Universidade de Lisboa
Faculdade de Ciências
Departamento de Biologia Animal

Connectivity among estuarine and coastal fish populations using otolith geochemistry

Susanne Eva Tanner

Doutoramento em Biologia
Especialidade de Biologia Marinha e Aquacultura
2012

Universidade de Lisboa
Faculdade de Ciências
Departamento de Biologia Animal

Connectivity among estuarine and coastal fish populations using otolith geochemistry

Susanne Eva Tanner

Tese orientada por
Professor Auxiliar com Agregação Henrique Nogueira Cabral
Senior Scientist Dr. Simon R. Thorrold

Doutoramento em Biologia
Especialidade de Biologia Marinha e Aquacultura
2012

Doctoral dissertation in Biology
(specialization in Marine Biology and Aquaculture)
presented to the University of Lisboa

Dissertação apresentada à Universidade de Lisboa
para obtenção do grau de doutor
(especialidade Biologia Marinha e Aquacultura)

Susanne Eva Tanner

2012

TABLE OF CONTENTS

Abstract

Connectivity is a critical property of marine fish populations as it defines population replenishment, colonization patterns and populations' resilience to harvesting. Otolith geochemistry is one of the techniques with greatest potential to determine fish movements, especially at early life stages. The accurate use of otolith geochemical signatures as natural tags in connectivity and population structure studies is tied to a thorough assessment of the variability of these markers at different spatio-temporal scales. Additionally, understanding the relationships between otolith geochemical composition and physicochemical properties of ambient water is necessary for tracking fish movement patterns. Several otolith elemental and isotopic ratios were identified as potentially useful proxies for temperature or salinity in estuarine environment which might allow the accurate reconstruction of habitat use of juvenile *Solea solea*. The significant differences between the geochemical composition of otolith core and edge observed in *S. solea* and *Merluccius merluccius* were possibly related to ontogenetic and environmental factors. Otolith geochemical signatures of juvenile *S. solea* and *Solea senegalensis* varied significantly between and within years yet without interfering in the spatial discrimination of estuaries. Juvenile soles were accurately assigned to their estuaries of origin and the established geochemical signatures constitute the baseline data for assessments of connectivity between juvenile and adult populations. The estimated relative contributions from estuarine source areas to adult coastal populations allowed the successful identification of the estuarine systems that accounted most for the replenishment of adult stocks of both sole species in two years. The integration of otolith geochemistry and microsatellite DNA markers, applied in a hierarchical manner, enhanced population structure estimates of *M. merluccius* since the two markers act at different spatio-temporal scales. Overall, the present results should promote the development of effective conservation strategies for ecologically important habitats and integrated fisheries management plans for these commercially important species.

Keywords: otolith geochemistry, natural markers, spatio-temporal variability, population structure, connectivity

Resumo

A conectividade é uma característica importante de populações de peixes marinhos, pois determina a reposição populacional, os padrões de colonização e a resiliência das populações à pesca. A geoquímica de otólitos é uma das técnicas com maior potencial para determinar movimentos de peixes, sobretudo nos estados de vida iniciais. A aplicação correcta das assinaturas geoquímicas de otólitos como marcadores naturais depende duma avaliação abrangente da sua variabilidade a diferentes escalas espácio-temporais. Além disso, é necessário um conhecimento sobre as relações entre a composição geoquímica e as propriedades físico-químicas da água para seguir os movimentos dos peixes. Diferentes elementos e isótopos foram identificados como potenciais indicadores de temperatura ou salinidade em estuários, que poderão permitir a determinação do padrão de utilização por habitats de juvenis de *Solea solea*. As diferenças significativas entre a composição geoquímica do núcleo e da margem de otólitos de *S. solea* e *Merluccius merluccius* estão provavelmente associadas a factores ontogenéticos e ambientais. As assinaturas químicas dos juvenis de *S. solea* e *Solea senegalensis* variaram significativamente entre anos e meses, porém sem interferir com a discriminação espacial entre estuários. Os juvenis foram correctamente atribuídos aos seus estuários de origem e as assinaturas estabelecidas constituem as bases de referência para avaliar a conectividade entre populações de juvenis e adultos. As estimativas de contribuições relativas das áreas estuarinas para as populações adultas na costa permitiram a identificação dos estuários que mais contribuem para a reposição dos mananciais das duas espécies de linguado em dois anos distintos. A integração de geoquímica de otólitos e marcadores genéticos melhorou as estimativas de estrutura populacional de *M. merluccius*, quando aplicada hierarquicamente, pois os dois marcadores naturais têm resoluções espácio-temporais diferentes. Globalmente, os resultados obtidos poderão ajudar a promover o desenvolvimento de estratégias de conservação eficientes e planos de gestão integrados para estas espécies.

Palavras-chave: geoquímica de otólitos, marcadores naturais, variabilidade espácio-temporal, estrutura populacional, conectividade

Resumo alargado

Muitas espécies de peixes marinhos utilizam estuários, baías ou áreas costeiras para completar o seu ciclo de vida. Os juvenis destas espécies podem passar meses ou até anos nestes ambientes mais abrigados e com maior disponibilidade de alimento, antes de se juntarem às populações de adultos ao largo da costa. Um melhor conhecimento sobre os padrões e taxas de movimento de peixes entre diferentes habitats, ou seja sobre a conectividade destas populações, pode fornecer dados importantes relativamente às dinâmicas populacionais, aos padrões de colonização de novos habitats e à resiliência das populações ao impacto da pesca. Estas informações são essenciais para o desenvolvimento adequado de estratégias de conservação e planos de gestão de recursos.

A quantificação do movimento individual e da conectividade entre populações de peixes constitui uma tarefa difícil, sobretudo em fases iniciais do ciclo de vida. Neste contexto, a análise de assinaturas geoquímicas de otólitos de peixes e a sua utilização como marcadores naturais representa um avanço notável e é, sem dúvida, uma das metodologias com maior potencial na quantificação da conectividade e na sua caracterização espácio-temporal.

O presente estudo tem como objectivo principal a avaliação da variabilidade espácio-temporal das assinaturas geoquímicas de otólitos de peixes em ambiente estuarino e costeiro. Pretende-se utilizar estas assinaturas na estimativa das contribuições relativas das áreas estuarinas utilizadas por juvenis para os mananciais de adultos, bem como determinar os padrões de movimento e a estrutura populacional de peixes marinhos.

A presente tese é composta por oito capítulos, seis dos quais correspondem a artigos científicos elaborados de forma a responder directamente aos objectivos propostos, estando publicados ou em revisão em revistas internacionais com arbitragem científica e incluídas no *Science Citation Index*. Estes capítulos são precedidos de um capítulo de introdução geral e seguidos de um capítulo de discussão geral com sugestão de desenvolvimentos futuros.

Na introdução geral (Capítulo I) é apresentado um enquadramento teórico do tema da presente tese, focando a importância da avaliação da conectividade em populações de organismos marinhos, com uma descrição de abordagens directas e

indirectas aplicadas ao longo das últimas décadas. É dado particular destaque às assinaturas geoquímicas de otólitos, enquanto marcadores naturais, e à integração de diferentes marcadores naturais (i.e. geoquímica de otólitos e marcadores genéticos) em estudos de conectividade e estrutura populacional de peixes.

As relações entre a composição geoquímica de otólitos de juvenis de linguado legítimo (*Solea solea*) e as propriedades físico-químicas da água do estuário (i.e. temperatura, salinidade e composição química) são exploradas no Capítulo II. A utilização da geoquímica de otólitos para seguir migrações de peixes ou reconstruir o uso de habitats em ambientes estuarinos exige que as relações entre a composição geoquímica dos otólitos e o meio ambiente sejam significativas. No presente estudo, os resultados indicaram uma relação negativa entre a incorporação de lítio (Li) nos otólitos e a temperatura, salientando assim o potencial deste elemento na reconstituição de padrões de exposição à temperatura ao longo da vida dos peixes. De modo similar, as razões de Ba:Ca, $\delta^{13}C$ e $\delta^{18}O$ foram identificadas como possíveis marcadores de uso de habitat em ambientes eurihalinos. Estes elementos apresentaram padrões de mistura significativos ao longo do gradiente salino do estuário, evidenciando a relação entre as suas incorporações e a disponibilidade e concentração dos respectivos elementos na água.

O Capítulo III é relativo à investigação da variabilidade espacial e ontogenética das assinaturas geoquímicas dos otólitos de juvenis de *S. solea*, recolhidos nos principais estuários da costa portuguesa. No que diz respeito à variabilidade espacial, foram encontradas diferenças significativas tanto entre estuários, como entre locais dentro de um mesmo estuário. As diferenças encontradas entre a composição química do núcleo e da margem do otólito foram provavelmente influenciadas por factores ambientais, bem como por efeitos ontogenéticos. As variações espaciais observadas entre estuários e entre locais de um mesmo estuário indicam que as assinaturas geoquímicas de otólitos são marcadores naturais apropriados para esta espécie. Porém, a variação ontogenética deve ser considerada em estudos de conectividade e estrutura populacional desta espécie.

Antes de utilizar as assinaturas geoquímicas de otólitos para determinar a residência estuarina ou origem natal de indivíduos, é necessária uma avaliação completa da variabilidade espácio-temporal, que é apresentada no Capítulo IV deste trabalho. Determinar a variação temporal a diferentes escalas é particularmente importante, uma vez que esta pode confundir a discriminação espacial entre os estuários. As assinaturas geoquímicas dos otólitos de juvenis de linguado legítimo (*S. solea*) e de linguado do Senegal (*Solea senegalensis*), recolhidos durante dois anos em estuários portugueses, mostraram variabilidade em todas as escalas temporais examinadas sem, no entanto, interferir com a discriminação espacial. Em geral, os juvenis foram correctamente classificados nos seus estuários de origem, embora variando entre anos e espécies. As assinaturas geoquímicas determinadas nos otólitos de juvenis de *S. solea* e *S. senegalensis* constituem as bases de referência para subsequentes avaliações de conectividade entre populações de juvenis e de adultos nestas duas espécies de linguado.

A variabilidade das assinaturas geoquímicas de otólitos a larga escala espacial foi investigada usando a pescada (*Merluccius merluccius*). Em resposta a algumas lacunas acerca da estrutura populacional desta espécie, certos aspectos fundamentais para esta espécie são tratados e desenvolvidos nos Capítulos V e VI. A geoquímica de otólitos foi utilizada para examinar movimentos de pescada entre populações locais no Atlântico Norteste e no Mediterrâneo (Capítulo V). A composição geoquímica encontrada no núcleo dos otólitos distinguiu-se da determinada nas suas margens. Estas diferenças estão possivelmente associadas a alterações ontogenéticas da fisiologia e da ecologia alimentar desta espécie. Os resultados obtidos sugeriram que existe movimento de indivíduos entre as populações locais dentro do Atlântico Norteste, bem como dentro do Mediterrâneo. No entanto, não foram encontrados indícios de migração através do Estreito de Gibraltar, que indicasse a ligação entre as populações das duas regiões. Os padrões de movimento de pescada observados não coincidiram com os limites de mananciais implementados no Atlântico Norte. Recomenda-se, portanto, uma reavaliação das unidades de gestão desta espécie demersal, de forma a tornar a sua gestão mais adequada e eficiente.

No Capítulo VI, a estrutura populacional e os padrões de movimento de *M. merluccius*, considerando sete locais no Atlântico Norteste e no Mediterrâneo, foram investigados utilizando uma abordagem integradora baseada em assinaturas geoquímicas e marcadores genéticos (microssatélites). As assinaturas geoquímicas do núcleo dos otólitos, utilizadas para investigar a separação espacial nas fases iniciais do ciclo de vida, não foram suficientemente robustas para identificar grupos em toda a escala geográfica estudada. No entanto, dentro de cada região hidrogeográfica (Atlântico Norteste e Mediterrâneo) foram identificados três grupos com base nesta metodologia. De modo semelhante, os microssatélites não esclareceram a estrutura genética da população a uma escala mais reduzida (dentro de cada região hidrogeográfica), tendo sido encontrados indícios de uma separação genética evidente próximo do Estreito de Gibraltar que divide claramente as populações do Atlântico Norteste e do Mediterrâneo. A integração das duas técnicas melhorou as estimativas de estrutura populacional da espécie, quando aplicadas de maneira hierárquica, pois os dois marcadores naturais actuam a escalas espácio-temporais diferentes.

No Capítulo VII, apresenta-se a determinação dos estuários de origem dos indivíduos adultos recolhidos ao longo da costa portuguesa, utilizando as bases de referência das assinaturas geoquímicas dos juvenis de *S. solea* e *S. senegalensis* de duas classes anuais diferentes (apresentadas no Capítulo IV). As contribuições relativas estimadas variaram entre os dois anos analisados. No entanto, foram identificados sistemas estuarinos que, durante o período estudado, forneceram muitos indivíduos para as populações de adultos (e.g. Ria de Aveiro) e outros que não tiveram um papel importante nestas contribuições (e.g. Mira). As comparações das assinaturas geoquímicas de juvenis de áreas de viveiro estudadas com as de adultos indicaram a possibilidade de existirem áreas de viveiro desconhecidas, não incluídas na base de referência, e que possam ter contribuído para as populações de adultos. Porém, as estimativas das contribuições relativas das áreas estuarinas para as áreas ao largo da costa permitiram a identificação dos sistemas estuarinos que mais contribuíram para a reposição dos mananciais das duas espécies de linguado durante os dois anos estudados.

Finalmente, no Capítulo VIII é apresentada uma discussão geral que engloba as principais conclusões dos capítulos anteriores e são delineadas perspectivas futuras de investigação. São também tecidas algumas considerações finais acerca da relevância do presente trabalho para o conhecimento geral sobre a conectividade entre populações e a estrutura populacional de peixes marinhos, bem como a integração de diferentes marcadores naturais.

LIST OF PAPERS

This thesis is comprised by the papers listed below, each corresponding to a chapter (Chapter II to VII). The author of this thesis is the first author in all papers and was responsible for conception and design of the work, field surveys, sample collection and processing, laboratory analytical procedures, data analysis and manuscript writing of all the papers. Remaining authors collaborated in some or several of these procedures. All papers published or in press were included with the publishers' agreement.

CHAPTER II: Does otolith geochemistry record ambient environmental conditions in estuaries?
Susanne E. Tanner, Patrick Reis-Santos, Rita P. Vasconcelos, Vanessa Fonseca, Susana França, Henrique N. Cabral & Simon R. Thorrold
In review in Journal of Experimental Marine Biology and Ecology

CHAPTER III: Spatial and ontogenetic variability in the chemical composition of juvenile common sole (*Solea solea*) otoliths
Susanne E. Tanner, Rita P. Vasconcelos, Patrick Reis-Santos, Henrique N. Cabral & Simon R. Thorrold
Published in Estuarine, Costal and Shelf Science (2011) 91: 150-157

CHAPTER IV: Otolith geochemistry discriminates among estuarine nursery areas of *Solea solea* and *S. senegalensis* over time
Susanne E. Tanner, Patrick Reis-Santos, Rita P. Vasconcelos, Susana França, Simon R. Thorrold & Henrique N. Cabral
Published in Marine Ecology Progress Series (2012) 452: 193-203

CHAPTER V: Testing an otolith geochemistry approach to determine population structure and movements of European hake in the northeast Atlantic Ocean and Mediterranean Sea
Susanne E. Tanner, Rita P. Vasconcelos, Henrique N. Cabral & Simon R. Thorrold
Published in Fisheries Research (2012) 125-126: 198-205

CHAPTER VI: Integrating microsatellite DNA markers and otolith geochemistry to assess stock structure of European hake (*Merluccius merluccius*)
Susanne E. Tanner, Montse Pérez, Pablo Presa, Simon R. Thorrold & Henrique N. Cabral
In review in Fisheries Oceanography

CHAPTER VII: Population connectivity of *Solea solea* and *Solea senegalensis* over time
Susanne E. Tanner, Patrick Reis-Santos, Rita P. Vasconcelos, Simon R. Thorrold & Henrique N. Cabral
In review in Journal of Sea Research

CHAPTER I

General introduction
Aims and importance of the thesis
Thesis outline

General introduction
Aims and importance of the thesis
Thesis outline

GENERAL INTRODUCTION

Connectivity, or exchange among geographically separated groups, is a critical component in marine fish populations as it determines population replenishment, colonization patterns and the resilience of populations to harvesting (Thorrold et al. 2001, Cowen et al. 2007). Understanding this link provides important information on the dynamics, genetic structure and biogeography of populations (Cowen et al. 2000). Furthermore, it contributes 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) which are major concerns since marine species are under considerable and increasing stress from human activities (Pauly et al. 1998, Jackson et al. 2001). These anthropogenic pressures significantly contribute to the deterioration and fragmentation of estuarine and coastal habitats, biodiversity loss and stock depletion (Jackson 2008).

Many marine fish species occupy spatially separated habitats during their life history cycle. Adults generally show broad and continuous distributions, whereas juveniles use more discrete geographic areas such as estuaries, bays or shallow coastal areas that favor survival and growth of early life stages of fish (Beck et al. 2001, Gillanders et al. 2003, Able 2005). Juveniles remain in these sheltered areas for months or years, depending on the species, before migrating to the adult populations offshore (Beck et al. 2001, Able 2005). The concept of a population is central to the fields of ecology, evolutionary biology, and conservation biology, and many definitions can be found in the literature. In the present work, population refers to a group of juvenile or adult individuals of a species that occupy the same space at a particular time (reviewed by Waples & Gaggiotti 2006). Following this definition, the present work will use the terms adult population and juvenile population.

Quantifying the movement of fishes is a challenging task that, over the decades, has been tackled by a variety of indirect and direct approaches. Although only direct approaches can provide quantitative measurements of individuals shifting between groups, a large number of studies has used indirect approaches including differences in distribution, abundance and size structure (reviewed by Gillanders et al. 2003). Artificial and natural tags are direct approaches that provide estimates of rate and extent of movement between habitats. Despite technological advances, conventional artificial tagging techniques are logistically difficult to execute in early life stages, such as larvae and juveniles due to small body size, high mortality and low recapture rates. However, artificial tags in calcified tissues of fish using chemical (fluorescent compounds, elemental tags, enriched isotopes) or physical markers have been used more routinely and have provided insight into movement patterns in early life stages of fish (reviewed by Thorrold et al. 2002). Recently, transgenerational otolith marking using enriched isotopes has emerged as a promising artificial chemical tagging technique (e.g. Thorrold et al. 2006, Almany et al. 2007, Munro et al. 2009). This technique relies on the premise that the chemical tags are passed from the female to the eggs during oocyte development and are subsequently incorporated into embryonic otoliths. However, this technique is technically and logistically difficult to implement, labor-intensive and depends on the species and systems under study which is reflected in the low number of studies that have used otolith transgenerational marking in the field (Thorrold et al. 2006, Almany et al. 2007, Planes et al. 2009). The numerous difficulties associated with artificial tags have led to an increased interest in the use of natural tags to track movement patterns in fish. Naturally occurring markers (chemical, genetic and parasitic tags) are particularly interesting since they depend on differences in environmental conditions or genetic heterogeneity to produce group-specific tags and inevitably every individual in a group is tagged (Thorrold et al. 2002).

Amongst natural tags, otolith geochemistry has developed into one of the most promising techniques to determine population connectivity and quantify movement patterns of fish (Gillanders 2005, Elsdon et al. 2008). The first studies investigating geochemical composition of otoliths date back up to four decades (Degens et al. 1969, Gauldie et al. 1986, Radtke 1987) and mainly focused on the potential use of otolith chemical composition to reconstruct environmental life histories of fish. Since then otolith geochemistry has been applied in a wide variety of studies, namely to estimate stock identity and population structure (e.g. Thresher 1999, Newman et al. 2009, Steer et al. 2010), assess migration patterns (e.g. Hamer et al. 2006, Suzuki et al. 2008, Limburg et al. 2011), identify natal origin or nursery areas (e.g. Vasconcelos et al. 2007, Clarke et al. 2009, Cuveliers et al. 2010) and to retrospectively determine the origin of adults (e.g. Thorrold et al. 2001, Vasconcelos et al. 2008, Wright et al. 2010).

This wide array of applications is based on the metabolic inertness of otoliths (Campana & Neilson 1985), which results in the permanent retention of the geochemical tag. Calcium carbonate (usually in the form of aragonite) and proteins are continuously accreted to the otolith surface forming daily and annual increment patterns that can be used as chronological recorders of environmental conditions (Campana & Thorrold 2001). Finally, the incorporation of at least some elements is influenced by environmental conditions (e.g. water temperature, salinity, chemical composition of the water) while other elements are under physiological or genetic control (Campana 1999). Hence, fish occupying habitats with different environmental conditions may present distinct otolith geochemical signatures. To use otolith geochemical signatures as natural tags in connectivity and population structure studies three assumptions should be met. First, otolith geochemical signatures of fish from different groups of interest must differ significantly. Second, the geochemical signatures of all possible groups contributing to the mixture need to be characterized and third, the geochemical signatures should remain stable over the time period during which group assignments are to be made (Campana 1999, Campana et al. 2000). By complying with these criteria, otolith geochemistry is an outstanding tool to address a number of core challenges of marine ecology, such as determining the spatio-temporal scale of connectivity, the underlying processes of dispersal, the impacts of connectivity on population structure and dynamics and effectively applying this information to management (Cowen & Sponaugle 2009).

Genetic markers are another natural tag that is well established in studies of population structure and connectivity in marine populations (Hellberg et al. 2002, Hedgecock et al. 2007). Marine fish are expected to show low genetic differentiation and high gene flow due to large population sizes, broad geographic distributions, high fecundity and high dispersal potential (DeWoody & Avise 2000). Highly variable codominant microsatellite DNA markers have been used in many population genetic studies to identify genetic structure in populations with low genetic differentiations such as marine fish species (White et al. 2010). The ecological implications of low but significant genetic structure in fish populations are difficult to assess, especially because estimates of gene flow are generally made on recent and/or past evolutionary time scales rather than an ecological time frame over which most management decisions are made (Palumbi 2003).

Population connectivity and structure are influenced by behavioural and physical processes that act over a range of temporal scales. Therefore the use of multiple and complementary techniques that integrate over different scales might provide the best inference on population connectivity and structure (Thorrold et al. 2002, Lowe & Allendorf 2010). Several studies have combined genetic markers and otolith geochemistry as these two natural tags provide complementary information on population structure and connectivity patterns over evolutionary and ecological time scales, respectively (Miller et al. 2005, Bradbury et al. 2008, Woods et al. 2010). The chronological properties of otoliths may provide information on demographic connectivity patterns of fish, whereas genetic markers may resolve population structure over various time scales.

Along the Portuguese coast, few studies have addressed fish population structure (Correia et al. 2011, Correia et al. 2012) or population connectivity (Vasconcelos et al. 2007, Vasconcelos et al. 2008) using otolith geochemistry. The latter confirmed the applicability of otolith geochemistry to investigate the movement of individuals between estuarine and coastal environments and provided the first estimates of estuarine contributions to adult populations. However, spatio-temporal variability of otolith geochemical signatures has not been evaluated. A sound assessment of spatio-temporal variability at different scales is vital to accurately use otolith geochemistry in connectivity and population structure studies. Only with geochemical baselines that are spatially-explicit, can the relative contributions of different juvenile areas to the adult population be feasibly determined. Genetic markers have commonly been employed to address fish population structure in Portuguese waters (e.g. Castilho & McAndrew 1998, Cabral et al. 2003, Correia et al. 2006) however, combining them with other natural markers has not been attempted yet. The integration of different natural markers (i.e. genetic markers and otolith geochemistry) that act at different temporal scales has shown very promising results that complement our understanding of connectivity and structure of natural populations even though it is still in its infancy.

AIMS AND IMPORTANCE OF THE THESIS

The present work aims to assess the spatio-temporal variability in otolith geochemical signatures in estuarine and coastal fish populations, and to use these signatures to evaluate relative contributions of estuarine areas inhabited by juveniles to coastal adult stocks as well as to determine movement patterns and population structure of marine fish.

Otolith geochemistry is used in a wide variety of studies, although still many uncertainties persist regarding the factors affecting element incorporation into otoliths (e.g. Elsdon et al. 2008, Thorrold & Swearer 2009). An in situ approach is employed to address the effects of environmental factors on otolith geochemical composition. Such approaches are highly valuable since the high variability of the physicochemical properties of the water in estuaries can complement finding from laboratory experiments.

Variation in geochemical composition of otoliths at different temporal scales (e.g. inter-annual, seasonal) may confound spatial discrimination and lead to inadequate interpretations of geochemical data (e.g. Gillanders 2002, Hamer et al. 2003). Moreover, ontogenetic effects may also contribute to variations in otolith geochemistry. Therefore, a thorough assessment of the spatio-temporal variability in otolith geochemical signatures at different scales is essential to correctly and feasibly apply them as natural tags in connectivity studies. The identified spatially-explicit signatures will constitute the geochemical baselines used to evaluate the rate and extent of connectivity between juvenile source areas and coastal adult populations.

Integrating natural markers that provide information at different ecological and evolutionary time scales complements and enhances our understanding of demographic and genetic connectivity in marine systems. The determination of quantitative estimates of relative contributions from juvenile source areas to adult populations identifies the areas that most account for stock replenishment, and focusing on two different years quantifies inter-annual variability. Both approaches should produce much needed multifaceted information to prioritize conservation strategies towards areas identified as essential to fish recruitment and stock maintenance, as well as sustainable management of marine capture fisheries.

THESIS OUTLINE

The present thesis comprises six scientific papers published or in review in peer reviewed international journals. Each paper corresponds to a chapter.

In chapter II, otolith geochemical composition of juvenile *Solea solea* is related to the physicochemical properties of estuarine water at a small spatio-temporal scale. Significant relationships between otolith geochemical composition and environmental factors are established and potential proxies for temperature and salinity are identified.

Spatial and ontogenetic variability in the otolith geochemical composition of juvenile *S. solea* is evaluated in chapter III. The accuracy of assignment of individuals to their estuary of origin is assessed and the environmentally- and ontogenetically-induced differences in otolith geochemical composition of juvenile common sole are discussed.

Otolith geochemistry is used to discriminate estuarine nursery areas of *S. solea* and *Solea senegalensis* over time in chapter IV. Temporal variation in otolith geochemical signatures is assessed at different scales and its potentially confounding effect on spatial discrimination is explored. The established elemental signatures constitute the baseline data for the subsequent assessments of connectivity between juvenile and adult populations of the two sole species.

In chapter V, movement of *Merluccius merluccius* among local populations is examined at a large spatial scale and the accuracy of current stock boundaries for this species is tested using otolith geochemistry. Differences between otolith core and edge composition are appraised and discussed with respect to ontogenetic changes in physiology and feeding habits of *M. merluccius*.

An integrative approach based on otolith core and edge geochemical composition and microsatellite DNA is used to investigate population structure and movement patterns of *M. merluccius* in chapter VI. The complementarity of the information on population structure and connectivity patterns obtained using the two markers is discussed.

In chapter VII, relative contributions of estuarine nursery areas to the coastal adult populations of *S. solea* and *S. senegalensis* are estimated based on otolith geochemical signatures. Inter-annual stability of estuary-specific contributions is examined and important nursery areas, in terms of high contribution proportions, are identified.

In the final chapter, a general discussion addresses the major conclusions obtained in the different studies regarding otolith geochemistry as a valuable tool in connectivity studies and stock structure assessment. Further, the potential advantages of integrating different natural markers are discussed. Perspectives for future research are also included in this chapter.

LITERATURE CITED

Able KW (2005) A re-examination of fish estuarine dependence: Evidence for connectivity between estuarine and ocean habitats. Estuar Coast Shelf Sci 64:5-17

Almany GR, Berumen ML, Thorrold SR, Planes S, Jones GP (2007) Local Replenishment of Coral Reef Fish Populations in a Marine Reserve. Science 316:742-744

Beck MW, Heck Jr. KL, Able KW, Childers DL, Eggleston DB, Gillanders BM, Halpern BS, Hays CG, Hoshino K, Minello TJ, Orth RJ, Sheridan PF, Weinstein MP (2001) The Identification, Conservation, and Management of Estuarine and Marine Nurseries for Fish and Invertebrates. BioScience 8:633-641

Bradbury IR, Campana SE, Bentzen P (2008) Estimating contemporary early life-history dispersal in an estuarine fish: integrating molecular and otolith elemental approaches. Mol Ecol 17:1438-1450

Cabral HN, Castro F, Linhares D, Alexandrino P (2003) Genetic differentiation of *Solea solea* (Linnaeus, 1758) and *Solea senegalensis* Kaup, 1858, (Pisces: Pleuronectiformes) from several estuarine systems of the Portuguese coast. Sci Mar 67:43-52

Campana SE, Neilson JD (1985) Microstructure of fish otoliths. Can J Fish Aquat Sci 42:1014-1032

Campana SE (1999) Chemistry and composition of fish otoliths: pathways, mechanisms and applications. Mar Ecol Prog Ser 188:263-297

Campana SE, Chouinard GA, Hanson JM, Fréchet A, Brattey J (2000) Otolith elemental fingerprints as biological tracers of fish stocks. Fish Res 46:343-357

Campana SE, Thorrold SR (2001) Otoliths, increments, and elements: keys to a comprehensive understanding of fish populations? Can J Fish Aquat Sci 58:30-38

Castilho R, McAndrew BJ (1998) Population structure of seabass in Portugal: evidence from allozymes. J Fish Biol 53:1038-1049

Clarke LM, Walther BD, Munch SB, Thorrold SR, Conover DO (2009) Chemical signatures in the otoliths of a coastal marine fish, *Menidia menidia*, from the northeastern United States: spatial and temporal differences. Mar Ecol Prog Ser 384:261-271

Correia AT, Faria R, Alexandrino P, Antunes C, Isidro EJ, Coimbra J (2006) Evidence for genetic differentiation in the European conger eel Conger conger based on mitochondrial DNA analysis. Fish Sci 72:20-27

Correia AT, Pipa T, Goncalves JMS, Erzini K, Hamer PA (2011) Insights into population structure of Diplodus vulgaris along the SW Portuguese coast from otolith elemental signatures. Fish Res 111:82-91

Correia AT, Gomes P, Goncalves JMS, Erzini K, Hamer PA (2012) Population structure of the black seabream Spondyliosoma cantharus along the south-west Portuguese coast inferred from otolith chemistry. J Fish Biol 80:427-443

Cowen RK, Lwiza KMM, Sponaugle S, Paris CB, Olson DB (2000) Connectivity in marine populations: Open or closed? Science 287:857-859

Cowen RK, Gawarkiewicz G, Pineda J, Thorrold SR, Werner FE (2007) Population connectivity in marine systems: an overview. Oceanography 20:14-21

Cowen RK, Sponaugle S (2009) Larval dispersal and marine population connectivity. Annu Rev Mar Sci 1:443–466

Cuveliers EL, Geffen AJ, Guelinckx J, Raeymaekers JAM, Skadal J, Volckaert FAM, Maes GE (2010) Microchemical variation in juvenile *Solea solea* otoliths as a powerful tool for studying connectivity in the North Sea. Mar Ecol Prog Ser 401:211-220

Degens ET, Deuser WG, Haedrich RL (1969) Molecular structure and composition of fish otoliths. Mar Biol 2:105-113

DeWoody JA, Avise JC (2000) Microsatellite variation in marine, freshwater and anadromous fishes compared with other animals. J Fish Biol 56:461-473

Elsdon TS, Wells BK, Campana SE, Gillanders BM, Jones CM, Limburg KE, Secor DH, Thorrold SR, Walther BD (2008) Otolith chemistry to describe movements and life-history parameters of fishes: hypotheses, assumptions, limitations and inferences. Oceanogr Mar Biol Annu Rev 46:297-330

Gauldie RW, Fournier DA, Dunlop DE, Coote G (1986) Atomic emission and proton microprobe studies of the ion content of otoliths of chinook salmon aimed at recovering the temperature life history of individuals. Comp Biochem Physiol A: Physiol 84:607-615

Gillanders BM (2002) Temporal and spatial variability in elemental composition of otoliths: implications for determining stock identity and connectivity of populations. Can J Fish Aquat Sci 59:669-679

Gillanders BM, Able KW, Brown JA, Eggleston DB, Sheridan PF (2003) Evidence of connectivity between juvenile and adult habitats for mobile marine fauna: an important component of nurseries. Mar Ecol Prog Ser 247:281-295

Gillanders BM (2005) Otolith chemistry to determine movements of diadromous and freshwater fish. Aquat Living Resour 18:291-300

Hamer PA, Jenkins GP, Gillanders BM (2003) Otolith chemistry of juvenile snapper *Pagrus auratus* in Victorian waters: natural chemical tags and their temporal variation. Mar Ecol Prog Ser 263:261-273

Hamer PA, Jenkins GP, Coutin P (2006) Barium variation in *Pagrus auratus* (Sparidae) otoliths: A potential indicator of migration between an embayment and ocean waters in south-eastern Australia. Estuar Coast Shelf Sci 68:686-702

Hedgecock D, Barber PH, Edmands S (2007) Genetic approaches to measuring connectivity. Oceanography 20:70-79

Hellberg ME, Burton RS, Neigel JE, Palumbi SR (2002) Genetic assessment of connectivity among marine populations. Bull Mar Sci 70:273-290

Jackson JBC, Kirby MX, Berger WH, Bjorndal KA, Botsford LW, Bourque BJ, Bradbury RH, Cooke R, Erlandson J, Estes JA, Hughes TP, Kidwell S, Lange CB, Lenihan HS, Pandolfi JM, Peterson CH, Steneck RS, Tegner MJ, Warner RR (2001) Historical overfishing and the recent collapse of coastal ecosystems. Science 293:629-638

Jackson JBC (2008) Ecological extinction and evolution in the brave new ocean. Proc Natl Acad Sci 105:11458-11465

Limburg KE, Olson C, Walther Y, Dale D, Slomp CP, Høie H (2011) Tracking Baltic hypoxia and cod migration over millennia with natural tags. Proc Natl Acad Sci 108:E177-E182

Lowe WH, Allendorf FW (2010) What can genetics tell us about population genetics? Mol Ecol 19:3038-3051

Miller JA, Banks MA, Gomez-Uchida D, Shanks AL (2005) A comparison of population structure in black rockfish (*Sebastes melanops*) as determined with otolith microchemistry and microsatellite DNA. Can J Fish Aquat Sci 62:2189-2198

Munro AR, Gillanders BM, Thurstan S, Crook DA, Sanger AC (2009) Transgenerational marking of freshwater fishes with enriched stable isotopes: a tool for fisheries management and research. J Fish Biol 75:668-684

Newman SJ, Buckworth RC, Mackie MC, Lewis PD, Wright IW, Williamson PC, Bastow TP, Ovenden JR (2009) Spatial subdivision among assemblages of Spanish mackerel, *Scomberomorus commerson* (Pisces: Scombridae) across northern Australia: implications for fisheries management. Global Ecol Biogeogr 18:711-723

Palumbi SR (2003) Population genetics, demographic connectivity, and the design of marine reserves. Ecol Appl 13:S146-S158

Pauly D, Christensen V, Dalsgaard J, Froese R, Torres Jr. F (1998) Fishing down marine food webs. Science 279:860-863

Planes S, Jones GP, Thorrold SR (2009) Larval dispersal connects fish populations in a network of marine protected areas. Proc Natl Acad Sci 106:5693-5697

Radtke RL (1987) Age and growth information available from the otoliths of the Hawaiian snapper, *Pristipomoides filamentosus*. Coral Reefs 6:19-25

Steer MA, Halverson GP, Fowler AJ, Gillanders BM (2010) Stock discrimination of Southern Garfish (*Hyporhamphus melanochir*) by stable isotope ratio analysis of otolith aragonite Environ Biol Fishes 89:369-381

Suzuki KW, Kasai A, Ohta T, Nakayama K, Tanaka M (2008) Migration of Japanese temperate bass *Lateolabrax japonicus* juveniles within the Chikugo River estuary revealed by $\delta^{13}C$ analysis. Mar Ecol Prog Ser 358:245-256

Thorrold SR, Latkoczy C, Swart PK, Jones CM (2001) Natal homing in marine fish metapopulation. Science 291:297-299

Thorrold SR, Jones GP, Hellberg ME, Burton RS, Swearer SE, Neigel JE, Morgan SG, Warner RR (2002) Quantifying larval retention and connectivity in marine populations with artificial and natural markers. Bull Mar Sci 70:291-308

Thorrold SR, Jones GP, Planes S, Hare JA (2006) Transgenerational marking of embryonic otoliths in marine fishes using barium stable isotopes. Can J Fish Aquat Sci 63:1193-1197

Thorrold SR, Zacherl DC, Levin LA (2007) Population connectivity and larval dispersal using geochemical signatures in calcified structures. Oceanography 20:80-89

Thorrold SR, Swearer SE (2009) Otolith chemistry. In: Green BS, Mapstone BD, Carlos G, Begg GA (eds) Tropical Fish Otoliths: Information for Assessment, Management and Ecology. Springer, Netherlands, pp. 249-295

Thresher RE (1999) Elemental composition of otoliths as a stock delineator in fishes. Fish Res 43:165-204

Vasconcelos RP, Reis-Santos P, Tanner S, Fonseca V, Latkoczy C, Günther D, Costa MJ, Cabral H (2007) Discriminating estuarine nurseries for five fish species through otolith elemental fingerprints. Mar Ecol Prog Ser 350:117-126

Vasconcelos RP, Reis-Santos P, Tanner S, Maia A, Latkoczy C, Günther D, Costa MJ, Cabral H (2008) Evidence of estuarine nursery origin of five coastal fish species along the Portuguese coast through otolith elemental fingerprints. Estuar Coast Shelf Sci 79:317-327

Waples RS, Gaggiotti O (2006) What is a population? An empirical evaluation of some genetic methods for identifying the number of gene pools and their degree of connectivity. Mol Ecol 15:1419-1439

White C, Selkoe KA, Watson J, Siegel DA, Zacherl DC, Toonen RJ (2010) Ocean currents help explain population genetic structure. Proc R Soc B 277:1685-1694

Woods RJ, Macdonald JI, Crook DA, Schmidt DJ, Hughes JM (2010) Contemporary and historical patterns of connectivity among populations of an inland river fish species inferred from genetics and otolith chemistry. Can J Fish Aquat Sci 67:1098-1115

Wright PJ, Tobin D, Gibb FM, Gibb IM (2010) Assessing nursery contribution to recruitment: relevance of closed areas to haddock *Melanogrammus aeglefinus*. Mar Ecol Prog Ser 400:221-232

CHAPTER II

Does otolith geochemistry record ambient environmental conditions in estuaries?

Susanne E. Tanner[1], Patrick Reis-Santos[1], Rita P. Vasconcelos[1], Vanessa Fonseca[1], Susana França[1], Henrique N. Cabral[1] & Simon R. Thorrold[2]

[1]Centro de Oceanografia, Faculdade de Ciências da Universidade de Lisboa, Campo Grande, 1749-016 Lisboa, Portugal
[2]Biology Department MS 50, Woods Hole Oceanographic Institution, Woods Hole, MA 02543, USA

In review in Journal of Experimental Marine Biology and Ecology

Does otolith geochemistry record ambient environmental conditions in estuaries?

ABSTRACT The use of otolith geochemistry to track fish migration patterns and establish past habitat use in estuarine environments relies on the presence of significant correlations between the physicochemical properties of ambient water and otolith composition. To test this assumption, juvenile *Solea solea* and ambient waters were sampled at fortnightly and monthly intervals in six sites within the Tejo estuary (Portugal) from May to October 2009. A number of element:Ca ratios (Li:Ca, Mg:Ca, Mn:Ca, Cu:Ca, Sr:Ca, Ba:Ca) along with $\delta^{13}C$ and $\delta^{18}O$ values were measured in otoliths and water samples. The distributions of most element:Ca ratios (with the exception of Mn:Ca and Sr:Ca) and both $\delta^{13}C$ of dissolved inorganic carbon (DIC) and $\delta^{18}O_{water}$ values were correlated with salinity in the estuarine waters. Otolith Ba:Ca, $\delta^{13}C$ and $\delta^{18}O$ ratios were significantly correlated with their respective ambient water values. Each of these variables showed distinctive mixing patterns along the salinity gradient of the estuary and therefore may be valuable tracers of habitat use in euryhaline environments. Surprisingly, only Li:Ca ratios in otoliths were significantly negatively correlated with ambient temperature. While more work is obviously needed, Li:Ca may be a useful, but currently overlooked, temperature proxy in fish otoliths. Our results highlight the difficulties of linking measurements of otolith geochemistry to individual water masses or habitats in estuarine systems with large physicochemical gradients over relatively small spatial and temporal scales.

Keywords: otolith geochemistry, estuary, *Solea solea*, temperature, salinity, water chemistry

---

INTRODUCTION

Otolith geochemistry has been used in a wide variety of studies to identify fish stock structure (e.g. Kalish 1996, Campana et al. 2000, Bergenius et al. 2005), assess migration patterns (e.g. Secor et al. 1995, Tsukamoto & Arai 2001, Hamer et al. 2006) and to retrospectively assign natal origins of adult fishes (e.g. Gillanders & Kingsford 1996, Thorrold et al. 2001, Vasconcelos et al. 2008). These applications are typically based on geographic variability in otolith geochemistry, the chronological record provided by referencing daily and annual increments present in otoliths, and the assumption that otoliths are metabolically inert and therefore their composition remains unaltered after deposition. Potential mechanisms generating geographic differences in otolith geochemistry include variability in physicochemical properties of ambient waters (Bath et al. 2000), genetic differences among populations (Clarke et al. 2011), and physiological effects that may or may not be correlated with temperature, salinity or metabolic activity (Elsdon et al. 2008). In many instances it is not necessary to understand the mechanisms generating geographic variability in otolith geochemistry provided it is possible to compile an atlas of this variability for locations of interest. Nonetheless, most studies to date have at least implicitly assumed that physicochemical properties of ambient water masses have generated observed geographic variability in otolith chemistry. Indeed, the prospect of retrospectively determining habitat use based on reconstructing ambient water mass characteristics at different life history stages motivated the initial studies on otolith chemistry (Radtke 1987, Radtke et al. 1996).

It is, however, by no means certain that environmental variability plays a major or even significant role in determining otolith geochemical composition. Dissolved ions in the ambient waters must cross intestinal and endolymphatic membranes before being available for deposition at the otolith surface. There are opportunities for considerable isotopic and elemental fractionation during ion transport across both these membranes. Otolith composition is therefore, not necessarily a direct reflection of the chemical composition of the surrounding water and the factors affecting elemental and stable isotope uptake in otoliths remain obscure (Campana 1999, Campana & Thorrold 2001). Laboratory studies have provided the most definite results linking otolith chemistry to physicochemical properties of ambient water (e.g. Farrell & Campana 1996, Thorrold et al. 1997, Bath et al. 2000, Martin & Thorrold 2005). However, even under controlled experimental conditions there appear to be few general responses of otolith chemistry to environmental variability. Otolith Sr:Ca has, for instance, been shown to be positively, negatively and uncorrelated with ambient temperature (e.g. Townsend et al. 1995, Bath et al. 2000, Martin et al. 2004).

The lack of a predictive and mechanistic understanding of ion uptake in otoliths makes it difficult to extrapolate the results of laboratory studies to field collections (Elsdon & Gillanders 2005). In laboratory experiments, many factors that potentially affect otolith composition are controlled and maintained stable (e.g. diet, oxygen, light, temperature and salinity). However, in field situations these same factors may vary enough over short temporal scales to confound comparisons among locations. The problem is exacerbated in estuaries where sites will likely see distinct water masses at frequencies ranging from tidal cycles to inter-annual variability in freshwater discharge (Dorval et al. 2005, Dorval et al. 2007).

