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Otolith elemental fingerprints indicate the importance of estuarine nursery areas for European anchovy in the Gulf of Cadiz

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ABSTRACT

Understanding which habitats function as nurseries is essential for assessing population connectivity and managing exploited marine fishes. Early life stages often concentrate in estuarine and coastal environments, yet high larval abundance does not necessarily translate into recruitment to adult stocks. Here, we compared otolith chemistry and field-based abundance data to evaluate the nursery role of major estuarine systems (Guadiana, Odiel–Tinto, Guadalquivir, Cadiz Bay) and adjacent coastal areas for European anchovy (*Engraulis encrasicolus*) in the Gulf of Cadiz during 2016–2018. Using LA-ICPMS, we obtained elemental fingerprints from postlarvae as young as ~20 days old, representing the first application of this technique at such an early developmental stage for this species. Despite some interannual variability, otolith elemental signatures differed consistently among locations, allowing accurate classification (80.1%) using Random Forest models. Zn, Ba, Cu, P and Pb were the most important predictors and showed positive relationships with ambient water concentrations. Adult anchovies sampled offshore in 2018 were assigned to their most probable nursery origins, revealing that ~20% of the adult stock had used estuarine habitats, mainly the Guadiana estuary and Cadiz Bay. Importantly, the estuarine contribution did not correlate with postlarval density, particularly in the Guadalquivir estuary, highlighting the role of hydrodynamic processes in decoupling abundance from recruitment success. The heavily mining-impacted Odiel–Tinto system showed elevated metal concentrations in water and otoliths, negligible contribution to the adult stock, and evidence that Ba-based salinity marker can be confounded by contamination. Overall, this approach improves understanding of anchovy dynamics in ICES Division 9a-South.

1. Introduction

Estuaries are recognized for their high biological productivity and for providing optimal conditions for the early development of many fish species (Strydom et al., 2003). These environments often offer refuge from predators and an abundant food supply, which can enhance larval and juvenile survival rates (Elliot and Hemingway, 2002). However, for an area to be considered an effective nursery ground, it is not sufficient to only host high densities of early life stages (Beck et al., 2001; Freeman et al., 2024) individuals must reach adulthood or significantly contribute to population recruitment (Dahlgren et al., 2006).

One of the main challenges in evaluating marine connectivity and the relative contribution of estuaries to adult populations lies in the difficulty of tracking individual movements throughout their life cycle. Although artificial tracking tools are available, they often have temporal limitations and are rarely applicable to small organisms or early developmental stages. In this context, over the last 30 years, otoliths (earstones) have emerged as a key tool in connectivity studies, as these calcified structures record the chemical signature of the environment in which a fish inhabits, allowing the reconstruction of its environmental history (Campana et al., 1994; Hüssy et al., 2021). Elemental composition of otoliths have been used to determine natal origin of individuals

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and to reconstruct ontogenetic habitat use patterns along the salinity gradient of estuaries (Elsdon and Gillanders, 2005; Walther and Limburg, 2012). Additionally, this approach has been used to estimate the relative contribution of different estuaries to adult stocks based on natal origin assignments (Reis-Santos et al., 2013; Tanner et al., 2012; Anstead et al., 2016). However, the utility of trace element markers depends on the geochemical variability of the environment in relation to habitat use and migration of the species as well as potential physiological influences on element incorporation (Sturrock et al., 2012). Consequently, context-specific validation studies are typically needed to determine whether otolith elemental markers can provide information on fish connectivity in a specific environmental and management context (Sturrock et al., 2012).

Understanding the critical habitats and connectivity patterns supporting fish populations is crucial for implementing effective conservation strategies. This is especially important for commercially valuable species such as the European anchovy (*Engraulis encrasicolus*), whose populations are declining due to multiple stressors including overfishing, habitat degradation, alteration in plankton community, pollution, microplastics, and climate change (ICES, 2024a; Mutalipassi et al., 2024). The European anchovy is a small pelagic fish characterized by rapid growth and a relatively short lifespan (4–5 years). It forms large schools and is widely distributed from the North Sea to Central Africa, including the entire Mediterranean basin (FAO, 2025). Although it is primarily considered a marine species, it is frequently associated with river plumes and estuarine systems (Palomera et al., 2007), particularly during its early developmental stages, where it exhibits an estuarine-opportunistic behavior and uses these ecosystems as nursery habitats (Faria et al., 2006; Drake et al., 2007). Previous studies have identified spatial structuring within European anchovy, often linked to differences in shelf geomorphology and associated environmental conditions (Montes et al., 2016). In particular, a broad distinction has been made between genetic lineages associated with wide continental shelves, generally linked to more oceanic environments, and those associated with narrow shelves and upwelling systems, which tend to exhibit more coastal-oriented behaviors (Zarraonaindia et al., 2012; Montes et al., 2016). The degree of population structuring varies geographically: while in some regions (e.g. the Bay of Biscay or Adriatic Sea) different lineages may co-occur (Aldanondo et al., 2010; Montes et al., 2016), in others, (e.g. the Ibero-Atlantic region), anchovy populations appear to be dominated by a single lineage (Catanese et al., 2017).

Furthermore, morphometric and genetic studies suggest a differentiation between anchovy populations associated with different genetic lineages in the Atlantic and Mediterranean regions (Ivanova and Dobrovolov, 2006; Zarraonaindia et al., 2012; Bacha et al., 2014; Jemaa et al., 2015), although significant uncertainties remain regarding the ecological population structure in the Ibero-Atlantic region (i.e. ICES Division 9a). The Gulf of Cadiz, located in the southernmost part of this region (Division 9a South), represents a particularly complex area where Atlantic and Mediterranean influences converge. This subregion has recently been subdivided into the South-Portugal and South-Spain subareas due to the still-uncertain stock structure. Managing both components under a single total allowable catch could risk the overexploitation of one population with distinct dynamics (ICES, 2024b). In the Gulf of Cadiz, anchovy is of great importance to the fishing industry (Ruiz et al., 2017; Marti, 2018) which relies heavily on age-0 individuals, making it particularly vulnerable to overexploitation and environmental changes (Ruiz et al., 2009). During its reproductive period (April–September), anchovy spawn in coastal areas (at depths of 50–100 m), with eggs and larvae generally found near the mouths of major rivers (Baldó et al., 2006), although their distribution varies depending on meteorological conditions (Ruiz et al., 2006).

In this context, the anchovy population in the Gulf of Cadiz is considered part of the Southern Iberian-Atlantic group, associated with the narrow-shelf lineage, and is therefore expected to exhibit predominantly coastal-oriented behaviors (Zarraonaindia et al., 2012; Montes et al., 2016). This suggests that a large proportion of individuals (unknown to date) may, at some stage of their development, make use of estuaries and their adjacent areas. This region contains several estuaries that could play a key role in the development of European anchovy (Miró et al., 2020), potentially contributing to other nearby regions (Casaucao et al., 2021). Previous studies have shown that estuaries in the Gulf of Cadiz host high abundances of early anchovy stages [e.g., Guadiana River (Faria et al., 2006), Guadalquivir River (Drake et al., 2007), Cadiz Bay (Drake and Arias, 1991)]. However, the salinity tolerance of larval anchovies and the extent to which these estuaries serve as effective nursery habitats supporting recruitment remains unknown.

