

Otolith geochemistry discriminates among estuarine nursery areas of *Solea solea* and *S. senegalensis* over time

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ABSTRACT

Otolith geochemistry is used increasingly as a natural tag to retrospectively determine habitat use in marine fishes. It is necessary to first conduct a thorough assessment of spatio-temporal variability before attempting to use the approach to determine estuarine residency or natal origins. In particular, knowledge of temporal variation at different scales is important when such variability may confound spatial discrimination. We assayed elements and calculated the elemental ratios to Ca (Li:Ca, Mg:Ca, Mn:Ca, Cu:Ca, Sr:Ca, Ba:Ca, Pb:Ca) in otoliths of juvenile *Solea solea* and *Solea senegalensis*, collected over several months in 2006 and 2009 in Portuguese estuaries, using laser ablation inductively coupled plasma mass spectrometry (LA ICP-MS). The elemental compositions of the otoliths varied significantly between and within years in both of the species, although the within-year variability did not interfere in spatial discrimination. The overall classification accuracy of juveniles to their estuaries of origin varied among the years and species, ranging from 71.0% to 80.1%. Established elemental signatures constitute the baseline data for future assessments of connectivity between juvenile and adult populations of the two sole species.

Keywords: otolith chemical composition, temporal variability, Soleidae, connectivity, natural tags

INTRODUCTION

Movement of individuals among geographically separated subpopulations, or connectivity, is an essential component of the understanding of fish population dynamics, colonization patterns and resilience to harvesting (Cowen et al. 2007). Many marine fishes undertake movements among habitats at different times during their life history cycle. For instance, juveniles often use estuaries as nursery grounds before recruiting to adult populations offshore. Knowledge of these movement patterns can contribute significantly to the identification of ecologically important habitats, the sustainable management of fish populations and the design of marine protected areas (Thorrold et al. 2007).

Quantifying the movement of fishes is a difficult undertaking that, over the decades, has been tackled using a variety of indirect approaches, including the comparison of distribution, abundance and size structure of study species as well as artificial and natural tagging methods (reviewed by Gillanders et al. 2003). Conventional tagging methodologies are logistically difficult to execute due to the small size, high mortality rates and low recapture rates of juveniles. Hence, natural tags have become more commonly used, and in particular, otolith geochemistry has developed into a promising technique to quantify rates of movement (Thorrold et al. 2002, Elsdon et al. 2008). Otoliths are useful natural tags due to their continuous growth and, metabolic inertness and the fact that the incorporation of at least some elements is influenced by environmental conditions, while other elements are under physiological or genetic control (Campana 1999). Otoliths have been widely used in age structure and growth studies and, more recently, to estimate population structure (e.g. Campana 1999, Thresher & Procter 2007), assess migration patterns (Hamer et al. 2006, Fairclough et al. 2011), identify natal origin or nursery areas (Vasconcelos et al. 2007b, Clarke et al. 2009) and retrospectively classify adults to their areas of origin (e.g. Thorrold et al. 2001, Vasconcelos et al. 2008).

Otolith geochemistry must show significant spatial variation among the areas of interest

for otoliths to be effectively used as natural tags in population connectivity studies. Variability at the appropriate temporal scales may also need to be investigated because several studies have shown differences in otolith composition among years (Gillanders 2002, Kerr et al. 2007) and within years (Thorrold et al. 1998, Hamer et al. 2003). Information on temporal variation, particularly the inter-annual stability of otolith geochemistry, is necessary for retrospective determination of adult origins. Furthermore, variation at smaller time scales, e.g. among seasons, may confound spatial discrimination, leading to misinterpretation of geochemical data. The discriminatory power of otolith geochemistry in estuarine systems along the Portuguese coast and their applicability in connectivity studies has already been demonstrated (Vasconcelos et al. 2007b, Vasconcelos et al. 2008). However, little is known about the inter-annual stability of otolith geochemistry from marine migrant species along the Portuguese coast, and temporal variation at smaller scales has yet to be investigated.

The common sole, *Solea solea* (Linnaeus, 1758), and the Senegalese sole, *Solea senegalensis*, Kaup, 1858, are flatfishes of commercial importance throughout Europe. Their distribution is sympatric from the Bay of Biscay to Senegal and the western Mediterranean Sea (Quéro et al. 1986). The two species are very similar in terms of morphology and ecology. Spawning and larval development takes place in shelf waters with metamorphosis, and the resulting shift to a benthic life form occurs during transport from spawning to nursery grounds (Marchand 1991). Several cohorts of *S. solea* colonize Portuguese estuaries beginning in May, whereas estuarine colonization by *S. senegalensis* starts later and lasts longer (June to October) (Cabral 2003). Juveniles of both species spend up to two years in estuarine nursery grounds before migrating to shelf waters (Quéro et al. 1986, Koutsikopoulos et al. 1989), where they reach sexual maturity at 3 to 4 years of age (Ramos 1982). The Portuguese coast has several estuaries with well-established nurseries for juveniles of *S. solea* and *S. senegalensis* (Cabral et al. 2007, Vasconcelos et al. 2010). Within an estuary, juveniles of both species are known to occur in a single nursery area or in multiple, segregated nursery

areas (Vasconcelos et al. 2010).

The main objectives of the present study are to: (1) determine the temporal variation in otolith composition of the two species between and within years and assess its implications on the spatial separation and classification of juveniles to their estuaries of origin, (2) evaluate differences in otolith geochemistry between the two sole species and (3) assess the accuracy of the classification of juveniles to their estuaries of origin to use the data as a baseline for future retrospective determination of estuary origin of adult *S. solea* and *S. senegalensis*.

