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Using Otolith Microchemistry of *Haemulon flavolineatum* (French Grunt) to Characterize Mangroves and Coral Reefs Throughout Turneffe Atoll, Belize: Difficulties at Small Spatial Scales

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ABSTRACT: We investigated whether the otolith chemistry of *Haemulon flavolineatum* (French grunt), a nocturnally active fish, could be used as a means to differentiate individuals occupying mangrove and coral reef habitats. In 2003, adults were collected from 9 mangrove and 10 coral reef sites throughout Turneffe Atoll, Belize. Concentrations of trace elements were measured at the edge of sagittal otoliths by laser ablated inductively coupled plasma mass spectrometry. Results of a two-factor nested MANCOVA (sites nested within habitat and covariate of fish size), used to investigate whether significant differences in otolith elemental concentrations existed between habitats (i.e., mangrove versus reef) and among sites, indicated significant differences between habitats, in terms of lithium, magnesium, zinc, and rubidium (fish from mangroves had greater concentrations than those from coral reefs), as well as among sites (for several elements). Because elemental variability existed between habitats and among sites, we asked whether this variability was sufficient to differentiate habitats and sites using separate linear discriminant function analyses (LDFA). LDFA indicated that fish were classified to the habitat (mangrove or reef) from which they were collected with a moderate degree of accuracy (correct classification of 74% and 79% for mangrove and coral reef fish, respectively), but were poorly classified to the site from which they were collected (average correct classification of 46% with a range of 0–89%). Otolith microchemical investigations of *H. flavolineatum* at Turneffe Atoll can be used to identify movement between habitats, yet due to the lack of unique site-specific chemical signatures likely caused by the nocturnal movement of individuals, it will not be possible to identify specific sites from which reef fish originated.

Introduction

Shallow water habitats such as mangroves have traditionally been regarded as areas that provide food and shelter for developing fish and crustaceans, as well as sources of recruits for nearby coral reefs (Beck et al. 2001; Gillanders et al. 2003; Sheridan and Hays 2003). The extent of connectivity (i.e., the demographic link maintained between populations of a species due to the movement of individuals; Mora and Sale 2002) between potential nursery (mangrove) and adult (coral reef) habitat is relevant to fisheries conservation and management throughout the world. Although numerous ecological processes, such as competition, predation, and responses to abiotic factors, determine the distribution and abundance of reef fish populations, direct quantification of connectivity remains a particularly significant gap in our understanding.

Because the chemical nature of an otolith reflects the environment in which a fish resides (various elements have shown correspondence between the otolith and environmental concentration; strontium [Sr]: Farrell and Campana 1996; Gallahar and Kingsford 1996; Bath et al. 2000; Kennedy et al. 2002; Eldson and Gillanders 2003; barium [Ba]: Bath et al. 2000; Milton and Chenery 2001; Eldson and Gillanders 2003; lithium [Li]: Milton and Chenery 2001; lead [Pb]: Geffen et al. 1998), otolith microchemistry can provide information on habitat use and the nursery potential of mangroves. By using otolith trace element concentrations, several studies have successfully investigated the spatial arrangement of fish (Edmunds et al. 1989; Dove and Kingsford 1998; Patterson et al. 1999; Kingsford and Gillanders 2000; Rooker et al. 2003), as well as the migration of individuals and resulting connectivity among populations (Swearer et al. 1999; Kafemann et al. 2000; Milton et al. 2000; Thorrold et al. 2001; Forrester and Swearer 2002).

Many otolith microchemical studies have observed substantial differences in trace element concentrations among sites separated by more than 10 km (see Thorrold et al. 1998; Milton et al. 2000;

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Table 2 in Gillanders et al. 2001), and a growing number of studies have successfully detected differences in otolith microchemistry at smaller distances. Dove and Kingsford (1998) and Kingsford and Gillanders (2000), working at sites separated by 0.5–3 km, reported microchemical differences in the otoliths of *Parma microlepis* (white-ear scalyfin) in Australia. Yamashita et al. (2000) observed microchemical differences in otoliths of *Platichthys bicoloratus* (stone flounder) from reef and estuary sites separated by 5 km in Japan, and used this spatial variability to identify individuals that originated from estuarine nursery grounds. Chittaro et al. (2004) working among mangrove and reef sites that were separated by distances of 0.25–7 km in Belize and Bahamas, reported differences in the otolith microchemistry of caged *Haemulon flavolineatum* (French grunt). Using this chemical variability among habitats, they determined that 36% of 39 adults taken from a reef had elemental signatures from juvenile portions of their otoliths that were representative of a nearby mangrove site.

