



Evidence of estuarine nursery origin of five coastal fish species along the Portuguese coast through otolith elemental fingerprints

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ABSTRACT

Connectivity is a critical property of marine populations, particularly for species with segregated juvenile and adult habitats. Knowledge of this link is fundamental in understanding population structure and dynamics. Young adults of commercially important fish species *Solea solea*, *Solea senegalensis*, *Platichthys flesus*, *Diplodus vulgaris* and *Dicentrarchus labrax* were sampled off the Portuguese coast in order to establish preliminary evidence of estuarine nursery origins through otolith elemental fingerprints. Concentrations of Li, Na, Mg, K, Mn, Cu, Zn, Sr, Ba and Pb in the otolith section corresponding to juvenile's nursery life period were determined through laser ablation inductively coupled plasma mass spectrometry. Element: Ca ratios in coastal fish differed significantly amongst collection areas, except for *Platichthys flesus*, and were compared with the elemental fingerprints previously defined for age 0 juveniles in the main estuarine nurseries of the Portuguese coast. Identification of nursery estuaries was achieved for four of the species. Assigned nursery origins varied amongst species and differences in the spatial scale of fish dispersal were also found. *Diplodus vulgaris* was not reliably assigned to any of the defined nurseries. Overall, results give evidence of the applicability of estuarine habitat tags in future assessments of estuarine nursery role. Research developments on the links between juvenile and adult habitats should contribute for the integrated management and conservation of nurseries and coastal stocks.

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1. Introduction

Estuaries and their associated coastal habitats provide nursery grounds for juveniles of several fish species that occupy different habitats from the adults. Larvae or early juveniles move to near shore and estuarine habitats and remain there during months or years, depending on the species, until recruiting to marine stocks (Gibson, 1973; Desaunay et al., 1981; Miller et al., 1985; Beck et al., 2001; Able, 2005). A nursery is defined as a habitat that produces relatively more adult recruits per unit of area than other juvenile habitats used by a species (Beck et al., 2001). To identify estuarine nurseries it is then essential to recognize areas with high juvenile densities, enhanced growth and survival rates and evaluate the connectivity between estuaries and coastal areas.

The exchange of individuals amongst geographically separated groups, or connectivity, is a critical property of marine populations

(Cowen et al., 2000). Beck et al. (2001) stressed that it is essential to measure the movement of individuals from juvenile to adult habitats and that this is a vital missing link in our understanding of nurseries. Estimating connectivity is ecologically relevant in estuaries since they, or even habitats within them, may differ greatly in terms of their nursery role, contributing disproportionately to adult populations and influencing the structure of these populations (Gillanders, 2005). Such information is decisive for managing coastal fish populations, identifying ecologically important habitats and their resources, functions and services (Beck et al., 2001). Failure by fishery managers to consider stock complexity, namely origin and composition may lead to depletion of particular components with ecological consequences (Stephenson, 1999) which can critically affect the long-term stability and sustainability of entire stocks (Fritsch et al., 2007).

Artificial tagging techniques have been widely used for determining movements of fish (see reviews by Gillanders et al., 2003; Pittman and McAlpine, 2003) and although they are in constant development, particularly due to the advances in the miniaturisation of artificial tags and telemetry, they are not usually viable in small juveniles. On the other hand, recent studies with natural tags consisting of elemental composition in calcified

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structures show great promise for studying populations connectivity.

The chemical composition of fish otoliths is a valuable natural tag of habitat use due to specific otolith properties. They are metabolically inert, grow continuously and the chemical elements of the organism's surrounding environment are accreted onto the growing surface of the otolith and permanently retained (Campana, 1999; Thresher, 1999; Elsdon and Gillanders, 2003; Gillanders, 2005). The environmental history of a fish at a specific time period in the past can therefore be determined by analysing the chemical composition of the portion of otolith that corresponds to that specific time period (Brown, 2006a). Thus, coastal fish that spent their juvenile period in an estuary are expected to have an otolith elemental fingerprint which reflects their estuarine life period. To assess habitat use with natural tags, these should be consistent over time and all possible source habitats characterized (Campana, 1999).

Juveniles from distinct habitat types (Gillanders and Kingsford, 1996; Forrester and Swearer, 2002; Brown, 2006b) and different estuaries (Thorrold et al., 1998; De Pontual et al., 2000; Gillanders and Kingsford, 2000; Gillanders and Kingsford, 2003; Dorval et al., 2005; Vasconcelos et al., 2007a) have been discriminated using elemental signatures suggesting that nursery origin of adult fish can be identified through the chemical analysis of the juvenile section of adult otoliths. This has allowed the determination of the proportion of adult population that resided in different juvenile habitat types (Gillanders and Kingsford, 1996; Brown, 2006a) as well as in diverse estuaries (Thorrold et al., 2001; Gillanders, 2002). The classification of individuals of unknown origin to known populations is commonly done through discriminant function analysis (DFA) where a high frequency of correctly classified samples (i.e. greater than classification expected by chance alone) is essential (Cadurin, 2000; White and Ruttenberg, 2007).

Along the Portuguese coast several estuaries have been defined as putative nursery areas for various commercially important fish species, namely: common sole *Solea solea* (Linnaeus, 1758), Senegal sole *Solea senegalensis* Kaup, 1858, flounder *Platichthys flesus* (Linnaeus, 1758), common two-banded sea bream *Diplodus vulgaris* (Geoffroy Saint-Hilaire, 1817) and sea bass *Dicentrarchus labrax* (Linnaeus, 1758) (Costa and Cabral, 1999; Pombo et al., 2002; Vinagre et al., 2005; Veiga et al., 2006; Cabral et al., 2007; Martinho et al., 2007; Ribeiro et al., 2008). Adults of these species spawn offshore, mainly during winter. Larvae are passively transported towards the shore and in spring late-larvae and post-larvae enter inshore nursery areas such as estuaries. Juveniles remain in these nursery areas until autumn, when most leave to coastal and offshore areas even though some remain in estuarine grounds for longer periods. Whilst nurseries in shallow coastal areas have been described for these species in other geographical areas, for example *S. solea* and *P. flesus* in northern Europe (Kerstan, 1991; Riou et al., 2001; Van der Veer et al., 2001), along the Portuguese coast high juvenile densities of sole, Senegal sole, flounder, two-banded sea bream and sea bass have only been reported in estuaries. These species are amongst some of the most valuable to Portuguese fisheries and although their average landings represent only 2.2% of the total weight of marine fish captured in mainland Portugal, they represent over 14.0% of total revenue (DGPA – national directorate of fisheries and aquaculture).

In view of the commercial importance of these species and their use of or dependence on estuarine environments, it is fundamental to address the contribution of Portuguese estuarine nurseries to adult populations. To comprehensively evaluate effective nursery function, it is also important to analyse connectivity collectively for several species, rather than for a single species.

The present study tests the applicability of the juvenile estuarine fingerprints described in Vasconcelos et al. (2007a) to

identify the estuarine nursery origin of young adult fish of five commercially important fish species caught along the Portuguese coast. Multi-element fingerprints in the otolith portion that corresponds to the juvenile life period were determined. Spatial patterns of fingerprints were analysed and subsequently estuarine origin of fish was classified based on the natural tags previously defined for the eight main estuaries of the Portuguese coast.

