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Black seabream, *Spondyliosoma cantharus*: stock structure in the eastern Atlantic and characterisation of the biology and fishery in the Portuguese coast

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Ana Margarida Antunes Neves

Tese orientada por:
Prof. Doutor Leonel Serrano Gordo

Documento especialmente elaborado para a obtenção do grau de doutor

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Abstract

In this thesis the stock structure of the black seabream, *Spondyliosoma cantharus*, along the Eastern Atlantic is explored using 4 different methodologies, body morphometry, otolith shape and stable isotopes ratio and genetics (mitochondrial and nuclear markers). Samples were gathered for five European areas (English Channel, Bay of Biscay, Galicia, west Portuguese coast – Peniche, and south Portuguese coast – Algarve), and two areas from African coast (Canary Islands and Angola).

Results from morphometric analyses support the existence of different phenotypic stocks in each sampled area, while stable isotope ratios show fuzzier results with only 3 areas clearly distinguished: Angola, Canary Islands and Bay of Biscay.

Genetic analyses were enhanced with samples from Mediterranean Sea and Cape Verde. A clear structuration is present between regions: North-east Atlantic, Mediterranean Sea, Cape Verde and Angola, not being detected any population structuring within each region; however high levels of private haplotypes for all populations were observed, which can indicate that not enough dispersal/gene flow exists to homogenize more recent mutations at the ecological timescale.

Integrating results from all methods, in a holistic stock structure analyses, the scenario with 6 different stock units in the North-eastern Atlantic is the most probable.

Life history parameters for the species were also evaluated for the west Portuguese coast, since these are key parameters for a conscientious stock assessment.

Maximum age of 17 years was assigned for a specimen with 38 cm. Age at growth was best described with the L_∞ hyperbolic modification of von Bertalanffy curve. Under this model, a change in growth occurs around 8 years, which corresponds roughly to the average age for sex reversal in the species.

The estimated exploitation rate was relatively high (0.43 – 0.62), indicating that although the species is not the main target of the fisheries, its management needs careful attention.

Considering the reproductive strategy of the species, half of the females' population was mature at 18.41 cm and changed sex at 25.62 cm. Sex change takes only a brief period of time, since transitional individuals were scarce and most of them showed oocytes regressing into cystic structures. The species presents a clear indeterminate fecundity type with massive atresia happening at the end of the spawning season. Mean values of 203 oocytes and 5431 oocytes by gram of eviscerated female were estimated for relative batch fecundity and relative annual fecundity, respectively.

Keywords: Sparidae; stock structure; life history parameters; protogyny; Atlantic

Resumo

As pescas são um sector com grande importância económica e social em Portugal e, embora cerca de 370 espécies de peixes sejam comercialmente exploradas a nível nacional, a informação biológica para efeitos de gestão está disponível apenas para um pequeno número, realçando a falta de informação existente para a maioria das espécies capturadas. Um elemento essencial para uma gestão sustentável das pescas é a identificação de mananciais, visto que é sobre esta unidade que os parâmetros da população são avaliados e que as medidas de gestão são aplicadas.

Esta tese tem como tema de estudo a choupa, *Spondyliosoma cantharus*, uma espécie hermafrodita protogínica, que matura primeiro como fêmea mudando posteriormente para macho. Esta espécie tem uma vasta distribuição ao longo do Atlântico Este e Mediterrâneo e, embora seja explorada comercialmente ao longo de toda a sua distribuição geográfica, não existe nenhum estudo sobre a estrutura dos seus mananciais. Neste contexto foi recolhida informação de várias áreas onde a espécie está presente, concretamente, de cinco áreas Europeias (Canal da Mancha, Golfo da Biscaia, Galiza, Peniche e Algarve) e duas da Costa Africana (Ilhas Canárias e Angola), para elucidar a estrutura dos mananciais da espécie ao longo do Atlântico Este.

Neste trabalho foram aplicadas quatro metodologias (morfometria do corpo, forma do otólito, análise dos ratios de isótopos estáveis ($\delta^{13}C$ e $\delta^{18}O$) e análise genética) aos indivíduos de cada área de forma a investigar a existência de mananciais separados. Todas as técnicas foram aplicadas aos mesmos indivíduos, embora nem todos tenham sido analisados com todas as técnicas.

Os resultados obtidos pela análise da morfometria do corpo e da forma do otólito suportam a existência de diferentes mananciais fenotípicos em cada área amostrada, enquanto na análise dos ratios de isótopos estáveis apenas três áreas foram claramente distintas das restantes (Angola, Ilhas Canárias e Golfo da Biscaia).

