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DISCRIMINATION OF *TRISOPTERUS LUSCUS* (LINNAEUS 1758) STOCKS IN THE NORTHERN OF PORTUGAL USING OTOLITH ELEMENTAL FINGERPRINTS

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Dissertação de candidatura ao grau de Mestre em Ciências do Mar – Recursos Marinhos submetida ao Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto.

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“Due to some of their characteristics…, and the dependence of these properties on the variation of the factors of the environment in which the animal lives, (otoliths) are currently among the anatomical parts of the fish that have higher utility, and whose study a larger number of practical applications allows.”

(Carlos Assis, 2004)

Abstract

The pout, *Trisopterus luscus* (Linnaeus, 1758), is one of the most important fish species captured by mainland Portugal traditional fisheries, mainly in the North coast, where the species is very abundant and commercialized. The sampling process took place between February and March 2010, with nearly 100 specimens of *T. luscus* being captured in the main three fishery grounds of the Portuguese north coast (Viana do Castelo, Matosinhos and Aveiro). Fishery took place in shallow waters along the coastline (distance less than 12 nautical miles from the shore line). From each of the three sites, we used 30 pout, with lengths ranging between 17.9 and 25 cm. For the elemental chemical analysis, we selected individuals from age group 2+, through the reading of its annual increments in the sagittal otoliths. The bulk elemental composition of otoliths was analyzed by Inductively Coupled Plasma Mass Spectometry - Solution Base (ICPMS-SB). Otolith fingerprint analysis detected the presence of several informative trace elements (Ca, Sr, Ba, Mn, Mg, Ni and Li). The main objective of this work was to evaluate the use of otolith elemental fingerprints in the discrimination of pout stocks from the Northern Portugal. Molar concentrations for each site were analyzed through uni and multivariate statistical tests. Sr, Ba, Li and Mg differed significantly among locations (One Way ANOVA, $P < 0.05$). The results allow us to discriminate adult stocks and determine, with a good percentage of classification (69%), the original sampling areas of the pout. The otolith chemical signatures of the three locations suggest some sedentary life strategy of the species in relation to their growing areas. However some connectivity between the three fishing areas exists probably as result of an important migratory flow of individuals between adjacent areas. This new data will allow us to make a more rational and sustainable management of this important northern Portuguese fishery resource.

Resumo

A faneca, *Trisopterus luscus* (Linnaeus, 1758), é um dos principais peixes capturados pela pesca artesanal em Portugal continental, principalmente na região Norte do país. Entre Fevereiro e Março de 2010 foram capturados cerca de uma centena de exemplares juvenis de *T. luscus* em três regiões piscatórias da Costa Norte portuguesa (Viana do Castelo, Matosinhos e Aveiro). De cada um dos três locais foram seleccionados 30 indivíduos, pertencentes ao grupo de idades 2+, com comprimentos entre 17.9 e 25 cm. A estimativa de idades foi obtida por leitura dos seus incrementos anuais por via dos otólitos *sagitta*. As assinaturas químicas elementares dos seus otólitos foram analisadas pela técnica de ICPMS–SB (Inductively Coupled Plasma Mass Spectometry – Solution Base). A análise química detectou a presença de vários elementos traço e vestigiais informativos (Ca, Sr, Ni, Ba, Mn, Li e Mg) cujas concentrações para cada local foram analisadas através de técnicas estatísticas uni e multivariadas. O principal objectivo do presente trabalho foi avaliar a utilidade das assinaturas químicas elementares dos otólitos na diferenciação dos stocks de faneca da costa Norte de Portugal. O Sr, Ba, Li e Mg apresentaram diferenças significativas entre os diferentes locais (análise de variância unifactorial, $P < 0.05$). Os resultados obtidos permitem-nos discriminar os stocks e determinar, com uma boa percentagem de classificação (69%), as áreas originais de captura das fanecas. As assinaturas químicas dos otólitos dos três locais sugerem algum sedentarismo da espécie em relação ao seu local de crescimento, embora com indícios de alguma conectividade entre as três zonas piscatórias, fruto de uma migração dos indivíduos entre áreas adjacentes. Os resultados obtidos irão contribuir para uma gestão mais racional e sustentável deste recurso haliêutico.

Résumé

Le tacaud, *Trisopterus luscus* (Linnaeus, 1758), c'est une des espèces de poissons les plus collectées par la pêche traditionnelle au Portugal continental, principalement dans la région Nord du pays, où l'espèce est très abondante et commercialisée. Le processus d'échantillonnage a eu lieu entre février et mars 2010, avec une centaine d'individus capturés en trois communautés de pêcheurs de la côte Nord du Portugal (Matosinhos, Viana do Castelo et Aveiro). On a obtenu de chacun des sites d'échantillonnage 30 individus appartenant au groupe d'âges 2+, avec une longueur totale entre 17.9 et 25 cm, capturés dans les zones côtières, entre 11 et 12 miles nautiques. L'estimation de l'âge a été obtenue par la lecture de leurs accroissements annuels à partir des otolithes *sagitta*. Les signatures chimiques élémentaires de leurs otolithes ont été analysées par la technique ICPMS-SB (Inductively Coupled Plasma Mass Spectometry – Solution Base). Après tout le processus de laboratoire effectué avec tous les spécimens, les deux otolithes *sagitta* de chacun des 90 tacauds ont été extraits aux fins suivantes: les otolithes *sagitta* droits ont été utilisés pour estimer l'âge des animaux, tandis que les otolithes *sagitta* gauches ont été chimiquement dissous en HNO_3 pour permettre l'analyse des éléments vestigiaux par ICPMS–SB. Cette analyse multi-élémentaire a détecté la présence de plusieurs éléments trace et vestigiaux informatifs (Ca, Sr, Ni, Ba, Mg, Li et Mn) dont les concentrations ont été analysées selon des méthodes statistiques uni et multivariées pour chaque endroit. L'objectif principal de ce travail est d'évaluer l'utilité des empreintes élémentaires des otolithes pour la discrimination des stocks de tacauds au Nord du Portugal. Les éléments Sr, Li et Mg ont présenté des différences significatives entre les divers lieux (analyse de variance unifactorielle, $P < 0.05$). L'utilisation de ces données de l'analyse de la composition totale des otolithes *sagitta* des tacauds, nous permet de distinguer les stocks adultes et de déterminer les zones originelles de leur capture, avec un bon pourcentage de précision (69%). Le site spécifique de l'ensemble des signatures chimiques des otolithes des trois zones de pêche suggèrent l'existence d'une certaine sédentarité de l'espèce par rapport à son lieu de croissance. Cependant, il y a des indices d'une certaine connectivité entre ces trois lieux, à cause de la migration des individus parmi les zones adjacentes où les tacauds, dans la phase juvénile ou adulte, semblent se déplacer. Cette étude démontre que l'analyse de la composition chimique des otolithes de *T. luscus* est un outil important qui nous permet de prendre des conclusions importantes sur la connectivité parmi les zones côtières habitées par

l'espèce. Les résultats obtenus à partir de cette étude, favoriseront une gestion plus rationnelle et soutenable de cette ressource halieutique.

Index

Index of Figures

Index of Tables

1 – Introduction

1.1 – Biology of the pout

The pout, *Trisopterus luscus* (Linnaeus, 1758), is a marine teleost that belongs to the GADIDAE family (Chevey, 1929). The genus *Trisopterus* (Rafinesque, 1814) has two more fish species: *Trisopterus minutus* (Linnaeus, 1758) and *Trisopterus esmarkii* (Nilsson, 1855). The pout body is covered by a thin layer of scales and presents three dorsal fins, two anal fins, two pectoral fins, two pelvic fins and a caudal fin (Chevey, 1929). Dorsal fins contact on their bases or show only a very small interspace between them, just like the anal fins (Cohen, 1990). At the base of the pectoral fin appears a dark spot, typical in pout (Desmarchelier, 1986). The dorsal region has a silver-brown color and the ventral region is lighter, showing a silver-white colour. Concerning the pout head, the eyes have a relatively large size and there is the presence of a well-developed jaw barbell. The lateral line appears longitudinally along the fish body, somewhat curved near the head and a darker color than the surrounding back, being visibly distinguishable (figure 1).

