their first few weeks of life. Additionally, all four fish that exhibited evidence of immigration to riverine habitats at age-0 based on Sr:Ca analysis by LA-ICPMS (a much higher-resolution technique than the one used for δD) had a floodplain pond otolith core δD signature (including one age-9 largemouth bass). All 79 individuals that exhibited a riverine otolith core δD signature possessed an otolith core Sr:Ca ratio consistent with that expected for riverine-resident fish. Consistency of otolith core δD and Sr:Ca results does not eliminate the possibility that δD analysis may have misclassified origin of some individuals, as the two markers do not differentiate among identical habitat types in our study area (δD distinguishes floodplain pond- from riverine-resident fish, whereas Sr:Ca differentiates between residence in high-salinity habitats (including some floodplain ponds) and low-salinity areas). However, results at least indicate no evidence that mistakes were made. For future applications of otolith δD analysis, advancement of microsampling techniques such as ion microprobe technology (Weber et al. 2002) would be valuable for improving temporal resolution. However, substantial improvement in analytical precision of δD measurements by ion microprobe (currently ~10‰) would be required.

We were unable to include an independent set of fish of known environmental history to validate our assignments of source habitat for individual fish. We attempted a transplant experiment to verify our ability to recognize the signature of a previously-

occupied environment in otolith cores by stocking into a fishless, isolated pond. Unfortunately, that experiment failed due to a complete summer kill. The availability of fish of known environmental history in our study area is also quite limited given the open, highly connected nature of river-floodplain systems. The source of any fish collected in the Colorado River is inherently unknown, and therefore individuals obtained there could not be used for model validation. Very few completely isolated floodplain ponds are present in our study area; all of these ponds were sampled for fish that were used in a regression of otolith δD on water δD (Whitledge et al. 2006) that served as the basis for our classification model. Transferring fish from floodplain ponds to cages placed in the Colorado River for the purpose of generating validation data was also impractical given the probabilities of flooding and vandalism.

Directing centrarchid control efforts

Pinpointing locations within the study area that contribute large numbers of nonnatives will be important for directing control efforts to problem areas. The greater proportion of fishes with floodplain pond otolith core δD signatures collected below in comparison to above the Gunnison River confluence is not likely the result of the Gunnison River contributing substantial numbers of pond-origin fish to the Colorado River, as the density of ponds along the Gunnison River is relatively low (1.2/river km; Martinez and Nibbelink 2004). Rather, the higher incidence of centrarchids emigrating from ponds to the Colorado River below the Gunnison River confluence is likely related to the relatively high density of ponds along the Colorado River in the Grand Valley downstream from where the Gunnison River enters (6.2/river km), coupled with the relative abundance of irrigation ditches and washes that enter the Colorado River downstream from the Gunnison River confluence (Martinez and Nibbelink 2004). Another contributing factor may be that the generally larger, deeper, more structurally complex backwaters found below the Gunnison River confluence may be more attractive to centrarchids or more conducive to their growth or survival than the generally smaller, shallower, and structurally simpler backwaters found above the Gunnison confluence.

Efforts to control centrarchid escapement from floodplain ponds to the Colorado River should be focused on the reach below the Gunnison River confluence. However, such actions should be secondary to management activities in riverine habitats given that the majority of fish examined in this study exhibited riverine otolith core δD signatures. Placing physical barriers in irrigation ditches and washes represents one possible strategy for controlling centrarchid immigration to critical riverine habitats (Tyus and Saunders 2000). However, such barriers could negatively impact native fishes that also use ditches and washes in our study area (A. Martinez and L. Martin, Colorado Division of Wildlife, personal observation). Physical or chemical control or outlet screening of individual ponds would have less impact on native fishes (Tyus and Saunders 2000; Martinez 2004), but may be impractical for achieving substantial reductions in centrarchid escapement due to the large number of floodplain ponds in the Grand Valley below the Gunnison River confluence, many of which are privately owned. Reinvasion by centrarchids is also common in Grand Valley floodplain ponds in which nonnative fishes had previously been eradicated (Martinez 2004).

The high proportion (83%) of pond emigrants that left floodplain ponds with water δD values $\leq$ -100 ‰ likely reflects a higher probability of fish immigration to riverine habitats from floodplain ponds that are closely associated with the Colorado River compared to ponds that are more distant from the river. Increased connectivity between large rivers and off-channel floodplain lakes can enhance fish passage between these habitats (Galat et al. 1998). Centrarchids with floodplain pond δD signatures in their otolith cores exhibited a wide range of otolith core Sr:Ca ratios, reflecting emigration from ponds with differing salinities (predicted range 0-5 ‰). Most individuals that exhibited evidence of emigration from high-salinity habitats were collected below the Gunnison River confluence, reflecting the relative abundance of high-salinity ponds and washes in that area. Significantly higher median otolith core Sr:Ca ratio for black crappie compared to the other three species may indicate a greater tendency for black crappie to originate in high-salinity ponds, but our sample size for black crappie was relatively small. Black crappie have been collected in waters having salinities as high as 4.7 ‰ (Edwards et al. 1982), although other centrarchids collected in this study are also at least as salinity tolerant (Musselman et al. 1995; Susanto and Peterson 1996).

While results of otolith core δD analyses indicate that any efforts to control centrarchid escapement from floodplain ponds should be directed primarily toward locations closely associated with the river, our findings do not provide any more specific evidence that particular ponds or groups of ponds are disproportionately contributing to centrarchid abundance in riverine habitats. No clear pattern with respect to age at immigration was evident from Sr:Ca data. However, our results indicate that centrarchids have the capacity to move into riverine habitats from age-0 to at least age-3.

Lack of a significant association between relative frequencies of individuals collected in backwaters with pond, riverine, and uncertain otolith core δD signatures and presence or absence of direct inflowing ditches or washes to backwaters suggests that centrarchids that immigrate to riverine habitats may be selecting the best available habitats rather than simply occupying those closest to their point of entry to the river. Species collected in this study are typically associated with structurally complex habitats (Scott and Crossman 1998; Barwick 2004) and tend to be most abundant in backwaters that are large, relatively deep, and possess plentiful cover (Bundy and Bestgen 2001). Control efforts in riverine habitats for centrarchids included in this study should emphasize backwaters that contain abundant structure irrespective of presence or absence of direct tributaries rather than focusing on those with inflowing washes or ditches.

Relation between fish age and source habitat

The proportion of centrarchids with floodplain pond otolith core δD signatures increased with fish age, and individuals with floodplain pond otolith core δD signatures had greater median total lengths compared to fish with riverine otolith core δD signatures for three of the four species examined. These findings may be consequences of differential mortality of riverine- and floodplain pond-origin fish or inter-annual variation in river hydrology and its potential effects on centrarchid reproduction, larval nursery, and immigration to the river. The upper Colorado River basin has experienced below average precipitation and mean annual discharge was below average from 2000-2004 (USGS 2005). During dry years, decreased river-pond connectivity (Galat et al. 1998) and increased temporal and spatial extent of low-velocity

habitat in the river would be expected. Such conditions could be more favorable for centrarchid reproduction and recruitment in riverine habitats due decreased probability of scouring flows and flushing of larvae from nesting sites (Brown and Ford 2002) while simultaneously limiting access to the river for pond-dwelling fish. Warmer temperatures during years of reduced snowmelt runoff may also be more optimal for centrarchid reproduction in the river. Thus, the recent drought may explain why the majority of the smallest, youngest fish carried a riverine δD signature in the otolith core.