Na análise genética, foram usados dois marcadores, um mitocondrial, citocromo *b*, e um nuclear, primeiro intrão da proteína ribossomal S7, e foi efetuado um reforço de amostras do Mediterrâneo, da Bélgica e de Cabo Verde. Esta técnica permitiu verificar que a choupa apresenta uma clara estruturação, com quatro regiões bem definidas: Norte Atlântico, Mediterrâneo, Cabo Verde e Angola. Dentro de cada região, não foi detetada estruturação populacional; no entanto a ocorrência de um elevado número de haplótipos

privados em cada população indica que não deverão existir níveis suficientes de dispersão/fluxo de genes para homogeneizar mutações ocorridas mais recentemente à escala ecológica.

Integrando os resultados obtidos pelas várias técnicas, o cenário mais provável parece ser a existência de 6 diferentes mananciais no Nordeste Atlântico.

Para uma gestão apropriada de cada manancial, é vital obter informação sobre os parâmetros de história de vida da espécie. A informação de idade e crescimento permite, por exemplo, calcular taxas de crescimento, mortalidade e recrutamento que serão posteriormente utilizadas em modelos de gestão de pescas. A fiabilidade da informação é por isso assaz importante.

De forma a permitir uma melhor perceção da deposição dos incrementos, a validação do primeiro incremento anual foi feita através do acompanhamento numa área de viveiro, a Lagoa de Óbidos, da coorte do primeiro ano. Foi possível desta forma estimar o comprimento médio total, 7,8 ± 0,9 cm e do raio do otólito, 1,65 ± 0,16 mm, no final do primeiro ano. A validação de idades foi também realizada através da comparação das estimativas de idades de dois leitores independentes. A idade máxima atribuída foi de 17 anos a um indivíduo com 38 cm de comprimento total.

Tendo em conta a estratégia de vida peculiar da espécie (protoginia), vários modelos foram comparados de forma a perceber qual o mais apropriado para descrever o seu crescimento. O que obteve o melhor ajuste aos dados foi a modificação hiperbólica do L_∞ da curva de von Bertalanffy, que incorpora uma alteração do crescimento perto dos 8 anos, que é aproximadamente a idade estimada para a transição de sexo.

Os valores estimados para a taxa de mortalidade natural e mortalidade por pesca, para a costa oeste Portuguesa, foram semelhantes para os dois anos analisados, verificando-se uma taxa de exploração relativamente elevada. De facto, embora a espécie não seja o alvo principal dos pescadores, é capturada com frequência por diversos tipos de artes.

A biologia reprodutiva é outro aspeto crucial para a caracterização da dinâmica populacional da espécie e consequentemente para a sua gestão. Em espécies hermafroditas a avaliação deste parâmetro é particularmente exigente uma vez que o mesmo indivíduo funciona como fêmea e macho em diferentes fases da vida.

De forma a compreender a dinâmica reprodutiva da espécie, foram analisados cortes histológicos da maior parte dos indivíduos tendo, todas as fêmeas durante a época de postura e todos os indivíduos que apresentassem características dúbias sobre os seu estado (em transição ou com estruturas císticas), sido analisados minuciosamente.

Com esta análise detalhada, verificou-se que apenas uma pequena fração dos indivíduos analisados foram considerados em transição, indicando que a mudança de sexo nesta espécie será um processo rápido. O elevado número de estruturas císticas observado, principalmente em espécimes em transição e machos, indicia que estas estruturas devem de alguma forma estar relacionadas com o processo de transição de sexo.

A época de reprodução da espécie foi definida de fevereiro a maio para a costa ocidental Portuguesa. O comprimento à primeira maturação foi estimado em 18,41 cm, correspondendo a uma idade de 3,8 anos, e o comprimento ao qual metade das fêmeas muda para macho aos 25,62 cm, que corresponde a uma idade de 7,94 anos. O facto de terem sido observados machos com 20 cm e fêmeas com 35 cm, parece evidenciar que esta espécie pode apresentar machos primários e fêmeas que não mudam de sexo. No entanto, a amostragem obtida para os intervalos de comprimento que poderiam elucidar esta questão não foi suficiente para se obter uma conclusão substanciada. No que diz respeito à investigação da existência de machos primários, teria de se fazer um esforço de amostragem em indivíduos imaturos e em desenvolvimento, entre 15 e 20 cm, que não são apanhados pela pesca comercial, tornando assim especialmente difícil a clarificação deste tópico.