The objective of this study was to determine the importance of the estuaries from the Spanish subregion of the Gulf of Cadiz in the life cycle of European anchovy populations. To achieve this, we i) collected a reference library of postlarval anchovies from the main estuaries and adjacent coastal areas during three consecutive years (2016, 2017, 2018), characterizing their otolith chemical signatures to assess spatial differences and temporal consistency, and to train and test assignment models for each nursery habitat. We then ii) tested whether water elemental signatures were correlated with postlarval otolith signatures and/or salinity; and iii) applied our assignment models to adults sampled in 2018 to estimate the relative contribution of each nursery habitat to the fishery.

2. Material and methods

2.1. Study area and field sampling

The study area was located in the Gulf of Cadiz, in the southwest of the Iberian Peninsula (Fig. 1). Samples were taken from 3 stations of the four main estuaries: Guadiana (GN), Odiel-Tinto (OT), Guadalquivir (GQ), and Cadiz Bay (CB). These estuaries differ in freshwater input, where the Guadiana and Guadalquivir estuaries have higher freshwater inputs resulting in a more marked salinity gradient, while more marine conditions prevail in the Odiel-Tinto and Cadiz Bay estuaries. In addition, samples were collected from four coastal stations located in the adjacent offshore areas of each estuary (O). These stations were selected at a sufficient distance from the estuary mouths and under environmental conditions expected to reflect predominantly coastal marine waters with reduced direct estuarine influence. Given the similarity in their environmental characteristics across systems, these stations were pooled into a single analytical group (OUT) for subsequent analyses. Postlarval anchovies (n = 156, Table 1) were sampled using a plankton net of 1 m diameter and 1 mm mesh size equipped with a flowmeter. Anchovies were preserved in 70% ethanol. Sampling was carried out in June-July for three consecutive years (2016, 2017, 2018) (Table 1). Full station details are provided in Table S1 and more information on postlarval sampling methods and estuary conditions in Miró et al. (2020). Adult anchovies (TL = 115-125 mm) were collected in the fishery ground of the Gulf of Cadiz approximately 30 km offshore by commercial fishermen from Punta Umbria (PU) and Sanlucar de Barrameda (SL), which accounted for 13% and 28%, respectively, of total regional catches, during August-September 2018 (Table 1).

2.2. Otolith preparation and elemental analysis

Sagittal otoliths were extracted under a stereoscope. Left or right otoliths were randomly selected then mounted sulcus side up on a glass petrographic slide with thermoplastic glue (Crystalbond 509). Postlarval otoliths were too thin and fragile to polish, but adults were ground with 1500 silicon carbide papers, and later polished with 3-μm and 1-μm lapping films and Milli-Q water until the core was visible. Nineteen elements (^{7}Li, ^{23}Na, ^{24}Mg, ^{31}P, ^{39}K, ^{44}Ca, ^{52}Cr, ^{55}Mn, ^{56}Fe, ^{59}Co, ^{60}Ni, ^{63}Cu, ^{66}Zn, ^{85}Rb, ^{88}Sr, ^{107}Ag, ^{111}Cd, ^{138}Ba, ^{208}Pb) were measured by

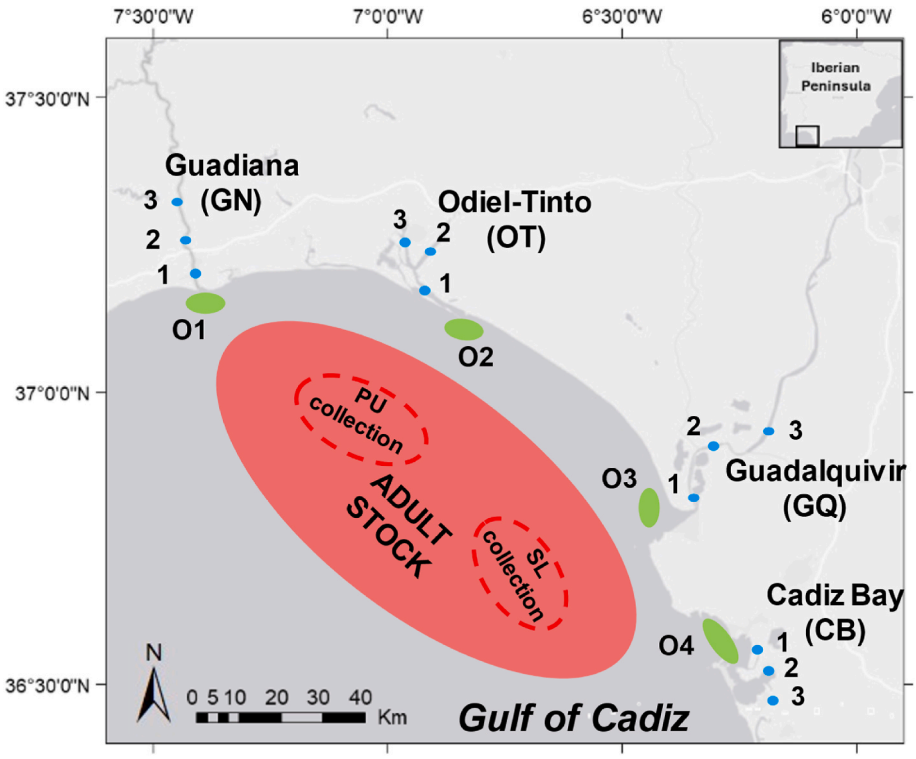

Fig. 1. Study area in the Gulf of Cadiz with three water and anchovy sampling stations within the main estuaries (blue) and four broader sampling areas along the coastline (O1-4) outside the estuary mouth (green). Dashed red circles represent the fishing grounds for Punta Umbria (PU) and Sanlucar de Barrameda (SL) used for adult anchovy collections in 2018. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Data of sampling locations (salinity, life stage, size of anchovies, and otoliths analysed) during the studied period (2016-2018). Location codes: Guadiana (GN), Odiel-Tinto (OT), Guadalquivir (GQ), Cadiz Bay (CB) and coastal areas outside the estuary mouth (OUT) (Fig. 1).