Age-4 and older fish had the highest proportion of individuals with floodplain pond otolith core δD signatures, and the largest individuals of three species (particularly largemouth bass and black crappie) almost always carried a floodplain pond otolith core δD signature. These results suggest that, although the percentage of pond-origin fish in riverine habitats was relatively low at the time of our collections, it may have been higher prior to the current drought and could increase again during years with normal or above average precipitation and river discharge. During wetter years, increased river-pond connectivity (Galat et al. 1998) and a reduction in temporal and spatial extent of low-velocity habitat in the river would be expected. These conditions would be anticipated to be detrimental to centrarchid reproduction and recruitment in riverine habitats (Brown and Ford 2002), while enhancing access to the river for pond-dwelling fish. Our results suggest that centrarchid control efforts in the upper Colorado River should be focused on riverine habitats when hydrologic conditions are similar to those during this study, but reevaluation of relative proportions of riverine-dwelling centrarchids with pond and riverine otolith core δD signatures is recommended during and immediately following years of above average precipitation and river discharge. Possible effects of increased precipitation on riverine and floodplain pond water δD signatures should be assessed as part of this effort. Such a follow-up study would be useful for determining whether management of centrarchid abundance in critical habitat should always be focused within riverine habitats themselves, or if additional emphasis should be placed on controlling centrarchid escapement from ponds to curtail immigration to riverine habitats during high-water years.

Our findings corroborate those of a few other recent studies that demonstrated that otolith microchemistry and isotopic analysis represent powerful techniques for

retrospectively describing environmental history of fishes that reside solely in freshwaters, including lakes (Joukhadar et al. 2002; Brazner et al. 2004; Munro et al. 2005; Ludsin et al. 2006), streams (Wells et al. 2003), and small rivers (Bickford and Hannigan 2005). Results of this study demonstrate that otolith elemental and isotopic assays can also be applied to gain valuable insight into fish movement between large rivers and associated lentic floodplain habitats that would be difficult to obtain by other means. The ability to track movement of relatively large numbers of individual fish between lotic and lentic habitats will likely prove beneficial to management of both native and nonnative fishes in other large river-floodplain ecosystems.

CONCLUSIONS

Otolith microchemistry provides a powerful tool for determining movements of fishes among riverine and pond habitats in the Colorado River. While the relationship between environmental and otolith selenium concentrations was obscure, the high spatial variation in selenium concentrations in the Upper Colorado River Basin presents an exciting opportunity to investigate a potential new trace element marker for studying origins and movement patterns of fishes, while at the same time yielding valuable ecotoxicological knowledge of fish exposure history in the wild. We would be very interested in pursuing this avenue of investigation in the future if the Recovery Program was interested in supporting such research

Hydrogen isotopes and strontium abundance showed large variation among habitats and in otoliths. These isotopes and elements were preferable to stable isotopes of carbon and nitrogen because of the strong influence of food on the composition of the latter in fish tissues. The stable isotope of hydrogen (deuterium, ^{2}H) proved to be an excellent naturally occurring marker for discriminating fishes originating from pond versus riverine habitats. Ponds and “riverine” habitats possessed distinct deuterium signatures (δD) with low temporal variation, and there was a strong and consistent relationship between hydrogen signatures of the environment and the isotopic composition in otoliths. Whereas δD has been used to track movements of migratory terrestrial animals on a continental scale, here we showed, for the first time, that δD was capable of discriminating locations of origin for fishes on a much smaller

scale (m-km). δD can be used to indicate centrarchids of pond origin or that have spent the majority of their first year of life in “riverine” habitats.

Relationships between both fish muscle and otolith δD and water δD encompassed a wide range of water δD signatures and were consistent across distantly related species and a wide range of fish sizes. We expect that δD will likely be applicable as an environmental tracer for fishes in locations outside the Upper Colorado River basin as well. Otolith strontium concentration is a useful adjunct to hydrogen analyses because strontium can identify fish that have resided in high-salinity ponds, washes, and irrigation ditches.

Successful development of microchemical markers allowed us to determine the origins and movements of centrarchids in the study area, and the proportion of centrarchids in backwaters within the study area that originated from out-of-channel ponds versus in-channel habitats. The relative abundance of fish with riverine otolith core δD signatures and Sr:Ca ratios indicated that low-velocity backwater and beaver pond habitats were likely the primary source of three of the four species of centrarchids included in this study. Black crappie was the only species for which the majority of individuals collected showed evidence of having emigrated from ponds.

The greater proportion of fishes with pond otolith core δD signatures collected below in comparison to above the Gunnison River confluence is likely related to the relatively high density of ponds along the Colorado River in the Grand Valley downstream from where the Gunnison River enters coupled with the relative abundance of irrigation ditches and washes that enter the Colorado River downstream from the Gunnison River confluence. Another contributing factor may be that the generally larger, deeper, more structurally complex backwaters found below the Gunnison River confluence may be more attractive to centrarchids or more conducive to their growth or survival than the generally smaller, shallower, and structurally simpler backwaters found above the Gunnison confluence.

Most pond emigrants left ponds with relatively depleted water δD values ($\leq$ -100 $^{o}/_{oo}$), suggesting a higher probability of emigration from ponds that are closely associated hydrologically with the Colorado River (the river had the most depleted or “lightest” δD signature, Figure 4). Centrarchids with pond δD signatures in their otolith

cores exhibited a wide range of otolith core Sr:Ca ratios, reflecting emigration from ponds with differing salinities (predicted range 0-5 $^{o}/_{oo}$). Most individuals showing evidence of emigration from high-salinity habitats were collected below the Gunnison River confluence, reflecting the relative abundance of high-salinity ponds and washes in that area. Significantly higher mean otolith core Sr:Ca ratio for black crappie compared to the other three species may indicate a greater tendency for black crappie to originate in high-salinity ponds, although our sample size for black crappie was relatively small.

Although our δD analyses indicated that centrarchid escapement from ponds occurred primarily from ponds closely associated with the river, we did not find evidence that particular ponds or groups of ponds are disproportionately contributing to centrarchid abundance in the river. Further, no clear pattern in age at immigration was evident in the Sr:Ca data, although results indicated that centrarchids were able to move into riverine habitats during age-0 to at least age-3.

Lack of a significant association between relative frequencies of individuals collected in backwaters and beaver ponds with pond, riverine, and uncertain otolith core δD signatures and presence or absence of direct inflowing ditches or washes to backwaters or beaver ponds suggests that centrarchids that immigrate to riverine habitats may be selecting the best available habitats rather than simply occupying those closest to their point of entry to the river.

Most of the fish examined in this study were determined to have originated from riverine habitats, but the proportion of centrarchids with pond otolith core δD signatures increased with fish age and length for three of the four species. We believe this pattern may be a consequence of inter-annual variation in river hydrology and its effects on centrarchid recruitment and immigration to the river. The upper Colorado River basin experienced below average precipitation and discharge during 2000-2004. During dry years, less river-pond connectivity and more low-velocity habitat in the river would be expected. Such conditions could be more favorable for centrarchid reproduction and recruitment in riverine habitats, while simultaneously limiting access to the river for pond-dwelling fish. Thus, the drought may explain why the majority of the smallest, youngest fish carried a riverine δD signature in the otolith core.

