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Note

Discrimination of *Trisopterus luscus* stocks in northern Portugal using otolith elemental fingerprints

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Abstract – The pout, *Trisopterus luscus*, is one of the most important gadoid fish captured by northern Portuguese traditional fisheries. In spite of a substantial decrease in fish catches, little data are available either on the population structure or on the management of the species. In this study, chemical analysis with inductively coupled plasma mass spectrometry of whole otoliths of 90 pouts (age group 2, total length: 17.9–25.0 cm) provided location-specific elemental signatures. Sampling took place in shallow waters along the coastline in three fishing grounds off the Portuguese north coast (Viana do Castelo, Matosinhos and Aveiro) between February and March 2010. Otolith fingerprint analysis detected the presence of several informative trace elements. Molar concentrations for each site were analysed through uni- and multivariate statistical tests. Strontium, barium, magnesium and lithium (Sr, Ba, Mg and Li) differed significantly among locations, while no significant differences were found for manganese (Mn) and nickel (Ni). Canonical analysis allowed us to discriminate the tested individuals with respect to their sampling origin with a mean classification accuracy of 69%. The observed site-specific elemental differences in pout otoliths suggest a high level of site-fidelity in relation to their growing/feeding areas. Pouts from these locations can be regarded as a single, although not necessary homogenous, stock. Furthermore, this study also suggests that the populations of juvenile fish mix partially and, therefore, cannot be assumed to be separate units for fisheries management purposes.

Key words: *Trisopterus luscus* / Otolith fingerprint / Trace metal / Stock discrimination / NE Atlantic

Introduction

The pout, *Trisopterus luscus* (Linnaeus, 1758), is a marine demersal gadoid fish usually found in shallow coastal waters at depths between 30 and 100 metres (Svetovidov 1986). *T. luscus* is a benthopelagic species of cold waters with a wide distribution along the coasts of the North Atlantic Ocean, from the British Isles to the coast of Morocco, extending into the western Mediterranean (Cohen at al. 1990). This species is of major commercial importance for the artisanal fleets of some European countries, namely France, Portugal and Spain (Alonso-Fernández et al. 2008). In Portuguese waters, the estimated landings in 2009 totaled 3280 tons, but had a relatively low commercial value (1.32 € kg^{-1} at first auction) (DAT-APESCAS 2009). On the north Portuguese coast, the species is abundant and plays an important role in the local fisheries economy. The commercial landings of fresh fish in the northern Portuguese trade delegations of Viana do Castelo, Matosinhos and Aveiro show that pout is one of the four main landed fish species (INE 2010).

Histological studies have shown that pout has determinate fecundity and asynchronous ovary development (Alonso-Fernández et al. 2008), allowing several spawning events per individual over the whole reproductive season (Murua and Saborido-Rey 2003). Spawning appears to occur from December to March/April in the Atlantic Ocean and from January to July in the Mediterranean Sea (Cohen at al. 1990; Merayo 1996; Alonso-Fernández et al. 2008). After a very short pelagic larval stage (Chevey 1929), the early juveniles actively migrate inshore towards shallow coastal areas (Hamerlynck and Hostens 1993). After spending the first year of life in these coastal nursery areas, pout migrate offshore to

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Table 1. Location, date of collection, sample size (N), fish size range (TL, cm), mean total length (TL, cm) and eviscerated weight (g) (mean ± SE) of pout (age group 2) used for otolith fingerprint analysis.