Location	Year	Life stage	Salinity range (PSU)	Otoliths analysed (n)	Fish total length (mm; mean ± SD)
GN	2016	Postlarvae	6.9-24.6	13	20.6 ± 2.1
	2017	Postlarvae	4.6-24	11	17.4 ± 4.6
	2018	Postlarvae	4-22.9	8	19.8 ± 3.8
OT	2016	Postlarvae	35.5-36.4	6	9 ± 0.9
	2017	Postlarvae	36.9-37	4	9.2 ± 2.5
	2018	Postlarvae	36-36.1	5	12 ± 1.5
GQ	2016	Postlarvae	4-25.1	5	19.2 ± 3
	2017	Postlarvae	5.4-22	23	20.9 ± 1.5
	2018	Postlarvae	5.2-22.7	13	20.1 ± 2.3
CB	2016	Postlarvae	37.4-38.7	13	17.3 ± 4
	2017	Postlarvae	37-38.1	8	13.6 ± 1.8
	2018	Postlarvae	35.6-37.1	11	15.3 ± 1.5
OUT	2016	Postlarvae	36.4-37.3	0	-
	2017	Postlarvae	36.8-37	24	14.3 ± 3.2
	2018	Postlarvae	35.7-36.3	12	13.3 ± 3.2
PU	2018	Adult	36.1-36.3	18	120.2 ± 5
SL	2018	Adult	36.1-36.3	19	121.5 ± 5

laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) using a 7900 Agilent ICP-MS coupled to a New Wave 193 nm excimer laser system at the University of Essex. All samples were pre-ablated to remove surface contamination (Table 2). NIST 612 and NIST 610 glass certified standards were analysed using the same settings and pattern as the otoliths at the beginning and end of every run, and after every 60 min of analysis, and were used to convert raw intensity counts to concentrations, correct for instrument drift, and assess analytical precision using Iolite software (Paton et al., 2011). Ca was used as an internal standard, assuming a constant concentration of 388,

Table 2
LA-ICPMS settings for raster in postlarval growth bands to identify nursery area in juvenile and adult otoliths. *Otolith samples with a radius <50 μm (16%) were set with a beam diameter of 17 μm in preablation and 15 μm in ablation.

	Circular Raster	
	Preablation	Ablation
Beam diameter	23 μm*	20 μm*
Energy	5%	40%
Repetition rate	10 Hz	20 Hz
Fluence	NA	5.9 J/cm2
Scan speed	50 μm/s	30 μm/s

000 μg/g in the otoliths. Limits of detection (LOD) were calculated for each element according to (Longerich et al., 1996). Elements with more than 40% of measurements below LOD were excluded from further analysis (^{7}Li, ^{85}Rb, ^{107}Ag, ^{111}Cd; Table S2). Remaining concentrations below LOD were replaced with zero. Outliers were identified using the Rosner Test (<1%) and replaced by the average of all remaining measurements of this element within the same estuary, in order to preserve the spatial structure of otolith elemental signatures and avoid dilution of estuary-specific geochemical variability.

In postlarval otoliths, to obtain a chemical fingerprint for their capture site, we ablated a circular raster just inside the outer edge of the otolith (Fig. 2A; Table 2), exhibiting a mean ratio of 75.8 μm (31.7 SD, n = 156). The ages of the postlarvae were estimated (21.7 ± 4.3 days; mean ± SD) using the Gompertz growth equation ($L_t = L_\infty\ exp^{-exp^{-K\ (t-t0)}}$; Ricker, 1979), following the formulation and parameters reported by Aldanondo et al. (2011) for anchovy larvae from the Bay of Biscay (as no larval growth data are available for the Gulf of Cadiz). The estimation was based on the relationship between otolith radius and body length, applying the parameters ($L_\infty = 1759$ μm; $k = 0.034$; $t_0 = 56.03$ days), considering the otolith radius as an equivalent to L_t. Using this model, the average duration of the fish's life that was integrated by the laser

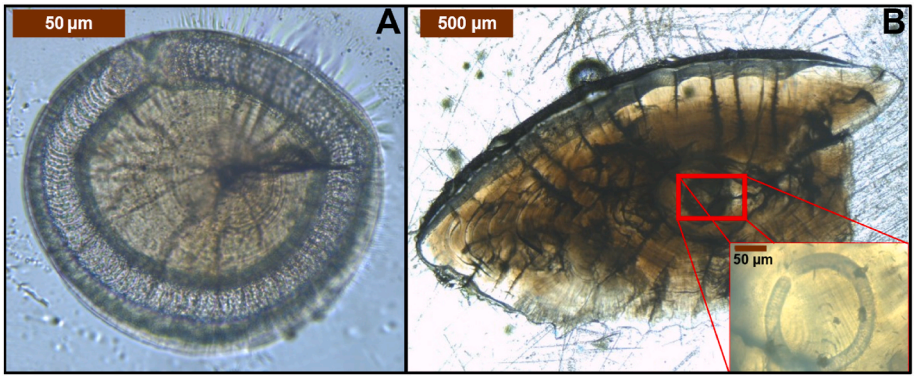

Fig. 2. Micrographs showing laser ablation circular raster on early life stages (A) and adult (B) otoliths of European anchovy (*Engraulis encrasicolus*).

beam (20 µm diameter) was approximately 3.6 ± 2.1 days (mean ± SD).

In adult otoliths, the same raster was used to analyse the postlarval region of the otolith (80.7 ± 10.8 µm equivalent to 22.8 ± 2.1 days (mean ± SD); Fig. 2B) to match age periods between sample types for assigning the adults to a nursery area. For adults, due to annual growth increments were not clearly distinguishable in all individuals, the age was estimated (1.13 ± 0.05 years; mean ± SD; i.e., all belonging to cohort 2017) using the von Bertalanffy growth equation, in accordance with the formulation and parameters provided by Bellido et al. (2000) for the Gulf of Cadiz anchovy stock, derived using the Powell-Wetherall method ($L_\infty = 18.69$; $k = 0.9$; $t_0 = -0.01635$). In this case, the model was applied to standard length measurements rather than otolith radius, as adult growth is more accurately represented by length-based data. Nonetheless, according to Bellido et al. (2000), the size range of our adult samples (115 to 135 mm) may include some slower growing individuals from cohorts 2016 and 2017.

2.3. Water elemental analysis

Water samples were taken at mid-depth in each station with an acid washed Niskin bottle. Fifteen elements (^{23}Na, ^{24}Mg, ^{31}P, ^{39}K, ^{44}Ca, ^{52}Cr, ^{55}Mn, ^{56}Fe, ^{59}Co, ^{60}Ni, ^{63}Cu, ^{66}Zn, ^{88}Sr, ^{138}Ba, ^{208}Pb) were measured by ICP-OES (Varian ICP 720-ES) equipped with ultrasonic nebulizer CETAC U5000AT + after filtration through Nylon filters (pore size = 0.45 µm). They were acidified using trace metal grade HNO_3 to 2% of sample volume then analysed after one month. Procedural blanks were collected using Milli-Q water instead of seawater. Calibration and Quality Control solutions were prepared from an ICP multi-element standard solution IV Certipur obtained from Merck and Spectrascan certified reference solution from LGC Standards GmbH (Wesel, Germany). The calibration for all elements evaluated was from 0,005 to 1 ppm, and for the highly concentrated elements (Ca, Fe, K, Mg, Mn and Na) up to 250 ppm. When concentrations exceeded the highest calibration standard, samples were diluted as necessary and re-analysed, with final concentrations corrected by the corresponding dilution factor. The accuracy of the analytical methods was assessed through reference water sample (TR-434 Trace of metals in drinking water) from INTER 2000 Program (Trace Elements in Estuarine Water CRM 505 No. 048). The recoveries were 89.2–109.4% for all the elements. The differences in element concentrations between analysed and certified values were generally <10%. Consumables were acid washed by soaking in 2% v/v HNO_3 then rinsed twice with Milli-Q water.

