



Otolith shape and elemental signatures provide insights into the connectivity of euryhaline *Chelon labrosus* inhabiting two close estuaries with different burdens of xenoestrogens in the Southern Bay of Biscay

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ABSTRACT

Intersex gonads have been observed in thicklip grey mullet *Chelon labrosus*, inhabiting estuaries with high burdens of xenoestrogens in the Southeast Bay of Biscay, but knowledge of population connectivity among estuaries is lacking for this euryhaline fish species. This study investigates the population structure of *C. labrosus* using otolith shape and elemental signatures of 60 adults (overall length ~ 38 cm) from two estuaries 21 nautical miles apart, one with a high incidence of intersex condition (Gernika), and the other one pristine (Plentzia). Otolith shape analyses were performed using elliptical Fourier descriptors, while elemental signatures of whole sagittae were obtained by inductively coupled plasma mass spectrophotometry. Univariate and multivariate statistics were applied to determine if otolith signatures show patterns of homogeneity between estuaries. The data indicated significant differences in the otolith shape and elemental composition between mullets of Gernika and Plentzia. Elemental differences were mainly driven by Sr, Li (both higher in Plentzia) and Ba (higher in Gernika). The 98% re-classification success rate obtained from stepwise linear discriminant function analysis suggests that Gernika and Plentzia individuals form separated population units. The limited connectivity between these two close estuaries would indicate a different life history of exposure to chemicals, which might explain the high prevalence of intersex condition in Gernika and its absence in Plentzia.

1. Introduction

Otoliths are paired metabolically inert bio-mineralized crystalline-organic complex structures found in the inner ears of teleost fishes which are used for balance and hearing (Thomas and Swearer, 2019). They are mainly composed of calcium carbonate (~98%) and an organic matrix (~2%), and grow continuously, resulting in conspicuous growth bands generated by seasonal variations in the aragonitic matrix as they grow (Hüssy et al., 2021). During growth, otoliths incorporate different minor

and trace elements from the local aquatic environment through which a fish passes during its lifetime, thus providing a record of their life history and habitat residency (Hüssy et al., 2021; Thomas and Swearer, 2019). Otolith shape and elemental composition may show intra-specific geographic variations depending on the genetics, environmental conditions (water temperature, salinity and others), feeding regime, fish condition, growth, maturation and reproduction (Hüssy et al., 2021; Izzo et al., 2018; Sturrock et al., 2015). However, the extent to which these processes influence otolith chemistry is still poorly understood

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(Hoff et al., 2022; Moreira et al., 2018; Reis-Santos et al., 2018). Differences in shape and elemental composition in otoliths provide a method to unravel the population structure and habitat connectivity of fish that may reflect the level of connectivity between different individuals of one single species (Moreira et al., 2022; Schroeder et al., 2022; Soeth et al., 2019).

The thicklip grey mullet *Chelon labrosus* (Risso, 1827) is a member of the family Mugilidae, which consists of approximately 25 genera and 80 species widely distributed around the world in tropical and temperate regions (Xia et al., 2016). It is a long-lived and slow-growing euryhaline demersal fish species broadly distributed along the eastern Atlantic Ocean from southern Scandinavia and Iceland to Senegal and Cape Verde, and across the Mediterranean and Black Seas (Freyhof and Kottelat, 2008; Froese and Pauly, 2022; Turan, 2015). It occupies inshore areas, estuaries, rivers, coastal lagoons and seas, thereby supporting artisanal and recreational fisheries (Freyhof and Kottelat, 2008; Froese and Pauly, 2022; Turan, 2015). *C. labrosus* is known to use estuaries as nursery areas or foraging habitats, migrating daily in and out of the estuaries with the tidal regime (Froese and Pauly, 2022; Whitfield, 2020). It is euryhaline, spawning yearly at sea in coastal surface waters during the winter season (December–February), and pelagic eggs hatch in 3–4 days, with the pelagic larval stage lasting approximately four weeks (González-Castro and Minos, 2016). In coastal environments, juveniles form dense schools, migrating to inshore coastal waters and shallow estuaries, that in the Mediterranean Sea, occurs during the first and second month of life for *C. labrosus* (Crosetti and Cataudella, 1995; Hickling, 1970; Koutrakis, 2015; Mićković et al., 2010). The species can survive in highly polluted environments, making it a good choice as an estuarine pollution sentinel organism (Ortiz-Zarragoitia et al., 2014).

Strong xenoestrogenic effects have been reported in *C. labrosus* individuals from contaminated estuaries along the Basque coast (Bizarro et al., 2014; Diaz De Cerio et al., 2012; Ortiz-Zarragoitia et al., 2014; Puy-Azurmendi et al., 2013; Valencia et al., 2017). This has been mainly linked to exposure to alkylphenols, pesticides and other xenoestrogens from wastewater treatment plant (WWTP) effluents that are discharged into estuaries, resulting in the development of intersex individuals (Bizarro et al., 2014; Ortiz-Zarragoitia et al., 2014; Puy-Azurmendi et al., 2013). Intersex gonads show the simultaneous occurrence of male and female reproductive stages in a gonochoristic species, mostly characterized by the presence of oocytes in testicular tissue (ovotestes) (Bahamonde et al., 2013). An abnormally high number of intersex testes (up to 83%) has been identified in *C. labrosus* males sampled in the Urdaibai estuary near Gernika, and these individuals have also shown accumulation of xenoestrogenic compounds such as bisphenol A (up to 177.5 ng mL⁻¹), estradiol (up to 27 ng mL⁻¹) and nonylphenol (up to 1142 ng mL⁻¹) (Bizarro et al., 2014; Ortiz-Zarragoitia et al., 2014; Puy-Azurmendi et al., 2013; Ros et al., 2015). Additionally, varying prevalences of intersex condition have been reported in *C. labrosus* males captured from Bilbao (Nerbioi-Ibaizabal estuary), near the WWTP of Galindo (9%) and the Arriluze marina (10%), the fishing port of Ondarroa (50%), the port of Deba (20%) and the industrial harbour of Pasaia (56%) at the mouth of Oiartzun River (Bizarro et al., 2014; Diaz De Cerio et al., 2012; Ortiz-Zarragoitia et al., 2014; Puy-Azurmendi et al., 2013; Ros et al., 2015; Valencia et al., 2017). However, mullets sampled from the leisure port of Plentzia have shown normally developing testes with no incidences of intersex condition (Bizarro et al., 2014; Ortiz-Zarragoitia et al., 2014; Ros et al., 2015). The development of intersex condition thus appears to be estuarine-specific depending on the pollution load present in each estuary (Bizarro et al., 2014; Diaz De Cerio et al., 2012; Ortiz-Zarragoitia et al., 2014; Puy-Azurmendi et al., 2013).

The limited knowledge about the population structure and connectivity of *C. labrosus* in the Southern Bay of Biscay (SBB), prevents the understanding of the history of chemical exposure and the development of gonadal alterations in mullets. It could be perfectly feasible that an adult mullet from Plentzia might travel to Gernika after any of its yearly

reproductive migrations. Several methods, including natural tags (body morphometrics and meristics, otoliths, scales count, presence of parasites, population genetic structure) and applied or artificial markers (mark-recapture), can be used to identify and discriminate fish population (Cadurin et al., 2013; Marengo et al., 2017). In genetic approaches, highly polymorphic microsatellite DNA markers provide great resolution for identifying population structure (Durand et al., 2013; Ward, 2000), however, genetic variation between population groups may be inadequate, especially where low and inconsistent levels of genetic differentiation exist (Marengo et al., 2017; Moreira et al., 2019a; Ward, 2000). For such marine fish populations, with long larval stages, without physical barriers and high genetic flow, other approaches such as those studying otoliths might be useful in distinguishing geographically separated populations by discriminating the heterogeneous environmental conditions the fish has lived in (Correia et al., 2012; Marengo et al., 2017; Soeth et al., 2019).

Thus, the use of otolith shape and elemental signatures could provide insights into whether *C. labrosus* constitute a single interconnected population in the Basque coast or whether a meta-population structure exists. Interpreting the migratory patterns of *C. labrosus* within and among estuaries may unravel the time during which fish are first exposed to xenoestrogens and intersex condition is developed. Therefore, it is important to define the mobility pattern of *C. labrosus* between and within estuaries in the Basque coast to better understand the reproductive constraints that exposure to xenoestrogens at the local level could have on the populations.

2. Materials and methods

2.1. Study area

Two estuaries located in the Basque coast in the SBB in northern Spain (c. 21 nautic miles apart) were selected as the sampling areas: the Plentzia estuary (43°24'25.794" N, 2°56'46.921" W) located in the resort town of Plentzia, and the Urdaibai estuary (43°19'26.389" N, 2°40'25.46" W) located within the Urdabai Biosphere Reserve (declared by UNESCO in 1984) in the town of Gernika (Fig. 1). In between these two estuaries, there lies a small estuary (c. 9 miles from Plentzia and c. 12 miles from Gernika) in the town of Bakio (43° 25'51" N, 2° 48'40" W) that is drained inland by the river Estepona (Marigómez et al., 2013).

Plentzia estuary is a relatively shallow meso-tidal estuary (tidal variation ~2.5 m) that forms the tidal part of the 7.9 km long Butron River (Leorri et al., 2013), and has a small leisure port near the tidal inlet and the adjacent beaches of Plentzia and Gorliz. Pollution inputs are minimal and the local WWTP that collects urban wastewater from ~10,000 inhabitants discharges its effluent outside the estuary through a submarine pipe extending ~1 km offshore at a depth of ~18 m (Mijangos et al., 2018). Urdaibai estuary is also a shallow (mean depth of 3 m) meso-macrotidal estuary that is 12.5 km long and receives freshwater input from the Oka River (Iriarte et al., 2015). Major impacts can be attributed to the direct discharge of effluent from the Gernika WWTP at a narrow and very shallow stretch of the river within the estuary. This old WWTP receives domestic and industrial wastes from the town of Gernika and its surrounding areas (~26,000 inhabitants).