Sites	Location	Date	N	Size range TL (cm)	TL (cm)	Weight (g)
Viana do Castelo	41°41'N 8°54'W	17 February 2010	30	17.9-23.4	20.2 ± 0.3	81.30 ± 3.61
Matosinhos	41°10'N 8°44'W	12 February 2010	30	18.2-25.0	19.9 ± 0.3	80.12 ± 4.44
Aveiro	40°38'N 8°50'W	3 March 2010	30	18.4-22.7	20.5 ± 0.2	86.02 ± 2.40

deeper waters (Cohen at al. 1990; Hamerlynck and Hostens 1993). The pout is a species with a short lifespan of about 3-4 years (Chevey 1929; Cohen at al. 1990), although some individual variation may occur (Gherbi-Barré 1983; Ferreiro and Labarta 1988; Puente 1988; Merayo and Villegas 1994). Sexual maturation generally occurs between the first and second year of life (Chevey 1929) at 22 and 24 cm in length for females and males, respectively (Merayo 1996; Alonso-Fernández et al. 2008).

The English Channel coast is known to be a major breeding area where spawning occurs annually from January to July (Chevey 1929). The high abundance of age 0+ pout individuals in the adjacent coastal areas of some Portuguese estuaries, namely in the Douro (França et al. 2004) and Tejo rivers (Costa and Cabral 1999; Cabral et al. 2000) suggests that these grounds are important nursery areas for the species. While estuaries are well studied and recognized as fundamental nursery areas for this species, the contribution of the adjacent coastal areas as recruitment grounds remains unknown (França et al. 2004). Juvenile and adult pouts rarely coexist in the same habitat: juveniles are more abundant in shallow coastal areas and can penetrate estuarine regions; adults normally inhabit areas with sandy bottoms at greater depths and far from the shore (Desmarchelier 1986; Cabral et al. 2000; Tanner et al. 2009). In a recent study, significant variations in some anatomical characteristics among specimens of *T. luscus* collected in Huelva, Ría de Vigo and Alborán Sea suggested the existence of genetic separation between the Mediterranean and Atlantic populations (Miramontes-Sequeiros et al. 2009). However, some aspects of their biology remain poorly known, particularly population structure and coastal recruitment processes.

Otoliths are acellular inner-ear fish structures that play an important role in the major system that controls balance and hearing in teleost fishes (Popper et al. 2005). Otoliths have several specific characteristics that make them excellent natural markers of fish habitat and valuable tools for studies of fish life history and movements: a) otoliths grow by the addition of calcium carbonate and by the successive uptake of chemical elements present in the water surrounding the fish; b) otoliths are structures deposited continuously throughout the life of the fish; and c) otoliths do not suffer reabsorption, therefore allowing a complete environmental record throughout the entire fish life history (Campana and Thorrold 2001). The combination of the these characteristics means that, within their structure, otoliths record chemical elements acquired from the different bodies of water inhabited by fishes throughout their life (Elsdon et al. 2008). This is especially the case if fish reside in a particular environment long enough to incorporate a detectable chemical tag in their otoliths (Campana et al. 2000). Therefore, bulk chemical otolith analysis can provide a tag of the environment inhabited by fish over the entire life, from the earliest embryonic stages until death (Elsdon et al. 2008). Otoliths have proved useful for the determination of stock structures (e.g. Campana et al. 1994; Thresher 1999; Volpedo and Cirelli 2006), to identify the natal origin of fish and the contribution of nurseries to adult populations (e.g. Gillanders 2002; Hamer et al. 2005; Vasconcelos et al. 2008), and to infer migration pathways (e.g. Sandin et al. 2005; Secor and Piccoli 2007; Campana et al. 2007).

The main aim of this study was to evaluate the use of the elemental composition of otoliths from *T. luscus* specimens as a tool to determine whether these otolith fingerprints are site-specific and can be used to assess the degree of separation between stocks, and to investigate population connectivity between adjacent fishing grounds.