In this study, we expand on previous work by Chittaro et al. (2004), to further examine the utility of using otolith microchemistry of *H. flavolineatum* in population discrimination and connectivity between potential nursery and adult habitats. To assess whether detectable chemical variability existed between adjacent habitats and whether otolith microchemistry is a feasible technique to investigate fish movement between habitats, Chittaro et al. (2004) placed *H. flavolineatum* in cages within mangrove and reef sites in Belize and Bahamas. Since *H. flavolineatum* is nocturnally active, the caging experiment ensured that the microchemistry at the otolith edge reflected the site fish were held and prevented any confounding effect that may result from fish movement. Results reported by Chittaro et al. (2004) indicated that otolith microchemical differences were sufficient to identify adjacent sites and further investigations were encouraged. In this study, we assess the ability to differentiate individuals of *H. flavolineatum,* in the absence of captivity, collected from sites throughout Turneffe Atoll, Belize: 9 mangrove sites and 10 coral reef sites (adjacent sites were separated by 0.8–20 km). We investigate whether it is possible to identify the habitat (i.e., mangrove or coral reef) and at a finer resolution, the sites from which individuals were collected.

Materials and Methods

Our sampling location, Turneffe Atoll, Belize, is a large (50 km long and 16 km wide) complex of cayes that are isolated from the mainland (51 km) and the Belize barrier reef (14 km) by a 275–300 m deep channel. Turneffe Atoll is an ideal location for investigations of nursery habitats and movements of fish, since the numerous cayes that form the atoll are covered with an extensive mangrove forest (covering 74.2 km^2), while the perimeter of Turneffe Atoll is made of a barrier reef.

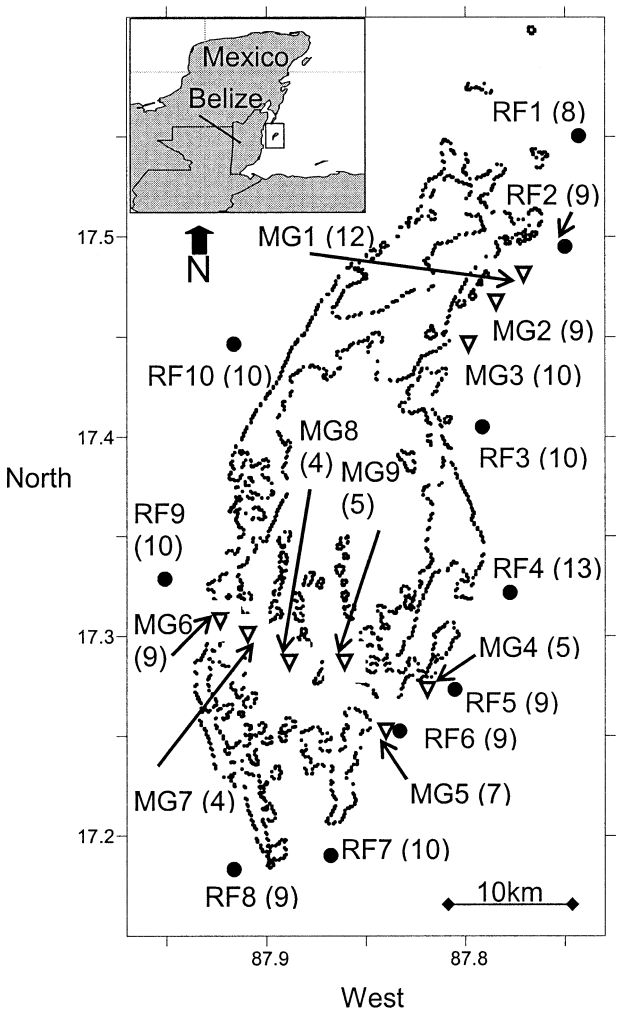

Fig. 1. Geographic position (in decimal degrees) of mangrove and coral reef sites throughout Turneffe Atoll, Belize. Triangles and circles indicate mangrove (MG) and coral reef (RF) sites, respectively. Site sample sizes are in the parentheses by the sample stations.