Age-4 and older fish had the highest proportion of individuals with pond otolith core δD signatures and the largest individuals of three species almost always carried a pond otolith core δD signature. This finding suggests that although the percentage of pond-origin fish in riverine habitats was relatively low at the time of our collections, it may have been higher prior to the drought and could increase again during years with normal or above average precipitation and river discharge. While fish of pond origin were fewer than fish of riverine origin during this study, the data suggest that ponds may sometimes be important sources of centrarchids, allowing them to repopulate riverine habitat after periods of unfavorable hydrologic conditions in the river itself. This hypothesis must be evaluated to fully interpret the implications of the present study.

Our findings corroborate those of other recent studies and demonstrate that otolith elemental and isotopic assays can be applied to gain valuable insight into fish movement between large rivers and associated lentic floodplain habitats that would be difficult to obtain by other means. The ability to track movement of relatively large numbers of individual fish between lotic and lentic habitats will likely prove beneficial to management of both native and nonnative fishes in other large river-floodplain ecosystems, including the Green, White, Gunnison, Duchesne and Yampa Rivers. Additionally, our work did not address provenance of smallmouth bass found in the Colorado River but techniques developed here could be very useful for determining the degree of smallmouth bass recruitment originating in reservoirs versus within the rivers downstream of reservoirs.

Two important caveats must be stressed. First, our work was conducted during a relatively dry period, when connectivity between ponds and the river would be expected to be less than during wetter hydrologic conditions. Conclusions about habitats contributing the majority of the centrarchids to the river should be restricted to time periods exhibiting river hydrology similar to that of the period of study. Therefore, management of nonnative fishes by control of immigration sources should not be directed exclusively at riverine habitats, at least until a similar study of nonnative fish provenance is conducted and it can be shown that ponds do not supply significant numbers to the burden of nonnative fish in critical habitat. Second, sample mass requirements of analytical methods currently available for δD determination limit

temporal resolution within the otolith to approximately the entire first year of life. Thus, the possibility exists that we misclassified individuals that emigrated from ponds very early during age-0 as being of riverine origin, because material indicative of riverine residence could dominate the otolith core δD signature under such a scenario. However, largemouth bass, bluegill, and green sunfish exhibit parental care, which would likely limit the extent of emigration from ponds by early age-0 individuals of these species. At present, we cannot draw conclusions about the degree of emigration of small age-0 fishes from ponds.

RECOMMENDATIONS

1. Although results of this project indicate that centrarchid control efforts in the upper Colorado River should focus on riverine habitats when hydrologic conditions are similar to those during this study, reevaluation of relative proportions of river-dwelling centrarchids with pond and riverine otolith core signatures is recommended during and immediately following years of above average precipitation and river discharge. Such a follow-up study would be essential for assessing whether management of centrarchid abundance in critical habitat should always be focused within riverine habitats themselves or if additional emphasis should be placed on controlling centrarchid escapement from ponds to curtail immigration to riverine habitats during high-water years.

2. Efforts to control abundance of centrarchids (except black crappie and smallmouth bass) in critical habitat for native threatened and endangered fishes should emphasize backwaters and beaver ponds that contain abundant structure irrespective of presence or absence of tributaries rather than focusing on those with inflowing washes or ditches.

3. Efforts to control centrarchid escapement from ponds to the Colorado River should focus on the reach downstream of the Gunnison River confluence, although such actions should be secondary to management activities in riverine

habitats given that the majority of centrarchids examined in this study exhibited riverine otolith core δD signatures.

4. If additional control measures were deemed necessary to control movement of largemouth bass from ponds, such efforts could be applied on ponds with a salinity < 1.8 ‰, thus narrowing the number of candidate ponds for treatment.

5. Management of black crappie abundance, in particular, within critical habitat would require an emphasis on restricting escapement from ponds; however, black crappie were the least numerous of the five centrarchids present in our study area.

6. Placing physical barriers in irrigation ditches and washes represents one possible strategy for controlling centrarchid immigration to critical habitat, although such barriers could negatively impact native fishes that also use ditches and washes in that area. Physical or chemical control or outlet screening of individual ponds would have less impact on native fishes, but may be impractical for achieving substantial reductions in centrarchid escapement due to the large number of ponds in the Grand Valley downstream of the Gunnison River confluence, many of which are privately owned.

7. Advancement of microsampling techniques such as ion microprobe technology would be valuable for improving temporal resolution and would make future applications of otolith δD analysis more powerful. Further experimental research to refine our understanding of sources and behavior of stable hydrogen isotopes in fishes and other aquatic food web components is also warranted.

8. Efforts to track movement of nonnative fishes between lotic and lentic habitats elsewhere in the Colorado River basin should consider applying techniques developed here. Further, otolith microchemistry should be applied to determining the proportion of smallmouth bass and other nonnative fishes found in critical

habitat that originated in reservoirs versus those that reproduced within the rivers downstream of reservoirs.

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Table 1. Locations sampled for fishes, latitude and longitude, species collected (BCR-black crappie (*Pomoxis nigromaculatus*), BGL-bluegill (*Lepomis macrochirus*), CUT-cutthroat trout (*Oncorhynchus clarki*), GSF-green sunfish (*Lepomis cyanellus*), HAL-Pacific halibut (*Hippoglossus stenolepis*), KOK-kokanee (*Oncorhynchus nerka*), LMB-largemouth bass (*Micropterus salmoides*), NPK-northern pike (*Esox lucius*), SMB-smallmouth bass (*Micropterus dolomieu*), WHS-white sucker (*Catostomus commersoni*), YLP-yellow perch (*Perca flavescens*)), number of individuals collected (n), samples collected (M-muscle, O-otolith) and mean water δD (‰, ± 1 standard error (SE)) for each location. n=3 water samples collected during different seasons for Horsethief Pond and Bounds Pond. n=2 water samples collected during different seasons for the Government Highline Canal, Blue Mesa Reservoir, the Research Hatchery, College Lake, and Audubon Pond. n=1 water sample for all other locations.

Location	Latitude/Longitude	Species	n	Samples	Water δD (SE)
Govt. Highline Canal	N 39° 07’ W 108° 22’	WHS	20	O	-124.68 (2.11)
Highline Reservoir	N 39° 16’ W 108° 50’	LMB	5	M, O	-118.97
Blue Mesa Reservoir	N 38° 28’ W 107° 15’	KOK	7	O	-115.36 (0.16)
Lake Powell	N 37° 39’ W 110° 31’	SMB	5	O	-114.00
Horsethief Pond	N 39° 10’ W 108° 47’	BGL, GSF, LMB	7	O	-112.91 (2.67)

Table 1. Continued.

Location	Latitude/Longitude	Species	n	Samples	Water δD (SE)
Research Hatchery	N 40° 37’ W 105° 10’	CUT	10	M, O	-110.58 (1.24)
College Lake	N 40° 34’ W 105° 08’	LMB, NPK, SMB, YLP	23	M, O	-97.96 (1.19)
Dixon Reservoir	N 40° 33’ W 105° 08’	LMB	8	M, O	-93.33
Bounds Pond	N 39° 05’ W 108° 23’	GSF, LMB	10	M, O	-79.35 (4.24)
Audubon Pond	N 39° 04’ W 108° 36’	BCR, BGL, GSF, LMB	20	M, O	-45.40 (2.27)
Cook Inlet	N 59° 56’ W 152° 20’	HAL	10	M, O	-12.73

Table 2. Selenium:calcium (Se:Ca) ratios (μmol/mol) of sagittal otoliths for 13 individual fishes collected at 4 locations in western Colorado. 30 Road Pond represents a high-selenium environment, the Colorado (CO) River below the Gunnison River confluence (Gunn) represents a moderate-selenium environment, and the Colorado River above the Grand Valley (GV) and Blue Mesa Reservoir represent low-selenium environments.