Materials and methods

Biological sampling

Fish were sampled from catches landed at three main fishing harbours in the northern region of the Portuguese coast (Viana do Castelo, Matosinhos and Aveiro) between mid-February and early March 2010 (Table 1). These collection sites were chosen because *T. luscus* is very abundant and widely commercialized in these areas (DATAPESCAS 2009). To ensure that the location of capture was accurate, fish were bought straight from fishing boats and this information was requested from the fishermen. Pout fishing was done in shallow coastal waters (up to 75 m water depth) near the coast (up to 6 nautical miles from the shoreline) by small local fishing boats using gillnets (60 mm mesh size). An attempt was made to collect individuals from the same cohort by choosing fishes of similar length. Subsequently, age was confirmed by counting the otolith annual growth increments. A sub-sample of 30 individuals of age group 2+ was selected from each site for chemical analyses. The collected fishes were stored in ice after landing and the total length (0.1 cm) and gutted weight (0.01 g) were recorded at the laboratory for all specimens.

Otolith age estimates

Otoliths were extracted from the fish heads by using plastic forceps, they were then washed with distilled water, dried with paper and stored in eppendorf tubes. The preparation of otoliths for age estimates was adapted from procedures of previous studies (Puente 1988; Merayo and Villegas 1994).

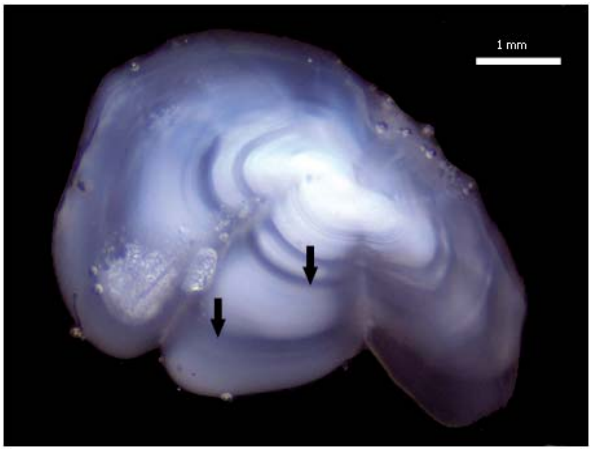

Fig. 1. Transverse section from the right sagittal otolith of a two-year-old pout (TL = 20.6 cm) The arrows represent the annuli. Scale bar: 1 mm, 25× magnification.

Right sagittal otoliths were embedded in epoxy resin (Struers, Epofix) and all the templates were left for 5 min in a hot oven (60 °C) to release air bubbles and to dry the mixture, after which polymerization took place overnight at room temperature. Transversal sections (~2 mm) of the blocks were cut through the core region using a diamond saw (Buehler, Diamond Saw 15HC series) lubricated with Milli-Q-Water at 3000 rpm. The otoliths were ground with 500, 1000 and 1200 silicon carbide papers to a thickness of approximately 0.8 mm. Afterwards, the otolith sections were fine polished using alumina solution (1:20) in order to clean the scratches from the surface. Thin otolith sections were immersed in a clearing mixture of ethanol and glycerol (1:1) and the annual increments (translucent and opaque bands) were counted under a stereoscopic microscope (Meiji, EMZ-13TR) under reflected light against a dark background at 25 × magnification. Otolith annual increments were identified from digital photographs (Olympus, SC 30) (Fig. 1). It has been previously demonstrated for this species that an opaque and a translucent increment in the otolith is deposited annually (Blacker 1974; Desmarchelier 1986). Each otolith image was read twice by two independent readers. When readings did not agree, the otolith was read a third time. The value of two concordant readings was accepted as the best estimate of age. The overall percentage of concordance between the different readers was 82%. The age was calculated by counting the translucent increments, but taking into consideration the date of birth (1st January following the rules in the northern hemisphere) and the date of capture (Panfili et al. 2002).

Otolith chemical analyses

Prior to the chemical analyses, the left sagittal otoliths were cleaned and decontaminated in an ultrasonic cleaner for 5 min in ultrapure water (Milli-Q-Water) to remove the adherent biological tissues, followed by immersion in 3% analytical grade hydrogen peroxide (H_2O_2) for 15 min to dissolve the remaining biological residues. Then the otoliths were immersed in 1% nitric acid (HNO_3) solution for 10 s to remove superficial contamination, followed by a double-immersion in Milli-Q Water for 5 min to remove the acid (Rooker et al. 2001). The otoliths were stored in new, previously decontaminated, eppendorf microcentrifuge tubes, where they were allowed to air dry in a laminar flow fume hood (Patterson et al. 1999).