Figure 1 – Photography of a pout, *Trisopterus luscus*, with 22.4 cm long.

T. luscus is a demersal fish usually found in shallow coastal waters, at depths between 30 and 100 meters (Svetovidov, 1986) reaching in some cases about 250 to 300 meters of depth (Merayo, 1993). Juveniles and adults pout rarely coexist in the same habitat: juveniles are abundant in coastal areas and can penetrate in estuarine regions; adults normally inhabit areas with sandy bottoms, far from the shore (Desmarchelier, 1986). *T. luscus* is regarded as a essentially benthopelagic species of cold waters (Cohen, 1990), with a wide along the Atlantic Ocean, mainly in the North Atlantic, between 29° and 60° of latitude (Chevey, 1929). This species is also frequently found in the Atlantic coasts from the British Isles to the coast of Morocco (Alonso-Fernández et al., 2008), being found in France, Spain and Portugal (Merayo, 1996; Miramontes-Sequeiros, 2009). Pout specimens also occurs in the Mediterranean Sea, especially in West side (Alonso-Fernández et al., 2008; Cohen, 1990). The distribution of *T. luscus* does not extend north of the Shetland Islands (Chevey, 1929) due to the presence of a large pit near the Norwegian coast, which acts as a natural barrier for the species (Wheeler, 1969). The area of greatest abundance of pout is in the region of the English Channel and Atlantic coasts of France (Chevey, 1929) (figure 2).

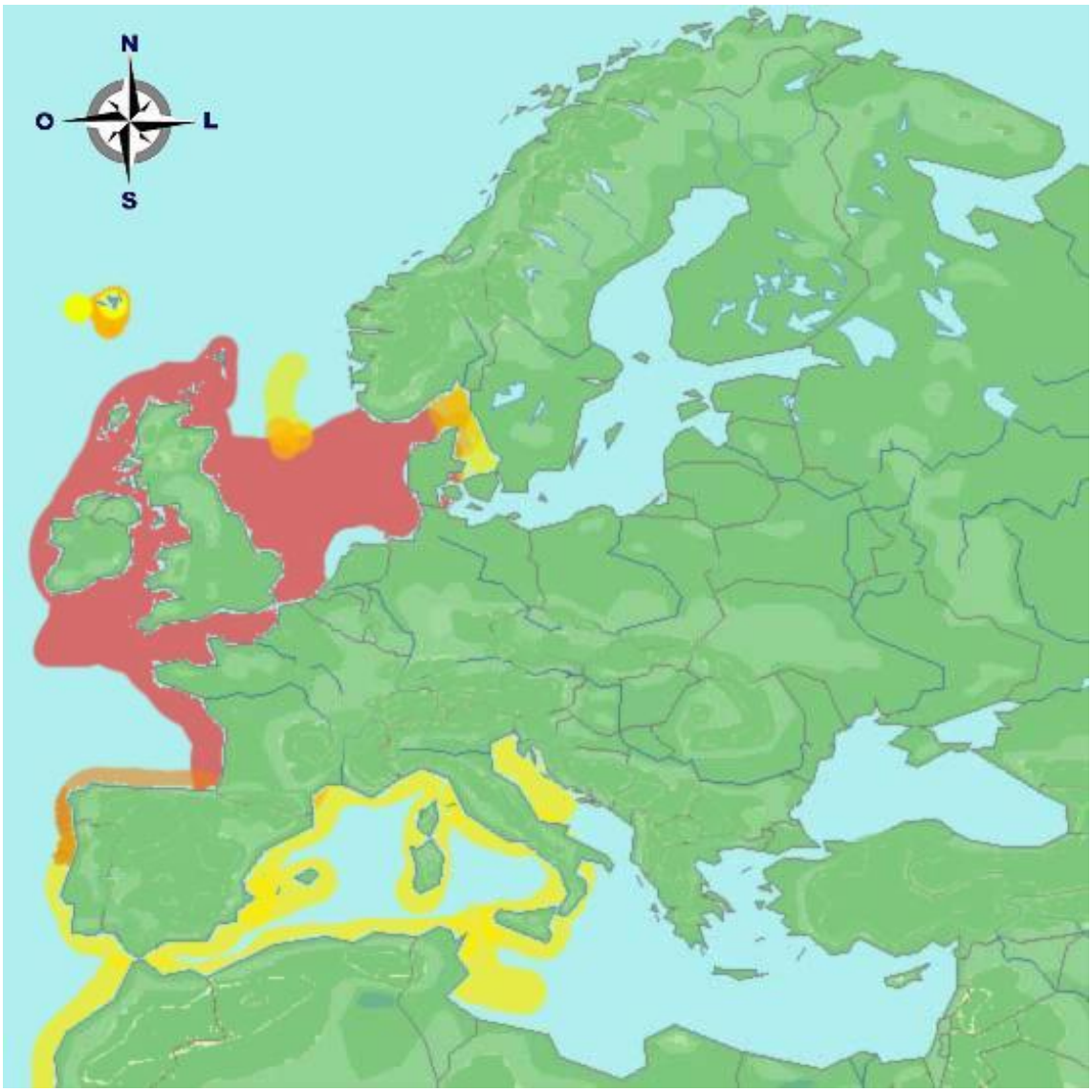

Figure 2 – Distribution map of the pout, *Trisopterus luscus*, on the North-East Atlantic and Mediterranean Sea. From yellow to red there is an increasing in the species abundance.

Based on several technical reports (DGPA, 2010) concerning the distribution of fish species in Portugal, it seems that *T. luscus* species has a greater abundance in the north region.

In the Douro estuary region, the abundance of age 0+ pout is higher in the adjacent coastal areas of the estuary (França, 2004), suggesting a preference of the species for these coastal sites during the early stages of life. The presence of the species in the Tejo estuary has suffered recently a strong decrease (Costa and Cabral, 1999; Cabral et al., 2000), possibly due to a reduction of the water quality mainly because of the de strong presence of industries and residential areas near the estuary jointly with the reduction of the river flow due to dams (Costa and Cabral, 1999). Although, the Tejo estuary has suffered some reductions in the amount of pout

newborns, the coastal areas adjacent to the estuary provide a greater number of juveniles pout, being the estuary an alternative nursery area for *T. luscus* and the coastal regions adjacent to estuaries the main nursery areas (Cabral et al., 2000). Both estuaries and adjacent coastal regions have nursery functions, but for different types of marine species (Prista et al., 2003). While estuaries are well studied and recognized as fundamental nurseries areas, the importance of adjacent coastal areas remains little studied (França et al., 2004). Additional studies are needed to access the contribution of these areas for the recruitment grounds.

The pout is a main target species for the artisanal fleets of some European countries, namely France, Spain and Portugal (Alonso-Fernández et al., 2008, 2010). There is a great abundance of the species in the Portuguese waters (estimated landings of about 3.139 and 3.278 tons in 2008 and 2009, respectively) although with a low commercial value per kilo at first auction (1.58 €/kg in 2008, 1.32 €/kg in 2009) (DATAPESCAS, 2009), and 2-4 times more at fish market. It is in the Portuguese north coast that pout gains more preponderance in the national fisheries and shows to be more abundant (DGPA, 2010).

The commercial landings of fresh fish in the Portuguese north trade delegations of Viana do Castelo, Matosinhos and Aveiro shows that pout is within the four main discharged species. In the central region of the country is also common the landing of pout (Nazaré). However at south of Nazaré and in the Portuguese autonomous regions of Madeira and Azores, the pout is no longer part of the predominantly discharged species, demonstrating the remarkable predominance of this species in waters of northern Portugal (table 1).

Table 1 – The four most captured species in Portugal (DGPA, 2010).