2. Material and methods

2.1. Fish collection

Collection of young adults of *Solea solea*, *Solea senegalensis*, *Platichthys flesus*, *Diplodus vulgaris* and *Dicentrarchus labrax* was carried out along the Portuguese coast in July 2006 (Fig. 1). Since species distribution varies along the coast, specimens of each species were sampled in four coastal areas within their areas of occurrence. Sampling was directed towards the expected fish lengths, at this point in time, for fish previously sampled in estuaries by Vasconcelos et al. (2007a). Fish were obtained directly from professional fishermen operating locally in the coastal waters of the continental shelf, transported on ice to the laboratory and preserved frozen until dissection.

2.2. Otolith preparation

Ten young adults of each species, of similar length and weight, were selected from each collection area. Sagittal otoliths were

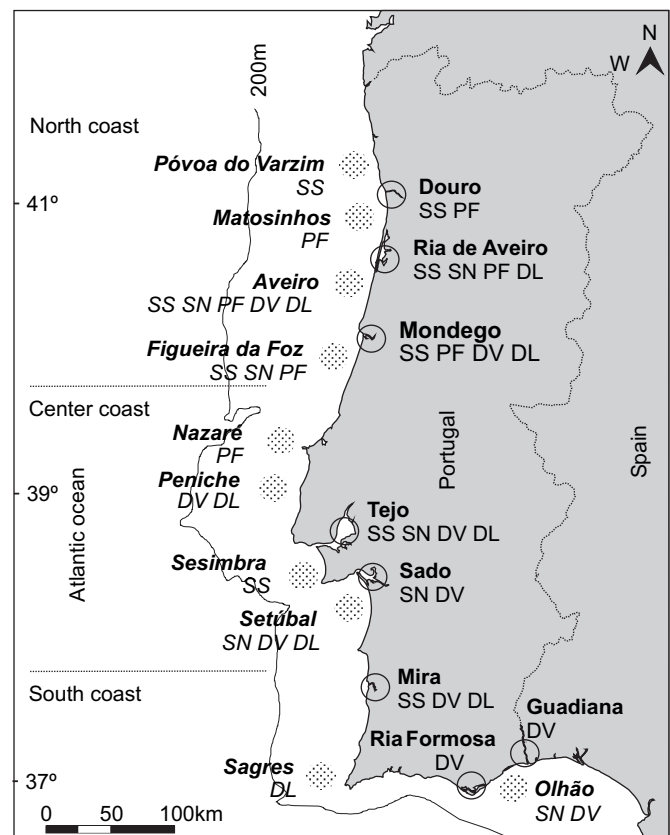


Fig. 1. Coastal sampling areas off the Portuguese coast (dotted circles ● and names in italic font) for: *Solea solea* (SS), *Solea senegalensis* (SN), *Platichthys flesus* (PF), *Diplodus vulgaris* (DV) and *Dicentrarchus labrax* (DL). Also shown, estuarine systems of the Portuguese coast (white circles ○ and names in regular font) where estuarine otolith fingerprints for juveniles of these species were previously discriminated (Vasconcelos et al., 2007a).

extracted using plastic forceps and were preserved in decontaminated Eppendorf microcentrifuge tubes. All glassware, plastic containers and implements were previously decontaminated by an acid wash in 10% analytical grade nitric acid (HNO₃) (Merck KGaA, Germany) for 24 h, rinsed with Normapur water (Prolabo, France) and dried in a laminar flow positive pressure fume hood.

Upon extraction, otoliths were cleaned and decontaminated in a laminar flow positive pressure fume hood, following a protocol adapted from Rooker et al. (2001): (1) immersion in ultrapure water to hydrate biological residues adherent to the surface of the otoliths; (2) immersion in 3% hydrogen peroxide (H₂O₂) p.a. (Pancreac, Spain) for 15 min to dissolve biological residues; (3) immersion in 1% HNO₃ solution for 10 s to remove superficial contamination; (4) double-immersion in ultrapure water for 5 min to remove acid; (5) stored in new previously decontaminated Eppendorf microcentrifuge tubes and left open until dry. The nitric acid used was p.a. quality further purified by a Sub-Boiling Distillation System (duoPUR, Milestone-MLS GmbH, Germany) and diluted with ultrapure 18.2 MΩ cm⁻¹ water (Milli-Q Element A10 Ultrapure Water Purification System, Millipore, Switzerland). For laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) analysis of the juvenile section of the otoliths, these were embedded in disc shaped moulds with epoxy resin. Once dry, otoliths were ground from the dorsal convex side surface until the core was exposed using resin bonded diamond discs, with a Struers LabPol-5 rotary polisher (600 grit wet/dry sand paper, followed by 6 μm, then 1 μm diamond powder slurries) and cleaned with ultrapure water.

2.3. Sample analysis

In this work, a Nd:YAG 266 nm laser system (LSX-500, Cetac Technologies, Omaha, NE, USA) was utilized (Guillong et al., 2002). The laser was used in scanning mode with a 200 μm laser spot diameter and a scan speed of 100 μm s⁻¹. Sampling area on the polished otolith was chosen manually around the otolith core and up to 2 × 2 mm. This area was defined to cover the otolith section formed while fish are in a nursery area and corresponds to the otoliths sampled in age 0 juveniles by Vasconcelos et al. (2007a) and is hereafter referred to as otolith juvenile section. The laser was operated with a repetition rate of 10 Hz, a laser pulse width of <6 ns and a constant fluence of 12 J cm⁻². The volume of the ablation cell was 28 cm³ and the tube length to the ICP-MS was approximately 50 cm. Helium was used as ablation gas, leading to less condensation of ablated particles on the sample surface (Eggins et al., 1998). Prior to introducing the helium gas flow, containing the laser-induced aerosol into the ICP-MS, an argon gas flow was admixed after the ablation cell to the laser-generated aerosol (Günther and Heinrich, 1999).