2.2. Sample collection

Adult thicklip grey mullets (*Chelon labrosus*) were caught using a fishing rod in Plentzia (PL; n = 30) and Gernika (GE; n = 30) within a period of three weeks in June 2020. The fish were anaesthetized in a saturated benzocaine/seawater bath and preserved in ice before being taken to be processed in the laboratory. The individuals were measured for total length (TL, 1 mm) and weighed (W, 1 g). Sagittal otoliths from each fish were removed using plastic forceps to avoid metallic contamination, cleaned of any adherent tissues with ultrapure water (Milli-Q water, 0.52 µS cm⁻¹) and air-dried in clean and labelled

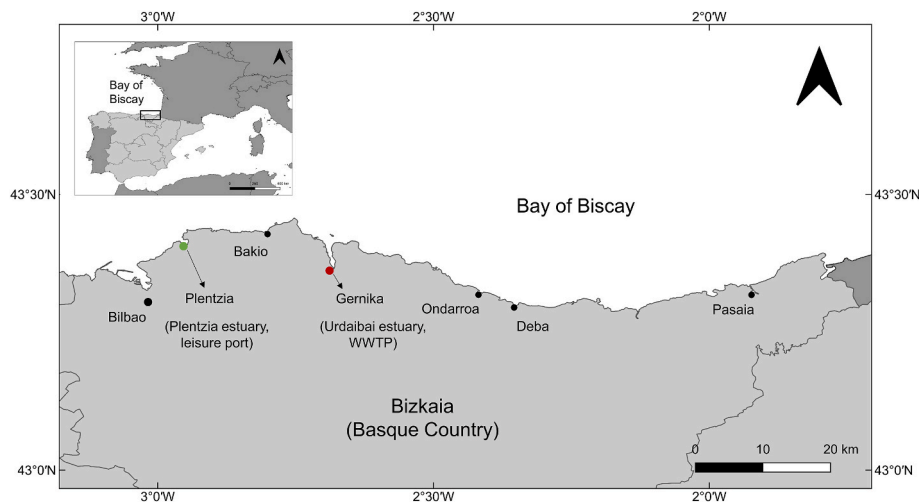


Fig. 1. Overview of sampling locations for *Chelon labrosus* along the Basque coast. Brackets indicate the main activity in the location and nearby wastewater treatment plants (WWTP) indicated. The green dot marks the location with no prevalence of intersex conditions (Plentzia) while the red dot marks the location where intersex condition was prevalent (Gernika). This figure was produced with the GADM database (www.gadm.org), version 4.1, 16 July 2022 and QGIS version 3.18.0-Zürich.

Eppendorf tubes. The right otolith was used for shape analysis, while the left otolith was used for microchemistry analysis. All fishing activities and handling procedures were carried out in accordance with the Ethics Committee for Animal Experimentation of the UPV/EHU and with the permission for sampling activities of the regional authorities.

2.3. Otolith shape analyses (Elliptic Fourier descriptors)

Right sagittal otoliths were placed with the sulcus acusticus up (convex side down) and the rostrum to the left side. Orthogonal two-dimensional digital images were captured using a stereomicroscope at 10× magnification (Meiji Techno EMZ-13TR, Meiji Techno Co. Ltd, Saitama, Japan) coupled to a USB digital camera (Olympus SC30) with the analysis getIT software (Olympus Soft Imaging Solutions GmbH, Münster, Germany). Full colour (*.jpg) and high-resolution (2048 × 1532) microphotographs of each otolith were captured using reflected light against a dark background (Fig. 2A).

To determine whether the shape of the otoliths varied between locations, elliptic Fourier descriptors (EFD) was used to describe the shape outline of each otolith. EFD fits a closed curve to an ordered set of data points and decomposes the contour into a sum of harmonically related ellipses (Kuhl and Giardina, 1982) giving consistent results suitable for use in statistical comparisons between samples (Mérigot et al., 2007; Moura et al., 2020; Muniz et al., 2020).

The digitized otolith images were analysed using the R package *shapeR* (Libungan and Pálsson, 2015). The outlines were detected by transforming the images into grey-scale and converting them to black and white images using a threshold pixel value. The outlines were later superimposed on the original image to verify consistency and a weighted moving average over three successive coordinate points used to eliminate pixel noise from the captured outline (Fig. 2B) (Libungan and Pálsson, 2015). Mean EFD were obtained by rotating all otoliths positioned horizontally along the longest axis, setting the area equal in all otoliths and drawing a polar axis (radial) horizontally from the otolith centroid to the right which corresponds to the 0° angle of the otolith outline (Fig. 2C). Fourier coefficients standardized to otolith size (i.e. otolith length) were used to obtain the normalized EFD (Libungan and Pálsson, 2015). A level of 95% of accumulated variance was used to select the minimum number of harmonics. The first 12 harmonics reached >95% of the cumulative power and gave 45 normalized Fourier coefficients (48–3 = 45), with the first three coefficients (a1, b1 and c1) omitted due to standardisation in relation to size, rotation and starting

point. One coefficient showed significant interactions between populations and otolith length ($p < 0.05$) (i.e. otolith length having an effect on site-specific differences) and was thus excluded from further analysis. While applying the Bonferroni adjustment to account for increased alpha error due to multiple testing of the different coefficients (Sokal and Rohlf, 1995), all coefficients were included. The inclusion of the one coefficient did not affect the results of the overall analysis.

2.4. Otolith elemental analyses (Element:Ca)

Right otoliths were cleaned in an ultrasonic bath for 5 min in ultrapure water (Thermo Scientific, Lab Tower EDI 15, Thermo Electron LED GmbH, Langensfeld, Germany), followed by immersion in 200 µL of 3% analytical grade hydrogen peroxide solution (H₂O₂, Fluka Analytical) for 15 min to remove any adherent biological tissues. The otoliths were then rinsed in ultrapure 1% nitric acid (HNO₃ solution (Fluka Trace Select, >69%) for 10 s and triple-washed in ultrapure water for 5 min to remove the acid (Rooker et al., 2001). Finally, otoliths were stored in pre-decontaminated Falcon tubes and allowed to dry overnight in a laminar flow hood (Moura et al., 2020; Patterson et al., 1999; Rooker et al., 2001). The decontaminated otoliths were then weighed (otolith mass: OM, 0.00001 g) on an analytical balance (Secura225D-1S, Sartorius Lab Instruments GmbH, Goettingen, Germany), dissolved for 15 min in 300 µL of ultrapure nitric acid (HNO₃, Fluka Trace Select, >69%) and then diluted with ultrapure water to a final volume of 15 mL [2% HNO₃ (v/v) and 0.02% TDS (m/v)] before stirring with a vortex (Moura et al., 2020).

Twelve elements (¹³⁷Ba, ⁴³Ca, ¹¹¹Cd, ⁵⁹Co, ⁶⁵Cu, ⁷Li, ²⁶Mg, ⁵⁵Mn, ⁶⁰Ni, ²⁰⁸Pb, ⁸⁸Sr, ⁶⁶Zn), were analysed by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) using an iCAP™ Q (Thermo Fisher Scientific, Bremen, Germany) instrument equipped with a concentric glass nebulizer, a Peltier-cooled baffled cyclonic spray chamber, a standard quartz torch and a two-cone interface design (nickel sample and skimmer cones). Argon of high-purity (99.9997%, Gasin II, Leça da Palmeira, Portugal) was used as the nebulizer and plasma gas. The Qtegra™ software (Thermo Fisher Scientific, Bremen, Germany) was used for instrument control and data acquisition. The instrument was operated under the following conditions: RF power, 1550 W; argon flow rate, 14 L min⁻¹; auxiliary argon flow rate, 0.8 L min⁻¹; nebulizer flow rate, 0.98 L min⁻¹. Indium (¹¹⁵In), Scandium (⁴⁵Sc), Yttrium (⁸⁹Y) and Terbium (¹⁵⁹Tb) were monitored as internal standards. Otolith samples were analysed in random order to avoid possible sequence effects.