H. flavolineatum is an abundant Caribbean fish (Class Actinopterygii, Order Perciformes, Family Haemulidae) of moderate commercial value, which is known to occupy mangrove and reef habitat (Billings and Munro 1974; Mumby et al. 2004) and suspected to move between them (Brothers and McFarland 1981; Nagelkerken et al. 2000a,b). Individuals of *H. flavolineatum* (4–13 per site) were collected at 19 sites (9 mangrove and 10 reef sites) at Turneffe Atoll, over 20 d (July 21–August 9, 2003; Fig. 1) using both hand spear and gill net (5

× 2 m monofilament barrier net, 1 cm stretched mesh). Coral reef sites were located on back reef sections of the large continuous reef surrounding Turneffe Atoll and were at a depth of approximately 0.75–1.5 m. Mangrove sites were chosen based on their accessibility as well as their proximity to coral reef sites (although desired, paired mangrove and coral reef sites were not always possible; e.g., reef site 1, 7, 8, and 10 did not have a corresponding mangrove site) and were approximately 1–3 m deep (each site encompassed a total area of c. 200 m^2). Adjacent sites were separated by 0.8–20 km, with most sites separated by > 4 km (55 km separated the most distant sites). Immediately after *H. flavolineatum* collection, we measured their standard length and removed sagittal otoliths, which were then stored dry in individual vials.

At the University of Windsor, we embedded sagittal otoliths in epoxy resin (Gougeon) and sectioned them in a transverse plane, using a low speed diamond saw (Buehler), to a width of 350 μm. In a class 100 clean room we mounted multiple otolith sections (up to 30) to a microscope slide, sonicated in a milli-Q water bath for 2.5 min, triple rinsed in 95% ethanol, triple rinsed in milli-Q water, and dried in a laminar flow HEPA filtered fume hood. Otoliths were chemically analyzed at the Great Lakes Institute of Environmental Research, University of Windsor, using Laser Ablation Inductively Coupled Plasma Mass Spectrometry (LA-ICP-MS). A Thermo Elemental X7 ICP-MS was operated at low resolution using argon as the carrier gas. The laser sampling system is a purpose-built system (Fryer et al. 1995) based on a non-homogenized, high power, frequency quadrupled (266 nm) Nd:YAG (neodymium-doped yttrium aluminium garnet) laser. The laser beam is focused onto the sample using an Olympus BX-51 petrographic microscope and an Optics For Research 266 nm 10× objective lens. A 1.5 mm pinhole beam constrictor was used to increase the spatial resolution of the laser sampling (beam diameter was approximately 15–20 μm). The sampling system is more fully described in Crowe et al. (2003).

The otolith edge (which corresponds to the site from which fish were collected, but see discussion) was targeted using an automated microscope stage resulting in a contour of approximately 80–120 μm in length (speed of the stage varied between 3–5 μm s^{-1}). Data acquisition lasted 100 s with 60 s of background acquisition at the start of each ablation. Trace element doped glass standards (National Institute of Standards and Technology, NIST, 610) were analyzed at the beginning and end of each sample set to correct for instrument drift. Calcium was used as an internal standard to compensate for signal variation caused by differences in the amount of ablated material.

In total, 20 isotopes were analyzed by LA-ICP-MS and chemical concentrations and detection limits (parts per million) were calculated using Lamtrace software (van Achterbergh et al. 2001). Elements that met the following two criteria were included in statistical analyses: concentrations of NIST samples were determined with a satisfactory precision (coefficient of variation less than 10%), and concentrations in otoliths were greater than the detection limit for more than 50% of otoliths analyzed. Prior to any analysis, we removed outliers for each site if their value was greater than three times the interquartile distance (see Fowler et al. 1995; StatSoft 2001). The remaining data were $\log_{10}$ transformed to improve normality for multivariate analyses (see below).