We addressed the influence of environmental variability on otolith chemistry in an estuarine environment using common sole (*Solea solea*) as our focal species. Along the Portuguese coast, juveniles of common sole use estuaries as nursery grounds for up to two years before migrating to coastal waters (Quéro et al. 1986, Koutsikopoulos et al. 1989, Cabral et al. 2007, Vasconcelos et al. 2010). Estuarine colonization of *S. solea* begins in May and several cohorts may recruit to estuaries along the Portuguese coast (Cabral 2003).

The aim of the present study was: 1) to assess variability of water chemistry and otolith composition at small spatio-temporal scales (within an estuary and within a recruitment season); 2) to determine how changes in water chemistry, salinity and temperature affect otolith composition in the field; 3) and to identify element:Ca and stable isotope ratios that may be useful in tracking fish migration pathways and habitat use.

MATERIAL AND METHODS

Sample and environmental data collection

Our study was conducted in the Tejo estuary, the largest estuarine system along the Portuguese coast with an approximate area of 320 km^2 with several important nursery areas for juvenile *S. solea* (Costa & Bruxelas 1989, Cabral & Costa 1999, Cabral et al. 2007, Vasconcelos et al. 2010).

Juvenile *S. solea* were collected from May to October 2009, fortnightly for the first ten weeks and then monthly for the following three months. Six sites within the Tejo estuary (Fig. 1) were sampled using a beam trawl. Fish were stored and transported on ice to the laboratory and preserved frozen until dissection. Total length of each individual was determined and sagittal otoliths were removed, cleaned of adhering tissue and dried (Table 1). Right otoliths were mounted on glass slides with cyanoacrylic glue and ground to the midplane using 30 µm and 3 µm lapping film for elemental analysis; left otoliths were preserved whole for isotope analysis. 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.

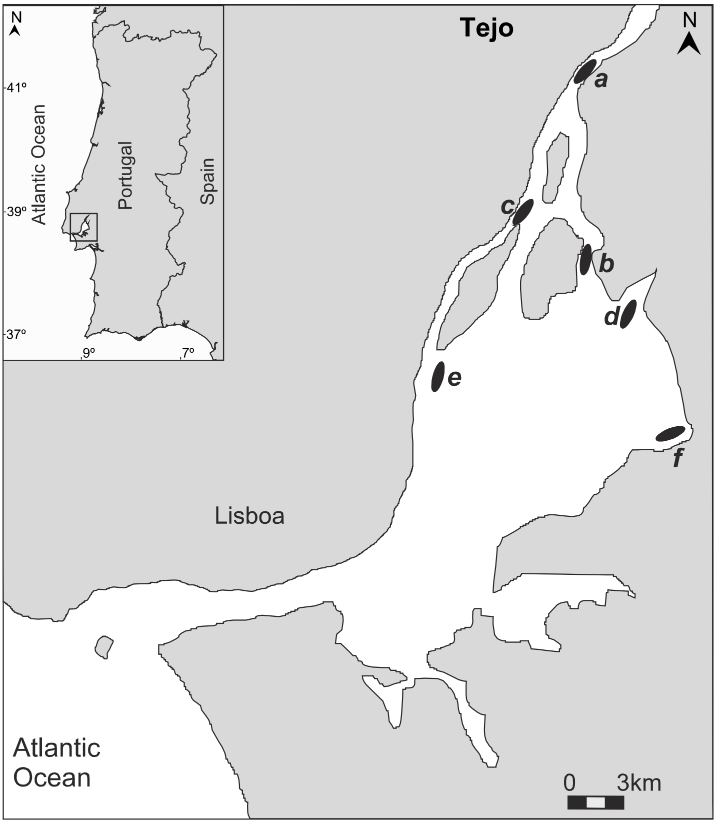

Fig. 1. Sampling sites (a – f) of juvenile *Solea solea* within the Tejo estuary, Portugal.

Water samples (3 replicates) were collected at each site within the Tejo estuary concurrently with fish collections. All glass and plastic material used in this study was initially acid washed in a 10% nitric acid (HNO_3) bath. Water samples were collected in glass sample jars. The samples were stored on ice until return to the laboratory and then filtered using polyvinylidene difluouride (PVDF) syringe filters. Filtered 50 ml samples (n=144) were acidified by adding 1 ml of concentrated ultrapure HNO_3 for elemental analysis while 20 ml samples (n=144) were transferred to glass vials and poisoned with 40 µl saturated mercury chloride ($HgCl_2$), for stable C and O isotope analysis. All samples were stored refrigerated at 4°C until analysis.

Water temperature (°C) was measured hourly by temperature loggers (HOBO Water Temp Pro v2 Logger) that were deployed at each sampling site in May 2009. Salinity was measured at each site using a portable multiparameter instrument (YSI 6820) during fish collections.

Table 1. Mean values and standard error (SE) of fish total length (Lt in mm) and sample size (n) of *Solea solea* at each sampling occasion during 2009 in the different sampling sites in the Tejo estuary. Sampling occurred fortnightly for the first ten weeks passing to monthly for the following three months.

Sampling occasion	Sampling month	Site																	
		a			b			c			d			e			f		
		Lt	SE	n	Lt	SE	n	Lt	SE	n	Lt	SE	n	Lt	SE	n	Lt	SE	n
t1	May	53.3	1.9	8	44.8	2.9	10	52.6	1.8	10	-	-	-	48.1	1.2	7	-	-	-
t2	May	-	-	-	55.1	1.4	10	52.6	1.5	10	63.6	2.1	10	54.1	1.4	10	-	-	-
t3	June	64.9	2.3	9	65.9	2.0	11	64.3	1.2	10	-	-	-	67.0	6.5	3	-	-	-
t4	June	69.0	1.6	11	69.5	5.0	6	72.0	1.6	10	71.9	2.2	10	71.6	6.1	5	74.4	3.0	7
t5	July	81.8	2.3	9	79.6	1.9	11	79.2	1.3	9	86.1	0.7	10	75.9	3.0	9	86.7	2.6	3
t6	August	88.4	2.0	10	-	-	-	88.0	7.8	6	106.6	6.5	7	-	-	-	92.1	2.7	10
t7	September	94.0	3.1	3	94.0	9.0	2	85.2	5.3	6	92.0	5.5	5	-	-	-	-	-	-
t8	October	98.8	5.7	8	84.8	3.8	10	106.5	5.5	2	112.5	5.5	2	-	-	-	-	-	-

Otolith analysis

Otoliths were analysed for Li:Ca, Mg:Ca, Mn:Ca, Cu:Ca, Sr:Ca, Ba: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 sectioned 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 µl min^{-1} nebulizer. The analyte was then transported to the ICP-MS via an Ar carrier gas.

The elemental ratios were quantified by measuring ^{7}Li, ^{25}Mg, ^{48}Ca, ^{55}Mn, ^{63}Cu, ^{88}Sr and ^{138}Ba in the ablated otolith material. Instrumental blanks (2% HNO_3) were run at the beginning and end of each set of 10 otoliths. Blank correction of all measured raw isotope counts was done by linear interpolation between the measured blanks. A dissolved otolith certified reference material (CRM) (Sturgeon et al. 2005), diluted to a Ca concentration of 40 µg g^{-1}, was used to correct for instrument mass bias. Instrument precision was assessed by running another CRM (Yoshinaga et al. 2000). External precision (relative standard deviations) for this reference material (n=60) were as follows: Li:Ca=1.9%, Mg:Ca=13.3%, Mn:Ca=12.0%, Cu:Ca=6.0%, Sr:Ca=0.8%, Ba:Ca=1.4%.

Left otoliths were analysed for $\delta^{13}C$ and $\delta^{18}O$ using isotope ratio monitoring mass spectrometry (irm-MS). Otoliths were crushed and the resulting powder was transferred to acid washed vials. Samples were analyzed on a Thermo Finnigan MAT253 equipped with a Kiel III carbonate device following methods outlined by Ostermann and Curry (2000). Isotopic values were expressed in standard δ notation and reported relative to Vienna Pee Dee belemnite (VPDB). Long-term precision estimates of the mass spectrometer based on analyses of NBS19 are ±0.03 for $\delta^{13}C$ and ±0.07 for $\delta^{18}O$.

Water sample analysis

Filtered and acidified water samples were diluted 1:20 with 2% HNO_3 and analyzed using solution-based ICP-MS to quantify ^{7}Li, ^{25}Mg, ^{48}Ca, ^{55}Mn, ^{63}Cu, ^{88}Sr and ^{138}Ba using the instrument described above. Instrumental blanks (2% HNO_3) were run every 8 samples. Measured blanks were used for correction as described for the otoliths. Two seawater CRMs were run; a simulated seawater standard (High Purity Standards) was used to correct for instrument mass bias and SLEW-3 (National Research Council of Canada) was used to assess instrument precision. External precision (relative standard deviations) for this CRM (n=20) were as follows: Li:Ca=8.8%, Mg:Ca=5.5%, Mn:Ca=5.5%, Cu:Ca=7.3%, Sr:Ca=4.1%, Ba:Ca=3.8%.

Values of $\delta^{13}C$ of dissolved inorganic carbon ($\delta^{13}C_{DIC}$) in the water samples was quantified by generating CO_2 via H_3PO_4 acidification of a 5 cm^3 aliquot and then measuring $\delta^{13}C$ using a Finnigan-MAT 251 irm-MS. All $\delta^{13}C_{DIC}$ values were reported relative to VPDB and the analytical precision measure was ±0.1‰. Values of $\delta^{18}O$ of the ambient water samples ($\delta^{18}O_{water}$) were quantified by equilibrating 1 cm^3 of sample with CO_2 at atmospheric pressure following the method described by Epstein and Mayeda (1953). The resulting gas was transferred from the water equilibration system to a Europa GEO 20-20 dual-inlet mass spectrometer. All $\delta^{18}O_{water}$ values were reported relative to Vienna Standard Mean Ocean Water (VSMOW) and the analytical precision measure based on an internal standard was ±0.08% (Swart & Price 2002).

Data analysis

Pearson's correlation coefficient was used to determine if fish total length was significantly correlated with the measured element:Ca and stable isotope ratios. None of the variables analysed showed significant correlations with fish total length. Similarly, temperature and salinity were not significantly correlated considering a significance level of 0.01.

Hourly measured temperatures at the different collection sites in the Tejo estuary were averaged over the time between two sampling occasions, i.e. temperature of t_x was the average between t_x and t_{x-1}, with the exception of t_1. For t_1 a daily mean temperature of the first sampling day was used.

Site-specific partition coefficients (D_{Me}) were calculated for each sampling occasion (t) by dividing the element:Ca ratio of an otolith by the mean element:Ca ratio measured in the water samples collected in each site during each sampling occasion (Morse & Bender 1990).

$$D_{Me} = \frac{[\text{element} : \text{Ca}]_{otolith}}{[\text{element} : \text{Ca}]_{water}} \qquad (1)$$

The oxygen isotope fractionation is expressed as 1000ln(α) where:

$$\alpha = \frac{\delta^{18}O_{otolith} + 1000}{\delta^{18}O_{water} + 1000} \qquad (2)$$

The conversion equation of the VSMOW standard (for water samples) to the VPDB standard (for carbonate samples) of Coplen et al. (1983) was used:

$$\delta^{18}O_{(VSMOW)} = 1.03091 * \delta^{18}O_{(VPDB)} + 30.91 \qquad (3)$$

For stable C isotopes, $\Delta^{13}C$ was calculated by subtracting the $\delta^{13}C_{DIC}$ values measured in the water samples from the $\delta^{13}C$ values of the otoliths.

Linear regression was used to investigate the influence of ambient water temperature, salinity and chemistry on otolith composition. For all regression analyses, otolith elemental and isotopic ratios, partition coefficients and fractionation factors were averaged per sampling site and sampling occasion. R^2 values were used for the assessment of fitness and t-tests of slope parameters were performed in order to evaluate if the slope was significant.

RESULTS

Water temperature at study sites in the Tejo estuary varied between 17.9°C and 27.8°C during the 6 months of sampling (Fig. 2). Throughout the study period only small differences were observed among sampling sites, although one site (site f) showed a broader temperature range while remaining largely in phase with within-season temperature variability at the other sites.

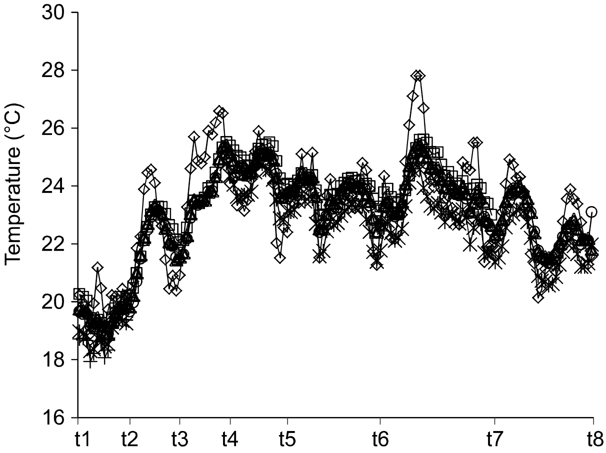

Fig. 2. Daily mean temperature from May to October during sampling occasions (t1 – t8) at the different sampling sites (□ – a, Δ - b, ○ – c, + - d, * - e, ◊ - f).

Mean salinity at sampling sites ranged from 4.46 (site a) to 23.42 (site e) with the largest amplitude observed at site b (Table 2). A number of variables were significantly correlated with ambient salinity, including Li:Ca_{water}, Mg:Ca_{water} and Cu:Ca_{water}, along with both $\delta^{13}C_{DIC}$ and $\delta^{18}O_{water}$ (Fig. 3a). Alternatively, Ba:Ca_{water} showed a significant negative relation with salinity (Fig. 3a). Interestingly, Sr:Ca_{water} was not significantly correlated to salinity, while Mn:Ca_{water} values were dominated by high values at one site (site f). Finally, $\delta^{13}C_{DIC}$ was significantly (negatively) related with temperature (Fig. 3b).

Table 2. Mean, standard deviation (in brackets), minimum and maximum salinity, element:Ca and stable isotope ratios in the ambient water in sampling sites in the Tejo estuary.

	Site					
	a	b	c	d	e	f
Salinity	4.46 (2.16) 1.00-6.84	16.40 (4.78) 8.55-25.15	8.79 (4.37) 4.85-16.00	21.32 (3.55) 16.00-26.90	23.42 (3.09) 18.13-29.10	21.45 (5.61) 18.13-29.10
Li:Ca$_{water}$ (mmol mol^{-1})	2.11 (0.38) 1.61-2.75	2.23 (0.20) 1.86-2.55	2.07 (0.11) 1.92-2.22	2.27 (0.12) 2.07-2.41	2.36 (0.17) 2.07-2.57	2.32 (0.08) 2.23-2.49
Mg:Ca$_{water}$ (mol mol^{-1})	3.43 (0.79) 1.65-4.34	4.62 (0.25) 4.18-4.89	4.14 (0.47) 3.42-4.68	4.92 (0.18) 4.63-5.15	5.07 (0.34) 4.53-5.65	5.02 (0.22) 4.74-5.45
Mn:Ca$_{water}$ (µmol mol^{-1})	16.00 (19.16) 3.96-61.55	3.76 (4.16) 0.76-12.83	5.37 (6.18) 1.06-19.63	14.14 (17.73) 1.88-44.37	2.33 (2.01) 0.76-6.75	225.49 (173.55) 52.29-565.38
Cu:Ca$_{water}$ (µmol mol^{-1})	7.43 (1.37) 5.67-9.08	11.53 (2.42) 8.08-16.04	9.75 (1.67) 7.66-12.98	13.27 (2.55) 10.14-17.35	13.53 (1.79) 11.03-16.34	12.58 (1.75) 10.60-15.46
Sr:Ca$_{water}$ (mol mol^{-1})	7.80 (0.85) 6.51-9.33	7.83 (0.47) 7.04-8.75	7.59 (0.29) 7.16-7.92	8.03 (0.28) 7.75-8.60	8.19 (0.45) 7.50-8.92	7.99 (0.43) 7.43-8.81
Ba:Ca$_{water}$ (µmol mol^{-1})	60.55 (16.23) 48.88-90.69	25.80 (12.93) 14.13-56.82	37.16 (7.92) 21.29-47.54	16.26 (3.09) 13.05-21.42	13.29 (2.14) 8.81-15.11	33.64 (9.08) 21.65-45.13
$\delta^{13}C_{DIC}$ (‰ VPDB)	-4.62 (1.32) -6.33- -2.89	-3.89 (1.09) -6.00- -2.32	-4.62 (1.33) -6.09- -2.96	-2.60 (0.54) -3.41- -1.70	-2.67 (0.86) -3.78- -1.26	-3.89 (2.03) -7.72- -0.73
$\delta^{18}O_{water}$ (‰ VSMOW)	-3.52 (0.39) -3.90- -2.92	-1.50 (0.71) -2.46- -0.61	-2.70 (0.77) -3.73- -1.57	-0.79 (0.67) -1.64-0.10	-0.41 (0.63) -1.52-0.51	0.43 (0.74) -0.37-1.83

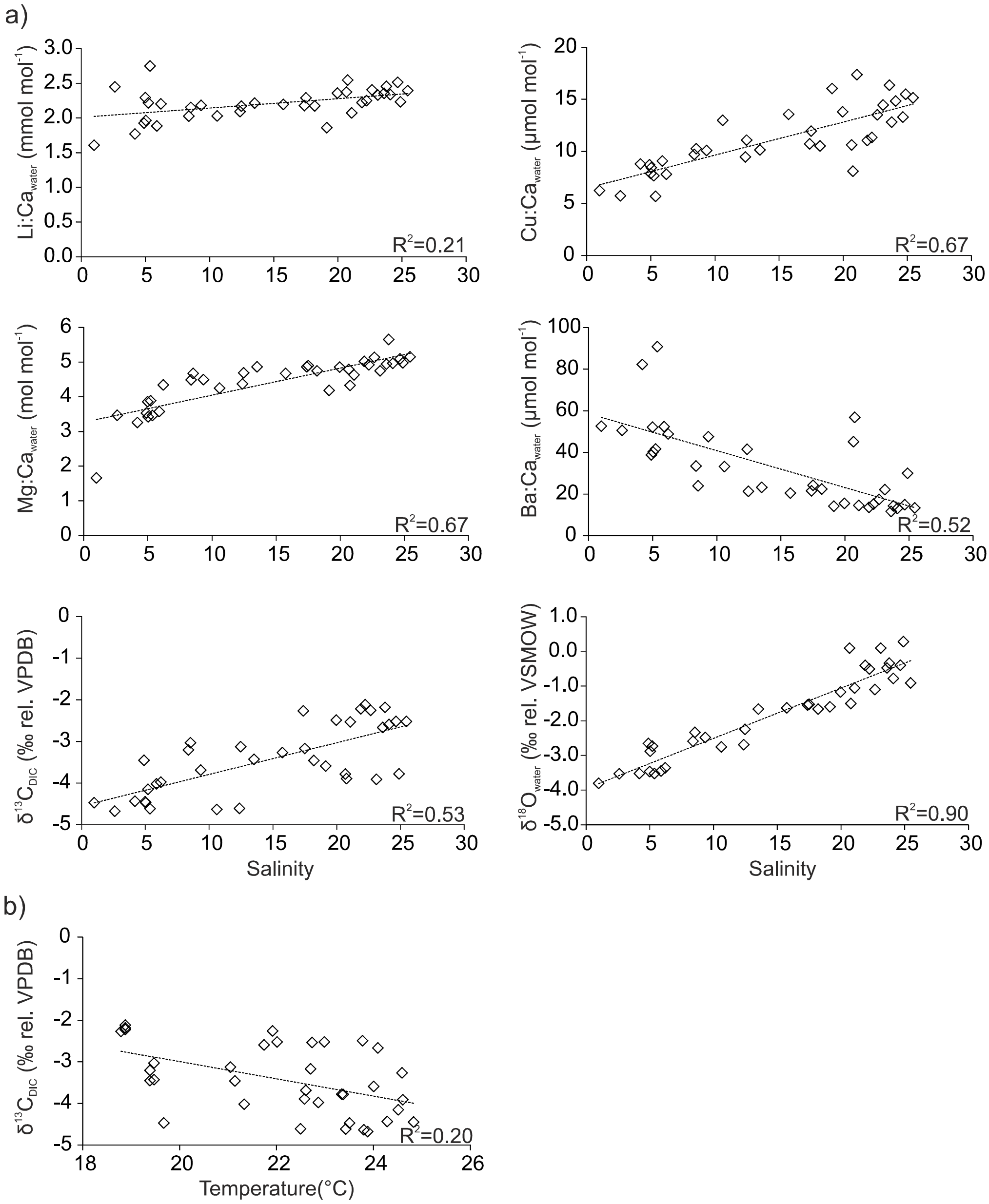

Fig. 3. Relationship between element:Ca and isotope ratios in the ambient water of the Tejo estuary and salinity (a) or temperature (b). Values represent means per sampling site and sampling occasion. R^2 values are given. Only significant regressions (t-test, p<0.01) are shown.

Element:Ca and stable isotope ratios in otoliths of juvenile *S. solea* showed significant variability both across sites and over time (Fig. 4). Some of these variations were correlated with similar patterns in water chemistry. For instance, otoliths from individuals collected at the site with the highest Mn:Ca$_{water}$ (site f) also had the highest values of Mn:Ca$_{otolith}$. Similarly, for Ba:Ca$_{otolith}$ the lowest and highest values were recorded in site e and a, respectively, which had the highest and lowest Ba:Ca$_{water}$ values (Fig. 5). Finally, both otolith δ^{13}C and δ^{18}O showed significant and positive relationships with the concentration of δ^{13}C$_{DIC}$ and δ^{18}O$_{water}$, respectively (Fig. 6).

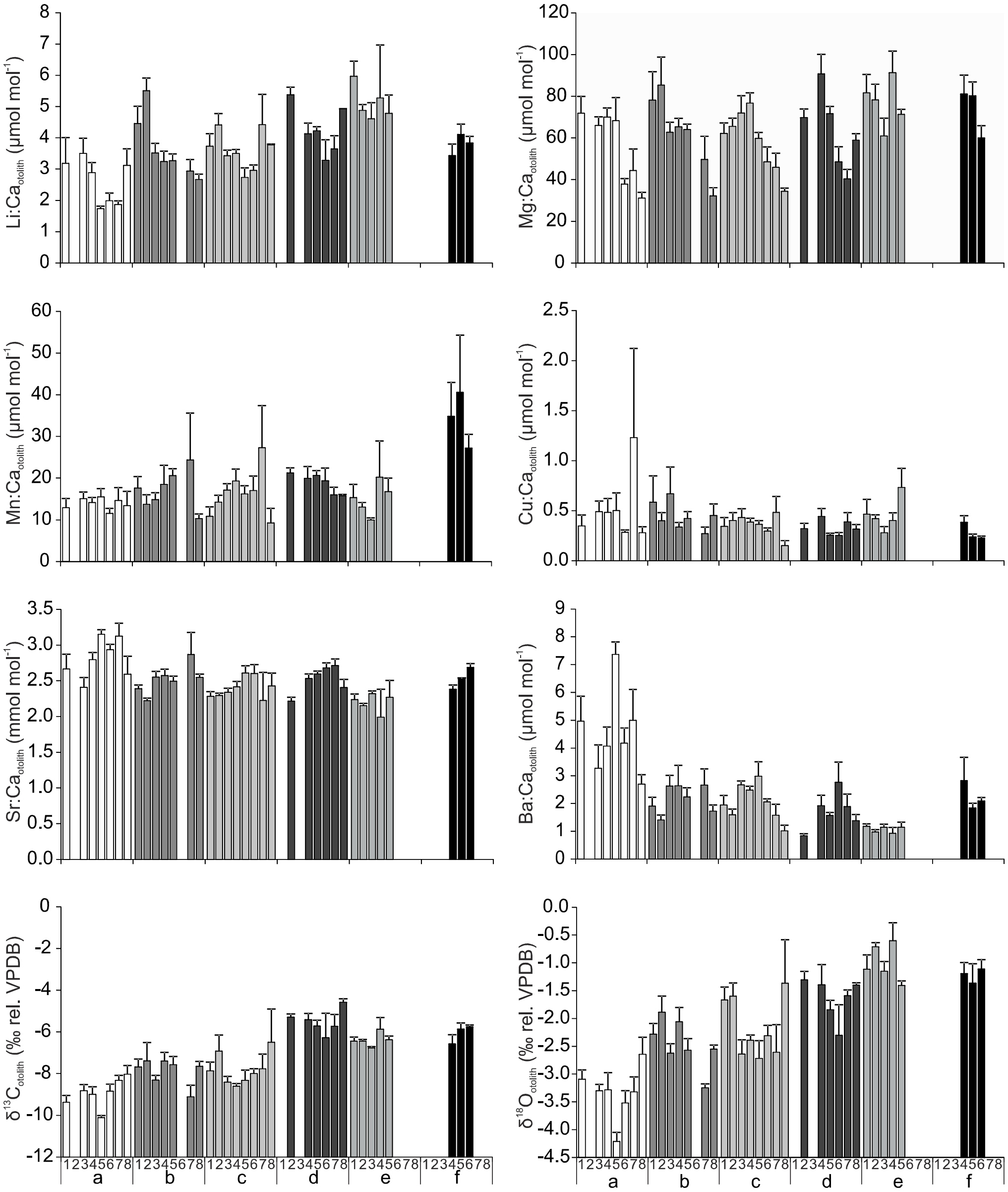

Fig 4. Mean values and standard error of element:Ca and isotope ratios (Li:Ca, Mg:Ca, Mn:Ca, Cu:Ca, Sr:Ca, Ba:Ca, δ^{13}C, δ^{18}O) in otoliths of juvenile *Solea solea* in the different sampling sites in the Tejo estuary during the sampling occasions (t1 – t8).

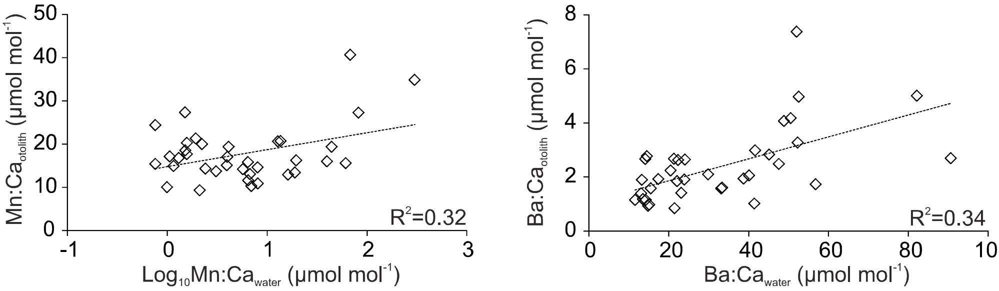

Fig. 5. Relationship between element:Ca in otoliths of juvenile *Solea solea* and in the ambient water of the Tejo estuary. Values represent means per sampling site and sampling occasion. R^2 values are given. Only significant regressions (t-test, $p<0.01$) are shown.

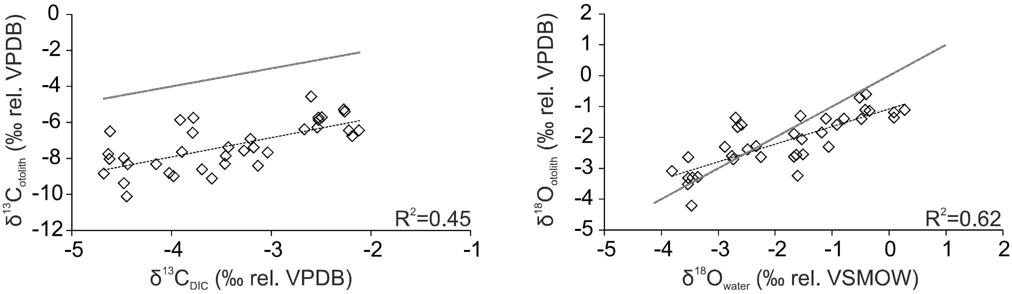

Fig. 6. Relationship between isotope ratios in otoliths of juvenile *Solea solea* and in the water of the Tejo estuary. Values represent means per sampling site and sampling occasion. Isotopic equilibrium lines are shown in dashed grey. R^2 values are given. Only significant regressions (t-test, $p<0.01$) are shown.

Analysis of otolith chemistry, accounting for the ambient composition of the water, revealed non-linear uptake for one element (Mn) and a number of significant correlations with temperature and salinity (Table 3). Manganese ions were apparently actively incorporated in otoliths at low dissolved Mn:Ca levels in ambient waters ($D_{Mn} > 1$), but were discriminated against at high levels at site f ($D_{Mn} < 1$). Partition coefficients of all other elements were less than 1 and ranged from 0.36 (D_{Sr}) to 1.2×10^{-5} (D_{Mg}), indicating a broad range of elemental discrimination across the intestinal and endolymphatic membranes. We also detected significant, negative, temperature effects on fractionation for both D_{Li} and 1000lnα (Fig. 7). Finally, salinity was negatively correlated with D_{Cu}, D_{Sr} and 1000lnα, and positively correlated with $\Delta^{13}C$ (Fig. 7).

Table 3. Mean, standard deviation (in brackets), minimum and maximum of element partition coefficients and isotope fractionation factors in otoliths of juvenile *Solea solea* in the different sampling sites in the Tejo estuary.

	Site					
	a	b	c	d	e	f
D_{Li} (10^{-3})	1.27 (0.48) 0.76-1.98	1.67 (0.47) 1.05-2.49	1.76 (0.33) 1.24-2.18	1.87 (0.36) 1.53-2.47	2.17 (0.31) 1.88-2.69	1.64 (0.17) 1.45-1.77
D_{Mg} (10^{-5})	1.85 (1.15) 0.96-4.35	1.34 (0.33) 0.74-1.76	1.41 (0.32) 0.79-1.76	1.28 (0.35) 0.78-1.77	1.51 (0.27) 1.08-1.81	1.53 (0.28) 1.20-1.69
D_{Mn}	1.66 (1.22) 0.25-3.81	10.81 (10.55) 1.47-32.00	6.96 (6.53) 0.83-18.08	4.13 (4.64) 0.40-11.02	11.58 (6.57) 1.93-20.20	0.35 (0.24) 0.12-0.60
D_{Cu}	0.05 (0.01) 0.05-0.06	0.04 (0.02) 0.02-0.06	0.04 (0.01) 0.02-0.05	0.02 (0.01) 0.01-0.03	0.04 (0.01) 0.02-0.04	0.02 (0.01) 0.01-0.04
D_{Sr}	0.36 (0.05) 0.28-0.43	0.32 (0.04) 0.28-0.41	0.32 (0.02) 0.30-0.34	0.31 (0.02) 0.28-0.35	0.27 (0.02) 0.24-0.28	0.32 (0.01) 0.30-0.33
D_{Ba}	0.08 (0.03) 0.03-0.14	0.10 (0.05) 0.03-0.19	0.06 (0.03) 0.02-0.13	0.12 (0.05) 0.04-0.19	0.08 (0.02) 0.06-0.10	0.07 (0.01) 0.06-0.08
$\Delta^{13}C$	-4.06 (1.18) -5.31- -2.13	-4.06 (1.20) -5.08- -1.64	-3.18 (1.30) -5.43- -1.90	-2.90 (0.85) -3.70- -1.46	-3.72 (0.94) -4.44- -2.34	-2.11 (0.60) -2.63- -1.45
$1000\ln\alpha$	30.73 (0.46) 30.13-31.42	29.35 (0.86) 27.80-30.50	30.98 (0.87) 29.37-31.75	29.49 (0.79) 28.62-30.60	29.93 (0.46) 29.29-30.41	28.64 (0.45) 28.13-28.99

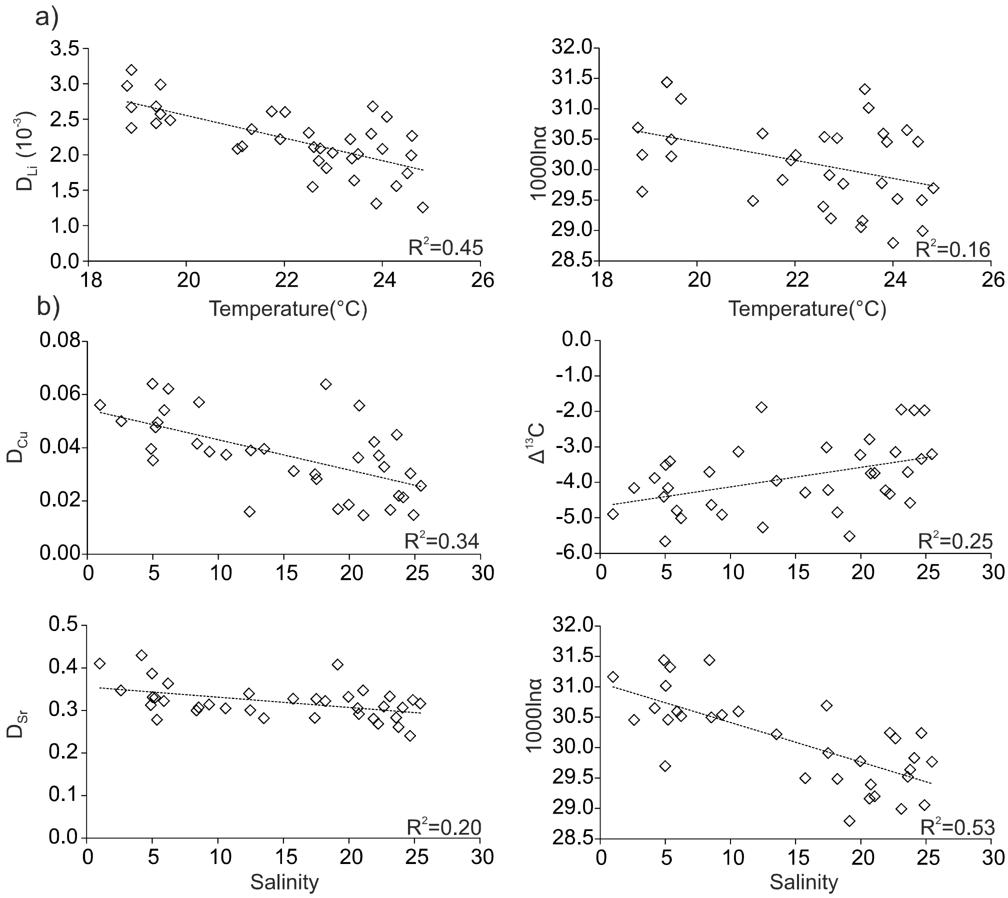

Fig. 7 Relationship between partition coefficients and fractionation factors in otoliths of juvenile *Solea solea* and temperature (a) or salinity (b). Values represent means per sampling site and sampling occasion. R^2 values are given. Only significant regressions (t-test, $p<0.01$) are shown, with the exception of 1000lnα vs. temperature (t-test, $p=0.02$).

DISCUSSION

The influence of environmental variations on the element and isotope composition of juvenile *S. solea* otoliths was assessed in field collections from the Tejo estuary. While commonly investigated in the laboratory, few studies have examined the relation between the geochemical composition of otoliths and the physicochemical characteristics of the water in the field (Wells et al. 2003, Kraus & Secor 2004, Elsdon & Gillanders 2005, Dorval et al. 2007). Our results highlight the difficulties of interpreting otolith geochemistry data from dynamic environments when both fish and water masses may be making significant excursions over short time scales. Nonetheless, we detected significant correlations between otolith composition and water chemistry, temperature and salinity. Physicochemical properties of ambient water masses are therefore a major, if not the only, determinant of otolith chemistry in juvenile *S. solea*.

Dissolved Li:Ca and Sr:Ca ratios both showed conservative mixing in the Tejo estuary, with generally higher and stable values in seawater, and lower but variable values in freshwaters (e.g. Huh et al. 1998, Pogge von Strandmann et al. 2008). Hicks et al. (2010) found a significant positive correlation between otolith Li:Ca and salinity in two laboratory-reared amphidromous galaxiids and suggested that Li:Ca could be a valuable tracer for diadromous fish migration. However, Li:Ca in freshwater environments varies greatly and therefore the freshwater endmember under study would need to be determined before using otolith Li:Ca as a proxy for salinity. More interestingly, we found that Li partition coefficients in otoliths were significantly correlated with ambient temperature. While more work is clearly needed, Li:Ca thermometry may be possible in juvenile *S. solea*. On the other hand, we found no indication that D_{Sr} was related to temperature, even though several other studies documented significant relationships between otolith Sr:Ca and temperature (e.g. Bath et al. 2000, Collingsworth et al. 2010). Rather, D_{Sr} was negatively correlated with salinity and, therefore, ambient Sr concentration ($[Sr]_{water}$). Martin et al. (2004) documented the opposite pattern, whereby D_{Sr} was positively correlated with $[Sr]_{water}$ and they suggested that this may be related to non-linear inhibition of Ca ions by Sr ions at uptake sites on branchial and intestinal membranes (Chowdhury & Blust 2001, 2002). More definite resolution of this discrepancy will likely require laboratory studies where it is possible to isolate individual effects of these variables on otolith chemistry.

Dissolved and particulate Mn varies largely in estuaries, especially during summer, due to increased microbial activity in the tidal flats, anoxic conditions and increased Mn quantities in the pore water (Eaton 1979, Dellwig et al. 2007). A significant relationship between $Mn:Ca_{otolith}$ and $Mn:Ca_{water}$ of juvenile sole was observed in the Tejo estuary. However, if we excluded a single site with very high Mn:Ca levels in water and otoliths (site f) the correlation was no longer significant. Analysis of Mn partition coefficients revealed that Mn was discriminated against during otolith deposition at very high levels of ambient Mn while an active Mn uptake occurred at low ambient levels. These patterns of discrimination and active uptake of Mn are in accordance with those found in laboratory reared black bream (Elsdon & Gillanders 2003). Otolith Ba:Ca levels were consistently related to $Ba:Ca_{water}$ values, as has been shown in several laboratory and field studies (Elsdon & Gillanders 2003, Hamer et al. 2006). Moreover, Ba:Ca$_{water}$ was linearly (negatively) related to salinity in the Tejo estuary, with deviating values found at site f. These higher values of Ba:Ca$_{water}$ may be related with the deposition of Ba-enriched freshwater sediments at site f during high freshwater input (winter) and the desorption of Ba with rising salinity during low freshwater input (summer) (Carroll et al. 1993). Furthermore, extensive salt marshes surround site f and this habitat has been identified as a potential storage of particulate Ba (Coffey et al. 1997). Based on the linear increase of Ba:Ca in otoliths and water, Ba:Ca$_{otolith}$ of juvenile sole appeared to be a valid tracer for movements along the salinity gradient in the Tejo estuary and should be included in the suite of elements when attempting to resolve fish migrations or environmental histories of this species. The linear relationship between Ba:Ca$_{water}$ and salinity was in contrast to the lack of any significant correlation between Sr:Ca$_{water}$ and salinity. Freshwater endmember values for Sr:Ca$_{water}$ in the Tejo were similar to the global marine Sr:Ca$_{water}$ value, and therefore otolith Sr:Ca was not a useful proxy for salinity in this system.

Stable C and O isotopes in the Tejo estuary demonstrated conservative behaviour along the length of the estuary that reflected mixing between riverine and marine C and O sources. The negative relation of $\delta^{13}C_{DIC}$ with temperature in the Tejo estuary may be related to an increase of respiration with temperature, causing a negative isotopic shift due to the oxidation of organic matter**,** which has a more negative $\delta^{13}C_{DIC}$ signature (Mook & Tan 1991). Otolith $\delta^{13}C$ is rarely incorporated close to equilibrium with $\delta^{13}C_{DIC}$ because 20% - 80% of the carbon in otoliths is derived from metabolic sources (Kalish 1991, Thorrold et al. 1997). The contribution of isotopically depleted metabolic sources to otoliths almost always results in more negative otolith $\delta^{13}C$ values than $\delta^{13}C_{DIC}$ (McMahon et al. 2011). Studies investigating food web dynamics in the Tejo estuary showed that values of $\delta^{13}C$ in main prey items of juvenile soles and muscle tissue of fish were more depleted in an upstream location (close to site a) than in a mesohaline site (site f) (Vinagre et al. 2008, França et al. 2011). Therefore, because $\delta^{13}C_{otolith}$ of juvenile common sole is strongly related to $\delta^{13}C_{DIC}$ across the salinity gradient and the metabolic carbon sources appeared to mirror this trend, $\delta^{13}C_{otolith}$ could greatly contribute to resolve habitat use and movement patterns in the Tejo estuary. Kerr et al. (2007) also identified otolith $\delta^{13}C$ in white perch as a useful proxy for salinity due to the large range of response across estuarine habitats.

Oxygen isotopes in otoliths are generally deposited at or close to equilibrium with ambient waters with a small temperature effect (Thorrold et al. 1997, Høie et al. 2004), making otolith $\delta^{18}O$ values a useful tracer in euryhaline environments. We were, therefore, surprised to find a significant inverse relationship between 1000lnα and salinity which suggested some degree of disequilibrium between $\delta^{18}O_{otolith}$ and $\delta^{18}O_{water}$. The most parsimonious explanation for this disequilibrium is movement of juvenile soles within the Tejo estuary between habitats with different $\delta^{18}O_{water}$. The observation that we measured both high and low $\delta^{18}O_{otolith}$ values comparatively to the ambient $\delta^{18}O_{water}$ in the low and high salinity sites, respectively, suggested that fish moved in both directions along the estuarine gradient. The generally high $\delta^{18}O_{otolith}$ values in low salinity sites (a and c) pointed to upstream movement while the high $\delta^{18}O_{otolith}$ values in the remaining sites indicated downstream movement.

In conclusion, our results highlighted the need to determine ambient concentrations of element:Ca and isotope ratios to identify potential geochemical tracers in otoliths from fishes resident in estuaries. Additionally, due to the complexity of the estuarine environment as well as the movement patterns of fish, the simultaneous use of multiple geochemical tracers is advisable to accurately reconstruct habitat use and movement of euryhaline fish. The assessment and identification of otolith element:Ca and stable isotope ratios in the natural environment that have the power to resolve past habitat use and track movement patterns of fish may lead to a better understanding of the spatial distribution of juveniles in estuaries. Ultimately, knowledge on how juveniles use estuaries is important towards the establishment of more concerted conservation directives and management plans, as estuaries provide several habitats that are vital to early life stages of many commercially important coastal fish species.

ACKNOWLEDGEMENTS

We would like to thank all involved in the sampling surveys and S. Birdwhistell for help in sample analysis. This study was funded by 'Fundação para a Ciência e a Tecnologia' (FCT), through PEst- OE/MAR/UI0199/2011 and other projects. S. E. Tanner was funded with a PhD grant from FCT (SFRH/BD/40313/2007).

