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Otolith morphometry and microchemistry as habitat markers for juvenile *Mugil cephalus* Linnaeus 1758 in nursery grounds in the Valencian community, Spain

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Summary

The aim of this study was to identify and characterize juvenile *Mugil cephalus* (flathead grey mullet) habitats in the Valencian community by means of otolith morphometry and microchemistry. Specimens (total length: 250–350 mm) were obtained from October 2011 to March 2012 with gill nets in two protected wetlands: the Parque Nartural de l'Albufera de Valencia (AV) ($n = 45$), a Mediterranean lake; and the Parque Natural Salinas de Santa Pola (SP) ($n = 37$), a coastal salt marsh. Otolith shape indices (circularity, rectangularity, aspect ratio, surface occupied by *sulcus*, ellipticity and form factor) and microchemistry (Sr/Ca and Ba/Ca ratios) were measured and compared as area markers. The chemical composition of the water in both areas was also obtained. Morphometric results showed, by an ANOVA with Bonferroni contrasts, that saccular otoliths from AV individuals had more edge complexity, hence a higher circularity index ($p < .001$), but that there was less otolith percentage occupied by the *sulcus* ($p < .001$). When analyzing the morphometric variables simultaneously, both sites differed significantly (Hotelling's $T^2 < 0.001$). A paired t-test among sites of the microchemical variables showed that otoliths of AV presented higher values of Ba/Ca ratios and lower Sr/Ca ratios ($p < .001$). This coincides with water values obtained and could be associated with the low salinity observed in the lake. The opposite pattern was observed in SP, both for otolith and water samples, this being associated with the high-salinity waters of the area. Results obtained in the present research suggest, by the use of otolith morphometry and microchemistry, that the nursery grounds of juvenile *M. cephalus* in the Valencian community could be differentiated. Even though habitats could be separated using otolith morphometry, only a few of the studied shape indices were important in area differentiation. Nevertheless, the use of both methodologies simultaneously could be robust habitat markers for this species.

1 | INTRODUCTION

The Mugilidae family is widely distributed throughout the world. In the Mediterranean Sea there are eight mullet species: *Chelon labrosus* (Risso, 1827), *Liza aurata* (Risso, 1810), *Liza ramada* (Risso, 1827), *Liza saliens* (Risso, 1810), *Mugil cephalus* Linnaeus, 1758, *Oedalechilus labeo* (Cuvier, 1829), *Liza carinata* (Valenciennes, 1836), and *Liza haematocheila* Temminck and Schelegel, 1845) (Thomson, 1997). The flathead grey mullet, *Mugil cephalus*, is the most common and has an important economic value in the region (Whitfield, Panfili, & Durand, 2012). After spawning off–shore, *M. cephalus* larvae migrate to estuarine or freshwater areas where juveniles remain until they reach sexual maturity (Chang & Iizuka, 2012; Whitfield et al., 2012). Several authors have studied the biology, life history traits, and the movements

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and migration of this cosmopolitan species with its distribution range mainly between lat. 42°N and 42°S (Hsu & Tzeng, 2009; Wang, Hsu, Chang, You, & Tzeng, 2010; Whitfield et al., 2012).

Studies of nurseries and habitat use in diadromous fish are very important, not only to analyze fish biology but also to generate proper management regulations for these preferred fish areas (Beck et al., 2001; Payne Wynne, Wilson, & Limburg, 2015). Numerous studies have analyzed the influence of habitat features on the otolith and fish body shape in various commercially important or threatened species such as American eel, Japanese sea bass and blueblack herring (Benchetrit et al., 2015; Payne Wynne et al., 2015; Secor, Ohta, Nakayama, & Tanaka, 1998). However, the identification of nurseries used by *M. cephalus* in its early stages has not yet been deeply studied.

The analysis of otolith morphology, morphometry and chemical composition has been used to identify stocks of important commercial species, and has facilitated the study of fish movements and migration (Avigliano, Martinez, & Volpedo, 2014; Avigliano, Villatarco, & Volpedo, 2015; Tracey, Lyle, & Duhamel, 2012). Otoliths are complex carbonate structures located in the inner ear of the fish and associated with balance, and given their features they serve as natural tags (Campana, 1999). The morphometrical study of these structures allows one to describe and compare their shapes quantitatively (Lestrel, 1997). Moreover, the use of Sr/Ca and Ba/Ca otolith ratios has been simultaneously studied by some authors as indicators of habitats, and for stock and migration studies (Avigliano, Callicó Fortunato, Buitrago, & Volpedo, 2015; Schuchert, Arkhipkin, & Koenig, 2010; Tabouret et al., 2010).