To investigate patterns of elemental concentrations between mangrove and reef habitats we used a multivariate approach, so tests of homogeneity of slopes, homogeneity of variance, and normality were required. If assumptions were met then a nested MANCOVA was used with sites (9 mangrove sites and 10 reef sites) nested within habitats. Since trace element incorporation may be related to growth and the size and age of fish (Begg et al. 1998), the interpretation of habitat or spatial variation may be confounded if fish are of different ages or sizes. To reduce any influence of fish size, fish standard length was used as a covariate. Dependent variables were the elemental concentrations and independent variables were habitat (mangrove or reef) and sites. If significant differences among sites within habitats were detected, a Tukey's HSD post hoc test for unequal sample sizes would be used to determine which sites were significantly different from each other.

We performed two linear discriminant function analyses (LDFA); one at the level of habitats and the other at the level of sites. One LDFA tested whether otolith microchemical differences were substantial enough to differentiate fish collected from mangrove and coral reef habitats, while the other determined if there was sufficient variability in elemental concentrations to identify the sites (within mangrove and coral reef habitats) from which fish were collected. A classification matrix and partial Wilks' lambda statistic were determined for both LDFAs; the former indicates the percent of fish that were correctly identified to the habitat and site they were collected from, while the latter indicates the element(s) that explained the greatest degree of separation between or among habitats and sites (StatSoft Inc. 2001).

TABLE 1. Average coefficient of variation (CV) and the percentage of samples greater than detection limit for those elements that met two criteria; the CV of NIST samples was < 10% and > 50% of otolith samples had concentrations that were greater than the detection limit. For each element, average (standard deviation) detection limit and concentration (both measured as parts per million) are provided. Average CV is the mean of CV of 15 different sets of otolith ablations.

Isotope Measured (atomic mass)	Average CV	% of Samples > Detection Limit	Average Detection Limit by Sample	Average Concentration
Li (7)	5.27 (0.21)	89	0.02 (0.01)	0.12 (0.03)
Mg (25)	1.84 (0.08)	99	0.96 (0.19)	14.48 (6.21)
Cu (65)	4.24 (0.26)	60	0.31 (0.23)	1.63 (1.53)
Zn (66)	3.80 (0.17)	76	0.17 (0.17)	1.67 (1.93)
Rb (85)	3.28 (0.13)	69	0.03 (0.01)	0.12 (0.03)
Sr (86)	1.32 (0.05)	99	1.10 (0.26)	3272 (534)
Sn (120)	3.54 (0.12)	99	0.02 (0.000	0.77 (0.63)
Ba (138)	1.79 (0.08)	61	0.01 (0.00)	4.44 (2.12)
Pb (208)	4.00 (0.18)	86	0.01 (0.03)	0.15 (0.20)