LITERATURE CITED

Bath GE, Thorrold SR, Jones CM, Campana SE, McLaren JW, Lam JWH (2000) Strontium and barium uptake in aragonitic otoliths of marine fish. Geochim Cosmochim Acta 64:1705-1714

Bergenius MAJ, Mapstone BD, Begg GA, Murchie CD (2005) The use of otolith chemistry to determine stock structure of three epinepheline serranid coral reef fishes on the Great Barrier Reef, Australia. Fish Res 72:253-270

Cabral H (2003) Differences in growth rates of juvenile *Solea solea* and *Solea senegalensis* in the Tagus estuary, Portugal. J Mar Biol Assoc UK 83:861-868

Cabral HN, Costa MJ (1999) Differential use of nursery areas within the Tagus estuary by sympatric soles, *Solea solea* and *Solea senegalensis*. Environ Biol Fishes 56:389-397

Cabral HN, Vasconcelos RP, Vinagre C, França S, Fonseca V, Maia A, Reis-Santos P, Lopes M, Ruano M, Campos J, Freitas V, Costa MJ (2007) Relative importance of estuarine flatfish nurseries along the Portuguese coast. J Sea Res 57:209-217

Campana SE (1999) Chemistry and composition of fish otoliths: pathways, mechanisms and applications. Mar Ecol Prog Ser 188:263-297

Campana SE, Chouinard GA, Hanson JM, Fréchet A, Brattey J (2000) Otolith elemental fingerprints as biological tracers of fish stocks. Fish Res 46:343-357

Campana SE, Thorrold SR (2001) Otoliths, increments, and elements: keys to a comprehensive understanding of fish populations? Can J Fish Aquat Sci 58:30-38

Carroll J, Falkner KK, Brown ET, Moore WS (1993) The role of the Ganges-Brahmaputra mixing zone in supplying barium and ^{226}Ra to the Bay of Bengal. Geochim Cosmochim Acta 57:2981-2990

Chowdhury MJ, Blust R (2001) A mechanistic model for the uptake of waterborne strontium in the common carp (Cyprinus carpio L.). Environ Sci Technol 35:669-675

Chowdhury MJ, Blust R (2002) Bioavailability of waterborne strontium to the common carp, Cyprinus carpio, in complexing environments. Aquat Toxicol 58:215-227

Clarke LM, Thorrold SR, Conover DO (2011) Population differences in otolith chemistry have a genetic basis in *Menidia menidia*. Can J Fish Aquat Sci 68:105-114

Coffey M, Dehairs F, Collette O, Luther G, Church T, Jickells T (1997) The behaviour of dissolved barium in estuaries. Estuar Coast Shelf Sci 45:113-121

Collingsworth PD, Van Tassell JJ, Olesik JW, Marschall EA (2010) Effects of temperature and elemental concentration on the chemical composition of juvenile yellow perch (*Perca flavescens*) otoliths. Can J Fish Aquat Sci 67:1187-1196

Coplen TB, Kendall C, Hopple J (1983) Comparision of stable isotope reference material. Nature 302:236-238

Costa MJ, Bruxelas A (1989) The structure of fish communities in the Tagus estuary, Portugal, and its role as a nursery for commercial fish species. Sci Mar 53:561-566

Dellwig O, Bosselmann K, Kölsch S, Hentscher M, Hinrichs J, Böttcher ME, Reuter R, Brumsack H-J (2007) Sources and fate of manganese in a tidal basin of the German Wadden Sea. J Sea Res 57:1-18

Dorval E, Jones CM, Hannigan R (2005) Chemistry of surface waters: Distinguishing fine-scale differences in sea grass habitats of Chesapeake Bay. Limnol Oceanogr 50:1073-1083

Dorval E, Jones CM, Hannigan R, van Montfrans J (2007) Relating otolith chemistry to surface water chemistry in a coastal plain estuary. Can J Fish Aquat Sci 64:411-424

Eaton A (1979) The impact of anoxia on Mn fluxes in the Chesapeake Bay. Geochim Cosmochim Acta 43:429-432

Elsdon TS, Gillanders BM (2003) Relationship between water and otolith elemental concentrations in juvenile black bream *Acanthopagrus butcheri*. Mar Ecol Prog Ser 260:263-272

Elsdon TS, Gillanders BM (2005) Consistency of patterns between laboratory experiments and field collected fish in otolith chemistry: an example and applications for salinity reconstructions. Mar Freshw Res 56:609-617

Elsdon TS, Wells BK, Campana SE, Gillanders BM, Jones CM, Limburg KE, Secor DH, Thorrold SR, Walther BD (2008) Otolith chemistry to describe movements and life-history parameters of fishes: hypotheses, assumptions, limitations and inferences. Oceanogr Mar Biol Annu Rev 46:297-330

Epstein S, Mayeda T (1953) Variation of ^{18}O content of waters from natural sources. Geochim Cosmochim Acta 4:213-224

Farrell J, Campana SE (1996) Regulation of calcium and strontium deposition on the otoliths of juvenile tilapia, *Oreochromis niloticus*. Comp Biochem Physiol A: Physiol 115:103-109

França S, Vasconcelos RP, Tanner S, Máguas C, Costa MJ, Cabral HN (2011) Assessing food web dynamics and relative importance of organic matter sources for fish species in two Portuguese estuaries: A stable isotope approach. Mar Environ Res 72:204-215

Gillanders BM, Kingsford MJ (1996) Elements in otoliths may elucidate the contribution of estuarine recruitment to sustaining coastal reef populations of temperate reef fish. Mar Ecol Prog Ser 141:13-20

Hamer PA, Jenkins GP, Coutin P (2006) Barium variation in *Pagrus auratus* (Sparidae) otoliths: A potential indicator of migration between an embayment and ocean waters in south-eastern Australia. Estuar Coast Shelf Sci 68:686-702

Hicks AS, Closs GP, Swearer SE (2010) Otolith microchemistry of two amphidromous galaxiids across an experimental salinity gradient: A multi-element approach for tracking diadromous migrations. J Exp Mar Biol Ecol 394:86-97

Høie H, Otterlei E, Folkvord A (2004) Temperature-dependent fractionation of stable oxygen isotopes in otoliths of juvenile cod (*Gadus morhua* L.). ICES J Mar Sci 61:243-251

Huh Y, Chan L-H, Zhang L, Edmond JM (1998) Lithium and its isotopes in major world rivers: Implications for weathering and the oceanic budget. Geochim Cosmochim Acta 62:2039-2051

Kalish JM (1991) ^{13}C and ^{18}O isotopic disequilibria in fish otoliths: metabolic and kinetic effects. Mar Ecol Prog Ser 75:191-203

Kalish JM (1996) Trace elements in the otoliths of New Zealand blue grenadier (*Macruronus novaezelandiae*) as an aid to stock discrimination. Mar Freshw Res 47:537-542

Kerr LA, Secor DH, Kraus RT (2007) Stable isotope (δ^{13}C and δ^{18}O) and Sr/Ca composition of otoliths as proxies for environmental salinity experienced by an estuarine fish. Mar Ecol Prog Ser 349:245-253

Koutsikopoulos C, Desaunay Y, Dorel D, Marchand J (1989) The role of coastal areas in the life history of sole (*Solea solea* L.) in the Bay of Biscay. Sci Mar 53:567-575

Kraus RT, Secor DH (2004) Incorporation of strontium into otoliths of an estuarine fish. J Exp Mar Biol Ecol 302:85– 106

Martin GB, Thorrold SR, Jones CM (2004) Temperature and salinity effects on strontium incorporation in otoliths of larval spot (*Leiostomus xanthurus*). Can J Fish Aquat Sci 61:34-42

Martin GB, Thorrold SR (2005) Temperature and salinity effects on magnesium, manganese, and barium incorporation in otoliths of larval and early juvenile spot *Leiostomus xanthurus*. Mar Ecol Prog Ser 293:223-232

McMahon KW, Fogel ML, Johnson BJ, Houghton LA, Thorrold SR (2011) A new method to reconstruct fish diet and movement patterns from δ^{13}C values in otolith amino acids Can J Fish Aquat Sci 68:1330-1340

Mook WG, Tan FC (1991) Stable Carbon Isotopes in Rivers and Estuaries. In: Degens ET, Kempe S, Richey JE (eds) Biogeochemistry of Major World Rivers, Scope 42. John Wiley & Sons, Chichester, pp. 245-264

Morse JW, Bender ML (1990) Partition coefficients in calcite: Examination of factors influencing the validity of experimental results and their application to natural systems. Chem Geol 82:265-277

Ostermann DR, Curry WB (2000) Calibration of stable isotopic data: an enriched δ^{18}O standard used for source gas mixing detection and correction. Paleoceanography 15:353-360

Pogge von Strandmann PAE, James RH, van Calsteren P, Gílason SR, Burton KW (2008) Lithium, magnesium and uranium isotope behaviour in the estuarine environment of basaltic islands. Earth Planet Sci Lett 274:462-471

Quéro JC, Dessouter M, Lagardère F (1986) Soleidae. In: Whitehead P, Bauchot M-L, Hureau J-C, Nielsen J, Tortonese E (eds) Fishes in the North-Eastern Atlantic and the Mediterranean. UNESCO, Paris, pp. 1308-1342

Radtke RL (1987) Age and growth information available from the otoliths of the Hawaiian snapper, *Pristipomoides filamentosus*. Coral Reefs 6:19-25

Radtke RL, Showers W, Moksness E, Lenz P (1996) Environmental information stored in otoliths: insights from stable isotopes. Mar Biol 127:161-170

Secor DH, Henderson-Arzapalo A, Piccoli PM (1995) Can otolith microchemistry chart patterns of migration and habitat utilization in anadromous fishes? J Exp Mar Biol Ecol 192:15-33

Sturgeon RE, Willie SN, Yang L, Greenberg R, Spatz RO, Chen Z, Scriver C, Clancy V, Lam JW, Thorrold SR (2005) Certification of a fish otolith reference material in support of quality assurance for trace element analysis. J Anal At Spectrom 20:1067-1071

Swart PK, Price R (2002) Origin of salinity variations in Florida Bay. Limnol Oceanogr 47:1234-1241

Thorrold SR, Campana SE, Jones CM, Swart PK (1997) Factors determining $\delta^{13}C$ and $\delta^{18}O$ fractionation in aragonitic otoliths of marine fish. Geochim Cosmochim Acta 61:2909-2919

Thorrold SR, Latkoczy C, Swart PK, Jones CM (2001) Natal homing in marine fish metapopulation. Science 291:297-299

Townsend DW, Radtke RL, Malone DP, Wallinga JP (1995) Use of otolith strontium:calcium ratios for hind-casting larval cod *Gadus morhua* distributions relative to water masses on Georges Bank. Mar Ecol Prog Ser 119:37-44

Tsukamoto K, Arai T (2001) Facultative catadromy of the eel *Anguilla japonica* between freshwater and seawater habitats. Mar Ecol Prog Ser 220:265-276

Vasconcelos RP, Reis-Santos P, Tanner S, Maia A, Latkoczy C, Günther D, Costa MJ, Cabral H (2008) Evidence of estuarine nursery origin of five coastal fish species along the Portuguese coast through otolith elemental fingerprints. Estuar Coast Shelf Sci 79:317-327

Vasconcelos RP, Reis-Santos P, Maia A, Fonseca V, França S, Wouters N, Costa MJ, Cabral HN (2010) Nursery use patterns of commercially important marine fish species in estuarine systems along the Portuguese coast. Estuar Coast Shelf Sci 86:613-624

Vinagre C, Salgado J, Costa MJ, Cabral HN (2008) Nursery fidelity, food web interactions and primary sources of nutrition of the juveniles of *Solea solea* and *S. senegalensis* in the Tagus estuary (Portugal): A stable isotope approach. Estuar Coast Shelf Sci 76:255-264

Wells BK, Rieman BE, Clayton JL, Horan DL, Jones CM (2003) Relationships between Water, Otolith, and Scale Chemistries of Westslope Cutthroat Trout from the Coeur d’Alene River, Idaho: The Potential Application of Hard-Part Chemistry to Describe Movements in Freshwater. Trans Am Fish Soc 132:409-424

Yoshinaga J, Nakama A, Morita M, Edmonds JS (2000) Fish otolith reference material for quality assurance of chemical analyses. Mar Chem 69:91-97

CHAPTER III

Spatial and ontogenetic variability in the chemical composition of juvenile common sole (*Solea solea*) otoliths

Susanne E. Tanner[1], Rita P. Vasconcelos[1], Patrick Reis-Santos[1], Henrique N. Cabral[1], Simon R. Thorrold[2]

[1]Centro de Oceanografia, Faculdade de Ciências da Universidade de Lisboa, Campo Grande, 1749-016 Lisboa, Portugal
[2]Biology Department MS 50, Woods Hole Oceanographic Institution, Woods Hole, MA 02543, USA

Estuarine, Costal and Shelf Science (2011) 91: 150-157

Spatial and ontogenetic variability in the chemical composition of juvenile common sole (*Solea solea*) otoliths

ABSTRACT A description of variations in the chemical composition of fish otoliths at different spatial scales and life history stages is a prerequisite for their use as natural tags in fish population connectivity and migration studies. Otolith geochemistry of juvenile common sole (*Solea solea*), a marine migrant species collected in six Portuguese estuaries was examined. Elemental ratios (Mg:Ca, Mn:Ca, Cu:Ca, Sr:Ca, Ba:Ca, Pb:Ca) were analyzed in two zones of the right otolith (corresponding to late larval and juvenile stages) using laser ablation inductively coupled plasma mass spectrometry (ICP-MS). Stable carbon and oxygen isotopes ($\delta^{13}C$ and $\delta^{18}O$) were determined in left otoliths using isotopic ratio monitoring mass spectrometry (irm-MS). Significant differences in otolith geochemical signatures were found among estuaries, among sites within estuaries and between otolith zones. Several elemental ratios (Mg:Ca, Mn:Ca, Cu:Ca and Sr:Ca) showed consistent patterns between otolith zones and were likely influenced by environmental factors and ontogenetic effects associated with physiological changes during metamorphosis. Assignment of individuals to their collection estuary based on the otolith geochemical signatures was more accurate at the site level (81%) than among estuaries (69%). Site temperature was not correlated with any of the elemental or isotope ratios, but salinity was significantly correlated with Ba:Ca, $\delta^{13}C$ and $\delta^{18}O$. Observed spatial variations among estuaries and sites within estuaries indicate that geochemical signatures in otoliths are accurate natural tags of estuarine habitat in common sole. Nevertheless, the significant variations observed between otolith zones should be taken into account in the design of population connectivity studies.

Keywords: otolith geochemical composition, life stage variability, spatial variation, *Solea solea*

INTRODUCTION

Otoliths are paired calcified structures located in the inner ear of most fishes, and have been widely used in marine ecology and fisheries research, particularly to understand age structure and growth patterns, but also to define population structure (e.g. Campana 1999, Patterson et al. 2004, Thresher & Procter 2007), identify natal sources and nursery areas (e.g. Gillanders & Kingsford 2000, Thorrold et al. 2001, Vasconcelos et al. 2008) and study migration patterns (e.g. Tsukamoto & Arai 2001, Hamer et al. 2006, Rooker et al. 2008). Otoliths are suited for these applications because they grow continuously, forming daily or annual increments and are metabolically inert. Elements and stable isotopes are incorporated into the calcium carbonate and protein matrix of otoliths and otolith chemical composition is often different in fish collected from areas with distinct water chemistry. However, the incorporation of elements and isotopes into otoliths is more complex than their mere availability and concentration in the ambient water since it is affected by several factors including environmental conditions (temperature, salinity and water chemistry) (Bath et al. 2000, Elsdon & Gillanders 2002, Martin & Thorrold 2005), physiology (Kalish 1989, Payan et al. 1999) and otolith crystal structure (Chesney et al. 1998, Brown & Severin 1999). While some elements and isotopes (e.g. Sr, Ba, O isotopes) appear to directly reflect ambient concentrations, others (e.g. Na, K, Zn) are physiologically regulated and less likely to reflect the surrounding environmental conditions (Campana 1999). Nonetheless, natural variations in otolith chemistry have been widely used to identify natal origins and reconstruct habitat associations of individual fish (reviewed by Elsdon et al. 2008).

Estuarine systems are particularly amenable for studies using geochemical signatures as natural tags since differences in water chemistry are often more pronounced among estuaries than adjacent coastal ocean waters. Variation of otolith elemental fingerprints in *Solea solea* has been reported among estuaries along the Portuguese coast (Vasconcelos et al. 2007) however, significant differences have also been observed among sites within estuaries (Thorrold et al. 1998, Gillanders & Kingsford 2003). A sound assessment of the variation at different spatial scales (intra- and inter-estuaries) and throughout larval and juvenile development is vital to accurately use otolith chemistry to retrospectively identify juvenile habitat use. Moreover, ontogenetic effects may also contribute to variations in otolith chemistry even if strong gradients in environmental variables exist among locations. De Pontual et al. (2003) reported that Sr:Ca ratios in early stage common sole (*Solea solea*) were influenced both by ontogenetic and environmental factors. Similarly, Fowler et al. (1995) concluded that temperature, salinity and ontogeny explained a significant amount of variance in otolith chemistry of Atlantic croaker (*Micropogonius undulatus*) larvae reared in the lab under constant conditions. Alternatively, ontogenetic effects on Sr:Ca and Ba:Ca incorporation in lab-reared black bream (*Acanthopagrus butcheri*) were not documented by Elsdon and Gillanders (2005). Ultimately, knowledge of the variations in otolith chemistry at different spatial scales combined with an understanding of possible ontogenetic effects will facilitate the interpretation of otolith geochemical data.

The common sole, *Solea solea*, is a flatfish of commercial importance in Europe.

Its distribution extends from Norway and the western Baltic Sea to Senegal, including the Mediterranean Sea. Spawning and larval development occurs in shelf waters with metamorphosis and the consequent shift to a benthic life form, occurring during transport from spawning to nursery grounds (Marchand 1991). Along the Portuguese coast estuarine colonization of *S. solea* begins in May and generally several cohorts occur (Cabral 2003). Juvenile soles spend up to two years in estuarine nursery grounds before migrating to coastal waters up to 200 m (Quéro et al. 1986, Koutsikopoulos et al. 1989) where they reach sexual maturity at 3-4 years age (Ramos 1982). The Portuguese coast has several estuaries with well-established nurseries for juveniles of multiple species (Cabral et al. 2007, Vasconcelos et al. 2010). Within an estuary juvenile *S. solea* are known to occur in a single nursery area (e.g. Tejo) or in multiple, segregated nursery areas (e.g. Mira) (Vasconcelos et al. 2010).

The purpose of the present study was to determine: (1) variability in otolith elemental composition between two different life history stages of *S. solea* (late larval and juvenile); and (2) variability in otolith chemistry among estuaries and sites within estuaries along the Portuguese coast. The obtained data were used to assess the accuracy of natural geochemical signatures in otoliths to distinguish juveniles collected in distinct sites within several estuaries along the Portuguese coast which might, in turn, be used to determine the estuaries or sites within estuaries that contribute most to the replenishment of fish stocks.

MATERIAL AND METHODS

Sample collection, preparation and environmental data

Sampling of 0-group juvenile specimens of *S. solea* was carried out using a beam trawl during July 2006 (corresponding to the peak densities of juveniles of this species in Portuguese estuaries), in the main estuarine nursery areas where the species occurs along the Portuguese coast (Fig. 1): Douro (D), Ria de Aveiro (RA), Mondego (Mo), Tejo (T), Mira (Mi) and Guadiana (G). In the Ria de Aveiro (RA 1 and RA 2), Mondego (Mo 1 and Mo 2) and Mira (Mi 1 and Mi 2) estuaries common sole juveniles occur in high densities in two distinct nursery areas, hence juveniles were collected in both areas (Fig. 1). Fish were stored and transported on ice to the laboratory and preserved frozen until dissection.

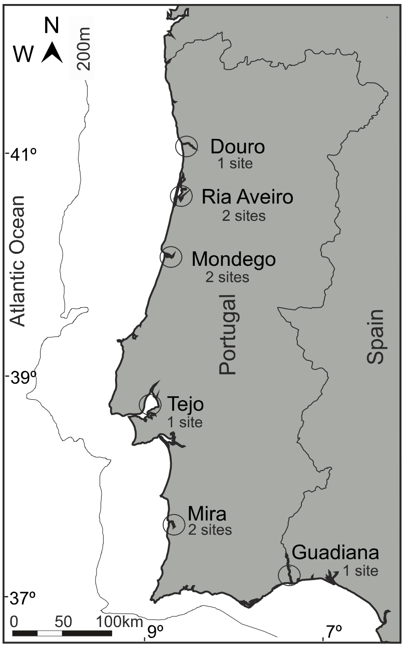

Fig. 1. Estuaries sampled along the Portuguese coast and number of collection sites within each estuary.

Water temperature (°C), salinity, dissolved oxygen (mg L^{-1}) and conductivity (mS cm^{-1}) were measured using a portable multiparameter instrument (YSI 6820). Mean site temperature (°C) and salinity were calculated based on data collected in May and July 2006 (Table 1).

Table 1. Collection site, mean value and standard error (SE) of temperature (°C) and salinity in May and July 2006. Also shown total fish length (Lt, mm) and sample size (*n*) of juveniles of *Solea solea* along the Portuguese coast.

Collection site	Temperature (°C)				Salinity				Lt (mm)		*n*
	May 2006		July 2006		May 2006		July 2006				
	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	77.3	2.4	6
Ria Aveiro 1	21.1	0.1	24.5	0.3	18.6	1.1	34.6	0.1	85.3	7.9	5
Ria Aveiro 2	26.6	6.0	25.2	0.1	13.9	1.4	20.5	0.3	100.1	2.9	9
Mondego 1	18.8	0.0	22.9	0.3	4.1	0.1	11.0	0.9	73.5	1.3	12
Mondego 2	22.3	0.3	23.9	0.2	9.6	0.7	19.2	1.8	81.4	2.1	12
Tejo	20.3	0.0	23.8	0.1	0.4	0.1	3.1	0.7	88.1	2.3	12
Mira 1	21.9	0.2	26.7	0.1	10.3	0.6	23.0	1.4	75.7	1.5	10
Mira 2	21.6	0.2	27.1	0.0	3.6	1.3	4.4	0.5	75.5	1.7	12
Guadiana	23.1	0.2	27.0	0.2	6.9	1.5	8.9	1.3	82.7	2.5	11

Total length of each individual was determined (Table 1) and sagittal otoliths were extracted using plastic tweezers, cleaned of adhering tissue in ultrapure water (18.2 MΩ cm^{-1}) and stored in plastic microcentrifuge tubes. All glassware and plasticware used were previously decontaminated with an acid wash in 10% nitric acid (HNO_3) for 24 h, rinsed with ultrapure water and dried in a laminar flow positive pressure fume hood.

Geochemical analyses

Laser ablation ICP-MS

Right (eyed side of fish) otoliths were mounted individually on a petrographic slide with cyanoacrylic glue. Otoliths were ground to the midplane on 30 mm and 3 mm lapping film and then immersed in ultrapure water and sonicated for 2 min to remove surface contamination. After a triple-rinse with ultrapure water, the otoliths were left to dry in a class 100 laminar flow hood. Once dry, sets of 10 otoliths were re-mounted on new slides using double-sided tape. Elemental analysis was performed using a Thermo Finnigan Element2 single collector inductively coupled plasma mass spectrometer (ICP-MS) coupled to a New Wave Research 213 nm laser ablation system. The laser software was used to trace two 600 µm lines: (1) along increments deposited during the late larval stage and (2) along the otolith edge incorporating the most recently deposited material laid down corresponding to juvenile estuary residency (Lagardère & Troadac 1997). 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 ml min^{-1} nebulizer. The analyte was then transported to the ICP-MS via an Ar carrier gas.

Elemental ratios of Mg:Ca, Mn:Ca, Cu:Ca, Sr:Ca, Ba:Ca and Pb:Ca were quantified by measuring ^{25}Mg, ^{48}Ca, ^{55}Mn, ^{63}Cu, ^{88}Sr, ^{138}Ba and ^{208}Pb in the ablated otolith material. At the beginning and end of each set of 10 otoliths instrumental blanks (2% HNO_3) and standards were analyzed. All raw measured values were blank-corrected by calculating a blank value for each sample by linear interpolation between measured blanks. A dissolved otolith certified reference material (FEBS 1 - Sturgeon et al. 2005), diluted to a Ca concentration of 40 mg g^{-1}, was used to correct for instrument mass bias. Elemental mass bias was calculated from the known values of FEBS-1 and then interpolated and applied to each of the blank-corrected elemental ratios. No samples and elements were below limits of detection, calculated using the 3 sigma criterion (3 x SD of blank samples) and expressed as percent of mean sample intensity: ^{25}Mg=3.0%, ^{48}Ca=0.007%, ^{55}Mn=4.8%, ^{63}Cu=47.1%, ^{88}Sr=0.03%, ^{138}Ba=2.56% and ^{208}Pb=40.2%. Measurement precision was assessed by running another otolith certified reference material (Yoshinaga et al. 2000) every 10 samples as an unknown. External precision (relative standard deviations) for this reference material (n=34) were as follows: Mg:Ca=6.3%, Mn:Ca=28.0%, Cu:Ca=1.7%, Sr:Ca=0.6%, Ba:Ca=3.0% and Pb:Ca=3.9%. While our estimate of precision for Mn:Ca values was relatively high, Mn intensities in the reference material were considerably lower than in either the FEBS-1 standard or the analyzed common sole otoliths. Therefore external precision in otolith samples was assumed to be sufficient and Mn:Ca ratios were kept in all subsequent analyses.

Isotope ratio monitoring mass spectrometry

Left (blind-side of fish) otolith of each individual was analyzed for δ^{13}C and δ^{18}O using isotope ratio monitoring mass spectrometry (irm-MS). Whole otoliths were individually crushed and the resulting powder was transferred to acid washed vials. Samples were then analyzed on a Thermo Finnigan MAT253 equipped with a Kiel III carbonate device following methods outlined by Ostermann and Curry (2000). Isotopic values were reported relative to Vienna Pee Dee belemnite (VPDB) and expressed in standard δ notation. Long-term precision estimates of the mass spectrometer based on analyses of NBS19 are ±0.03 for δ^{13}C and ±0.07 for δ^{18}O.

Data analysis

Raw data for each element:Ca and stable isotope ratio were checked for normality and homogeneity of variances and all variables were normally distributed after $\log_{10}$ transformation. Analysis of variance (ANOVA) was used to examine variation between otolith zones and among estuaries and sites nested within estuaries, whenever more than one site per estuary was sampled. Posthoc multiple comparisons were conducted with Tukey test when significant differences were observed among otolith zones, estuary and site combinations. Multi-element fingerprints were analyzed using multivariate analysis of variance (MANOVA), using the same designs as in the univariate ANOVA.

Linear discriminant function analysis (LDFA) was used to classify juvenile soles to their collection site or estuary using log-transformed Ca ratios of elements (Mg, Mn, Cu, Sr, Ba and Pb) from otolith edges (juvenile zone) and δ^{13}C and δ^{18}O values from whole otoliths. Different LDFA were run to test elemental and isotopic ratios combined and separately. Classification accuracy of the discriminant functions was evaluated by calculating cross-validated classification success using a jackknife (leave-one-out) approach. Log_{10} transformed data met all assumptions of LDFA, i.e. normality and homogeneity of variance-covariance matrices.

Finally, the potential influence of temperature and salinity on otolith geochemistry of juvenile *S. solea* was examined. Correlation between both temperature and salinity measured at each of the collection sites (in May and July 2006) and each of the six elemental ratios measured in the juvenile zone of the otoliths along with $\delta^{13}C$ and $\delta^{18}O$ measured in otoliths were tested using Pearson product moment correlations. Further, correlations between elemental and isotope ratios were also examined.

RESULTS

Significant differences in geochemical signatures were found between larval and juvenile zones of common sole otoliths for all estuaries and sites combined (Wilks' Lambda test, $F_{6,171}$=112.2, P<0.001). Results of ANOVA comparisons showed that element:Ca ratios, with the exception of Pb:Ca, varied significantly between the two zones for all locations combined (Table 2).

Table 2. Results of ANOVA comparisons of element:Ca ratios from otolith zones corresponding to late larval and juvenile stages of juveniles of *Solea solea* collected in estuarine sites along the Portuguese coast. Non-significant p-values are indicated by "ns".

Source	Otolith zone	
df	1, 176	
	F	*P*
Mg:Ca	418.40	<0.001
Mn:Ca	85.70	<0.001
Cu:Ca	42.90	<0.001
Sr:Ca	141.10	<0.001
Ba:Ca	6.50	<0.05
Pb:Ca	1.00	ns

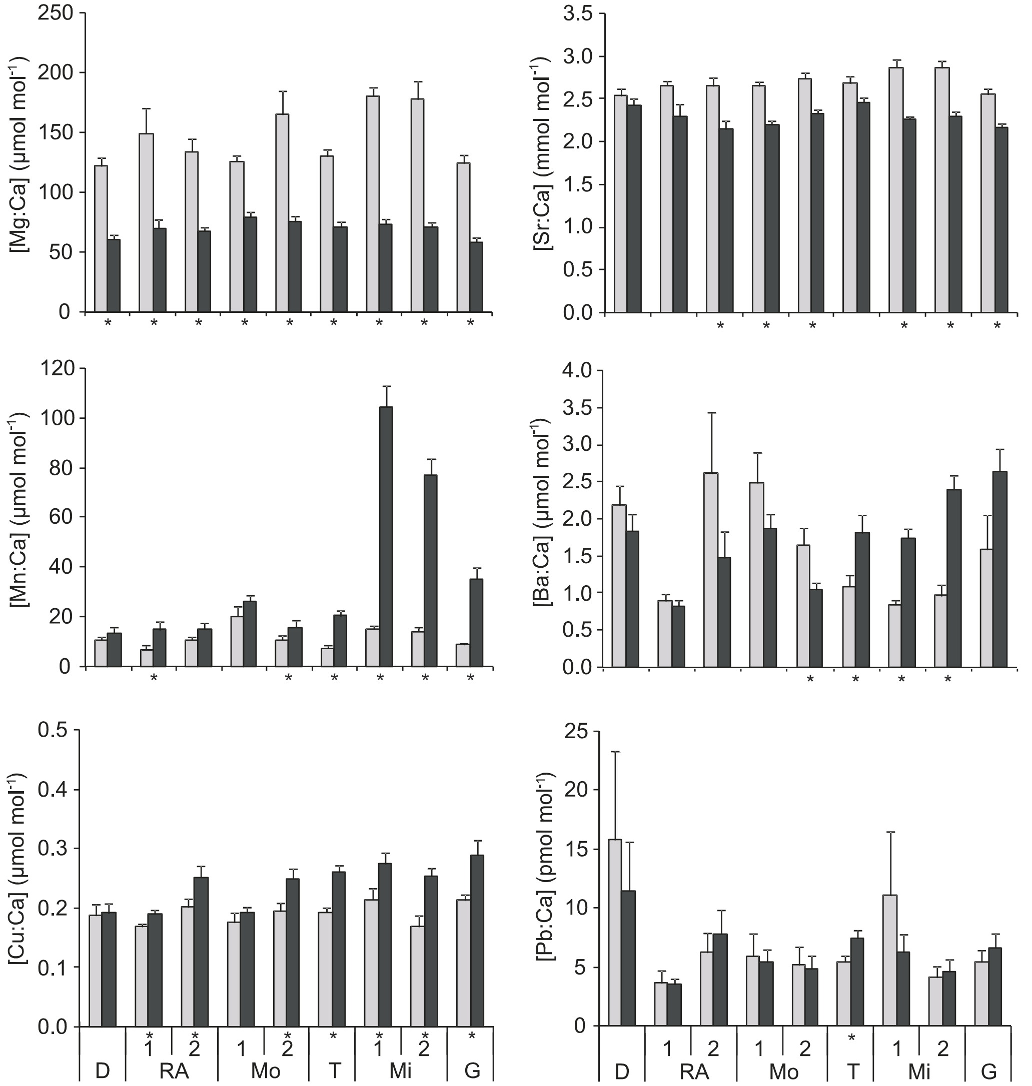

Fig. 2. Mean (and standard error) bars of element:Ca ratios (Mg, Mn, Cu, Sr, Ba, Pb) in the late larval (grey bars) and juvenile (dark bars) zone of otoliths of juvenile *Solea solea* collected in nine estuarine sites along the Portuguese coast. Asterisks indicate significant differences between otolith zones in a collection site. See text for site abbreviations.

Differences were particularly consistent for Mg:Ca and Sr:Ca, with higher values of both ratios associated with the late larval life stage in comparison to the juvenile estuarine residency (Fig. 2). Mg:Ca ratios showed significant differences between larval and juvenile zones in all sites along the Portuguese coast while differences in Sr:Ca ratios between otolith zones were observed at six of the sites. The opposite pattern was found for Mn:Ca and Cu:Ca, with higher values for both ratios measured during juvenile residency in estuaries. Otolith Mn:Ca ratios were significantly higher in the juvenile zone in Ria de Aveiro 1, Mondego 2 and in the three more southern estuaries. Cu:Ca ratios were also higher in the juvenile zone in Mondego 2 and all sites within Ria de Aveiro, Tejo, Mira and Guadiana estuaries (Fig. 2). Finally, Ba:Ca ratios were also in general higher in the juvenile zones of fish collected in the southern Portuguese estuaries, but higher in larval zones from fish collected in the northern estuaries.

Elemental signatures in larval (Wilks' Lambda test, $F_{30,302}=8.10$, $P<0.001$) and juvenile (Wilks' Lambda test, $F_{30,302}=3.60$, $P<0.001$) zones of sole otoliths varied significantly among estuaries. Individually, only Cu:Ca in the larval zone and Pb:Ca in both zones did not differ significantly among estuaries (Table 3).

Table 3. Results of ANOVA comparisons of element:Ca (Mg, Mn, Cu, Sr, Ba, Pb) in otoliths' late larval zone (a), juvenile zone (b) and isotope ratios (δ^{13}C, δ^{18}O) in whole otoliths (c) of juveniles of *Solea solea* collected in six estuaries (multiple sites within an estuary were pooled) along the Portuguese coast. For estuaries with more than one site, sites were nested within estuaries. Non-significant p-values are indicated by "ns".

a) Late larval zone

Source	Estuary		Site(Estuary)	
df	5, 80		3, 80	
	F	*P*	*F*	*P*
Mg:Ca	6.00	<0.001	2.10	ns
Mn:Ca	5.65	<0.001	3.86	<0.05
Cu:Ca	0.80	ns	3.20	<0.05
Sr:Ca	3.40	<0.01	0.30	ns
Ba:Ca	7.17	<0.001	3.64	<0.05
Pb:Ca	1.54	ns	1.08	ns

b) Juvenile zone

Estuary		Site(Estuary)	
5, 80		3, 80	
F	*P*	*F*	*P*
3.40	<0.01	0.10	ns
50.15	<0.001	5.11	<0.01
5.50	<0.001	4.80	<0.01
3.70	<0.01	1.70	ns
7.75	<0.001	6.18	<0.001
2.04	ns	0.99	ns

c) Whole otoliths

Source	Estuary		Site(Estuary)	
df	5, 80		3, 80	
	F	*P*	*F*	*P*
δ^{13}C	41.12	<0.001	113.63	<0.001
δ^{18}O	30.89	<0.001	59.14	<0.001

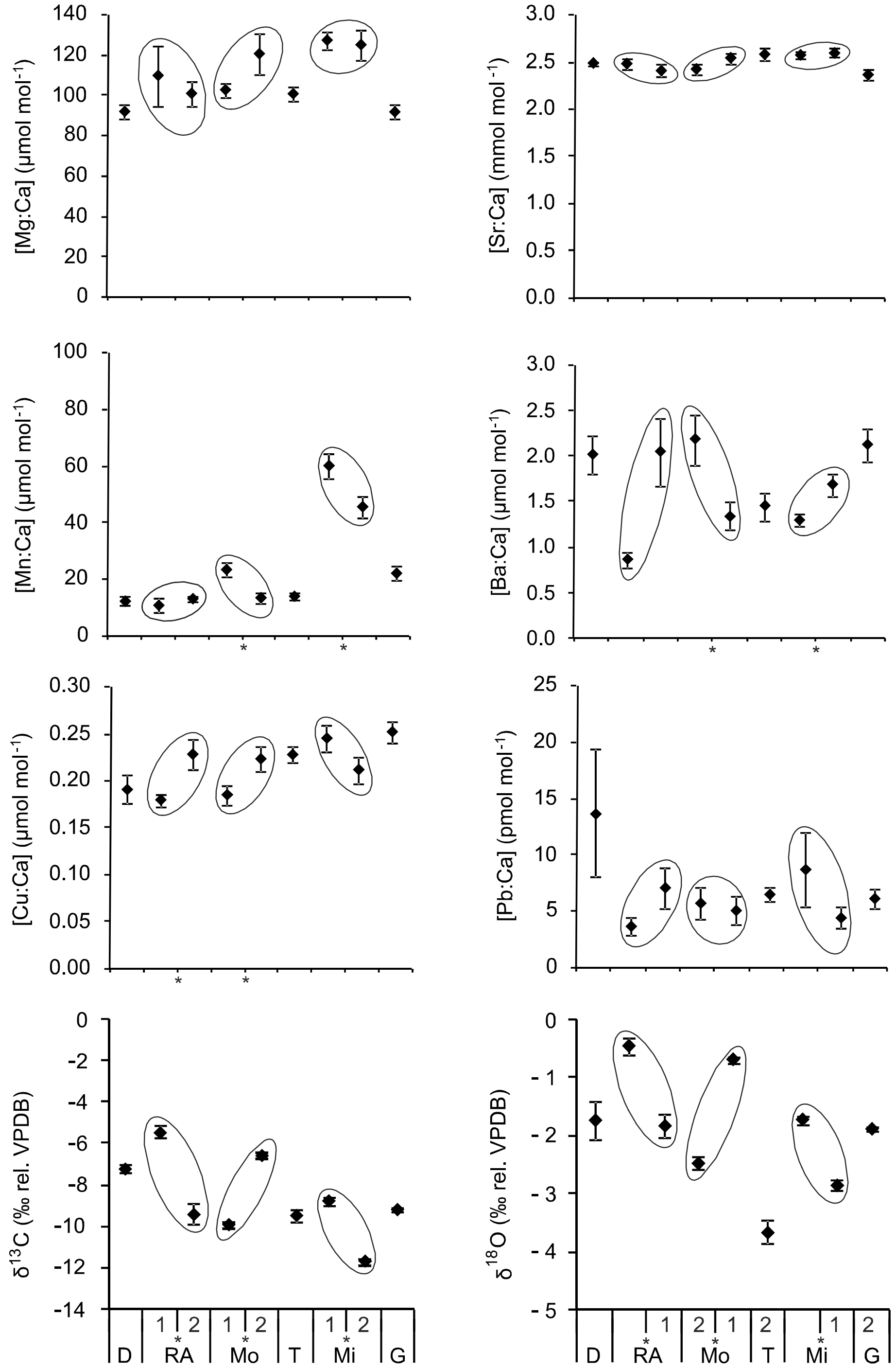

Fig. 3. Mean values (and standard error) of element:Ca ratios (Mg, Mn, Cu, Sr, Ba, Pb) of the otolith juvenile zone and isotope ratios (δ^{13}C and δ^{18}O) of juvenile *Solea solea* collected in nine estuarine sites along the Portuguese coast. Sites within an estuary are marked within ovals and asterisks indicate significant differences between sites within an estuary. See text for site abbreviations.

All remaining element:Ca ratios varied among estuaries and Mn:Ca, Cu:Ca and Ba:Ca differed significantly between sites within estuaries (Table 3, Fig. 3). In the LDFA performed to classify individuals to collection estuary based on elemental signatures of the juvenile zone of the otoliths, the first two canonical variates of the LDFA explained 90% of total variance, with discrimination among estuaries based primarily on Mn:Ca and Pb:Ca. However, classification to collection estuary was only moderately successful with an average cross-validated accuracy of 57% (Table 4).

Table 4. Summary of cross-validated classification results (% of correctly classified cases) of discriminant function analysis of juvenile *Solea solea*. Classification to an estuary (sites within the same estuary were pooled) (a) and to a collection site (b) was based on multi-element fingerprints (Mg:Ca, Mn:Ca, Cu:Ca, Sr:Ca, Ba:Ca, and Pb:Ca) in juvenile zones of otoliths, isotope ratios (δ^{13}C, δ^{18}O) of otoliths and the combination thereof. See text for site abbreviations.

a)

Predicted estuary	Elements (juvenile zone)	Isotopes	Combined
D	50.0	50.0	83.3
RA	46.2	15.4	38.5
Mo	32.0	8.0	44.0
T	41.7	83.3	83.3
Mi	95.5	54.5	95.5
G	72.7	90.9	81.8
Total	**57.3**	**43.8**	**68.5**

b)

Predicted site	Elements (juvenile zone)	Isotopes	Combined
D	33.3	83.3	83.3
RA 1	25.0	50.0	50.0
RA 2	22.2	22.2	44.5
Mo 1	46.2	76.9	76.9
Mo 2	41.7	83.3	83.3
T	41.7	83.3	83.3
Mi 1	70.0	50.0	100.0
Mi 2	41.7	91.7	100.0
G	63.6	54.5	81.8
Total	**44.9**	**68.5**	**80.7**

Some of this error may have been generated by the above mentioned differences between sites within estuaries. Therefore LDFA was repeated with all sites considered separately. In this instance classification accuracy to site of collection was, however, even lower (45%) than to collection estuary.

δ^{13}C in whole otoliths ranged from -12‰ to -4‰ and δ^{18}O values ranged from -4‰ to 0‰ (Fig. 3). There were obvious geographic differences in otolith isotope signatures among estuaries but variability between sites within estuaries was as large as among-estuary variations (Table 3, Fig. 3).

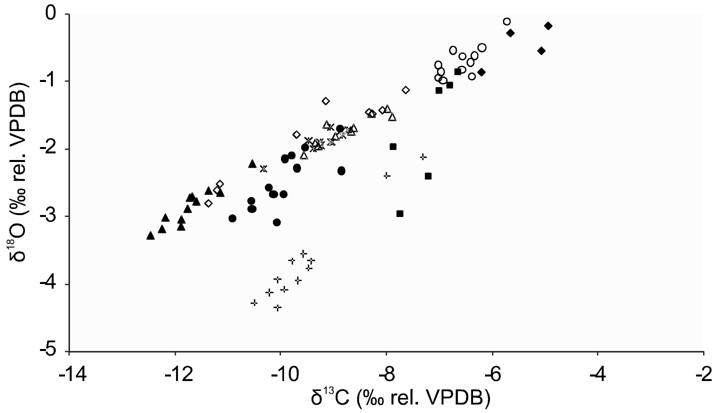

Fig. 4. Dual isotope plot with δ^{18}O plotted against δ^{13}C values from otoliths of *juvenile Solea solea* collected in nine estuarine sites along the Portuguese coast: D (■), RA 1 (♦), RA 2 (◊), Mo 1 (●), Mo 2 (○), T (+), Mi 1 (▲), Mi 2 (Δ) and G (*). See text for site abbreviations.

The two isotope ratios were also significantly correlated (r= 0.79, $P<0.001$, Fig. 4). The success of LDFA classifications to collection estuary improved considerably when δ^{13}C and δ^{18}O values were added to elemental ratios in the analysis, with an average accuracy of 69% among estuaries (Table 4). High classification accuracy was achieved for the Douro (83.3%), Tejo (83.3%) and Mira estuaries (95.5%) whilst classification accuracies were low for the Ria de Aveiro and Mondego estuaries (38.5% and 44.0%, respectively). The first two canonical discriminant functions of the LDFA explained 90% of total variance and discrimination was mainly based on Mn:Ca and δ^{18}O. When considering individual sites separately in the LDFA, individuals were correctly classified to their collection site with an average accuracy of 81%. In this instance the first two canonical discriminant functions of LDFA accounted for 81% of total variance and discrimination was mainly based on δ^{13}C, δ^{18}O and Mn:Ca.

Temperature did not correlate with any of the geochemical variables analyzed. However, Ba:Ca (May 2006: r=-0.33, $P<0.01$; July 2006: r =-0.43, $P<0.001$), δ^{13}C (May 2006: r=0.44, $P<0.001$; July 2006: r=0.61, $P<0.001$), and δ^{18}O (May 2006: r= 0.69, $P<0.001$; July 2006: r=0.74, $P<0.001$) were significantly correlated with salinity (Fig. 5).