MATERIAL AND METHODS

Sampling surveys and otolith analysis

Juvenile *S. solea* and *S. senegalensis* were collected in May and July 2006 and in May, July and October 2009 in the main estuarine nursery areas where these species occur along the Portuguese coast (Fig. 1, Table 1). In total, seven estuaries were sampled: Douro, Ria Aveiro, Mondego, Tejo, Sado, Mira and Guadiana. The main geomorphologic and hydrologic characteristics of these estuarine systems were described by Cabral et al. (2007), and the anthropogenic pressures in these estuaries were explored by Vasconcelos et al. (2007a). In some of the estuarine systems, juveniles of *S. solea* and *S. senegalensis* occur in high densities in two or three distinct nursery areas; hence, juveniles were collected in all of the areas within a system (Vasconcelos et al. 2010). Individuals obtained from multiple nursery sites within an estuary were pooled because the spatial level of interest in the present study was the estuary scale. Several measurements of water temperature (°C) and salinity were taken during sampling surveys at each collection site using a portable multi-parameter instrument (YSI 6820) and averaged for each collection location and sampling date (Table 2).

Fish were collected with a beam trawl, stored and transported on ice to the laboratory and preserved frozen until dissection. The total length of each individual was determined (Table 1), and sagittal otoliths were removed, cleaned of adhering tissue and dried. Right otoliths

were mounted on glass slides with cyanoacrylic glue and ground to the midplane using 30 μm and 3 μm lapping film. Surface contamination was removed by immersing the otoliths in ultrapure water, sonicating for 2 min and triple-rinsing with ultrapure water in a class-100 clean room.

Otoliths were analyzed for Li:Ca, Mg:Ca, Mn:Ca, Cu:Ca, Sr:Ca, Ba:Ca and Pb:Ca on a Thermo Finnigan Element2 single collector inductively coupled plasma mass spectrometer (ICP-MS) coupled to a New Wave 193 nm excimer laser ablation system. The laser software was used to trace 450 μm lines along the posterior, dorsal edge of the otoliths. The ablated material was transported via a He gas stream to the dual-inlet quartz spray chamber, where it was mixed with a 2% HNO_3 aerosol from a self-aspirating PFA 20 $\mu\text{l min}^{-1}$ nebulizer. The analyte was then transported to the ICP-MS via an Ar carrier gas.

Elemental ratios were quantified by measuring ^7Li , ^{25}Mg , ^{48}Ca , ^{55}Mn , ^{63}Cu , ^{88}Sr , ^{138}Ba and ^{208}Pb in the ablated otolith material. ^7Li was not quantified in the otoliths of juvenile *S. solea* collected in July 2006. Instrumental blanks (2% HNO_3) were run at the beginning and end of each set of 10 otoliths. A blank correction of all of the measured raw values was done by calculating a blank value for each sample by linear interpolation of the measured blanks. A dissolved otolith certified reference material (CRM) (Sturgeon et al. 2005), diluted to a Ca concentration of 40 $\mu\text{g g}^{-1}$, was used to correct for instrument mass bias. The instrument precision was assessed by running another CRM (Yoshinaga et al. 2000), similarly dissolved and diluted to a Ca concentration of 40 $\mu\text{g g}^{-1}$. The external precision (relative standard deviation) for this reference material (n=122) was as follows: Li:Ca: 1.9%, Mg:Ca: 3.5%, Mn:Ca: 12.4%, Cu:Ca: 8.6%, Sr:Ca: 0.5%, Ba:Ca: 2.0%, Pb:Ca: 25.3%.

Data analysis

The raw data for each element:Ca ratio were \log_{10} transformed to achieve normality and homogeneity of variance-covariance matrices. Differences in otolith elemental composition

between and within years were evaluated using multivariate analysis of variance (MANOVA). Inter-annual variation was addressed using estuary and year as factors and short-term variability was investigated using estuary and month as factors. For the MANOVA tests, Wilks' lambda was used as the test statistic. Elemental differences at these temporal scales were investigated by considering subsets of the data for both *S. solea* and *S. senegalensis* independently. Variation between years was evaluated considering all estuaries sampled in both years, and the two months sampled in both years (May and July) were pooled. The assessment of seasonal variability (months) for both species was based on datasets that included estuaries sampled in at least two months during the 2009 sampling survey. Canonical discriminant analysis (CDA) was then used to display the multivariate data in reduced space when the MANOVA found significant effects of location or time. An additional CDA was carried out to evaluate otolith chemistry differences between the two sole species in estuaries where they co-occurred, based on samples collected in 2009.

Linear discriminant function analysis (LDFA) was used to classify juveniles of the two sole species to their estuary of origin. After \log_{10} transformation, the element:Ca ratios met the assumptions of LDFA, i.e. normality and homogeneity of variance-covariance matrices. The classification accuracy of the discriminant functions was evaluated by calculating cross-validated classification success using a jackknife (leave-one-out) approach. Otolith elemental data from all of the sampled months within each year were pooled for the LDFAs and all of the estuaries sampled were included in this analysis. October was included in the data of 2009 to ensure that the elemental signatures used in the analysis encompassed all of the assessed temporal variability within an estuary. We subsequently pooled the four northern and central estuaries (Douro, Ria Aveiro, Mondego and Tejo) for *S. solea* collected in 2006, based on weaker results for these data.

RESULTS

Significant differences were found in the otolith elemental composition of the two sole species among the different temporal scales (between and within years). The range of values of the different element:Ca ratios was similar in the two years considered, both in *S. solea* and *S. senegalensis* (Fig. 2). No significant correlations were found between the fish total length and element:Ca ratio in either of the years or species.