Delegation	Four most captured species (tons)			
Viana do Castelo	Common octopus (56.5)	European hake (9)	**Pout (8.8)**	Horse mackerel (6.7)
Matosinhos	Sardine (1077.7)	Horse mackerel (102.5)	**Pout (88.7)**	Common octopus (27.2)
Aveiro	Common edible cockle (179.1)	Sardine (136.3)	Horse mackerel (84.7)	**Pout (60)**
Nazaré	Horse mackerel (69.6)	European hake (21.8)	Common octopus (16.1)	**Pout (13.5)**
Peniche	Sardine (335.8)	Horse mackerel (81.49	Chub mackerel (75.8)	Common octopus (68.5)
Sesimbra	Sardine (416.2)	Black scabbardfish (128.1)	Common octopus (99.2)	Chub mackerel (56.1)
Sines	Sardine (251.6)	Chub mackerel (22.3)	Common octopus (14.6)	Two-banded seabream (6.7)
Portimão	Sardine (128)	Horse mackerel (76.3)	Common octopus (58.1)	Chub mackerel (55.9)
Olhão	Chub mackerel (414.2)	Common octopus (149.3)	Sardine (77.7)	Common cuttlefish (57.7)
Azores	Blue Jack mackerel (85.4)	Veined squid (37.3)	Chub mackerel (30.6)	Blackspot seabream (30.4)
Madeira	Black scabbardfish (127.9)	Blue Jack mackerel (40.9)	Leafscale gulper shark (13.8)	Chub mackerel (11)

On the reproductive biology of the species, histological studies on *T. luscus* female gonads revealed that the species has a determinate fecundity and an asynchronous development of ovaries (Barbosa, 2002; Fernández-Alonso, 2008). It means that pout have a potential of fertility clearly established prior to onset of spawning and that remains the same, year after year (Murua and Saborido-Rey, 2003). Ovaries possess oocytes at all stages of development (Murua and Saborido-Rey, 2003), allowing several spawning periods per individual over a reproductive season (Costa, 2004).

The English Channel coast is one of the areas with greatest abundance of pouts and is known as a major breeding area, where occurs annually frequent spawns from January to July (Chevey, 1929). The pout have an extended spawning season from December to March/April in the Atlantic Ocean (Cohen, 1990; Merayo, 1996; Ré 1999; Alonso-Fernández et al., 2008) and from January to July in the Mediterranean Sea (Cohen, 1990), always in shallow waters (Cohen, 1990).

The pout eggs are spherical and pelagic with non-segmented yolk and pout larvae hatch with 3.0 mm long (Ré, 1999). Until reach 18.0 mm, the larvae assume a very short pelagic life, floating in the water column until develop into more advanced life stages and move down to greater depths waters (Chevey, 1929). After the larval life, the early juvenile shows an active inshore migration towards shallow coastal areas (Hamerlynck and Hostens, 1993). After spending the first year of life in these coastal nursery areas, pouts migrate again, but this time to more distant and deeper waters (Cohen, 1990; Hamerlynck and Hostens, 1993). The high density of age 0+ pout existing in shallow water areas, coupled with the fact that adult pouts are predominantly found in waters of greater depths, shows that this is a species with different juvenile and adult habitats (Cabral, 2000; Tanner, 2009).

The pout is a species with a short lifespan, reaching about 3-4 years old (Chevey, 1929; Cohen, 1990), although some individual variation may occur. Pout can easily reach older ages such as 5 to 9 years old (Labarta and Ferreiro,1988; Gerby-Barré, 1983; Puente, 1988; Merayo and Villegas, 1994).

T. luscus initial growth is extremely fast and followed by an abrupt slowdown when entering the sexual maturation (Chevey, 1929; Puente, 1988). This generally occurs between the first and second year of life (Chevey, 1929; Merayo, 1996). Pout may reach 21-25, 23-27 and 28-33 cm during the first, second and third year of age, respectively. However, they usually do not exceed 30 cm long, although they can reach a maximum length of 45 cm, (Cohen 1990). Females have a faster growth than males (Puente, 1988; Merayo and Villegas, 1994).

Results obtained from macroscopic inspection of gonads revealed a length at sexual maturity of 22 cm for the females (Merayo 1996; Alonso-Fernández et al., 2008) and of 23.4 cm for the males (Merayo, 1996) but this length depends highly to external factors (Domínguez-Petit et al., 2008).

Feeding activity in pout occurs in the late afternoon and early evening (Last, 1978). During daytime pouts minimize their energy expenditure, leaving the task of feeding to the night, probably due to a greater prey availability (Fowler et al., 1999). The pout is a predator species since the earliest stages of life (Last, 1978). After the yolk sc reabsorption early juveniles begin to feed exogenously, being diatoms, dinoflagellates, tintinnids, nauplii and copepodites larvae of crustaceans, the main food supplies (Last, 1978). For juvenile pouts, both in estuaries and in adjacent coastal areas, crustaceans are the main source of food (Costa and Cabral, 1999; Oliveira, 2002; França et al., 2004). To pouts with greater sizes, fishes and crustaceans are the main preys (Santos, 1989; Hamerlynk and Hostens, 1993).

1.2 – Otoliths as useful tools for ichthyologic studies

Otoliths are acellular inner-ear fish structures formed by calcium carbonate ($CaCO_3$) in the mineral form of aragonite (90%) and a protein matrix keratin-type, called otolin (10%) (Degens et al., 1969; Elsdon et al., 2008). During otolith formation, different chemical elements can be incorporated in their structure (Gillanders and Kingsford, 2003). The main elements presented in the otoliths are: calcium, carbon and oxygen. Other elements (e.g. Sr, Ba, Mn, Mg) occur in the otoliths structure, but at lower (> 100 ppm) or even trace (< 100 ppm) levels of detection (Campana, 1999).

The inner ear is a labyrinthic compartment located in a dorsal and lateral position to the fish brain, where otoliths are (Campana, 1999; Thresher, 1999; Tuset et al., 2008). The teleosts inner ear consists of three semi-circular canals, each of which with an otolith inside: the *utriculus* contains inside the *lapillus* otolith; ventrally to *utriculus* there is the *sacculus*, containing the larger otolith, the *sagitta*, and finally, there is *lagena*, housing inside the *asteriscus* otolith (Härkönnen, 1986; Popper et al., 2005). Due to their larger dimensions and ease of obtaining, *sagitta* are the more frequently used otoliths (Thresher, 1999; Tuset et al., 2008), except for the Ostariophysi group, where *asteriscus* are the larger otoliths (Morales-Nin and Panfili, 2002). In the inner ear, all the otoliths are surrounded by a semipermeable membrane and bathed in an endolymphatic fluid (Campana, 1999), whose chemical properties will influence the whole process of formation and calcification of these structures (Borelli et

al., 2003). Otoliths are part of a major system that controls the balance and hearing in teleost fishes (Campana, 1999; Ling et al., 2005; Popper et al., 2005).

The more classical use of otoliths in fish science is the age determination (Gauldie, 1988, 2008). This purpose has more than a hundred years and started with Reibisch's observation of plaice (*Pleuronectes platessa*) *annuli* in 1899 (Campana, 1999). Since then, much of the attention given to the otoliths is largely due to the precision of the estimated ages based on the counting of growth rings formed in the otoliths structure (Campana & Thorrold, 2001). In 1971, with Pannella's discovery of daily growth increments in *Merluccius bilinearis* otoliths, there was the emergence of new studies on otoliths microstructure (Campana, 1999). Nowadays, otoliths are of interest to investigators from several disciplines including systematic, evolution, auditory neuroscience, fisheries and general biology of fishes (Popper et al., 2005). In the last decade, significant advances have occurred in the protocols for determining the age of fish through otolith analysis (Campana, 2001) and this estimation involves several stages, from collection and preparation of these structures to the observation of the growing bands (Morales-Nin and Panfili, 2002).

Fish larvae do not have annual or seasonal growth bands and the determination of their age through otoliths is only possible due to the daily growth increments. For some juveniles it happens the same but, for adults, determining the age by counting daily growth rings is not advisable and would be a complicated and unreliable task, being used for this purpose annual or seasonal growth marks (Morales-Nin and Panfili, 2002).