A commercially available double-focusing sector field ICP-MS (ICP-SF-MS, Element 2, Thermo Fisher Corporation, Germany) was used for the mass analysis of the laser-induced aerosols. Improved settings, like fast-scan speed and optimized ion generation processes of the ICP were utilized to increase the detection power of the ICP-MS in combination with high spatial resolved laser ablation and sampling (Latkoczy and Günther, 2002). The instrument was operated in medium mass resolution ($m/\Delta m = 4000$) to avoid spectral interferences and the following isotopes were selected for the analysis: ⁷Li, ²³Na, ²⁵Mg, ³⁹K, ⁴²Ca, ⁴⁸Ca, ⁵⁵Mn, ⁶⁵Cu, ⁶⁶Zn, ⁸⁶Sr, ⁸⁸Sr, ¹³⁷Ba and ²⁰⁸Pb. The interference of the main isotope of ⁴⁸Ti with ⁴⁸Ca can be neglected, since titanium is only present in ultra trace amounts and therefore does not significantly affect the signal intensities of ⁴⁸Ca in otolith samples. Two isotopes were read for Ca and Sr in order to cross-check the occurrence of any possible interferences (e.g. ⁴²Ca/²⁶Mg¹⁶O, ⁸⁶Sr/⁴⁰Ar¹⁴N¹⁶O¹⁶O). For quantification of calcium and strontium, the isotopes ⁴²Ca and

⁸⁸Sr were used. The optimization procedure of the LA-ICP-SF-MS was performed using a standard reference glass material (NIST SRM 612) and included maximum signal-to-noise ratio for Li, In, and U together with lowest oxide formation in the plasma, monitored on the ThO⁺/Th⁺ ratio (Wang et al., 2006). The procedure for data acquisition and calculation of transient analyte signals is derived from Longerich et al. (1996) employing the factory supplied time resolved software and transferring the raw data to a spreadsheet program for evaluation and quantification. Otolith samples were read in random order. For external quantification, a fish otolith reference material FEBS-01 (National Research Council Canada, Institute for National Measurement Standards, Canada) pressed into a pellet was used (Sturgeon et al., 2005). Calcium was used as internal standard element. The calcium concentrations for all investigated otolith samples were determined prior by liquid ICP-MS analysis. For quality control of the measurements, another pressed pellet fish otolith standard reference material, NIES-022 (National Institute for Environmental Studies, Environment Agency of Japan, Japan), was included into the analysis sequence (Yoshinaga et al., 2000). The precision or reproducibility ($n = 10$) for the reference samples was 2–10% relative standard deviation (RSD) for the elements Na, Mg, K, Sr and Ba with corresponding deviations from the reference values between 3 and 8%. For the lower abundant trace elements (Li, Mn, Cu, Zn and Pb), the precision was up to 35% with deviations from the reference value up to 25%. These limited values for the trace elements can be explained by the lack of well certified values for these elements in the certification process. The given reference values in the certificate are based on analytical values obtained in a single laboratory and are, therefore, of limited reliability. Thus, these high deviations do not reflect the quality of the obtained analytical data for lower abundant trace elements. A summary of both laser and ICP-MS parameters is given in Table 1.

Table 1

Instrumental parameters for otolith analysis by laser ablation inductively coupled plasma sector field mass spectrometry (LA-ICP-SF-MS)

LA parameters (266 nm Nd:YAG)	
Spot diameter (μm)	200
Repetition rate (Hz)	10
Energy density (J cm ⁻²)	20
Helium carrier gas flow (L min ⁻¹)	0.8
Ablation cell volume (cm ³)	28
Tube length (cm)	50
Measuring mode	Scanning
Scan speed (μm s ⁻¹)	100
Distance between lines (μm)	200
Scanned area (μm ²)	2000 × 2000
ICP-SF-MS parameters (element 2)	
RF power (W)	1250
Argon make up gas flow rate (L min ⁻¹)	0.9
Argon auxiliary gas flow rate (L min ⁻¹)	0.6
Argon cooling gas flow rate (L min ⁻¹)	16.0
Sampling and skimmer cones	Platinum
Guard electrode	Platinum (off)
Acquisition mode	E – scan
Mass window (%)	100
Sample time (ms)	2
Segment duration (ms)	40
Scan duration (ms)	1100
ThO ⁺ /Th ⁺ (%) (measured on NIST SRM 612)	<0.2
Mass resolution ($m/\Delta m$)	4000

2.4. Data analysis

Concentrations of trace elements (originally in pg g^{-1}) were transformed to ratio to Ca concentration (element: Ca ratio). Raw data of each element were checked for normality and homogeneity of variances and all elements were normally distributed after \log_{10} transformation. Throughout the statistical analysis all assumptions, including normality, homocedasticity and homogeneity of variance–covariance matrices, were met and the fiducial limit set at 0.05.

2.4.1. Differences in elemental composition of the juvenile section of coastal fish otoliths

Univariate analysis of variance (ANOVA) and multivariate analysis of variance (MANOVA) were used to explore the spatial patterns of single and multi-element fingerprints in the young adults of each of the five fish species along the Portuguese coast, in order to detect if the composition of the otolith juvenile section differed amongst fish from several coastal areas.

2.4.2. Nursery origin of coastal fish based on estuarine otolith elemental fingerprints

In a previous study, estuarine nursery otolith fingerprints were identified for juveniles of *Solea solea*, *Solea senegalensis*, *Platichthys flesus*, *Diplodus vulgaris* and *Dicentrarchus labrax* in July 2005 in the estuaries of their occurrence, within the eight main estuaries of the Portuguese coast: Douro, Ria de Aveiro, Mondego, Tejo, Sado, Mira, Ria Formosa and Guadiana (Vasconcelos et al., 2007a). In that study, concentrations of Li, Na, Mg, K, Ca, Mn, Cu, Zn, Sr and Ba were determined using solution based ICP-MS in whole otoliths (c. 10 individuals per estuary and species) and estuarine fingerprints characterized using linear discriminant function analysis (LDFA). Jackknife procedure was used to estimate reclassification success and null hypothesis that the observed reclassification success is no better than expected by random chance was checked with the randomization technique described in White and Ruttenberg (2007). Classification of unknowns in the discriminant analysis was done with uniform priors since there is no information on prior probabilities (White and Ruttenberg, 2007). In average 82% of juveniles were correctly classified to their estuaries of origin and results varied with species (from 70.2% in *S. solea* to 92.0% in *P. flesus*) and also with estuary (in average from 100.0% for the Douro and 72.7% for the Mondego). In general, element concentration varied with species and estuary. All elements were used in the discrimination of estuaries and the set of elements most relevant for statistical discrimination differed with species. Average posterior group membership probability of correctly classified juveniles was 0.965.

In order to identify the juvenile nursery estuary of the fish caught off the coast, the elemental composition of their otolith juvenile section was classified through the linear discriminant functions previously parameterized with juveniles' otoliths. Since element concentrations in otoliths of juveniles and adults were determined using different ICP-MS techniques, residuals were

calculated by subtracting the grand mean (across all locations) to an individual value for a given element (Thorrold et al., 2001) and used to run the LDFA. Mahalanobis distance was used in the LDFA and classification results were organized by collection area. Frequency distributions of predicted group membership probabilities of the LDFA classification results were also determined.

3. Results

3.1. Differences in elemental composition of the otolith juvenile section of coastal fish

Significant differences in otolith composition amongst coastal sites were found with ANOVA in *Solea solea* (Zn and Pb), *Solea senegalensis* (Li, Mg, K, Zn, Sr, Pb) and *Dicentrarchus labrax* (K, Cu, Ba, Pb) (Table 2). Otolith multi-element composition in sampled areas was significantly different with MANOVA in four species (Table 3). Otolith composition differed significantly amongst *S. solea* from the four areas ($F_{10,30} = 3.6$; $P < 0.001$), namely concerning fish from Sesimbra which differed from all others. The same occurred for *S. senegalensis* ($F_{10,30} = 3.89$; $P < 0.001$), with individuals from Figueira different from remaining areas. Otolith composition in *Platichthys flesus* showed no significant differences in sampled coastal areas ($F_{10,30} = 0.85$; $P > 0.05$) while in *Diplodus vulgaris* ($F_{10,30} = 4.61$; $P < 0.001$) only between Aveiro and Setúbal were no differences found. For *D. labrax*, differences were found amongst the four coastal areas ($F_{10,30} = 2.0$; $P < 0.05$), namely between Aveiro and Peniche.