Both of the sole species showed significant inter-annual variation in otolith elemental composition (Table 3a). For *S. solea*, each of the estuaries sampled in two years was generally separated in the CDA (Fig. 3a). Only the Guadiana estuary showed overlap of the 95% confidence ellipses of the mean. The separation of the estuaries and years on the first canonical variate was mainly based on differences in Mn:Ca, while Mg:Ca contributed most to the separation on the second canonical variate. Inter-annual variability was also observed in the otolith elemental composition of *S. senegalensis*, with the two years separated in Ria Aveiro and Sado (Fig 3b). The Tejo estuary showed some overlap between the 2006 and 2009 data. Separation on the first and second canonical variates was driven by Ba:Ca and Mg:Ca values, respectively.

Regarding the spatial separation of *S. solea*, the Douro, Ria Aveiro, Mondego and Tejo estuaries occupied similar canonical space, especially in 2006, while clearly separated from the Mira and Guadiana estuaries (Fig. 3a). In the case of *S. senegalensis*, samples from Ria Aveiro and Sado were in close proximity to each other in both years (Fig. 3b). Both of the sole species showed generally higher Mn:Ca values in 2006 in the estuaries where the species occurred (Fig. 2), while *S. senegalensis* otoliths had higher Li:Ca and Mg:Ca ratios in 2009 in all of the estuaries sampled (Fig. 2b). Furthermore, Mn:Ca ratios were noticeably higher in the Mira estuary (up to 5 times the average) in both of the species and throughout the two sampling years (Fig. 2).

Seasonal variation in otolith elemental composition was significant in the two sole species

(Table 3b). In *S. solea*, samples from later months (July and October) were generally similar and significantly different from the initial sampling month (May) (Fig. 4a). The separation on the first canonical variate was mainly driven by Mn:Ca ratios, and the second canonical variate was dominated by Li:Ca and Ba:Ca ratios. Seasonal variation was also observed for the otolith elemental signatures of *S. senegalensis*, particularly in the Tejo and Sado estuaries (Fig. 4b). Samples in these two estuaries showed little variation within months based on the small confidence ellipses, but mean canonical scores among months were dispersed throughout the CDA plot. In contrast, samples from Ria Aveiro and Mira occupied rather similar locations on the CDA plot in the two months that these locations were visited. Separation on the first two canonical variates was driven primarily by Sr:Ca and Mn:Ca ratios, respectively.

Some seasonal patterns were visible in the individual elemental ratios for *S. solea* (Fig. 5a), with Li:Ca values highest in May and diminishing over the following two months while Sr:Ca showed the opposite pattern. Otolith elemental values for *S. senegalensis* showed no consistent seasonal patterns (Fig. 5b). The CDA plot of elemental signatures of the two sole species in the estuaries where they co-occurred in 2009 indicated that variability among estuaries was larger than differences between species, with *S. solea* and *S. senegalensis* grouped together for each of the three estuaries where these species co-occurred (Ria Aveiro, Tejo and Mira) (Fig. 6).

Finally, we examined the effectiveness of using elemental values in otoliths to accurately identify juvenile habitat residency using LDA. Classification accuracy varied between the two years as well as between the two species (Table 4). Elemental values from *S. solea* collected in 2006 did not satisfactorily classify the juveniles to the estuaries in which they were captured (overall cross-validated correct classification: 54.0%) (Table 4a). Based on the CDA plot of the estuaries in the two years of sampling (Fig. 3a), the northern and central estuaries (Douro, Ria Aveiro, Mondego and Tejo) were then pooled. The classification

accuracy in the resulting LDFA for *S. solea* juveniles collected in 2006 improved to 80.1%. In 2009, the overall cross-validated correct classification of individuals to the estuary where they were collected was 71.0%. The highest classification accuracy was attained in the Mondego estuary, with 95.0% of the individuals correctly classified, and the Tejo estuary presented the lowest correct classifications, with 57.6% accuracy. Juveniles of *S. senegalensis* collected in 2006 were correctly classified to the estuary where they were collected with 71.6% accuracy (Table 4b). The highest accuracy was achieved for the Tejo estuary with 83.3%, while 60.0% of the individuals from the Ria Aveiro estuary were correctly classified. In 2009, 72.9% of juvenile *S. senegalensis* were correctly classified to the estuary where they were collected. All of the individuals sampled from the Mira estuary were correctly assigned. The lowest classification accuracy was obtained in the Tejo estuary, with 62.7% of the juveniles correctly classified. Generally, the overall accuracy of the LDFA based on otolith elemental signatures varied considerably between years in *S. solea* juveniles. In contrast, while overall classification accuracy of juveniles of *S. senegalensis* did not differ much between years, the accuracy of the classification to individual estuaries varied. The Tejo estuary presented the highest classification accuracy in 2006 and the lowest in 2009. Similarly, Ria Aveiro had the lowest correct classification in 2006 and one of the highest in 2009.

DISCUSSION

Inter-annual variations in otolith geochemistry have been documented around the globe from freshwater to marine environments (Rooker et al. 2001, Kerr et al. 2007, Walther et al. 2008). Significant variability has also been documented at shorter time scales among months or seasons (Thorrold et al. 1998, Hamer et al. 2003, Patterson & Kingsford 2005). Assessing temporal variation in otolith composition, at different scales, is of paramount importance when these values are used to constrain baseline data for connectivity studies (Gillanders 2002). We found that, while some of the estuaries along the Portuguese coast showed little

inter-annual variation in terms of otolith composition, there was a significant overall effect of collection year. Knowledge of the inter-annual variation of elemental values in these two sole species will be particularly important if present data is to be used as a baseline to retrospectively determine the juvenile estuarine use of adults. In this case, the adults that are to be assigned to the estuaries must match the juveniles' year classes to determine the importance of different estuarine systems along the Portuguese coast.