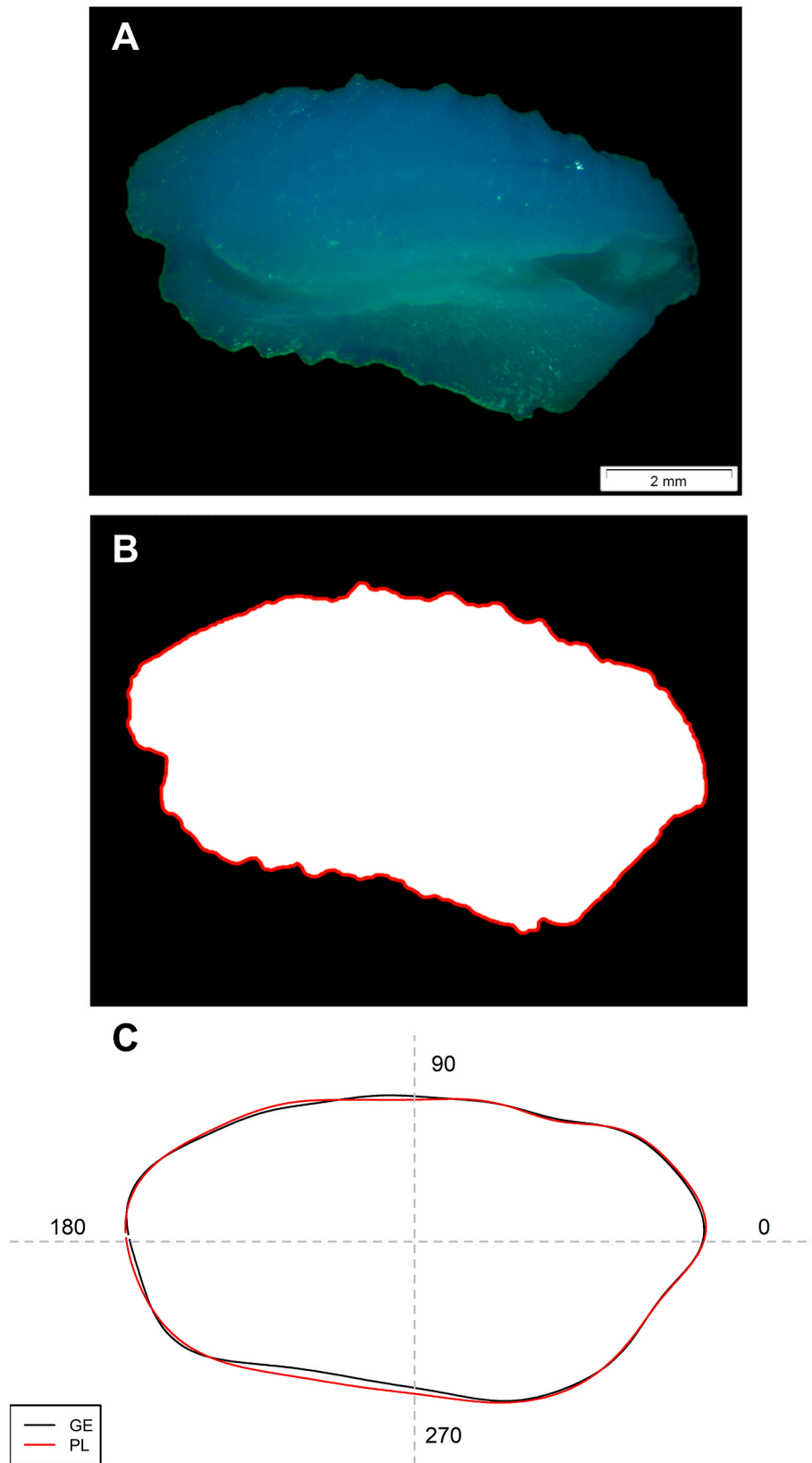


Fig. 2. Medial view of the right sagittal otolith microphotograph of *Chelon labrosus*: (A) the original image with $\times 10$ magnification, (B) the corresponding binary image and (C) the averaged outlined contour for each location based on Elliptical Fourier Descriptor (EFD) reconstructions. (GE = Gernika; PL = Plentzia; Scale bar = 2.0 mm).

Procedural blanks and Fish Otolith Certified Reference Material (CRM; NIES 22, National Institute for Environmental Studies; <http://www.nies.go.jp/>) were similarly prepared for blank corrections, accuracy of calculations and quality precision of the analysis of selected trace elements in fish otolith. The precision of individual elements was determined from the percentage of the relative standard deviation (RSD, %) of three replicate measurements. The accuracy, expressed as a percentage of the recovery rate (RR, %), was checked using the NIES22. The limit of detection (LOD) were obtained from the individual calibration of the curves using the three sigma criteria. Nine elements (^{137}Ba , ^{43}Ca , ^{59}Co , ^{65}Cu , ^7Li , ^{26}Mg , ^{55}Mn , ^{60}Ni , ^{88}Sr) were consistently above the LOD: Ba ($0.021 \mu\text{g L}^{-1}$), Ca ($8630 \mu\text{g L}^{-1}$), Co ($0.016 \mu\text{g L}^{-1}$), Cu ($0.060 \mu\text{g L}^{-1}$), Li ($0.013 \mu\text{g L}^{-1}$), Mg ($0.096 \mu\text{g L}^{-1}$), Mn ($0.016 \mu\text{g L}^{-1}$), Ni ($0.014 \mu\text{g L}^{-1}$) and Sr ($0.025 \mu\text{g L}^{-1}$). The precision expressed as the relative standard deviation (RSD) ranged between 0.98% and 3.49%, and the accuracy expressed as the recovery rate (RR) varied from 82% to 110%; both values (RSD and RR) are within the analytical accepted values (Dove et al., 1996: RSD: <20% and RR: 75%–125%). The trace element concentrations, originally in $\mu\text{g element/L}$ solution, were thereafter transformed to $\mu\text{g element/g}$ otolith and finally to $\mu\text{g element/g}$ calcium (Higgins et al., 2013).

2.5. Data analysis

The mean EFD reconstruction was used to evaluate the main differences in otolith contours among the two different locations (Libungan and Pálsson, 2015). Spatial differences in otolith shape variability were visualized using the otolith mean length-standardized elliptic Fourier descriptors with the Canonical Analysis of Principal Coordinates (CAP) (Anderson and Willis, 2003) using the *vegan* package (Oksanen et al., 2022) in R software (R Core Team, 2020). CAP analyses were based on Euclidean distance (modified Gower dissimilarity index) and an ANOVA-like permutation test for Canonical Correspondence Analysis (CCA), also in *vegan*, was used to test the partition of variation among groups (Libungan and Pálsson, 2015). P-values were generated using 1000 permutations.

Before any statistical analyses, elemental signatures (element:Ca) were checked for normality (Shapiro-Wilk test, $P > 0.05$) and homogeneity of variances (Levene's test, $P > 0.05$), and the few outliers that were detected and trunked using Grubb's test. Ba:Ca, Mg:Ca and Mn:Ca were $\log(x + 1)$ transformed to meet these assumptions. To ensure that site-specific differences in otolith chemistry were not confounded by otolith mass (OM) (Gerard and Muhling, 2010), the relationship between elemental ratios and OM was evaluated using analysis of covariance (ANCOVA), with OM as a covariate and location as a fixed factor (Campana et al., 2000; Correia et al., 2021; Moura et al., 2020). This relationship was significant for Ba:Ca (positive relationship, $r^2 = 0.347$, $P = 0.000$), Mg:Ca (negative relationship, $r^2 = 0.240$, $P = 0.065$), Mn:Ca (negative relationship, $r^2 = 0.294$, $P = 0.184$) and Sr:Ca (positive relationship, $r^2 = 0.76$, $P = 0.000$). The effect of OM on otolith chemistry was thus removed by subtracting the product of the common within-group linear slope and OM from the observed elemental ratios:

$$[X]_D = [X] - (b \times \text{covariate})$$

where $[X]_D$ is the detrended elemental concentration, $[X]$ the original element concentration and b the ANCOVA slope value (Campana et al., 2000).

Analysis of variance (ANOVA) was used to explore differences in individual shape and elemental variables between the two locations ($P < 0.05$). Multivariate analysis of variance (MANOVA) was used to test for differences in the otolith shape and multi-elemental signatures from different locations and approximate F-ratio statistic (Pillai's trace) was reported for MANOVA (Correia et al., 2012, 2021). Stepwise linear discriminant function analysis (SLDFA) was used to visualize differences in locations and to examine the re-classification accuracy of fish to their

original location, verified through the percentage of correct re-classification of the discriminant functions using a jack-knifed ("leave one out") matrix (Correia et al., 2014, 2021). All statistical analyses were performed using open access R software version 3.6.3 (R Core Team, 2020) and Systat v.12 software (SYSTAT Software Inc., San Jose, CA, USA). A level of significance (α) level of 0.05 was used for all two-tailed statistical tests, with data presented as means \pm standard errors (SE).

3. Results

3.1. Otolith shape analyses (Elliptic Fourier analyses)

The mean total length and weight of individuals selected (\pm Standard Error) were 38.41 ± 0.70 cm and 778.07 ± 36.00 g for Gernika, and 37.45 ± 0.93 cm and 617.13 ± 37.49 g for Plentzia. The mean Fulton's condition factor, $K_F = [100 \cdot (W/TL^3)]$, of the selected individuals, was 1.37 ± 0.05 for Gernika and 1.15 ± 0.04 for Plentzia.

Three individual EFD (c2, a5 and c5) (Table 1) presented significant differences between individuals from Gernika and Plentzia (One-Way ANOVA, $P < 0.05$). MANOVA statistical tests performed on the EFD showed significant differences between Gernika and Plentzia (MANOVA, Pillai's Trace $F_{2,101} = 0.48$, $P < 0.05$). The canonical analysis of principal coordinates (CAP) plot displayed a clear separation between individuals from Gernika and Plentzia (Fig. 3A), identifying two groups (One-Way ANOVA, $P < 0.05$). CAP and stepwise linear discriminant function analysis (SLDFA) showed distinct population units of *C. labrosus* from Gernika and Plentzia, although two individuals from Gernika were re-classified by the SLDFA to Plentzia and one individual the other way around (Table 2).

3.2. Otolith elemental analyses (Element:Ca)

Significant differences among locations for Ba:Ca, Li:Ca and Sr:Ca (One-Way ANOVAs, $P < 0.05$) were observed (Fig. 4). Ba:Ca showed lower mean ratio values for Plentzia, while Li:Ca and Sr:Ca showed higher mean ratio values (Fig. 4). No significant differences were found between the locations for Co:Ca, Cu:Ca, Mg:Ca, Mn:Ca, and Ni:Ca (Fig. 4B, C and 4E–G) (One-Way ANOVAs, $P < 0.05$).

Table 1

C. labrosus otolith elliptic Fourier descriptors (EFD) values for Gernika and Plentzia sampling locations. Eighteen EFD at 95% accumulation were selected, with the first three (a1, b1 and c1 constants) excluded. For each EFD, overall significant differences between Gernika and Plentzia populations (One-Way ANOVA, $P < 0.05$) were highlighted in bold. Data are presented as mean values \pm SE.