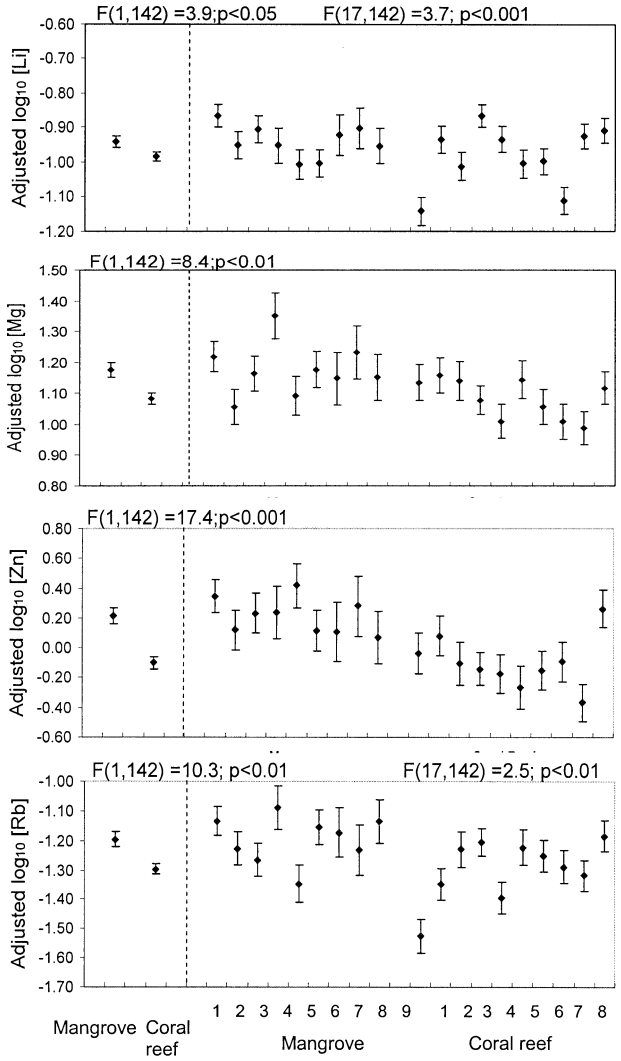

Fig. 2. Adjusted $\log_{10}$ transformed otolith elemental concentrations (ppm) of lithium (Li), magnesium (Mg), zinc (Zn), and rubidium (Rb) per habitat and site. Mean and standard error are plotted. Statistical significance from the nested MANCOVA is indicated (i.e., comparisons between habitats and among sites within habitats).

Results

Based on the two criteria (concentrations of NIST samples with a coefficient of variation less than 10% and concentrations in otoliths were greater than the detection limit for more than 50% of otoliths analyzed), several elements were retained for statistical analysis. Lithium (Li), magnesium (Mg), copper (Cu), zinc (Zn), rubidium (Rb), Strontium (Sr), tin (Sn), barium (Ba), and lead (Pb) were at concentrations sufficiently above detection limit to permit meaningful interpretations (Table 1). Outlier analysis removed 11 fish from the 173 that were collected, resulting in 65 fish analyzed for mangrove sites and 97 analyzed for coral reef sites.

Since assumptions of homogeneity of slopes, homogeneity of variance, and normality were met, a nested MANCOVA was performed (Sn and Sr showed a lack of normality even after transformation and were excluded from this analysis). There were significant differences in elemental concentrations of fish from mangrove and coral reef habitats (Wilks' lambda = 0.10; df = 153, 1090; F = 2.34; $p < 0.001$). We observed statistical significance of the univariate analyses between habitats in terms of Li, Mg, Zn, and Rb, such that concentrations of these elements were greater in mangroves than on reefs (Figs. 2 and 3). We also observed significant variability in the concentrations of most elements (Li, Cu, Rb, Sr, Sn, Ba, and Pb) among certain sites within habitats (Fig. 2 indicates the univariate results of the site comparison for Li, Mg, Zn, and Rb, but only Li and Rb were significant). Tukey's HSD post hoc test revealed only a small number of pair-wise comparisons were significant (i.e., for Li, Cu, Rb, Sr, Sn, Ba, and Pb, 10, 2, 9, 23, 6, 10, and 4 pair-wise comparisons, respectively, were significantly different out of 171 possible comparisons), and a limited spatial pattern in otolith microchemistry was observed. The highest Li concentration was observed to be at a