2.4. Data analysis

2.4.1. Spatial and temporal variation in water and postlarval otolith element concentrations

Comparisons of water and postlarval otolith element concentrations were conducted using permutational multivariate analysis of variance (PERMANOVA) (Anderson, 2001), with location (5 levels: GN, OT, GQ, CB, OUT; Fig. 1) and years (3 levels: 2016, 2017, 2018) as fixed factors, and station (nested in location and time) as a random factor. Elements were log (x + 1) transformed, and PERMANOVA was based on Euclidean distance dissimilarity matrices. A similar process was used for univariate analyses of each element but without data transformation. When significant differences were found in multivariate analyses, pairwise comparisons among all levels of a term were also examined. These analyses were performed using the software PRIMERv6.1.11 and PERMANOVA + v1.0.1 statistical package (Clarke and Gorley, 2006). In addition, Pearson's correlation coefficient was used to determine if otolith elements were significantly correlated with water elements.

2.4.2. Nursery area assignment

To assess the ability of estuarine elemental fingerprints to successfully classify early life stages of fish to their estuary of origin, the random forest (RF) classification method was used (Brieman, 2001). The main advantage of RF models is that they make no assumptions on variable distributions or linear relations between variables (Mercier et al., 2011). Therefore, all elements were used as predictors. For each of the individual measures performed on the otoliths, the RF predicts habitat origin by running it through 5000 trees of a classifier (built from known signatures from all habitats). Different combinations of cohorts were used to train the Random Forest models in order to assess the potential influence of temporal variability on classification performance. Given that adults sampled in 2018 could originate from either the 2016 or 2017 cohorts based on size-derived age estimates, we first explored cohort-specific assignments consistent with this biological uncertainty. In addition, because age estimates based on body size indicated a predominant contribution of the 2017 cohort, this year was also considered as a single-cohort scenario. Finally, a pooled dataset including 2016, 2017, and 2018 was used to construct a generalized model not constrained by year-specific baselines, thereby increasing sample size and allowing evaluation of overall temporal robustness. In our case, for every cohort group, a portion of the dataset (2/3) was randomly selected to build the RF classifier, and the remaining portion (1/3) was used to calculate the global classification accuracy and the percentage of correct re-assignments for each location (cross validation procedure). The contribution of each element to the global success of discrimination was evaluated using the mean decrease in the Gini index (Archer and Kimes, 2008): the higher it is, the more discriminative the element. In addition, to improve the reliability of the classifications and predictions, multiple RF models were applied (n = 1000) changing the dataset selected in every cohort group for the construction and cross-validation for every model, and the average and standard deviation of their parameters generated. Every model was applied to predict the nursery area used by adults collected in each fishing grounds (PU and SL) at the same size and time period using the *predict* function of 'RandomForest' package (Liaw and Wiener, 2002) performed in R 4.3.3 (R Core Team, 2024).

3. Results

3.1. Spatial and temporal variation in anchovy abundances

The abundance of early life stages of anchovy varied markedly among locations (Fig. 3). The Guadalquivir estuary consistently showed the highest densities across all years, clearly exceeding those observed in the other locations. The Guadiana estuary presented the second highest values, although this pattern was mainly driven by a pronounced peak in 2017. In contrast, Cadiz Bay, Odiel-Tinto, and the OUT locations exhibited comparatively low densities throughout the study period (2016-2018).

3.2. Spatial and temporal variation in otolith element concentrations

Otolith elemental concentrations differed significantly among locations (GN, OT, GQ, CB and OUT), both in terms of individual elements and as a multivariate fingerprint (Fig. 4, Table 3). Specifically, P, K, Fe, Zn and Ba differed significantly among locations, with K being consistently higher in fresher estuaries (GQ and GN) and Ba being lower in the more saline estuary (CB) and the outer sampling sites (OUT). Conversely, otolith Sr concentrations did not differ significantly among locations. Interestingly, otoliths from OT exhibited significantly higher concentrations of Ba, Fe, Cu, Zn, and Pb. Pairwise comparisons of multivariate elemental fingerprints among locations showed that OT estuary was different from all other locations, and GQ was different from all other locations except GN (Table 4).

The PERMANOVA analysis also revealed temporal variations in the multivariate otolith fingerprint (Tables 3 and 4), with pairwise comparisons indicating no difference between 2016 and 2018, both years being signfiicantly different to 2017. Temporal inconsistencies at the individual element level were observed for Na, K, Co and Ba. Cu and Pb exhibited significant location × year interactions, while most elements showed additional spatiotemporal heterogeneity, often involving significant interactions between location, year and/or station (Table 3).

3.3. Relationship between water and postlarval otolith element concentrations

The chemical composition of water, in its multi-element comparison, varied spatially but not temporally (Table S3, Fig. S1 and S2). Locations with a longitudinal salinity gradient, such as GQ and GN, differed from the rest but not from each other, as did the marine areas CB and OUT (Table S4). Estuaries receiving a higher freshwater input (GQ and GN) exhibited lower concentrations of Ca, K, Na, Mg, and Sr but higher levels of Ba. OT, however, was the only location distinct from all others, containing higher levels of metals such as Cu, Mn, and Zn. At the individual element level, all elements showed spatial variations except Pb, while temporally, no differences were observed for Ca, Mn, and Sr.

The correlation revealed consistent patterns in the relationship between elemental concentrations in water and in the postlarval otoliths of anchovies (Fig. S3). Cu, Zn, Sr, Pb, Ba, and Fe consistently show significant positive correlations, suggesting that these elements are incorporated systematically from the environment into the otoliths, regardless of whether the data are standardized by Ca. Also, P showed a positive correlation, which became higher and statistically significant only after standardization by Ca. Similarly, K and Mg consistently show significant negative correlations in both cases.