We also found evidence of seasonal variation in the otolith composition of individuals sampled in the same estuary in May, July and October 2009. As with many marine migrants, *S. solea* and *S. senegalensis* have protracted estuarine colonization periods, and a single year-class consists of several cohorts (Cabral 2003). Over this time, individuals may experience considerable environmental variation that may result in differences in the elemental composition of the otoliths. Seasonal variation in the elemental signatures of *S. solea* and *S. senegalensis* had a limited influence on the overall spatial separation of the estuaries analyzed in this study. However, the differences in otolith elemental signatures among these first months of estuarine use by the two sole species were significant. It therefore seems advisable to develop baseline values that incorporate short-term temporal variation to ensure that the signatures are representative of the year class. Samples should be based on juveniles of a year class collected at several occasions during the period of estuarine colonization. If juveniles are sampled at only one occasion, sampling should target individuals that are not all of the same size (Hamer et al. 2003).

The significant inter-annual and seasonal variability in otolith composition in this study likely reflects differences in the temperature, salinity and chemical composition of the water (reviewed by Elsdon et al. 2008). These parameters vary extensively in dynamic systems such as estuaries. Water temperatures in the sampled estuarine systems along the Portuguese coast were generally higher in 2006 than 2009. No consistent pattern in salinity over the two sampling years was observed; however, precipitation was higher during 2006, especially in

spring (Instituto de Meteorologia 2007, 2010). Higher precipitation rates potentially influence the chemical composition of the water due to higher freshwater input into estuaries. Monthly variation of temperature and salinity was also pronounced in 2009, with lowest values observed in May. Some of the elements analyzed notably varied spatially or temporally at the scales examined. The fact that Mn:Ca ratios were lower in 2006 than 2009 in the otoliths of both species of sole suggest that the difference was caused by an environmental factor related either directly (e.g. dissolved Mn:Ca ratios in ambient waters) or indirectly (e.g. through an effect of temperature on Mn:Ca ratios in otoliths of both species) to otolith composition. Similarly, the consistently high Mn:Ca ratios observed in the Mira estuary in both species and sampling years, as well as in an otolith chemistry study conducted in 2005 (Vasconcelos et al. 2007b), suggests that juveniles are exposed to elevated dissolved Mn:Ca levels in the estuary. While we can only speculate as to the source of the Mn ions that were found in the juvenile sole otoliths, the only functioning iron-manganese ore mine in Portugal is located a few km north of the Mira estuary (Cercal).

Consistent and negatively correlated patterns in Li:Ca and Sr:Ca ratios from *S. solea* juveniles were found. Ambient Sr:Ca levels are often highly correlated with otolith Sr:Ca (e.g. Bath et al. 2000, Elsdon & Gillanders 2003). However, the non-linear mixing profiles of Sr and Ca ions in estuaries implies that this pattern was not a function of variable Sr:Ca ratios in the ambient water. A previous study of otolith elemental signatures of this species conducted in the same estuaries similarly found no correlation between Sr:Ca and salinity (Tanner et al. 2011). Temperature has been shown to affect Sr incorporation into otoliths positively, particularly at temperatures around 20°C (e.g. Elsdon & Gillanders 2004, Martin et al. 2004). A response of otolith chemistry to increases in water temperatures in the estuaries from late spring to late summer therefore provides the most likely explanation for the Sr:Ca patterns. If this explanation is accurate, the observation that Li:Ca and Sr:Ca were negatively correlated suggests that Li:Ca ratios in otoliths may be negatively correlated with temperature. However,

very little is known about the factors influencing Li:Ca ratios in otoliths, even though Li is often included in the suite of elements used in otolith chemistry studies (Brown 2006). While it remains to be seen if indeed Li:Ca may be a useful proxy for temperature, understanding what are likely complex mechanisms underlying element incorporation into otoliths is not required to use elemental differences in otoliths as estuary-specific natural tags (Thorrold et al. 1998).

The results obtained confirm that otolith geochemistry in juvenile *S. solea* and *S. senegalensis* are suitable natural markers to discriminate estuaries along the Portuguese coast and identify the nursery origin of adults as suggested by Vasconcelos et al. (2007b, 2008). In addition, otolith compositions of *S. solea* have been successfully used to distinguish estuarine and coastal areas in the Thames estuary (Leakey et al. 2009) and to discriminate among four known nursery areas in the North Sea (Cuveliers et al. 2010). However, none of these studies assessed the temporal variation of otolith chemistry. The large differences observed in classification accuracy of juveniles of *S. solea* collected in 2006 and 2009 demonstrate that some years produce stronger spatial differences in elemental signatures than others. Moreover, elemental ratios in otoliths from an estuary may show strong spatial differences from the other estuaries in one year and have spatially similar signatures in another year, as observed in the elemental signatures of juvenile *S. senegalensis* in the Tejo estuary. These data highlight the observation that the application of otolith elemental signatures in assessing the importance of juvenile nursery habitats to adult populations is a 2-step process (Cook 2011). First, otolith geochemistry of juveniles must show spatial differences among the estuaries of interest. Second, adult fish that match the juvenile year-class of the baseline data are then compared to the juvenile baseline data. The elemental values in otoliths of the two sole species assayed in this study constitute the baseline data for the future assessment of movements between juvenile and adult populations.

The comparison of elemental signatures of the two sole species in the estuaries where

they co-occurred also evidenced the strong spatial variation, particularly in 2009. Otolith elemental signatures have been shown to be species specific (Swearer et al. 2003) and only some similarities may exist among species with close phylogeny and ecology (Reis-Santos et al. 2008). Still, spatial differences in the otolith elemental signatures of *S. solea* and *S. senegalensis* among the estuaries outweighed the species specific variation.