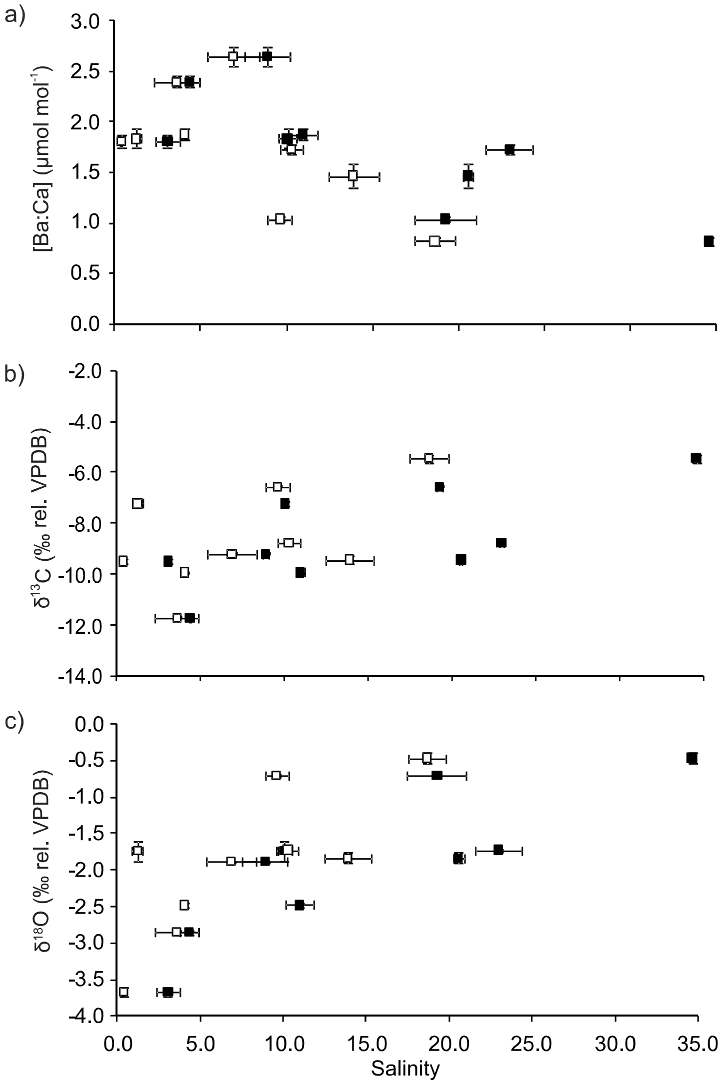

Fig. 5. Variation of mean values (and standard error) of (a) Ba:Ca ratios, (b) $\delta^{13}C$ and (c) $\delta^{18}O$ isotope ratios of otoliths of juvenile *Solea solea* and salinity in the nine sites sampled along the Portuguese coast in May 2006 (light squares) and July 2006 (dark squares).

DISCUSSION

Otolith chemistry of juvenile common soles varied significantly over each of the spatial (among estuaries and sites within them) and temporal (between life history stages) scales investigated. However, results show that statistically significant variability among locations does not necessarily imply that the differences are sufficient for the accurate classification of individuals to a particular location. Acceptable discrimination accuracy (>70%) was only achieved by adding $\delta^{13}C$ and $\delta^{18}O$ to the multivariate classification analysis. Several studies have also documented improved discrimination among fish groups by using otolith $\delta^{13}C$ and $\delta^{18}O$ in estuarine systems (Thorrold et al. 1998, Dorval et al. 2005). Nonetheless, our results confirm that estuarine systems are particularly good systems for using geochemical signatures in otoliths as natural tags of habitat residency (Elsdon et al. 2008).

Several of the analyzed elemental ratios showed consistent patterns between the otolith zones in the nine estuarine collection sites along the Portuguese coast. These differences either reflect differences in water temperature or chemistry between coastal habitats adjacent to estuaries inhabited by larvae and estuarine habitats used by juveniles, or alternatively are related to physiological effects occurring around the time of larval metamorphosis. The most noticeable differences between the analyzed otolith zones were observed for Mg:Ca and Sr:Ca ratios, with higher values of both ratios found in the larval zones of otoliths. Both elemental ratios are typically higher in seawater than in freshwater (e.g. Martin & Thorrold 2005, Kerr et al. 2007) providing a ready explanation for the observed patterns. However, we found no significant correlation between salinity at collection site and either Mg:Ca or Sr:Ca in the juvenile zone of *S. solea* otoliths. No clear and consistent relationship has been established between environmental factors and otolith concentrations of Mg (e.g. Elsdon & Gillanders 2002, Dorval et al. 2007) and at least one study has found a (negative) correlation between fish growth rate and Mg:Ca ratios in otoliths (Martin & Thorrold 2005). Physiological factors, and not ambient water chemistry, are therefore more likely to explain the documented patterns in otolith Mg:Ca.

A number of studies have found a significant positive relationship between Sr:Ca and salinity (reviewed by Secor & Rooker 2000). The lack of correlation between Sr:Ca ratios in the juvenile zones of *S. solea* otoliths and ambient salinity was somewhat surprising given that otolith Sr:Ca was higher in larval compared with juvenile zones. Changes in Sr:Ca ratios in the ambient water explain some, but not all, of the variability in otolith Sr:Ca in laboratory studies (Bath et al. 2000, Kraus & Secor 2004). Temperature has been shown to be positively correlated with Sr:Ca ratios in otoliths (e.g. Bath et al. 2000, Martin et al. 2004) but estuarine waters along the Portuguese coast are typically warmer than adjacent coastal water masses. It is possible that the observed variations in temperature were too small to reveal any relationship. It is also likely that physiological processes associated with metamorphosis influenced the ontogenetic pattern observed and indeed de Pontual et al. (2003) suggested that trends in Sr:Ca ratios in the otoliths of *S. solea* were due to physiological effects.

Higher Mn:Ca and Cu:Ca in the juvenile zone of *S. solea* otoliths laid down during estuarine residency, presumably reflecting differences in bioavailability between coastal and estuarine waters (Milton & Chenery 2001, Forrester & Swearer 2002). However, because neither Mn:Ca or Cu:Ca were correlated with salinity these ions likely came from either sediment pore waters or point-source pollution with elevated concentrations of these metals. On the other hand, otolith Ba:Ca did not differ consistently between larval and juvenile growth zones but was significantly negatively correlated with salinity in the estuaries. Previous studies have shown that otolith Ba:Ca is highly correlated with dissolved Ba:Ca in the ambient water and that the main source of Ba in otoliths is water rather than food (Walther & Thorrold 2006). This in turn implies that Ba:Ca ratios are indeed higher in freshwater endmembers of the Portuguese estuaries sampled and that this pattern was recorded in the *S. solea* otoliths.

The addition of $\delta^{13}C$ and $\delta^{18}O$ values clearly improved the performance of the discriminant function analysis to correctly classify individuals to their collection estuary. Both isotope ratios were significantly correlated with salinity and with each other. The dual isotope plot revealed a similar pattern to that found by Thorrold et al. (1998) with otoliths from each of the estuaries plotting on mixing curves between different freshwater endmembers and relatively homogeneous coastal ocean values. Oxygen isotopes in otoliths are laid down close to isotopic equilibrium and therefore reflect ambient values in the water with a small temperature effect (Thorrold et al. 1997, Høie et al. 2004) making otolith $\delta^{18}O$ a particularly useful tracer in euryhaline environments. While $\delta^{13}C$ in otoliths is rarely in isotopic equilibrium with ambient dissolved organic carbon ($\delta^{13}C_{DIC}$), otolith $\delta^{13}C$ values often vary systematically with salinity (Kerr et al. 2007). Presumably the relative contribution of metabolic carbon remains relatively constant and therefore otolith $\delta^{13}C$ values track the consistent differences in $\delta^{13}C_{DIC}$ with salinity. Different isotopic compositions of freshwater and saltwater endmembers and the relative stability in the mixing ratios of two different water masses within an estuary highlight the efficacy of stable isotopes as tracers of salinity (Kerr et al. 2007).

Two estuarine systems, Ria de Aveiro and Mondego showed rather poor classification accuracies to estuary of collection. Vasconcelos et al. (2007) obtained similar results in a study conducted in Portuguese estuaries in 2005 using otolith elemental signatures to discriminate nursery areas of five species including *S. solea*.

These authors reported that overall classification accuracy of *S. solea* was 70.2% and the lower percentages of individuals of this species correctly classified to their nursery estuary (only one collection site) were observed in Mondego (12.5%) and in Ria de Aveiro (66.7%). Gillanders and Kingsford (2003) obtained good discrimination results for three sparid species when pooling sites within estuaries and grouping nearby estuaries. However, our results clearly indicate that pooling sites in Ria de Aveiro and Mondego does not produce good classification accuracies and may lead to misclassification of individuals and to inadequate data interpretations. On the other hand, individuals from the Mira estuary with multiple collection sites achieved very good discrimination results whether pooled to classify to collection estuary or separated to classify to collection site.

Successful discrimination of nursery sites and estuaries using geochemical signatures is the first assumption that must be met before these signatures can be used as natural habitat tags in connectivity and migration studies (Campana 1999). Several studies have documented significant temporal variability in geochemical signatures at a single site (Gillanders 2002, Hamer et al. 2003). *S. solea* colonize Portuguese estuaries generally in several cohorts thus, to ensure that the otolith signatures of this species in the different sites are well characterized, future studies should include sampling of all cohorts of a year-class accounting for temporal variations over months and should be carried out over several years to analyze inter-annual temporal variations. Knowledge of the temporal variations in otolith chemical fingerprints at different scales will be required to determine their use and applicability in studies of connectivity between juvenile and adult life stages. Moreover, the large variations observed among otolith zones in this species should always be considered in the design of studies that use geochemical signatures as natural tags. Overall, the spatial variations observed among estuaries and sites within estuaries indicate that otolith elemental and isotopic fingerprints are accurate natural habitat tags and their use in future studies of metapopulation connectivity or migration patterns of this species is promising.

ACKNOWLEDGEMENTS

We thank all involved in fish sampling. This study was co-funded by the European Union through the FEDER e Portuguese Fisheries Programme (MARE), as well as by the ‘Fundação Luso-Americana’ (FLAD). S. E. Tanner was funded with a PhD grant by

‘Fundação para a Ciência e a Tecnologia’ (FCT).

LITERATURE CITED

Bath GE, Thorrold SR, Jones CM, Campana SE, McLaren JW, Lam JWH (2000) Strontium and barium uptake in aragonitic otoliths of marine fish. Geochim Cosmochim Acta 64:1705-1714

Brown R, Severin KP (1999) Elemental distribution within polymorphic inconnu (*Stenodus leucichthys*) otoliths is affected by crystal structure. Can J Fish Aquat Sci 56:1898-1903

Cabral H (2003) Differences in growth rates of juvenile *Solea solea* and *Solea senegalensis* in the Tagus estuary, Portugal. J Mar Biol Assoc UK 83:861-868

Cabral HN, Vasconcelos RP, Vinagre C, França S, Fonseca V, Maia A, Reis-Santos P, Lopes M, Ruano M, Campos J, Freitas V, Costa MJ (2007) Relative importance of estuarine flatfish nurseries along the Portuguese coast. J Sea Res 57:209-217

Campana SE (1999) Chemistry and composition of fish otoliths: pathways, mechanisms and applications. Mar Ecol Prog Ser 188:263-297

Chesney EJ, McKee BM, Blanchard T, Chan L-H (1998) Chemistry of otoliths from juvenile menhaden *Brevoortia patronus*: evaluating strontium, strontium:calcium and strontium isotope ratios as environmental indicators. Mar Ecol Prog Ser 171:261-273

de Pontual H, Lagardère F, Amara R, Bohn M, Ogor A (2003) Influence of ontogenetic and environmental changes in the otolith microchemistry of juvenile sole (*Solea solea*). J Sea Res 50:199–210

Dorval E, Jones CM, Hannigan R, van Montfrans J (2005) Can otolith chemistry be used for identifying essential seagrass habitats for juvenile spotted seatrout, *Cynoscion nebulosus*, in Chesapeake Bay? Mar Freshw Res 56:645-653

Dorval E, Jones CM, Hannigan R, van Montfrans J (2007) Relating otolith chemistry to surface water chemistry in a coastal plain estuary. Can J Fish Aquat Sci 64:411-424

Elsdon TS, Gillanders BM (2002) Interactive effects of temperature and salinity on otolith chemistry: challenges for determining environmental histories of fish. Can J Fish Aquat Sci 59:1796-1808

Elsdon TS, Gillanders BM (2005) Consistency of patterns between laboratory experiments and field collected fish in otolith chemistry: an example and applications for salinity reconstructions. Mar Freshw Res 56:609-617

Elsdon TS, Wells BK, Campana SE, Gillanders BM, Jones CM, Limburg KE, Secor DH, Thorrold SR, Walther BD (2008) Otolith chemistry to describe movements and life-history parameters of fishes: hypotheses, assumptions, limitations and inferences. Oceanogr Mar Biol Annu Rev 46:297-330

Forrester GE, Swearer SE (2002) Trace elements in otoliths indicate the use of open-coast versus bay nursery habitats by juvenile California halibut. Mar Ecol Prog Ser 241:201-213

Fowler AJ, Campana SE, Jones CM, Thorrold SR (1995) Experimental assessment of the effect of temperature and salinity on elemental composition of otoliths using laser ablation ICPMS. Can J Fish Aquat Sci 52:1431-1441

Gillanders BM, Kingsford MJ (2000) Elemental fingerprints of otoliths of fish may distinguish estuarine 'nursery' habitats. Mar Ecol Prog Ser 201:273-286

Gillanders BM (2002) Connectivity between juvenile and adult fish populations: do adults remain near their recruitment estuaries? Mar Ecol Prog Ser 240:215-223

Gillanders BM, Kingsford MJ (2003) Spatial variation in elemental composition of otoliths of three species of fish (family Sparidae). Estuar Coast Shelf Sci 57:1049–1064

Hamer PA, Jenkins GP, Gillanders BM (2003) Otolith chemistry of juvenile snapper *Pagrus auratus* in Victorian waters: natural chemical tags and their temporal variation. Mar Ecol Prog Ser 263:261-273

Hamer PA, Jenkins GP, Coutin P (2006) Barium variation in *Pagrus auratus* (Sparidae) otoliths: A potential indicator of migration between an embayment and ocean waters in south-eastern Australia. Estuar Coast Shelf Sci 68:686-702

Høie H, Otterlei E, Folkvord A (2004) Temperature-dependent fractionation of stable oxygen isotopes in otoliths of juvenile cod (*Gadus morhua* L.). ICES J Mar Sci 61:243-251

Kalish JM (1989) Otolith microchemistry: validation of the effects of physiology, age and environment on otolith composition. J Exp Mar Biol Ecol 132:151-178

Kerr LA, Secor DH, Kraus RT (2007) Stable isotope ($\delta^{13}C$ and $\delta^{18}O$) and Sr/Ca composition of otoliths as proxies for environmental salinity experienced by an estuarine fish. Mar Ecol Prog Ser 349:245-253

Koutsikopoulos C, Desaunay Y, Dorel D, Marchand J (1989) The role of coastal areas in the life history of sole (*Solea solea* L.) in the Bay of Biscay. Sci Mar 53:567-575

Kraus RT, Secor DH (2004) Incorporation of strontium into otoliths of an estuarine fish. J Exp Mar Biol Ecol 302:85– 106

Lagardère F, Troadac H (1997) Age estimation in common sole *Solea solea* larvae: validation of daily increments and evaluation of a pattern recognition technique. Mar Ecol Prog Ser 155:223-237

Marchand J (1991) The influence of environmental conditions on settlement, distribution and growth of 0-group sole (*Solea solea* (L.)) in a macrotidal estuary (Vilaine, France). Neth J Sea Res 27:307-316

Martin GB, Thorrold SR, Jones CM (2004) Temperature and salinity effects on strontium incorporation in otoliths of larval spot (*Leiostomus xanthurus*). Can J Fish Aquat Sci 61:34-42

Martin GB, Thorrold SR (2005) Temperature and salinity effects on magnesium, manganese, and barium incorporation in otoliths of larval and early juvenile spot *Leiostomus xanthurus*. Mar Ecol Prog Ser 293:223-232

Milton DA, Chenery SR (2001) Sources and uptake of trace metals in otoliths of juvenile barramundi (*Lates calcarifer*). J Exp Mar Biol Ecol 264:47-65

Ostermann DR, Curry WB (2000) Calibration of stable isotopic data: an enriched $\delta^{18}O$ standard used for source gas mixing detection and correction. Paleoceanography 15:353-360

Patterson HM, McBride RS, Julien N (2004) Population structure of red drum (*Sciaenops ocellatus*) as determined by otolith chemistry. Mar Biol 144:855–862

Payan P, Edeyer A, De Pontual H, Borelli G, Boeuf G, Mayer-Gostan N (1999) Chemical composition of saccular endolymph and otolith in fish inner ear: lack of spatial uniformity. Am J Physiol Regul Integr Comp Physiol 46:R123-R131

Quéro JC, Dessouter M, Lagardère F (1986) Soleidae. In: Whitehead P, Bauchot M-L, Hureau J-C, Nielsen J, Tortonese E (eds) Fishes in the North-Eastern Atlantic and the Mediterranean. UNESCO, Paris, pp. 1308-1342

Ramos J (1982) Contribuición al estudio de la sexualidad del lenguado, *Solea solea* (Linneo, 1758) (Pisces, Soleidae). Investigacion Pesquera 46:275-286

Rooker JR, Secor DH, De Metrio G, Schloesser R, Block BA, Neilson JD (2008) Natal homing and connectivity in Atlantic bluefin tuna populations. Science 322:742-744

Secor DH, Rooker JR (2000) Is otolith strontium a useful scalar of life cycles in estuarine fishes? Fish Res 46:359-371

Sturgeon RE, Willie SN, Yang L, Greenberg R, Spatz RO, Chen Z, Scriver C, Clancy V, Lam JW, Thorrold SR (2005) Certification of a fish otolith reference material in support of quality assurance for trace element analysis. J Anal At Spectrom 20:1067-1071

Thorrold SR, Campana SE, Jones CM, Swart PK (1997) Factors determining $\delta^{13}C$ and $\delta^{18}O$ fractionation in aragonitic otoliths of marine fish. Geochim Cosmochim Acta 61:2909-2919

Thorrold SR, Jones CM, Swart PK, Targett TE (1998) Accurate classification of juvenile weakfish *Cynoscion regalis* to estuarine nursery areas based on chemical signatures in otoliths. Mar Ecol Prog Ser 173:253-265

Thorrold SR, Latkoczy C, Swart PK, Jones CM (2001) Natal homing in marine fish metapopulation. Science 291:297-299

Thresher RE, Procter CH (2007) Population structure and life history of orange roughy (*Hoplostethus atlanticus*) in the SW Pacific: inferences from otolith chemistry. Mar Biol 152:461-473

Tsukamoto K, Arai T (2001) Facultative catadromy of the eel *Anguilla japonica* between freshwater and seawater habitats. Mar Ecol Prog Ser 220:265-276

Vasconcelos RP, Reis-Santos P, Tanner S, Fonseca V, Latkoczy C, Günther D, Costa MJ, Cabral H (2007) Discriminating estuarine nurseries for five fish species through otolith elemental fingerprints. Mar Ecol Prog Ser 350:117-126

Vasconcelos RP, Reis-Santos P, Tanner S, Maia A, Latkoczy C, Günther D, Costa MJ, Cabral H (2008) Evidence of estuarine nursery origin of five coastal fish species along the Portuguese coast through otolith elemental fingerprints. Estuar Coast Shelf Sci 79:317-327

Vasconcelos RP, Reis-Santos P, Maia A, Fonseca V, França S, Wouters N, Costa MJ, Cabral HN (2010) Nursery use patterns of commercially important marine fish species in estuarine systems along the Portuguese coast. Estuar Coast Shelf Sci 86:613-624

Walther BD, Thorrold SR (2006) Water, not food, contributes the majority of strontium and barium deposited in the otoliths of a marine fish. Mar Ecol Prog Ser 311:125-130

Yoshinaga J, Nakama A, Morita M, Edmonds JS (2000) Fish otolith reference material for quality assurance of chemical analyses. Mar Chem 69:91-97

CHAPTER IV

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

Susanne E. Tanner[1], Patrick Reis-Santos1, Rita P. Vasconcelos[1], Susana França[1], Simon R. Thorrold[2] & Henrique N. Cabral[1]

[1]Centro de Oceanografia, Faculdade de Ciências da Universidade de Lisboa, Campo Grande, 1749-016 Lisboa, Portugal
[2]Biology Department MS 50, Woods Hole Oceanographic Institution, Woods Hole, MA 02543, USA

Marine Ecology Progress Series (2012) 452:193-203

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

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. 2007a, 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. 2007a, 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. (2007b). 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).

Table 1. Sampled estuaries, mean and standard error (SE) of fish total 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	*n*	Lt	*n*	Lt	*n*	Lt	*n*	Lt	*n*
S. solea										
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
S. senegalensis										
Ria Aveiro (RA)	-	-	73.1 (3.6)	20	-	-	57.8 (7.3)	13	60.2 (7.9)	5
Tejo (T)	109.3 (5.8)	6	-	-	49.2 (1.0)	26	74.2 (3.4)	21	56.4 (5.6)	18
Sado (S)	50.1 (1.6)	15	86.5 (3.4)	26	47.2 (2.1)	34	74.6 (2.3)	39	57.9 (4.0)	26
Mira (Mi)	-	-	-	-	-	-	69.3 (15.3)	6	101 (6.1)	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.

	2006				2009					
	Temperature (°C)		Salinity		Temperature (°C)			Salinity		
Estuary	May	July	May	July	May	July	October	May	July	October
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)

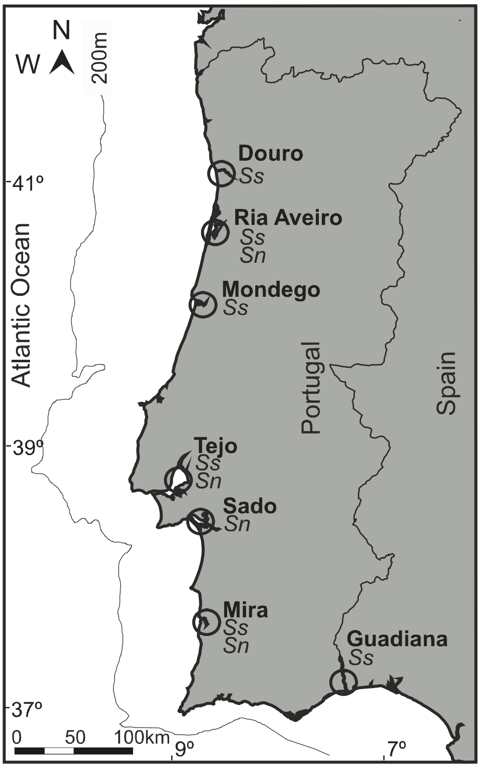

Fig. 1. Estuaries sampled along the Portuguese coast in 2006 and 2009. Species occurrences are also indicated, *Solea solea* (*Ss*) and *Solea senegalensis* (*Sn*).

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 µl min^{-1} nebulizer. The analyte was then transported to the ICP-MS via an Ar carrier gas.

Elemental ratios were quantified by measuring ^{7}Li, ^{25}Mg, ^{48}Ca, ^{55}Mn, ^{63}Cu, ^{88}Sr, ^{138}Ba and ^{208}Pb in the ablated otolith material. ^{7}Li 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 µg 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 µg 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.

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

	S. solea			*S. senegalensis*		
	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

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.

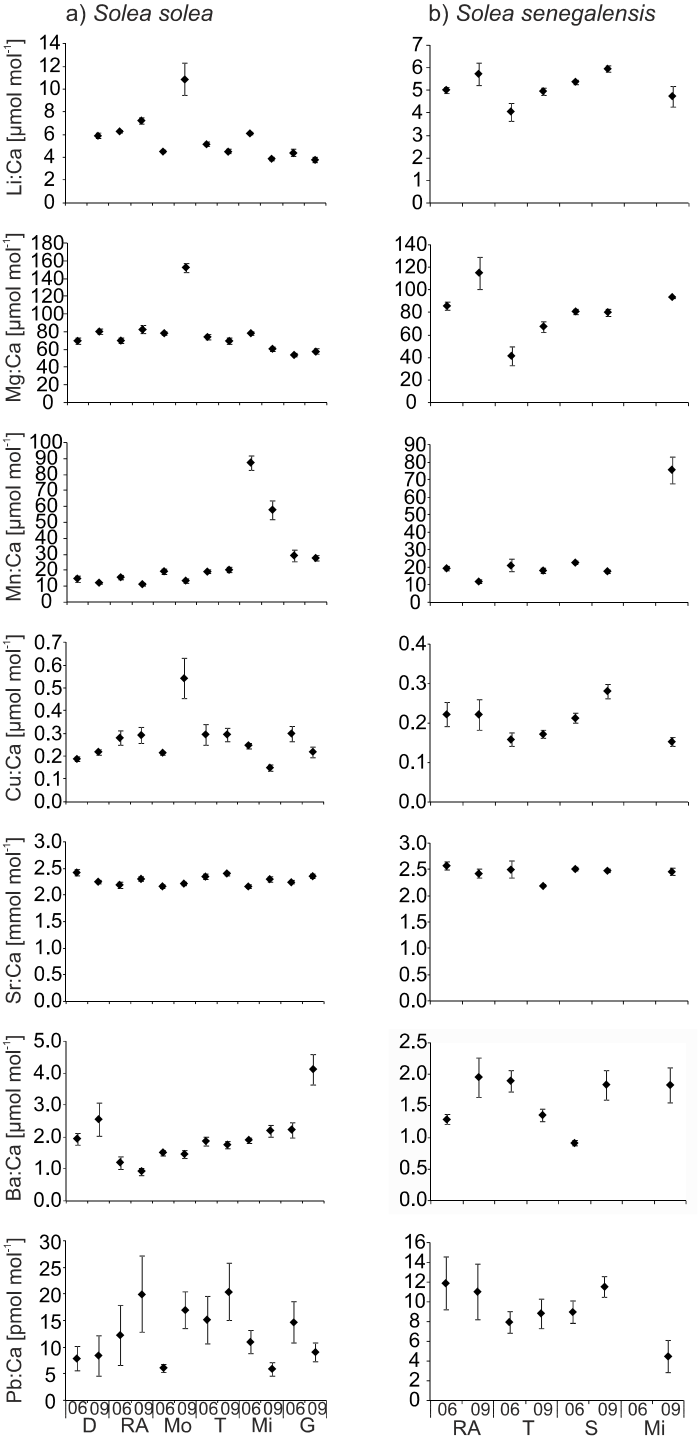

Fig. 2. Mean and standard error of element:Ca ratios (Li, Mg, Mn, Cu, Sr, Ba, Pb) in otoliths of juvenile *Solea solea* (a) and *Solea senegalensis* (b) in the estuarine systems sampled in 2006 and 2009. Data from May and July were pooled in each year. See Table 1 for estuary abbreviations.

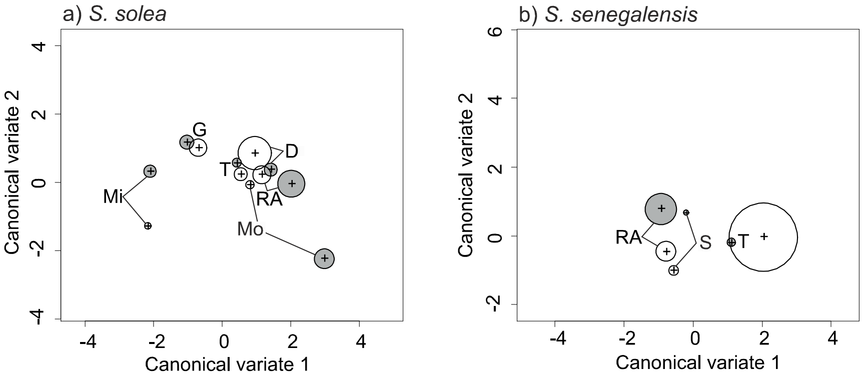

Fig. 3. Canonical discriminant analysis plot summarizing variation in otolith elemental signatures in juvenile *Solea solea* (a) and *Solea senegalensis* (b) in the estuarine systems along the Portuguese coast in 2006 and 2009. Circle enclosures represent 95% confidence limits for the mean. White and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

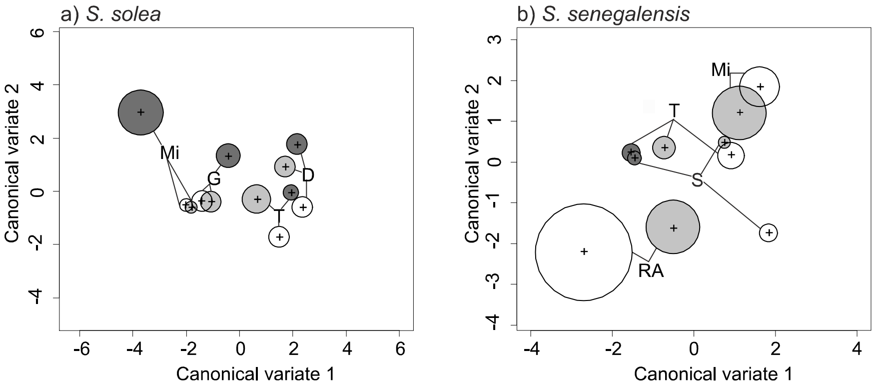

Fig. 4. Canonical discriminant analysis plot summarizing variation in otolith elemental signatures in juvenile *Solea solea* (a) and *Solea senegalensis* (b) in the estuarine systems along the Portuguese coast in May, July and October 2009. Circle enclosures represent 95% confidence limits for the mean.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

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).

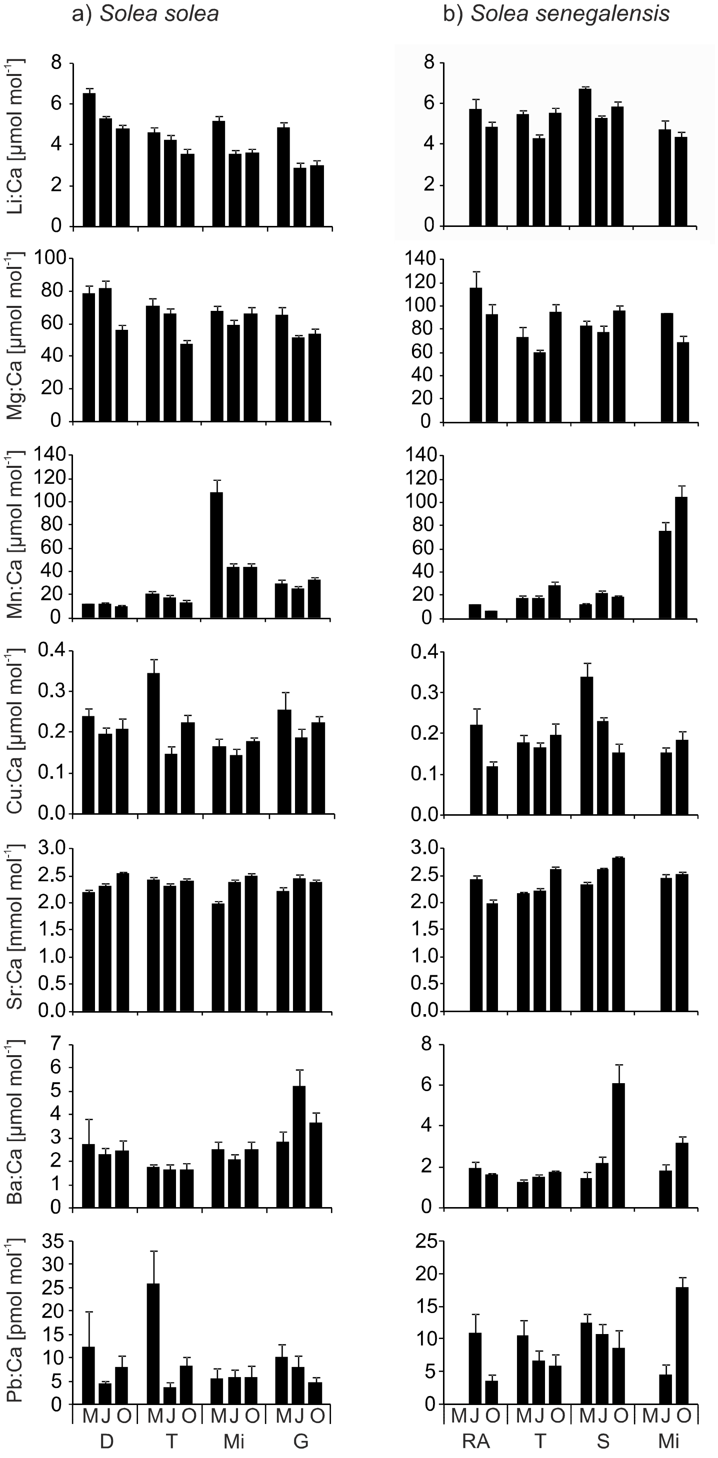

Fig. 5. Mean and standard error of element:Ca ratios (Li, Mg, Mn, Cu, Sr, Ba, Pb) in otoliths of juvenile *S. solea* (a) and *S. senegalensis* (b) in the estuarine systems sampled in May (M), July (J) and October (O) 2009. See Table 1 for estuary abbreviations.

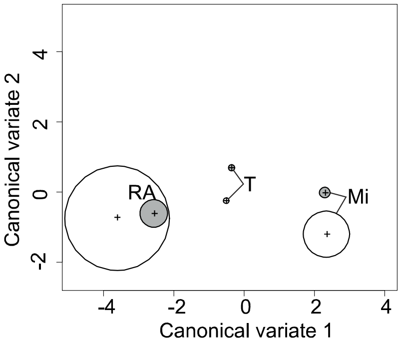

Fig. 6. Canonical discriminant analysis plot summarizing variation in otolith elemental signatures in the estuarine systems along the Portuguese coast where juvenile *Solea solea* and *Solea senegalensis* co-occur in 2009. Circle enclosures represent 95% confidence limits for the mean. Grey and white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

Finally, we examined the effectiveness of using elemental values in otoliths to accurately identify juvenile habitat residency using LDFA. 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.

Table 4. Cross-validated classification accuracy of discriminant function analysis of juvenile *Solea solea* (a) and *Solea senegalensis* (b) in 2006 and 2009. Individuals were classified to an estuary based on otolith elemental signatures. Juveniles of *S. solea* sampled in 2006 in northern (Douro, Ria de Aveiro, Mondego) and central estuaries (Tejo) were pooled in Group North. Months were pooled within each year. Values in bold represent individuals correctly classified to the estuary of origin.

Predicted estuary (% of individuals)

a) *S.solea*

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: 71.0%

Predicted estuary (% of individuals)

b) *S. senegalensis*

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%

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. 2007a), 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. (2007a, 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.

ACKNOWLEDGEMENTS

We would like to thank all involved in the fish sampling and S. Birdwhistell for help in sample analysis. This study was funded by the 'Fundação para a Ciência e a Tecnologia' (FCT), PEst-OE/MAR/UI0199/2011. S. E. Tanner was funded with a PhD grant from FCT (SFRH/BD/40313/2007).

LITERATURE CITED

Bath GE, Thorrold SR, Jones CM, Campana SE, McLaren JW, Lam JWH (2000) Strontium and barium uptake in aragonitic otoliths of marine fish. Geochim Cosmochim Acta 64:1705-1714

Brown JA (2006) Using the chemical composition of otoliths to evaluate the nursery role of estuaries for English sole *Pleuronectes vetulus* populations. Mar Ecol Prog Ser 306:269–281

Cabral H (2003) Differences in growth rates of juvenile *Solea solea* and *Solea senegalensis* in the Tagus estuary, Portugal. J Mar Biol Assoc UK 83:861-868

Cabral HN, Vasconcelos RP, Vinagre C, França S, Fonseca V, Maia A, Reis-Santos P, Lopes M, Ruano M, Campos J, Freitas V, Costa MJ (2007) Relative importance of estuarine flatfish nurseries along the Portuguese coast. J Sea Res 57:209-217

Campana SE (1999) Chemistry and composition of fish otoliths: pathways, mechanisms and applications. Mar Ecol Prog Ser 188:263-297

Clarke LM, Walther BD, Munch SB, Thorrold SR, Conover DO (2009) Chemical signatures in the otoliths of a coastal marine fish, *Menidia menidia*, from the northeastern United States: spatial and temporal differences. Mar Ecol Prog Ser 384:261-271

Cook GS (2011) Changes in otolith microchemistry over a protracted spawning season influence assignment of natal origin. Mar Ecol Prog Ser 423:197-209

Cowen RK, Gawarkiewicz G, Pineda J, Thorrold SR, Werner FE (2007) Population connectivity in marine systems: an overview. Oceanography 20:14-21

Cuveliers EL, Geffen AJ, Guelinckx J, Raeymaekers JAM, Skadal J, Volckaert FAM, Maes GE (2010) Microchemical variation in juvenile *Solea solea* otoliths as a powerful tool for studying connectivity in the North Sea. Mar Ecol Prog Ser 401:211-220

Elsdon TS, Gillanders BM (2003) Relationship between water and otolith elemental concentrations in juvenile black bream *Acanthopagrus butcheri*. Mar Ecol Prog Ser 260:263-272

Elsdon TS, Gillanders BM (2004) Fish otolith chemistry influenced by exposure to multiple environmental variables. J Exp Mar Biol Ecol 313:269–284

Elsdon TS, Wells BK, Campana SE, Gillanders BM, Jones CM, Limburg KE, Secor DH, Thorrold SR, Walther BD (2008) Otolith chemistry to describe movements and life-history parameters of fishes: hypotheses, assumptions, limitations and inferences. Oceanogr Mar Biol Annu Rev 46:297-330

Fairclough DV, Edmonds JS, Lenanton RCJ, Jackson G, Keay IS, Crisafulli BM, Newman SJ (2011) Rapid and cost-effective assessment of connectivity among assemblages of *Choerodon rubescens* (Labridae), using laser ablation ICP-MS of sagittal otoliths. J Exp Mar Biol Ecol 403:46-53

Gillanders BM (2002) Temporal and spatial variability in elemental composition of otoliths: implications for determining stock identity and connectivity of populations. Can J Fish Aquat Sci 59:669-679

Gillanders BM, Able KW, Brown JA, Eggleston DB, Sheridan PF (2003) Evidence of connectivity between juvenile and adult habitats for mobile marine fauna: an important component of nurseries. Mar Ecol Prog Ser 247:281-295

Hamer PA, Jenkins GP, Gillanders BM (2003) Otolith chemistry of juvenile snapper *Pagrus auratus* in Victorian waters: natural chemical tags and their temporal variation. Mar Ecol Prog Ser 263:261-273

Hamer PA, Jenkins GP, Coutin P (2006) Barium variation in *Pagrus auratus* (Sparidae) otoliths: A potential indicator of migration between an embayment and ocean waters in south-eastern Australia. Estuar Coast Shelf Sci 68:686-702

Instituto de Meteorologia (2007) Instituto de Meteorologia, Caracterização climática ano 2006, Ministério da Ciência Tecnologia e Ensino Superior, Lisboa, Portugal. Technical Report. pp. 40

Instituto de Meteorologia (2010) Instituto de Meteorologia, Boletim climatológico anual - ano 2009, Ministério da Ciência Tecnologia e Ensino Superior, Lisboa, Portugal. Technical Report. pp 12

Kerr LA, Secor DH, Kraus RT (2007) Stable isotope ($\delta^{13}C$ and $\delta^{18}O$) and Sr/Ca composition of otoliths as proxies for environmental salinity experienced by an estuarine fish. Mar Ecol Prog Ser 349:245-253

Koutsikopoulos C, Desaunay Y, Dorel D, Marchand J (1989) The role of coastal areas in the life history of sole (*Solea solea* L.) in the Bay of Biscay. Sci Mar 53:567-575

Leakey CDB, Attrill MJ, Fitzsimons MF (2009) Multi-element otolith chemistry of juvenile sole (*Solea solea*), whiting (*Merlangius merlangus*) and European seabass (*Dicentrarchus labrax*) in the Thames Estuary and adjacent coastal regions. J Sea Res 61:268-274

Marchand J (1991) The influence of environmental conditions on settlement, distribution and growth of 0-group sole (*Solea solea* (L.)) in a macrotidal estuary (Vilaine, France). Neth J Sea Res 27:307-316

Martin GB, Thorrold SR, Jones CM (2004) Temperature and salinity effects on strontium incorporation in otoliths of larval spot (*Leiostomus xanthurus*). Can J Fish Aquat Sci 61:34-42

Patterson HM, Kingsford MJ (2005) Elemental signatures of *Acanthochromis polyacanthus* otoliths from the Great Barrier Reef have significant temporal, spatial, and between-brood variation. Coral Reefs 24:360-369

Quéro JC, Dessouter M, Lagardère F (1986) Soleidae. In: Whitehead P, Bauchot M-L, Hureau J-C, Nielsen J, Tortonese E (eds) Fishes in the North-Eastern Atlantic and the Mediterranean. UNESCO, Paris, pp. 1308-1342

Ramos J (1982) Contribuición al estudio de la sexualidad del lenguado, *Solea solea* (Linneo, 1758) (Pisces, Soleidae). Investigacion Pesquera 46:275-286

Reis-Santos P, Vasconcelos RP, Ruano M, Latkoczy C, Günther D, Costa MJ, Cabral HN (2008) Interspecific variations of otolith chemistry in estuarine fish nurseries. J Fish Biol 72:2435-2717

Rooker JR, Secor DH, Zdanowicz VS, Itoh T (2001) Discrimination of northern bluefin tuna from nursery areas in the Pacific Ocean using otolith chemistry. Mar Ecol Prog Ser 218:275-282

Sturgeon RE, Willie SN, Yang L, Greenberg R, Spatz RO, Chen Z, Scriver C, Clancy V, Lam JW, Thorrold SR (2005) Certification of a fish otolith reference material in support of quality assurance for trace element analysis. J Anal At Spectrom 20:1067-1071

Swearer SE, Forrester GE, Steele MA, Brooks AJ, Lea DW (2003) Spatio-temporal and interspecific variation in otolith trace-elemental fingerprints in a temperate estuarine fish assemblage. Estuar Coast Shelf Sci 56:1111-1123

Tanner SE, Vasconcelos RP, Reis-Santos P, Cabral HN, Thorrold SR (2011) Spatial and ontogenetic variability in the chemical composition of juvenile common sole (*Solea solea*) otoliths. Estuar Coast Shelf Sci 91:150-157

Teixeira CM, Cabral HN (2009) Time series analysis of flatfish landings in the Portuguese coast. Fish Res 96:252-258

Thorrold SR, Jones CM, Campana SE, McLaren JW, Lam JWH (1998) Trace element signatures in otoliths record natal river of juvenile American shad (*Alosa sapidissima*). Limnol Oceanogr 43:1826-1835

Thorrold SR, Latkoczy C, Swart PK, Jones CM (2001) Natal homing in marine fish metapopulation. Science 291:297-299

Thorrold SR, Jones GP, Hellberg ME, Burton RS, Swearer SE, Neigel JE, Morgan SG, Warner RR (2002) Quantifying larval retention and connectivity in marine populations with artificial and natural markers. Bull Mar Sci 70:291-308

Thorrold SR, Zacherl DC, Levin LA (2007) Population connectivity and larval dispersal using geochemical signatures in calcified structures. Oceanography 20:80-89

Thresher RE, Procter CH (2007) Population structure and life history of orange roughy (*Hoplostethus atlanticus*) in the SW Pacific: inferences from otolith chemistry. Mar Biol 152:461-473

Vasconcelos RP, Reis-Santos P, Tanner S, Fonseca V, Latkoczy C, Günther D, Costa MJ, Cabral H (2007a) Discriminating estuarine nurseries for five fish species through otolith elemental fingerprints. Mar Ecol Prog Ser 350:117-126

Vasconcelos RP, Reis-Santos P, Fonseca V, Maia A, Ruano M, França S, Vinagre C, Costa MJ, Cabral H (2007b) Assessing anthropogenic pressures on estuarine fish nurseries along the Portuguese coast: A multi-metric index and conceptual approach. Sci Total Environ 374:199-215

Vasconcelos RP, Reis-Santos P, Tanner S, Maia A, Latkoczy C, Günther D, Costa MJ, Cabral H (2008) Evidence of estuarine nursery origin of five coastal fish species along the Portuguese coast through otolith elemental fingerprints. Estuar Coast Shelf Sci 79:317-327

Vasconcelos RP, Reis-Santos P, Maia A, Fonseca V, França S, Wouters N, Costa MJ, Cabral HN (2010) Nursery use patterns of commercially important marine fish species in estuarine systems along the Portuguese coast. Estuar Coast Shelf Sci 86:613-624

Walther BD, Thorrold SR, Olney JE (2008) Geochemical signatures in otoliths record natal origins of American shad. Trans Am Fish Soc 137:57-69

Yoshinaga J, Nakama A, Morita M, Edmonds JS (2000) Fish otolith reference material for quality assurance of chemical analyses. Mar Chem 69:91-97

CHAPTER V

Testing an otolith geochemistry approach to determine population structure and movements of European hake in the northeast Atlantic Ocean and Mediterranean Sea

Susanne E. Tanner[1], Rita P. Vasconcelos[1], Henrique N. Cabral[1] & Simon R. Thorrold[2]

[1]Centro de Oceanografia, Faculdade de Ciências da Universidade de Lisboa, Campo Grande, 1749-016 Lisboa, Portugal
[2]Biology Department MS 50, Woods Hole Oceanographic Institution, Woods Hole, MA 02543, USA

Fisheries Research (2012) 125-126: 198-205

Testing an otolith geochemistry approach to determine population structure and movements of European hake in the northeast Atlantic Ocean and Mediterranean Sea

---

ABSTRACT Population structure of European hake (*Merluccius merluccius*) remains poorly understood despite the need for such information for effective fisheries management of the species. Otolith geochemistry was used to examine movements of individuals among local populations and test the accuracy of current stock boundaries for the species. Age-1 European hake were collected at 7 locations in the Atlantic Ocean and the Mediterranean Sea. Otolith geochemistry (Mg:Ca, Mn:Ca, Sr:Ca, Ba:Ca, $\delta^{13}C$, and $\delta^{18}O$) was assayed in cores and edge zones. Both otolith core and edge values varied significantly among the collection locations in the Atlantic Ocean and the Mediterranean Sea. The significant differences in the geochemical signatures between the two otolith zones (Sr:Ca, Ba:Ca, $\delta^{13}C$, $\delta^{18}O$) were possibly associated with ontogenetic changes in physiology and feeding habits of hake. Otolith edge signatures proved to be a valuable tool for classifying individuals to the collection locations. Individuals were assigned to three different groups in the Atlantic Ocean and in the Mediterranean Sea based on otolith core values using nonmetric multi-dimensional scaling. Our results suggested movement of individuals among local populations within the Atlantic Ocean and also within the Mediterranean Sea. However, there appeared to be little or no movement of hake through the Strait of Gibraltar. The movement patterns observed do not support the stock boundary established in the northeast Atlantic Ocean but rather advocate for a re-evaluation of the most adequate and effective management units for this valuable demersal species.