Variable	Gernika	Plentzia	One-Way ANOVA	
			F-statistic	P-value
EFD				
d1	-0.587 ± 0.005	-0.596 ± 0.006	1.233	0.271
a2	-0.012 ± 0.003	-0.009 ± 0.002	0.760	0.387
b2	-0.004 ± 0.003	-0.014 ± 0.005	3.052	0.086
c2	0.015 ± 0.002	0.028 ± 0.005	4.505	0.038
d2	0.007 ± 0.002	0.001 ± 0.003	1.354	0.249
a3	-0.061 ± 0.002	-0.063 ± 0.002	1.164	0.285
b3	-0.001 ± 0.002	-0.005 ± 0.002	2.004	0.162
c3	-0.024 ± 0.002	-0.023 ± 0.002	0.516	0.475
d3	-0.058 ± 0.002	-0.057 ± 0.002	0.015	0.902
a4	0.002 ± 0.001	0.000 ± 0.001	1.072	0.305
b4	0.008 ± 0.001	0.008 ± 0.001	0.024	0.877
c4	0.010 ± 0.002	0.007 ± 0.002	1.928	0.170
d4	-0.015 ± 0.001	-0.012 ± 0.002	1.981	0.165
a5	-0.011 ± 0.001	-0.014 ± 0.001	4.965	0.030
b5	-0.002 ± 0.001	-0.003 ± 0.001	0.291	0.591
c5	0.012 ± 0.001	0.007 ± 0.001	6.399	0.014
d5	-0.006 ± 0.001	-0.004 ± 0.002	1.461	0.232
a6	0.007 ± 0.001	0.006 ± 0.001	0.215	0.644

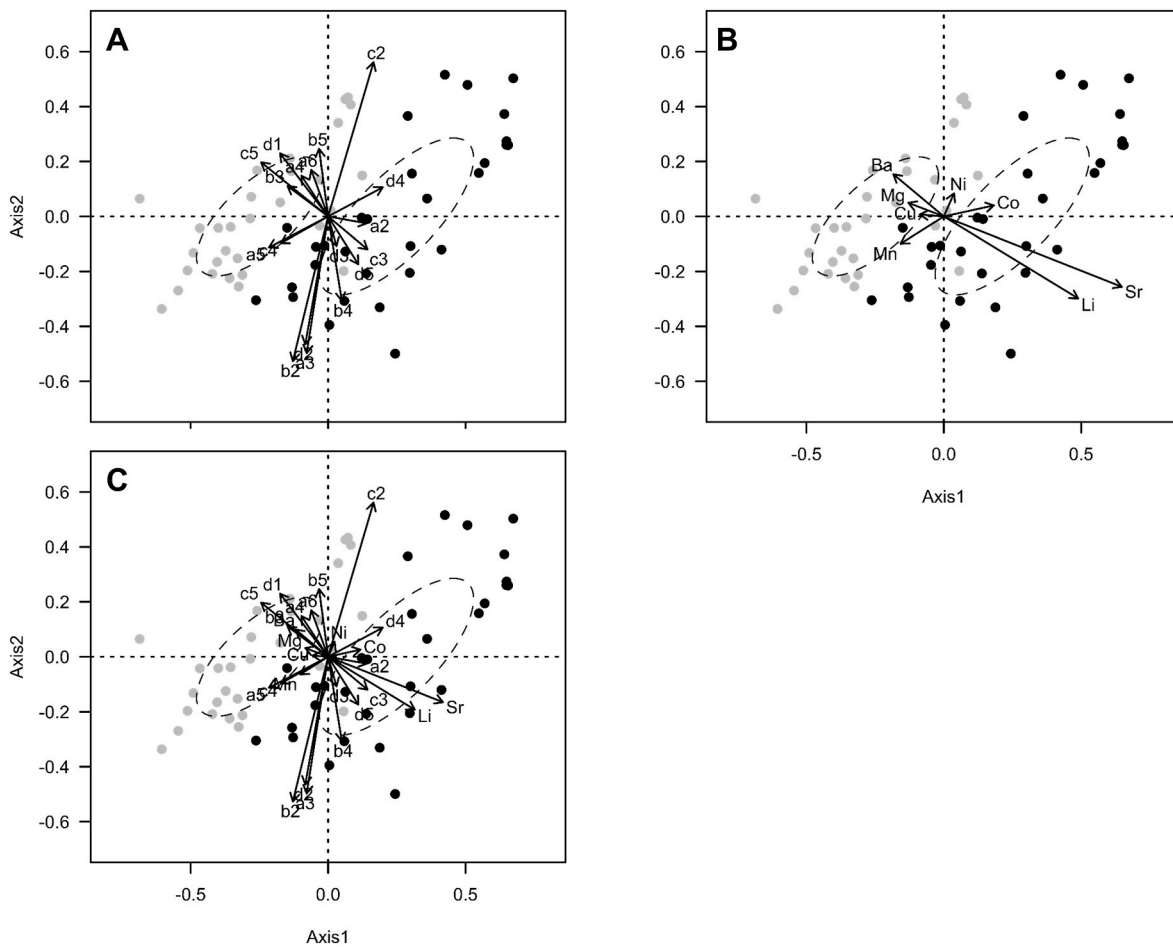


Fig. 3. Canonical analysis of principal coordinates (CAP) plots for otolith shape analyses (EFD) and otolith elemental analyses (MES) from *C. labrosus* individuals collected from the estuaries of Gernika (GE) and Plentzia (PL). (A) CAP with EFD, (B) CAP with MES and (C) CAP with EFD and MES. Ellipses represent 95% confidence intervals. Each data point represents an individual fish from Gernika (grey dots) and Plentzia (black dots).

Table 2

Jack-knifed re-classification matrix resulting from stepwise discriminant linear function analyses (SLDFA) using elliptical Fourier descriptors (EFD). Multi-elemental signatures (MES) and a combination of both (EFD and MES) for *Chelon labrosus* populations from the Bay of Biscay (correct reclassification in bold).

Original Location	N	Predicted Location		% Correct
		Gernika	Plentzia	
A. EFD				
Gernika	30	23	7	77
Plentzia	30	8	22	73
Total	60	31	29	75
B. MES				
Gernika	30	28	2	93
Plentzia	30	0	30	100
Total	60	28	32	97
C. EFD + MES				
Gernika	30	29	1	97
Plentzia	30	0	30	100
Total	60	29	31	98

3.3. Otolith shape and elemental analyses

Stepwise linear discriminant function analysis (SLDFA) (Table 2) for otolith shape analyses (EFD), otolith elemental analyses (MES) and all data combined (EFD + MES) showed a clear separation between individuals from Gernika and Plentzia. Jack-knife re-classification accuracies for EFD showed a good overall re-classification success of 75% of

individuals to the estuary of origin, with individuals from Gernika (77%) showing the best re-classification success (Table 2A). Regarding MES, a better overall jack-knife re-classification success (97%) was observed. With individuals from Plentzia showing the best re-classification success of 100% (Table 2B). Using both otolith shape and elemental analyses, the allocation of samples increased the overall re-classification success to 98%. Full discrimination was obtained for individuals from Plentzia (100%) with a 97% re-classification success for Gernika (Table 2C). The canonical analysis of principal coordinates (CAP) plot displayed a clear distinction between individuals from Gernika and Plentzia using EFD + MES (Fig. 3C).

4. Discussion

This study aimed at examining differences in otolith shape and elemental signatures of *C. labrosus* individuals inhabiting two estuaries with distinct burdens of xenoestrogens in the Southern Bay of Biscay, and to assess their potential to delineate the structure of *C. labrosus* populations. Elliptic Fourier descriptors (EFD) and multi-elemental signatures (MES) of whole otoliths distinguished individuals from the two estuaries sampled, suggesting that there is more than one local population unit along the Basque coast. Moreover, univariate tests on MES indicate that the differences between the two locations were largely driven by Ba, Li and Sr content. These results suggest that the individuals from Gernika and Plentzia have limited connectivity despite their geographical proximity.

Otolith shape and elemental analyses provide powerful means to

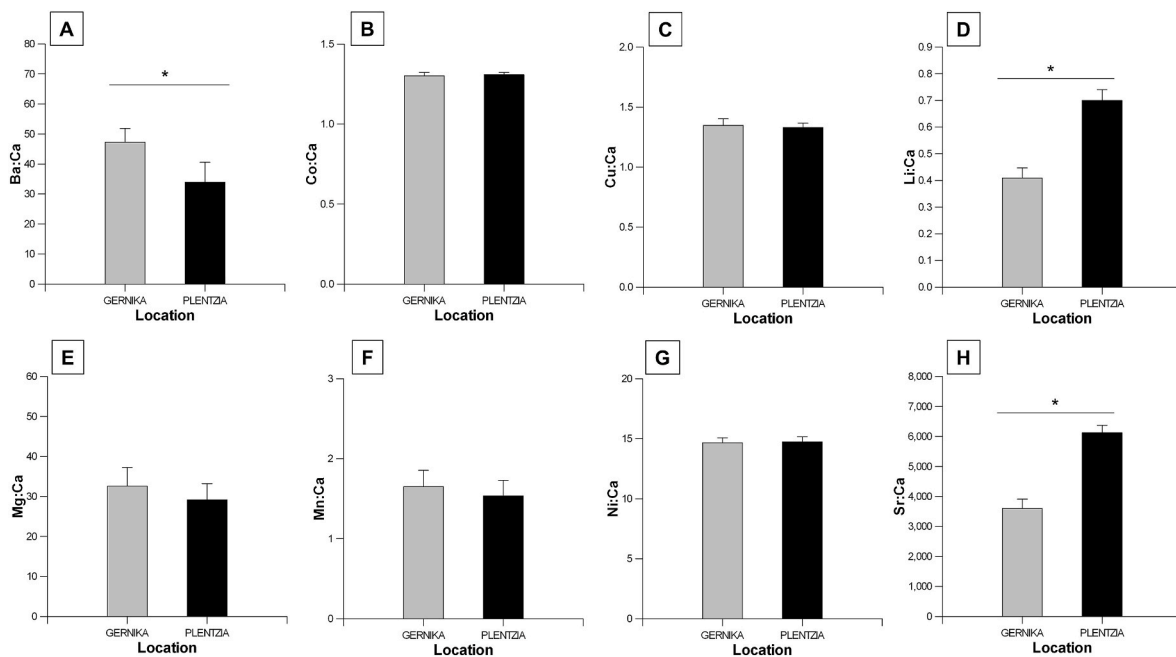


Fig. 4. Element:Ca ratios in whole otoliths of *C. labrosus* collected in the Southeast Bay of Biscay. Unit of measurement is given as μg element g^{-1} calcium. An asterisk (*) above the bars indicates statistically significant different results (One-Way ANOVA, $P < 0.05$). For Ba:Ca, Mg:Ca, Mn:Ca and Sr:Ca detrended values were shown. Data are presented as mean values \pm SE.