Flatfish fisheries are widely spread along the Portuguese coast and play an important socio-economic role (Teixeira & Cabral 2009). Although flatfishes account for less than 4% of the total fish biomass landed, these species, and particularly soles, account for almost 11% of the economic value of the landings (source: DGPA - National Directorate of Fisheries and Aquaculture; www.dgpa.min-agricultura.pt). The importance of soles to small-scale fisheries in Portuguese waters and the increasing fishing pressures on these stocks call for a more holistic understanding of the ecology of these species. In particular, knowledge of the population structure and exchange rates among spatially separated subpopulations of juveniles and adults is essential for the development of effective fisheries management plans and conservation strategies.

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Table 1. Sampled estuaries, mean values and standard error (SE) of total fish length (Lt) in mm and sample size (*n*) of juveniles of *Solea solea* and *Solea senegalensis* along the Portuguese coast during 2006 and 2009.

Estuary	2006						2009								
	May			July			May			July			October		
	Lt	SE	<i>n</i>	Lt	SE	<i>n</i>	Lt	SE	<i>n</i>	Lt	SE	<i>n</i>	Lt	SE	<i>n</i>
<i>S. solea</i>															
Douro (D)				77.3	2.2	12	50.2	2.5	15	83.7	2.0	15	111.9	7.0	15
Ria Aveiro (RA)	61.8	10.2	4	91.1	4.6	18	41.6	2.0	15						
Mondego (Mo)	58.2	2.2	15	77.2	1.8	30	51.9	4.3	20						
Tejo (T)	45.5	3.0	15	88.5	2.7	15	66.6	3.7	33	76.0	3.9	11	89.8	4.0	15
Mira (Mi)	37.8	0.9	31	76.9	1.5	28	47.0	2.0	7	85.7	2.6	25	95.6	2.0	24
Guadiana (G)	77.6	10.1	9	83.5	3.0	14	45.1	2.7	13	78.4	1.6	15	106.8	2.3	15
<i>S. senegalensis</i>															
Ria Aveiro (RA)				73.15	3.585	20				57.77	7.286	13	60.2	7.889	5
Tejo (T)	109.3	5.777	6				49.19	1.027	26	74.22	3.424	21	56.41	5.638	18
Sado (S)	50.07	1.62	15	86.46	3.419	26	47.21	2.11	34	74.59	2.271	39	57.88	4.034	26
Mira (Mi)										69.33	15.28	6	101	6.11	6

Table 2. Mean values and standard errors (SE) of temperature and salinity in the estuarine systems sampled along the Portuguese coast in 2006 and 2009.

Estuary	2006								2009											
	Temperature (°C)				Salinity				Temperature (°C)				Salinity							
	May		July		May		July		May		July		October		May		July		October	
	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE
Douro	17.1	0.1	24.1	0.2	1.2	0.3	10.1	0.5	18.3	0.1	23.7	0.2	19.8	0.0	2.0	0.3	2.4	0.5	2.2	0.4
Ria Aveiro	23.8	3.1	24.9	0.2	16.3	1.3	27.6	0.2	16.7	0.2	24.9	0.3	19.6	0.1	20.9	2.3	30.1	0.7	19.8	0.6
Mondego	20.5	0.2	23.4	0.2	6.8	0.4	15.1	1.3	19.1	0.2	22.7	0.2			8.1	1.2	5.4	1.0		
Tejo	20.0	0.1	25.5	0.1	9.8	0.5	13.5	1.1	15.7	0.3	21.5	0.3	21.5	0.2	25.1	0.8	18.6	1.1	5.1	0.1
Sado	21.7	0.2	26.0	0.1	25.5	0.4	29.3	0.7	15.3	0.7	25.5	0.6	22.2	0.2	30.7	0.8	28.8	0.3	29.0	1.3
Mira	21.7	0.2	26.9	0.1	6.9	1.0	13.7	1.0	19.1	0.0	25.7	0.1	23.4	0.0	12.8	0.5	21.5	0.9	27.4	0.3
Guadiana	23.1	0.2	27.0	0.2	6.9	1.5	8.9	1.3	19.8	0.6	25.4	0.0	22.0	0.1	12.8	1.2	14.7	0.7	14.2	0.5

Table 3. Results of MANOVA examining temporal variation of otolith elemental signatures between years (a) and within one year (b) for *S. solea* and *S. senegalensis*.

	<i>S. solea</i>			<i>S. senegalensis</i>		
	df	F	p	df	F	p
a) Between years comparison; 2006 and 2009						
Estuary	30	28.1	<0.001	14	7.9	<0.001
Year	6	7.0	<0.001	7	7.7	<0.001
Estuary x Year	30	7.5	<0.001	14	5.0	<0.001
b) Within year comparison; 2009						
Estuary	21	24.7	<0.001	7	14.6	<0.001
Month	14	12.5	<0.001	7	2.3	<0.001
Estuary x Month	42	3.6	<0.001	28	5.2	<0.001

Table 4. Cross-validated classification accuracy of discriminant function analysis of juvenile *S. solea* and *S. senegalensis* in 2006 and 2009. Individuals were classified to an estuary based on otolith elemental signatures. Juveniles of *S. solea* sampled in 2006 in Northern and central estuaries were pooled to Group North. Months were pooled within each year. Values in bold represent individual correctly classified to the estuary of origin.