Keywords: *Merluccius merluccius*, otolith, geochemistry, stock structure, movement

---

INTRODUCTION

European hake, *Merluccius merluccius* (Linnaeus, 1758), is a demersal and benthopelagic species found mainly between 70 and 370 m depth from Norway to the Gulf of Guinea in the northeast Atlantic Ocean and throughout the Mediterranean and Black Seas, with highest abundances reached from the British Isles to southern Spain (Murua 2010). Adult hake spawn offshore around the continental shelf break and juveniles are usually found closer to the coast (Kacher & Amara 2005). The species supports important commercial fisheries throughout its distribution range and is especially important for Spanish and French fishing fleets due to its high market value (Otxotorena et al. 2010).

The Mediterranean and Atlantic populations of European hake are usually considered as different stocks due to geographic variability in morphology and population genetics (Abaunza et al. 2001, Lo Brutto et al. 2004, Mellon-Duval et al. 2010). However, there is evidence of some gene flow between the Mediterranean and the Atlantic in the vicinity of the Strait of Gibraltar (Roldán et al. 1998). In the northeast Atlantic, the International Council for Exploration of the Sea (ICES) considers two stocks for European hake (northern and southern stocks) divided at the Capbreton canyon (southern Bay of Biscay, SE France). The boundaries of the two stocks were defined based on management considerations without biological basis (ICES 2011). Based on recent studies of population genetics, there appears to be some movement between the two stocks (e.g. Pita et al. 2011). However, there is at present a lack of data to quantify the extent and rate of exchange between the putative stocks. Knowledge of stock structure is valuable to an understanding of population dynamics and sustainable management in marine-capture fisheries. Ignoring stock structure may lead to dramatic changes in the biological characteristics and productivity rates of a species, as well as in its genetic diversity (Begg et al. 1999). Mixing and exchange rates among geographically separated populations determine stock structure and these movements can in turn occur at different life history stages through dispersal of eggs and larvae or through movement of juveniles or adults (Fowler et al. 2005).

Over the last few decades, a wide variety of techniques have been applied to identify stock structure in marine fish populations, including distribution and abundance data, morphometrics and meristics, life history parameters, population genetics, and artificial and natural tags (Pawson & Jennings 1996). Among the natural tags, otolith chemistry has been used successfully to address issues related to stock identity and fish movements (e.g. Campana et al. 2000, Ashford et al. 2008, Steer et al. 2010). Fish otoliths are metabolically inert aragonite structures with a composition that is influenced by the physical and chemical properties of the environment (Elsdon et al. 2008). Furthermore, otoliths grow continuously and record information on life histories in a chronological manner, making it possible to retrieve information on environmental conditions experienced by individual fish from hatching to capture (Campana 1999).

Otolith chemical composition has already been used to address stock separation in European hake. Based on the analysis of whole otoliths, Swan et al. (2006) classified individuals to their capture locations in the northeast Atlantic and the Mediterranean Sea with an accuracy of 79.0% and 65.5%, respectively. The whole otolith assays necessarily integrated environmental variations experienced by individual European hake over their entire lifetimes. This approach is problematic when trying to identify population structure as it may fail to account for situations where individuals are separated for much of their life history but come together to spawn or move to geographically separated locations at some point in their life history (Ashford et al. 2006).

The present study sought to further resolve movement patterns and stock structure of European hake based on otolith geochemistry by targeting specific zones at the core and edge of otoliths. The otolith edge provides information on the environment experienced by the individuals prior to capture and on the suitability and discriminatory power of this natural tag. The otolith core, on the other hand, represents larval and early pelagic juvenile life stages and may contain information on spatial separation during these life stages. Multivariate comparisons of geochemical variables from core and edge zones in otoliths were used to evaluate movement and mixture of European hake at these two different life stages.

MATERIAL AND METHODS

Fish sampling

Specimens of European hake, *Merluccius merluccius* (Linnaeus, 1758) were obtained from research surveys at seven different locations in the northeast Atlantic Ocean and the Mediterranean Sea (Fig. 1). Total length of the individuals was determined (Table 1) and the *sagittal* otoliths were extracted. Otoliths were rinsed with water, cleaned from adhering tissue, dried and stored in plastic microcentrifuge tubes or paper envelopes. All individuals used in this study belonged to age-class 1 (Piñeiro & Saínza 2003, Kacher & Amara 2005).

Geochemical analysis

For trace element analysis, 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 (18.2 MΩ cm^{-1}), sonicating for 2 min and triple-rinsing with ultrapure water in a class-100 clean room. Elemental analysis was performed using a Thermo Finnigan Element2 single collector inductively coupled plasma mass spectrometer (ICP-MS) coupled to a New Wave Research 193 nm excimer laser ablation system.

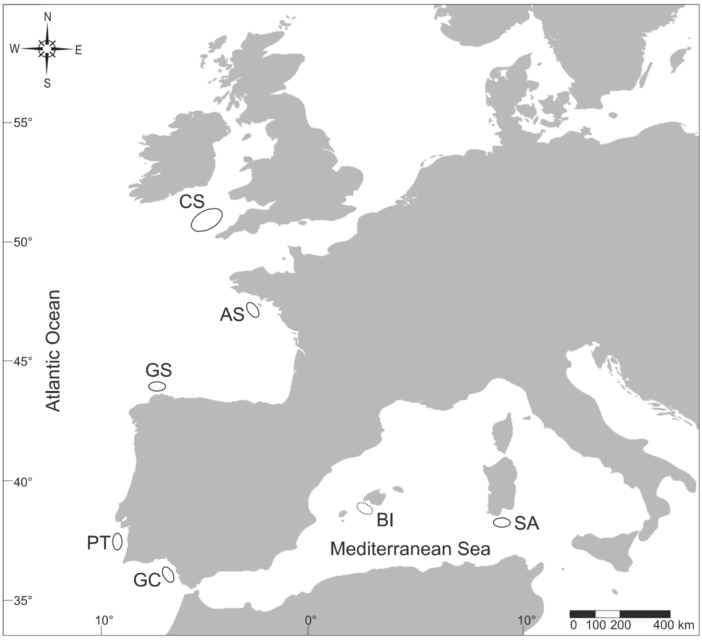

Fig. 1. Collection locations of *Merluccius merluccius* in the Atlantic Ocean and Mediterranean Sea. CS–Celtic Sea, AS–Armorican Shelf, GS–Galician Shelf, PT–Portugal, GC–Gulf of Cádiz, BI–Balearic Islands, SA–Sardinia.

The laser software was used to trace a raster 80 x 150 µm in the core of the otolith and a 390 µm line along the dorsal edge of the otolith. The laser ablated otolith material using a 5 Hz pulse rate and moved at 5 µm s^{-1}. The ablated material was transported via a He gas stream to the dual-inlet quartz spray chamber where it was mixed with a 2% nitric acid (HNO_3) aerosol from a self-aspirating PFA 20 ml min^{-1} nebulizer. The analyte was then transported to the ICP-MS via an Ar carrier gas. Elemental ratios of Mg:Ca, Mn:Ca, Sr:Ca and Ba:Ca were quantified by measuring ^{25}Mg, ^{48}Ca, ^{55}Mn, ^{88}Sr and ^{138}Ba in the ablated otolith material. Instrumental blanks (2% HNO_3) and certified reference materials (CRM - Yoshinaga et al. 2000, Sturgeon et al. 2005) were analyzed every four otoliths and used to correct for background intensities and instrument mass drift (for details see Tanner et al. 2011). External precision (relative standard deviations) for the CRM (n = 42) was as follows: Mg:Ca = 1.2%, Mn:Ca = 18.6%, Sr:Ca = 0.9% and Ba:Ca = 1.5%.

For the stable isotope analysis, a computer-controlled micromill was used to remove otolith material from the core and edge of each left otolith. In order to achieve optimal minimum sample size requirements of the ratio mass spectrometer used, about 0.025 mm^3 material was extracted in the core and along the dorsal edge of each otolith (Hidalgo et al. 2008). The mean mass of the removed otolith powder (n=140) was

47.5 µg (SD=8 µg) using 250 µm milling depth. Samples were then analyzed on a Thermo Finnigan MAT253 equipped with a Kiel III carbonate device following methods outlined by Ostermann and Curry (2000). Isotopic values were reported relative to Vienna Pee Dee belemnite (VPDB) and expressed in standard δ notation. Long-term precision estimates of the mass spectrometer based on analyses of NBS19 are ±0.03‰ for $\delta^{13}C$ and ±0.07‰ for $\delta^{18}O$.

Table 1. Collection location, date of collection, mean and standard deviation (SD) of fish total length (Lt) in cm and sample size (*n*) of *Merluccius merluccius*.

Collection location	Abbreviation	Collection date	Lt (cm) Mean	SD	*n*
Celtic Sea	CS	November 2010	22.1	1.4	10
Armorican Shelf	AS	May 2010	22.7	1.0	10
Galician Shelf	GS	October 2010	22.5	1.5	10
Portugal	PT	June 2010	20.9	0.8	10
Gulf of Cádiz	GC	November 2010	22.4	0.7	10
Balearic Islands	BI	May 2010	21.3	1.3	10
Sardinia	SA	October 2010	23.6	1.3	10

The otolith core area sampled for both trace element and stable isotope analysis was defined as the region between the primordium and the accessory growth centers that correspond to the larval and early juvenile pelagic pre-settlement period (Morales-Nin & Moranta 2004, Hidalgo et al. 2008). The otolith material sampled along the edge corresponds to the carbonate laid down immediately prior to capture.

Statistical analysis

Each element:Ca and stable isotope ratio was $\log_{10}$ transformed in order to achieve normality and homogeneity of variances. A repeated measure univariate and multivariate analysis of variance (ANOVA and MANOVA, respectively) was used to determine variation between the otolith core and edge zones of European hake collected in the northeast Atlantic Ocean and the Mediterranean Sea. One-way ANOVA and MANOVA were used to assess variation among collection locations using core and edge element:Ca and stable isotope ratios separately.

Canonical discriminant analysis (CDA) was used to display the geochemical data of the otolith core and edge material in reduced space and to obtain the relative importance of each variable to the separation patterns observed. Several CDAs were performed using: the core and edge otolith signatures simultaneously; and only the edge signatures. In both cases, CDAs were based on the Atlantic and the Mediterranean datasets separately. Individuals from the Gulf of Cádiz were included in both the Atlantic and the Mediterranean datasets in order to evaluate the potential migration of European hake through the Strait of Gibraltar.

Linear discriminant function analysis (LDFA) was used to classify European hake to their collection locations using geochemical values at otolith edges. As for the CDA, separate LDFAs were run to evaluate classification success for individuals collected in the Atlantic Ocean and in the Mediterranean Sea. Classification accuracy of the discriminant functions was evaluated by calculating cross-validated classification success using a jackknife (leave-one-out) approach. The assumptions of LDFA, i.e. normality and homogeneity of variance-covariance matrices, were met after $\log_{10}$ transformation of the variables.

European hake collected in a location did not necessarily originate in early life from the same location and therefore LDFA was not appropriate to investigate the larval and early pelagic juvenile life stages of individuals based on otolith core signatures. The data was examined graphically using nonmetric multidimensional scaling (nMDS) in order to visually identify discrete groups in the Atlantic Ocean and the Mediterranean Sea. Euclidean distances were used to construct a dissimilarity matrix. Ward's independent, agglomerative hierarchical clustering procedure was also performed to corroborate the grouping obtained in the nMDS (Ashford et al. 2006). The appropriate number of groups in the Atlantic and Mediterranean populations using the clustering procedure was assessed visually and using distances between clusters.

RESULTS

Significant differences in the otolith geochemical signatures were found among the collection locations for the otolith core (Wilks' Lambda test, $F_{36,258} = 8.26$, $p<0.001$) and edge zone (Wilks' Lambda test, $F_{36,258} = 11.65$, $p<0.001$). Results of ANOVA comparisons showed that all the element:Ca and stable isotope ratios differed significantly among the collection locations, with the exception of Mg:Ca and Mn:Ca in the otolith core zone (Table 2). Significant differences in the geochemical signatures were also observed between the two otolith zones for all the collection locations combined (Wilks' Lambda test, $F_{1,69} = 15.39$, $p<0.001$) All the element:Ca and stable isotope ratios, with exception of Mn:Ca showed significant differences between the otolith zones. Variation between the otolith zones was mainly based on the differences in Sr:Ca, Ba:Ca and $\delta^{13}C$ (Fig. 2).

Table 2. Results of ANOVA examining variation of geochemical signatures in the otolith core and edge of *Merluccius merluccius* among the collection locations in the Atlantic Ocean and the Mediterranean Sea. Non-significant p-values are indicated by "ns".

	otolith core		otolith edge	
df	6, 63		6, 63	
	F	p	F	p
Mg:Ca	1.49	ns	14.42	<0.001
Mn:Ca	1.96	ns	17.06	<0.001
Sr:Ca	7.48	<0.001	7.86	<0.001
Ba:Ca	6.01	<0.001	6.74	<0.001
$\delta^{13}C$	8.91	<0.001	7.83	<0.001
$\delta^{18}O$	18.58	<0.001	14.38	<0.001

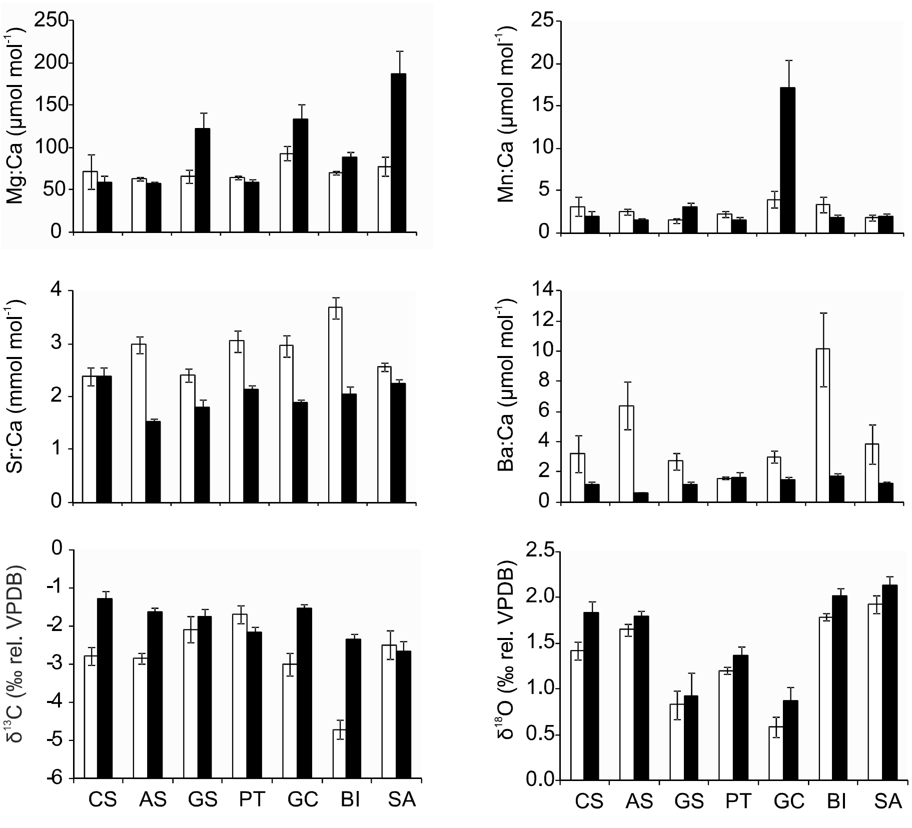

Fig. 2. Mean (and standard error) bars of element:Ca and stable isotope ratios (Mg, Mn, Sr, Ba, $\delta^{13}C$, $\delta^{18}O$) in the core (white bars) and edge (dark bars) zone of otoliths of *Merluccius merluccius* collected at the locations in the Atlantic Ocean and the Mediterranean Sea. See Figure 1 for collection location abbreviations.

In almost all the collection locations of this study, Sr:Ca and Ba:Ca were higher in the otolith core than at the edge. Conversely, δ^{18}O values were higher at the otolith edge than in the core. Generally, δ^{13}C showed lower values in the otolith core than at the edge. Finally, no common patterns were observed between the otolith zones in the different collection locations for Mg:Ca and Mn:Ca (Fig. 2).

Clear differences between the two otolith zones were also observed in the CDA plots (Fig. 3). Core and edge otolith zones were clearly separated in multivariate space along the first canonical variate (CV 1) for Atlantic Ocean locations (Fig. 3a). Differences along CV 1 were generated primarily by Sr:Ca and Ba:Ca ratios while Mn:Ca and δ^{18}O contributed most to the second canonical variate (CV 2). The separation of the core and edge geochemical signatures in the Mediterranean Sea dataset was mainly based on the differences in δ^{18}O and Mn:Ca on CV 1 and by Sr:Ca and δ^{13}C on CV 2 (Fig. 3b).

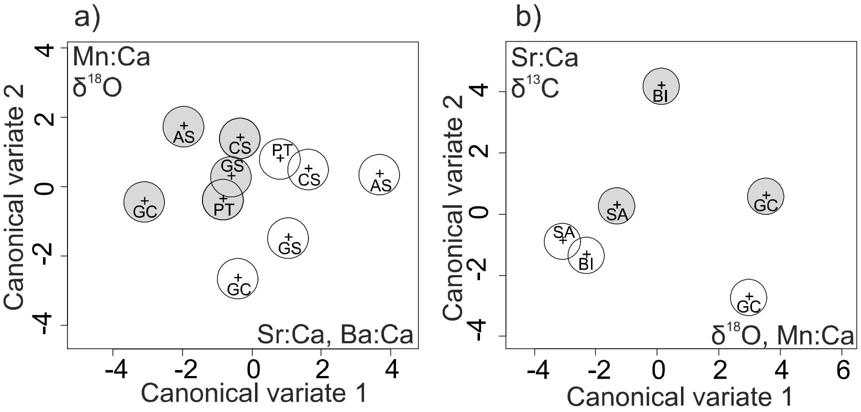

Fig. 3. Canonical discriminant analysis plot summarizing variation in geochemical signatures of otolith core (grey circles) and edge material (white circles) of *Merluccius merluccius* in the Atlantic Ocean (a) and the Mediterranean Sea (b). Circle enclosures represent 95% confidence limits for the mean. See Figure 1 for collection location abbreviations.

Collection locations in the Atlantic Ocean and the Mediterranean Sea were clearly separated based on data from otolith edges in the CDA plot (Fig. 4). Separation of the Atlantic locations was mainly based on Sr:Ca, Mg:Ca, Ba:Ca and Mn:Ca (Fig. 4a), whereas separation in the Mediterranean was due to variability in δ^{18}O, Mn:Ca, Mg:Ca and Sr:Ca ratios (Fig. 4b). The clear separation of collection locations was also reflected in the overall correct classification accuracy of European hake to their collection locations with cross-validated accuracies of 72% and 90% for the Atlantic Ocean and the Mediterranean Sea, respectively. In the Atlantic Ocean, individuals from the Armorican Shelf and Portugal achieved the best classification results while the Celtic Sea and Galician Shelf showed the lowest accuracies (Table 3). The discriminant model for the Mediterranean Sea correctly classified 80% of the individuals collected in the Balearic Islands and 90% collected in Sardinia were correctly classified, while all the individuals from the Gulf of Cádiz were correctly assigned to their collection location (Table 3).

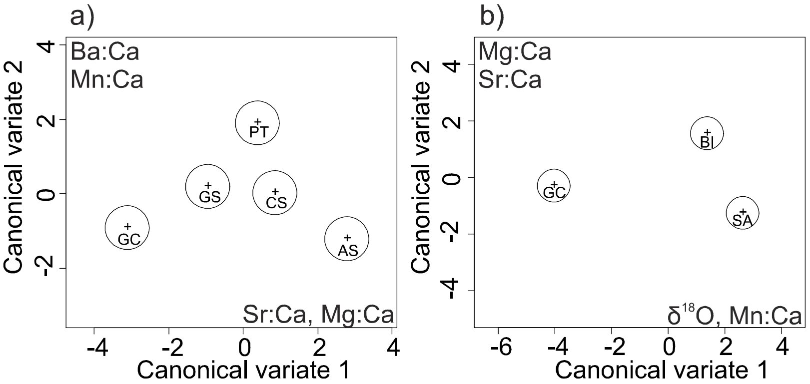

Fig. 4. Canonical discriminant analysis plot summarizing variation in geochemical signatures of otolith edge material of *Merluccius merluccius* in the Atlantic Ocean (a) and the Mediterranean Sea (b). Circle enclosures represent 95% confidence limits for the mean. See Figure 1 for collection location abbreviations.

Table 3. Cross – validated classification results of discriminant function analysis of *Merluccius merluccius* collected in the Atlantic Ocean and in the Mediterranean Sea. Individuals were classified to a collection location based on the geochemical signature of the otolith edge material. Values in bold represent individuals correctly classified to their collection location.

Atlantic Ocean

Collection location group	Predicted group membership (%)				
	CS	AS	GS	PT	GC
Celtic Sea (CS)	**60**	10	0	20	10
Armorican Shelf (AS)	10	**90**	0	0	0
Galician Shelf (GS)	20	0	**50**	20	10
Portugal (PT)	0	10	0	**90**	0
Gulf of Cádiz (GC)	0	0	30	0	**70**

a)Overall cross-validated correct classification: 72%

Mediterranean Sea

Collection location group	Predicted group membership (%)		
	GC	BI	SA
Gulf of Cádiz (GC)	**100**	0	0
Balearic Islands (BI)	0	**80**	20
Sardinia (SA)	0	10	**90**

b)Overall cross-validated correct classification: 90%

Three groups were detected in both nMDS analyses based on otolith core geochemistry from the Atlantic Ocean and Mediterranean Sea (Fig. 5). The cluster analysis also produced three groups based on distances between the clusters and visual assessment.

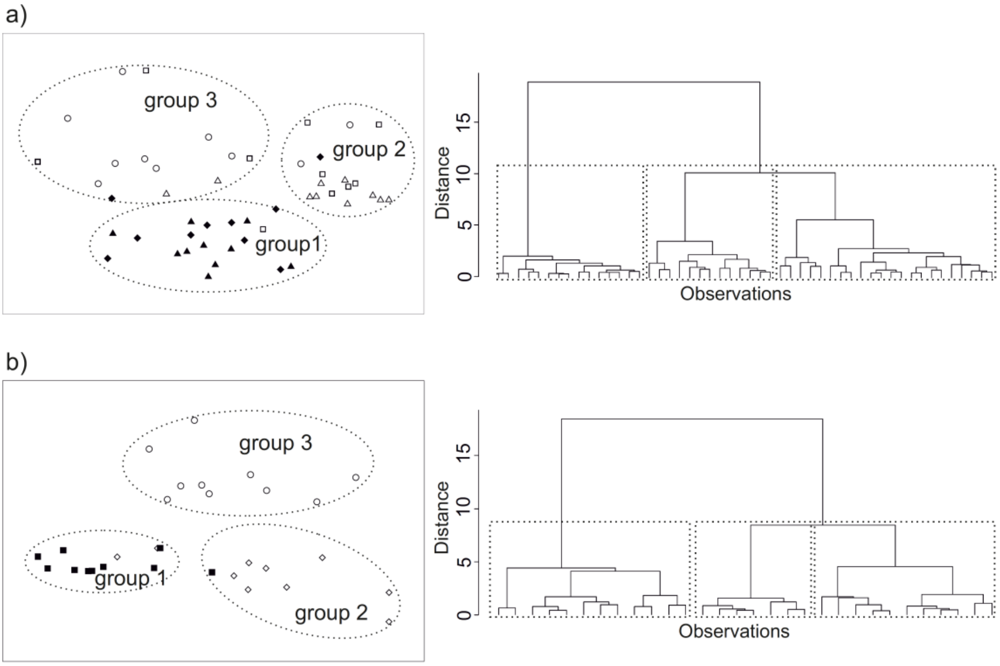

Fig. 5. Nonmetric multidimensional scaling (nMDS) and dendrogram of hierarchical cluster analysis based on otolith core geochemical signatures material of *Merluccius merluccius* in the Atlantic Ocean (a) and the Mediterranean Sea (b). Dashed lines delineate different groups identified. ♦ - Celtic Sea, ▲ – Armoricain Shelf, □ - Galician Shelf, ∆ - Portugal, ○ – Gulf of Cádiz, ■ – Balearic Islands, ◊ - Sardinia.

In the Atlantic Ocean, group 1 was mainly composed of individuals collected in the Armorican Shelf and Celtic Sea and one individual from the Galician Shelf, individuals collected in Portugal and Galician Shelf were clustered in group 2, along with two individuals collected in the Gulf of Cádiz and one in the Celtic Sea (Fig. 6a). In group 3, most individuals were collected in the Gulf of Cádiz, Portugal, Galician Shelf and one individual in the Celtic Sea (Fig. 6a). In the Mediterranean Sea, group 1 was mainly composed of hake collected in the Balearic Islands and two individuals in Sardinia (Fig. 6b). Similarly, most individuals in group 2 were collected in Sardinia and one in Balearic Islands. Group 3 was composed solely by hake collected in the Gulf of Cádiz, indicating little or no connection through the Strait of Gibraltar (Fig. 6b).

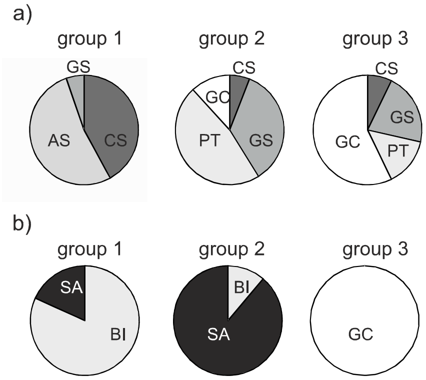

Fig. 6. Percentage of individuals of *Merluccius merluccius* assigned to group 1, 2, or 3 identified in the nonmetric multidimensional scaling (nMDS) plot based on otolith core geochemical signatures collected in the different locations in the Atlantic Ocean (a) and the Mediterranean Sea (b). See Figure 1 for collection location abbreviations.

DISCUSSION

Otolith geochemistry of European hake showed significant variation among the collection locations in the Atlantic Ocean and Mediterranean Sea, and between core and edge zones within otoliths. Variability among otolith edge geochemical signatures was sufficient to produce good results, classifying individuals to their location of collection and based on otolith core signatures three groups could be discerned in the Atlantic Ocean and in the Mediterranean Sea.

Some of the element:Ca and stable isotope ratios showed a consistent pattern between the otolith core and edge zones in all the locations sampled. Such differences may be related to distinct physicochemical properties of the ocean water in the habitats occupied by individuals of European hake during the different life history stages. Alternatively, consistent differences between otolith zones may reflect physiological changes occurring during ontogeny or variability in feeding habits and diet of the different life history stages. Both Sr:Ca and Ba:Ca ratios were significantly higher in the otolith core than at the edge. Strontium is a conservative element in oceanic environment and Sr:Ca ratios show little variations spatially in ocean and coastal waters (de Villiers 1999). It is, therefore, unlikely that the ontogenetic pattern in otolith Sr:Ca reflect variability in ambient Sr:Ca ratios. Temperature and salinity have also been shown to influence Sr uptake in otoliths (e.g. Farrell & Campana 1996, Bath et al. 2000, Martin et al. 2004). While salinity is likely not important for otolith Sr:Ca, the fact that $\delta^{18}O$ co-varies with Sr:Ca in our data argues that temperature plays some role in determining the ontogenetic patterns in Sr:Ca that we observed. Morales-Nin et al.

(2005) and Tomás et al. (2006) also observed high Sr:Ca values in otolith cores of European hake and suggested that they were related to physiological changes occurring during settlement of juveniles to the ocean floor. The inner ear of hake does indeed undergo major changes during metamorphosis (Lombarte & Popper 2004), although it is not clear how these physiological changes would lead to dissolved Sr:Ca variations in the endolymphatic fluid.

We also found that Ba:Ca ratios were higher in core zones than at otolith edges. Ambient Ba:Ca levels are generally considered to exert the most influence on otolith Ba:Ca (Bath et al. 2000). Barium has a nutrient-type distribution in ocean waters and is more depleted in ocean surface waters than in the deep ocean (Wolgemuth & Broecker 1970). Hake larvae and early pelagic juveniles are most abundant between 100 m – 200 m depth (Olivar et al. 2003, Alvarez et al. 2004) while hake of age 1 are found in the highest abundance at depth ranging from 25 m to 125 m (Woillez et al. 2007). Therefore ambient Ba:Ca values seem unlikely to explain otolith Ba:Ca, at least in terms of the ontogenetic patterns documented here. Several other studies have documented high Ba:Ca and Mn:Ca levels in otolith cores that were possibly related to the presence of organic molecules involved in aragonite nucleation (e.g. Brophy et al. 2004, Ruttenberg et al. 2005). Thorrold et al. (2006) documented the movement of Ba ions from gravid females to embryonic otoliths, but we lack a full understanding of potential mechanisms generating high elemental ratios in otolith cores.

Stable C and O isotope values also varied significantly among locations and with ontogeny. Otolith $\delta^{13}C$ values are rarely laid down close to isotopic equilibrium with $\delta^{13}C$ of dissolved inorganic carbon ($\delta^{13}C_{DIC}$), since 20% - 80% of the carbon in otoliths derived from metabolic sources (Kalish 1991, Thorrold et al. 1997). We noted generally, although not consistently, lower $\delta^{13}C$ values in otolith cores compared to edges with differences between zones as large as 3 in the Balearic Islands. These results are remarkably consistent with earlier work on European hake from waters off Mallorca that also documented a shift in $\delta^{13}C$ values from otolith cores to edges (Hildago et al. 2008). This shift in $\delta^{13}C$ may reflect ontogenetic variety in diets as larval and early pelagic juvenile life stages are zooplanktivorous, whereas hake over 20 cm in length feed mainly on small fish (Mahe et al. 2007). However, $\delta^{13}C$ typically varies by no more than 1-2 with trophic level in marine fish (Elsdon et al. 2010), and DIC dilution will reduce any trophic effect on bulk otolith $\delta^{13}C$ values even further (McMahon et al. 2011). Rather, we believe that the ontogenetic $\delta^{13}C$ trend is driven by individuals incorporating varying proportions of metabolic carbon and DIC at some, but not all, locations. On the other hand, oxygen isotopes are deposited at or very close to equilibrium with ambient waters (Thorrold et al. 1997, Høie et al. 2004). Differences in bathymetric preferences and associated temperatures of the different life history stages therefore provide the most parsimonious explanation for consistently higher $\delta^{18}O$ values observed in the otolith edge material. The patterns of element:Ca and stable isotope ratios in hake otoliths potentially could provide insights on the incorporation of certain elements. However, geochemical data obtained over several years would be necessary in order to discover consistent patterns and support meaningful interpretations.

Otolith geochemistry has been successfully applied in a number of marine species to reveal population structure, migration and spatial isolation (e.g. Ashford & Jones 2007, Arkhipkin et al. 2009). Our results suggest that the approach is also useful for European hake, although spatial resolution varied regionally. We found reasonably high error rates at some locations in the northeast Atlantic Ocean, especially for individuals collected in the Celtic Sea and on the Galician Shelf. It is possible that these individuals moved to the collection sites shortly prior to capture and that the residence time was not sufficient to acquire the site-specific signatures. Alternatively, these errors may simply reflect the fact that environmental conditions at the two locations were not sufficiently distinct to generate unique geochemical values in hake otoliths. European hake from the Mediterranean Sea were classified with very high accuracy to their collection locations. Hake populations in the Atlantic Ocean are clearly separated from the ones in the Mediterranean and there was no evidence of migration through the Strait of Gibraltar.

Although spawning areas of European hake extend all along the western margin of Europe and throughout the Mediterranean Sea (Alvarez et al. 2001), otolith core geochemistry identified three discrete groups of European hake in the Atlantic Ocean and Mediterranean Sea. In the Atlantic Ocean, samples from the northern stock (Celtic Sea and Armorican Shelf) were grouped together while individuals collected from locations within the southern stock (Galician Shelf, Portugal and Gulf of Cádiz) were segregated into two groups. Little exchange was observed between Balearic Islands and Sardinia, while the Gulf of Cádiz did not seem to be connected to the Mediterranean populations by larval dispersal. However, the complexity of the structure and connectivity in the Mediterranean populations of European hake needs to be further investigated. We did find some evidence for movement of at least some individuals among locations. For instance, a number of individuals collected in the Celtic Sea were assigned to the groups dominated by southern stock local populations while some individuals collected on the Galician Shelf were grouped with the northern stock. Several population genetic studies have similarly shown that there are no significant genetic divergences between the northern and southern stocks of hake within in the northeast Atlantic (Roldán et al. 1998, Castillo et al. 2005, Pita et al. 2011). Taken together, these movement patterns do not support the management boundary established in the Atlantic Ocean to separate the European hake stocks but rather advocate for a re-evaluation of the most adequate and effective management units for the species due to the high complexity of European hake population structure.

In summary, otolith geochemistry of European hake showed a strong ontogenetic signal that must be considered when using them as natural tags in connectivity or migration studies. Both otolith core and edge geochemical signatures provided strong evidence of movement of European hake between the local populations in the Atlantic Ocean and the Mediterranean Sea. Nonetheless, the results were still insufficient to provide unequivocal information on population structure and connectivity of European hake. Future studies should increase sample size and will need to focus on otoliths from larvae or early pelagic juveniles to provide baseline values that could then be used to assign natal origins of adults. These data will be invaluable to future management directives aimed at improving the sustainability of the exploration of this valuable demersal species.

ACKNOWLEDGEMENTS

The authors would like to thank Marie-Laure Bégout, Fátima Cardador, Jim Ellis, Beatriz Guijarro, Matteo Murenu, Carmen Piñeiro, Ignacio Sobrino and Francisco Velasco for providing the otolith samples. This study was funded by ‘Fundação para a Ciência e a Tecnologia’ (FCT), Pest-OE/MAR/UI0199/2011 and other projects. S.E. Tanner was funded with a PhD grant by FCT (SFRH/BD/40313/2007).