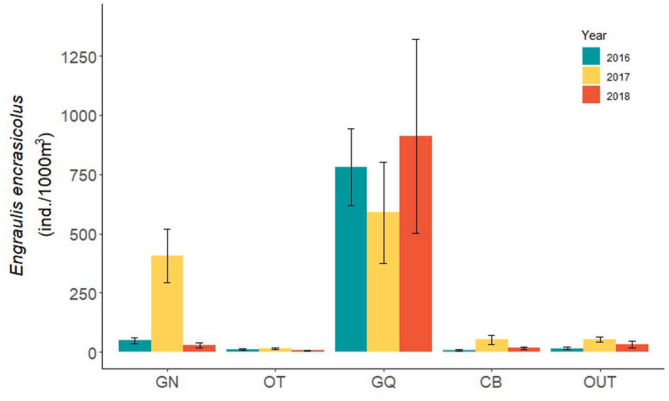

Fig. 3. Total abundance (mean ± SE) of early life stages of *Engraulis encrasicolus* per location and year during the studied period (2016–2018). Location codes: Guadiana (GN), Odiel-Tinto (OT), Guadalquivir (GQ), Cadiz Bay (CB) and coastal areas outside the estuary mouth (OUT) (Fig. 1).

3.4. Nursery area assignment model performance

The performance of the RF models, based on a subsample of postlarvae (test dataset) excluded from the training dataset, was generally high (accuracy: 72.5-80.1%), but varied slightly depending on the cohort(s) used for training the model (Table 5 and S7). As the models trained with the largest sample size (all cohorts combined, 2016–2018) showed the highest classification accuracy (80.1%; Out-Of-Bag: 20.9%) we used this for the assignment of the adults (see below). Balanced accuracy to each source was also consistently highest across locations in the combined-cohort model (Table 5).

Across all cohort training groups, assignment accuracy indicated by a confusion matrix was generally high for the different locations (Table S6). In general, the fresher estuaries (GQ and GN) were most frequently misclassified between, and the same for the more saline estuaries and outer coast location (CB, OT and OUT) (Table S6).

Among the otolith elements that were most important for discriminating among locations (inferred via the Gini index; Fig. S4), Zn was the most important element, followed by Ba and Cu. Consistently across all models, Zn, Ba, Cu, P and Pb were among the most important predictors of location, and also exhibited positive correlations with water concentrations (Fig. S3). Conversely, otolith Fe, Sr, Ni and Co showed low importance for location discrimination.

3.5. Nursery area assignments of adult samples

Overall, the percentage of adults that were assigned to an estuarine nursery areas GN, GQ, OT and CB (excluding the OUT assignment given that these likely also represent any coastal area) was 21.4%. This appeared to differ among fishing areas, with the adults fished from Punta Umbria area showing higher estuarine use (26% of individuals) than those caught in Sanlucar de Barrameda (17.5% of individuals). The remaining individuals (74-82.5%) were assigned to the outer coast sites, and thus may have grown up in the estuary mouths and/or along coastal beaches (that were not included in our baseline). Guadalquivir and Odiel-Tinto showed negligible contribution across all model scenarios (Table 5, Table S7). While some variations in estuarine contribution rates were observed between PU and SL, the contribution rate rankings were consistent for both fishing areas. While differences in the baseline were observed among years (Table 3), suggesting that a cohort-matched baseline sample (2017 only) would be best, the low numbers of samples created instability in the 2017-only models (Table S7) and the assignment results were very similar, independent of which cohorts were included in the training models. Cadiz Bay showed the most variable contribution rates depending on the cohort, ranging from 6.7% in the models used (all years combined) to 32.3% using the 2017-only models.

4. Discussion

Despite the ecological and commercial importance of European anchovy (*Engraulis encrasicolus*), their use of estuarine and nearshore habitats throughout early life stages remains poorly understood. For