		Predicted estuary				
<i>S. solea</i>						
2006						
	Group North	Mira	Guadiana			
Group North	79.1	4.5	16.4			
Mira	5.2	86.2	8.6			
Guadiana	21.7	8.7	69.6			
Overall cross-validated correct classification: 80.1%						
2009						
	Douro	Ria Aveiro	Mondego	Tejo	Mira	Guadiana
Douro	68.9	8.9	4.4	17.8	0	0
Ria Aveiro	6.7	86.7	6.7	0	0	0
Mondego	0	0	95.0	6.7	0	0
Tejo	16.9	6.8	3.4	57.6	13.6	1.7
Mira	0	0	0	10.7	71.4	17.9
Guadiana	2.3	0	2.3	7.0	14.0	74.4
Overall cross-validated correct classification: 71%						
<i>S. senegalensis</i>						
2006						
	Ria Aveiro	Tejo	Sado			
Ria Aveiro	60.0	0	40.0			
Tejo	16.7	83.3	0			
Sado	24.4	0	75.6			
Overall cross-validated correct classification: 71.6%						
2009						
	Ria Aveiro	Tejo	Sado	Mira		
Ria Aveiro	83.3	0	16.7	0		
Tejo	21.6	62.7	7.8	7.8		
Sado	7.1	15.2	73.7	4.0		
Mira	0	0	0	100		
Overall cross-validated correct classification: 72.9%						

Figure legend

Figure 1. Estuaries sampled along the Portuguese coast in 2006 and 2009. Species occurrences are also indicated, *Solea solea* (*Ss*) and *Solea senegalensis* (*Sn*).

Figure 2. Mean and standard error of element:Ca ratios (Li, Mg, Mn, Cu, Sr, Ba, Pb) in otoliths of juvenile *Solea solea* (a) and *Solea senegalensis* (b) in the estuarine systems sampled in 2006 and 2009. Data from May and July were pooled in each year. See Table 1 for estuary abbreviations.

Figure 3. Canonical discriminant analysis plot summarizing variation in otolith elemental signatures in juvenile *Solea solea* (a) and *Solea senegalensis* (b) in the estuarine systems along the Portuguese coast in 2006 and 2009. Circle enclosures represent 95% confidence limits for the mean. White and grey circles represent individuals sampled in 2006 and 2009, respectively. See Table 1 for estuary abbreviations.

Figure 4. Canonical discriminant analysis plot summarizing variation in otolith elemental signatures in juvenile *Solea solea* (a) and *Solea senegalensis* (b) in the estuarine systems along the Portuguese coast in May, July and October 2009. Circle enclosures represent 95% confidence limits for the mean. Dark grey, light grey and white circles represent individuals sampled in May, July and October 2009, respectively. See Table 1 for estuary abbreviations.

Figure 5. Mean and standard error of element:Ca ratios (Li, Mg, Mn, Cu, Sr, Ba, Pb) in otoliths of juvenile *Solea solea* (a) and *Solea senegalensis* (b) in the estuarine systems sampled in May (M), July (J) and October (O) 2009. See Table 1 for estuary abbreviations.

Figure 6. Canonical discriminant analysis plot summarizing variation in otolith elemental signatures in the estuarine systems along the Portuguese coast where juvenile *Solea solea* and *Solea senegalensis* co-occur in 2009. Circle enclosures represent 95% confidence limits for the mean. Grey and white circles represent juvenile *S. solea* and *S. senegalensis*, respectively.