LITERATURE CITED

Abaunza P, Mattiucci S, Nascetti G, Magoulas A, Cimmaruta R, Bullini L (2001) Morphometric and meristic variation in European hake, *Merluccius merluccius*, from the Northeast Atlantic and Mediterranean Sea. ICES Document CM 2001/J:01:pp. 20

Alvarez P, Motos L, Uriarte A, Egaña J (2001) Spatial and temporal distribution of European hake, *Merluccius merluccius* (L.), eggs and larvae in relation to hydrographical conditions in the Bay of Biscay. Fish Res 50:111-128

Alvarez P, Fives J, Motos L, Santos M (2004) Distribution and abundance of European hake *Merluccius merluccius* (L.), eggs and larvae in the North East Atlantic waters in 1995 and 1998 in relation to hydrographic conditions. J Plankton Res 26:811-826

Arkhipkin AI, Schuchert PC, Danyushevsky L (2009) Otolith chemistry reveals fine population structure and close affinity to the Pacific and Atlantic oceanic spawning grounds in the migratory southern blue whiting (*Micromesistius australis australis*). Fish Res 96:199-194

Ashford J, Jones C (2007) Oxygen and carbon stable isotopes in otoliths record spatial isolation of Patagonian toothfish (*Dissostichus eleginoides*). Geochim Cosmochim Acta 71:87–94

Ashford JR, Arkhipkin AI, Jones CM (2006) Can the chemistry of otolith nuclei determine population structure of Patagonian toothfish *Dissostichus eleginoides*? J Fish Biol 69:708–721

Ashford JR, Jones CM, Hofmann EE, Moreno CA, Duhamel G, Williams R (2008) Otolith chemistry indicates population structuring by the Antarctic Circumpolar Current. Can J Fish Aquat Sci 65:135-146

Bath GE, Thorrold SR, Jones CM, Campana SE, McLaren JW, Lam JWH (2000) Strontium and barium uptake in aragonitic otoliths of marine fish. Geochim Cosmochim Acta 64:1705-1714

Begg GA, Friedland KD, Pearce JB (1999) Stock identification and its role in stock assessment and fisheries management: an overview. Fish Res 43:1-8

Brophy D, Jefferies TE, Danilowicz BS (2004) Elevated manganese concentrations at the cores of clupeid otoliths: possible environmental, physiological, or structural origins. Mar Biol 144:779-786

Campana SE (1999) Chemistry and composition of fish otoliths: pathways, mechanisms and applications. Mar Ecol Prog Ser 188:263-297

Campana SE, Chouinard GA, Hanson JM, Fréchet A, Brattey J (2000) Otolith elemental fingerprints as biological tracers of fish stocks. Fish Res 46:343-357

Castillo AGF, Alvarez P, Garcia-Vasquez E (2005) Population structure of *Merluccius merluccius* along the Iberian Peninsula coast. ICES J Mar Sci 62:1699-1691

de Villiers S (1999) Seawater strontium and Sr/Ca variability in the Atlantic and Pacific oceans. Earth Planet Sci Lett 171:623-634

Elsdon TS, Wells BK, Campana SE, Gillanders BM, Jones CM, Limburg KE, Secor DH, Thorrold SR, Walther BD (2008) Otolith chemistry to describe movements and life-history parameters of fishes: hypotheses, assumptions, limitations and inferences. Oceanogr Mar Biol Annu Rev 46:297-330

Elsdon TS, Ayvazian S, McMahon KW, Thorrold SR (2010) Experimental evaluation of stable isotope fractionation in fish muscle and otoliths. Mar Ecol Prog Ser 408:195-205

Farrell J, Campana SE (1996) Regulation of calcium and strontium deposition on the otoliths of juvenile tilapia, *Oreochromis niloticus*. Comp Biochem Physiol A: Physiol 115:103-109

Fowler AJ, Gillanders BM, Hall KC (2005) Relationship between elemental concentration and age from otoliths of adult snapper (*Pargus auratus*, Sparidae): implications for movement and stock structure. Mar Freshw Res 56:661-676

Hidalgo M, Tomás J, Høie H, Morales-Nin B, Ninnemann US (2008) Environmental influences on the recruitment process inferred from otolith stable isotopes in *Merluccius merluccius* off the Balearic Islands. Aquat Biol 3:195-207

Høie H, Otterlei E, Folkvord A (2004) Temperature-dependent fractionation of stable oxygen isotopes in otoliths of juvenile cod (*Gadus morhua* L.). ICES J Mar Sci 61:243-251

ICES (2011) Report of the Working Group on the Assessment of Southern Shelf stocks of Hake, Monk and Megrim (WGHMM). ICES CM 2011/ACOM:11 pp. 625

Kacher M, Amara R (2005) Distribution and growth of 0-group European hake in the Bay of Biscay and Celtic Sea: a spatial and inter-annual analyses. Fish Res 71:373-378

Kalish JM (1991) ^{13}C and ^{18}O isotopic disequilibria in fish otoliths: metabolic and kinetic effects. Mar Ecol Prog Ser 75:191-203

Lo Brutto S, Arculeo M, Parrinello N (2004) Congruence in genetic markers used to describe Mediterranean and Atlantic populations of European hake (*Merluccius merluccius* L. 1758). J Appl Ichthyol 20:81-86

Lombarte A, Popper AN (2004) Quantitative changes in the otolithic organs of the inner ear during the settlement period in European hake *Merluccius merluccius*. Mar Ecol Prog Ser 267:233-240

Mahe K, Amara R, Bryckaert T, Kacher M, Brylinski JM (2007) Ontogenetic and spatial variation in the diet of hake (*Merluccius merluccius*) in the Bay of Biscay and the Celtic Sea. ICES J Mar Sci 64:1210-1219

Martin GB, Thorrold SR, Jones CM (2004) Temperature and salinity effects on strontium incorporation in otoliths of larval spot (*Leiostomus xanthurus*). Can J Fish Aquat Sci 61:34-42

McMahon KW, Fogel ML, Johnson BJ, Houghton LA, Thorrold SR (2011) A new method to reconstruct fish diet and movement patterns from δ^{13}C values in otolith amino acids Can J Fish Aquat Sci 68:1330-1340

Mellon-Duval C, De Pontual H, Métral L, Quemener L (2010) Growth of European hake (*Merluccius merluccius*) in Gulf of Lions based on conventional tagging. ICES J Mar Sci 67:62-70

Morales-Nin B, Moranta J (2004) Recruitment and post-settlement growth of juvenile *Merluccius merluccius* on the western Mediterranean shelf. Sci Mar 68:399-409

Morales-Nin B, Swan SC, Gordon JDM, Palmer M, Geffen AJ, Shimmield T, Sawyer T (2005) Age-related trends in otoliths chemistry of *Merluccius merluccius* from the north-eastern Atlantic Ocean and the western Mediterranean Sea. Mar Freshw Res 56:599-607

Murua H (2010) The Biology and Fisheries of European Hake, *Merluccius merluccius*, in the North-East Atlantic. Adv Mar Biol 58:97-154

Olivar MP, Quílez G, Emelianov M (2003) Spatial and temporal distribution and abundance of European hake, *Merluccius merluccius*, eggs and larvae in the Catalan coast (NW Mediterranean). Fish Res 60:321-331

Ostermann DR, Curry WB (2000) Calibration of stable isotopic data: an enriched $\delta^{18}O$ standard used for source gas mixing detection and correction. Paleoceanography 15:353-360

Otxotorena U, Díez G, López de Abechuco E, Santurtún M, Lucio P (2010) Estimation of age and growth of juvenile hakes (*Merluccius merluccius* Linneaus, 1758) of the Bay of Biscay and Great Sole by means of the analysis of macro and microstructure of the otoliths. Fish Res 106:337-343

Pawson MG, Jennings S (1996) A critique of methods for stock identification in marine capture fisheries. Fish Res 25:203-217

Piñeiro C, Saínza M (2003) Age estimation, growth and maturity of the European hake (*Merluccius merluccius* (Linnaeus, 1758)) from Iberian Atlantic waters ICES J Mar Sci 60:1086-1102

Pita A, Pérez M, Cerviño S, Presa P (2011) What can gene flow and recruitment dynamics tell us about connectivity between European hake stocks in the Eastern North Atlantic? Cont Shelf Res 31:376-387

Roldán MI, García-Marín JL, Utter FM, Pla C (1998) Population genetic structure of European hake, *Merluccius merluccius*. Heredity 81:327-334

Ruttenberg BI, Hamilton SL, Hickford MJH, Paradis GL, Sheehy MS, Standish JD, Ben-Tzvi O, Warner RR (2005) Elevated levels of trace elements in cores of otoliths and their potential for use as natural tags. Mar Ecol Prog Ser 297:273-281

Steer MA, Halverson GP, Fowler AJ, Gillanders BM (2010) Stock discrimination of Southern Garfish (*Hyporhamphus melanochir*) by stable isotope ratio analysis of otolith aragonite Environ Biol Fishes 89:369-381

Sturgeon RE, Willie SN, Yang L, Greenberg R, Spatz RO, Chen Z, Scriver C, Clancy V, Lam JW, Thorrold SR (2005) Certification of a fish otolith reference material in support of quality assurance for trace element analysis. J Anal At Spectrom 20:1067-1071

Swan SC, Geffen AJ, Morales-Nin B, Gordon JDM, Shimmield T, Sawyer T, Massut E (2006) Otolith chemistry: an aid to stock separation of *Helicolenus dactylopterus* (bluemouth) and *Merluccius merluccius* (European hake) in the Northeast Atlantic and Mediterranean. ICES J Mar Sci 63:504-513

Tanner SE, Vasconcelos RP, Reis-Santos P, Cabral HN, Thorrold SR (2011) Spatial and ontogenetic variability in the chemical composition of juvenile common sole (*Solea solea*) otoliths. Estuar Coast Shelf Sci 91:150-157

Thorrold SR, Campana SE, Jones CM, Swart PK (1997) Factors determining $\delta^{13}C$ and $\delta^{18}O$ fractionation in aragonitic otoliths of marine fish. Geochim Cosmochim Acta 61:2909-2919

Thorrold SR, Jones GP, Planes S, Hare JA (2006) Transgenerational marking of embryonic otoliths in marine fishes using barium stable isotopes. Can J Fish Aquat Sci 63:1193-1197

Tomás J, Geffen AJ, Millner RS, Piñeiro CG, Tserpes G (2006) Elemental composition of otolith growth marks in three geographically separated populations of European hake (Merluccius merluccius). Mar Biol 148:1399–1413

Woillez M, Poulard J-C, Rivoirard J, Petitgas P, Bez N (2007) Indices for capturing spatial patterns and their evolution in time, with application to European hake (*Merluccius merluccius*) in the Bay of Biscay. ICES J Mar Sci 64:537-550

Wolgemuth K, Broecker WS (1970) Barium in sea water. Earth Planet Sci Lett 8:372-378

Yoshinaga J, Nakama A, Morita M, Edmonds JS (2000) Fish otolith reference material for quality assurance of chemical analyses. Mar Chem 69:91-97

CHAPTER VI

Integrating microsatellite DNA markers and otolith geochemistry to assess stock structure of European hake (*Merluccius merluccius*)

Susanne E. Tanner[1], Montse Pérez[2], Pablo Presa[3], Simon R. Thorrold[4] & Henrique N. Cabral[1]

[1]Centro de Oceanografia, Faculdade de Ciências da Universidade de Lisboa, Campo Grande, 1749-016 Lisboa, Portugal
[2]Instituto Español de Oceanografia (IEO), Centro Oceanográfico de Vigo, 36390 Vigo, Spain
[3]University of Vigo, Department of Biochemistry, Genetics and Immunology, 36310 Vigo, Spain
[4]Biology Department MS 50, Woods Hole Oceanographic Institution, Woods Hole, MA 02543, USA

In review in Fisheries Oceanography

Integrating microsatellite DNA markers and otolith geochemistry to assess stock structure of European hake (*Merluccius merluccius*)

ABSTRACT Population structure and movement patterns of European hake were investigated using an integrative approach based on otolith core and edge geochemical composition and microsatellite DNA markers. Age-1 hake were collected in seven locations in the northeast Atlantic Ocean and the Mediterranean Sea, otolith geochemical core and edge composition was determined (Mg:Ca, Mn:Ca, Sr:Ca, Ba:Ca, $\delta^{13}C$ and $\delta^{18}O$) and five microsatellites were genotyped. Otolith edge geochemical composition proved to be a suitable natural tag to discriminate among collection locations at this spatial scale by correctly assigning 74.3% of the individuals. Otolith core geochemical values used to investigate spatial separation of early life stages were not sufficiently powerful at the full geographical range however within each hydrogeographic region (Atlantic Ocean and Mediterranean Sea) three distinct groups were identified. The composition of these groups indicated migration among locations within both regions but no evidence of movement of hake through the Strait of Gibraltar was found. Microsatellites did not resolve population genetic structure within the hydrogeographic regions, but evidence of a major genetic split was found in the vicinity of the Strait of Gibraltar, separating the Atlantic and the Mediterranean populations. Overall, the integration of the two techniques enhanced population structure estimates of European hake when applied in a hierarchical manner, since the two markers act at different spatio-temporal scales. The integration of genetic and otolith chemical markers, combined where possible with artificial tagging, shows great promise to resolve population structure and connectivity patterns of European hake at different life stages.

Keywords: movement, population structure, otolith geochemistry, microsatellites, European hake

INTRODUCTION

Knowledge of stock structure is a necessary prerequisite for the sustainable management of marine capture fisheries (Begg & Waldman 1999). Lack of such information can lead to scientists missing changes in biological characteristics and productivity rates of a species that would otherwise trigger new management actions (Begg et al. 1999). A wide array of methodological approaches have been employed to assess stock structure in marine fish populations, including distribution and abundance data, morphometrics and meristics, life history patterns, genetics, and artificial and natural tags (Pawson & Jennings 1996). Among these techniques, genetic markers and otolith geochemistry represent the two most powerful and commonly used tools for determining population structure (Feyrer et al. 2007).

Genetic markers are well established in studies of population structure and connectivity in aquatic and terrestrial environments (e.g. Luikart et al. 2011, Pita et al. 2011). Highly variable codominant microsatellite DNA markers have proved particularly useful for identifying genetic structure in populations with low genetic differentiation such as marine fish species (White et al. 2010). The marine environment presents few physical barriers to dispersal of the different life history stages of fish allowing exchange of individuals among local groups of fish. Even very low exchange rates (~10 individuals per generation) can prevent the accumulation of large genetic differences among groups of fish (Slatkin 1987, Palumbi 2003). The ecological implications of low but significant genetic structure in marine fish populations are difficult to assess, especially because estimates of gene flow are generally made on recent and/or past evolutionary time scales rather than an ecological time frame over which most management decisions are made (Palumbi 2003).

Alternatively, otolith geochemistry has been used to examine stock structure (Campana et al. 2000, Bergenius et al. 2005), adults’ natal or juvenile origin (Thorrold et al. 2001, Hamer et al. 2011) and migration patterns (Campana et al. 2007, Walther et al. 2011) over demographic time scales. These applications are feasible due to the inertness of otoliths, their continuous growth, recording information of the individual life history in a chronological manner and the fact that their chemical composition is highly influenced by the physical and chemical properties of the surrounding water (Campana 1999).

Stock structure is influenced by behavioral and physical processes that act over a range of temporal scales and therefore the best inference on stock structure may be achieved by using multiple and potentially complementary techniques that integrate over different scales (Begg & Waldman 1999, Thorrold et al. 2002). Recently, a holistic approach integrating four different approaches (genetic markers, morphometry, parasites and life history traits) has provided reliable information on stock structure of Atlantic horse mackerel (*Trachurus trachurus*) throughout the species distribution range (Abaunza et al. 2008). More commonly, stock structure assessment integrates two different techniques, such as parasite assemblage composition combined with genetic markers or otolith shape analysis (McClelland et al. 2005, Vignon et al. 2008). The combination of genetic markers and otolith geochemistry has been increasingly used as they provide complementary information on population structure and connectivity patterns over evolutionary and ecological time scales, respectively (Miller et al. 2005, Bradbury et al. 2008, Woods et al. 2010). In some cases the two techniques produced conflicting estimates on population structure and connectivity (e.g. Thorrold et al. 2001), most likely due to different temporal scales at which genetic markers and otolith chemistry are informative. The chronological properties of otoliths may provide information on the aquatic environments experienced by an individual over its lifetime, whereas genetic markers may resolve population structure over various time scales depending on the rate that variation accumulates at a given locus (Woods et al. 2010).

Despite the progress in the application of different techniques used to identify stock structure, the problem of defining the management units of many commercially exploited species is far from resolved (e.g. Lleonart & Maynou 2003, Abaunza et al. 2008). European hake, *Merluccius merluccius* (Linnaeus, 1758), a commercially important demersal, benthopelagic species is one such case. The species is distributed from Norway to the Gulf of Guinea in the northeast Atlantic and throughout the Mediterranean and Black Sea, with highest abundances from the British Isles to southern Spain (Murua 2010). Although there is evidence of some gene flow between the Mediterranean and Atlantic in the vicinity of the Strait of Gibraltar (Roldán et al. 1998), the Mediterranean and Atlantic populations of hake are considered and managed as different stocks due to differences in biology, morphology and genetics (Abaunza et al. 2001, Lo Brutto et al. 2004, Mellon-Duval et al. 2010). Moreover, in the northeast Atlantic Ocean, the International Council for Exploration of the Sea (ICES) divides the hake population into the northern and southern stocks with Capbreton canyon (Bay of Biscay, SE France) delineating the boundary between them. While the establishment of these two stocks was based on management considerations without biological basis (ICES 2011), recent population genetics studies have presented evidence of a variable but consistent gene flow between these stocks (e.g. Pita et al. 2011).

The aim of the present study was to use genetic markers and otolith geochemistry to investigate population structure of European hake in the northeast Atlantic Ocean and the Mediterranean Sea, representing three currently implemented management units. Suitability and discriminatory power of otolith geochemistry were assessed using the chemical composition of otolith edges that is relative to the time prior to capture. Geochemical composition of otolith cores, representing larval and early pelagic juvenile life stages, was used to investigate spatial separation at these early life stages.

Genetic structure of hake populations was assessed using microsatellite DNA markers. Finally, the complementarity of the information on population structure obtained by genetic markers and otolith geochemistry was assessed.

MATERIAL AND METHODS

Fish sampling

Specimens of European hake were obtained from research surveys at seven different locations in the northeast Atlantic Ocean and the Mediterranean Sea (Fig. 1). Total length of the individuals was determined (Table 1), *sagittal* otoliths were extracted and fin tissue clips (ca. 1 cm^2) were obtained. Otoliths were rinsed with water, cleaned from adhering tissue and preserved dry. Fin tissue was stored in pure ethanol for genetic analysis. All individuals used in this study were classified into the age-class 1 using standardized methods (Piñeiro & Saínza 2003, Kacher & Amara 2005).

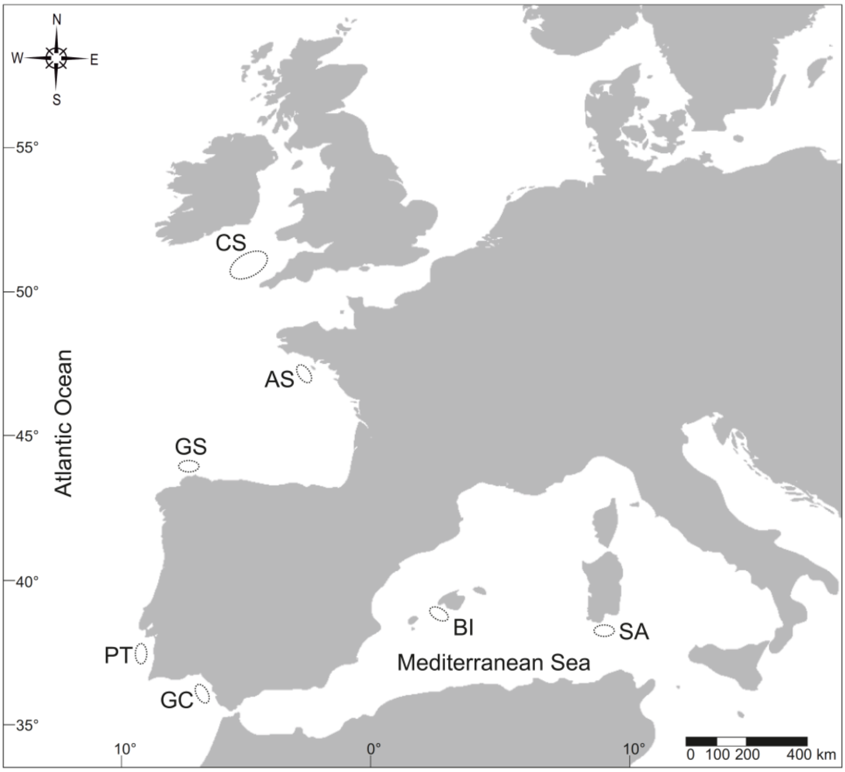

Fig. 1. Collection locations of *Merluccius merluccius* in the northeast Atlantic Ocean and Mediterranean Sea: CS – Celtic Sea, AS – Armorican Shelf, GS – Galician Shelf, PT – Portugal, GC – Gulf of Cádiz, BI – Balearic Islands, SA – Sardinia.

Table 1. Collection location, date of collection, mean and standard deviation (SD) of fish total length (Lt) in cm and sample sizes for geochemical (n_a) and genetic analysis (n_b) of *Merluccius merluccius*.

Collection location	Abbreviation	Date of collection	Lt (cm)		n_a	n_b
			Mean	SD		
Celtic Sea	CS	November 2010	22.1	1.4	10	50
Armorican Shelf	AS	May 2010	22.7	1.0	10	42
Galician Shelf	GS	October 2010	22.5	1.5	10	50
Portugal	PT	June 2010	20.9	0.8	10	50
Gulf of Cádiz	GC	November 2010	22.4	0.7	10	50
Balearic Islands	BI	May 2010	21.3	1.3	10	50
Sardinia	SA	October 2010	23.6	1.3	10	47

Otolith geochemistry

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 (18.2 MΩ cm^{-1}), sonicating for 2 min and triple-rinsing with ultrapure water in a class-100 clean room. Elemental analysis was performed using a Thermo Finnigan Element2 single collector inductively coupled plasma mass spectrometer (ICP-MS) coupled to a New Wave Research 193 nm excimer laser ablation system. The laser software was used to raster a 80 x 150 µm area at the core of the otolith and a 390 µm line along the dorsal edge of the otolith. The ablated material was transported via a He gas stream to the dual-inlet quartz spray chamber where it was mixed with a 2% nitric acid (HNO_3) aerosol from a self-aspirating PFA 20 ml min^{-1} nebulizer. The analyte was then transported to the ICP-MS via an Ar carrier gas. Elemental ratios of Mg:Ca, Mn:Ca, Sr:Ca and Ba:Ca were quantified by measuring ^{25}Mg, ^{48}Ca, ^{55}Mn, ^{88}Sr and ^{138}Ba in the ablated otolith material. Instrumental blanks (2% HNO_3) and certified reference materials (CRM - Yoshinaga et al. 2000, Sturgeon et al. 2005) were analyzed every four otoliths and were used to correct for background intensities and instrument mass drift (for details see Tanner et al. 2011). External precision (relative standard deviation) for the CRM (n = 42) was as follows: Mg:Ca = 1.2%, Mn:Ca = 18.6%, Sr:Ca = 0.9% and Ba:Ca = 1.5%.

For stable isotope analysis, a computer-controlled micromill was used to remove otolith material from the core and edge of each left otolith. In order to achieve optimal sample size for the isotope ratio monitoring mass spectrometer used, about 0.025 mm^3 material was extracted in the core and along the dorsal edge of each otolith (Hidalgo et al. 2008). The mean mass of the otolith powder sampled (n=140) was 47.5 µg (SD=8 µg). Samples were then analyzed on a Thermo Finnigan MAT253 equipped with a Kiel III carbonate device following methods outlined by Ostermann and Curry (2000). Isotopic values were reported relative to Vienna Pee Dee belemnite (VPDB) and expressed in standard δ notation. Long-term precision estimates of the mass spectrometer based on analyses of NBS19 are ±0.03‰ for $\delta^{13}C$ and ±0.07‰ for $\delta^{18}O$. The otolith core area sampled for both elemental and stable isotope analysis was defined as the region between the primordium and the accessory growth centers that correspond to the larval and early juvenile pelagic pre-settlement period (Morales-Nin & Moranta 2004, Hidalgo et al. 2008). The otolith material sampled along the edge corresponds to the carbonate laid down immediately prior to capture.

Suitability and discriminatory power of otolith geochemistry at the analyzed spatial scale was evaluated using linear discriminant function analysis (LDFA) to classify European hake to their collection locations based on geochemical variables at otolith edges. Classification accuracy of the discriminant functions was evaluated by calculating cross-validated classification success using a jackknife (leave-one-out) approach. The assumptions of LDFA, i.e. normality and homogeneity of variance-covariance matrices, were met after $\log_{10}$ transformation of the variables.

European hake collected in a location did not necessarily originate from the same location and, hence LDFA based on otolith core composition and collection locations was not appropriate to identify natal origins. Rather, the geochemical data from otolith cores were examined using nonmetric multidimensional scaling (nMDS) in order to visually identify discrete groups in the whole dataset, in the northeast Atlantic Ocean and the Mediterranean Sea separately. For this purpose, individuals from the Gulf of Cádiz were included in both the Atlantic and the Mediterranean datasets in order to evaluate the potential movement of European hake through the Strait of Gibraltar. Euclidean distances were used to construct a dissimilarity matrix. Ward's independent, agglomerative hierarchical clustering procedure was also performed to corroborate the grouping obtained in the MDS (Ashford et al. 2006). The appropriate number of groups using the clustering procedure was assessed visually and based on the distances among clusters.

Microsatellite marker analysis

For DNA extraction and purification of European hake fin tissue samples a commercial kit (MasterPure Complete DNA and RNA purification kit, EPICENTRE

Biotechnologies) was used. Five microsatellite markers (*Mmer* UEAHk3b, *Mmer* UEAHhk9b, *Mmer* UEAHk20, *Mmer* UEAHk29 and *Mmer* UEAHk34b) that were previously described for this species (Morán et al. 1999) were amplified following PCR reaction conditions outlined by Pita et al. (2011). The forward primer of each marker was fluorescently labeled: *Mmer* UEAHk3b and *Mmer* UEAHk29 with 6FAM, *Mmer* UEAHhk9b and *Mmer* UEAHk34b with HEX and *Mmer* UEAHk20 with NED. Amplified fragments were analyzed by capillary electrophoresis (Applied Biosystems) using an ABI 3130 automatic DNA sequencer and the internal sizer GeneScan 500 Rox Size Standard (Applied Biosystems). GeneMarker V1.97 software (SoftGenetics, LLC) was used to determine the allele size and genotype of all individuals. Genotypic scores were cross-checked among three independent readings to minimize putative genotyping errors.

Allele frequencies, observed and expected heterozygosities, and Hardy-Weinberg equilibrium tests were performed using Genepop 4.0 (Raymond & Rousset 1995). Fstat 2.9.2.3 (Goudet 1995) was used to calculate number of alleles, allelic richness, and fixation indices within samples (F_{IS}) and between samples (F_{ST}). Hierarchical analysis of molecular variance (AMOVA) implemented in Arlequin (ver. 3.11, Excoffier et al. 2005) was used to examine differences among groups of collection locations (F_{CT}) and among collection locations within groups (F_{SC}). In order to assess molecular variance at different spatial scales, collection locations were pooled into the two hydrogeographic regions (Atlantic Ocean and Mediterranean Sea) as well as to the currently implemented management units, i.e. northern and southern stocks in the Atlantic Ocean and the Mediterranean stock. A consensus star-like dendrogram describing the relationships among collection locations was generated with the neighbor-joining algorithm (Saitou & Nei 1987) using Cavalli-Sforza and Edwards (1967) chord distances implemented in PHYLIP package (Felsenstein 2005). One thousand bootstrap replicates of allele frequencies were used as nodal support of tree branches and the software TREEVIEW (Page 1996) was used for tree edition. Principal component analysis was computed to confirm the relationships among collection locations, using a correlation matrix of the allele frequencies.

A Bayesian clustering algorithm implemented in the R package Geneland version 2.0 (Guillot et al. 2005) was used to determine the most probable number of genetic clusters (*k*) within the dataset. K was selected a priori ranging from 1 to 7 populations and a correlated allele frequency model was chosen. Five independent runs were completed using 100000 MCMC iterations with 20000 burn-in and 1000 thinning-out. Genetic data was considered co-dominant and geographic information (GPS) of the collection locations was included in the analysis.

RESULTS

Otolith geochemistry

Otolith geochemistry at otolith edges varied significantly among locations (Fig. 2).

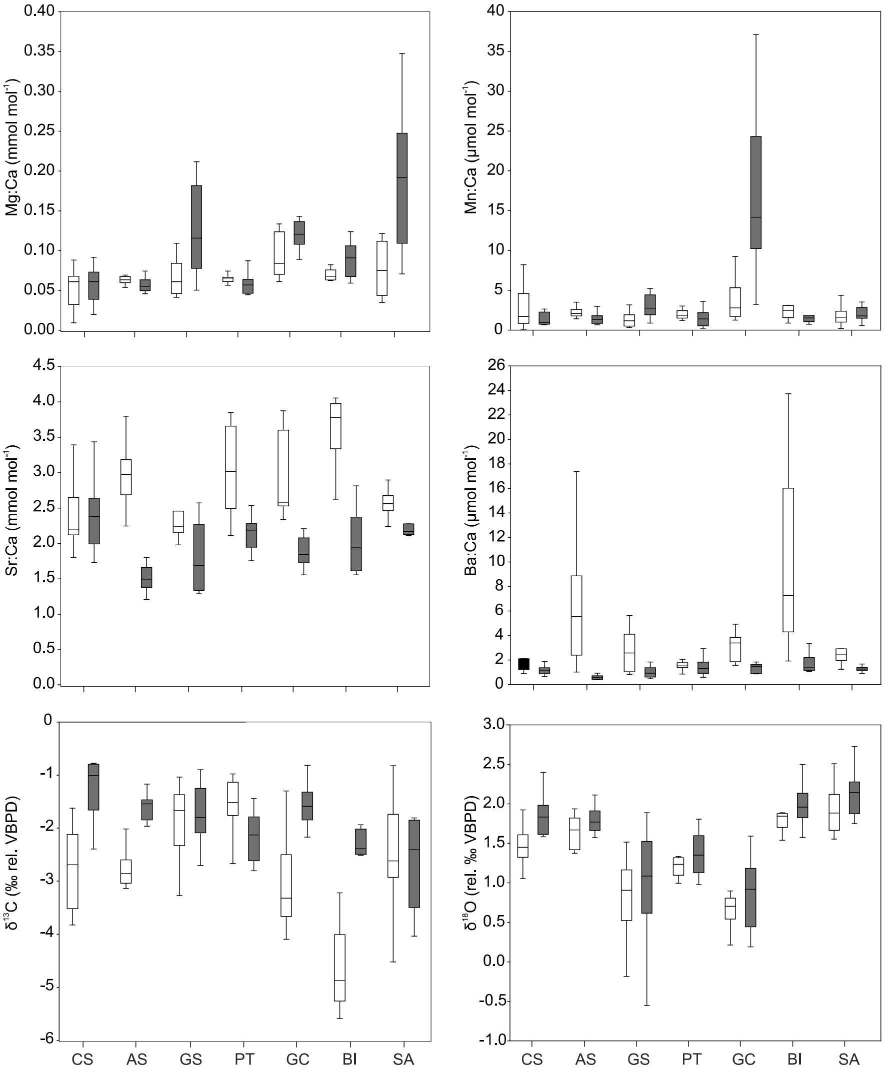

Fig. 2. Box plots of element:Ca and stable isotope ratios in otoliths of *Merluccius merluccius* in all the collection locations. White boxes indicate values obtained in otolith cores and grey boxes values obtained in otolith edges. The center line of each box represents the median, the box indicates the 25th and 75th percentiles, and the whisker delimits the non-outlier range.

Based on these values, overall classification accuracy of European hake to their collection locations based on elemental and isotope ratios at otolith edges was 74.3% (Table 2). Individuals collected in Celtic Sea and Galician Shelf recorded the lowest correct classification with 60% and 50%, respectively. In the remaining collection locations, 70% to 90% of the individuals were correctly assigned.

Table 2. Cross – validated classification results of discriminant function analysis of *Merluccius merluccius* collected in the northeast Atlantic Ocean and the Mediterranean Sea. Individuals were classified to a collection location based on the geochemical composition of the otolith edge. Values in bold represent percentage of individuals correctly classified to their collection location.

Collection location	Predicted collection location (%)						
	CS	AS	GS	PT	GC	BI	SA
Celtic Sea (CS)	**60**	10	0	10	10	10	0
Armorican Shelf (AS)	10	**90**	0	0	0	0	0
Galician Shelf (GS)	10	10	**50**	20	10	0	0
Portugal (PT)	0	10	0	**80**	0	10	0
Gulf of Cádiz (GC)	0	0	30	0	**70**	0	0
Balearic Islands (BI)	0	0	0	10	0	**80**	10
Sardinia (SA)	10	0	0	0	0	0	**90**
Overall cross-validated correct classification: 74.3 %							

We found no clear distinction between the Atlantic and Mediterranean populations of hake based on the geochemical composition of otolith cores (Fig. 3). While individuals collected in Balearic Islands were separated from other locations in the nMDS plot, individuals collected in Sardinia overlapped with individuals from the more northern locations in the Atlantic Ocean, Celtic Sea and Armorican Shelf. When only considering Atlantic Ocean locations, three groups were detected in the nMDS plot as well as in the cluster analysis (Fig. 4). Group 1 was mainly composed of individuals collected in the northern stock populations (Celtic Sea and Armorican Shelf) along with a single individual collected in the Galician Shelf. Hake collected in Portugal and Galician Shelf were clustered in group 2, along with two individuals collected in the Gulf of Cádiz and one in the Celtic Sea. In group 3, most fish were collected in the Gulf of Cádiz, Portugal, and Galician Shelf along with one individual from the Celtic Sea.

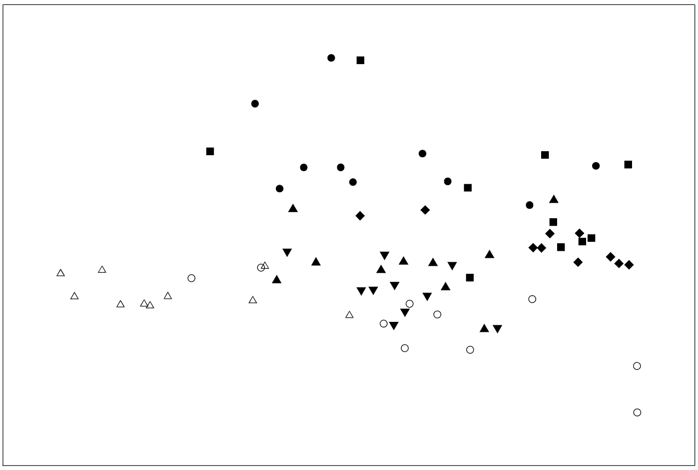

Fig. 3. Nonmetric multidimensional scaling (nMDS) plot based on otolith core geochemical composition of *Merluccius merluccius* from all collection locations in the northeast Atlantic Ocean and the Mediterranean Sea. ▲-Celtic Sea, ▼–Armorican Shelf, ■-Galician Shelf, ♦-Portugal, ●–Gulf of Cádiz, Δ–Balearic Islands, ○-Sardinia.

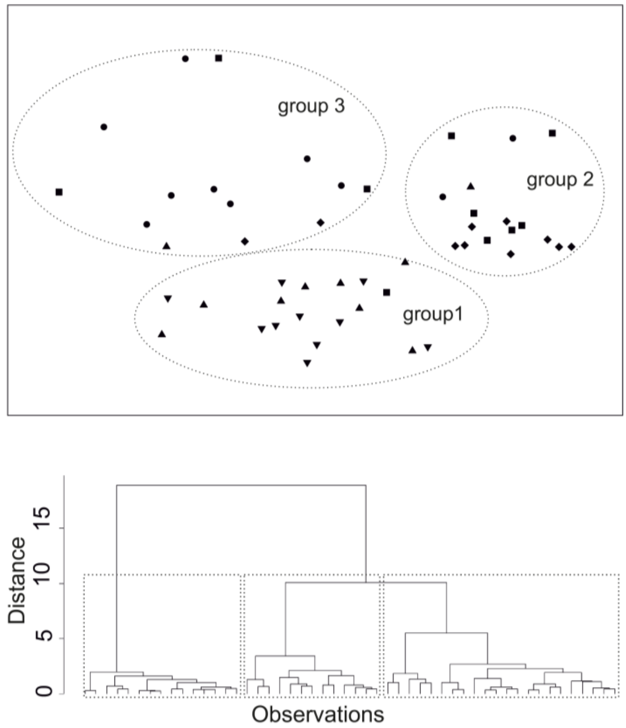

Fig. 4. Nonmetric multidimensional scaling (nMDS) plot and dendrogram of hierarchical cluster analysis based on otolith core geochemical composition of *Merluccius merluccius* in the locations sampled in the northeast Atlantic Ocean: ▲-Celtic Sea, ▼–Armorican Shelf, ■-Galician Shelf, ♦-Portugal, ●–Gulf of Cádiz. Dashed lines delineate different groups identified.

In the nMDS plot and cluster analysis based on the collection locations in the Mediterranean Sea and Gulf of Cádiz three groups were also identified (Fig. 5). Groups 1 and 2 were mainly composed of individuals collected in Balearic Islands and Sardinia, respectively, while hake collected in the Gulf of Cádiz were found in group 3.

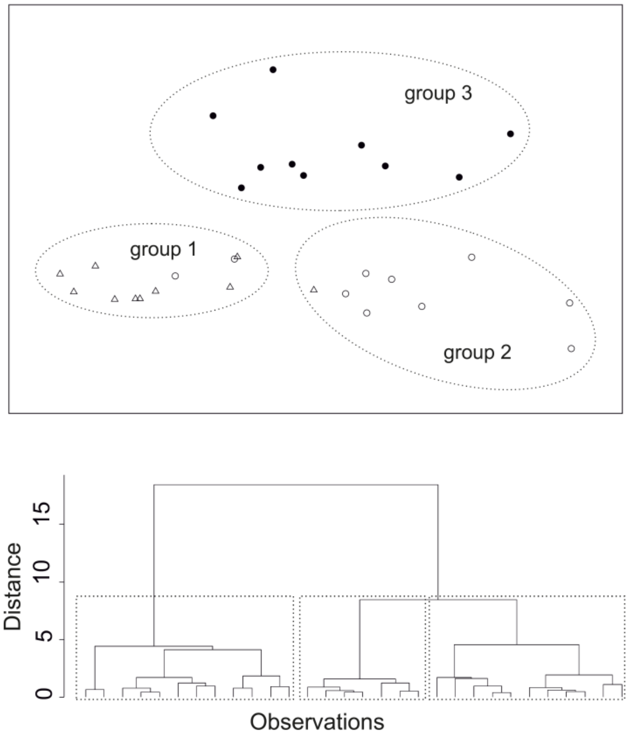

Fig. 5. Nonmetric multidimensional scaling (nMDS) plot and dendrogram of hierarchical cluster analysis based on otolith core geochemical composition of *Merluccius merluccius* in the locations sampled in the Mediterranean Sea: ●–Gulf of Cádiz, ∆–Balearic Islands, ○-Sardinia. Dashed lines delineate different groups identified

Microsatellite markers

Genetic parameters including number of alleles (A), gene diversity (H_e) and allelic richness (R_S) did not differ significantly among collection locations (Appendix 1). Systematic deviations from Hardy-Weinberg expectations were observed in most populations at two loci, *Mmer* UEAHk9b and *Mmer* UEAHk29b. Such deviations consisted in high heterozygote deficits and were probably caused by null alleles which are common to microsatellite markers (e.g. O'Connell & Wright 1997).

Global differentiation test among collection locations (100000 Markov chain iterations) was not significant (P=1.0). Pair-wise comparison of F_{ST} distance revealed that European hake collected in the Mediterranean Sea were genetically differentiated from the collection locations in the Atlantic Ocean with the exception of the Gulf of Cádiz (Table 3).

Table 3. Pair-wise F_{ST}-distance between collection locations of *Merluccius merluccius* in the northeast Atlantic Ocean and the Mediterranean Sea. Asterisks indicate p-values smaller than the adjusted nominal level for multiple comparisons α=0.0023 obtained after 420 permutations.

Collection location	CS	AS	GS	PT	GC	BI	SA
Celtic Sea (CS)	-	0.0016	0.0016	0.0066	0.0191*	0.0485*	0.0402*
Armorican Shelf (AS)		-	-0.0013	0.0048	0.0145	0.0372*	0.0292*
Galician Shelf (GS)			-	0.0041	0.0150	0.0384*	0.0301*
Portugal (PT)				-	0.0061*	0.0214*	0.0167*
Gulf of Cádiz (GC)					-	0.0084	0.0070
Balearic Islands (BI)						-	-0.0003
Sardinia (SA)							-

Within the Atlantic Ocean, only the Gulf of Cádiz was significantly different from Celtic Sea and Portugal. AMOVA analyses showed that most variation among collection locations (2.25%) was due to differences between the Atlantic Ocean and Mediterranean Sea or among the three management units (1.60%) currently implemented (Table 4). The neighbor-joining dendrogram showed a major branching between the Atlantic and Mediterranean collection locations with 98.8% bootstrap support (Fig. 6a). Within the branch of the Atlantic locations, the Gulf of Cádiz population was positioned apart with 83.2% bootstrap support. Similarly, the PCA showed two main clusters, one composed of the Atlantic locations, except the Gulf of Cádiz and the other composed by the two Mediterranean locations (Balearic Islands and Sardinia) (Figure. 6b).

Table 4. Hierarchical analysis of molecular variance (AMOVA) among the whole dataset, the hydrographic regions (northeast Atlantic Ocean and Mediterranean Sea) and the management units (northern and southern stock in the Atlantic Ocean and Mediterranean Sea). Asterisk indicates *p*<0.01.

Hierarchical level	Source of variation	df	Sum of Squares	Variance components	% of variation	Fixation indices
Whole dataset	Among locations	6	37.13	0.0412	1.85	F_{ST}= 0.018*
	Within locations	671	1472.50	2.1945	98.15	
Hydrogeographic regions	Among groups	1	17.97	0.5103	2.25	F_{CT}= 0.0225*
	Among locations	5	19.16	0.0169	0.75	F_{SC}= 0.0075*
	Within locations	671	1472.50	2.1994	97.00	F_{ST}= 0.0300*
Management units	Among groups	2	22.91	0.0359	1.60	F_{CT}= 0.0160*
	Among locations	4	14.22	0.0140	0.63	F_{SC}= 0.0063*
	Within locations	671	1472.50	2.1944	97.77	F_{ST}= 0.0223*

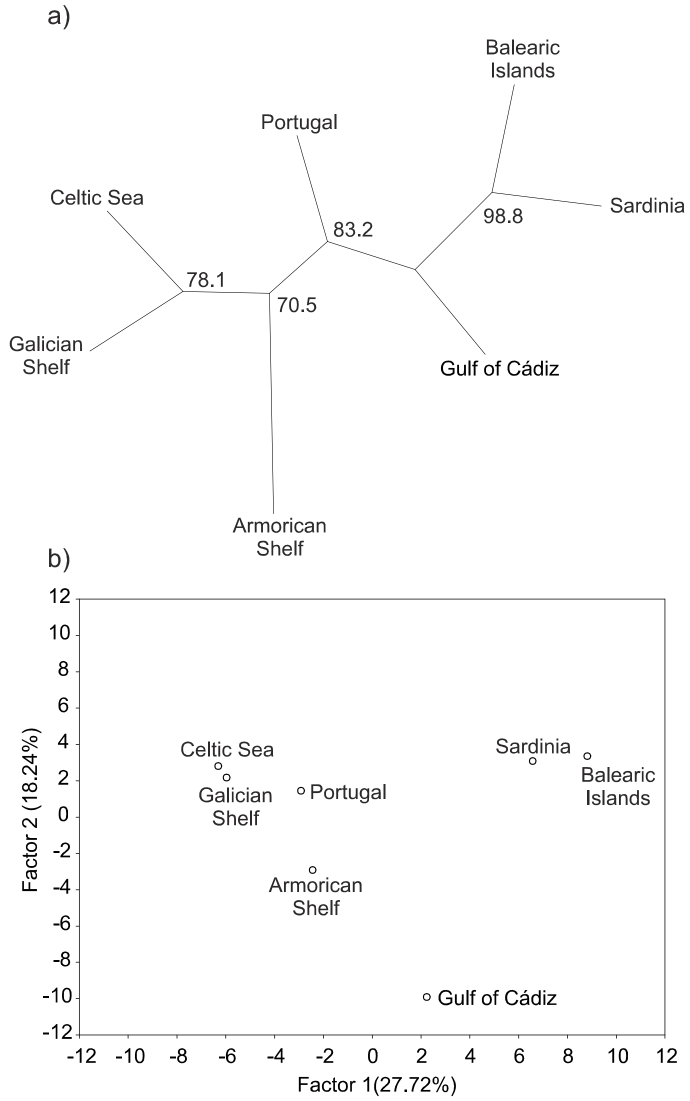

Fig. 6. Relative relationships between *Merluccius merluccius* collected in several locations in the northeast Atlantic Ocean and the Mediterranean Sea according to two methods: (a) Unrooted neighbour-joining tree, based on Cavalli-Sforza and Edwards chord distances. Bootstrap support was generated from 1000 replicates. (b) Principal component analysis (PCA) based on correlation matrix of allele frequencies.

These findings were congruent with the results obtained from the Bayesian clustering algorithm implemented by Geneland. The existence of two genetically homogeneous sub-units (*k*=2) of European hake had the highest probability. The first sub-unit was composed by the individuals collected in the Atlantic and the two Mediterranean locations (Balearic Islands and Sardinia) formed the second sub-unit

(Fig. 7).

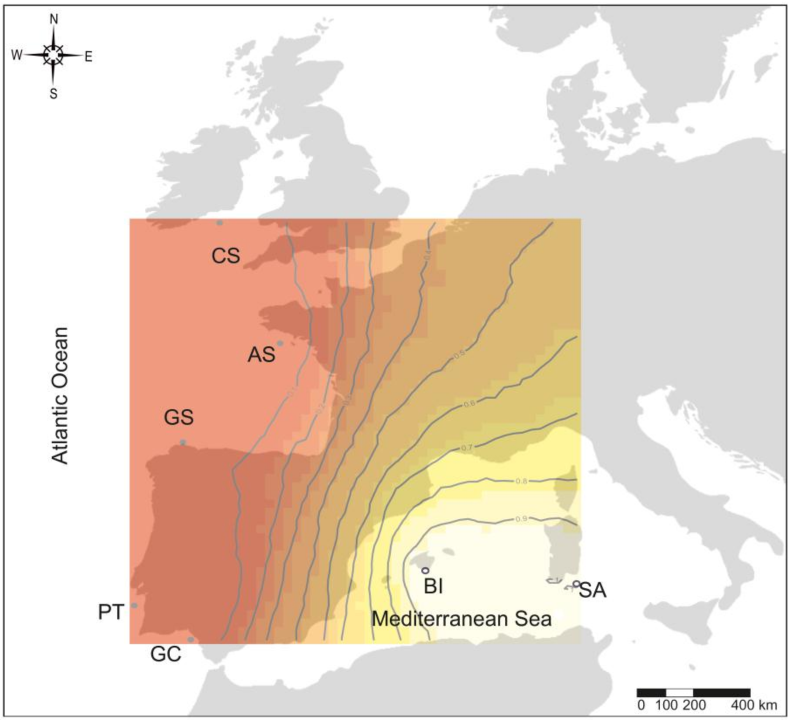

Fig. 7. Geographically constrained Bayesian model from Geneland. Probability isoclines identify two genetically distinct sub-units of *Merluccius merluccius* in the Atlantic Ocean and Mediterranean Sea. The populations marked in grey (northeast Atlantic Ocean) were identified as one sub-unit and the populations marked in white (Mediterranean Sea) as the other sub-unit.

DISCUSSION

Integrating otolith geochemistry and microsatellite DNA markers improved stock structure estimates of European hake when applied in a hierarchical manner. Otolith geochemistry generally provided information on finer spatial scales than microsatellite markers. Alternatively, microsatellite DNA markers report on gene flow from past or recent evolutionary time scales and therefore act on a broader spatio-temporal resolution. In this instance, Atlantic and Mediterranean hake populations were clearly distinguished based on microsatellite markers while otolith geochemistry differed significantly among locations within both regions.

Otolith geochemical values from otolith edge regions that reflect the environment experienced by fish prior to capture showed sufficient spatial variation to correctly classify individuals to their locations of collection. Overall classification accuracy (74.3%) confirmed the suitability of otolith geochemistry as a tracer of fish groups in

European hake. The discriminatory power achieved in the present study was similar to other studies with marine fish (Ashford et al. 2005, Castro 2007) and outperformed an earlier study that also used otolith chemistry on European hake by almost 10% (Swan et al. 2006). The high misclassification rate observed in the Celtic Sea and Galician Shelf may be related to possible movement of these individuals to the collection locations shortly prior to capture. Alternatively, otolith chemistry at the two locations might not be sufficiently distinct from the remaining locations to allow for accurate classification.

While otolith geochemistry proved to be a useful natural tag in European hake, disentangling individuals' natal origins based on otolith core values over the full geographical range of our study was not possible. Elemental and isotope ratios in otolith cores of individuals collected in the most distant locations were confusingly close in multivariate space. Similarly, values of the isotopic ratio ^{87}Sr/^{86}Sr were of limited utility in identifying Chinook salmon natal origin at a broad geographic scale (Barnett-Johnson et al. 2010).