Chemical composition of whole juvenile otoliths was determined using a solution- based inductively coupled plasma mass spectrometry (ICPMS-SB) method. Decontaminated otoliths were weighed on an analytical balance (0.0001 g), dissolved for 15 min in 1 ml ultra pure HNO_3 and diluted with Milli-Q Water to a final acid concentration of 10% (wt/wt). ICPMS-SB analyses were made using a double focusing magnetic sector field instrument ICP-SF-MS (Thermo ICP-MS x series, Thermo Electron Corporation). This instrument is equipped with a compact double-focusing magnetic sector mass spectrometer of reversed Nier-Johnson geometry. All measurements were made at a medium resolution setting ($m/\Delta m$ = 4000) to avoid false readings from spectral interferences. The instrument was equipped with a micro flow nebulizer (PFAAR35-1-C1E, Glass Expansion), operated in the self aspirating mode (sample uptake rate ~0.93 L min^{-1}). Quantification of trace elements was based on the external calibration method, preparing multi-element standards that contained the elements of interest in the expected concentration range. To minimize the effect of any plasma fluctuations or different nebulizer aspiration rates between the samples, ^{115}In of a known concentration was added to all samples and standards as an internal standard. Concentrations were calculated by linear interpolation (sum of least squares) based on normalization with the internal standard, and on calibration curves made from single element standards (Merck KGaA) covering the individual expected concentration ranges. A calibration was made at the beginning of each session. The matrix of both the blank and the standard solutions was 1% HNO3.

A preliminary analysis was made to determine the most abundant and informative elements present in whole otoliths of *T. luscus* using ten extra specimens. Seven elements (^{48}Ca, ^{88}Sr, ^{137}Ba, ^{55}Mn,^{26}Mg, ^{62}Ni and ^{7}Li) were detectable in whole otoliths of pout and they were used for further ICPMS-SB analysis. For ^{65}Cu, ^{66}Zn and ^{208}Pb, concentrations were below the limit of detection. Otolith samples were analysed in random order to avoid possible sequence effects. For quality control of the measurements, two fish otolith reference materials were analysed: (1) NIES-022 (National Institute for Environmental Studies and Environment Agency of Japan, Tsukuba, Ibaraki, Japan) and (2) FEBS-01 (National Research Council Canada, Institute for National Measurement Standards, Ottawa, Ontario, Canada). Accuracy on both standard materials ranged between 2 and 5% relative standard deviation (RSD). RSD for elements ranged between 8.5 and 1.3%. The limits of detection were calculated from the individual calibration curves using the three sigma criteria and were (in ppb): ^{48}Ca (10), ^{88}Sr (0.25), ^{137}Ba (0.25),^{55}Mn (0.25), ^{26}Mg (10), ^{62}Ni (1) and ^{7}Li (1).

Statistical analyses

ICPMS-SB concentrations of trace elements, originally in μg element L^{-1} solution were transformed to μg element g^{-1}

Table 2. Molar elemental concentrations (mean ± SE), expressed in μmol element mol^{-1} calcium, in whole otoliths of pout collected in three locations along the NW coast of Portugal (Viana do Castelo, Matosinhos and Aveiro). For each element: Ca ratio, the values marked with the same letter are not significantly different ($p > 0.05$).