characterise the natal origin and movements of fish (Adelir-Alves et al., 2018; Moura et al., 2020; Soeth et al., 2019) and to distinguish between groups of fish experiencing geographic separation (Correia et al., 2011, 2014; Smoliński et al., 2020). Even when no significant genetic differences can be found (Correia et al., 2012; Marengo et al., 2017; Moreira et al., 2019a). Polymorphic microsatellite DNA markers provide great resolution for identifying population structure (Durand et al., 2013; Moreira et al., 2020; Ward, 2000); however, genetic variation between population groups may be so low that individuals appear as members of a single interbreeding population (Marengo et al., 2017; Moreira et al., 2019b; Ward, 2000). Recently, *C. labrosus* individuals from five Basque estuaries were analysed by applying 10 different microsatellite markers and by comparing them with two out-groups from the Southern Atlantic Ocean and the Mediterranean Sea; however, no genetic differences were found, suggesting that all mullets from the SBB to the Greek Mediterranean form a single panmictic population (Nzioka et al., 2023). This geographically-extense gene flow could be due to the existence of common spawning grounds at sea with high dispersion rates of adult individuals and long larval stages with high dispersion rates on homing into estuaries in the absence of physical barriers (DeWoody and Avise, 2000; Karaiskou et al., 2004; Martinez et al., 2018; Moreira et al., 2018). Despite this, *C. labrosus* mullets display phenotypic differences associated to the estuaries they live within the Biscay Bay. In this sense, male mullets from the Gernika estuary show up to 83% prevalence of intersex condition due to the exposure to xenoestrogens discharged by the WWTP of Gernika, while in the nearby pristine Plentzia estuary, not a single case has been reported (Bizarro et al., 2014; Ortiz-Zarragoitia et al., 2014; Valencia et al., 2017). The absence of genetic differences between Plentzia and Gernika individuals does not rule out the possibility that individuals in such estuaries are not lifelong residents of such estuaries, and that migration to the sea, for instance, for reproduction, is always followed by a return to the same estuary, with intersex individuals always returning to the xenoestrogenic loaded environment.

Some studies have been able to establish site-specific differences in otolith shape, even in the presence of limited genetic heterogeneity (Marengo et al., 2017; Neves et al., 2021; Soeth et al., 2019). Reef species such as the yellowstripe goatfish *Mulloidichthys flavolineatus* from the South-West Indian Ocean show recruitment back into native reefs

and this is reflected in site-specific differences in otolith morphometric features observed in two population units sampled in two close sites in Reunion Island (Pothin et al., 2006). Genetic differences influence otolith shape, but environmental conditions have been shown to alter otolith growth rates, which in turn affected otolith shape. For instance, in the case of the Atlantic cod *Gadus morhua* from the Faroe islands (Cardinale et al., 2004), Atlantic cod individuals were significantly separated into Bank and Plateau stocks with environment-linked classification rates above 85% depending on the age class (Cardinale et al., 2004). In the absence of genetic differences, environment-related differences in otolith shape have also allowed the identification of different stocks/groups of the Brazilian sardine (*Sardinella brasiliensis*) in the South-West Atlantic (Schroeder et al., 2021), the common dentex (*Dentex dentex*) in the Mediterranean Sea (Marengo et al., 2017), the blue jack mackerel (*Trachurus picturatus*) (Moreira et al., 2019a), the chub mackerel (*Scomber colias*) (Muniz et al., 2020) and the European sardine (*Saldina pilchardus*) in the North-East Atlantic Ocean (Neves et al., 2021). However, few studies have explored the effect of local habitat variability (physical and chemical) on otolith shape over short geographical distances in the absence of genetic differences (Vignon, 2012). In the catadromous and panmictic European eel (*Anguilla anguilla*), otolith shape differences in adult individuals have been attributed to variability in environmental factors, with local population units being successfully separated into brackishwater estuarine and riverine freshwater population units in Italy (Capoccioni et al., 2011) and in waters of the North-West Iberian Peninsula (Moura et al., 2022). The difference in shape among the two locations studied hereby (only 21 miles apart) provided an overall re-classification accuracy of 75% (Table 2), a value considered acceptable for separating different fish populations (Stransky et al., 2008). Otolith shapes are generally more uniform and linked to fish size during the early life history stages due to an ontogenetically determined development. And show a more divergent and constant pattern in relation to fish size once fish reach sexual maturity (Capoccioni et al., 2011; Vignon, 2012). Considering that otolith grow by incremental layers through the deposition of calcium carbonate, differential otolith growth rates as a result of environmental factors that strongly influence fish growth (salinity, water temperature, substrate type and feeding regime) will have a strong influence on

otolith shape (Capoccioni et al., 2011; Soeth et al., 2019; Vignon, 2012). *C. labrosus* used in this study were mature adults (overall total length ~ 38 cm) (Tsikliras and Stergiou, 2014) and thus, the observed variations in otolith shape comparing mullets between the close estuaries of Gernika and Plentzia probably results from the influences of local environmental conditions (Capoccioni et al., 2011; Cardinale et al., 2004; Correia et al., 2014; Hüsey, 2008; Vignon and Morat, 2010). In any case, this reflects different habitat residencies and life histories for the individuals collected in the two estuaries.

The concentration of five elements (Co, Cu, Mg, Mn and Ni) in the otoliths of *C. labrosus* individuals from the two sampled locations did not show any significant differences between Gernika and Plentzia. These elements (including Cd, Pb and Zn, which were below the LOD) are usually found in domestic and industrial wastes, and occur naturally in freshwater terrestrial run-off (Gillanders and Kingsford, 1996). They are well suited for otolith elemental fingerprint studies, as they could be reflecting the distinctive geology in an estuary and/or the level of exposure to domestic and industrial wastewater effluents (Søndergaard et al., 2015; Vasconcelos et al., 2011; Vrdoljak et al., 2020). However, differences between Gernika and Plentzia and their environmental concentrations have not greatly affected the chemical element composition in otoliths of *C. labrosus*. Instead elemental signatures of Ba, Li and Sr resulted in a clear separation of individuals from Gernika and Plentzia (overall re-classification accuracy of 95%. Table 2) into two distinct population units.

Incorporation of Ba, Li and Sr in otoliths is influenced by water temperature, salinity and primary productivity and growth rate (Bath et al., 2000; Grammer et al., 2017; Hüsey et al., 2021; Sturrock et al., 2015; Walther and Thorrold, 2006). Sr and Li are positively correlated with salinity, hence higher Sr and Li concentrations reflect higher salinities as a result of greater marine water influence relative to freshwater (Bath et al., 2000; Hicks et al., 2010; Hüsey et al., 2021). In this study, the higher Sr and Li concentrations observed in Plentzia is to be expected, considering that mullets in Plentzia were sampled in the small leisure port in the outer part of the relatively shallow estuary of the Butron river. This estuary experiences strong wave energy fluxes in its tidal range with strong tidal currents during ebb and flood that generate an equilibrium in the tidal inlet and bay area (Leorri et al., 2013; Lomonaco et al., 2003). Although growth and reproduction have also been shown to affect Sr:Ca ratios in marine fish (Grammer et al., 2017; Hüsey et al., 2021; Sturrock et al., 2015), these factors do not seem to establish differences within each of the studied population units. On the other hand, the lower Sr and Li and higher Ba otolith contents in Gernika may be due to freshwater input and terrestrial enrichment influencing changes in water chemistry. The sampling area is known to have a low-salinity water lens formed by a stronger axial gradient towards the head, where it receives freshwater input from the Oka River, as well as effluent discharges from the Gernika WWTP (Iriarte et al., 2015, 2016; Villate et al., 2008). Ba has been linked to terrestrial run-off and freshwater/river discharge in estuaries (Elsdon and Gillanders, 2004; Hicks et al., 2010), and some studies report an inverse relationship between Ba:Ca otolith content and water salinity (Hüsey et al., 2021; Izzo et al., 2018; Moreira et al., 2018), consistent with the Ba results found in the present study.

Otolith analysis therefore suggests lack of connectivity between mullets from Plentzia and Gernika although genetic markers identified one single *Chelon labrosus* population from the Southern Bay of Biscay to the Thermoikos Gulf in the Mediterranean. Genetic analysis thus, suggests that individuals from the Basque estuaries may reproduce among themselves and/or that marine larvae show a broad range of geographical dispersion for incorporation into estuaries (Nzioka et al., 2023). This apparent contradiction between both datasets is not such. Panmixia as shown by microsatellites does not exclude that adults reproducing at sea, irrespective of who they reproduce with or where their larvae go to, show fidelity to their estuary of origin. A finer spatial structure was also found for whitemouth croaker (*Micropogonias furnieri*)

populations along the Rio de Janeiro coast, Brazil, when using otolith's shape and elemental signatures compared with nuclear microsatellites (Franco et al., 2023).