Through the microscope visualization of the otoliths it is possible to differentiate the existence of two different zones with distinct optical characteristics, opaque and translucent zones (Hunt, 1980). Opaque zones are extensive areas of rapid growth, corresponding to the large deposition of aragonite during the warm months and, translucent zones, are narrow areas of slow growth, corresponding to the lower deposition of aragonite during the cold months (Hunt, 1980). To an opaque zone follows a translucent zone and the set of these two areas must correspond to an annual growth increment or *annuli* (Hunt, 1980; Smith and Deguara, 2003). However, differentiation between different growth zones in otoliths is not always a simple and objective task. The identification of seasonal increments, or even daily ones, is never easy due to the presence of discontinuities and false rings, corresponding to events in the life of fish, such as migration or spawning, that may affect the interpretations (Morales-Nin and Panfili, 2002). The reproduction, for example, requires from the individuals a lot of energy and calcium reserves, leading to a decrease in somatic

growth and affecting the growth of calcified structures such as otoliths (Geffen et al., 2002).

The process of age estimation is easily incorporated by several types of errors, some of which may strongly influence the results (Campana, 2001). Yet, some techniques are frequently used to diminish the probability of some errors occurrence. The utilization of staining techniques or computer specialized software (like imaging programs) is frequent and it helps to improve the reading of growth increments.

Most of the works realized with otoliths are possible only due to some particular characteristics of these structures, which make them excellent natural markers of the fish habitat and valuable tools for studies of fish life history and movements (Campana, 1999; Campana and Thorrold, 2001; Elsdon et al., 2008): 1) otoliths grow by the addition of calcium carbonate and by the successive uptake of chemical elements present in the water surrounding the fish; 2) otoliths are structures deposited continuously since the first until the last phases of life of the fish; and 3) they do not suffer resorption and this is a key factor to the preservation of the growth and complete environmental record (Clear and Kalish, 1999; Campana and Thorrold, 2001). This last property is perhaps the most important characteristic because it is not shared with any other calcified structures of fish or other vertebrates (Campana and Thorrold, 2001). However, it is the combination of the three characteristics that enables otoliths to record in their structure chemical elements acquired from the different bodies of water inhabited by fishes throughout their life (Campana et al., 1995).

Different water bodies are regulated by different physical, chemical and biological parameters that vary both in space and time (Dorval and Jones, 2005; Kerr et al., 2007; Elsdon et al., 2008). During the otolith growth, chemical elements present in the water environment are deposited in its structure. Since the otoliths are not in direct contact with water, the incorporation of elements appears to be related to endolymphatic fluid composition (Campana, 1999; Elsdon and Gillanders, 2003).

Otolith aragonite crystallizes from the endolymphatic fluid (Bath et al., 2000), whose composition is regulated by membranes that separate the external environment from the blood plasma and the blood plasma from the endolymphatic fluid (Kalish, 1991). In other words, chemical elements present in the water will be transported through the gills to the blood plasma, then into the endolymphatic fluid that surrounds otoliths and finally to the mineral structure of the otolith (Bath et al., 1999; Campana, 1999), replacing calcium (Ca^{2+}) in the aragonite calcium carbonate (Milton and Chenery, 2001).

The aquatic environment has a strong influence on the otolith structure (Campana and Thorrold, 2001), which has the capacity to act as a natural marker of

some properties of aquatic ecosystems (Gillanders and Kingsford, 1996; Bath et al., 1999; Elsdon and Gillanders, 2003). This is particularly valid if fish reside in a particular environment long time enough to incorporate a detectable chemical tag in their otoliths (Elsdon et al., 2008). However, factors such as temperature, salinity, element concentration in water and issues related to the physiology and ontogeny of the fish can influence the incorporation of chemical elements in otoliths and lead to a misinterpretation of results (Clear and Kalish, 1999; Elsdon and Gillanders, 2002; Hamer et al., 2003; Elsdon and Gillanders, 2004).

Chemical analysis of the whole otolith gives results about the elemental fingerprint experienced by the fish throughout his life, from the earliest embryonic stages until death. Therefore, chemical bulk analysis acts like a tag of the environment inhabited by fish over the entire life (Campana et al., 2000; Elsdon et al., 2008). On the other hand, the chemical analysis of the otolith fraction deposited during the earliest stages of life, such as the core region, allows us to take conclusions about the fish origin (Clear and Kalish, 1999). Thus, along the otolith surface, from the core to the edge, it is possible to observe ontogenetic changes through the otolith elemental composition (Geffen et al., 2002).

Lately, many types of studies have been done based on otoliths chemical properties, showing a great utility for the determination of stock structures (e.g. Campana and Gagné, 1995, Campana et al., 2000; Tresher, 1999), the study of the early life stages of the fishes (Cooper et al., 2004), the origin of fish and the contribution of estuaries to the adult populations (e.g. Gillanders, 2005; Hamer et al., 2005; Vasconcelos et al., 2007, 2008) or the study of migratory behavior (e.g. Clear and Kalish, 1999; Secor and Rooker, 2000; Secor and Piccoli, 2007).

1.3 – Objectives

The main purpose of this work was to evaluate the use of the elemental composition of *T. luscus* otoliths from individuals collected in three main fishery grounds from the species in northern Portugal (Viana do Castelo, Matosinhos and Aveiro) to determine whether these otoliths fingerprint are site-specific and can be used to assess, or not, the degree of separation between stocks and to investigate population connectivity between these areas.

2 – Materials and methods

2.1 – Biological Sampling

The pouts used in this work were collected between middle February and early March 2010 by artisanal fishing boats landing at the three main fishing harbors (Viana do Castelo, Matosinhos and Aveiro) in the North region of the Portuguese coast (figure 3).

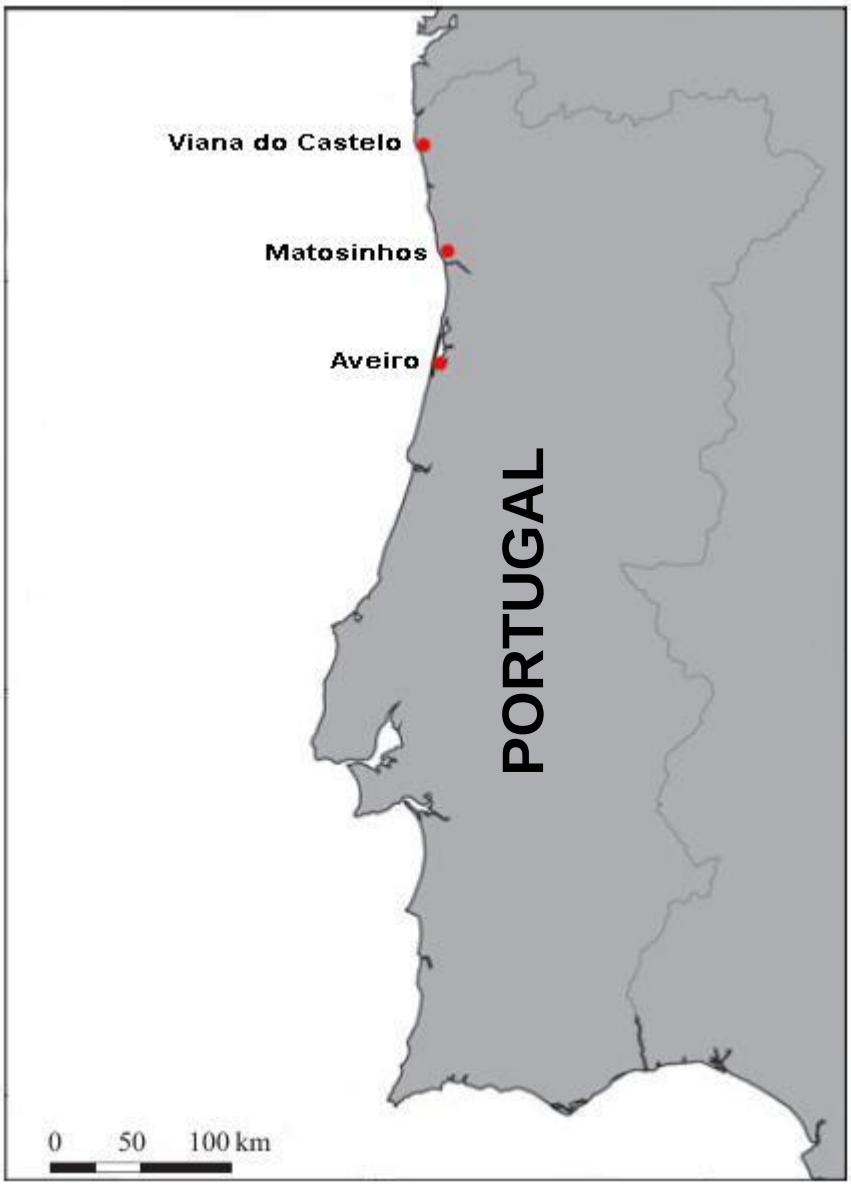

Figure 3 – Fishing harbors in the NW Portugal (Viana do Castelo, Matosinhos and Aveiro) where the pout specimens were collected.