Molar Ratio	Viana do Castelo	Matosinhos	Aveiro
Sr/Ca	2200 ± 50^a	2644 ± 55^b	2370 ± 46^a
Ba/Ca	2.18 ± 0.11^a	3.08 ± 0.13^b	2.50 ± 0.11^a
Mn/Ca	2.76 ± 0.09^a	2.68 ± 0.09^a	2.76 ± 0.09^a
Mg/Ca	85.0 ± 1.9^a	90.6 ± 1.6^b	77.5 ± 1.2^c
Ni/Ca	0.41 ± 0.01^a	0.43 ± 0.01^a	0.43 ± 0.01^a
Li/Ca	5.24 ± 0.07^a	4.97 ± 0.10^a	4.57 ± 0.07^b

otolith, and then to molar ratios (μmol element mol^{-1} Ca). Raw data for each element were checked for normality, homoscedasticity and homogeneity of variance-covariance matrices prior to statistical analysis. These assumptions were met after log 10 transformation.

Although there were no significant differences in the mean lengths of fish among locations (One-Way ANOVA, $n = 90$, $p < 0.05$), we tested for relationships between elemental concentration and fish size (otolith weight) with analysis of covariance (ANCOVA, otolith weight as co-variate). Otolith elemental concentrations were significantly correlated with otolith mass for all elements, with the exception of Ba ($r^2 = 0.00$, $n = 90$, $p > 0.05$). Sr presented a positive relationship ($r^2 = 0.12$, $n = 90$, $p < 0.05$), which was the opposite to Mn ($r^2 = 0.18$), Mg ($r^2 = 0.46$), Ni ($r^2 = 0.18$) and Li ($r^2 = 0.38$), all of which showed negative relationships. To ensure that differences in fish size among samples did not confound any site-specific differences in otolith chemistry, concentrations of elements were weight-detrended by subtraction of the product of the common within-group linear slope multiplied by the otolith weight from the observed concentration (Campana et al. 2000).

One-way analysis of variance (ANOVA) was used to explore individual elemental fingerprint differences between locations. If significant differences were found, this was followed by a Tukey post hoc test. Multivariate analysis of variance (MANOVA) was used to explore multi-elemental fingerprints and detect differences in the multi-elemental otolith composition from different locations. For the MANOVA, we reported the approximate F-ratio statistic for the most robust test of multivariate statistics (Pillai's trace). Post-hoc multivariate pairwise comparisons between locations were performed using the Hotelling T-square test. Multi-element compositions of otoliths were analysed with a linear discriminant function analysis (LDFA). Only the elements that differed significantly between locations using univariate tests were used for LDFA by running a complete analysis. LDFA functions allow us to classify individuals according to the original locations from which pout were collected. Classification accuracies of the discriminant functions for each site were evaluated through the percentage of correctly classified individuals using jackknifed cross-validations.

All these statistical analysis were performed using Systat (version 13.0). The statistical level of significance (α) was 0.05. Data are presented as mean values ± standard errors.

Results

There were no significant differences in fish size or weight between locations (one-way ANOVA, $n = 90$, $p > 0.05$; Table 1).

The molar ratios of Sr, Ba, Mg and Li differed significantly among locations (one-way ANOVA, $n = 90$, $p < 0.05$). Matosinhos was the location with the highest ratios of Sr, Ba and Mg (Tukey-test, $n = 90$, $p < 0.05$); Viana do Castelo had the highest ratio of Li, although it was not statistically different from Matosinhos (Tukey-test, $n = 90$, $p > 0.05$). No significant differences were found among areas for Mn or Ni concentration ratios (one-way ANOVA, $n = 90$, $p > 0.05$) (Table 2).

MANOVA indicated the existence of significant differences in the multi-element signatures of the whole otoliths (Pillai trace, F12.269 = 0.732, $p < 0.05$) and all pair comparisons (Hotelling's T-square) gave significant differences ($p < 0.05$). Furthermore, LDFA depicted three separate groups, but with a significant overlapping of the signals from fish collected in the different locations (Fig. 2). LDFA was able to discriminate fish between original locations with a high degree of accuracy (87%, 63% and 57% of Aveiro, Matosinhos and Viana do Castelo fishes were correctly classified, respectively) (Table 3).