Location	Species	Se:Ca (μmol/mol)
Blue Mesa Reservoir	Kokanee	1.6
Blue Mesa Reservoir	Kokanee	2.6
Blue Mesa Reservoir	Kokanee	2.3
CO River (above GV)	Smallmouth bass	0.2
CO River (above GV)	Smallmouth bass	0.3
CO River (above GV)	Smallmouth bass	0.4
CO River (below Gunn)	Smallmouth bass	0.3
CO River (below Gunn)	Smallmouth bass	0.9
30 Road Pond	Green sunfish	0.2
30 Road Pond	Green sunfish	0.3
30 Road Pond	Green sunfish	0.4
30 Road Pond	Green sunfish	0.6
30 Road Pond	Green sunfish	0.4

Table 3. Percentages of largemouth bass (LMB) and bluegills (BGL) collected from the Colorado River and its backwaters that possessed otolith core δD signatures indicative of floodplain pond (pond), uncertain, and riverine origins for individuals captured above versus below the Gunnison River confluence. n indicates number of individuals of each species sampled above and below the Gunnison River confluence.

	Location with respect to Gunnison River confluence							
	Above				Below			
Species	n	Pond	Uncertain	Riverine	n	Pond	Uncertain	Riverine
LMB	47	9%	4%	87%	72	25%	15%	60%
BGL	14	7%	0%	93%	29	24%	17%	59%

Table 4. Median and maximum otolith core Sr:Ca ratios (mmol/mol) for black crappie (BCR, n=11), bluegill (BGL, n=23), green sunfish (GSF, n=104), and largemouth bass (LMB, n=74). Estimated salinity (‰) associated with each Sr:Ca ratio is also shown. Salinities were calculated using the relationship shown in Figure 1.

Species	Median Sr:Ca	Median salinity	Maximum Sr:Ca	Maximum salinity
BCR	2.33	2.0	7.95	5.0
BGL	1.42	0.8	3.60	3.0
GSF	1.29	0.6	3.70	3.1
LMB	1.23	0.5	2.15	1.8

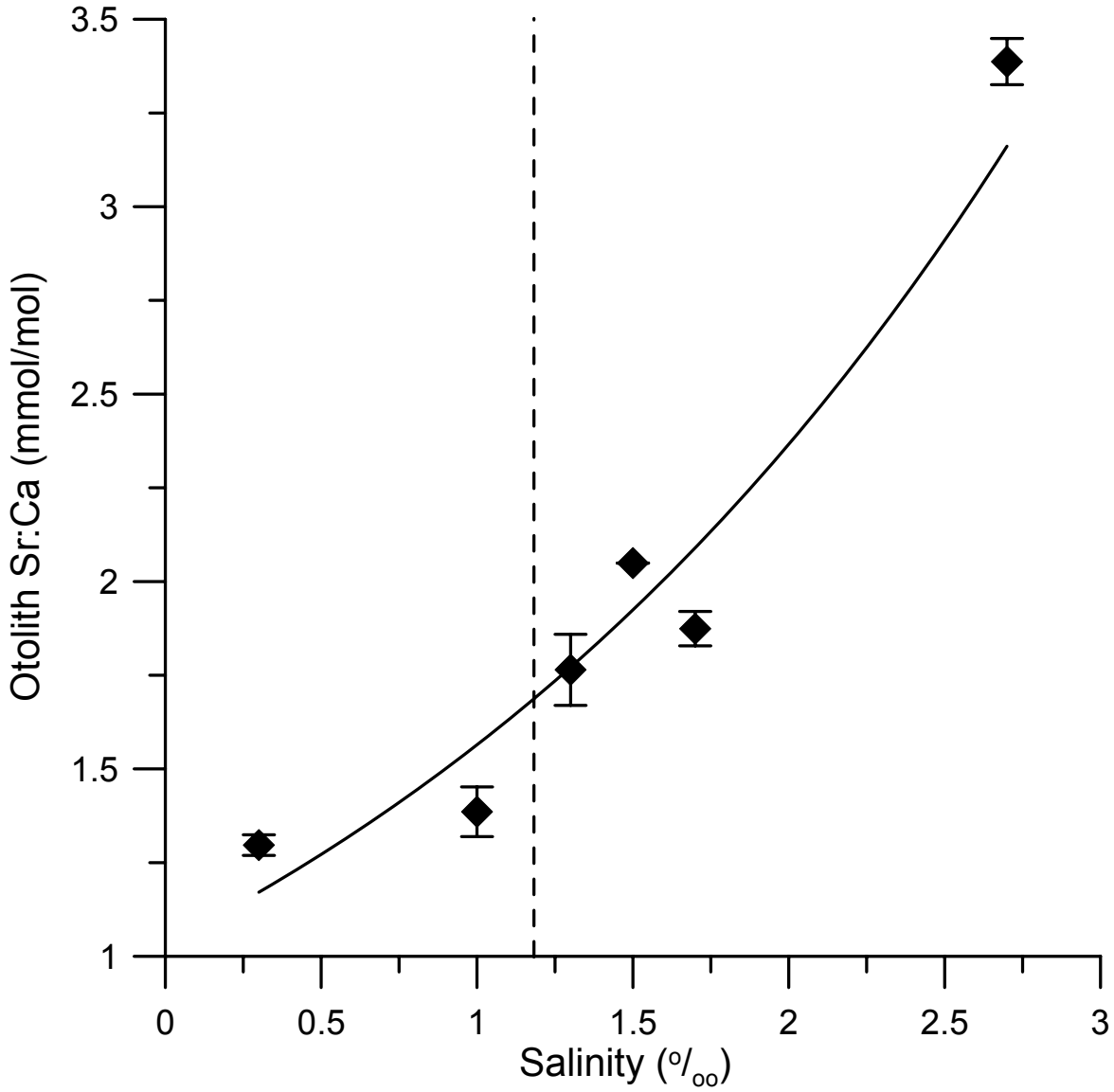

Figure 1. Relationship between otolith strontium:calcium ratio (Sr:Ca, mmol/mol) and environmental salinity developed with centrarchids of known environmental history. Data points are means (n = 5 fish per point) ± SE. Solid line is an exponential function fit to data (ln Sr:Ca = 0.413 salinity + 0.034, $r^2 = 0.92$, $P < 0.005$). Vertical dashed line indicates upper limit of measured salinity values for riverine habitats.