These collection sites were chosen because pout is very abundant and widely commercialized in these areas (DATAPESCAS, 2009).

The pout were caught in costal shallow waters (until 75 m of water depth) and near the coast line (up to 12 nautic miles from the shoreline) using small local fishing boats equipped with gillnets (60 mm mesh size). An effort to obtain pout specimens from the same cohort was made by choosing individuals with similar length. Subsequently pout age was confirmed by counting the annual growth increments formed in otoliths.

The collected fish was stored in ice after landing. In the laboratory, total length (0.1 cm), gutted weight (0.01 g), gonad weight (0.01g) and liver weight (0.01g) were recorded for all specimens.The sex of each pout was indentified through macroscopic visualization of their gonads (Barbosa, 2002). Sex ratio was determined through the female/male relation for the three sampling locations.

2.2 – Gonadosomatic and Hepatosomatic indexes

Gonadosomatic [(gonads weight / eviscerated body weight) * 100] (Kartas and Quignard, 1984) and hepatosomatic index [(liver weight / eviscerated body weight) * 100] (Nikolsky, 1963) were computed for all specimens.

2.3 – Otolith Age Estimates

Otoliths were extracted with plastic forceps from pout head, washed with distilled water, dried with paper and stored in eppendorf tubes. Otoliths are differentiated in left and right *sagitta* according with the position of the *sulcus acusticus* and the *rostrum* (Secor et al., 1991).

The preparation of pout otoliths for age estimates followed the procedures of previous works (Williams and Bedford, 1974; Puente, 1988; Merayo and Villegas, 1994).

Right sagittal otoliths were placed in moulds using an epoxy resin (Struers, Epofix). All the templates were left 5 min in the hot oven (60ºC) to release air bubbles and dry the mixture, after which the polymerization process occurred overnight at room temperature. Transversal sections (~2 mm) of the blocks were then taken through the core region with a diamond saw (Buehler, Diamond Saw 15HC series) lubricated with Milli-Q-Water at 3000 rpm. The otoliths were ground in 500, 1000 and 1200 silicon carbide papers to a thickness of approximately 0.8 mm. Afterwards the thin otolith sections were fine polished using alumina solution (1:20), in order to clean the

scratches on its surface. Otolith thin sections were immersed in a clearing mixture of ethanol and glycerol (1:1) and the annual increments (translucent and opaque bands) were counted using stereoscopic microscope, Meiji (EMZ-13TR), under reflected light against dark background at 2.5x magnification. Age readings were carried from microscope photographs took with a digital USB camera (Olympus SC 30).

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). Two independent observers made two readings each one and the percentage of concordance (82%) was calculated to obtain the age of each fish. The age was calculated by counting the translucent increments, but taking into consideration the date of birth and the date of capture. The birth date was assumed following the rules in North hemisphere, 1st of January (Panfili et al., 2002).

2.4 – Otolith Chemical Analyses

Prior to the chemical analyses, the otoliths were cleaned and decontaminated in an ultrasonic cleaner for 5 minutes in 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. Thereafter, otoliths were immersed in 1% HNO_3 solution for 10 s to remove superficial contamination, following a double-immersion in ultrapure water for 5 min to remove 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 technique for inductively coupled plasma mass spectrometry (ICPMS-SB). Decontaminated left otoliths were weighed on an analytical balance (0.0001 g), dissolved in 1 ml of nitric acid ultra pure during 15 minutes and diluted with ultra pure water (Milli-Q Water) to a final acid concentration of 10% HNO3 (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). The instrument is equipped with a compact double-focusing magnetic sector mass spectrometer of reversed Nier-Johnson geometry. All measurements were performed 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 containing 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, 115In at 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. The calibration was performed 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 (^{44}Ca, ^{88}Sr, ^{60}Ni, ^{137}Ba, ^{25}Mg, ^{7}Li and ^{55}Mn) were detectable in whole otoliths of pouts and they were used to further ICPMS-SB. For Cu, Zn and Pb, concentrations were below the limit of detection.

Otolith samples were analyzed sequentially in random sets and between sets, two fish otolith reference materials were analyzed for quality control: (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 2.0 and 5.0% relative standard deviation (R.S.D.). 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): ^{44}Ca (10), ^{88}Sr (0.25), ^{60}Ni (1), ^{137}Ba (0.25), ^{25}Mg (10), ^{7}Li (1) and ^{55}Mn (0.25).

2.5 – Statistical Analyses

ICPMS-SB concentrations of trace elements, originally in µg element/l solution were transformed to µg element/g otoliths and thereafter to molar ratios (µm element/ mol Ca).

Raw data for each element were checked for normality, homoscedasticity and homogeneity of variances-covariance matrices prior to statistical analysis. These assumptions were expected to be 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 weigth as co-variate). Otolith elemental concentrations were significantly correlated with otolith mass for all elements, with the exception of Ba. Sr presented a positive relationship (r^2=0.12, n=90, $P < 0.05$), opposite to Mn (r^2=0.18, n=90, $P<0.05$), Mg (r^2=0.46, n=90, $P<0.05$), Li (r^2=0.38, n=90, $P<0.05$) and Ni (r^2=0.18, n=90, $P<0.05$) that 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 elements fingerprint between locations. If significant differences exist, it was followed by a Tukey post hoc test**.**

Multivariate analysis of variance (MANOVA) was used to explore multi-element fingerprints and detect differences in the otoliths multi-elemental composition from different locations. For MANOVA we reported the approximate F-ratio statistic for the most robust test of multivariate statistics (Pillai's trace). Multi-element compositions of otoliths were analyzed using a classic linear discriminant function analysis (LDFA). Only the elements that differed significantly between locations using univariate tests were used in LDFA using a complete analysis. LDFAs functions allow us to classify individuals to the original locations from which pouts 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) and SigmaStat (version 3.5) softwares. The statistical level of significance (α) was 0.05.

3 – Results

There was no significant differences for size (One-Way Anova, n=90, $P<0.05$) and fish eviscerated weight (one-Way Anova, n=90, $P<0.05$) between locations. Mean total length was 20.2 cm, 19.9 cm and 20.5 cm for Viana do Castelo, Matosinhos and Aveiro, respectively.

The mean values of eviscerated weight were 81.30 g, 80.12 g and 86.02 g for the same locations, respectively. The GSI values were of 1.79%, 3.72% and 1.24% and the mean values of HSI were of 2.56%, 4.33% and 1.72% for Viana do Castelo, Matosinhos and Aveiro, respectively.

The sex ratio was of 0.5 in Viana do Castelo, 6.5 in Matosinhos and 0.25 in Aveiro (table 2).

Table 2 – Total length (TL), eviscerated weight (EW), gonadosomatic index (GSI), hepatosomatic index (HSI) (mean ± SE) and sex ratio for each of the three locations.