3.2. Nursery origin of coastal fish based on estuarine otolith elemental fingerprints

Successful classification of nursery estuary according to the discriminant functions generated by data from juvenile individuals (Vasconcelos et al., 2007a) was accomplished for all species except for *Diplodus vulgaris* (Fig. 2, Table 4).

Adult *Solea solea* from coastal samples were mainly classified to the nursery areas of the Mondego and Tejo estuaries (39.4% and 45.5%), with a lower percentage of classifications to the Mondego in samples collected in the central area of the coast. All fish from P. Varzim and Figueira were assigned to the Mondego and Tejo estuaries, which also occurred for most individuals from the remaining areas (70.0%). Few fish were assigned to the Douro and Ria de Aveiro and none to the Mira estuary.

Most *Solea senegalensis* were assigned to the Sado estuary (52.9%), but classified nursery origin of fish differed along the coast with many assigned to estuaries closer to them. Figueira was the only collection site where no fish were assigned to the Sado estuary. Individuals assigned to the Ria de Aveiro were caught mainly in the north coast and specimens classified to the Tejo were caught mainly in Figueira (75.0%) and Setúbal (50.0%). Senegal sole from the southern area of Olhão were assigned to the Sado (80.0%).

Most adult *Platichthys flesus* were assigned to the Douro estuary (53.3%) and lower percentages were identified to the Ria de Aveiro

Table 2
Results of ANOVA comparisons of mean concentrations of individual elements (Li, Na, Mg, K, Mn, Cu, Zn, Sr, Ba, Pb) in the otolith juvenile section of *Solea solea*, *Solea senegalensis*, *Platichthys flesus*, *Diplodus vulgaris* and *Dicentrarchus labrax*. Each species was collected in four coastal areas off the Portuguese coast. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Species	df	P									
		Li	Na	Mg	K	Mn	Cu	Zn	Sr	Ba	Pb
<i>S. solea</i>	3,29	0.694	0.022	0.071	0.642	0.030	0.816	**	0.714	0.594	***
<i>S. senegalensis</i>	3,27	***	0.112	***	*	0.196	0.203	***	***	0.420	***
<i>P. flesus</i>	3,32	0.217	0.468	0.111	0.975	0.487	0.935	0.577	0.231	0.844	0.849
<i>D. vulgaris</i>	3,35	0.131	0.688	0.254	0.082	0.473	0.200	0.132	0.161	0.081	0.453
<i>D. labrax</i>	3,23	0.075	0.156	0.120	*	0.119	*	0.349	0.78	**	**

Table 3

Results of MANOVA comparisons of mean element (Li, Na, Mg, K, Mn, Cu, Zn, Sr, Ba and Pb) concentrations in the otolith juvenile section of *Solea solea*, *Solea senegalensis*, *Platichthys flesus*, *Diplodus vulgaris* and *Dicentrarchus labrax*. Each species was collected in four coastal areas off the Portuguese coast. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

Species	Coastal areas											
<i>Solea solea</i>		P. Varzim <i>F</i>	<i>P</i>	Aveiro <i>F</i>	<i>P</i>	Figueira <i>F</i>	<i>P</i>	Sesimbra <i>F</i>	<i>P</i>			
	P. Varzim											
	Aveiro	2.51	0.194									
	Figueira	2.83	0.131	7.00	*							
	Sesimbra	9.75	**	7.10	*	18.6	***					
<i>Solea senegalensis</i>		Aveiro <i>F</i>	<i>P</i>	Figueira <i>F</i>	<i>P</i>	Setúbal <i>F</i>	<i>P</i>	Olhão <i>F</i>	<i>P</i>			
	Aveiro											
	Figueira	5.09	*									
	Setúbal	2.12	0.137	20.40	***							
	Olhão	2.09	0.367	293.90	*	7.18	0.128					
<i>Platichthys flesus</i>		Matosinhos <i>F</i>	<i>P</i>	Aveiro <i>F</i>	<i>P</i>	Figueira <i>F</i>	<i>P</i>	Nazaré <i>F</i>	<i>P</i>			
	Matosinhos											
	Aveiro	0.66	0.736									
	Figueira	0.86	0.596	0.94	0.543							
	Nazaré	2.18	0.202	0.38	0.909	0.614	0.761					
<i>Diplodus vulgaris</i>		Aveiro <i>F</i>	<i>P</i>	Peniche <i>F</i>	<i>P</i>	Setúbal <i>F</i>	<i>P</i>	Olhão <i>F</i>	<i>P</i>			
	Aveiro											
	Peniche	4.88	*									
	Setúbal	1.10	0.450	9.47	***							
	Olhão	12.89	***	4.00	*	7.61	**					
<i>Dicentrarchus labrax</i>		Aveiro <i>F</i>	<i>P</i>	Peniche <i>F</i>	<i>P</i>	Setúbal <i>F</i>	<i>P</i>	Sagres <i>F</i>	<i>P</i>			
	Aveiro											
	Peniche	18.90	*									
	Setúbal	9.40	0.080	4.00	0.068							
	Sagres	0.82	0.820	0.50	0.820	2.00	0.505					

and to the Mondego estuaries (23.3%). Only 20.0% of the fish sampled in Matosinhos were assigned to the nearby Douro estuary while flounder from the other three areas were mostly assigned to it (66.7–75.0%).

Classification of adult *Diplodus vulgaris* using the previously established juvenile estuarine tags presented very high Mahalanobis distances. These were up to 20-fold higher than those obtained between any juvenile individual and the centroid of its classification group. Considering this and the differences between adult and juvenile residuals (Fig. 3) these individuals could not be reliably assigned to any of the analysed estuarine nurseries.

Sampled *Dicentrarchus labrax* were mostly classified to the Mondego (41.4%) and Tejo (34.5%) estuaries and only the Mondego nursery was identified in all four areas. Fish from Aveiro and Sagres were predominantly assigned to the Mondego estuary, and fish from Peniche and Setúbal were mostly classified to the Tejo estuary. Only 6.9% of sea bass were identified as originating from the Mira nursery.

A similar pattern for some elements was observed between the otolith element residuals in juveniles of each estuary and the young adults assigned to those estuaries through LDFA (Fig. 3), namely for elements important in the discrimination of estuaries in Vasconcelos et al. (2007a). This was perceptible for *Solea senegalensis* (Na, Sr, Mn and Cu), *Platichthys flesus* (Li, Na, Mn) and *Dicentrarchus labrax* (Sr, Ba, Pb) and less for *Solea solea* (Li, Mn, Zn).