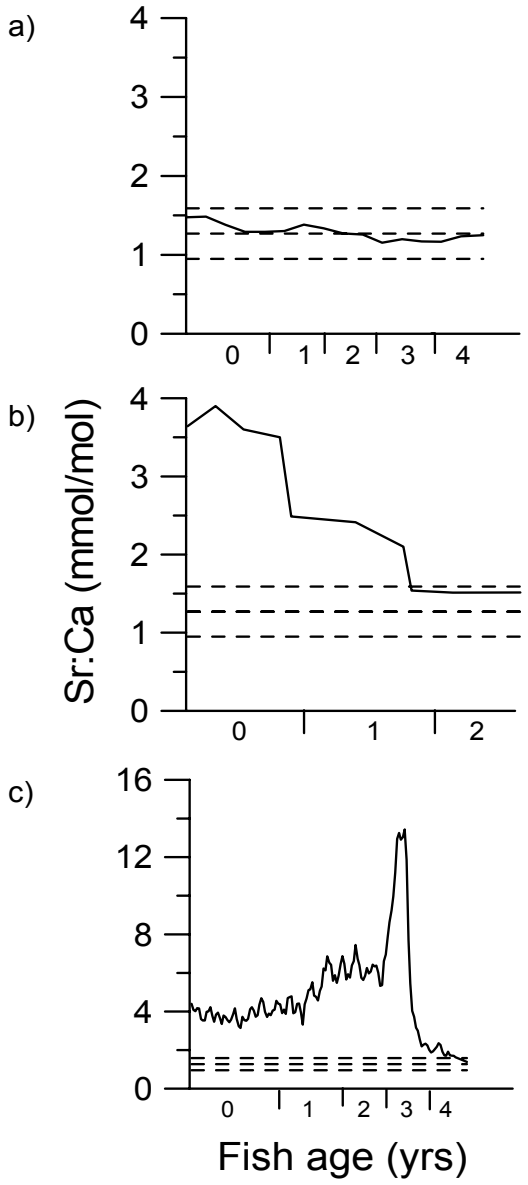

Figure 2. Representative patterns of otolith strontium:calcium ratio (Sr:Ca, mmol/mol) along laser-ablated transects from otolith core to edge for individual fish collected from the Colorado River and its backwaters. Data from an age-4 green sunfish (a), an age-2 green sunfish (b), and an age-4 black crappie (c) are shown. Dashed lines indicate mean Sr:Ca ratio (1.27 mmol/mol) ± SD (0.32 mmol/mol) for fish that possessed a riverine otolith core δD signature (n=79). Note different scale of y-axis in panel (c).

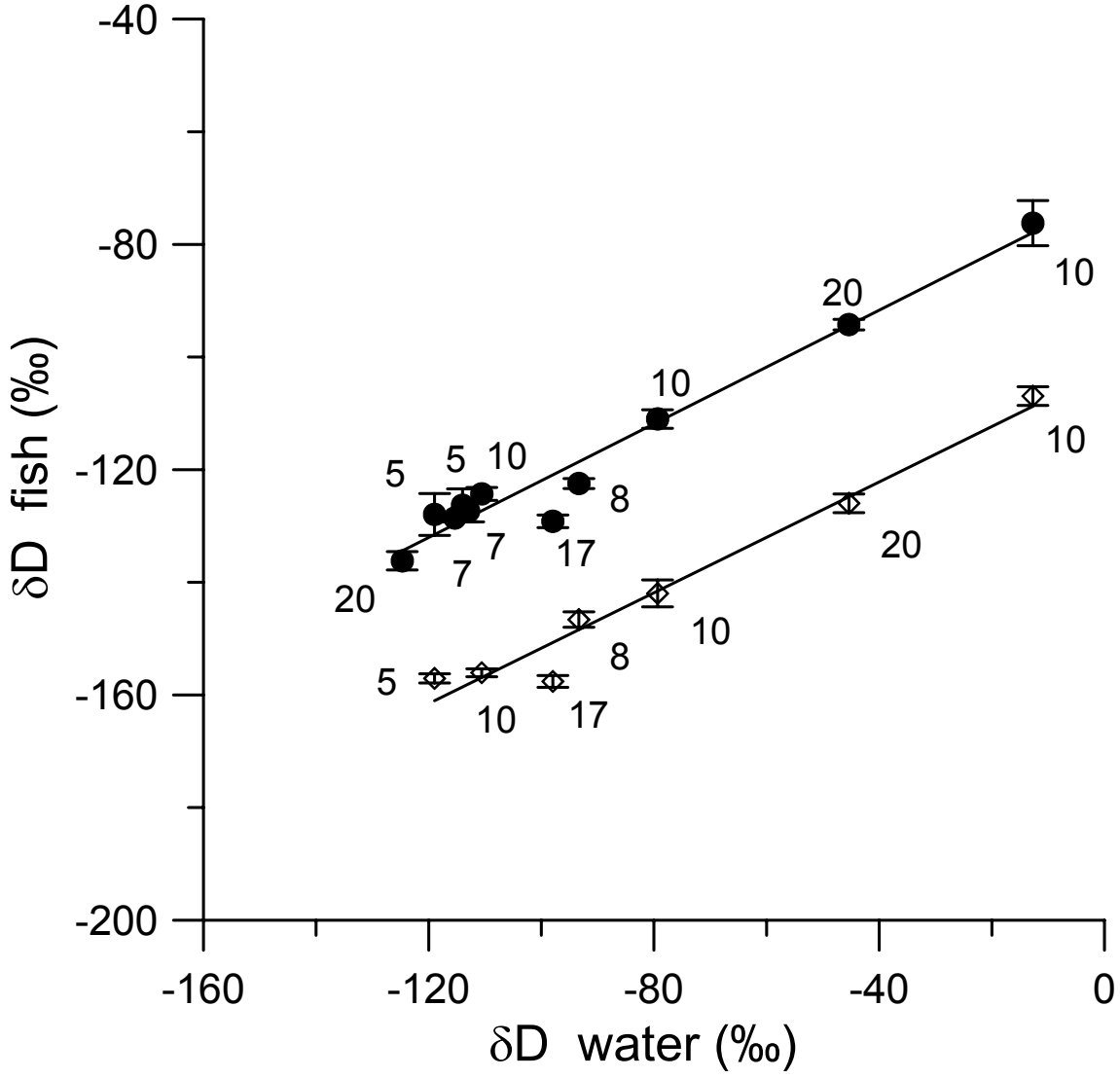

Figure 3. Mean otolith and muscle δD values in relation to mean water δD signatures. Solid circles represent means of otolith samples from each location ± 1 standard error (SE); open diamonds represent means of corresponding muscle samples ± 1 SE. Sample sizes are indicated next to each data point. Solid lines indicate least-squares linear regression functions fit to otolith data ($y = 0.50 x – 71.35$; $r^2 = 0.97$, $P < 0.0001$) and muscle data ($y = 0.49 x – 102.53$; $r^2 = 0.98$, $P < 0.0001$).