Location	N	TL (cm)	EW (g)	GSI (%)	HSI (%)	SR
Viana do Castelo	30	20.2 ± 0.30	81.30 ± 3.61	1.79 ± 0.34	2.56 ± 0.24	0.5
Matosinhos	30	19.9 ± 0.33	80.12 ± 4.44	3.72 ± 0.53	4.33 ± 0.33	6.5
Aveiro	30	20.5 ± 0.19	86.02 ± 2.40	1.24 ± 0.24	1.72 ± 0.09	0.25

The pout *sagitta* otoliths are thick, with an oval shape, a rounded anterior edge, a pointed posterior edge and with some typical zones easy to identify (figure 4). The inner face is convex and smooth, while the outer face is concave and formed by globular concretions of limestone (figure 5). On the otoliths inner face there is the presence of the *sulcus acusticus*, a slightly deep zone that crosses longitudinally the otolith body, dividing it into an upper and a lower area. Otolith thin sections observed in a stereoscopic microscope under reflected light allowed the pout age estimation. Both the observers responsible for the age reading through the count of translucent increments obtained a result of 2 years with a good concordance percentage of 82% (figure 6).

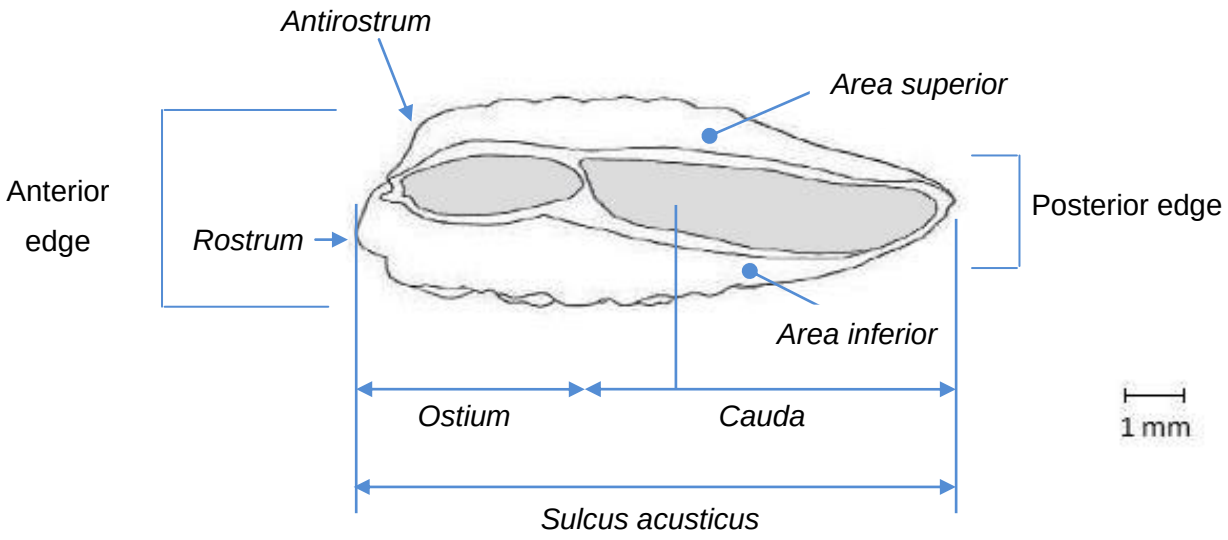

Figure 4 – View of the inner face of a *Trisopterus luscus sagitta* otolith, focusing the main morphological zones (modified from Assis, 2004).

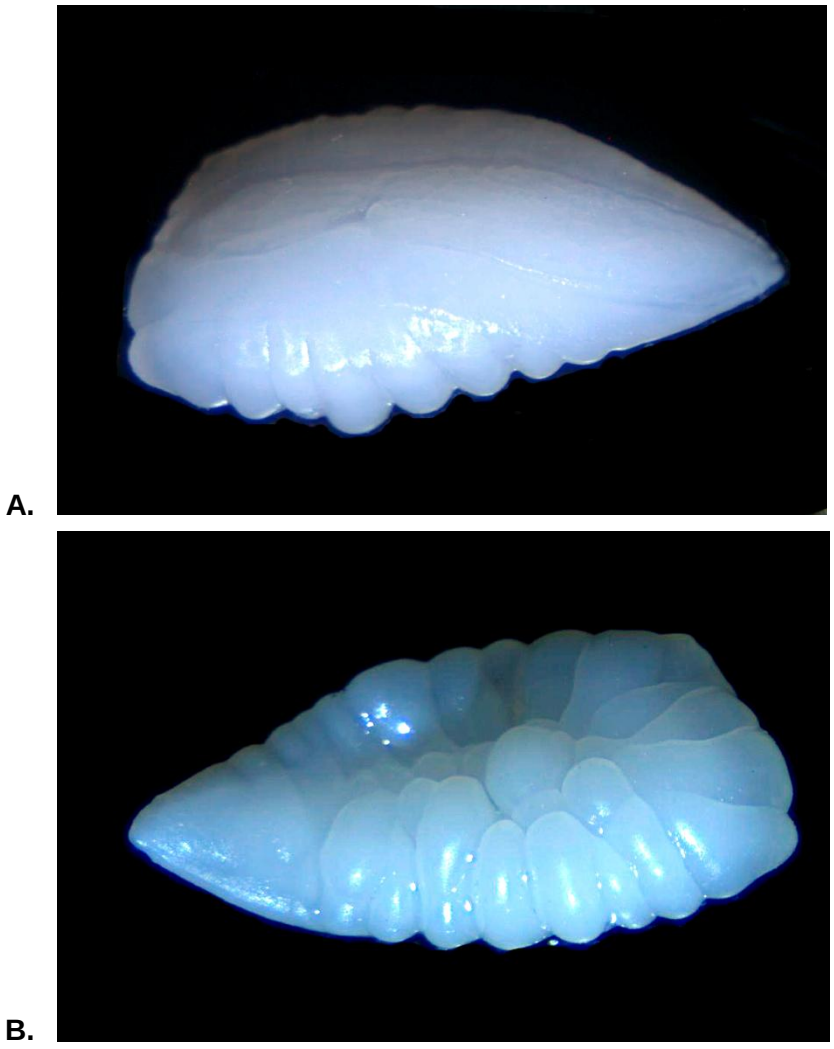

Figure 5 **–** Stereoscopic microscopic photography of a *Trisopterus luscus* right *sagitta* otolith showing: A) the inner convex face and *sulcus acusticus*; B) the outer concave face.

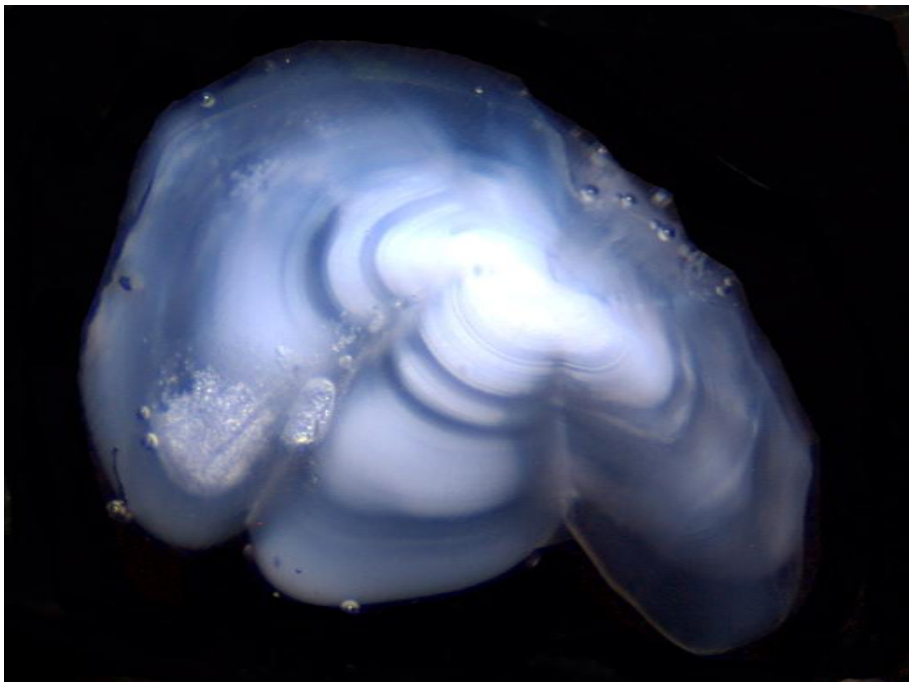

Figure 6 – Exemplary microscope photography of a thin *sagitta* section used for the pout age reading. This otolith is from a pout with 20.6 cm and 84.92 g, collected in Matosinhos.