In contrast, geochemical composition of otolith cores suggested connectivity at smaller spatial scales. Three different groups of individuals distinguished within the Atlantic region based on otolith core values indicated movement of individuals among local populations. Exchange of individuals was particularly obvious in samples in the Celtic Sea and the Galician Shelf. In the Mediterranean Sea, little exchange was observed between Balearic Islands and Sardinia. The Gulf of Cádiz, the southernmost Atlantic location sampled, was apparently not connected to the Mediterranean populations based on otolith core chemical values, suggesting little or no migration through the Strait of Gibraltar.

Genetic parameters of the microsatellite markers employed were within the range of previous studies in European hake (e.g. Lundy et al. 1999, Pita et al. 2011). The heterozygote deficit observed for two loci in most collection locations is an issue that has been previously reported for this set of markers (e.g. Lundy et al. 1999). Pita *et al.* (2011) suggested that the existence of multiple null alleles co-segregating at low-frequency as the most parsimonious explanation to the absence of null–null homozygotes in genotypes given that technical artifacts (e.g. drop-out effects) have been minimized. Heterozygote deficits caused by null alleles can introduce bias in estimates of divergence in highly structured species (i.e. different null alleles segregating at different frequencies in different populations). However, for closely related populations of highly homogeneous species such as hake, it may be assumed that the impact of null alleles is evenly distributed across samples and therefore the underestimation of gene diversity due to null alleles can be ignored (Lado-Insua et al. 2011) as it is usually done with homoplasy (Estoup et al. 1995).

Genetic differentiation observed among hake collection locations (F_{ST}=0.018) was in the range reported for this species, e.g. F_{ST}=0.026 (Lundy et al. 1999) and F_{ST}=0.018 (Pita et al. 2011). Evidence of a major genetic split was found in the vicinity of the Strait of Gibraltar, separating the Atlantic and the Mediterranean populations. However, there appears to be at least some gene flow across this partial barrier since individuals collected in the Gulf of Cádiz show an admixture of genetic attributes from the Atlantic and Mediterranean populations. This population subdivision is supported by the findings of other studies based on allozymes (Roldán et al. 1998) and microsatellite data (Lundy et al. 1999). These studies supported the hypothesis that unidirectional passive larval drift from the Atlantic Ocean into the Mediterranean Sea was probably responsible for the gene flow across the Strait of Gibraltar, as suggested in other marine organisms such as mussels (Diz & Presa 2008). However, the hypothesis of unidirectional dispersal of hake larvae from the Atlantic Ocean into the Mediterranean Sea has yet to be confirmed. We were unable to detect any genetic differentiation in the Atlantic or Mediterranean locations, with the single exception of the Gulf of Cádiz that was genetically divergent from some Atlantic populations but not from the Mediterranean populations. In the Atlantic Ocean, individuals collected in the Celtic Sea, Armorican Shelf and Galician Shelf showed very little genetic divergence. This result is in agreement with another study that has suggested significant gene flow between hake populations from Porcupine Bank and Great Sole Bank (Celtic Sea), and from northern Iberian Peninsula over a two year period (Pita et al. 2011).

The integrative application of otolith geochemistry and microsatellite DNA markers has provided information on population structure (Miller et al. 2005), early life stage dispersal (Bradbury et al. 2008) and natal origins of a number of species, particularly salmonids (Barnett-Johnson et al. 2010, Perrier et al. 2011). The results obtained in the present study using two techniques in European hake yielded complementary information on stock structure when applied hierarchically. Molecular markers distinguished populations collected in the northeast Atlantic Ocean from those collected in the Mediterranean Sea, while otolith geochemistry provided information on population structure on a finer spatial scale, within each of the two hydrogeographic regions. Multilocus genotypes did not resolve any genetic population structure within regions, and instead suggested a long history of genetic connectivity in the Atlantic Ocean. Similarly, geochemistry in otolith cores showed evidence of movement of individuals in the Atlantic Ocean, particularly for individuals collected in the Celtic Sea and Galician Shelf. Movement of European hake occurred in both south to north and the reverse direction. Individuals collected in the Celtic Sea appeared to have migrated north from areas around the Iberian Peninsula where they spent their first few months of life (pelagic larval and early juvenile stages). Northward and southward movements were also observed for individuals collected at the Galician Shelf. Some fish collected in this location apparently spent early life stages in a southern Iberian area while another individual seemed to originate from the northern stock as it clustered with the individuals from the Celtic Sea and Armorican Shelf.

Pita et al. (2011) proposed directional gene flow from northern to southern stocks based on the genetic similarity of Porcupine and Galician samples, large recruitment in the southern stock relative to its depleted spawning stock biomass and predominant current directions in the Bay of Biscay. Our results, however, suggested that movement of European hake might occur in both directions. Similarly, hake movement between the Balearic Islands and Sardinia were also bidirectional based on the otolith geochemistry results. Unfortunately we could say little about the extent or direction of probable movements through the Strait of Gibraltar, possibly related to the number of samples used in the otolith chemistry analysis. Analyzing a higher number of samples would increase the probability of possibly identifying migrants given that only a few migrants per generation are necessary to prevent genetic divergence among populations (Palumbi 2003). Nevertheless, our results add to the growing evidence highlighting the artificial nature of the barrier separating the northern and southern stocks of European hake in the northeast Atlantic Ocean and advocate for a re-evaluation of management units in the European hake fishery.

In conclusion, we confirmed otolith geochemistry is a useful technique to assess early life stage dispersal in populations with high gene flow and low genetic divergence (Campana 1999, Thorrold et al. 2001). Nevertheless, to obtain a reliable estimate of dispersal, hake larvae need to be sampled to constrain a baseline dataset for retrospective determination of natal origin of adults. The extent and direction of juvenile and adult movement might be further unraveled with additional otolith chemistry studies and using artificial tags given that successful tag-recapture experiments have been conducted with European hake with the objective of validating growth rate and age estimation based on otolith interpretation (e.g. De Pontual et al. 2003, Piñeiro et al. 2007). The integration of genetic and otolith chemical markers, combined where possible with artificial tagging, shows great promise to resolve population structure and connectivity patterns of European hake at different life stages.

ACKNOWLEDGEMENTS

The authors would like to thank Marie-Laure Bégout, Fátima Cardador, Jim Ellis, Beatriz Guijarro, Matteo Murenu, Carmen Piñeiro, Ignacio Sobrino and Francisco Velasco for providing the otolith and fin tissue samples. This study was funded by 'Fundação para a Ciência e a Tecnologia' (FCT), Pest-OE/MAR/UI0199/2011 and PTDC/MAR/117084/2010. S.E. Tanner was funded with a PhD grant from FCT (SFRH/BD/40313/2007).

LITERATURE CITED

Abaunza P, Mattiucci S, Nascetti G, Magoulas A, Cimmaruta R, Bullini L (2001) Morphometric and meristic variation in European hake, *Merluccius merluccius*, from the Northeast Atlantic and Mediterranean Sea. ICES Document CM 2001/J:01:pp. 20

Abaunza P, Murta AG, Campbell N, Cimmaruta R, Comesaña AS, Dahle G, García Santamaría MT, Gordo LS, Iverson SA, MacKenzie K, Magoulas A, Mattiucci S, Molloy J, Nascetti G, Pinto AL, Quinta R, Ramos P, Sanjuan A, Santos AT, Stransky C, Zimmermann C (2008) Stock identity of horse mackerel (*Trachurus trachurus*) in the Northeast Atlantic and Mediterranean Sea: Integrating the results from different stock identification approaches. Fish Res 89:196-209

Ashford JR, Jones CM, Hofmann EE, Everson I, Moreno C, Duhamel G, Williams R (2005) Can otolith elemental signatures record the capture site of Patagonian toothfish (*Dissostichus eleginoides*), a fully marine fish in the Southern Ocean? Can J Fish Aquat Sci 62:2832-2840

Ashford JR, Arkhipkin AI, Jones CM (2006) Can the chemistry of otolith nuclei determine population structure of Patagonian toothfish *Dissostichus eleginoides*? J Fish Biol 69:708–721

Barnett-Johnson R, Teel DJ, Casillas E (2010) Genetic and otolith isotopic markers identify salmon populations in the Columbia River at broad and fine geographic scales. Environ Biol Fishes 89:533-546

Begg GA, Friedland KD, Pearce JB (1999) Stock identification and its role in stock assessment and fisheries management: an overview. Fish Res 43:1-8

Begg GA, Waldman JR (1999) An holistic approach to fish stock identification. Fish Res 43:35-44

Bergenius MAJ, Mapstone BD, Begg GA, Murchie CD (2005) The use of otolith chemistry to determine stock structure of three epinepheline serranid coral reef fishes on the Great Barrier Reef, Australia. Fish Res 72:253-270

Bradbury IR, Campana SE, Bentzen P (2008) Estimating contemporary early life-history dispersal in an estuarine fish: integrating molecular and otolith elemental approaches. Mol Ecol 17:1438-1450

Campana SE (1999) Chemistry and composition of fish otoliths: pathways, mechanisms and applications. Mar Ecol Prog Ser 188:263-297

Campana SE, Chouinard GA, Hanson JM, Fréchet A, Brattey J (2000) Otolith elemental fingerprints as biological tracers of fish stocks. Fish Res 46:343-357

Campana SE, Valentin A, Sévigny J-M, Power D (2007) Tracking seasonal migrations of redfish (*Sebastes* spp.) in and around the Gulf of St. Lawrence using otolith elemental fingerprints. Can J Fish Aquat Sci 64:6-18

Castro BG (2007) Element composition of sardine (*Sardina pilchardus*) otoliths along the Atlantic Coast of the Iberian Peninsula. ICES J Mar Sci 64:512-518

Cavalli-Sforza LL, Edwards AWF (1967) Phylogenetic analysis: models and estimation procedures. Evolution 32:550-570

De Pontual H, Bertignac M, Battaglia A, Bavouzet G, Moguedet P, Groison A-L (2003) A pilot tagging experiment on European hake (*Merluccius merluccius*): methodology and preliminary results. ICES J Mar Sci 60:1318-1327

Diz AP, Presa P (2008) Regional patterns of microsatellite variation in *Mytilus galloprovincialis* from the Iberian Peninsula. Mar Biol 154:277-286

Estoup A, Garnery L, Solignac M, Cornuet J-M (1995) Microsatellite variation in honey bee (*Apis mellifera* L.) populations: hierarchical genetic structure and test of the infinite allele and stepwise mutation models. Genetics 140:679-695

Excoffier L, Laval G, Schneider S (2005) Arlequin ver. 3.0: an integrated software package for population genetics data analysis. Evol Bioinform Online 1:47-50

Felsenstein J (2005) PHYLIP - Phylogeny Interference Package (version 3.6). Distributed by the author Department of Genome Sciences, University of Washington, Seattle

Feyrer F, Hobbs J, Baerwald M, Sommer T, Yin QZ, Clark K, May B, Bennett W (2007) Otolith microchemistry provides information complementary to microsatellite DNA for a migratory fish. Trans Am Fish Soc 136:469–476

Goudet J (1995) Fstat (vers. 2.9.3): a computer program to calculate F-statistics. J Hered 86:485-486

Guillot G, Mortier F, Estoup A (2005) Geneland: A computer package for landscape genetics. Mol Ecol Notes 5:708-711

Hamer PA, Acevedo S, Jenkins GP, Newman A (2011) Connectivity of a large embayment and coastal fishery: spawning aggregations in one bay source local and broad-scale fishery replenishment. J Fish Biol 78:1090-1109

Hidalgo M, Tomás J, Høie H, Morales-Nin B, Ninnemann US (2008) Environmental influences on the recruitment process inferred from otolith stable isotopes in *Merluccius merluccius* off the Balearic Islands. Aquat Biol 3:195-207

ICES (2011) Report of the Working Group on the Assessment of Southern Shelf stocks of Hake, Monk and Megrim (WGHMM). ICES CM 2011/ACOM:11 pp. 625

Kacher M, Amara R (2005) Distribution and growth of 0-group European hake in the Bay of Biscay and Celtic Sea: a spatial and inter-annual analyses. Fish Res 71:373-378

Lado-Insua T, Pérez M, Diz AP, Presa P (2011) Temporal estimates of genetic diversity in some *Mytilus galloprovincialis* populations impacted by the Prestige oil spill. Cont Shelf Res 31:466-475

Lleonart J, Maynou F (2003) Fish stock assessments in the Mediterranean: state of the art. Sci Mar 67:37-49

Lo Brutto S, Arculeo M, Parrinello N (2004) Congruence in genetic markers used to describe Mediterranean and Atlantic populations of European hake (*Merluccius merluccius* L. 1758). J Appl Ichthyol 20:81-86

Luikart G, Amish SJ, Winnie J, Beja-Pereira A, Godinho R, Allendorf FW, Harris RB (2011) High connectivity among argali sheep from Afghanistan and adjacent countries: Inferences from neutral and candidate gene microsatellites. Conserv Genet 12:921-931

Lundy CJ, Moran P, Rico C, Milner RS, Hewitt GM (1999) Macrogeographical population differentiation in oceanic environments: a case study of European hake (*Merluccius merluccius*), a commercially important fish. Mol Ecol 8:1889-1898

McClelland G, Melendy J, Osborne J, Reid D, Douglas S (2005) Use of parasite and genetic markers in delineating populations of winter flounder from the central and south-west Scotian Shelf and north-east Gulf of Maine. J Fish Biol 66:1082-1100

Mellon-Duval C, De Pontual H, Métral L, Quemener L (2010) Growth of European hake (*Merluccius merluccius*) in Gulf of Lions based on conventional tagging. ICES J Mar Sci 67:62-70

Miller JA, Banks MA, Gomez-Uchida D, Shanks AL (2005) A comparison of population structure in black rockfish (*Sebastes melanops*) as determined with otolith microchemistry and microsatellite DNA. Can J Fish Aquat Sci 62:2189-2198

Morales-Nin B, Moranta J (2004) Recruitment and post-settlement growth of juvenile *Merluccius merluccius* on the western Mediterranean shelf. Sci Mar 68:399-409

Morán P, Lundy C, Rico C, Hewitt GM (1999) Isolation and charaterization of microsatellite loci European hake, *Merluccius merluccius* (Merlucidae, Teleostei). Mol Ecol 8:1357-1358

Murua H (2010) The Biology and Fisheries of European Hake, *Merluccius merluccius*, in the North-East Atlantic. Adv Mar Biol 58:97-154

O'Connell M, Wright JM (1997) Microsatellite DNA in fishes. Rev Fish Biol Fish 7:331-363

Ostermann DR, Curry WB (2000) Calibration of stable isotopic data: an enriched $\delta^{18}O$ standard used for source gas mixing detection and correction. Paleoceanography 15:353-360

Page RDM (1996) TREEVIEW: An application to display phylogenetic trees on personal computers. Comput Appl Biosci 12:357-358

Palumbi SR (2003) Population genetics, demographic connectivity, and the design of marine reserves. Ecol Appl 13:S146-S158

Pawson MG, Jennings S (1996) A critique of methods for stock identification in marine capture fisheries. Fish Res 25:203-217

Perrier C, Daverat F, Evanno G, Pécheyran C, Bagliniere J-L, Roussel J-M (2011) Coupling genetic and otolith trace element analyses to identify river-born fish with hatchery pedigrees in stocked Atlantic salmon (*Salmo salar*) populations. Can J Fish Aquat Sci 68:977-987

Piñeiro C, Saínza M (2003) Age estimation, growth and maturity of the European hake (*Merluccius merluccius* (Linnaeus, 1758)) from Iberian Atlantic waters ICES J Mar Sci 60:1086-1102

Piñeiro C, Rey J, De Pontual H, Goñi R (2007) Tag and recapture of European hake (*Merluccius merluccius* L.) off the Northwest Iberian Peninsula: First results support fast growth hypothesis. Fish Res 88:150-154

Pita A, Pérez M, Cerviño S, Presa P (2011) What can gene flow and recruitment dynamics tell us about connectivity between European hake stocks in the Eastern North Atlantic? Cont Shelf Res 31:376-387

Raymond M, Rousset F (1995) GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. J Hered 86:248-249

Roldán MI, García-Marín JL, Utter FM, Pla C (1998) Population genetic structure of European hake, *Merluccius merluccius*. Heredity 81:327-334

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406-425

Slatkin M (1987) Gene flow and the geographic structure of natural populations. Science 236:787-792

Sturgeon RE, Willie SN, Yang L, Greenberg R, Spatz RO, Chen Z, Scriver C, Clancy V, Lam JW, Thorrold SR (2005) Certification of a fish otolith reference material in support of quality assurance for trace element analysis. J Anal At Spectrom 20:1067-1071

Swan SC, Geffen AJ, Morales-Nin B, Gordon JDM, Shimmield T, Sawyer T, Massut E (2006) Otolith chemistry: an aid to stock separation of *Helicolenus dactylopterus* (bluemouth) and *Merluccius merluccius* (European hake) in the Northeast Atlantic and Mediterranean. ICES J Mar Sci 63:504-513

Tanner SE, Vasconcelos RP, Reis-Santos P, Cabral HN, Thorrold SR (2011) Spatial and ontogenetic variability in the chemical composition of juvenile common sole (*Solea solea*) otoliths. Estuar Coast Shelf Sci 91:150-157

Thorrold SR, Latkoczy C, Swart PK, Jones CM (2001) Natal homing in marine fish metapopulation. Science 291:297-299

Thorrold SR, Jones GP, Hellberg ME, Burton RS, Swearer SE, Neigel JE, Morgan SG, Warner RR (2002) Quantifying larval retention and connectivity in marine populations with artificial and natural markers. Bull Mar Sci 70:291-308

Vignon M, Morat F, Galzin R, Sasal P (2008) Evidence for spatial limitation of the bluestripe snapper *Lutjanus kasmira* in French Polynesia from parasite and otolith shape analysis. J Fish Biol 73:2305-2320

Walther BD, Dempster T, Letnic M, McCulloch MT (2011) Movements of diadromous fish in large unregulated tropical rivers inferred from geochemical tracers. PLOS one 6:1-12

Weir BS, Cockerham CC (1984) Estimating F-statistics for the analysis of population structure. Evolution 38:1358-1370

White C, Selkoe KA, Watson J, Siegel DA, Zacherl DC, Toonen RJ (2010) Ocean currents help explain population genetic structure. Proc R Soc B 277:1685-1694

Woods RJ, Macdonald JI, Crook DA, Schmidt DJ, Hughes JM (2010) Contemporary and historical patterns of connectivity among populations of an inland river fish species inferred from genetics and otolith chemistry. Can J Fish Aquat Sci 67:1098-1115

Yoshinaga J, Nakama A, Morita M, Edmonds JS (2000) Fish otolith reference material for quality assurance of chemical analyses. Mar Chem 69:91-97

Appendix 1. Genetic parameters (number of alleles (A), mean allele size ($\bar{A}$), allele size range (Range A), modal allele size (Modal A), allelic richness (R_S), expected heterozygosity (H_e), fixation index (F_{IS}) (Weir & Cockerham 1984)) of five microsatellite loci analyzed in *Merluccius merluccius* collected at seven locations in the Atlantic Ocean and the Mediterranean Sea. Bonferroni correction for significant departures from the Hardy-Weinberg expectations ($P<0.001$).

		Northern stock		Southern stock			Mediterranean stock	
Locus		Celtic Sea	Armoricain Shelf	Galician Shelf	Portugal	Gulf of Cádiz	Balearic Islands	Sardinia
Mmer-hk3b	A	10	10	14	11	11	10	10
	$\bar{A}$	334.9	335.5	336.2	334.8	334.1	332.4	332.6
	Range A	324-346	328-346	322-348	324-348	324-344	324-344	322-344
	Modal A	330-336	332-336	332-336	332-336	332	332	332
	R_s	9.68	10.00	13.18	10.66	10.79	9.29	9.76
	H_e	0.80	0.85	0.83	0.81	0.76	0.53	0.60
	F_{is}	0.038	0.139	0.001	0.002	0.004	-0.005	-0.013
Mmer-hk9b	A	32	29	34	29	35	36	34
	$\bar{A}$	150.5	144.1	147.5	146.3	150.3	155.4	147.5
	Range A	119-211	109-185	111-203	111-181	109-195	109-211	111-193
	Modal A	133-163	123-151	133-161	133-153	153	155	115-123
	R_s	30.55	29.00	32.67	27.98	33.23	34.06	33.04
	H_e	0.96	0.95	0.96	0.95	0.96	0.96	0.96
	F_{is}	0.028	0.059	0.245*	0.229*	0.219*	0.156*	0.169*
Mmer-hk20b	A	18	17	19	17	18	20	18
	$\bar{A}$	225.78	224.9	225.8	225.9	228.36	222.66	225.6
	Range A	213-249	213-247	213-251	213-249	213-247	211-253	211-253
	Modal A	221	221	221	221-237	221-237	223-239	221
	R_s	17.28	17.00	18.40	16.62	17.46	19.47	17.74
	H_e	0.89	0.90	0.88	0.89	0.92	0.91	0.90
	F_{is}	0.177	-0.016	-0.057	0.070	0.310*	0.005	-0.026

Continuance Appendix 1

		Northern stock		Southern stock			Mediterranean stock	
Locus		Celtic Sea	Armorican Shelf	Galician Shelf	Portugal	Gulf of Cádiz	Balearic Islands	Sardinia
Mmer-hk29b	A	12	13	15	14	13	15	15
	$\bar{A}$	162.98	164.9	157.8	164.1	158.62	162.84	162.6
	Range A	146-172	152-178	146-180	146-180	148-176	140-174	146-184
	Modal A	162	168	168	166	160-166	166	164
	R_s	11.79	13.00	14.57	13.60	12.98	14.58	14.55
	H_e	0.89	0.88	0.88	0.87	0.88	0.89	0.86
	F_{is}	0.400*	0.331*	0.534*	0.525*	0.407*	0.315*	0.467*
Mmer-hk34b	A	17	21	17	19	20	17	18
	$\bar{A}$	128.9	131.8333	127.7	130.88	131.5	131.08	133.6596
	Range A	128	128-136	128-138	130	130-140	130-136	130-140
	Modal A	110-152	108-160	112-156	110-158	106-160	112-152	114-154
	R_s	16.58	21.00	16.65	18.15	19.04	16.63	17.35
	H_e	0.90	0.92	0.90	0.90	0.92	0.90	0.89
	F_{is}	0.051	0.133	0.039	0.123	0.034	0.150	0.102

CHAPTER VII

Population connectivity of *Solea solea* and *Solea senegalensis* over time

Susanne E. Tanner[1], Patrick Reis-Santos[1], Rita P. Vasconcelos[1], Simon R. Thorrold[2] & Henrique N. Cabral[1]

[1]Centro de Oceanografia, Faculdade de Ciências da Universidade de Lisboa, Campo Grande, 1749-016 Lisboa, Portugal
[2]Biology Department MS 50, Woods Hole Oceanographic Institution, Woods Hole, MA 02543, USA

In review in Journal of Sea Research

Population connectivity of *Solea solea* and *Solea senegalensis* over time

ABSTRACT A better understanding of connectivity patterns between estuarine and coastal areas is fundamental towards sustainable management of estuarine associated fish and identification of ecologically important habitats. Otolith geochemistry (Li:Ca, Mg:Ca, Mn:Ca, Sr:Ca, Ba:Ca, Pb:Ca) determined in juveniles of *Solea solea* and *Solea senegalensis* in two year classes was used to determine the estuaries of origin of age 3+ and age 2+ fish sampled off the Portuguese coast. The contributions of estuarine nursery areas to coastal adult populations obtained using maximum likelihood estimation varied significantly over the two years. However, one estuary (Ria Aveiro) consistently contributed a large proportion (30% - 87%) to the adult populations of both sole species while a second estuary (Mira) contributed almost no adults (0% - 3%). Comparison of otolith geochemistry between juveniles from characterized estuarine nurseries and adults indicated that unknown nursery areas may have contributed to the coastal adult populations, particularly in one year class. Nevertheless, the estimated relative contributions, based on otolith geochemistry, successfully identified the estuarine systems that accounted for most of the recruitment to adult stocks in the two years. Ultimately, this information should promote the development of effective conservation strategies and integrated fisheries management plans for these two commercially important species.

Keywords: otolith geochemistry, Soleidae, connectivity, contribution, nursery, adult stock

INTRODUCTION

Many marine fish species use estuaries, bays or shallow coastal areas to complete their life cycles (Beck et al. 2001, Able 2005). Juveniles spend months and up to years in these more sheltered areas before recruiting to adult populations offshore. Movement patterns and connectivity between estuarine and coastal environments have important implications for understanding fish population dynamics, colonization patterns of new habitats and populations' resilience to harvest (Thorrold et al. 2001). Information on connectivity among these habitats is considered a fundamental component towards sustainable management of estuarine associated fishes and identification of ecologically important habitats yet, the rate and timing of movement at different spatial scales is still poorly understood for many marine species (e.g. Gillanders 2002, Secor & Rooker 2005, Rooker et al. 2010).

Quantifying the movement of fishes is a difficult undertaking that, over the decades, has been tackled by a variety of approaches such as artificial and natural tagging techniques (reviewed in Gillanders et al. 2003). Conventional artificial tagging methods are unlikely to be suitable for the assessment of connectivity between juvenile and adult habitats due to the logistical difficulty of tagging juveniles, high juvenile mortality and low recapture rates. Among the natural tagging techniques, otolith geochemistry has demonstrated to be a particularly useful tool to track movement patterns in fish (Thorrold et al. 2002, Elsdon et al. 2008). Otolith geochemical composition has been successfully used to discriminate among juvenile fish collected in different geographical areas (e.g. Vasconcelos et al. 2007a, Clarke et al. 2009, Cuveliers et al. 2010) and to determine the contribution, in terms of recruits, of different juvenile sources to adult populations (e.g. Thorrold et al. 2001, Hamer et al. 2005, Vasconcelos et al. 2008). The metabolic inertness and continuous growth of otoliths allows for these applications (Campana 1999). Calcium carbonate is continuously deposited at the otolith surface, and periodic deposition of protein and aragonite forms daily and annual increment patterns that provide, when combined with chemistry measurements of individual increments, a chronological record of environmental conditions (Campana & Thorrold 2001). Thus, the environmental conditions experienced by adult fish during their juvenile estuarine residency can be assessed by analyzing the portion of the otolith that corresponds to the juvenile life stage.

Understanding connectivity and quantifying the contributions, in terms of recruits, of different estuarine juvenile sources to the coastal adult population is particularly important for species that have commercial value. Since these populations are constantly harvested, the different sources of recruitment need to be identified and their relative importance evaluated. Flatfish fisheries, including the two sole species addressed in the present study, are widely spread along the Portuguese coast and play an important socio-economic role (Teixeira & Cabral 2009). Although flatfishes account for less than 4% in the total fish biomass landed, these species and particularly soles account for almost 11% of the economic value of all landings (source: DGPA - national directorate of fisheries and aquaculture). Common sole (*Solea solea*) and Senegalese sole (*Solea senegalensis*) occur sympatrically 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, occurring during transport from spawning to nursery grounds (Marchand 1991). Juveniles of both species spend up to two years in estuarine nursery grounds before migrating to shelf waters (Quéro et al. 1986, Cabral 2003, Dolbeth et al. 2008) where they reach sexual maturity at 3-4 years of age (Ramos 1982).

The aim of this study was to determine the relative contributions of several estuarine juvenile sources to the coastal adult populations of two commercially important sole species along the Portuguese coast. Furthermore, by assessing the contributions of two different year classes the inter-annual consistency of the importance of juvenile sources was investigated. A previous study established the geochemical baseline values of juvenile *S. solea* and *S. senegalensis* of the 2006 and 2009 year class in the major nursery areas along the Portuguese coast (Tanner et al. 2012). The high inter-annual variability of the otolith geochemical signatures in juveniles of both sole species implied that only adults belonging to the 2006 and 2009 year classes could be retrospectively assigned to the potential source estuaries (Tanner et al. 2012).

MATERIAL AND METHODS

Geochemistry of estuarine nursery areas

Baseline values for otolith geochemistry of estuarine nursery areas in *S. solea* and *S. senegalensis* were characterized previously from juveniles sampled in the major estuarine systems along the Portuguese coast in 2006 and 2009 (Tanner et al. 2012) (Fig. 1). While otolith geochemical values for juvenile *S. solea* and *S. senegalensis* showed strong variability among sampling years, we generally found good discrimination results among estuaries (average cross-validated accuracy of 74%, Tanner et al. 2012).

Sampling of adult soles

Length at age relationships in both sole species are well established for the Portuguese coast (Teixeira & Cabral 2010). We therefore collected age 3+ and age 2+ individuals of *S. solea* and *S. senegalensis* from the 2006 and 2009 year classes, respectively, based on size. Samples were obtained directly from professional fishermen working along the Portuguese coast during winter 2009/2010 and 2011/2012 (Fig. 1). Fish were transported on ice to the laboratory and dissected. Total length of each individual was determined and sagittal otoliths were extracted, cleaned of adhering tissue and stored dry.

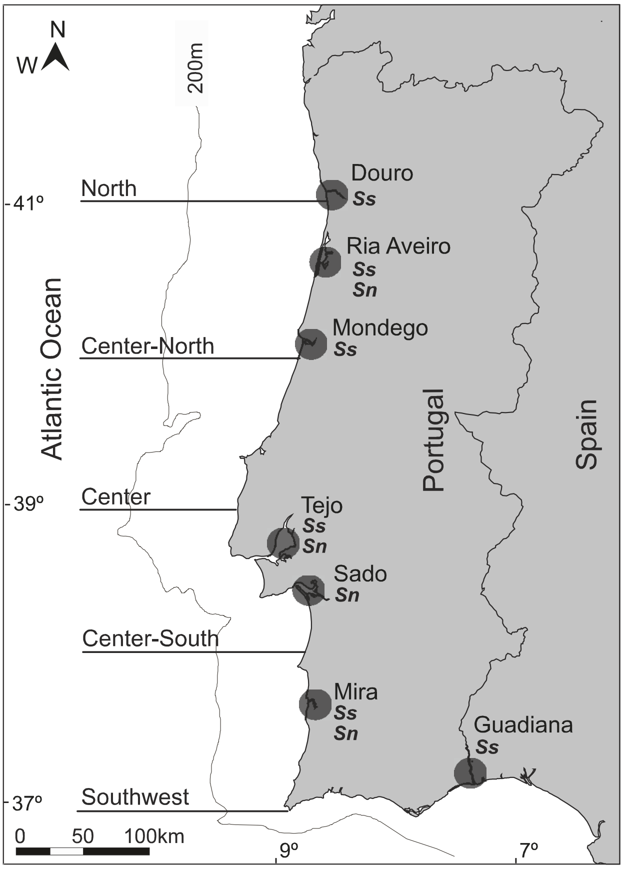

Fig. 1. Sampling of adult *Solea solea* (*Ss*) and *Solea senegalensis* (*Sn*) was carried out in 5 sectors off the Portuguese coast. Also shown estuaries (dark circles) where baseline geochemical signatures were previously obtained for juveniles of the two species (Tanner et al. 2012).

Geochemical analyses of adult otoliths

Preparation and analysis of otoliths from adults followed the same methods as those used to determine the baseline values of otolith geochemical composition in juveniles (Tanner et al. 2012). Briefly, after grinding otoliths to the midplane, possible surface contamination was removed by immersing otoliths in ultrapure water, sonicating for 2 min and triple-rinsing with ultrapure water in a class-100 clean room.

Otolith elemental ratios were quantified by measuring ^{7}Li, ^{25}Mg, ^{48}Ca, ^{55}Mn, ^{63}Cu, ^{88}Sr, ^{138}Ba and ^{208}Pb 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 rasters along the otolith increments that correspond to the juvenile life stage of the adult individuals. 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 µl min^{-1} nebulizer. The analyte was then transported to the ICP-MS via an Ar carrier gas. Instrumental blanks (2% HNO_3) were run at the beginning and end of each set of 10 otoliths. Blank correction of all 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 µg g^{-1}, was used to correct for instrument mass bias. Instrument precision was assessed by running another CRM (Yoshinaga et al. 2000), similarly dissolved and diluted to a Ca concentration of 40 µg g^{-1}. External precision (relative standard deviation) for this CRM (n=100) was as follows: Li:Ca=4.2%, Mg:Ca=5.0%, Mn:Ca=14.6%, Sr:Ca=0.7%, Ba:Ca=4.2%, Pb:Ca=8.7%. Due to low precision of Cu:Ca in the adult sole otoliths, this elemental ratio was excluded from further analyses.

Data analysis

Accurate estimation of relative contributions of different source nursery areas to a mixed stock is based on the assumption that all possible sources have been included in the baseline data (Campana 1999). The juvenile baseline datasets of *S. solea* and *S. senegalensis* included the geochemical signatures of all major nursery areas in estuaries along the Portuguese coast. However; we did not exhaustively sample smaller estuaries along the Portuguese coast due to logistical constraints. To reduce the potential bias of unknown source nursery areas contributing to the adult populations, the geochemistry of juvenile soles were compared to those of adults using principal component analysis (PCA) (Hamer et al. 2005, Chittaro et al. 2009). Adult individuals that fell outside the 95% confidence ellipse around the baseline data (juvenile geochemical values) were excluded from the adult dataset as they likely originated from a nursery area not characterized in the previous study (Tanner et al. 2012).

After excluding these individuals from the adult datasets, maximum likelihood estimation (MLE) approach was used to determine the relative contributions of the different nursery areas to the adult populations of *S. solea* and *S. senegalensis* along the Portuguese coast. Maximum likelihood estimation is preferred because it performs better than classification based estimators in mixed stock situations (Millar 1987, 1990a). All element:Ca ratios were $\log_{10}$ transformed to meet univariate normality. Direct maximum likelihood estimates of mixed-stock proportions and standard deviations were generated in HISEA (Millar 1990b) by bootstrapping with 1000 resampled baseline and adult datasets. The baseline groups for both species and years were individual estuaries with the exception of the 2006 year class of *S. solea*. In the latter, we pooled northern and central estuaries into a Group North due to only small differences in otolith chemistry among estuaries at these locations (Tanner et al. 2012).

RESULTS

Geochemical values in the core of adult otoliths for *S. solea* and *S. senegalensis* were mostly distributed within the 95% confidence ellipses around the baseline data of juveniles (Fig. 2 and 3). However, some differences in the geochemical signatures of adults belonging to the 2006 year class and the juvenile baseline geochemical signatures were observed. We therefore excluded 23% of adult *S. solea* (25 individuals) as they were placed outside the 95% confidence ellipse around the baseline data (Fig. 2a) and 32% of adult *S. senegalensis* (40 individuals) were similarly excluded (Fig. 3a). Otolith geochemical composition of adults and juveniles belonging to the 2009 year class were very similar: only 5 adult individuals (5%) of *S. solea* were excluded as their geochemical signatures differed from the baseline data (Fig. 2b); while 9% (10 individuals) of adult *S. senegalensis* were excluded from the adult data (Fig. 3b).

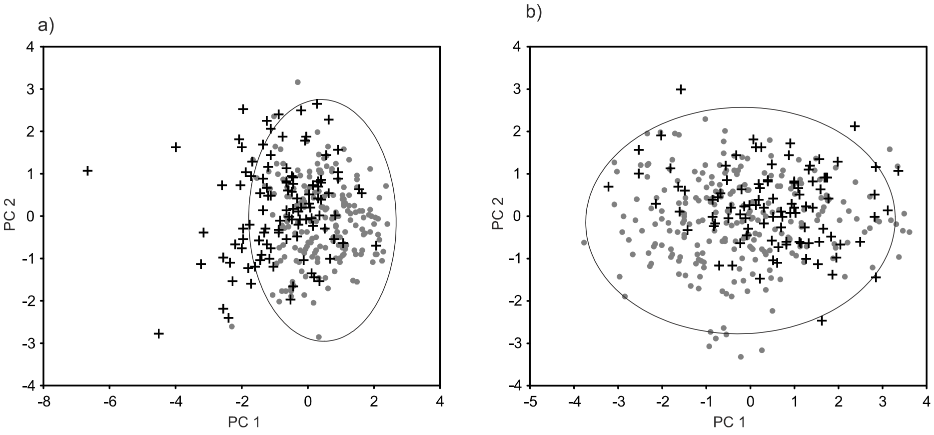

Fig. 2. Principal component plot comparing otolith geochemical composition of adult (mixed-stock group) (+) and juvenile *Solea solea* of known estuarine nursery origin (baseline groups) (●) belonging to the 2006 (a) and 2009 (b) year class. Ellipses are 95% confidence ellipses around the baseline group data.

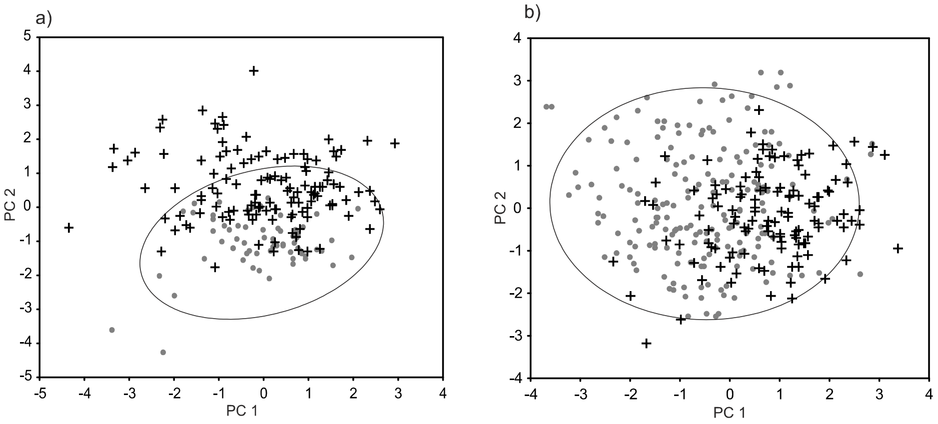

Fig. 3. Principal component plot comparing otolith geochemical composition of adult (mixed-stock group) (+) and juvenile *Solea senegalensis* of known estuarine nursery origin (baseline groups) (●) belonging to the 2006 (a) and 2009 (b) year class. Ellipses are 95% confidence ellipses around the baseline group data.

Estimates of relative contributions of the different nursery areas in the Portuguese estuaries to the mixed coastal populations using MLE showed that for *S. solea* the northern and central estuaries (Douro, Ria Aveiro, Mondego and Tejo) contributed the most in both year classes (Table 1).

Table 1. Relative contributions (%) of the different estuarine nursery areas where *Solea solea* and *Solea senegalensis* occurred to the adult populations captured along the Portuguese coast as determined using maximum likelihood estimation approach. Mean values, standard deviations (between brackets) and total number of adults (*n*) are given.

S. solea						
	Group North[1]	Mira	Guadiana			
year class 2006 *n*=84	88 (7)	1 (1)	11 (7)			
	Douro	Ria Aveiro	Mondego	Tejo	Mira	Guadiana
year class 2009 *n*=95	29 (7)	30 (5)	1 (1)	18 (7)	3 (2)	19 (5)
S. senegalensis						
	Ria Aveiro	Tejo	Sado			
year class 2006 *n*=86	87 (5)	3 (2)	10 (4)			
	Ria Aveiro	Tejo	Sado	Mira		
year class 2009 *n*=104	56 (7)	3 (3)	41 (8)	0 (0)		

[1] Douro, Ria Aveiro, Mondego and Tejo estuary were pooled in Group North

Guadiana, the most southern estuary, accounted for 11% and 19% for the 2006 and 2009 year class, respectively. Mira estuary made only small contributions to adult *S. solea* in both year classes. In detail, adults of *S. solea* belonging to the 2006 year class captured in the northern and central-northern sectors of the Portuguese coast came almost exclusively (98%) from the estuaries of Group North (Douro, Ria Aveiro, Mondego and Tejo) (Fig. 4a). The Group North estuaries contributed approximately two thirds to the adults captured in the central section of the coast while the remaining third originated from Guadiana estuary. The adults collected in the central-southern section of the coast also originated mostly from the Group North estuaries (58%) but with contributions from both the Mira (22%) and Guadiana (20%) estuaries (Fig. 4a). Previously established baseline values for *S. solea* collected in 2009 were sufficiently distinct that estuary specific contributions could be estimated. In the northern sector of the Portuguese coast, 48% of the adults belonging to the 2009 year class originated from the Douro estuary, 17% from Ria Aveiro, another 17% from Guadiana estuary and the remaining from Mondego (10%) and Mira estuaries (8%) (Fig. 4b). Ria Aveiro contributed the most *S. solea* adults captured in the central-northern sector (78%) followed by the Tejo estuary (13%). The contributions of Douro, Mondego and Guadiana to the adults from this sector were minor, ranging from 2% to 4%. Adult *S. solea* captured in the central section of the Portuguese coast mainly originated from the Tejo (47%), Ria Aveiro (26%) and Guadiana (19%) estuaries. In the central-southern section, Douro estuary showed a high contribution (57%) followed by Tejo estuary (28%) while in the southwestern sector of the coast the contributions from Guadiana (52%) and Tejo (26%) were most important (Fig. 4b).

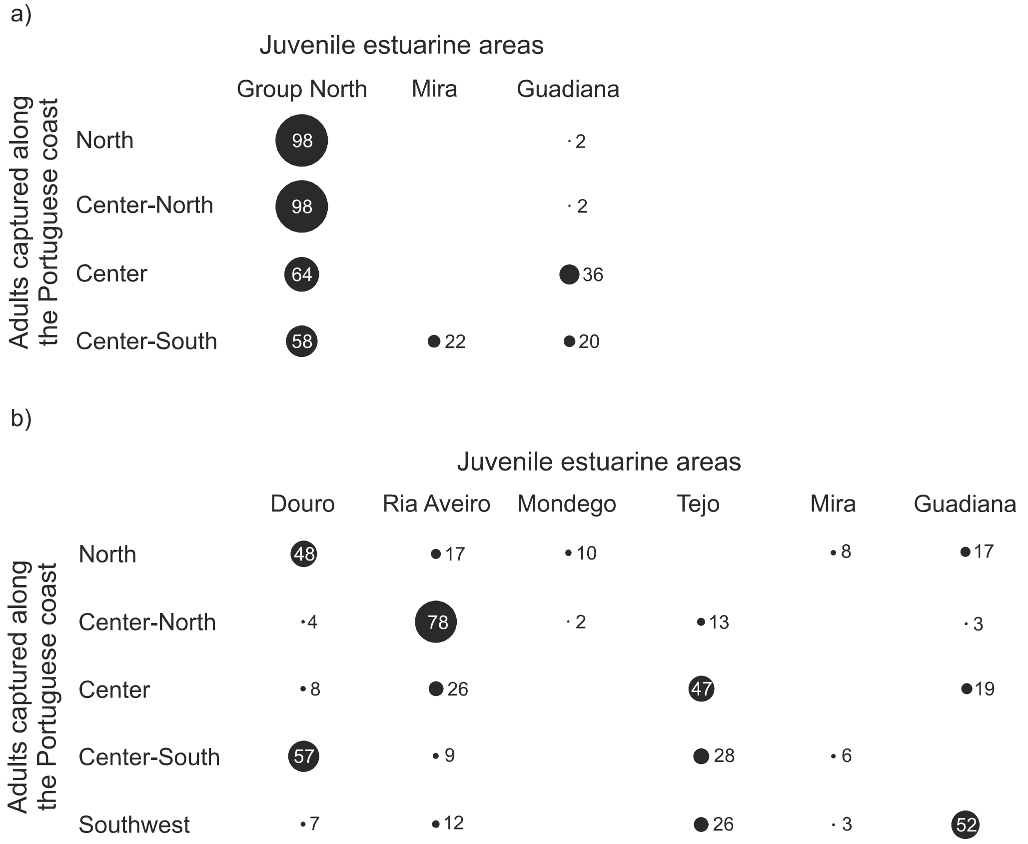

Fig. 4. Relative contributions (%) of the different estuaries where juvenile *Solea solea* occurred to the adult population captured in 5 sectors off the Portuguese coast determined using maximum likelihood estimation approach. Estimated contributions of the 2006 (a) and 2009 (b) year class are shown. Group North includes the Douro, Ria Aveiro, Mondego and Tejo estuaries.