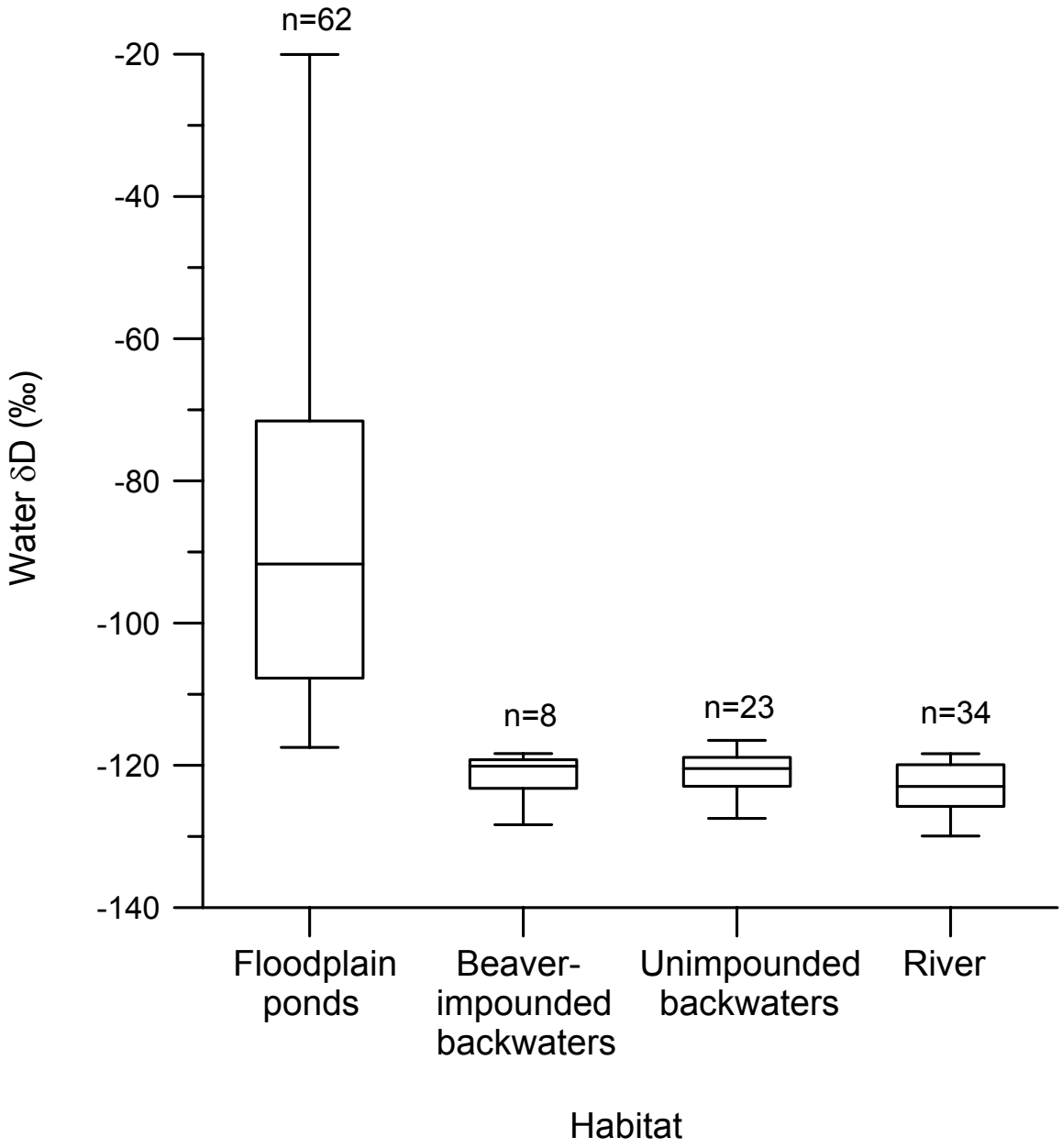

Figure 4. Box plots of water δD in floodplain ponds, beaver-impounded backwaters, un-impounded backwaters, and Colorado River main channel habitats. Median, inter-quartile range, and range of water δD values and number of samples (n) are shown for each habitat. Samples were collected during November 2003, April 2004, and July 2004.

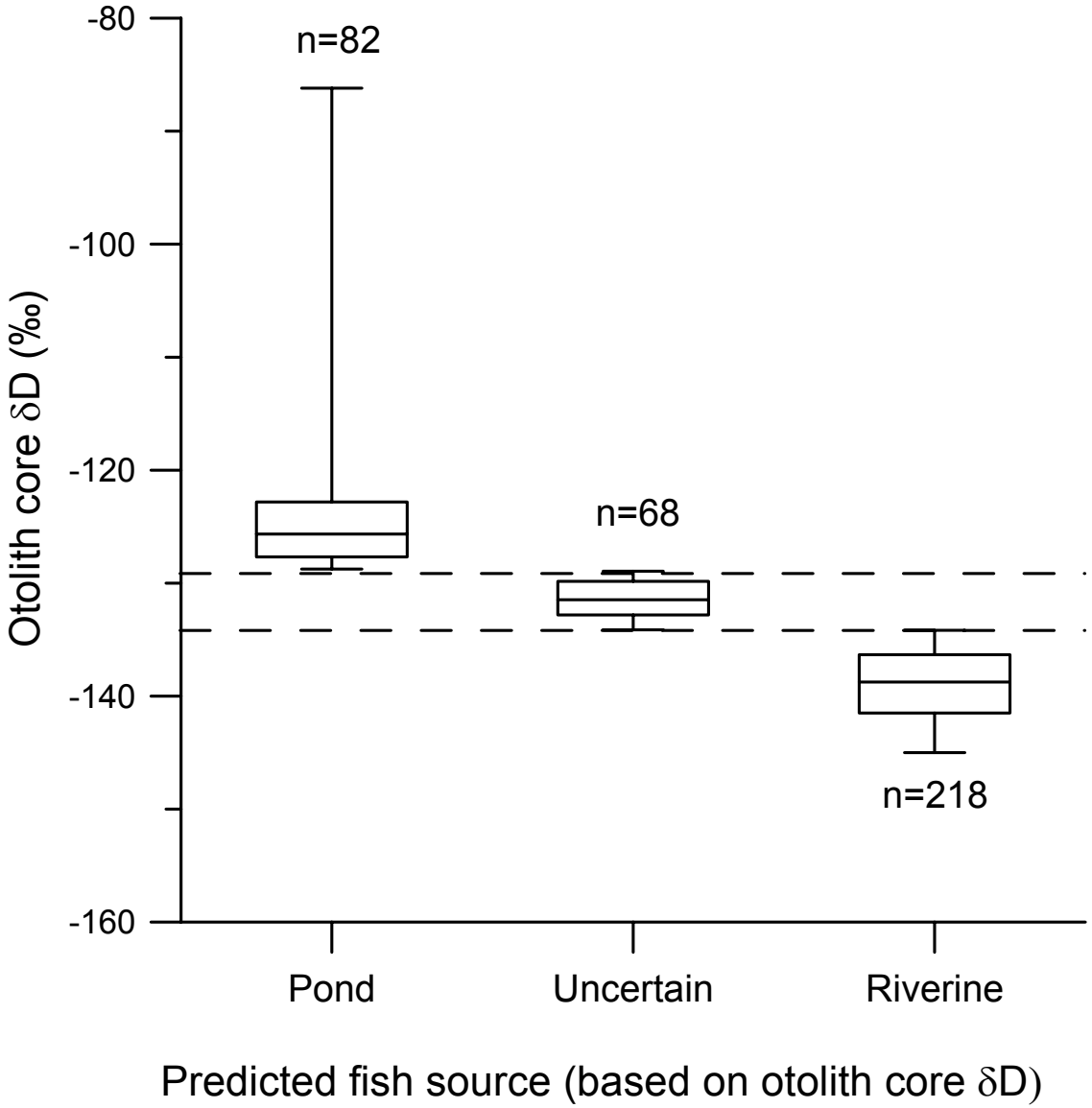

Figure 5. Box plots of otolith core δD values for fish collected in Colorado River backwater and main channel habitats. Medians, inter-quartile ranges, and ranges of otolith core δD values and number of individuals analyzed (n) are shown for fish classified as being of floodplain pond, uncertain, and riverine origin. Horizontal dashed lines indicate threshold δD values in the classification model that were used to assign location of origin (floodplain pond, uncertain, or riverine) to individual fish.