Frequency distributions of posterior group membership probabilities varied with species (Fig. 4). Posterior probabilities for *Solea solea* were in general lower than the ones found for the remaining species with only 6.1% of *S. solea* presenting probabilities above 0.7. For *Solea senegalensis*, *Platichthys flesus* and *Dicentrarchus labrax* posterior probabilities were above 0.8 for the majority of the individuals, 71.1%, 77.8% and 65.5%, respectively. No particular estuary

or coastal area was responsible for lower posterior group membership probabilities and no relationship was detected between lower posterior probabilities and an increase in the distance between collection area and estuarine nursery.

4. Discussion

Identification of estuarine nursery origin of coastal young adults collected in several areas off the Portuguese coast was successfully achieved for four of the analysed species based on the previously defined juvenile elemental fingerprints (Vasconcelos et al., 2007a). Results support the applicability of otolith elemental fingerprints to assess nursery function in this set of estuaries and species, providing a baseline and research directions for future assessments of the proportional contribution of these sites. Fish sampled in coastal areas had diverse nursery origins, both in and between collection areas, with different proportions of individuals assigned to each of the estuarine nurseries.

4.1. *Solea solea*

Young adults of common sole sampled along the Portuguese coast portrayed the existence of individuals with distinct nursery origins dispersed throughout the coast. This is consistent with reports for other areas, namely for the Bay of Biscay where offshore dispersal of late juveniles and adults resulted in a well mixed homogeneous population within the entire bay (Koutsikopoulos et al., 1995). Additionally, in the Irish Sea and coast of England Symonds and Rogers (1995) observed that juveniles from the nursery grounds also dispersed into wide areas but once adults recruited to an area they tended to remain there. It is not possible to infer, with current results, in what time frame migrations occur, or even if adult fish

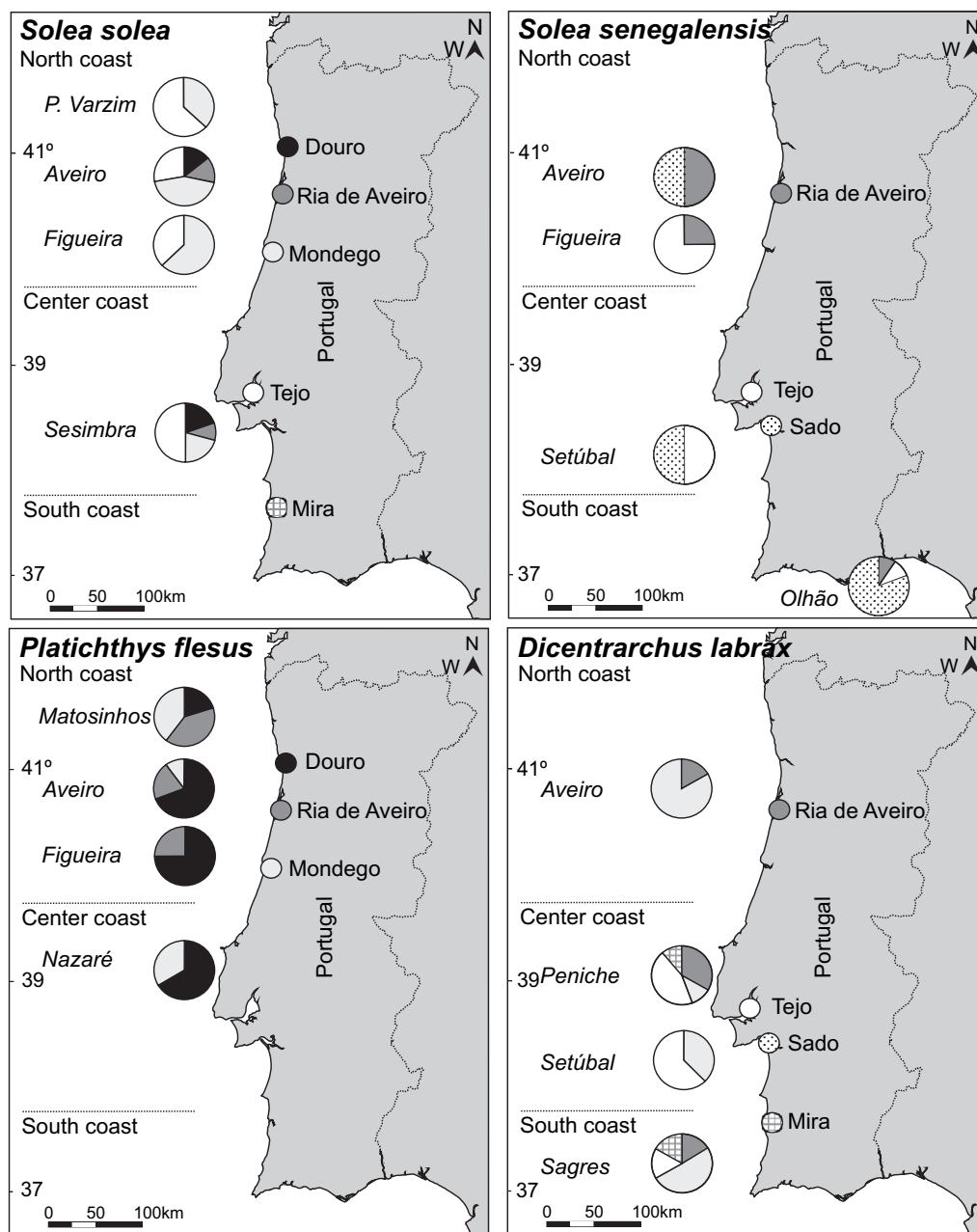


Fig. 2. Predicted estuarine nursery origin (%) of *Solea solea*, *Solea senegalensis*, *Platichthys flesus* and *Dicentrarchus labrax* sampled in each coastal area. Fish were collected in four coastal areas off the Portuguese coast and classified to a nursery estuary based on linear discriminant functions generated by otolith multi-element fingerprints of juveniles collected in the estuarine nursery areas of: Douro ●, Ria de Aveiro ●, Mondego ○, Tejo ○, Sado ○ and Mira ○ (Vasconcelos et al., 2007a). Results for *Diplodus vulgaris* are not shown, as they could not be reliably assigned to an estuarine nursery.

will remain in these specific areas, only that sampled *Solea solea* seem to have mixed nursery origins in the Portuguese coast.

Higher percentages of fish originating from an estuarine nursery area are most likely due to the exportation of a higher number of successful recruits from that estuary. Though results suggest the importance of the Mondego and Tejo estuaries some aspects should not be overlooked. Classification results of juvenile *Solea solea* to different nursery grounds (70.2%) were the lowest of the five species in Vasconcelos et al. (2007a), which may possibly explain the observed poorer posterior group membership probabilities in young adults classified both to the Mondego and Tejo. In contrast, classifications to the Douro were the most reliable even if few individuals were assigned. As emphasized in Vasconcelos et al. (2007a) further research should be directed towards enhancing

estuarine juvenile elemental fingerprints of *S. solea*. New data for juveniles, namely through analysis of other elements and isotopes, are necessary to improve the precision in the identification of nursery origin in adults.