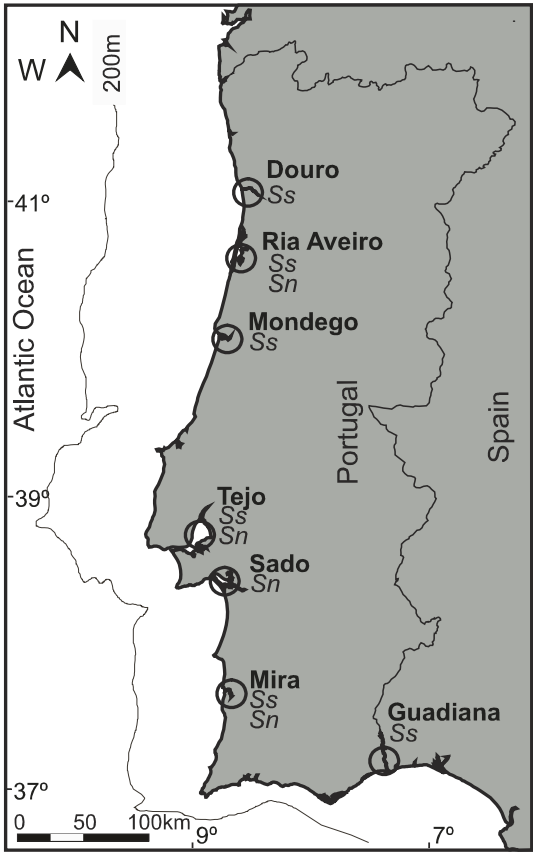


Fig. 1

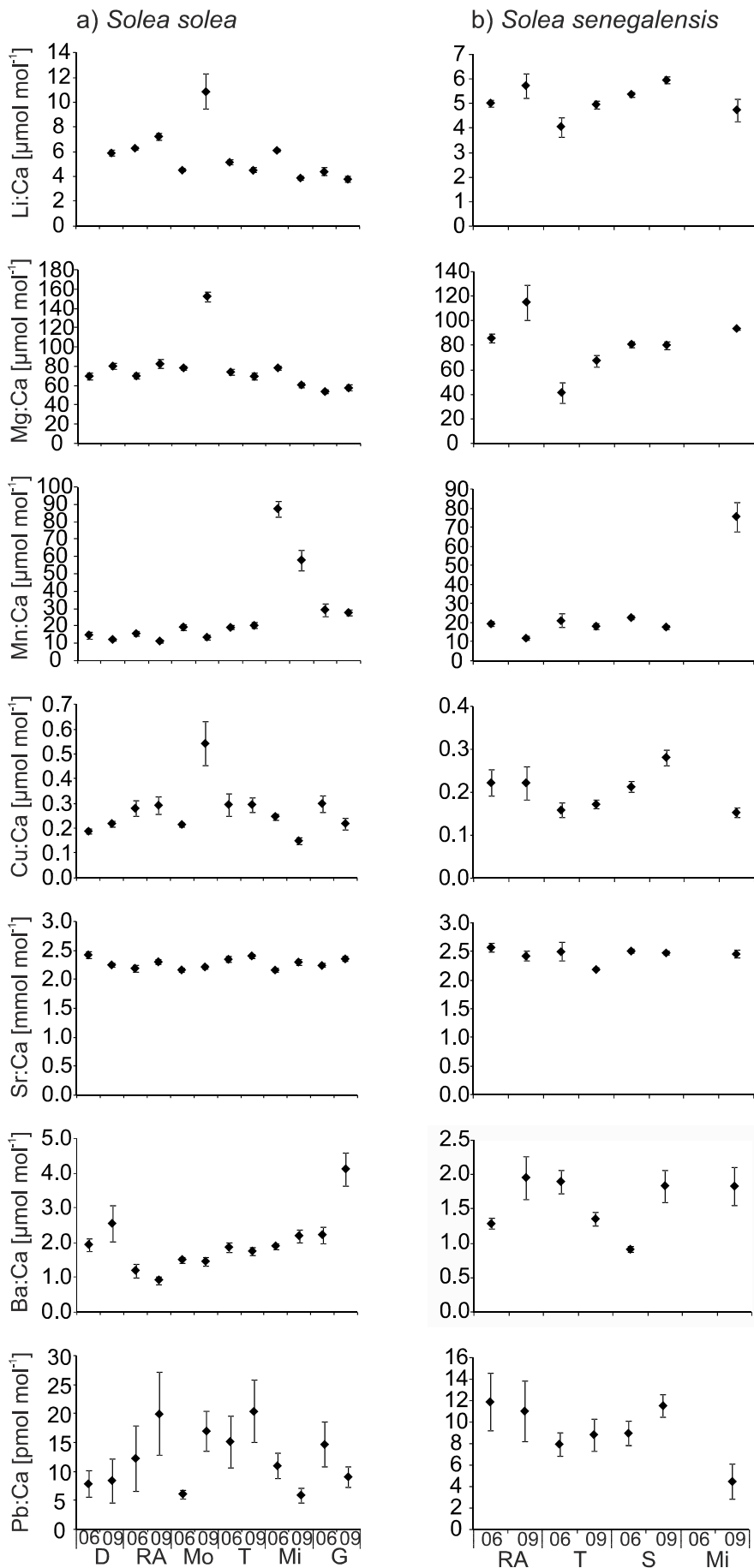


Fig.2

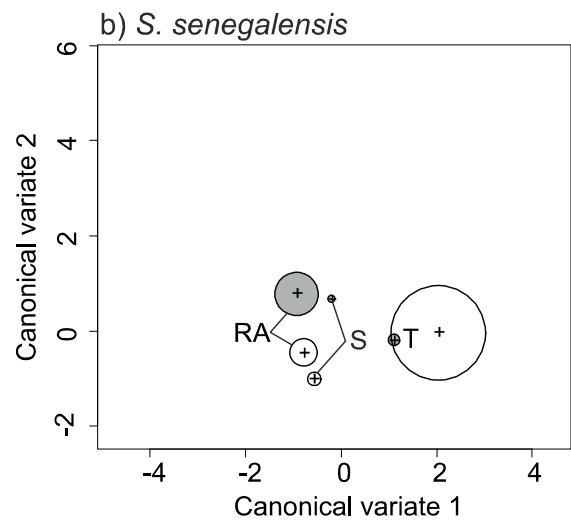
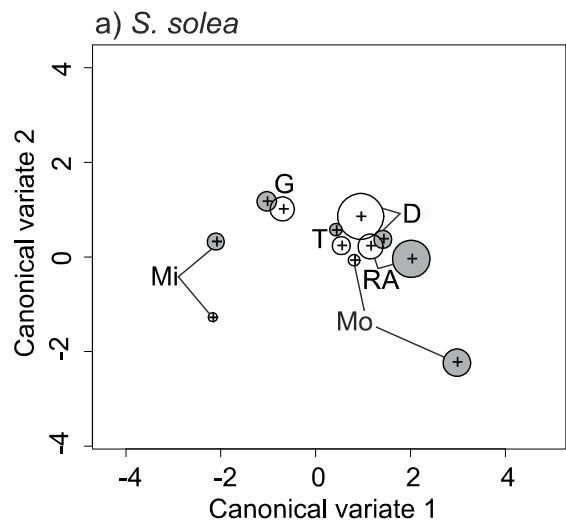