5. Conclusions

The high overall re-classification success of 98% (Table 2) obtained from combining both shape and elemental signatures in otoliths of mullets from Gernika and Plentzia indicated the presence of discrete and spatially structured phenotypic groups, which suggests that individuals passed enough time in distinct water compartments even if separated only by 21 miles of sea. Therefore, these mullets could be regarded as belonging to two different population units. These results, even with the spatial sampling limitations of this study, indicate restricted connectivity between mullets and suggest a limited degree of mixing of adults among estuaries in the Southern Bay of Biscay. This could also suggest that mullets migrating to the sea for reproduction every year would always return to their native estuary (the one they recruited to as larvae) and that the intersex condition that has been observed in Gernika is probably due to life-long exposure to xenoestrogens. However, the selection of only two locations is a limiting factor, considering the broader range of estuaries available for the species as nursery grounds. Future works should address the variations in informative minor and trace elements along the growth increments, from the otolith's core (natal origin) to the edge (moment of capture), to better understand the yearly pattern of brackishwater-marine migrations in *C. labrosus*. This could help us unravel the real fidelity of the adult individuals to their native estuary of recruitment.

Author contributions

Writing—original draft preparation, A.N. Investigation, A.N., O.D.D.C., I.C. and A.T.C. Methodology, A.N., A.A., E.P., O.D.D.C. and A.T.C. Formal analysis, A.N. Writing—review and editing, A.N., O.D.D.C., I.C. and A.T.C.; Funding acquisition, I.C. and A.T.C. Conceptualization, Coordination and Supervision, O.D.D.C., I.C. and A.T.C. All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

Data availability

Data will be made available on request.

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References

- Adelir-Alves, J., Daros, F.A.L.M., Spach, H.L., Soeth, M., Correia, A.T., 2018. Otoliths as a tool to study reef fish population structure from coastal islands of South Brazil. Mar. Biol. Res. 14, 973–988. <https://doi.org/10.1080/17451000.2019.1572194>.

- Anderson, M.J., Willis, T.J., 2003. Canonical analysis of principal coordinates: a useful method of constrained ordination for ecology. *Ecology* 84, 511–525. [https://doi.org/10.1890/0012-9658\(2003\)084\[0511:CAOPCA\]2.0.CO;2](https://doi.org/10.1890/0012-9658(2003)084[0511:CAOPCA]2.0.CO;2).
- Bahamonde, P.A., Munkittrick, K.R., Martyniuk, C.J., 2013. Intersex in teleost fish: are we distinguishing endocrine disruption from natural phenomena? *Gen. Comp. Endocrinol.* 192, 25–35. <https://doi.org/10.1016/j.ygcen.2013.04.005>.
- Bath, G.E., Thorold, S.R., Jones, C.M., Campana, S.E., McLaren, J.W., Lam, J.W.H., 2000. Strontium and barium uptake in aragonitic otoliths of marine fish. *Geochem. Cosmochim. Acta.* [https://doi.org/10.1016/S0016-7037\(99\)00419-6](https://doi.org/10.1016/S0016-7037(99)00419-6).
- Bizarro, C., Ros, O., Vallejo, A., Prieto, A., Etxebarria, N., Cajarville, M.P., Ortiz-Zarragoitia, M., 2014. Intersex condition and molecular markers of endocrine disruption in relation with burdens of emerging pollutants in thicklip grey mullets (*Chelon labrosus*) from Basque estuaries (South-East Bay of Biscay). *Mar. Environ. Res.* 96, 19–28. <https://doi.org/10.1016/j.marenvres.2013.10.009>.
- Cadrin, S.X., Kerr, L.A., Mariani, S., 2013. Stock identification methods: an Overview. *Stock Ident. Methods: Appl. Fish. Sci.: Sec. Ed.* 1–5. <https://doi.org/10.1016/B978-0-12-397003-9.00001-1>.
- Campana, S.E., Chouinard, G.A., Hanson, J.M., Fréchet, A., Brattey, J., 2000. Otolith elemental fingerprints as biological tracers of fish stocks. *Fish. Res.* 46, 343–357.
- Capoccioni, F., Costa, C., Aguzzi, J., Menesatti, P., Lombarte, A., Ciccotti, E., 2011. Ontogenetic and environmental effects on otolith shape variability in three Mediterranean European eel (*Anguilla anguilla*, L.) local stocks. *J. Exp. Mar. Biol. Ecol.* 397, 1–7. <https://doi.org/10.1016/j.jembe.2010.11.011>.
- Cardinale, M., Doering-Arjes, P., Kastowsky, M., Mosegaard, H., 2004. Effects of Sex, Stock, and Environment on the Shape of Known-Age Atlantic Cod (*Gadus morhua*) Otoliths. <https://doi.org/10.1139/F03-151>.
- Correia, A.T., Moura, A., Triay-Portella, R., Santos, P.T., Pinto, E., Almeida, A.A., Sial, N., Muniz, A.A., 2021. Population structure of the chub mackerel (*Scomber colias*) in the NE Atlantic inferred from otolith elemental and isotopic signatures. *Fish. Res.* 234, 105785. <https://doi.org/10.1016/j.fishres.2020.105785>.
- Correia, A.T., Pipa, T., Gonçalves, J.M.S., Erzini, K., Hamer, P.A., 2011. Insights into population structure of *Diplodus vulgaris* along the SW Portuguese coast from otolith elemental signatures. *Fish. Res.* 111, 82–91. <https://doi.org/10.1016/j.fishres.2011.06.014>.
- Correia, A.T., Ramos, A.A., Barros, F., Silva, G., Hamer, P., Morais, P., Cunha, R.L., Castilho, R., 2012. Population structure and connectivity of the European conger eel (*Conger conger*) across the north-eastern Atlantic and western Mediterranean: integrating molecular and otolith elemental approaches. *Mar. Biol.* 159, 1509–1525. <https://doi.org/10.1007/s00227-012-1936-3>.
- Correia, A.T., Hamer, P., Carocinho, B., Silva, A., 2014. Evidence for meta-population structure of Sardinia pilchardus in the Atlantic Iberian waters from otolith elemental signatures of a strong cohort. *Fish. Res.* 149, 76–85. <https://doi.org/10.1016/j.fishres.2013.09.016>.
- Crosetti, D., Cataudella, S., 1995. In: Nash, C.E. (Ed.), *Grey mullet culture, World Animal Science 34B: Production of Aquatic Animals*. Elsevier BV, Burlington, MA, USA, pp. 271–288.
- DeWoody, J.A., Avise, J.C., 2000. Microsatellite variation in marine, freshwater and anadromous fishes compared with other animals. *J. Fish. Biol.* 56, 461–473. <https://doi.org/10.1006/jfbi.1999.1210>.
- Diaz De Cerio, O., Rojo-Bartolomé, I., Bizarro, C., Ortiz-Zarragoitia, M., Cancio, I., 2012. 5S rRNA and accompanying proteins in gonads: powerful markers to identify sex and reproductive endocrine disruption in fish. *Environ. Sci. Technol.* 46, 7763–7771. <https://doi.org/10.1021/es301132b>.
- Dove, S.G., Gillanders, B.M., Kingsford, M.J., 1996. An investigation of chronological differences in the deposition of trace metals in the otoliths of two temperate reef fishes. *J. Exp. Mar. Biol. Ecol.* 205, 15–33. [https://doi.org/10.1016/S0022-0981\(96\)02610-X](https://doi.org/10.1016/S0022-0981(96)02610-X).
- Durand, J.D., Blel, H., Shen, K.N., Koutrakis, E.T., Guinand, B., 2013. Population genetic structure of *Mugil cephalus* in the mediterranean and black seas: a single mitochondrial clade and many nuclear barriers. *Mar. Ecol. Prog. Ser.* 474, 243–261. <https://doi.org/10.3354/meps10080>.
- Elsdon, T.S., Gillanders, B.M., 2004. Fish otolith chemistry influenced by exposure to multiple environmental variables. *J. Exp. Mar. Biol. Ecol.* 313, 269–284. <https://doi.org/10.1016/j.jembe.2004.08.010>.
- Franco, T.P., Vilasboa, A., Araújo, F.G., Gama, J.M., Correia, A.T., 2023. Identifying whitemouth croaker (*Micropogonias furnieri*) populations along the Rio de Janeiro coast, Brazil, through microsatellites and otoliths analyses. *Biology (Basel)*. In Press.