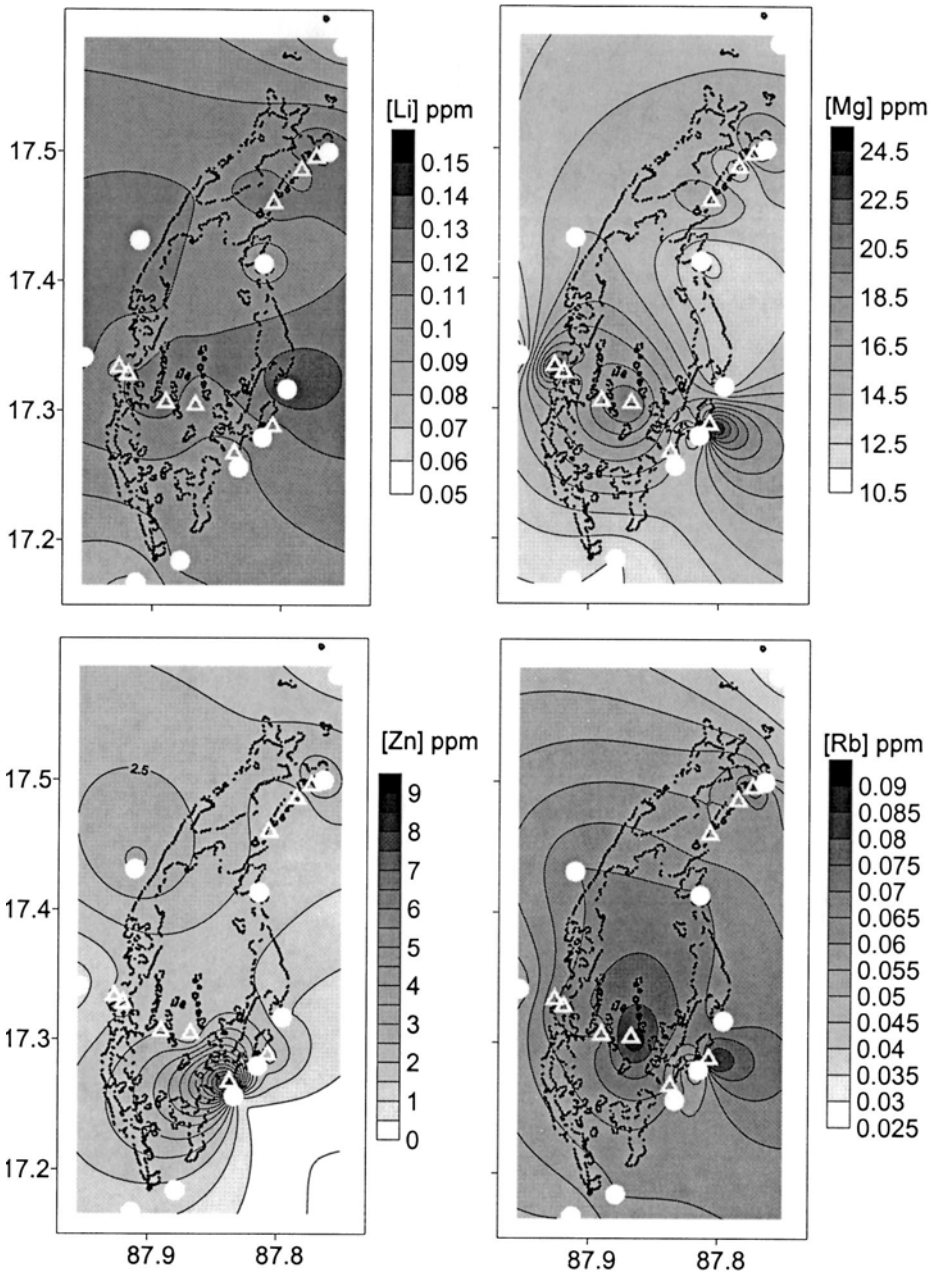

Fig. 3. Spatial gradient of otolith elemental concentrations (ppm) of lithium (Li), magnesium (Mg), zinc (Zn), and rubidium (Rb) throughout Turneffe Atoll, Belize. Contour plots are of untransformed concentrations (ppm). Triangles and circles indicate mangrove (MG) and coral reef (RF) sites, respectively.

central reef site (RF4), while concentrations were at their lowest at the northern and southern extremes (RF1 and RF8; Fig. 3). Otolith concentrations of Mg were relatively high throughout the central portion of the atoll with a slight decrease in concentrations moving from east to west. Concentrations of Zn and Rb were highest at the southeastern side of the atoll (MG4, MG5, RF5, and RF6).