Fig. 3

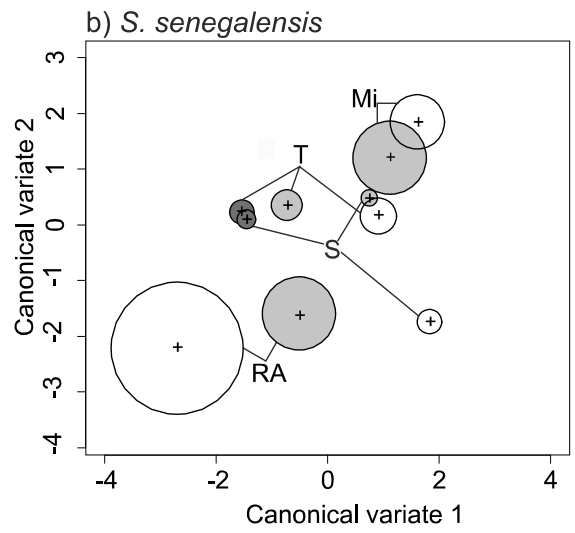
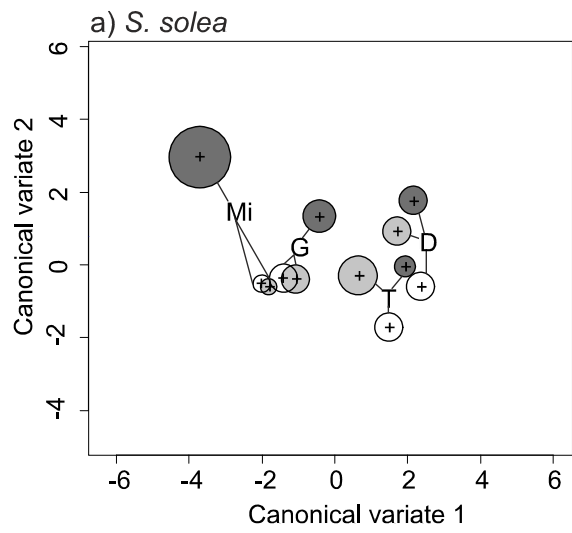


Fig. 4

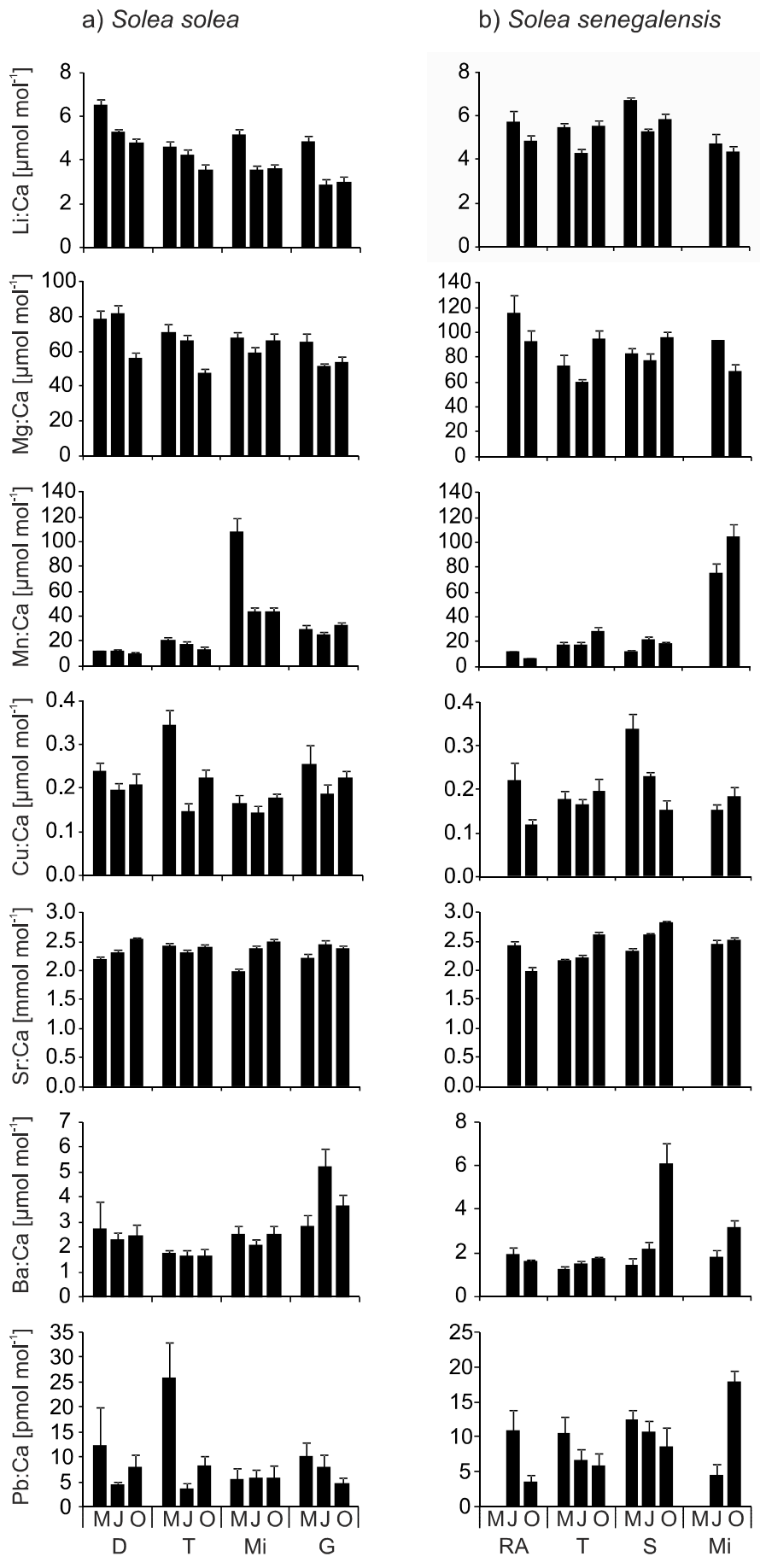


Fig. 5

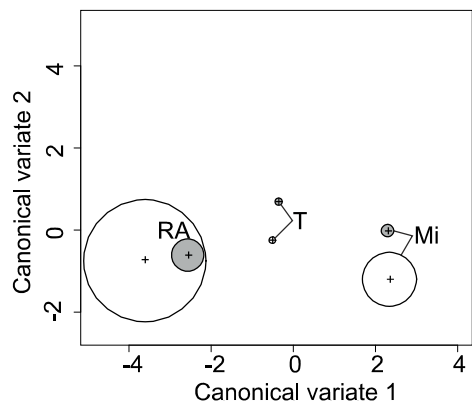


Fig. 6