- Freyhof, J., Kottelat, M., 2008. *Chelon labrosus*, thicklip grey mullet [WWW Document]. IUCN Red List. <https://doi.org/10.2305/IUCN.UK.2008.RLTS.T135689A4182365.en>.
- Froese, R., Pauly, D., 2022. FishBase [WWW Document]. World Wide Web electronic publication. <https://www.fishbase.se/search.php>. accessed 6.15.22.
- Gerard, T., Muhling, B., 2010. Variation in the isotopic signatures of juvenile gray snapper (*Lutjanus griseus*) from five southern Florida regions. *Fish. Bull.* 108, 98–105.
- Gillanders, B.M., Kingsford, M.J., 1996. Elements in otoliths may elucidate the contribution of estuarine recruitment to sustaining coastal reef populations of a temperate reef fish. *Mar. Ecol. Prog. Ser.* 141, 13–20. <https://doi.org/10.3354/meps141013>.
- González-Castro, M., Minos, G., 2016. In: Crosetti, D., Blaber, S. (Eds.), *Sexuality and reproduction of mugilidae, Biology, Ecology and Culture of Grey Mullet (Mugilidae)*. CRC Press, pp. 227–263.
- Grammer, G.L., Morrongiello, J.R., Izzo, C., Hawthorne, P.J., Middleton, J.F., Gillanders, B.M., 2017. Coupling biogeochemical tracers with fish growth reveals physiological and environmental controls on otolith chemistry. *Ecol. Monogr.* 87, 487–507. <https://doi.org/10.1002/ecm.1264>.
- Hickling, C.F., 1970. A contribution to the natural history of the English grey mullets [pisces, mugilidae]. *J. Mar. Biol. Assoc. U. K.* 50, 609–633. <https://doi.org/10.1017/S0025315400004914>.
- Hicks, A.S., Closs, G.P., Swearer, S.E., 2010. Otolith microchemistry of two amphidromous galaxiids across an experimental salinity gradient: a multi-element approach for tracking diadromous migrations. *J. Exp. Mar. Biol. Ecol.* 394, 86–97. <https://doi.org/10.1016/j.jembe.2010.07.018>.
- Higgins, R., Isidro, E., Menezes, G., Correia, A., 2013. Otolith elemental signatures indicate population separation in deep-sea rockfish, *Helicolenus dactylopterus* and *Pontinus kuhlii*, from the Azores. *J. Sea Res.* 83, 202–208. <https://doi.org/10.1016/j.seares.2013.05.014>.
- Hoff, N.T., Dias, J.F., Pinto, E., Almeida, A., Schroeder, R., Correia, A.T., 2022. Past and contemporaneous otolith fingerprints reveal potential anthropogenic interferences and allows refinement of the population structure of *Isopisthus parvipinnis* in the South Brazil Bight. *Biology (Basel)* 11, 1005. <https://doi.org/10.3390/biology11071005>.
- Hüssy, K., 2008. Otolith shape in juvenile cod (*Gadus morhua*): ontogenetic and environmental effects. *J. Exp. Mar. Biol. Ecol.* 364, 35–41. <https://doi.org/10.1016/J.JEMBE.2008.06.026>.
- Hüssy, K., Limburg, K.E., de Pontual, H., Thomas, O.R.B., Cook, P.K., Heimbrand, Y., Blass, M., Sturrock, A.M., 2021. Trace element patterns in otoliths: the role of biomineralization. *Rev. Fish. Sci. Aquac.* 29, 445–477. <https://doi.org/10.1080/23308249.2020.1760204>.
- Iriarte, A., Villate, F., Uriarte, I., Alberdi, L., Intxausti, L., 2015. Dissolved oxygen in a temperate estuary: the influence of hydro-climatic factors and eutrophication at seasonal and inter-annual time scales. *Estuar. Coast* 38, 1000–1015. <https://doi.org/10.1007/s12237-014-9870-x>.
- Iriarte, A., Villate, F., Uriarte, I., Arranz, S., 2016. Assessment of the climate and human impact on estuarine water environments in two estuaries of the Bay of Biscay. *Oceanol. Hydrobiol. Stud.* 45, 505–523. <https://doi.org/10.1515/ohs-2016-0043>.
- Izzo, C., Reis-Santos, P., Gillanders, B.M., 2018. Otolith chemistry does not just reflect environmental conditions: a meta-analytic evaluation. *Fish. Fish.* <https://doi.org/10.1111/FAF.12264>. John Wiley & Sons, Ltd.
- Karaiskou, N., Triantafyllidis, A., Triantafyllidis, C., 2004. Shallow genetic structure of three species of the genus *Trachurus* in European waters. *Mar. Ecol. Prog. Ser.* 281, 193–205. <https://doi.org/10.3354/meps281193>.
- Koutrakis, E., 2015. In: Crosetti, D., Blaber, S. (Eds.), *Biology and ecology of fry and juveniles of mugilidae, Biology, Ecology and Culture of Grey Mullet (Mugilidae)*. CRC Press, p. 264.
- Kuhl, F.P., Giardina, C.R., 1982. Elliptic Fourier features of a closed contour. *Comput. Graph. Image Process.* [https://doi.org/10.1016/0146-664X\(82\)90034-X](https://doi.org/10.1016/0146-664X(82)90034-X).
- Leorri, E., Cearreta, A., García-Artola, A., Irabien, M.J., Blake, W.H., 2013. Relative sea-level rise in the Basque coast (N Spain): different environmental consequences on the coastal area. *Ocean Coast Manag.* 77, 3–13. <https://doi.org/10.1016/j.ocecoaman.2012.02.007>.
- Libungan, L.A., Pålsson, S., 2015. ShapeR: an R package to study otolith shape variation among fish populations. *PLoS One* 10, 1–12. <https://doi.org/10.1371/journal.pone.0121102>.
- Lomonaco, P., Medina, R., Gyssels, P., Vidal, C., 2003. Application of a long-term morphologic evolution model to the Plencia tidal inlet and its adjacent beaches. In: *Proc. 3rd IAHR Symposium on Rivers, Coastal and Estuarine Morphodynamics*, pp. 401–413.
- Marengo, M., Baudouin, M., Viret, A., Laporte, M., Berrebi, P., Vignon, M., Marchand, B., Durieux, E.D.H., 2017. Combining microsatellite, otolith shape and parasites community analyses as a holistic approach to assess population structure of *Dentex dentex*. *J. Sea Res.* 128, 1–14. <https://doi.org/10.1016/j.seares.2017.07.003>.
- Marigómez, I., Garmendia, L., Soto, M., Orbea, A., Izagirre, U., Cajarville, M.P., 2013. Marine ecosystem health status assessment through integrative biomarker indices: a comparative study after the Prestige oil spill “mussel Watch”. *Ecotoxicology* 22, 486–505. <https://doi.org/10.1007/s10646-013-1042-4>.
- Martinez, A.S., Willoughby, J.R., Christie, M.R., 2018. Genetic diversity in fishes is influenced by habitat type and life-history variation. *Ecol. Evol.* 8, 12022–12031. <https://doi.org/10.1002/ece3.4661>.
- Mérigot, B., Letourneur, Y., Lecomte-Finiger, R., 2007. Characterization of local populations of the common sole *Solea solea* (Pisces, Soleidae) in the NW Mediterranean through otolith morphometrics and shape analysis. *Mar. Biol.* 151, 997–1008. <https://doi.org/10.1007/s00227-006-0549-0>.
- Mičković, B., Nikčević, M., Hegediš, A., Regner, S., Gačić, Z., Krpo-Četković, J., 2010. Mullet Fry (*Mugilidae*) in coastal waters of Montenegro, their spatial distribution and migration phenology. *Arch. Biol. Sci.* <https://doi.org/10.2298/ABS1001107M>.
- Mijangos, L., Ziarrusta, H., Ros, O., Kortazar, L., Fernández, L.A., Olivares, M., Zuloaga, O., Prieto, A., Etxebarria, N., 2018. Occurrence of emerging pollutants in estuaries of the Basque Country: analysis of sources and distribution, and assessment of the environmental risk. *Water Res.* 147, 152–163. <https://doi.org/10.1016/j.watres.2018.09.033>.
- Moreira, C., Correia, A.T., Vaz-Pires, P., Froufe, E., 2019a. Genetic diversity and population structure of the blue jack mackerel *Trachurus picturatus* across its western distribution. *J. Fish. Biol.* 94, 725–731. <https://doi.org/10.1111/jfbi.13944>.
- Moreira, C., Froufe, E., Sial, A.N., Caeiro, A., Vaz-Pires, P., Correia, A.T., 2018. Population structure of the blue jack mackerel (*Trachurus picturatus*) in the NE Atlantic inferred from otolith microchemistry. *Fish. Res.* 197, 113–122. <https://doi.org/10.1016/j.fishres.2017.08.012>.
- Moreira, C., Froufe, E., Vaz-Pires, P., Correia, A.T., 2019b. Otolith shape analysis as a tool to infer the population structure of the blue jack mackerel, *Trachurus picturatus*, in the NE Atlantic. *Fish. Res.* 209, 40–48. <https://doi.org/10.1016/j.fishres.2018.09.010>.