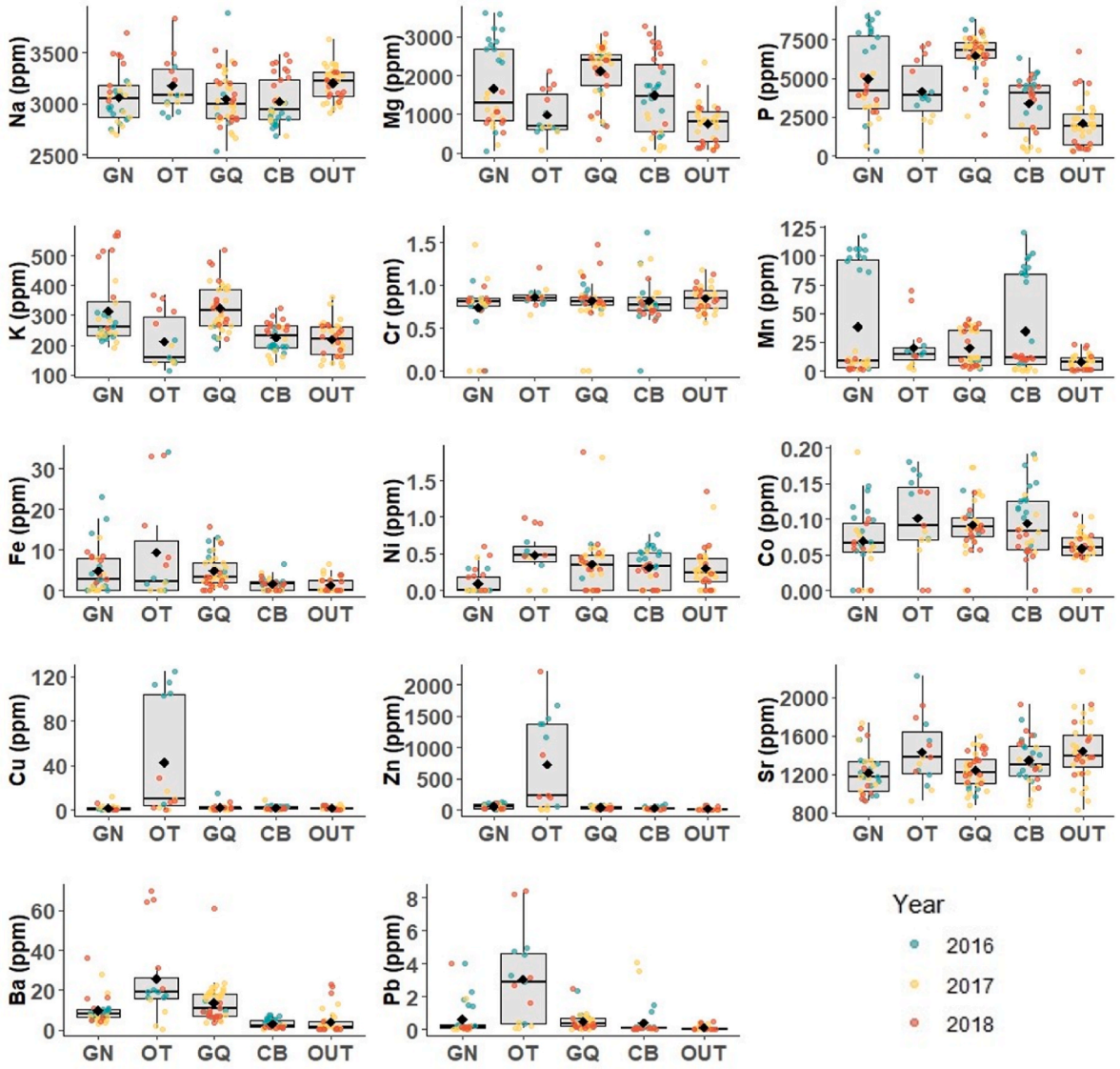

Fig. 4. Boxplots of element concentration (Na, Mg, P, K, Cr, Mn, Fe, Co, Ni, Cu, Zn, Sr, Ba, Pb) in the outermost growth layers of otoliths of postlarval anchovies sampled from four estuaries and the adjacent coast in 2016 (blue), 2017 (yellow) and 2018 (red). Black points indicate mean value of each location. Location codes: Guadiana (GN), Odiel-Tinto (OT), Guadalquivir (GQ), Cadiz Bay (CB) and coastal areas outside the estuary mouth (OUT) (Fig. 1). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

many marine fishes, estuaries are assumed to play an important nursery function, supporting larval and juvenile development. However, this is typically based on juvenile abundance data, and their contribution to the adult population is often uncertain (Beck et al., 2001; Freeman et al., 2024). Otolith chemistry provided us with unique insights into the relative contributions of estuarine and coastal nursery areas, where we find up to ~20% of adult anchovies in the Gulf of Cadiz are linked to estuary nurseries, with the remaining ~80% assigned to coastal sites sampled by estuary mouths. Collectively, these results highlight the importance of preserving, restoring and protecting functional estuarine and coastal habitats for sustainable management.

4.1. *Otolith fingerprints and classification*

Some studies have analysed the chemical composition of otoliths to establish nursery area signatures and estimate the relative contributions to adult stocks (e.g., Reis-Santos et al., 2013; Anstead et al., 2016). However, their application to early life stages of European anchovy had not been tested, despite the species' major ecological and economic importance along European and African coasts. The results of the present study demonstrate that this technique is a valid tool for discriminating among nursery areas, even when using very young individuals (~20 days old). These findings confirm the indications suggested by Guidetti et al. (2013), who detected different spawning and nursery areas in this species by analyzing the core region of adult otoliths from the Ligurian Sea. Nevertheless, the analyses showed potential annual variations in otolith composition, which is typical of these dynamic ecosystems. For this reason, several authors recommend that connectivity studies of this type be conducted on an annual basis (Gillanders, 2002; Reis-Santos et al., 2012). However, pooling data across multiple years, combined with careful selection of key elements, can achieve successful classification and allow broader application of the developed tool (Brown, 2006; Tournois et al., 2013). This is the case in the present study, where the most accurate and consistent classification models, including the correct assignments, were those trained with combined cohorts, particularly the pooled dataset from all years (2016, 2017 and 2018), which reached an average accuracy of up to 80%. This is likely due to the larger sample size available for both training and testing and

Table 3
Multivariate and univariate results of PERMANOVA testing spatial and temporal variation in otolith elements for postlarvae of European anchovy. Where 'location' represents capture estuary (CB, GN, GQ, OT) or coastal region (OUT), and 'station' represents the specific collection site, which was included as a random effect.

Source	df	Pseudo-F													
		All elements	Na	Mg	P	K	Cr	Mn	Fe	Co	Ni	Cu	Zn	Sr	Ba	Pb
Location	4	**4.63*****	1.59	2.69	**6.22****	**12.02*****	0.8	0.34	**4.22****	2.39	2.7	**102.4*****	**11.35***	2.03	**6.78****	**9.79****
Year	2	**3.16****	**9.42****	0.855	3.22	**17.06*****	0	3.13	4.17	**4.12***	1.9	**98.97*****	4.78	2.28	**4.28***	4.17
Loc*Year	7	1.99	1.12	1.54	1.74	2.48	0.9	1.89	2.98	1.05	1.5	**74.84*****	2.9	1.53	3.5	**4.39****
Station (Loc*Year)	22	**5.72*****	**3.17*****	**6.56*****	**5.37*****	**3.16*****	1.4	**14.91*****	1.92	**2.37****	1.8	0.4	**8.06****	**1.74***	**6.04*****	**2.80****
Res	120															

$^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

Table 4
Pair-Wise analysis of significant terms (a) Location (GN, OT, GQ, CB, OUT) and (b) Year (2016, 2017, 2018) from multivariate results of PERMANOVA in otolith elements for postlarvae of European anchovy.

a) Location			b) Year		
Groups	t	P(perm)	Groups	t	P(perm)
CB - OUT	0.92	0.4614	**2016–2017**	2.77	**0.0035**
CB - GN	1.42	0.137	2016 - 2018	0.98	0.3959
CB - GQ	2.84	**0.0013**	**2017–2018**	1.82	**0.0441**
CB - OT	3.71	**0.0004**			
OUT - GN	1.09	0.3119			
OUT - GQ	2.40	**0.0168**			
OUT - OT	2.39	**0.0154**			
GN - GQ	0.87	0.5017			
GN - OT	2.32	**0.014**			
GQ - OT	3.39	**0.0011**			

the generalized temporal consistency observed in the water chemical composition over the years. At the same time, the temporal differences observed in the otolith chemical composition of the 2017 cohort, compared to other years, align with the finding that the most important elements for its classification differ partially from those identified in the other cohorts.

In addition, multi-element analyses of the water samples did not reveal significant temporal differences, suggesting that inconsistencies in otolith composition might be influenced by variations in estuarine residence time prior to sampling, as well as by the temporally integrated nature of otolith elemental composition over longer periods. In contrast, spatial differences were evident, with characteristic and consistent patterns observed in the water chemistry at each location. Estuaries with greater freshwater input, such as GQ and GN, which generate marked salinity gradients, showed lower concentrations of Na, Mg, K, Ca and Sr, and higher levels of Ba, whereas marine environments like CB, OT and OUT exhibited the opposite pattern (except to Ba in OT).