This research proposes to study otolith morphometry and microchemistry as markers of juvenile nurseries of *M. cephalus* in the Valencian community. This is a preliminary study on the capability of these techniques to identify different habitats used by early-stages of the species.

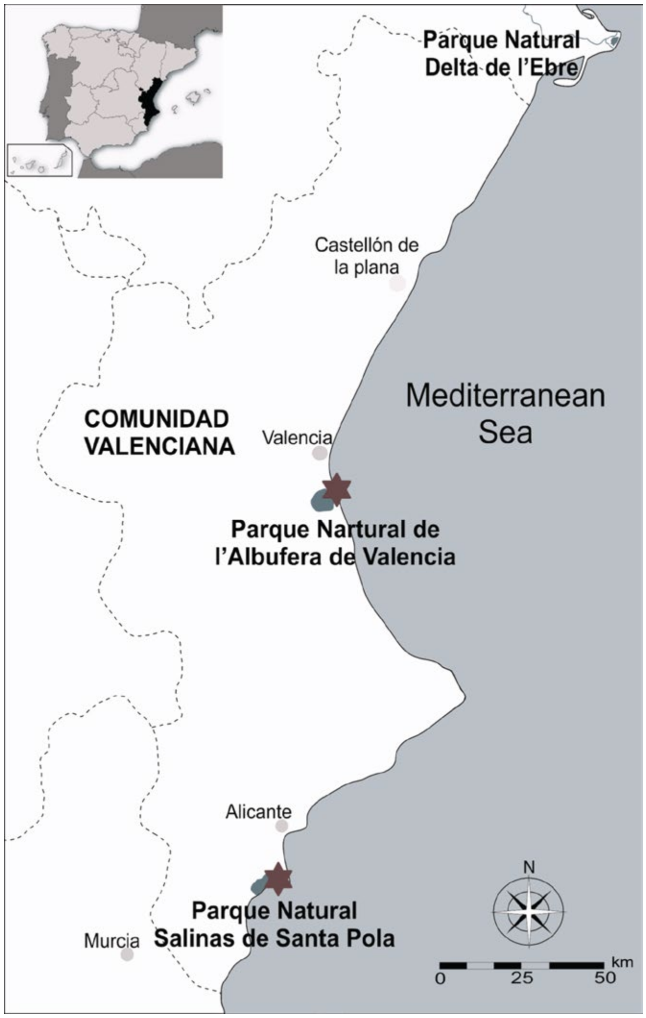

FIGURE 1 Location of two protected wetlands selected for the Valencian Community (stars) study

2 | MATERIALS AND METHODS

2.1 | Sample collection

Mugil cephalus (n = 82) juveniles were obtained in two protected wetlands having different ecological features: the Parque Nartural de l'Albufera de Valencia (AV) (n = 45) and the Parque Natural Salinas de Santa Pola (SP) (n = 37) (Figure 1). The first protected area sampled is the second largest wetland on the Mediterranean coast (Benedito Durà, Martín, Volpedo, & Santamalia, 2011) with a lake connected to the sea by three artificial channels that regulate water flow throughout the year (Soria, Miracle, & Vicente, 2002). The second studied area, SP, is a coastal salt marsh formed by channels, ponds and dams highly modified by human activities and connected to the Mediterranean Sea (Belda Antolí, Martínez-Pérez, Martín Cantarino, López Pomares, & Seva Román, 2008). The local fishing communities collected specimens bimonthly with gill nets between October 2011 and March 2012. Fish were stored at 4°C and brought to the laboratory; their total length (TL) was registered (in mm), and *sagitta* otoliths were extracted. Juvenile fish with total lengths between 250 and 350 mm were chosen. Specimen sizes among locations did not differ significantly (Mann–Whitney U-test, $p > .05$).

2.2 | Otolith morphometry

After extraction and cleaning, the innerface of all *sagittae* otoliths was photographed with a digital camera attached to a stereomicroscope (Leica® MZ16). Images were then analyzed and measured using image processing systems (Image-Pro Plus 4.5®). The morphometric variables registered were: otolith length (OL), otolith height (OH), otolith perimeter (OP) and *sulcus* perimeter (SP) in mm (Figure 2); and otolith area (OA) and *sulcus* area (SA) in mm^2. Using these measurements the shape indices were then calculated: circularity (OP^2/OA), rectangularity ($OA/[OL \times OH]$), aspect ratio (OH/OL; %), percentage of the otolith area occupied by the *sulcus* (SA/OA; %), ellipticity ($[OL - OH]/[OL + OH]$), and form factor ($[4\pi \times OA/OP^2]$) (Avigliano et al., 2014; De Carvalho, Vaz-dos-Santos, Spach, & Volpedo, 2015; Tuset, Lombarte, & Assis, 2008).