Although Sr and Sn did not meet assumptions of normality we included them in the LDFA because this analysis is relatively robust with respect to skew (McCune and Grace 2002). Significant discrimination (Wilks' lambda = 0.69; df = 9, 152; F = 7.39; $p < 0.001$) was observed for the LDFA comparing elemental concentrations of fish from mangrove and coral reef habitats, such that one significant function (chi-squared statistic = 56.44,

df = 9, $p < 0.001$) was produced explaining 55% of the variation. The partial Wilks' lambda was relatively high for all elements (the lowest values, 0.93–0.95, were for Zn, Rb, and Sr), suggesting that all elements contributed relatively weakly to the discrimination of fish between mangrove and reefs sites. The LDFA indicated that otolith microchemistry varied sufficiently between habitats to permit the correct classification of 74% and 79% of the fish to mangrove and coral reef habitat, respectively.

Results of the LDFA comparing otolith microchemistry among sites indicated significant discrimination (Wilks' lambda = 0.06; df = 162, 1110; F = 2.82; $p < 0.001$), such that four significant functions (χ^2 statistic for no roots removed = 410.38, df = 162, $p < 0.001$) were produced explaining 76% of the variation (first two functions accounted for 58% of the variation). The greatest discriminatory ability for this model (i.e., partial Wilks' lambda) resulted from Li, Sr, and Pb (0.63–0.70). The LDFA indicated a poor ability to correctly classify fish to the site they were collected (average correct classification of 46%; range = 0–89%). Misclassified reef fish were most often classified to other reefs sites (60%) than to mangrove sites (40%), while misclassified mangrove fish were most often classified to reef sites (74%) than to other mangrove sites (26%). Although larger sample sizes are ideal (average number of fish per site was 9.7 and 7.2 for coral reefs and mangroves, respectively), the lowest correct classifications, 0% and 22%, were from sites with low (MG9, 5 fish) to moderate (RF5, 9 fish) sample sizes. No spatial correlation in otolith chemistry was observed since only 8% of misclassified fish (7 of 84 individuals) were classified to adjacent sites (defined here as < 5 km).

Discussion

The elemental composition of otoliths of *H. flavolineatum* showed significant variation between mangrove and coral reef habitats. Although it was not possible to classify a quarter of all fish to the habitat from which they were collected, there was sufficient chemical variability to reliably separate habitats and provide a generalization of the chemical nature of mangrove and coral reef fish. The chemical identification of fish from mangrove and reef habitats was facilitated by Li, Mg, Zn, and Rb, which showed significantly greater concentrations in the mangrove habitat (Figs. 2 and 3).

Our analysis of otolith microchemistry among sites revealed that it was not possible to identify the site from which individuals were collected. The difficulty in assigning fish to specific sites is appreciated when the otolith concentrations of Li, Mg, Zn, and Rb were plotted spatially and the lack of strong elemental differences observed (see Fig. 3). The relatively unique elemental concentrations at four reef (RF2, RF5, RF7, and RF9) and two mangrove (MG2 and MG6) sites facilitated the correct classification of more than 62% of their individuals (i.e., 5–6 individuals out of a sample of 9–10) to the site from which they were collected. These unique concentrations may result from the local hydrology. Three of these reef sites and one mangrove site (RF2, RF5, and RF9 and MG6) were adjacent to boat channels (i.e., channels wide and deep enough to easily permit the passage of recreational boats), which may have acted as funnels through which larger volumes of trace element laden water would pass and consequently supply the otoliths of nearby fish.

Apart from the differences in otolith elemental concentrations at a few sites (see above), we observed substantial overlap elsewhere, which prevented the separation of collected fish. This limited discrimination was not surprising since the 19 sites were relatively close together (adjacent sites separated by 0.8–20 km) and were confined to an area (Turneffe Atoll) that lacks substantial inputs of terrigenous sediments more common in coastal locations. A similar lack of discriminatory ability was reported by Patterson et al. (1999) for collections of *Epinephelus striatus* (Nassau grouper) from three sites in Exuma Sound, Bahamas (sites were separated 50–150 km), as well as Gillanders et al. (2001) for populations of *Diplodus vulgaris* (two-branded bream; separated by 100s of m to 10s of km) in the Mediterranean. Both of these studies suggested that the difficulties discriminating populations were due to the lack of trace elemental sources, such as freshwater inputs and upwelling.