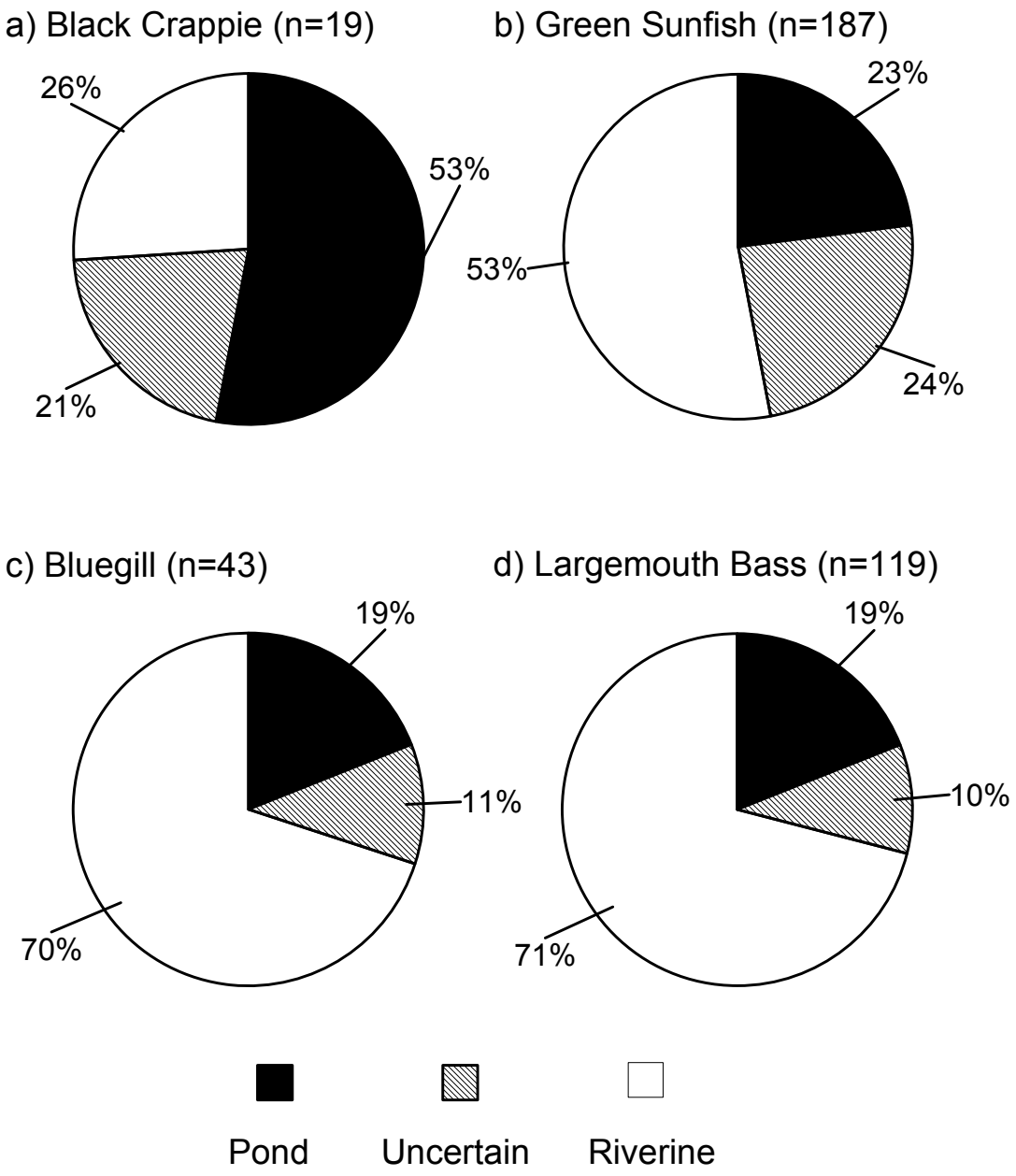

Figure 6. Relative proportions of black crappie (a), green sunfish (b), bluegill (c), and largemouth bass (d) collected in Colorado River backwater and main channel habitats with floodplain pond, uncertain, and riverine otolith core signatures. Number of individuals analyzed (n) is indicated for each species as are percentages contained within each slice.

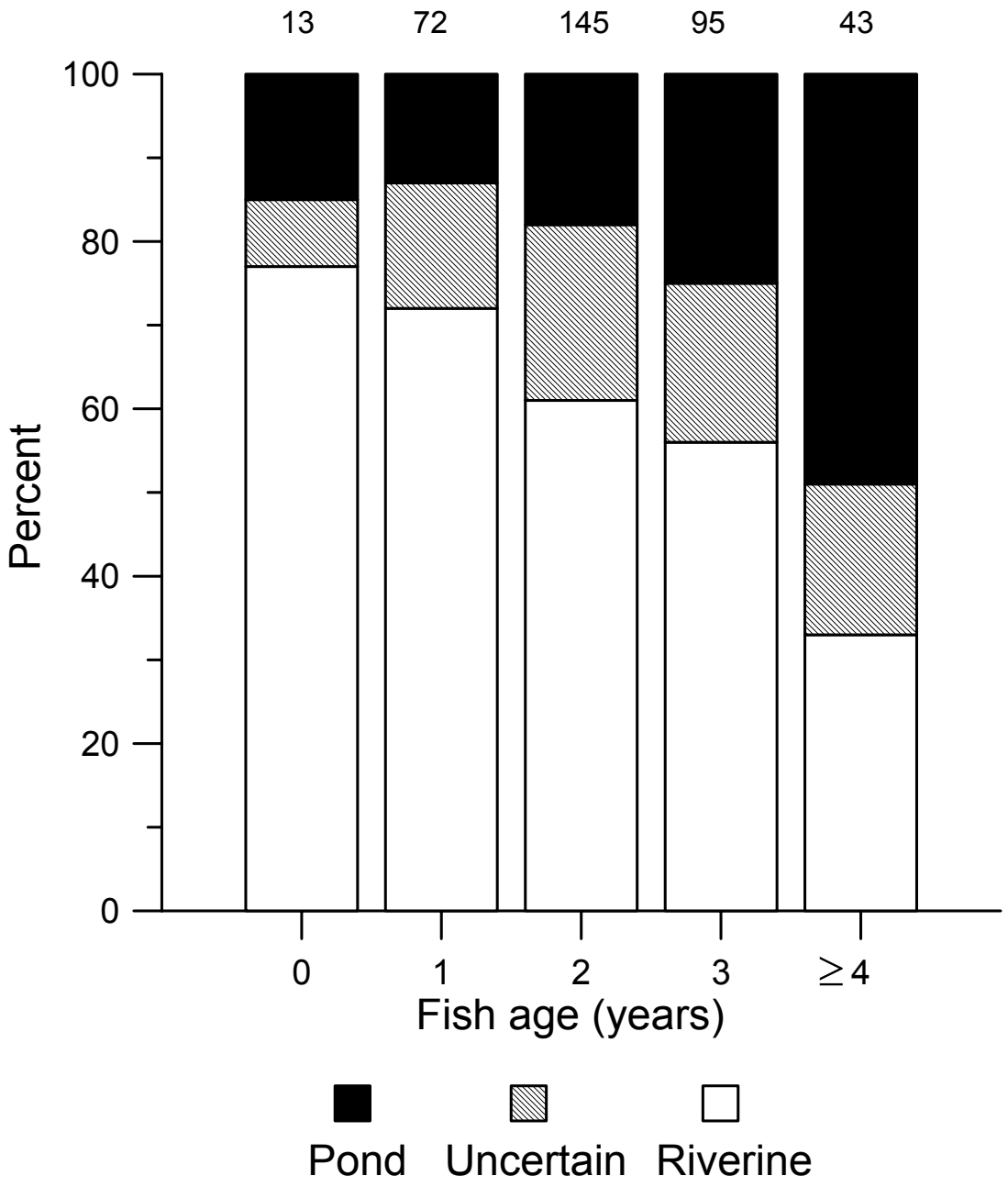

Figure 7. Relative proportions of centrarchids collected in Colorado River backwater and main channel habitats with floodplain pond, uncertain, and riverine otolith core signatures within fish age classes from age-0 to age ≥ 4 years. Values above bars indicate number of fish analyzed for each age class.