Concerning the univariate tests, Sr, Ba, Li and Mg differed significantly among locations (ANOVA, $P < 0.05$). Matosinhos was the location with the highest concentrations of Sr, Ba and Mg and Viana do Castelo has the highest concentrations of Li, although not statistically significant (table 3, figure 7).

Table 3 – Molar elemental concentrations (mean ± SE), expressed in µg mol element/mol calcium, in the whole otoliths of pout fishes collected in three locations along the NW of Portugal (Viana do Castelo, Matosinhos and Aveiro).

Element	Viana do Castelo	Matosinhos	Aveiro
Sr	2200 ± 50	2644 ± 55	2370 ± 46
Ni	0.41 ± 0.01	0.43 ± 0.01	0.43 ± 0.01
Ba	2.18 ± 0.11	3.08 ± 0.13	2.50 ± 0.11
Mn	2.76 ± 0.09	2.68 ± 0.09	2.76 ± 0.09
Li	5.24 ± 0.07	4.97 ± 0.10	4.57 ± 0.07
Mg	1.93 ± 0.01	1.96 ± 0.01	1.89 ± 0.01

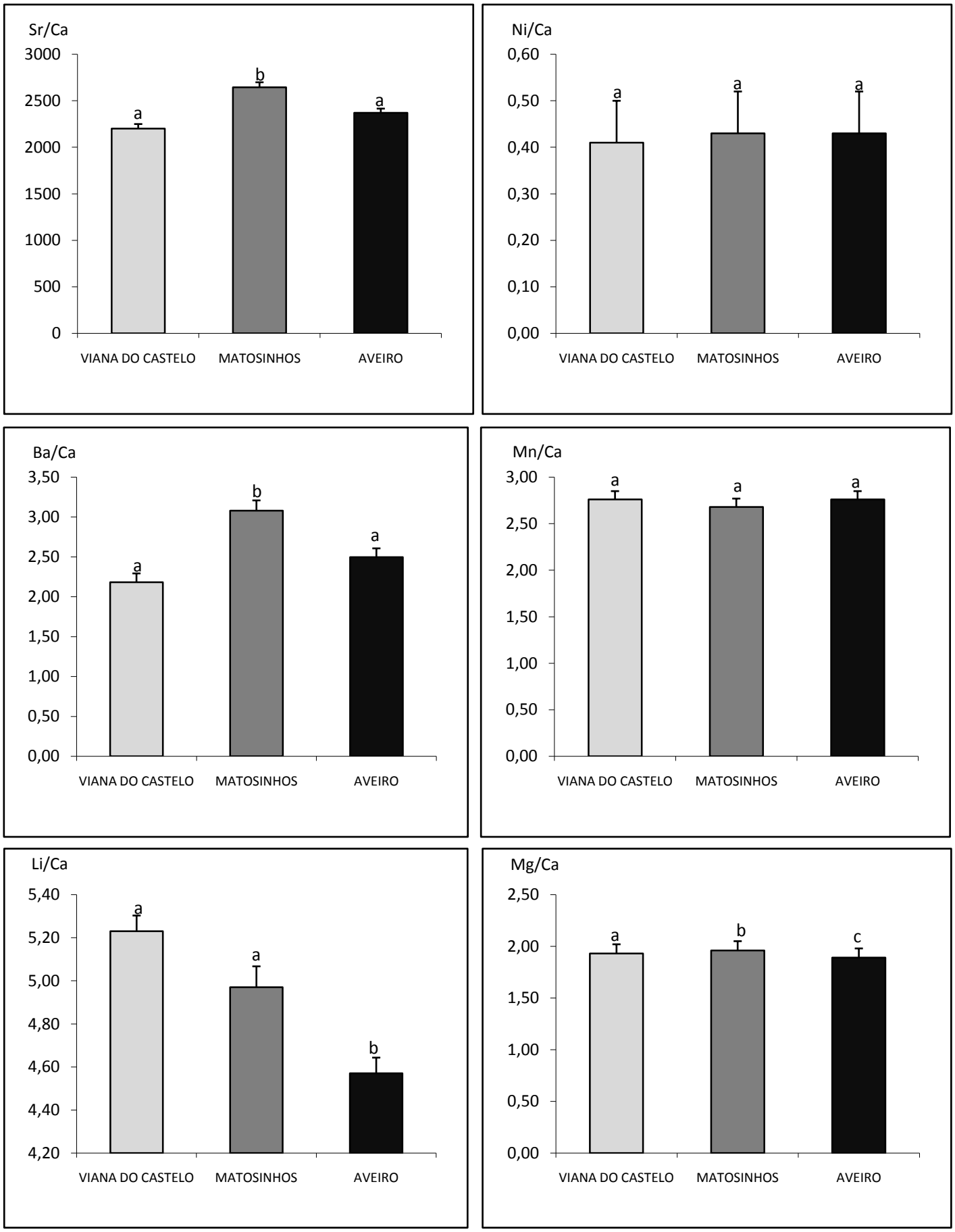

Figure 7 – Molar elemental concentrations (mean ± SE) in whole otoliths (ICPMS-SB) of pouts collected in three sampling locations (Viana do Castelo, Matosinhos and Aveiro) along the NW Portuguese coast. Ratios are given in µmol/mol Ca. The locations marked with the same letter above the error bars are not significantly diferent ($P > 0.05$).

MANOVA indicated the existence of significant differences in the multi-element signatures of the whole otoliths (Pillai Trace, $F_{12.269} = 0.732$, $P<0.005$). Furthermore, LDFA depicted a clear separation between fish locations, although with a significant overlapping between the three locations (figure 8).

LDFA was able to discriminate fish to the original location with a high degree of accuracy (87%, 63% and 57% of Aveiro, Matosinhos and Viana do Castelo fishes correctly classified) (table 4).

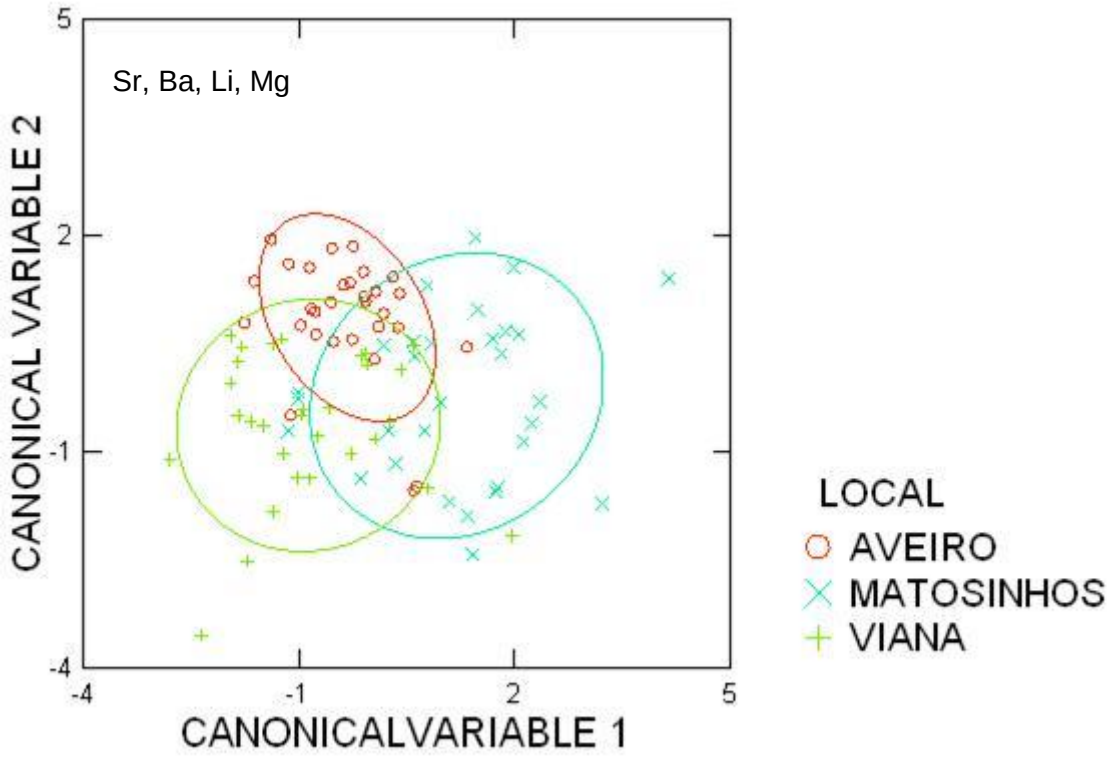

Figure 8 – Canonical variate plots displaying spatial differences in multi-elemental tags of pout whole otoliths from the three sampling locations along the NW Portuguese coast. Ellipses represent 95% confidence intervals around the data, and data points represent individual fish.