Barium (Ba) and Strontium (Sr) are among the most widely used markers for detecting fish migrations across salinity gradients (Walther and Limburg, 2012; Carlson et al., 2016), though their effectiveness can vary by species and systems (Sturrock et al., 2012). In European anchovy, while no controlled experiments have yet confirmed elemental incorporation patterns, several field studies have employed Sr as a salinity marker (Aldanondo et al., 2010; Morais et al., 2010; Catalán et al., 2014). In our study, higher Sr concentrations in water were observed in marine environments (OT, CB, and OUT) compared to brackish ones (GQ and GN), showing a direct relationship with salinity (Fig. S5A), as expected. However, significant among-estuary differences in Sr concentration were not found in otoliths, despite the positive correlation observed. Conversely, Ba concentration in water showed a strong negative relationship with salinity (Fig. S5B), with higher values in estuaries receiving greater freshwater input (GQ and GN). Ba also exhibited a weaker, yet significant, positive correlation with otolith concentration, and larger differences among locations were detected. This difference among elements likely reflects the importance of considering element:Ca ratios in the water rather than absolute concentrations, with the among-estuary differences in water Sr:Ca being far smaller than for water Ba:Ca (Fig. S2). Indeed, previous studies have observed unexpected relationships between otolith Sr:Ca and salinity in estuaries in different species and systems (Morales-Nin et al., 2012; Mohan et al., 2015), with most of the literature using otolith Sr as a salinity marker focused on diadromous species that move between fully fresh and marine waters (Sturrock et al., 2012). As in previous studies (e.g. Elsdon and Gillanders, 2005; Mohan et al., 2015), the results presented herein suggest that otolith Ba may be a more sensitive salinity marker for elucidating movement from sea to estuaries when the salinity difference is more subtle.

Interestingly, there was one clear outlier to this pattern, with the highest Ba concentrations observed at OT, an estuary characterized by

Table 5
Results from multiple random forest models (n seeds = 1000) trained using all cohorts (2016-2018) and their application to adult fish to estimate nursery contribution rates. OOB: out-of-bag.

% Accuracy (Mean ± SD)	% OOB error (Mean ± SD)	Location	% Balanced Accuracy	Estimation of Contribution rates (%)					
				ALL SAMPLES (PU + SL)		PUNTA UMBRIA (PU)		SANLUCAR (SL)	
				Mean ± SD	Range (max.-min.)	Mean ± SD	Range (max.-min.)	Mean ± SD	Range (max.-min.)
80.1 ± 5.4	**20.9 ± 2.7**	Guadiana	87.8 ± 5.9	14.0 ± 8.1	0-43	15.6 ± 10.1	0-53	12.6 ± 8.8	0-40
		Odiel-Tinto	90.5 ± 8.7	0.01 ± 0.4	0-11	0.02 ± 0.6	0-18	0.00 ± 0.2	0-5
		Guadalquivir	90.9 ± 4.9	0.7 ± 1.6	0-8	0.3 ± 1.4	0-12	1.1 ± 2.7	0-10
		Cadiz Bay	86.6 ± 6.7	6.7 ± 6.5	0-73	10.1 ± 8.9	0-65	3.8 ± 5.7	0-80
		OUT	87.1 ± 5.5	78.6 ± 7.7	27-95	74.0 ± 8.7	35-100	82.5 ± 8.3	20-100

marine-like salinity. This estuary is historically affected by mining activities (over 4500 years), draining the world's largest sulfide ore deposit (Nelson and Lamothe, 1993; Olías and Nieto, 2015). The mixture of acid mine drainage, industrial effluents and seawater has significantly influenced the chemical composition of the estuarine waters (Carro et al., 2019). Indeed, OT has the lowest mean pH recorded among estuaries in the Gulf of Cadiz (Miró et al., 2020), which may have increased Ba bioavailability, as free Ba^{2+} ions are negatively correlated with pH (Turner et al., 1981), thus could explain the higher Ba concentrations incorporated into the otoliths of some individuals. On the other hand, Ba concentration in sediments measured at several study sites during the summer of 2016 (personal data; Fig. S6) showed much higher concentrations in OT, indicating the source and accumulation material of this element. This pattern is consistent with the fact that sulfide reaction with barium salts produces barium sulfide, a solid that can precipitate and accumulate in sediments (Shikazono, 1994). These processes may help explain why high Ba levels in sediments are not reflected in the water column and may influence the correlations observed between water and otolith Ba concentrations.

The pattern observed for K deserves particular attention, as it was the only element showing clear and statistically significant differentiation (Table S5) between brackish (GQ, GN) and marine locations (OT, CB, OUT). Interestingly, this separation does not follow the expected direction based on its concentrations in the water, which exhibit a strong positive relationship with salinity given that it is a conservative element (Sturrock et al., 2012), confirmed by our own water sampling. Indeed, K exhibited a strong negative relationship between water and otolith concentrations. These patterns support previous studies suggesting that K concentrations in fish otoliths are primarily driven by physiological mechanisms (Sturrock et al., 2012), with these data suggesting a possible link to osmoregulation and Na^{+}/K^{+} pump activity, at least during early life stages.

The short lifespan of the post-larvae focused on in this study, together with uncertainty regarding the time spent in each water mass, may have reduced the clarity of otolith chemical signals despite the short (~3.6 days) temporal window analysed. Considering Ba and Sr as salinity markers for this species, Ba appears to be a sensitive marker, although caution is required because its concentrations are heavily influenced by freshwater inputs, which in turn can be affected by mining or industrial inputs, potentially complicating this signal. Here, otolith Sr showed a much weaker trend, likely due to the anchovies concentrating their distribution in brackish to marine waters, while the largest changes in water Sr tend to occur between salinities between 0 and 10 (Walther and Limburg, 2012). Overall, controlled experiments on European anchovy are needed to quantify how Ba and Sr incorporation responds to salinity under known residence times, and to distinguish environmental effects from physiological or ontogenetic influences. This would help clarify whether the weak Sr signal and the variability observed in Ba reflect environmental conditions or species-specific regulation of element uptake (Bath et al., 2000; de Vries et al., 2005).

Because the Odiel and Tinto rivers, as well as their tributaries, receive wastewater and acid drainage from numerous mines along the Iberian Pyrite Belt (one of the world's richest metallogenic regions) they are enriched in trace metals. Water samples showed high concentrations of Mn and Cu, consistent with other studies that also reported elevated levels of Co, Cd, Pb, Zn, Ni, and Mo (González-Ortegón et al., 2019). Consequently, numerous individuals from OT exhibited high otolith concentrations of Fe, Cu, Zn, and Pb. This distinct chemical signature made OT the only location clearly differentiated from all others, achieving higher balanced mean classification accuracies in the RF models (except in the 2017 cohort due to the low number of samples). Furthermore, it explains the high Gini index values observed for Zn, Ba, and Cu, where the most important elements for classification also corresponded to those with significant spatial variability in the univariate analyses (except for Fe). Additionally, positive correlations among these elements were observed, indicating their potential as markers of metal-contaminated environments. Similarly, other studies have reported comparable patterns in different species (e.g., high Mn in otoliths of juvenile sole and seabass from the Mira estuary, near an iron–manganese mine in the Iberian Pyrite Belt; Reis-Santos et al., 2012; Tanner et al., 2012) and in other regions (Arai et al., 2007; Ranaldi and Gagnon, 2008; Søndergaard et al., 2015; Andronis et al., 2017).

4.2. Contributions and nursery areas

Understanding larval and juvenile abundances in estuarine habitats, alongside their contributions to coastal adult stocks, is key for identifying effective nursery areas and assessing connectivity across life stages. This study integrates systematic field surveys and otolith chemical analyses to evaluate these processes in European anchovy populations in the Gulf of Cadiz.