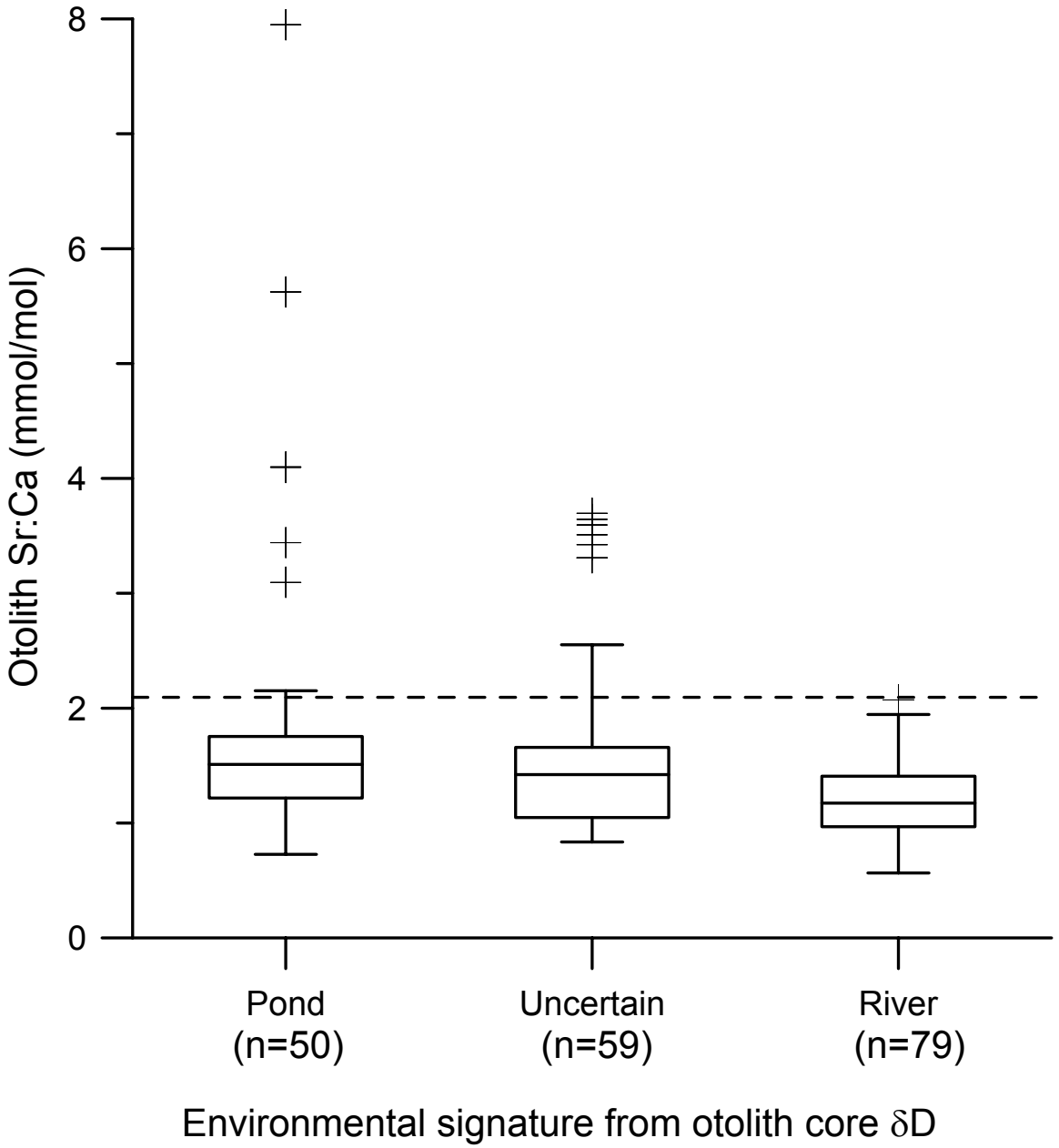

Figure 8. Box plots showing medians, inter-quartile ranges (IQR), and ranges of otolith core Sr:Ca ratios for fish with floodplain pond, uncertain, and riverine otolith core δD signatures. Values > 1.5·IQR from upper or lower quartiles are plotted as outliers (plus symbols). Horizontal dashed line indicates upper 95% confidence limit of Sr:Ca ratio expected for a riverine-resident fish (2.09 mmol/mol). n=number of samples analyzed.

APPENDICES

Table A1. Number of water samples analyzed for hydrogen stable isotope ratio from four habitat types in the floodplain of the Colorado River, in Colorado, from the town of Rifle (RM 241) downstream to the Loma Boat Ramp (LBR - RM 152). RM = river mile, RLD= Roller Dam, GUR = Gunnison River confluence.

Location	Number of water samples by habitat				Total no. of samples analyzed
	Pond	Mainstem	Backwater	Beaver pond	
Rifle to RLD (RM 193-241)	11	12	2	0	25
RLD to GUR (RM 171-193)	15	12	8	5	40
GUR to LBR (RM 152-171)	36	10	13	3	62
Total	62	34	23	8	127

Table A2. Number of otoliths analyzed for hydrogen stable isotope and strontium:calcium ratios obtained from four species of nonnative centrarchids in the floodplain of the Colorado River, in Colorado, from the town of Rifle (RM 241) downstream to the Loma Boat Ramp (LBR - RM 152). RM = river mile, GUR = Gunnison River confluence, BGL = bluegill, GSF = green sunfish, LMB = largemouth bass, BCR = black crappie.

Location	Habitat	BGL	GSF	LMB	BCR	Total
Hydrogen isotope ratio ($^2H/^1H$): whole or core						
Rifle to RLD (RM 193-241)	Pond	0	0	0	0	0
	Backwater	0	0	0	0	0
	Mainstem	1	34	20	0	55
RLD to GUR (RM 171-193)	Pond	0	5	5	0	10
	Backwater	5	51	27	0	83
	Mainstem	8	7	0	1	16
GUR to LBR (RM 152-171)	Pond	5	4	11	12	32
	Backwater	27	90	67	15	199
	Mainstem	2	5	5	3	15
Subtotal		48	196	135	31	410
Strontium:calcium ratio (Sr:Ca): thin section-LA						
Rifle to RLD (RM 193-241)	Pond	0	0	0	0	0
	Backwater	0	0	0	0	0
	Mainstem	0	0	0	0	0
RLD to GUR (RM 171-193)	Pond	0	5	10	0	15
	Backwater	3	31	16	0	50
	Mainstem	0	0	0	0	0
GUR to LBR (RM 152-171)	Pond	0	0	0	5	5
	Backwater	20	73	58	11	162
	Mainstem	0	0	0	0	0
Subtotal		23	109	84	16	232
Number of otoliths analyzed						
Rifle to RLD (RM 193-241)	Pond	0	0	0	0	0
	Backwater	0	0	0	0	0
	Mainstem	1	34	20	0	55
RLD to GUR (RM 171-193)	Pond	0	10	15	0	25
	Backwater	8	82	43	0	133