- Moreira, C., Froufe, E., Vaz-Pires, P., Triay-Portella, R., Méndez, A., Pisonero Castro, J., Correia, A.T., 2022. Unravelling the spatial-temporal population structure of *Trachurus picturatus* across the North-East Atlantic using otolith fingerprinting. *Estuar. Coast Shelf Sci.* 272, 107860 <https://doi.org/10.1016/j.ecss.2022.107860>.
- Moreira, C., Presa, P., Correia, A.T., Vaz-Pires, P., Froufe, E., 2020. Spatio-temporal microsatellite data suggest a multidirectional connectivity pattern in the *Trachurus picturatus* metapopulation from the Northeast Atlantic. *Fish. Res.* 225, 105–499. <https://doi.org/10.1016/j.fishres.2020.105499>.
- Moura, A., Dias, E., López, R., Antunes, C., 2022. Regional population structure of the European eel at the southern limit of its distribution revealed by otolith shape signature. *Fishes* 7, 135. <https://doi.org/10.3390/FISHES7030135/S1>.
- Moura, A., Muniz, A.A., Mullis, E., Wilson, J.M., Vieira, R.P., Almeida, A.A., Pinto, E., Brummer, G.J.A., Gaever, P.v., Gonçalves, J.M.S., Correia, A.T., 2020. Population structure and dynamics of the Atlantic mackerel (*Scomber scombrus*) in the North Atlantic inferred from otolith chemical and shape signatures. *Fish. Res.* 230, 105621 <https://doi.org/10.1016/j.fishres.2020.105621>.
- Muniz, A.A., Moura, A., Triay-Portella, R., Moreira, C., Santos, P.T., Correia, A.T., 2020. Population structure of the chub mackerel (*Scomber colias*) in the North-east Atlantic inferred from otolith shape and body morphometrics. *Mar. Freshw. Res.* 72, 341–352. <https://doi.org/10.1071/MF19389>.
- Neves, J., Silva, A.A., Moreno, A., Veríssimo, A., Santos, A.M., Garrido, S., 2021. Population structure of the European sardine *Sardina pilchardus* from Atlantic and Mediterranean waters based on otolith shape analysis. *Fish. Res.* 243 <https://doi.org/10.1016/j.fishres.2021.106050>.
- Nzioka, A., Madeira, M.J., Kokokiris, L., Ortiz-Zarragoitia, M., Diaz de Cerio, O., Cancio, I., 2023. Lack of genetic structure in euryhaline *Chelon labrosus* from the estuaries with anthropic pressure in the Southern Bay of Biscay to the coastal waters of the Mediterranean Sea. *Mar. Environ. Res.* 189, 106058 <https://doi.org/10.1016/j.marenvres.2023.106058>.
- Oksanen, J., Simpson, G.L., Blanchet, F.G., Kindt, R., Legendre, P., Minchin, P.R., O'Hara, R.B., Solyomos, P., Stevens, M.H.H., Szocs, E., Wagner, H., Barbour, M., Bedward, M., Bolker, B., Borcard, D., Carvalho, G., Chirico, M., De Caceres, M., Durand, S., Evangelista, H.B.A., FitzJohn, R., Friendly, M., Furneaux, B., Hannigan, G., Hill, M.O., Lahti, L., McGlenn, D., Ouellette, M.-H., Ribeiro Cunha, E., Smith, T., Stier, A., Ter Braak, C.J.F., Weedon, J., 2022. *Vegan: Community Ecology Package*.
- Ortiz-Zarragoitia, M., Bizarro, C., Rojo-Bartolomé, I., de Cerio, O.D., Cajaraville, M.P., Cancio, I., 2014. Mugilid fish are sentinels of exposure to endocrine disrupting compounds in coastal and estuarine environments. *Mar. Drugs* 12, 4756–4782. <https://doi.org/10.3390/md12094756>.
- Patterson, H.M., Thorrold, S.R., Shenker, J.M., 1999. Analysis of otolith chemistry in Nassau grouper (*Epinephelus striatus*) from the Bahamas and Belize using solution-based ICP-MS. *Coral Reefs* 18, 171–178. <https://doi.org/10.1007/s003380050176>.
- Pothin, K., Gonzalez-Salas, C., Chabanet, P., Lecomte-Finiger, R., 2006. Distinction between *Mulloidichthys flavolineatus* juveniles from Reunion Island and Mauritius Island (south-west Indian Ocean) based on otolith morphometrics. *J. Fish. Biol.* 69, 38–53. <https://doi.org/10.1111/j.1095-8649.2006.01047.x>.
- Puy-Azurmendi, E., Ortiz-Zarragoitia, M., Villagrasa, M., Kuster, M., Aragón, P., Atienza, J., Puchades, R., Maquieira, A., Domínguez, C., López de Alda, M., Fernandes, D., Porte, C., Bayona, J.M., Barceló, D., Cajaraville, M.P., 2013. Endocrine disruption in thicklip grey mullet (*Chelon labrosus*) from the Urdaibai Biosphere Reserve (bay of Biscay, Southwest Europe). *Sci. Total Environ.* 443, 233–244. <https://doi.org/10.1016/j.scitotenv.2012.10.078>.
- R Core Team, 2020. *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria.
- Reis-Santos, P., Vasconcelos, R.P., Tanner, S.E., Fonseca, V.F., Cabral, H.N., Gillanders, B. M., 2018. Extrinsic and intrinsic factors shape the ability of using otolith chemistry to characterize estuarine environmental histories. *Mar. Environ. Res.* <https://doi.org/10.1016/j.marenvres.2018.06.002>.
- Rooker, J.R., Zdanowicz, V.S., Secor, D.H., 2001. Chemistry of tuna otoliths: assessment of base composition and postmortem handling effects. *Mar. Biol.* 139, 35–43. <https://doi.org/10.1007/s002270100568>.
- Ros, O., Vallejo, A., Blanco-Zubiaguirre, L., Olivares, M., Delgado, A., Etxebarria, N., Prieto, A., 2015. Microextraction with polyethersulfone for bisphenol-A, alkylphenols and hormones determination in water samples by means of gas chromatography-mass spectrometry and liquid chromatography-tandem mass spectrometry analysis. *Talanta* 134, 247–255. <https://doi.org/10.1016/j.talanta.2014.11.015>.
- Schroeder, R., Schwingel, P.R., Correia, A.T., 2021. Population structure of the Brazilian sardine (*Sardinella brasiliensis*) in the Southwest Atlantic inferred from body morphology and otolith shape signatures. *Hydrobiologia* 7. <https://doi.org/10.1007/s10750-021-04730-7>.
- Schroeder, R., Schwingel, P.R., Pinto, E., Almeida, A., Correia, A.T., 2022. Stock structure of the Brazilian sardine *Sardinella brasiliensis* from Southwest Atlantic Ocean inferred from otolith elemental signatures. *Fish. Res.* 248, 106192 <https://doi.org/10.1016/j.fishres.2021.106192>.
- Smoliński, S., Schade, F.M., Berg, F., 2020. Assessing the performance of statistical classifiers to discriminate fish stocks using fourier analysis of otolith shape. *Can. J. Fish. Aquat. Sci.* 77, 674–683. <https://doi.org/10.1139/cjfas-2019-0251>.
- Soeth, M., Spach, H.L., Daros, F.A., Adelir-Alves, J., de Almeida, A.C.O., Correia, A.T., 2019. Stock structure of Atlantic spadefish *Chaetodipterus faber* from Southwest Atlantic Ocean inferred from otolith elemental and shape signatures. *Fish. Res.* 211, 81–90. <https://doi.org/10.1016/j.fishres.2018.11.003>.
- Sokal, R.R., Rohlf, F.J., 1995. *Biometry: the Principles and Practice of Statistics in Biological Research*, Third. ed. W. H. Freeman, New York.
- Søndergaard, J., Halden, N., Bach, L., Gustavson, K., Sonne, C., Mosbech, A., 2015. Otolith chemistry of common sculpins (*Myoxocephalus scorpius*) in a mining polluted Greenlandic fiord (Black Angel Lead-Zinc Mine, West Greenland). *Water Air Soil Pollut.* 226 <https://doi.org/10.1007/s11270-015-2605-1>.
- Stransky, C., Murta, A.G., Schlickeisen, J., Zimmermann, C., 2008. Otolith shape analysis as a tool for stock separation of horse mackerel (*Trachurus trachurus*) in the Northeast Atlantic and Mediterranean. *Fish. Res.* 89, 159–166. <https://doi.org/10.1016/j.fishres.2007.09.017>.
- Sturrock, A.M., Hunter, E., Milton, J.A., Johnson, R.C., Waring, C.P., Trueman, C.N., EIMF, 2015. Quantifying physiological influences on otolith microchemistry. *Methods Ecol. Evol.* 6, 806–816. <https://doi.org/10.1111/2041-210X.12381>.
- Thomas, O.R.B., Swearer, S.E., 2019. Otolith biochemistry — a review. *Rev. Fish. Sci. Aquacult.* 27, 1–32. <https://doi.org/10.1080/23308249.2019.1627285>.
- Tsikliras, A.C., Stergiou, K.I., 2014. Size at maturity of Mediterranean marine fishes. *Rev. Fish. Biol. Fish.* 24, 219–268. <https://doi.org/10.1007/s11160-013-9330-x>.
- Turan, C., 2015. In: Crosetti, D., Blaber, S. (Eds.), *Biogeography and distribution of mugilidae in the mediterranean and the black sea, and North-East Atlantic, Biology, Ecology and Culture of Grey Mullet (Mugilidae)*. CRC Press, pp. 116–127.
- Valencia, A., Rojo-Bartolomé, I., Bizarro, C., Cancio, I., Ortiz-Zarragoitia, M., 2017. Alteration in molecular markers of oocyte development and intersex condition in mullets impacted by wastewater treatment plant effluents. *Gen. Comp. Endocrinol.* 245, 10–18. <https://doi.org/10.1016/j.ygcen.2016.06.017>.
- Vasconcelos, R.P., Reis-Santos, P., Costa, M.J., Cabral, H.N., 2011. Connectivity between estuaries and marine environment: integrating metrics to assess estuarine nursery function. *Ecol. Indic.* 11, 1123–1133. <https://doi.org/10.1016/j.ecolind.2010.12.012>.
- Vignon, M., 2012. Ontogenetic trajectories of otolith shape during shift in habitat use: interaction between otolith growth and environment. *J. Exp. Mar. Biol. Ecol.* 420–421, 26–32. <https://doi.org/10.1016/j.jembe.2012.03.021>.
- Vignon, M., Morat, F., 2010. Environmental and genetic determinant of otolith shape revealed by a non-indigenous tropical fish. *Mar. Ecol. Prog. Ser.* 411, 231–241. <https://doi.org/10.3354/meps08651>.
- Villate, F., Aravena, G., Iriarte, A., Uriarte, I., 2008. Axial variability in the relationship of chlorophyll a with climatic factors and the North Atlantic oscillation in a Basque coast estuary, Bay of Biscay (1997–2006). *J. Plankton Res.* 30, 1041–1049. <https://doi.org/10.1093/plankt/fbn056>.
- Vrdoljak, D., Matic-Skoko, S., Peharda, M., Uvanović, H., Markulin, K., Mertz-Kraus, R., 2020. Otolith fingerprints reveals potential pollution exposure of newly settled juvenile *Sparus aurata*. *Mar. Pollut. Bull.* 160, 111695 <https://doi.org/10.1016/j.marpolbul.2020.111695>.
- Walther, B.D., Thorrold, S.R., 2006. Water, not food, contributes the majority of strontium and barium deposited in the otoliths of a marine fish. *Mar. Ecol. Prog. Ser.* 311, 125–130. <https://doi.org/10.3354/meps311125>.
- Ward, R.D., 2000. Genetics in fisheries management. *Hydrobiologia* 420, 191–201. <https://doi.org/10.1023/A:1003928327503>.
- Whitfield, A.K., 2020. Fish species in estuaries from partial association to complete dependency. *J. Fish. Biol.* 97, 1262–1264. <https://doi.org/10.1111/jfb.14476>.
- Xia, R., Durand, J.D., Fu, C., 2016. Multilocus resolution of Mugilidae phylogeny (Teleostei: Mugiliformes): implications for the family's taxonomy. *Mol. Phylogenet. Evol.* 96, 161–177. <https://doi.org/10.1016/j.ympev.2015.12.010>.