Ria Aveiro clearly contributed the most to the adult population of *S. senegalensis* off the Portuguese coast in both year classes whilst the contribution of the Sado estuary was also considerable for the 2009 year class (Table 1). For the 2006 year class of *S. senegalensis*, Ria Aveiro was the estuarine nursery area contributing most in all sections along the coast, ranging from 78% to 95%, the Tejo estuary contributed 15% to the adult population in the northern sector while its contributions to the remaining sectors were negligible (Fig. 5a). The contributions of Sado estuary were more important in the central, central-southern and southwestern sectors of the coast ranging from 13% to 22% (Fig. 5a). For the 2009 year class of *S. senegalensis*, the contributions of Ria Aveiro were most important in the northern (88%) and central-northern sectors (75%) of the coast (Fig. 5b). In the central, central-southern and southwestern sectors of the coast Ria Aveiro contributed with 42%, 52% and 40%, respectively. Tejo estuary only contributed low numbers to the adult population in the central-southern and southwestern sectors of the coast (5% and 18%, respectively). Contributions from Sado estuary were observed in all sectors along the coast with the most important contributions to the central (58%), central-southern (43%) and southwestern sectors (42%). The contribution of the Mira estuary to the adult population of *S. senegalensis* was insignificant (Fig. 5b).

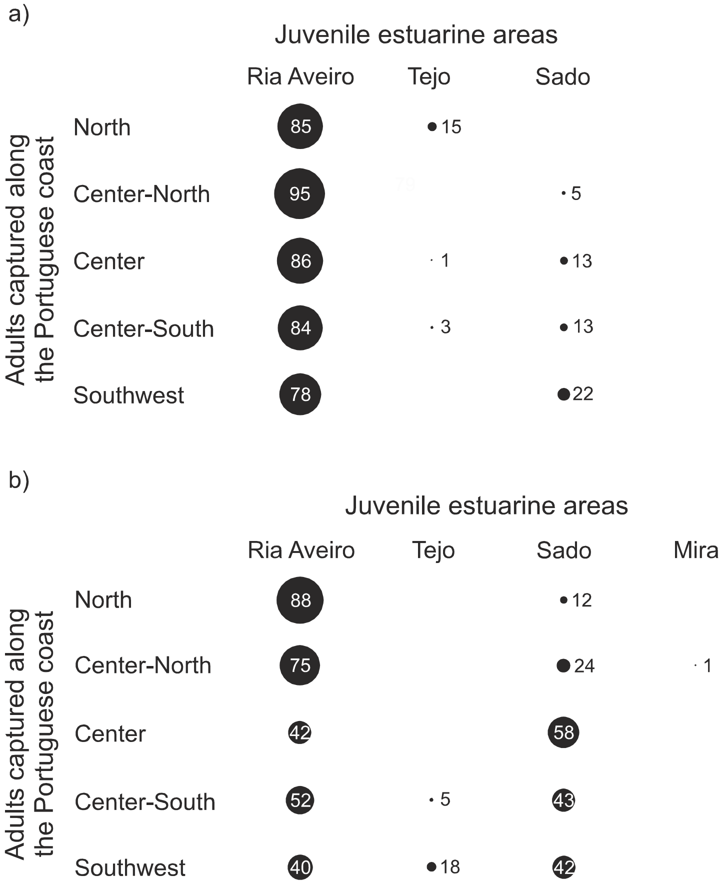

Fig. 5. Relative contributions (%) of the different estuaries where juvenile *Solea senegalensis* occurred to the adult population captured in 5 sectors off the Portuguese coast determined using maximum likelihood estimation approach. Estimated contributions of the 2006 (a) and 2009 (b) year class are shown.

DISCUSSION

Otolith geochemistry proved to be a valuable tool for estimating the contribution of estuarine nursery areas to coastal adult populations in *S. solea* and *S. senegalensis* populations along the Portuguese coast. While these contributions varied over time, particular source estuaries were nonetheless consistently identified as important contributors while others presented negligible contributions in both years analyzed.

A number of adult individuals belonging to the 2006 year class were excluded from the adult datasets of both sole species (23% and 32% of *S. solea* and *S. senegalensis*, respectively) because of geochemical values in otolith cores that were sufficiently different from all baseline data. After leaving juvenile nursery areas and recruiting to the coastal adult populations, 2006 year class adults (age 3+) had more time to disperse along the coast to locations further away from their nursery areas than adults belonging to the 2009 year class (age 2+). The sampled nursery areas constituting the geochemical baseline values of the two year classes were, with few exceptions, identical. The possible existence of alternative nursery areas along the Portuguese coast for the two sole species cannot be discarded. However, the large difference between the two age groups in the number of adults that possibly originated from uncharacterized sources suggested that many of the excluded 2006 year class adults were strays from nursery areas along the Spanish coast to the north and east of Portugal. Adult offshore movements, over several hundred kilometers, are well documented for *S. solea*, particularly in the Bay of Biscay and in coastal waters around the British Isles (Koutsikopoulos et al. 1995, Symonds & Rogers 1995). Furthermore, a recent population genetic structure study in *S. solea* showed that populations were genetically homogenous within hydrogeographic regions in the Northeast Atlantic Ocean (Cuveliers et al. 2012). No studies have assessed adult movements of either sole species along the Iberian coast. Nonetheless, although some of the sampled adults likely originated from uncharacterized sources, the majority of adult *S. solea* and *S. senegalensis* spent their juvenile life stage in one of the estuaries included in the baseline values.

Relative contributions of estuarine nursery areas to the coastal stocks of *S. solea* and *S. senegalensis* varied over the two years under study and between age classes, particularly when considering contributions to specific coastal sectors. Yet, certain estuarine nursery areas along the Portuguese coast showed consistently important or negligible contributions. Mira estuary was the nursery area that least contributed to the coastal adult populations in both year classes of *S. solea* and in the 2009 year class of *S. senegalensis*. This small estuary is considered the least impacted estuary along the Portuguese coast (Vasconcelos et al. 2007b) and supposedly provides favorable conditions for growth and survival of juvenile fish. While juvenile densities of *S. solea* have been reported to be very high in this system (Vasconcelos et al. 2010), this was not reflected in our contribution estimates of Mira estuary. Similarly, Mondego estuary generally had high juvenile densities of *S. solea* (Vasconcelos et al. 2010) but the contributions from this system to the coastal adult population was similarly very low. Ria Aveiro, on the other hand, consistently contributed large proportions to adult populations of both sole species, especially *S. senegalensis*. In contrast to Mira and Mondego estuaries, low densities of juvenile *S. solea* and *S. senegalensis* have been reported in Ria Aveiro (Pombo et al. 2007, Vasconcelos et al. 2010). For *S. solea* populations, Douro, Tejo and Guadiana estuaries showed high to intermediate contributions which is in agreement with juvenile densities found in these systems (Vasconcelos et al. 2010). In the case of *S. senegalensis*, Sado has high juvenile densities and also contributes significantly to adult populations. Alternatively, low contributions of Tejo estuary to adult populations was in contrast to the high densities of juvenile *S. senegalensis* typically found there (Vasconcelos et al. 2010). Our results, therefore, provided little evidence supporting the assumption that areas with dense aggregations of juveniles account for most adult recruits to coastal populations. This mismatch may be related to reduced growth through intraspecific competition in high density areas or increased predation pressure due to associated predator aggregations (Craig et al. 2007, Laurel et al. 2007, Temming et al. 2007). Clearly, further studies are needed to investigate the persistence of these uneven contributions of estuarine nursery areas to coastal adult populations as well as to determine if there are any specific underlying causes to lower contributions of high density areas.

A previous study also estimated connectivity patterns and nursery area origins along the Portuguese coast for same sole species analyzed here (Vasconcelos et al. 2008). Relative contributions of nursery areas to the sub-adult/adult populations in the coastal areas estimated by Vasconcelos et al. (2008) differed significantly from our contribution estimates in both year classes. This previous study determined that for *S. solea* Tejo and Mondego estuaries were the estuarine nursery areas with the highest relative contributions while for *S. senegalensis* Sado and Tejo estuaries together contributed over 75% to the coastal sub-adult/adult population. The large discrepancies between our estimates for two different year classes and the estimates of Vasconcelos et al. (2008) may reflect a high level of inter-annual variability in terms of relative contribution in both sole species. It is also possible that these differences might be related to the different analytical techniques and associated statistical methodologies employed in the two studies. The use of residuals of the otolith geochemical signatures to determine relative contributions to comply with different analytical techniques applied for otoliths of juveniles and adults in Vasconcelos et al. (2008) might have accounted for the large differences in relative contributions of estuarine systems along the Portuguese coast estimated in the two studies. This hypothesis was supported by the fact that relative contributions based on residuals calculated from our datasets yielded completely different contribution estimates compared to those based on $\log_{10}$-transformed element:Ca ratios. The use of two different analytical techniques and the associated statistical methodologies might introduce hardly discernible bias to the estimated contributions. In order to constrain the potential bias in estimated relative contributions from juvenile source areas to the adult populations it is advisable to determine both baseline and mixed datasets using the same analytical technique.

Several studies have used otolith geochemistry to determine relative contributions from juvenile nursery areas to adult populations (Gillanders 2002, Vasconcelos et al. 2008, Wright et al. 2010). However, most of these studies assessed contribution proportions and connectivity patterns for a single year class. There are only few studies that have estimated relative contributions of source areas over several years (Hamer et al. 2005, Chittaro et al. 2009, Hamer et al. 2011). Knowledge of the temporal stability of nursery area contributions to the adult population is critical, especially when constituting the basis for management decisions. Besides assessing the relative contributions and with it the importance of nursery areas for different year classes, adult individuals of different age belonging to the same year class should also be included as this would allow investigating age-related migration patterns, such as movement to spawning or foraging grounds.

Little information is available on the dispersal of early life stages (eggs and larvae) or movement of adults, for *S. solea* and *S. senegalensis* along the Portuguese or Iberian coast. Although local retention of eggs and larvae of marine fish species is likely more common than previously believed (Cowen et al. 2000), early life stages might be passively transported to distant locations before settlement in estuarine nursery areas. Furthermore, the well mixed adult coastal populations sampled in this study indicated a high movement potential in adult *S. solea* and *S. senegalensis*. Future research should focus on more reliably determining the rate and spatial extent of connectivity patterns at early and adult life stages of the two sole species. Nevertheless, the relative contributions estimated based on otolith geochemistry for *S.*

solea and *S. senegalensis* along the Portuguese coast successfully identified the estuarine systems that accounted most for the replenishment of adult stocks in two years. Ultimately, this information should promote the development of effective conservation strategies for these ecologically important habitats and integrated fisheries management plans.

ACKNOWLEDGEMENTS

We would like to thank all involved in fish sampling and S. Birdwhistell for help in sample analysis. This study was funded by 'Fundação para a Ciência e a Tecnologia' (FCT), PEst-OE/MAR/UI0199/2011 and PTDC/MAR/117084/2010. S.E. Tanner was funded with a PhD grant by FCT (SFRH/BD/40313/2007).

LITERATURE CITED

Able KW (2005) A re-examination of fish estuarine dependence: Evidence for connectivity between estuarine and ocean habitats. Estuar Coast Shelf Sci 64:5-17

Beck MW, Heck Jr. KL, Able KW, Childers DL, Eggleston DB, Gillanders BM, Halpern BS, Hays CG, Hoshino K, Minello TJ, Orth RJ, Sheridan PF, Weinstein MP (2001) The Identification, Conservation, and Management of Estuarine and Marine Nurseries for Fish and Invertebrates. BioScience 8:633-641

Cabral H (2003) Differences in growth rates of juvenile *Solea solea* and *Solea senegalensis* in the Tagus estuary, Portugal. J Mar Biol Assoc UK 83:861-868

Campana SE (1999) Chemistry and composition of fish otoliths: pathways, mechanisms and applications. Mar Ecol Prog Ser 188:263-297

Campana SE, Thorrold SR (2001) Otoliths, increments, and elements: keys to a comprehensive understanding of fish populations? Can J Fish Aquat Sci 58:30-38

Chittaro PM, Finley RJ, Levin PS (2009) Spatial and temporal patterns in the contribution of fish from their nursery habitats. Oecologia 160:49-61

Clarke LM, Walther BD, Munch SB, Thorrold SR, Conover DO (2009) Chemical signatures in the otoliths of a coastal marine fish, *Menidia menidia*, from the northeastern United States: spatial and temporal differences. Mar Ecol Prog Ser 384:261-271

Cowen RK, Lwiza KMM, Sponaugle S, Paris CB, Olson DB (2000) Connectivity in marine populations: Open or closed? Science 287:857-859

Craig JK, Rice JA, Crowder LB, Nadeau DA (2007) Density-dependent growth and mortality in an estuary-dependent fish: an experimental approach with juvenile spot *Leiostomus xanthurus*. Mar Ecol Prog Ser 343:251-262

Cuveliers EL, Geffen AJ, Guelinckx J, Raeymaekers JAM, Skadal J, Volckaert FAM, Maes GE (2010) Microchemical variation in juvenile *Solea solea* otoliths as a powerful tool for studying connectivity in the North Sea. Mar Ecol Prog Ser 401:211-220

Cuveliers EL, Larmuseau MHD, Verherstraeten SLNA, Maes GE (2012) Multi-marker estimate of genetic connectivity of sole (*Solea solea*) in the North-East Atlantic Ocean. Mar Biol 159:1239-1253

Dolbeth M, Martinho F, Viegas I, Cabral H, Pardal MA (2008) Estuarine production of resident and nursery fish species: Conditioning by drought events? Estuar Coast Shelf Sci 78:51-60

Elsdon TS, Wells BK, Campana SE, Gillanders BM, Jones CM, Limburg KE, Secor DH, Thorrold SR, Walther BD (2008) Otolith chemistry to describe movements and life-history parameters of fishes: hypotheses, assumptions, limitations and inferences. Oceanogr Mar Biol Annu Rev 46:297-330

Gillanders BM (2002) Connectivity between juvenile and adult fish populations: do adults remain near their recruitment estuaries? Mar Ecol Prog Ser 240:215-223

Gillanders BM, Able KW, Brown JA, Eggleston DB, Sheridan PF (2003) Evidence of connectivity between juvenile and adult habitats for mobile marine fauna: an important component of nurseries. Mar Ecol Prog Ser 247:281-295

Hamer PA, Jenkins GP, Gillanders BM (2005) Chemical tags in otoliths indicate the importance of local and distant settlement areas to populations of a temperate sparid, *Pagrus auratus*. Can J Fish Aquat Sci 62:623-630

Hamer PA, Acevedo S, Jenkins GP, Newman A (2011) Connectivity of a large embayment and coastal fishery: spawning aggregations in one bay source local and broad-scale fishery replenishment. J Fish Biol 78:1090-1109

Koutsikopoulos C, Dorel D, Desaunay Y (1995) Movement of sole (*Solea solea*) in the Bay of Biscay: coastal environment and spawning migration. J Mar Biol Assoc UK 75:109-126

Laurel B, Stoner AW, Hurst TP (2007) Density-dependent habitat selection in marine flatfish: the dynamic role of ontogeny and temperature. Mar Ecol Prog Ser 338:183-192

Marchand J (1991) The influence of environmental conditions on settlement, distribution and growth of 0-group sole (*Solea solea* (L.)) in a macrotidal estuary (Vilaine, France). Neth J Sea Res 27:307-316

Millar RB (1987) Maximum likelihood estimation of mixed stock fishery composition. Can J Fish Aquat Sci 44:583-590

Millar RB (1990a) Comparison of methods for estimating mixed stock fishery composition. Can J Fish Aquat Sci 47:2235-2241

Millar RB (1990b) A versatile computer program for mixed stock fishery composition estimation. Can Tech Rep Fish Aquat Sci 1753:1-29

Pombo L, Rebelo JE, Elliott M (2007) The structure, diversity and somatic production of the fish community in an estuarine coastal lagoon, Ria de Aveiro (Portugal). Hydrobiologia 587:253-263

Quéro JC, Dessouter M, Lagardère F (1986) Soleidae. In: Whitehead P, Bauchot M-L, Hureau J-C, Nielsen J, Tortonese E (eds) Fishes in the North-Eastern Atlantic and the Mediterranean. UNESCO, Paris, pp. 1308-1342

Ramos J (1982) Contribuición al estudio de la sexualidad del lenguado, *Solea solea* (Linneo, 1758) (Pisces, Soleidae). Investigacion Pesquera 46:275-286

Rooker JR, Stunz GW, Holt SA, Minello TJ (2010) Population connectivity of red drum in the northern Gulf of Mexico. Mar Ecol Prog Ser 407:187-196

Secor DH, Rooker JR (2005) Connectivity in the life histories of fishes that use estuaries. Estuar Coast Shelf Sci 64:1-3

Sturgeon RE, Willie SN, Yang L, Greenberg R, Spatz RO, Chen Z, Scriver C, Clancy V, Lam JW, Thorrold SR (2005) Certification of a fish otolith reference material in support of quality assurance for trace element analysis. J Anal At Spectrom 20:1067-1071

Symonds DJ, Rogers SI (1995) The influence of spawning and nursery grounds on the distribution of sole *Solea solea* (L.) in the Irish Sea, Bristol Channel and adjacent areas. J Exp Mar Biol Ecol 190:243-261

Tanner SE, Reis-Santos P, Vasconcelos RP, França S, Thorrold SR, Cabral HN (2012) Otolith geochemistry discriminates among estuarine nursery areas of *Solea solea* and *S. senegalensis* over time. Mar Ecol Prog Ser 452:193-203

Teixeira CM, Cabral HN (2009) Time series analysis of flatfish landings in the Portuguese coast. Fish Res 96:252-258

Teixeira CM, Cabral HN (2010) Comparative analysis of the diet, growth and reproduction of the soles, *Solea solea* and *Solea senegalensis*, occurring in sympatry along the Portuguese coast. J Mar Biol Assoc UK 90:995-1003

Temming A, Floeter J, Ehrich S (2007) Predation hot spots: Large scale impact of local aggregations. Ecosystems 10:865-876

Thorrold SR, Latkoczy C, Swart PK, Jones CM (2001) Natal homing in marine fish metapopulation. Science 291:297-299

Thorrold SR, Jones GP, Hellberg ME, Burton RS, Swearer SE, Neigel JE, Morgan SG, Warner RR (2002) Quantifying larval retention and connectivity in marine populations with artificial and natural markers. Bull Mar Sci 70:291-308

Vasconcelos RP, Reis-Santos P, Tanner S, Fonseca V, Latkoczy C, Günther D, Costa MJ, Cabral H (2007a) Discriminating estuarine nurseries for five fish species through otolith elemental fingerprints. Mar Ecol Prog Ser 350:117-126

Vasconcelos RP, Reis-Santos P, Fonseca V, Maia A, Ruano M, França S, Vinagre C, Costa MJ, Cabral H (2007b) Assessing anthropogenic pressures on estuarine fish nurseries along the

Portuguese coast: A multi-metric index and conceptual approach. Sci Total Environ 374:199-215

Vasconcelos RP, Reis-Santos P, Tanner S, Maia A, Latkoczy C, Günther D, Costa MJ, Cabral H (2008) Evidence of estuarine nursery origin of five coastal fish species along the Portuguese coast through otolith elemental fingerprints. Estuar Coast Shelf Sci 79:317-327

Vasconcelos RP, Reis-Santos P, Maia A, Fonseca V, França S, Wouters N, Costa MJ, Cabral HN (2010) Nursery use patterns of commercially important marine fish species in estuarine systems along the Portuguese coast. Estuar Coast Shelf Sci 86:613-624

Wright PJ, Tobin D, Gibb FM, Gibb IM (2010) Assessing nursery contribution to recruitment: relevance of closed areas to haddock *Melanogrammus aeglefinus*. Mar Ecol Prog Ser 400:221-232

Yoshinaga J, Nakama A, Morita M, Edmonds JS (2000) Fish otolith reference material for quality assurance of chemical analyses. Mar Chem 69:91-97

CHAPTER VIII

General discussion
Future perspectives

General discussion
Future perspectives

GENERAL DISCUSSION

The present study aimed to estimate connectivity patterns and population structure of marine fish using otolith geochemistry. Such estimates rely on a good understanding of the spatio-temporal variability of otolith geochemical signatures, which was examined at different scales. Furthermore, environmental factors potentially affecting element incorporation into otoliths were identified and an integrative approach based on otolith geochemistry and microsatellite DNA markers enhanced our knowledge of population structure and movement patterns in fish populations. Finally, the estimated relative contributions from estuarine source areas to offshore adult populations allowed the identification of the estuaries that most contributed to the replenishment of the stocks in two years.

Otolith geochemical signatures are used in a wide variety of studies in marine ecology and fisheries science, even though many uncertainties still linger on the incorporation of elements into otoliths and the factors affecting elemental uptake and incorporation. Various laboratory studies have addressed these questions focusing on a small variety of elemental and stable isotope ratios (e.g. Sr:Ca, Ba:Ca, Mg:Ca, Mn:Ca, $\delta^{18}O$) (e.g. Farrell & Campana 1996, Thorrold et al. 1997, Bath et al. 2000, Martin & Thorrold 2005). Unarguably, these studies have provided important information on the effects of environmental factors on the geochemical composition of otoliths. However, the feasibility of laboratory experiment-based predictions in wild fish is largely unknown (Elsdon & Gillanders 2005). The high variability of the physicochemical characteristics of the water in estuaries can potentially lead to major differences in findings from laboratory experiments and field studies (Dorval et al. 2007). In our study (Chapter II), six element:Ca ratios and two stable isotopes were assayed simultaneously in otoliths and water, including elements that have not been used frequently in laboratory studies (i.e. Li, Cu). Present results, revealed that Li might be a reliable tracer to reconstruct temperature history in juvenile *Solea solea*. Concentrations of Ba:Ca, $\delta^{13}C$ and $\delta^{18}O$ in otoliths were mainly determined by the respective ambient concentrations and since these concentrations show distinctive mixing patterns along the salinity gradient of the estuary they may be valuable tracers of habitat use in euryhaline environments. Due to the complexity of the estuarine environment as well as the movement patterns of fish, the simultaneous use of multiple geochemical tracers is advisable to accurately reconstruct habitat use and movement of euryhaline fish. Recently, Daverat et al. (2011) identified different colonization tactics of three catadromous species using Gaussian hidden Markov models based on Sr:Ca and Ba:Ca in otolith and ambient water. Yet, more research is needed, including laboratory experiments using this species and others, to confidently determine the utility of these elemental and stable isotope ratios as proxies for temperature or salinity and to assess the physiological and genetic regulation of element incorporation into otoliths. Nevertheless, understanding what are likely complex mechanisms underlying element incorporation into otoliths is not required to use differences in otolith geochemical signatures as group-specific natural markers (Thorrold et al. 1998a).

Knowledge of spatio-temporal variability in otolith geochemical signatures is a premise to use them as natural markers in connectivity or population structure studies. In addition, ontogenetic effects may also contribute to variations in otolith geochemistry even if strong gradients in environmental variables exist among locations (Fowler et al. 1995, de Pontual et al. 2003, Chapter III). The geochemical composition of otoliths varies over very different spatio-temporal scales (e.g. Gillanders 2002, Hamer et al. 2003, Swearer et al. 2003, Mateo et al. 2010) and the spatial range over which different groups of individuals can be discriminated based on otolith geochemical composition depends on the environment they inhabit. Estuaries are particularly amenable for otolith geochemistry studies and fine-scale spatial discrimination, among sites within estuaries, separated by only a few kilometers is possible (e.g. Thorrold et al. 1998b, Gillanders & Kingsford 2003, Chapter III). In contrast, the spatial discriminatory power of otolith geochemical signatures diminishes greatly in more homogenous environments such as the open ocean (Chapter V) and different groups can only be identified over hundreds of kilometers (Rooker et al. 2003, Jónsdóttir et al. 2006, Ashford et al. 2008). Similarly, temporal variability acts at different scales, so that fortnightly, monthly, seasonal and annual variation in otolith geochemical signatures occur (Chapter II and IV). Temporal variation of otolith geochemical signatures cannot be disregarded as it might confound spatial discrimination among groups and lead to inadequate interpretation of geochemical data. The geochemical signatures characterized in juvenile *S. solea* and *Solea senegalensis*, subsequently used to constrain the baseline values for the identification of the nursery estuaries of adults, showed seasonal and annual variation (Chapter IV). The seasonal variations in otolith geochemical composition did not interfere with spatial separation among estuaries. However, based on the seasonal variability found, we advise the development of baseline values that incorporate short-term temporal variation to ensure that the signatures are representative of the year class. Only a few studies have used individuals collected over more than one month to build the geochemical baselines (Thorrold et al. 1998a, Hamer et al. 2003) and rarely encompasses several seasons (Chapter IV). The inter-annual variability in the geochemical baseline values of the two sole species, markedly reflected in the power of spatial separation among estuaries, clearly evidenced that the geochemical baselines are year specific. This confirms the observation that assessing the importance of juvenile source areas to adult populations based on otolith geochemical signatures is a 2-step process (Cook 2011). First, otolith geochemical signatures 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.

In the second step of the process, the relative contributions of estuarine source areas to the adult populations offshore of two sole species (*S. solea* and *S. senegalensis*) over two years were estimated (Chapter VII). Only few studies have estimated connectivity rates between separated subpopulations over more than one year (Hamer et al. 2005, Chittaro et al. 2009, Hamer et al. 2011). In general, the relative contributions of the different source areas to the adult population likely show high inter-annual variability, as was observed in Chapter VII. Hence, estimates of relative contributions from estuarine source areas over more than one year are of paramount importance, particularly when these estimates form the basis for the identification of ecologically important habitats and subsequent conservation strategies. Since knowledge of the temporal stability of the relative contributions is critical, these must be assessed for different year classes. Another possibility to obtain more estimates of relative contributions involves including adult individuals with different age belonging to the same year class. The identification of the estuarine source area of origin of these adults would allow investigating age-related migration patterns, such as movement to spawning or foraging grounds. In the present study, the majority of adult *S. solea* and *S. senegalensis* spent their juvenile life stage in one of the estuaries included in the baseline values rendering the estimated relative contributions for the two sole species meaningful. Nevertheless, the possible existence of alternative juvenile source areas along the Portuguese coast for the two sole species must be contemplated. Further the hypothesis of strayers from source areas, not included in the baseline, along the Spanish coast to the north and east was considered possible. For *S. solea*, adult offshore movements, over several hundred kilometers, are well documented, particularly in the Bay of Biscay and in coastal waters around the British Isles (Koutsikopoulos et al. 1995, Symonds & Rogers 1995). In any case, to our knowledge, little or no information is available on the dispersal of early life stages (eggs and larvae) or on the migration of adults of *S. solea* and *S. senegalensis* in Iberian waters. Although local retention of eggs and larvae of marine fish species, particularly studied in the tropics (Cowen 2002, Almany et al. 2007), is more common than previously assumed (Cowen et al. 2000), early life stages might be passively transported to distant locations before settlement in estuarine nursery areas. The determination of the rate and spatial extent of connectivity patterns at early and adult life stages of the two sole species should be the focus of future research because these life history stages also need to be considered in the elaboration and implementation process of management directives for these commercially important species. Ultimately, information on relative contribution of estuarine source areas to the offshore adult population over more than one year should promote the development of effective conservation strategies for these ecologically important habitats and integrated fisheries management plans.

Population structure is influenced by behavioral and physical processes that act over a range of temporal scales. Therefore, the use of multiple and potentially complementary techniques that integrate information over different scales likely provide the best inference on population structure of fish (Begg & Waldman 1999, Thorrold et al. 2002). However, such integrative approaches have been rarely applied to address population structure (Miller et al. 2005, Bradbury et al. 2008, Woods et al. 2010, Perrier et al. 2011). Estimates of population structure of European hake (*Merluccius merluccius*) were improved through the integration of otolith geochemistry and microsatellite DNA markers in a hierarchical manner (Chapter VI). Otolith geochemistry generally provided information on finer spatial scales than microsatellite markers. Alternatively, microsatellite DNA markers report on gene flow from past or recent evolutionary time scales and therefore act on a broader spatio-temporal resolution. In this instance, Atlantic and Mediterranean populations of European hake were clearly distinguished based on microsatellite markers while otolith geochemistry differed significantly among locations within both regions. Otolith geochemistry is clearly a useful technique to assess early life stage dispersal in populations with high gene flow and low genetic divergence (Campana 1999, Thorrold et al. 2001). Nevertheless, more information on dispersal and movement throughout the life history of this species is necessary, using additional otolith geochemistry studies or artificial tagging techniques whenever possible, to further unravel the extent and direction of movement of European hake. The integration of different natural markers that have different spatio-temporal resolution, such as genetic and otolith chemical markers shows great promise and is an essential step towards resolving population structure and connectivity of many commercially important fish populations.

FUTURE PERSPECTIVES

The present work contributes to the continuing advances in estimating fish population structure and connectivity using otolith geochemistry, determining spatio-temporal variability in otolith geochemical tags, evaluating relationships of environmental factors that affect element incorporation into otoliths and integrating different natural markers to enhance stock structure estimates. Nevertheless, several other issues still need to be addressed in the near future to further disentangle the extent and rate of connectivity between sub-populations of fish.

For the large majority of elements, still very little is known about their incorporation into otoliths and the diverse factors affecting incorporation. More studies, both in laboratory settings and in the field are needed to advance our understanding of why otolith geochemical tags differ and if differences are likely to be found in predictable ways (Elsdon et al. 2008). Further, these studies should include a wide range of species to allow generalizations on the environmental effects on element incorporation.

The present work clearly evidenced the importance of a thorough assessment of the spatio-temporal variability of geochemical signatures in fish otoliths. Variability in otolith geochemistry at different spatio-temporal scales cannot be neglected, especially when the signatures are used to constrain baseline values. Estimated rates of connectivity are only reliable when based on sound markers which encompass the whole spatio-temporal scale considered.

The integration of different natural tags provides independent, but complementary, information on fish population connectivity and structure at different spatio-temporal resolutions. The use of multiple genetic markers that provide insights into contemporary and historical dispersal patterns combined with otolith geochemistry should become more commonly applied in connectivity studies (Woods et al. 2010). In addition, the application of artificial tagging techniques combined with natural markers should further enhance our understanding of how fish populations are connected.

Variations in contribution of source areas, population structure and habitat fragmentation due to human exploitation or environmental changes could have major implications in management and conservation needs. Scenario-based modeling should be used to predict how environmental changes or anthropogenic disturbances may affect the rates of connectivity and population structure as well as the effectiveness of distinct management and conservation directives. Model applications should shed light on the dynamics of fish populations and the consequences of environmental changes and anthropogenic disturbances, predicting population size, source areas, replenishment rates and stability. Ultimately, the output of these modeling approaches results should highlight where management and conservation efforts and resources should be invested more wisely towards greater returns.

LITERATURE CITED

Almany GR, Berumen ML, Thorrold SR, Planes S, Jones GP (2007) Local Replenishment of Coral Reef Fish Populations in a Marine Reserve. Science 316:742-744

Ashford JR, Jones CM, Hofmann EE, Moreno CA, Duhamel G, Williams R (2008) Otolith chemistry indicates population structuring by the Antarctic Circumpolar Current. Can J Fish Aquat Sci 65:135-146

Bath GE, Thorrold SR, Jones CM, Campana SE, McLaren JW, Lam JWH (2000) Strontium and barium uptake in aragonitic otoliths of marine fish. Geochim Cosmochim Acta 64:1705-1714

Begg GA, Waldman JR (1999) An holistic approach to fish stock identification. Fish Res 43:35-44

Bradbury IR, Campana SE, Bentzen P (2008) Estimating contemporary early life-history dispersal in an estuarine fish: integrating molecular and otolith elemental approaches. Mol Ecol 17:1438-1450

Campana SE (1999) Chemistry and composition of fish otoliths: pathways, mechanisms and applications. Mar Ecol Prog Ser 188:263-297

Chittaro PM, Finley RJ, Levin PS (2009) Spatial and temporal patterns in the contribution of fish from their nursery habitats. Oecologia 160:49-61

Cook GS (2011) Changes in otolith microchemistry over a protracted spawning season influence assignment of natal origin. Mar Ecol Prog Ser 423:197-209

Cowen RK, Lwiza KMM, Sponaugle S, Paris CB, Olson DB (2000) Connectivity in marine populations: Open or closed? Science 287:857-859

Cowen RK (2002) Larval dispersal and retention and consequences for population connectivity. In: Sale PF (ed) Coral Reef Fishes, Dynamics and Diversity in a Complex Ecosystem. Academic, San Diego, pp. 149-170

Daverat F, Martin J, Fablet R, Pécheyran C (2011) Colonisation tactics of three temperate catadromous species, eel *Anguilla anguilla*, mullet *Liza ramada* and flounder *Plathychtys flesus*, revealed by Bayesian multielemental otolith microchemistry approach. Ecol Freshw Fish 20:42-51

de Pontual H, Lagardère F, Amara R, Bohn M, Ogor A (2003) Influence of ontogenetic and environmental changes in the otolith microchemistry of juvenile sole (*Solea solea*). J Sea Res 50:199–210

Dorval E, Jones CM, Hannigan R, van Montfrans J (2007) Relating otolith chemistry to surface water chemistry in a coastal plain estuary. Can J Fish Aquat Sci 64:411-424

Elsdon TS, Gillanders BM (2005) Consistency of patterns between laboratory experiments and field collected fish in otolith chemistry: an example and applications for salinity reconstructions. Mar Freshw Res 56:609-617

Elsdon TS, Wells BK, Campana SE, Gillanders BM, Jones CM, Limburg KE, Secor DH, Thorrold SR, Walther BD (2008) Otolith chemistry to describe movements and life-history parameters of fishes: hypotheses, assumptions, limitations and inferences. Oceanogr Mar Biol Annu Rev 46:297-330

Farrell J, Campana SE (1996) Regulation of calcium and strontium deposition on the otoliths of juvenile tilapia, *Oreochromis niloticus*. Comp Biochem Physiol A: Physiol 115:103-109

Fowler AJ, Campana SE, Jones CM, Thorrold SR (1995) Experimental assessment of the effect of temperature and salinity on elemental composition of otoliths using laser ablation ICPMS. Can J Fish Aquat Sci 52:1431-1441

Gillanders BM (2002) Temporal and spatial variability in elemental composition of otoliths: implications for determining stock identity and connectivity of populations. Can J Fish Aquat Sci 59:669-679

Gillanders BM, Kingsford MJ (2003) Spatial variation in elemental composition of otoliths of three species of fish (family Sparidae). Estuar Coast Shelf Sci 57:1049–1064

Hamer PA, Jenkins GP, Gillanders BM (2003) Otolith chemistry of juvenile snapper *Pagrus auratus* in Victorian waters: natural chemical tags and their temporal variation. Mar Ecol Prog Ser 263:261-273

Hamer PA, Jenkins GP, Gillanders BM (2005) Chemical tags in otoliths indicate the importance of local and distant settlement areas to populations of a temperate sparid, *Pagrus auratus*. Can J Fish Aquat Sci 62:623-630

Hamer PA, Acevedo S, Jenkins GP, Newman A (2011) Connectivity of a large embayment and coastal fishery: spawning aggregations in one bay source local and broad-scale fishery replenishment. J Fish Biol 78:1090-1109

Jónsdóttir IG, Campana SE, Marteinsdottir G (2006) Stock structure of Icelandic cod *Gadus morhua* L. based on otolith chemistry. J Fish Biol 69:136-150

Koutsikopoulos C, Dorel D, Desaunay Y (1995) Movement of sole (*Solea solea*) in the Bay of Biscay: coastal environment and spawning migration. J Mar Biol Assoc UK 75:109-126

Martin GB, Thorrold SR (2005) Temperature and salinity effects on magnesium, manganese, and barium incorporation in otoliths of larval and early juvenile spot *Leiostomus xanthurus*. Mar Ecol Prog Ser 293:223-232

Mateo I, Durbin EG, Bengston DA, Kingsley R, Swart PK, Durant D (2010) Spatial and temporal variation in otolith chemistry for tautog (*Tautoga onitis*) in Narragansett Bay and Rhode Island coastal ponds. Fish Bull 108:155-161

Miller JA, Banks MA, Gomez-Uchida D, Shanks AL (2005) A comparison of population structure in black rockfish (*Sebastes melanops*) as determined with otolith microchemistry and microsatellite DNA. Can J Fish Aquat Sci 62:2189-2198

Perrier C, Daverat F, Evanno G, Pécheyran C, Bagliniere J-L, Roussel J-M (2011) Coupling genetic and otolith trace element analyses to identify river-born fish with hatchery pedigrees in stocked Atlantic salmon (*Salmo salar*) populations. Can J Fish Aquat Sci 68:977-987

Rooker JR, Secor DH, Zdanowicz VS, De Metrio G, Relini LO (2003) Identification of Atlantic bluefin tuna (*Thunnus thynnus*) stocks from putative nurseries using otolith chemistry. Fish Oceanogr 12:75-84

Swearer SE, Forrester GE, Steele MA, Brooks AJ, Lea DW (2003) Spatio-temporal and interspecific variation in otolith trace-elemental fingerprints in a temperate estuarine fish assemblage. Estuar Coast Shelf Sci 56:1111-1123

Symonds DJ, Rogers SI (1995) The influence of spawning and nursery grounds on the distribution of sole *Solea solea* (L.) in the Irish Sea, Bristol Channel and adjacent areas. J Exp Mar Biol Ecol 190:243-261

Thorrold SR, Campana SE, Jones CM, Swart PK (1997) Factors determining $\delta^{13}C$ and $\delta^{18}O$ fractionation in aragonitic otoliths of marine fish. Geochim Cosmochim Acta 61:2909-2919

Thorrold SR, Jones CM, Campana SE, McLaren JW, Lam JWH (1998a) Trace element signatures in otoliths record natal river of juvenile American shad (*Alosa sapidissima*). Limnol Oceanogr 43:1826-1835

Thorrold SR, Jones CM, Swart PK, Targett TE (1998b) Accurate classification of juvenile weakfish *Cynoscion regalis* to estuarine nursery areas based on chemical signatures in otoliths. Mar Ecol Prog Ser 173:253-265

Thorrold SR, Latkoczy C, Swart PK, Jones CM (2001) Natal homing in marine fish metapopulation. Science 291:297-299

Thorrold SR, Jones GP, Hellberg ME, Burton RS, Swearer SE, Neigel JE, Morgan SG, Warner RR (2002) Quantifying larval retention and connectivity in marine populations with artificial and natural markers. Bull Mar Sci 70:291-308

Woods RJ, Macdonald JI, Crook DA, Schmidt DJ, Hughes JM (2010) Contemporary and historical patterns of connectivity among populations of an inland river fish species inferred from genetics and otolith chemistry. Can J Fish Aquat Sci 67:1098-1115

Agradecimentos
Acknowledgements
Danksagung

A todas as pessoas que de algum modo contribuiram para o presente trabalho expresso o meu profundo e sincero agradecimento, especialmente:
To all who contributed in some way to the present work, I would like to express my sincere thanks, especially:
Aune, wo i irgendere Form, zu dere Arbeit hei bitreit, möcht i ganz härzlech danke, speziell:

Ao Professor Henrique Cabral, pela orientação, constante disponibilidade e aconselhamento objectivo em todas as fases deste trabalho, pela motivação e pela confiança que sempre me mostrou o caminho a seguir.

To Dr. Simon Thorrold, for the opportunity to work at WHOI. Thank you for all the support, trust and guidance throughout these years.

À Professora Maria José Costa pela oportunidade de integrar a equipa de Zoologia Marinha do Centro de Oceangrafia, FCUL.

Ao CO e à Faculdade de Ciências da Universidade de Lisboa por terem proporcionado as condições necessárias ao meu trabalho científico.

À Fundação pela Ciência e Tecnologia pela bolsa de doutoramento que possibilitou a execução desta tese.

À Doutora Montse Pérez e ao Doutor Pablo Presa por terem aceite de trabalhar comigo numa parte deste trabalho e de me terem apresentado o mundo fascinante da génetica populacional.

À Fátima Cardador, por me ter apoiado em todo trabalho realizado com a pescada e pelas amostras fornecidas da costa portuguesa.

To all researchers that provided samples of European hake, namely Marie-Laure Bégout, Fátima Cardador, Jim Ellis, Beatriz Guijarro, Matteo Murenu, Carmen Piñeiro, Ignacio Sobrino and Francisco Velasco a special thank you.

Aos pescadores, Lino, Mário, João, Manuel, Valdemar, Osvaldo, Mocadas e Mestre Antero, que colaboraram sempre de uma forma pronta e entusiasta nas campanhas de amostragens nos estuários, e também aos encarregados das lotas da Docapesca que possiblitaram as amostragens de adultos ao longo da costa portuguesa.

A todos os meus colegas da Zoologia Marinha do CO, em particular à Catarina G., Catarina V., Célia, Joana O., Marina, Mipá, Noémie, Rita G. e ao Valter pela amizade e ajuda pronta sempre que necessária.

A todos que passaram comigo noites longas e manhã frias a apanhar linguadinhos, Inês, Joana M., Marisa, Miguel, Patrick, Rita V., Sofia, Susana e Vanessa pela vossa ajuda sempre que foi preciso, pela amizade e pela boa disposição. Este trabalho não teria sido possivel sem esta equipa excelente.

A Rita V. e ao Patrick que acompanharam este trabalho de perto e sempre ajudaram em tudo que foi preciso, pela amizade e pelo incentivo.

Aos meus amigos, que me acompanharam ao longo dos anos de doutoramento como também ao longo dos anos aqui em Portugal pela vossa amizade, apoio e boa disposição e pelos fins de dia no Oliveira para desanuviar a cabeça.

A toda família Soares-Ribeiro pela simpatia e pelo apoio durante este trabalho.

Mine Fründe ir Schwyz, wo o us dr Distanz immer für mi sy da gsy, merci.

Mine Eutere, mim Brüetsch, Christian u ar Carine für ihri Ungerstützig us dr Ferni u ihres Vertroue i mi.

And last but not least, ao Lourenço, o meu companheiro e melhor amigo ao longo deste anos, pelo apoio e amor incondicinal e pela confiança. Sem ti não estaria onde estou hoje.

A autora desta tese foi financiada com uma bolsa de doutoramento (SFRH/BD/40313/2007) financiada por fundos nacionais do MCTES atribuida pela Fundação para a Ciência e a Tecnologia.

The author of this thesis was funded with a PhD grant (SFRH/BD/40313/2007) financed by “Fundação para a Ciência e a Tecnologia”.

O projecto onde se integra o presente trabalho foi financiado pela Fundação para a Ciência e a Tecnologia através do projecto BioConnect - Conectividade em populações de peixes estuarinos e costeiros: Marcadores naturais como um instrumento para gestão e conservação (PTDC/MAR/117084/2010).

This study was funded by the “Fundação para a Ciência e a Tecnologia” through the project BioConnect - Connectivity among estuarine and coastal fish populations: Natural tags as a tool for management and conservation (PTDC/MAR/117084/2010).

FCT Fundação para a Ciência e a Tecnologia
MINISTÉRIO DA CIÊNCIA, TECNOLOGIA E ENSINO SUPERIOR Portugal