4.2. *Solea senegalensis*

In the last decades the distribution area of Senegal sole has expanded further north along the NE Atlantic and it is expected to occur more frequently (Desaunay et al., 2006), including Portuguese estuaries and coastal areas, although no study has so far described its dispersal and movement patterns. Sampled individuals of Senegal sole presented assorted juvenile nursery origins throughout the coast. Identification of estuarine nursery

Table 4

Predicted estuarine nursery origin (%) of sampled *Solea solea*, *Solea senegalensis*, *Platichthys flesus* and *Dicentrarchus labrax*. Each species was collected in four coastal areas off the Portuguese coast and classified to an estuary based on multi-element linear discriminant functions of juveniles collected in the estuarine nursery areas of: Douro, Ria de Aveiro, Mondego, Tejo, Sado and Mira

Species ^a	Predicted estuarine nursery origin (%)				
<i>Solea solea</i>	Douro 9.1	Ria de Aveiro 6.1	Mondego 39.4	Tejo 45.4	Mira 0
<i>Solea senegalensis</i>	Ria de Aveiro 20.6	Tejo 26.5	Sado 52.9		
<i>Platichthys flesus</i>	Douro 53.3	Ria de Aveiro 23.3	Mondego 23.3		
<i>Dicentrarchus labrax</i>	Ria de Aveiro 17.2	Mondego 41.4	Tejo 34.5	Mira 6.9	

^a Results for *Diplodus vulgaris* are not shown, as they could not be reliably assigned to an estuarine nursery.

origin reliably assigned most sampled fish, namely to the estuaries closer to the areas where young adults were captured.

Solea senegalensis classified to the Sado estuary nursery were well dispersed throughout the coast. This is the most southern estuarine system in this coast with acknowledged nursery grounds for this species and results suggest that it could represent an

important source nursery for coastal populations, even though reported peak densities are lower than those described for the Tejo estuary (Cabral et al., 2007). On the contrary, sampled *S. senegalensis* identified as originating from the northerner Ria de Aveiro nursery were less represented and more locally distributed. Even though sample sizes were small, differences in the results for this

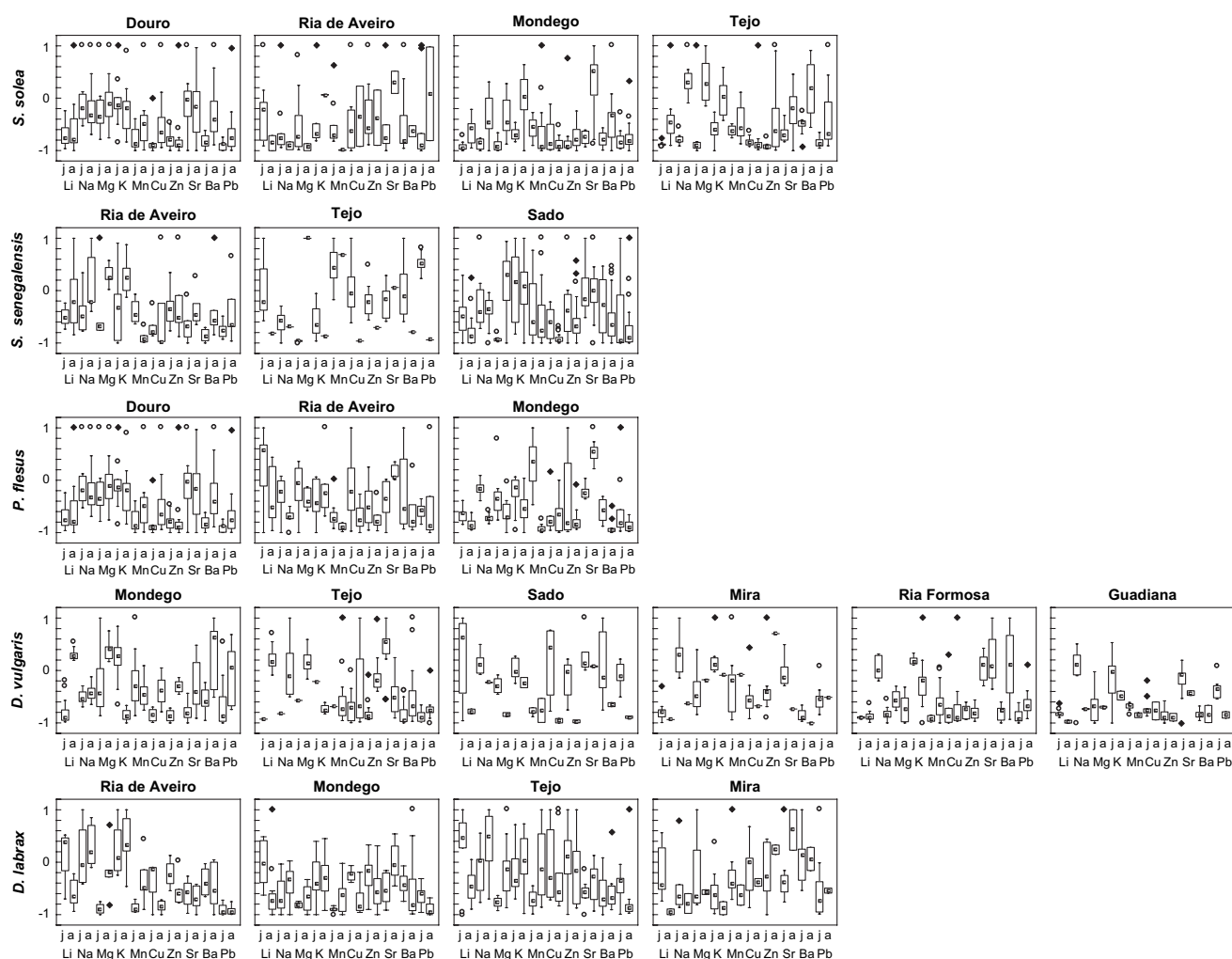


Fig. 3. Mean residual values (and standard error) of Li, Na, Mg, K, Mn, Cu, Zn, Sr, Ba and Pb concentrations in otoliths of *Solea solea*, *Solea senegalensis*, *Platichthys flesus*, *Diplodus vulgaris* and *Dicentrarchus labrax*: juvenile specimens (j) were collected in the estuarine nursery areas of Douro, Ria de Aveiro, Mondego, Tejo, Sado, Mira, Ria Formosa and Guadiana (Vasconcelos et al., 2007a); and young adult specimens (a) were collected in coastal areas off the Portuguese coast and classified to each nursery estuary based on multi-elemental fingerprints of juveniles. Outliers ○ and extremes ♦ are also represented.