Throughout otolith microchemistry literature, there are many examples of successful discrimination of populations separated by relatively small distances (e.g., < 10 km). The majority of these studies have been conducted in coastal systems where sources of trace elements are likely to be more numerous, thus resulting in greater spatial variability in otolith microchemistry. Along the coastline of Australia, significant otolith microchemical differences for *Pelates sexlineatus* (six-lined trumpeter; Gillanders and Kingsford 2000) and *Pagrus auratus* (Sparidae; Gillanders 2002; Hamer et al. 2003) were observed among sites (separated < 6 km) within estuaries, whereby the elevated concentrations of certain elements were linked to the local geology and pollution (see Dove and Kingsford 1998). Elevated concentrations of Ba in otoliths of *P. microlepis* and *P. auratus* were detected in Jervis Bay, within which existed a petroleum storage facility (Dove and Kingsford 1998; Gillanders

2002, respectively), while the increased levels of mercury at another location (Malabar) was correlated with a nearby sewage treatment plant (Dove and Kingsford 1998). Greater concentrations of manganese (Mn) at three locations (Terrigal, South Head, and Bundeena) were likely related to the proximity to freshwater input containing agricultural waste such as Mn-laden fertilizers (Dove and Kingsford 1998). The ability to discriminate fish at relatively small spatial scales is likely improved when sampling locations proximal to human development or areas of specific geology, both of which can influence concentrations of trace elements in the water and in turn within the otolith (de Pontual and Geffen 2002). Yet without likely sources of pollution nearby, Chittaro et al. (2004), working at very small spatial scales (as small as 0.25 km) at Turneffe Atoll, Belize, and Lee Stocking Island, Bahamas, observed significant differences in experimentally held fish among mangrove and reef sites.

Why was it possible for Chittaro et al. (2004) to discriminate *H. flavolineatum* from specific mangrove and coral reef sites at Turneffe Atoll (68–85% correct classification based on 3 sites), yet not for this study (46% correct classification based on 19 sites), the latter of which encompassed a much larger spatial scale (the majority of sites were separated by > 4 km) with an expected greater variability in elemental concentrations? Apart from the lack of terrigenous inputs at Turneffe Atoll relative to that of more coastal locations (as discussed above), we suspect that an important factor influencing the lack of discriminatory power among sites for this study (relative to that of Chittaro et al. 2004) resulted from fish movements that were unavoidably incorporated into the portion of the otolith targeted for LA-ICP-MS analysis. *H. flavolineatum* is a nocturnal predator that migrates into surrounding habitats (e.g., sandy areas) to forage on benthic invertebrates (Burke 1995), and any distinct elemental signature from their diurnal habitat and site (e.g., a specific mangrove or coral reef site) would be supplemented by new otolith growth that incorporated the chemistry resulting from their nocturnal movement. Because of this movement and the incorporation of elemental signatures from other areas, the variability in otolith elemental concentrations for fish from a given site would increase resulting in difficulties discriminating fish collected from different sites. Further complicating matters, is that the distance traveled during nocturnal foraging may vary among individuals (*H. flavolineatum* have been observed to remain relatively stationary or move up to 199 m away; see Burke 1995), which would again increase the variability in otolith elemental concentrations of fish collected from the same site, and make it more difficult to differentiate fish collected from multiple sites. To avoid this confounding influence of nocturnal movement, Chittaro et al. (2004) held *H. flavolineatum* in enclosures to ensure the chemical signature was from a known site. The discrimination that they reported was a result of the experimental design (i.e., a limitation to the movement of *H. flavolineatum*) and not necessarily what would have been observed if *H. flavolineatum* were allowed to conduct their natural nocturnal migrations.

This study determined that Li, Mg, Zn, and Rb characterized *H. flavolineatum* collected from mangroves and coral reefs, and suggests that these elements would be useful in examining movements between habitats throughout Turneffe Atoll, Belize. Because of the confounding effects of nocturnal fish movements, combined with the limited inputs of terrigenous sediments at Turneffe Atoll, site-specific variability in otolith microchemistry was not sufficient to classify fish to their site of collection. Otolith microchemistry is an effective technique that can be applied to assess movement of individuals between mangrove and coral reef habitats, but not at a finer spatial resolution at this location.

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