2.3 | Otolith microchemistry

To analyze the otolith chemical composition, 48 randomly selected specimens (n = 24 for each studied area) were chosen. Right otoliths

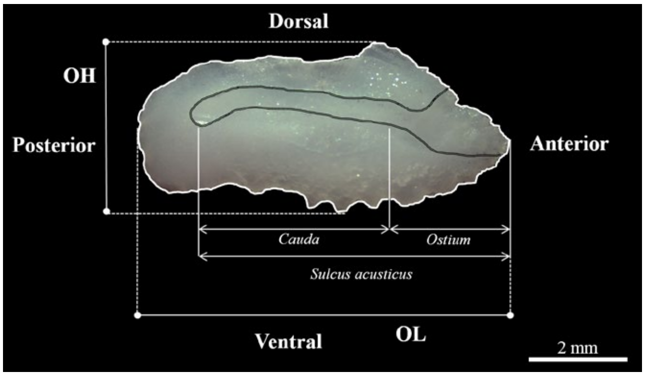

FIGURE 2 *Sagittae* otolith of *Mugil cephalus*. Features and measured variables: OL, otolith length; OH, otolith height; continuous white line: otolith perimeter (OP); continuous black line: *sulcus* perimeter (SP)

from each fish were weighed with an analytical balance. They were then digested with 10% nitric acid for 24 hrs to obtain solutions for analyses. Sr and Ba concentrations were determined using inductively coupled plasma–atomic emission spectrometry (ICP-OES, Perkin Elmer Optima 2000 DV optical emission spectrometer; method EPA 200.7). Ca concentrations were obtained by volumetric titration with ethylenediaminetetraacetic acid (EDTA) (APHA, 1995). Measurements were made in triplicate. Element/Ca relations were then calculated for comparisons among selected sites.

Water samples were taken along with fish in both sampled areas and preserved by the addition of 2 ml of nitric acid per liter of water (APHA, 1995; 3010 B method) and maintained at 4°C until further analysis. Sr, Ba and Ca water concentrations were determined as previously described.

2.4 | Statistical analysis

All morphometrical variables fitted a normal distribution and homogeneity of variance (Shapiro–Wilk, $p > .05$; Levene, $p > .05$), then an ANCOVA analysis was used to determine the effect of fish size on the morphometric indices. There was no effect of fish size in these variables ($p > .01$). They were first tested for correlation; those that were found to correlate strongly with others were eliminated from the analysis. The morphological indices were then analyzed by ANOVA, and Bonferroni contracts were used to evaluate the differences among sampling areas. Finally, a multivariate analysis of variance (MANOVA) with Hotelling's T^2 test was applied to evaluate differences among sites considering all morphometric variables simultaneously.

Microchemical variables fitted a normal distribution and homogeneity of variance (Shapiro–Wilk, $p > .05$; Levene, $p > .05$). A fish size effect was detected for Sr/Ca and Ba/Ca variables (ANCOVA analysis: $p < .01$). Thus, they were corrected using the common within-group slope (b) (Burke, Brophy, & King, 2008; Campana, Chouinard, Hanson, Fréchet, & Brattey, 2000; Galley, Wright, & Gibb, 2006). Constants used were: Sr/Ca, $b = 0.00086$; and Ba/Ca, $b = 0.00024$, successfully removing the significant correlation with fish length. Element/Ca relations were compared with a paired t-test and MANOVA with Hotelling's T^2 test, using both microchemical variables simultaneously, to analyze differences among sampled areas.

3 | RESULTS

3.1 | Otolith morphometry

Form factor was strongly correlated with circularity (r Pearson = −.67), with aspect ratio (r Pearson = .66) and with ellipticity (r Pearson = −.66), thus, this variable was eliminated from the analysis. Individuals of the selected studied areas differed in the circularity of their otoliths, Albufera had higher circularity values than Salinas de Santa Pola (Table 1). The percentage occupied by the *sulcus* also varied significantly, and SP individuals showed higher values (Table 1). When analyzing the morphometric variables simultaneously both sites differed significantly (Hotelling's $T^2 < 0.0001$).

3.2 | Otolith microchemistry

When analyzing the microchemical variables, significant differences were observed among the ratios studied for both sites (Table 1).