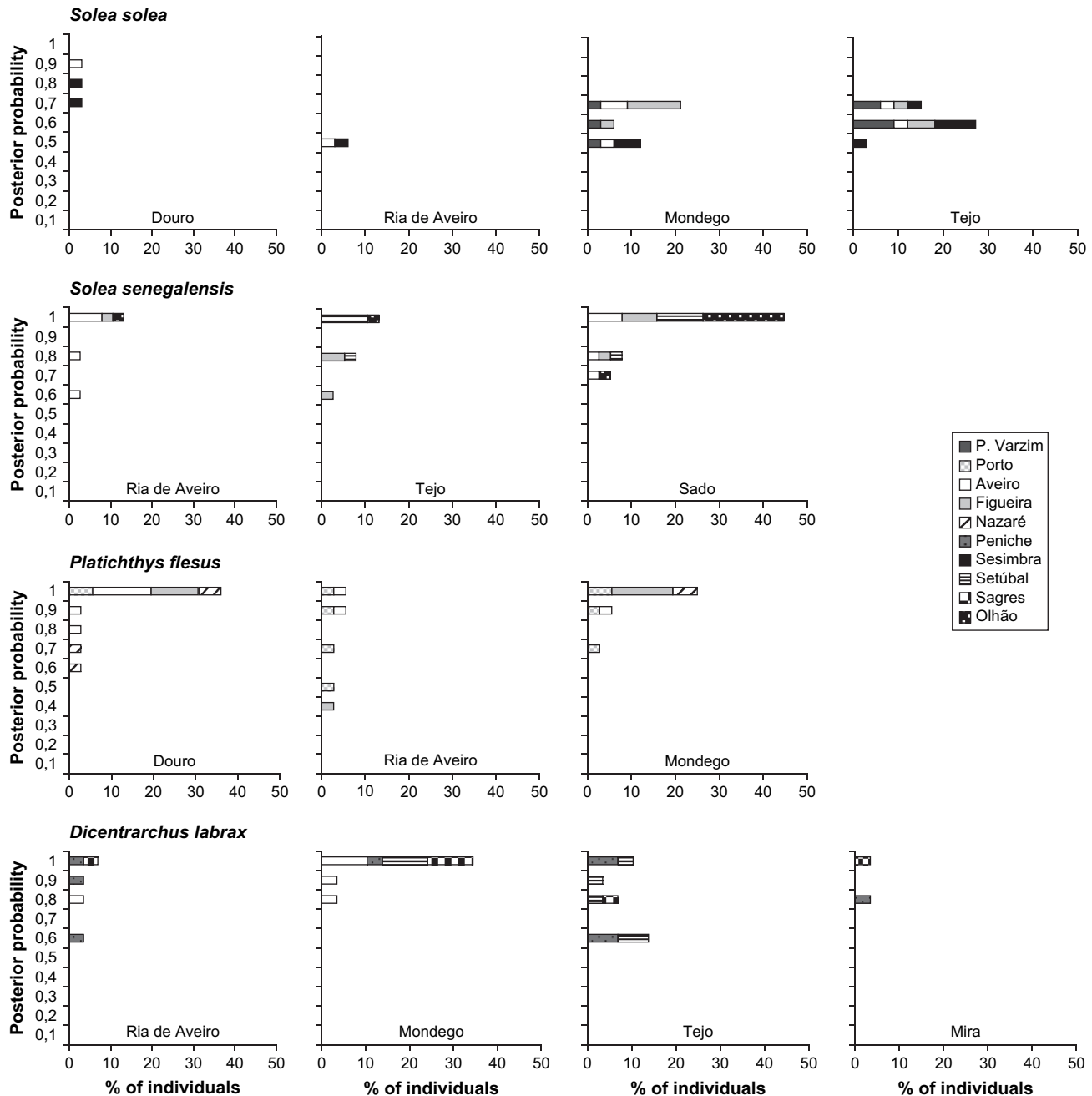


Fig. 4. Frequency of posterior group membership probabilities of the predicted estuarine nursery origin of *Solea solea*, *Solea senegalensis*, *Platichthys flesus* and *Dicentrarchus labrax*. Each species was collected in four areas off the Portuguese coast and assigned to each estuarine nursery through linear discriminant function analysis based on multi-element fingerprints of juveniles. Results for *Diplodus vulgaris* are not shown, as they could not be reliably assigned to an estuarine nursery.

species between northern and southern estuaries may arise from lower juvenile entry and success in possibly limiting environmental conditions of the more northern estuarine nurseries.

4.3. *Platichthys flesus*

Elemental compositions of the otolith juvenile section in *Platichthys flesus* sampled along the north Portuguese coast were similar and most fish were assigned to the Douro estuary. Beforehand, a high contribution from the Douro estuary to coastal populations could be expected given the particularly high abundance of this species (Vinagre et al., 2005; Cabral et al., 2007). High percentages of flounder assigned to the Douro contrast with the lower proportion of classifications to more southern estuaries which is also in

accord with reports on the decrease of flounder densities near its southern limit of distribution located along the center of the Portuguese coast (Cabral et al., 2001).

Tag recapture studies on plaice *Pleuronectes platessa* L., 1758, a Pleuronectidae species phylogenetically close to *Platichthys flesus* (Pardo et al., 2005), on the west coast of England and Wales pointed out common large scale movements in plaice (Dunn and Pawson, 2002). This study also outlined the existence of sub-stocks of plaice in each area and another contingent in all areas that undertook permanent dispersal to other areas, including migrations from the northern Irish Sea to the southern coast of the UK. Similar complex movement patterns of *P. flesus* may occur in the northern Portuguese coast. Flounder collected in Matosinhos, close to the Douro, were classified as originating from two other estuarine nurseries.

Moreover, adult individuals tagged inside the Douro estuary have been recaptured within a fortnight dispersed up to 30 nautical miles north and south of the estuary's mouth (Damasceno-Oliveira, personal communication).

4.4. *Diplodus vulgaris*

Differences found in the multi-element otolith composition of common two-banded sea bream sampled along the coast portrayed possible distinct nursery origins. Nevertheless, classification of coastal fish rendered high Mahalanobis distances and nursery origin of young adult *Diplodus vulgaris* could not be reliably assigned to the previously characterized estuaries, even though the main estuarine nursery grounds of this species in the Portuguese coast were well defined and discriminated; particularly since juveniles of *D. vulgaris* are found in high densities in these estuarine systems, above all in Ria Formosa (Monteiro et al., 1987; Ribeiro et al., 2008). However, considering the lack of match between the elemental fingerprints in sampled juveniles and young adults, juveniles' affinity for higher salinity areas, as well as the importance of coastal areas for juveniles of this species in other geographical areas, namely in the Mediterranean (Sea Vigliola, 1999; Gillanders et al., 2001), it is not possible to rule out the existence of coastal sites in the Portuguese coast that may contribute to the marine stocks. It is therefore essential to improve the knowledge on possible coastal nurseries for this species and, in case they are found along the Portuguese coast, characterize their elemental fingerprints. In coastal areas of the Mediterranean Sea site characteristic fingerprints were not identified in juveniles of *D. vulgaris*, probably due to environment homogeneity (Gillanders et al., 2001), however, successful coastal site discrimination in other species (Geffen et al., 2003; Castro, 2007) suggests that this should be possible for *D. vulgaris* along the Portuguese coast.