Table 4 – Jackknife classification matrix of pout specimens based on whole otolith fingerprints used in linear discriminant function analyses.

Original Location		Predicted Location			
		Aveiro	**Matosinhos**	**Viana do Castelo**	**% Correct**
	Aveiro	26	3	1	87
	Matosinhos	5	19	6	63
	Viana do Castelo	7	6	17	57
	Total	38	28	24	69

4 – Discussion and conclusion

To our knowledge, this is the first work that describes the concentration of trace elements in otoliths of *Trisopterus luscus* juveniles captured in the North coast of Portugal.

There were no significantly differences of the pout size and eviscerated weight between the three sampling locations.

The mean values of gonadosomatic and hepatosomatic index obtained in this work for *T. luscus* are in agreement to the results obtained in Barbosa (2002) for the months of February and March (4.21% and 3.49%, for GSI and HSI, respectively), also dealing with pout specimens captured on the Portuguese North coast (Aguda and Angeiras), using as well the eviscerated weight of pouts.

The sex ratio obtained for the three sampling locations in this work was of 0.88 females/male, showing a slightly dominance of males in the Northern Portugal during the two sampling months, February and March. In Barbosa (2002), the sex ratio for the two locations in the same two months of capture was similar, with a value of 0.97 females/male. At the present work, and despite the general dominance of *T. luscus* male specimens, Matosinhos was the single location with a high proportion of female individuals. In the other two locations, Viana do Castelo and Aveiro, males were in superior number.

In the pout growth, for each year of life are formed an opaque and a translucent ring in the otolith structure, like for most of marine teleosts from temperate zones (Blacker, 1974; Desmarchelier 1986). The thin otolith sections observed in a stereoscopic microscope allowed the age estimation of the studied specimens. Both the observers responsible for the age reading through the count of translucent increments obtained a result of 2 years (percentage of concordance of 82%), so the selected individuals were from age group 2+.

ICPMS is an analytical technique that offers numerous advantages in the developing of otolith elemental fingerprints in fishes, including a rapid and simultaneous determination of several elements (and isotopes of elements) with an unparalleled sensitivity (Campana and Gagné, 1995). Currently there are two main analytical methods used in the analysis of otolith fingerprints. In ICPMS–SB the purpose is to analyze the chemical content of the whole otoliths, meaning that all the trace elements incorporated in the otoliths from birth to dead (or capture), i.e. during the entire life of the fish, are recorded, regardless of the individual life history. In the case of the other method, ICPMS–LA (Inductively Coupled Plasma Mass Spectometry – Laser Ablation),

it allows just to analyze a particular section of the otolith, normally a growth band, the core or the otolith edge, representing a particular stage of the fish life (Eldson et al., 2008).

The existing differences in otoliths 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). Otolith elemental fingerprints were also successful in discriminating fishes from different geographic areas (e.g. Campana and Gagné, 1995; Campana et al., 2000; Gillanders and Kingsford, 2000).

The incorporation of trace elements in otoliths structure is a complex process which is still not fully studied and understood, but we know that several factors like salinity, temperature, concentrations of elements in the water or ontogenetic events (e.g. metamorphosis) have the ability to control the otolith rate of incorporation (Bath et al., 2000; Milton and Chenery, 2001; Gillanders and Kingsford, 2003). Some other factors, like feeding regime, metabolic rate and growth, may also influence otolith chemical composition (Kalish, 1991).

The seven trace elements obtained in this study (Ca, Sr, Ni, Ba, Mn, Li and Mg) were the ones that demonstrated to have a strong chemical signal, with a concentration above the limit of detection of the equipment. Our values are within the range of concentrations reported for other coastal marine fish species (Campana, 1999; Campana et al., 2000).

Univariate statistical techniques showed that the molar concentrations obtained in the bulk otoliths, namely for Sr, Ba, Li and Mg, showed significantly differences among the three locations. Sr differs considerably from the other two locations, being higher in Matosinhos; Ba possesses higher molar concentrations in Matosinhos; Li have smaller concentrations values in Aveiro; Mg showed significantly differences among the three sampling locations, being higher in Matosinhos and lower in Aveiro.

The concentrations of Sr, Ba and Mg are higher in Matosinhos location, although not necessarily statistically significant, in the last case. One possible reason for that may be the presence of a large river, the river Douro, nearby the sampling location. This river reaches the Atlantic Ocean only few kilometers south to Matosinhos, at Foz do Douro, and is the third longest river of the Iberian Peninsula, possessing the largest basin, with a total area of about 97.680 km^2, of which 18.643 km^2 (19%) are in Portugal and 78.960 km^2 (81%) in Spain (DEWA~Europe, 2004). Studies realized in the Douro estuary showed mercury pollution (Ramalhosa et al., 2005) and a substantial anthropogenic metal contamination (Mucha et al., 2004),

probably derived from emissions from the city of Porto through sewages, agriculture wastes or the extensive use by tourism and commercial boats (SedNet report, 2007). These factors contribute to a large quantity of contaminants flushed downstream towards the estuary and to the coastal adjacent areas, contributing to a great presence of trace elements on Matosinhos coastal waters.

By definition, a fish population is a unit of fish of the same species within a given area that has the potential to mix (Elsdon et al., 2008). If different fish populations inhabit different aquatic environments, the otolith elemental composition should serve as a natural tag of those groups, taking into account two important assumptions: 1) material deposited on the otolith is metabolically inert; 2) the physical and chemical environment surrounding otoliths influences the rate of trace element incorporation into their growing surface (Campana, 1999).

The multi-elemental analysis of whole otolith composition allowed toobtain additional information about the different adult stocks and stock mixing process of *Trisopterus luscus* in the NW Portuguese coast. Multi-elemental fingerprints obtained for each location had a good trace back result to the original location areas (69% of correct classification), demonstrating that LDFA analyses own a good specificity to the areas where fish were captured. The high classification values (57%, 63% and 87% to Viana do Castelo, Matosinhos and Aveiro, respectively) indicate that pout is a typically sedentary species, showing some fidelity to their growing area, despite of presenting some migratory displacements between adjacent relatively close areas (space range of about 120 km). The overlapping areas presented in the LDFA plot suggest the existence of some mixing between the individuals from the three different locations.

Our results indicate that elemental fingerprints of pout whole otoliths are site-specific and that can provide natural tags of their inhabited areas. The temporal stability of otolith chemistry is an issue that needs to be confirmed since some works indicate that specific fingerprints could vary over the years (Campana et al., 1994, 2000; Rooker et al., 2002). Long-term stability of otolith elemental fingerprints is not evident to date, suggesting that elemental fingerprints may serve only as short-term natural tags (1-3 years) (Rooker et al., 2002). By contrary, comparisons should be made only from fishes from the same year-classes or cohorts.

It is of the utmost importance to increase the existent information about the pout fish movements and their connectivity between nursery areas and adults grounds. Future works should include the study of the natal or nursery origin of pouts and the connectivity between spawning/nursery areas and feeding adults grounds in order to understand the population dynamics and preserve the habitats that most contribute to the adult coastal populations. This task could be easily accomplished using ICPMS-LA

through core analysis. This work with Portuguese pout will allow us to establish different strategies on the rational management of this fishery stock.

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