Discussion

ICPMS is an analytical technique that offers numerous advantages for the development of otolith elemental fingerprints in fishes, including a rapid and simultaneous determination of several elements with an unparalleled sensitivity (Campana et al. 1995). The purpose of ICPMS-SB is to analyse the chemical content of the whole otolith. It means that in bulk otolith analysis, all trace elements are recorded during the entire life of the fish regardless of the individual life history. The existing differences in otolith elemental fingerprints are frequently used to infer differences in stock structure (Gillanders and Kingsford 2003), but the most robust application of whole-otolith fingerprints is targeted at questions of stock mixing or for tracking stock migrations, in which the fingerprints are used as natural tags of fish movements (Campana 1999).

The trace elements obtained in this study (Sr, Ba, Mn, Mg, Ni and Li) were within the range of concentrations reported for other related coastal marine fish species

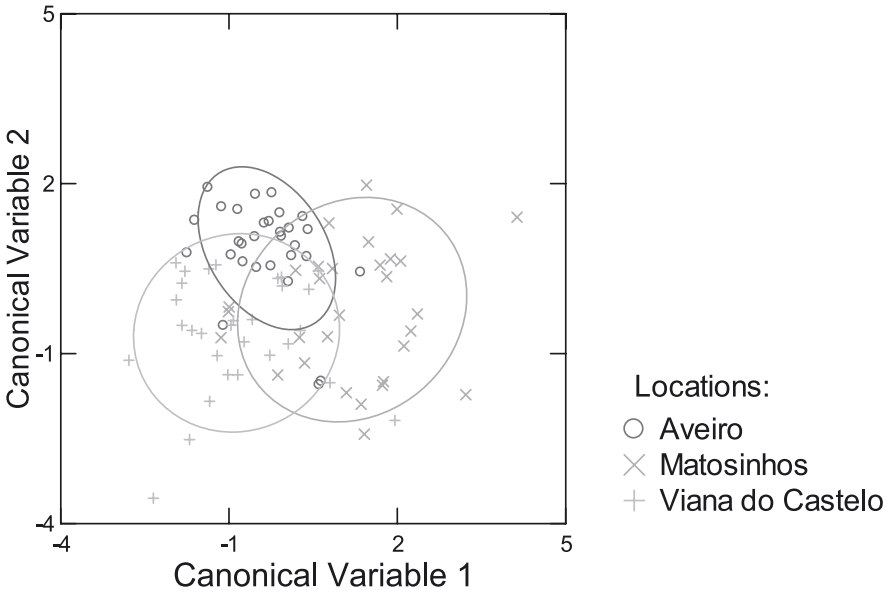

Fig. 2. Canonical variate plots displaying spatial differences in multi-elemental tags of whole pout otoliths from the three sampling locations along the NW Portuguese coast. Ellipses represent 95% confidence intervals around the data and symbols represent individual fish.

Table 3. Jackknife classification matrix of pouting specimens based on whole otolith fingerprints used in linear discriminant function analyses.

		Predicted Location			
		Viana Castelo	Matosinhos	Aveiro	% Correct
Real Location	Viana Castelo	17	6	7	57
	Mato sinhos	16	19	5	63
	Aveiro	1	3	26	87
	Total	24	28	38	69