4.5. *Dicentrarchus labrax*

Diverse juvenile origins were identified for sampled *Dicentrarchus labrax* along the Portuguese coast. The largest percentage of sampled individuals was reliably identified to the Mondego estuary. The Tejo estuary also had a high percentage of classifications albeit with some individuals classified with a lesser degree of confidence. To aid the application of natural tags in these cases, additional data enhancing fingerprint definition in the Tejo or of uncharacterized sources should be sought. Distant migrations of *D. labrax* have been reported in the Bay of Biscay and the English Channel (Fritsch et al., 2007) and in the coasts of England and Wales (Pickett et al., 2004). For example, in the latter, 300–500 km migrations of tagged sea bass were commonly found, with 22% of recaptures made outside the considered home regions. Results in both these studies pointed out a substantial mixing of individuals amongst regions and widespread movement of both juvenile and recruiting *D. labrax*. Present results suggest that similar widespread movements and migrations of sea bass are also likely to occur in the Portuguese coast. Hindsight might advise the use of a larger spatial spectrum of nursery sites to identify juvenile origin of species with such movement patterns.

4.6. Species comparison and overview

Identification of the estuarine nursery of origin for coastal individuals sampled along the Portuguese coast showed great promise in the application of habitat tags in four species, confirming its applicability in future assessments of the proportional contribution of these sites to coastal populations. Relevant guidelines for the application of these tags and future directions for such studies were also highlighted.

Preliminary evidence on nursery use was found in the present study. Species such as *Solea solea*, *Solea senegalensis* and *Dicentrarchus labrax* had diverse estuarine nursery origins dispersed along the coast, whilst sampled *Platichthys flesus* were found to have a predominant nursery origin. To achieve a comprehensive assessment of the nursery role of the estuarine systems along the Portuguese coast it will be fundamental to undertake a broader and comprehensive sampling design.

Studies in Europe have discriminated estuarine areas (De Pontual et al., 2000; Davaer et al., 2004; Vasconcelos et al., 2007a) and offshore stocks of marine species (Secor et al., 2002; Rooker et al., 2003; Stransky et al., 2005; Swan et al., 2006) but these have not been used to assess connectivity. Only a few studies, in North America and Australia, have identified connectivity between estuarine nursery habitats and coastal adult habitats. Estuarine origin of a Sparidae in the SE Australian coast was determined based on elemental fingerprints. Analysing 15 estuaries, results showed that an average of 89% adult fish collected in the coastal reefs originated from a group of estuaries closer to them (Gillanders, 2002). In the current study, although a smaller number of estuaries were considered the length of coast is similar (around 600 km) and even if many individuals were assigned to the estuaries closer to them, classification to distant estuaries in some species points towards wide dispersal movements.

When applying discriminant function analysis, or other methods as maximum likelihood estimation, aspects such as the possibility of classifications due to chance alone must be checked but are generally disregarded in several studies (White and Ruttenberg, 2007). Randomization tests in the present study confirmed the suitability of the discriminant functions and classification results were above those that would occur due to classification by chance alone. In addition, since all samples are necessarily classified as belonging to one of the previously established groups it is therefore essential to equate the hypotheses that some classifications might be false positives, particularly when the obtained Mahalanobis distances in the LDFA are very high, as in *Diplodus vulgaris*, or posterior group probabilities are low, as in some *Solea solea*. In nursery connectivity studies this may occur, amongst others, when: adults are from a nursery which was not previously characterized, juveniles spent a reduce time period in a nursery area or multiple nursery sites exist within one site and were not fully characterized.

To ensure the effectiveness of using elemental fingerprints in connectivity studies all possible contributing sources should be characterized (Campana, 1999). Based on this statement, elemental fingerprints characteristic of the main eight estuaries were previously accurately identified for the five species considered (Vasconcelos et al., 2007a) and provided the basis for the present study. The promising results in the identification of the nursery origin of adults suggest that, amongst other issues, future studies should undertake a more comprehensive sampling of adults, increasing sample sizes, to achieve an effective estimate of the relative contribution of each estuary to the overall coastal populations. In addition, an expansion of the study area in order to include the discrimination of estuaries in the south and northwest coasts of Spain may also be worthwhile, considering the dispersal ability of the analysed species.

Assessing contribution of estuarine vs. coastal nurseries (Gillanders and Kingsford, 1996; Brown, 2006a) for *Diplodus vulgaris*, is currently unattainable for the Portuguese coast, due to the lack of data confirming the existence of coastal nurseries. Nevertheless, this should not be disregarded and evaluated in future studies.

Elemental fingerprints have been shown to differ amongst years in some cases (Gillanders and Kingsford, 2000; Dorval et al., 2005). Analysis of elemental fingerprints of both juvenile and adults in subsequent years should focus on the temporal variability of the

tags and on the links between element concentrations in otoliths and the environment. This would allow to better understand broad scale spatial and temporal differences and to improve the applicability of natural tags.

It is necessary to combine connectivity results with density, growth and survival estimates of juvenile fish to fully understand the processes that determine the relative contribution of juvenile production from estuaries and recruitment to offshore adult stocks. This information should be used for the management of fish populations and classification of ecologically important habitats. Some of the estuaries identified as nursery of origin for most individuals are also greatly affected by anthropogenic pressures (Vasconcelos et al., 2007b). Combining information on the anthropogenic pressures affecting nursery areas with their connectivity data should promote a holistic and problem-orientated management of the selected nursery areas. Management and conservation efforts could also be more judiciously invested in particularly important habitats or estuaries, rather than focusing on all estuaries or seagrass, marsh or mangrove habitats within them (Gillanders et al., 2003), with priority given to habitats with high connectivity and nurseries for multiple species. Differential measures ought to be considered since present results suggest species contributions may differ amongst estuaries. When applicable, protection measures could include the establishment of protected areas for habitats that contribute significantly to the replenishment of adult populations.

Eggs and larvae of the studied species may be passively transported long distances before settlement and even if larval exchange rates are found to be more locally confined, as in Cowen et al. (2000), later life history stages present wide scale dispersal movements. Therefore coastal populations are most likely to be well mixed and the spatial scale of connectivity studies must be defined taking this into account if effective nursery estimates are to be obtained. The scale of migrations and movements identified in this and other studies recommend the elaboration of widespread protection plans for some of these commercially valuable species. The contribution of individual estuaries to fishing stocks and management of such resources and habitats may advocate the creation of internationally discussed and supported management plans, namely concerning the common protection of fisheries resources within the European Union framework. Stocks composed and replenished by organisms from specific nurseries that contribute to a wide coastal area, exploited by multi-national fleets, might justify a common protection by those which economically explore them.

5. Conclusions

The estuarine nursery origins of young adults of four fish species sampled along the Portuguese coast were successfully identified applying previously defined otolith elemental fingerprints. Overall, sampled fish had diverse estuarine nursery origins dispersed along the coast. Predominant nursery sources varied amongst species and differences in the spatial scale of fish dispersal were also found. To establish the effective proportional contribution of the Portuguese estuarine systems to coastal stocks further research is necessary. It is of the utmost importance to establish baseline data on juvenile densities, growth, condition, movements and survival from nursery habitats to fully understand connectivity dynamics. Ultimately, knowledge on the connectivity between estuaries and coastal marine stocks will allow the safeguard of the habitats that most contribute to the replenishment of adult coastal populations and promote integrated management plans.

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