TABLE 1 Mean and standard error of morphological indices (ANOVA statistical analysis: F [statistic] and p [significance]) and microchemical variables (paired t-test: T [statistic] and p [significance]) analyzed in *Mugil cephalus* otoliths among selected wetlands. Bold values represent significant differences ($p < 0.01$)

	AV ($n = 45$)	SP ($n = 37$)		
Morphometry indices	**Mean ± *SE***	**Mean ± *SE***	**Statistic**	***p***
Circularity (OP²/OA)	23.740 ± 0.230[a]	22.402 ± 0.250[b]	$F_{df = 1,82} = 15.61$	**.0002**
Rectangularity (OA/[OL × OH])	0.724 ± 0.004	0.714 ± 0.004	$F_{df = 1,82} = 3.62$	.0605
Aspect ratio (OH/OL)	0.445 ± 0.005	0.433 ± 0.010	$F_{df = 1,82} = 3.02$	.0859
Percentage occupied by the sulcus (SA/OA)	0.227 ± 0.002[a]	0.241 ± 0.002[b]	$F_{df = 1,82} = 18.20$	**.0001**
Ellipticity ([OL − OH]/[OL + OH])	0.384 ± 0.004	0.396 ± 0.010	$F_{df = 1,82} = 2.87$	.0943
Microchemistry ratios (mmol/mol)				
Sr/Ca	0.35 ± 0.02[a]	0.54 ± 0.03[b]	$T_{df = 46,48} = 3.47$	**<.001**
Ba/Ca	0.010 ± 0.001[a]	0.002 ± 0.001[b]	$T_{df = 46,48} = −5.52$	**<.0001**

Different letters = significant differences.

Albufera individuals had smaller Sr/Ca values and higher Ba/Ca ratios than those of Salinas de Santa Pola. When analyzing both ratios simultaneously, the locations differed significantly (Hotelling's $T^2 < 0.0001$).

Water chemistry also reflected differences among areas, Salinas de Santa Pola having a higher value of Sr/Ca ratio but a lower Ba/Ca ratio than Albufera (Sr/Ca = 12.65 and 9.63; Ba/Ca = 0.05 and 0.33 mmol/mol, respectively).

4 | DISCUSSION

Our results revealed that *sagittae* otoliths from Albufera individuals presented a more irregular shape, which increased the circularity index. In addition, these otoliths presented less area occupied by *sulcus* than those from Santa Pola. These morphological variations allow assertions that environmental conditions of the studied wetlands clearly affect the otolith shape, suggesting population differentiation of *M. cephalus* for the first ontogenetic stages. In this research, even though habitats could be separated using otolith morphometry, only a few of the studied shape indices were important in area differentiation. Although shape indices are not the best option to analyze otolith shape differences (Lombarte & Tuset, 2015), they have been used successfully in several studies (Avigliano, Villatarco et al., 2015; Briand, Mounaix, Fatin, Feunteun, & Pass, 2003; Tuset, Lozano, Pertusa, & García-Díaz, 2003).

The concentration of elements in the otolith is strongly related to the chemical composition of the water in which a fish swims (Campana, 1999; Reis-Santos et al., 2012; Sturrock, Trueman, Darnaude, & Hunter, 2012); the deposited chemicals represent a permanent record of the environmental conditions experienced by the fish at a particular time (Campana & Thorrold, 2001). Our findings showed that the higher values of Ba/Ca ratios of otoliths in the Parque Nartural de l'Albufera de Valencia specimens coincides with higher values of this ratio measured in the water; this could be associated with the low salinity values in this lake of around 1.5 PSU (Confederacion Hidrológica Jucar—CHJ, 2016). The Parque Natural Salinas de Santa Pola individuals showed an inverse pattern related to this ratio, both in otoliths and in water values. This marsh area also showed higher Sr/Ca ratios, associated with its high-salinity waters (37.3 PSU [pers. com.]). Other authors have observed that Sr/Ca and Ba/Ca ratios for *M. cephalus* were strongly associated with environments having different salinities (estuarine, offshore and freshwater) (Chang, Lin, Iizuka, & Tzeng, 2004; Górski, De Gruijter, & Tana, 2015; Wang, 2015) and have described mullet migration patterns associating microchemical ratios to water salinity. Using chemical tags to differentiate alternative juvenile habitats requires that elemental composition of the areas present marked differences that could be measured in otoliths of residing fish (Brown, 2006). In our study both areas presented environmental differences that were reflected in their elemental water composition and thus in the ratio values of the fish otoliths.

Present research results suggest that by means of otolith morphometry and microchemistry the nursery grounds of juvenile *M. cephalus* in the Valencian community could be differentiated. Thus, the use of both methodologies simultaneously could be robust as markers of habitat for this species. Although this is a preliminary work that should be widened to include more wetlands used by the species throughout the Mediterranean coast, this is a potential tool for the study of population dynamics and connectivity in this particular Mediterranean area. This would facilitate the proper management not only of the studied species but also of the protected coastal wetlands.

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