(Jónsdóttir et al. 2006; Gibb et al. 2007; Tobin et al. 2010). The incorporation of trace elements into otolith structure is a complex process which is still not fully studied and understood, but we know that several abiotic (e.g. salinity and temperature) and biotic (e.g. feeding regimes, metabolic rates and ontogenetic events) factors have the ability to control the rate of elemental incorporation into otoliths (Bath et al. 2000; Milton and Chenery 2001; Gillanders and Kingsford 2003). Univariate statistical techniques showed that the molar concentrations obtained in the otolith bulk analysis, namely for Sr, Ba, Mg and Li, showed significant differences among the three locations. Additionally, the concentrations of Sr, Ba and Mg were higher in fish collected from Matosinhos. One possible reason for this may be the presence of the river Douro close to the Matosinhos sampling location. The Douro river drains into the Atlantic Ocean at 41° 08′ N and 08° 42′ W, close to the Matosinhos area, with an average freshwater discharge of 488 m^3s^{-1} (Vieira and Bordalo 2000). Recent studies showed a substantial anthropogenic metal contamination in the Douro estuary (Mucha et al. 2004; Ramalhosa et al. 2005; Magalhães et al. 2007), probably derived from emissions from the city of Porto through sewage, agricultural waste or due to the extensive river use by tourism and commercial boats (Netzband 2006). Additionally, near the Matosinhos area there is also the Leça river, which drains directly into the fishing port (Porto de Leixões). The Leça river is considered one of the most polluted rivers on the Iberian Peninsula and is particularly rich in heavy metals (Couto 2010). These factors contribute to the input of a large quantity of contaminants flushed downstream towards the estuary and to the adjacent coastal areas, contributing to a greater abundance of trace elements in Matosinhos coastal waters and probably in otoliths of pout collected there. Furthermore, several studies have shown higher levels of heavy metals in otoliths consistent with an environmental exposure history of fish to aquatic contamination (e.g. Geffen et al. 2003; Arslan and Secor 2005; Ranaldi and Gagnon 2010). Although there is an important input of freshwater from the Douro and Lima Rivers in Matosinhos and Viana do Castelo, respectively, which could affect the Sr:Ca and Ba:Ca ratios as result of a change in the water salinity (Macdonald and Crook 2010), this relationship is not usual for marine fish (Brown and Severin 2009).

The knowledge of the underlying causes of the elemental composition of otolith carbonate is not necessary, however, for the use of measured differences as an aid in delineating fish stocks or populations. If different fish populations inhabit different aquatic environments, or at least have a very prolonged exposure to different water environments, the otolith elemental composition should serve as a natural tag for these groups (Campana 1999). There is no evidence to date of long-term stability of otolith elemental fingerprints and some studies indicate that specific fingerprints could vary over the years, suggesting that elemental fingerprints may serve only as short-term natural tags (1-3 years) (Campana et al. 1994, 2000;

Rooker et al. 2008). However, if otolith chemistry varies over time, we can always compare the otolith natural tags from individuals belonging to the same year-classes or cohorts, as in the present study (Eldson et al. 2008). The multi-elemental analysis of whole otolith composition allowed us to obtain additional information about the different adult stocks and the stock mixing process of *T. luscus* on the NW Portuguese coast. Multi-elemental fingerprints obtained for each location had a good trace-back result to the original location areas (69% mean correct classification) suggesting a limited movement among the areas where fish were captured. Our results indicate that elemental fingerprints of whole pout otoliths are site-specific and that they can provide natural tags of their feeding/growing areas. The high classification values (57%, 63% and 87% to Viana do Castelo, Matosinhos and Aveiro, respectively) indicate that pout shows some fidelity to its feeding/growing area. Furthermore, the overlapping areas presented in the LDFA plot indicate the existence of some mixing between the individuals from the three different locations. This means that *T. luscus* could make migratory displacements between adjacent fishery areas, at least in a spatial range of about 120 km (i.e. the geographic distance between the two extreme sampling points, Viana do Castelo and Aveiro). These results are somewhat contradictory to a recent morphological study that showed no interpopulation variation for Atlantic *T. luscus* specimens (Miramontes-Sequeiros et al. 2009). Pouts from these locations can, therefore, be regarded as a single, although not necessary homogenous, stock.

It is of the utmost importance to increase existing knowledge about pout movements and the connectivity between their nursery areas and adult grounds in the NE Atlantic and Mediterranean. In complement to genetics studies, future otolith fingerprint studies should be made including the analysis of the otolith core, which represents the early life stage, in order to identify the natal origin of pouts. Natal otolith elemental signatures can be used in the future to understand pout population dynamics, to study the connectivity between spawning/nursery areas and feeding/growing grounds, and to preserve the habitats that most contribute to adult coastal recruitment.

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