The results showed a higher contribution from the offshore (OUT), ranging from approximately 65.7% to 78.6%, despite this site exhibiting some of the lowest average densities among all locations. This is probably because its large water volume (encompassing the entire Gulf of Cadiz) supports a much greater total number of recruits compared to the estuaries. Even so, it would be interesting to determine whether, within this broad offshore area, individuals are uniformly distributed and widely dispersed across the continental shelf (Spanish and Portuguese components of Division 9a South), or whether they are concentrated in localized high-density areas, indicating the presence of specific marine nursery grounds, similar as described for eggs and larvae (Baldó et al., 2006). This information is essential for refining stock structure hypotheses in the Gulf of Cádiz, improving the accuracy of connectivity estimates between estuarine and marine habitats, and ultimately supporting more spatially explicit assessment and management strategies.

On the other hand, our results highlight the significant role of estuarine ecosystems in the early development of anchovy, with estuaries contributing on average between 21.4% and 34.3% of recruits to the adult stock, the main contributors being the Cadiz Bay and Guadiana estuaries. These findings are consistent with those of Morais et al. (2010), who, based on Sr otolith transects in adults, described that adult anchovies caught off the Guadiana coast likely originated in the estuary, showing early-life migrations from inner and low-salinity zones

towards more marine waters. Interestingly, each estuary presents distinct physicochemical and hydrogeomorphological characteristics (see Miró et al., 2020): GN has a marked salinity gradient and a linear, river-like morphology with strong currents, whereas CB is mainly marine in salinity and has a deltaic morphology with multiple creeks and saltmarshes. This reflects the adaptive plasticity of this species, considered primarily marine, but capable of opportunistically exploiting different types of estuarine environments during its early developmental stages.

In contrast, the OT estuary consistently showed the lowest contribution despite presenting hydrogeomorphological features relatively similar to those of CB, suggesting unsuitable nursery conditions, likely linked to its degraded physicochemical environment. Unexpectedly, the Guadalquivir estuary, while supporting the highest densities of anchovy early life stage, exhibited the second lowest average contributions (0-0.7%). This may reflect i) sample size limitations of the adult dataset or ii) impaired connectivity between early life stages within the estuary and the offshore adult stock.

On one hand, from an early age, anchovies tend to form small groups as a protective strategy (Palomera et al., 2007). However, the process by which these small groups, whose origin or nursery area is unknown, eventually aggregate into massive schools of thousands of individuals in the offshore remains unclear. It is possible that they all originate from a single location, or from multiple sources in varying and random proportions at the time of capture. To address potential biases linked to shared origin, efforts were made to collect adult samples from different locations (PU and SL) and times (August-September 2018). Nevertheless, it must be acknowledged that obtaining truly representative samples in a system hosting billions of individuals is inherently challenging.

On the other hand, natural mortality peaks during early fish development (Garrido et al., 2015), and several factors in the Gulf of Cadiz may disrupt recruitment success. Offshore spawning areas identified near the Odiel-Tinto estuary (Baldó et al., 2006; Catalán et al., 2006; Ruiz et al., 2006) may supply larvae to estuarine nurseries, with currents facilitating transport (García-Lafuente et al., 2006). In particular, the Guadalquivir estuary's hydrodynamics favor particle retention (Losada et al., 2017; Muñoz-Lopez et al., 2024), and its high productivity offers suitable conditions for early development (De Carvalho-Souza et al., 2018). However, episodic easterly winds may induce oligotrophic conditions and advect larvae offshore, reducing survival prospects (Ruiz et al., 2009; Casaucao et al., 2021). Indeed, a study conducted during the same sampling period (2016–2018) demonstrated that high freshwater discharges from the Guadalquivir River, particularly during freshet events, can flush anchovy postlarvae out of estuarine refuges, exposing them to predation and less productive oceanic environments, with no subsequent recovery of population abundance within the estuary (Miró et al., 2023). In addition, otolith geochemical data from Catalán et al. (2014) revealed the inherent complexity and limited ability of Sr/Ca ratios to resolve habitat differences in this species and system. Still, their results showed that all offshore anchovy juveniles (~6 months old, sampled at a site comparable to our SL location) exhibited Sr/Ca signatures indicative of exposure to fluvial influence. However, none displayed the low-salinity signals expected from true estuarine residence (with a marked salinity gradient), suggesting limited connectivity between the Guadalquivir estuary and the offshore population. Altogether, these processes and evidence observed likely explain the paradox of high larval densities but low adult stock contribution from the Guadalquivir estuary found during the study period. Still, this finding deserves further investigation, as this ecosystem could contribute significantly to the adult stock depending on environmental conditions of each year (Ruiz et al., 2009).

5. Conclusions

Estuaries are recognized as important nursery areas for many marine species, and new methods are helping to elucidate connectivity between early life stages and adult populations. Our results demonstrate that otolith chemistry is an effective tool to discriminate nursery areas and estimate the contributions of estuarine and offshore habitats to the adult stock of European anchovy in the Gulf of Cadiz, and can be used as a tool to assess the fishery stock, providing useful information for regional managers. Multi-year datasets and elemental fingerprinting revealed consistent spatial patterns, with Ba providing a more reliable tracer of salinity gradients than Sr, although interpretation must consider local anthropogenic influences such as those observed in the Odiel–Tinto estuary. In addition, K shows an inverse pattern between brackish and marine locations relative to environmental concentrations, suggesting a role for osmoregulatory processes. The offshore zone accounted for the highest contribution to the adult stock, likely reflecting its larger water volume and recruitment potential. In contrast, Cadiz Bay and Guadiana estuaries acted as key nurseries, whereas the Guadalquivir estuary, despite containing the highest larval and juvenile densities, showed low contribution, highlighting potential connectivity limitations. Integrating density data with contribution estimates proved particularly valuable for identifying connectivity bottlenecks or misassigned nursery origins. These findings underscore the adaptive plasticity of European anchovy, the importance of maintaining diverse functional habitats, and the need for controlled experiments and long-term environmental monitoring to refine otolith-based connectivity assessments and inform sustainable management.

CRediT authorship contribution statement

J.M. Miró: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft. **S.E. Tanner:** Methodology, Writing – review & editing. **C. Megina:** Conceptualization, Methodology, Writing – review & editing. **I. Donázar-Aramendía:** Investigation, Writing – review & editing. **J. Dawson:** Investigation, Writing – review & editing. **C.N. Trueman:** Writing – review & editing. **T.C. Cameron:** Writing – review & editing. **J.C. García-Gómez:** Funding acquisition. **A.M. Sturrock:** Formal analysis, Methodology, Supervision, Writing – review & editing.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marenvres.2026.108229.

Data availability

Data will be made available on request.

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