Um dos parâmetros usados para estudar o potencial reprodutivo de um manancial e essencial para compreender o processo de recrutamento em espécies exploradas comercialmente, é a determinação da fecundidade individual. Para ser possível calcular com rigor este parâmetro, é necessário primeiro determinar o tipo de fecundidade da espécie pelo que, neste contexto, foram seguidos 4 critérios apresentados por Hunter et al. (1992), Greer-Walker et al. (1994) e Murua e Saborido-Rey (2003). Embora estes critérios tenham de ser analisados com atenção, uma vez que para algumas espécies eles apresentem resultados contraditórios, continuam a ser a forma mais fiável de determinar o tipo de fecundidade da espécie. De salientar que, para a choupa, todos os critérios analisados apontaram para o mesmo resultado, ou seja, fecundidade claramente do tipo indeterminado.

Os valores de fecundidade estimados para a espécie foram, em média, de 203 ovócitos por grama de fêmea eviscerada para cada desova, tendo sido estimados 27 eventos de desova por fêmea ao longo da época de postura. Estes valores indicam que a espécie

produz em média um número potencial de descendentes relativamente elevado, de 5431 ovócitos por grama de fêmea eviscerada.

Os dados apresentados neste estudo constituem uma base de trabalho sólida que permitirão, de futuro, uma pesquisa mais aprofundada da pressão piscatória a que a choupa está sujeita. No entanto, numa análise mais empírica, a espécie parece estar, por agora, a ser capturada ainda dentro de limites adequados de exploração.

Palavras-chave: Sparidae; estrutura de mananciais; parâmetros de história de vida; protoginia; Atlântico

Table of Contents

List of Tables

List of Figures

CHAPTER 1

General Introduction

General Introduction

1.1 Fisheries and fish stock structure

Fisheries have been, for a long time, a major source of food for mankind, with high increase of landings until the 80s of last century. By the late 1980s it became clear that fisheries resources could not be sustainable under uncontrolled exploitation and development, and global marine catches have showed a stabilizing trend since then (FAO, 2016). Global fisheries production in 2014 was 93.4 million tonnes, of which 81.5 million tonnes from marine waters, with over 10% of this value caught in the Northeast Atlantic (FAO, 2016). Based on FAO's analysis of assessed stocks there has been a clear diminishing of underfished stocks and the increase of the number of stocks fished at unsustainable levels (overfished).

Portugal has been the third or fourth highest per capita consumer of fish in the world, depending on the years, with a fishing sector traditionally, socially and culturally important (Failler, 2007). The fishing fleet in Portugal consists of three main fleet components: trawl fisheries, purse-seine fisheries and multi-gear fisheries, with a production of 190,594 tonnes in 2016 (INE, 2017). Although approximately 370 fish species are commercially explored in Portuguese waters (Leitão et al., 2014), biological information for management purposes is available for a small number, stressing the lack of information for most of the captured species and the urgency of studies that gather such information.

An essential part of sustainable management of fisheries is the identification of biological fish stocks, as it is the unit at which population assessments are undertaken and management measures are applied (Begg et al., 1999; Abaunza et al., 2008). Determining whether post-juvenile populations of fish remain discrete and independent or whether there is mixing of these fish among different populations is critical to assessing the sustainability of fisheries (Welch et al., 2015).

Despite this knowledge, around 65% of global marine fisheries landings come from unassessed stocks (Ricard et al., 2012; Hilborn and Ovando, 2014), since population structure studies require comprehensive and interdisciplinary analyses, which normally imply the concerted effort of scientists with various research expertises (Fabrizio, 2005;

Cadrin et al., 2010). Such studies use techniques mainly based on phenotypic or genetic variation.

Phenotypic variation between stocks can indicate prolonged separation of postlarval fish in different environmental regimes providing an indirect basis for stock structure (Begg et al., 1999a). Several approaches have been used to inquiry phenotypic differences between populations: i) differences in vital population parameters as manifestation of life history modes to which fish stocks have evolved (e.g. Begg et al., 1999b; Sequeira et al., 2012; Barrios et al., 2017); ii) morphology, accounting for meristics (e.g. Turan et al., 2006; Erguden et al., 2009; Mir et al., 2013) and morphometric landmarks (e.g. Rodriguez-Mendoza et al., 2011; Ibáñez et al., 2017; Geladakis et al., 2018) and morphometric outline (e.g. Vieira et al., 2014; Mahe et al., 2016; Duncan et al., 2018); iii) otolith microchemistry (e.g. Heidemann et al., 2012; Higgins et al., 2013; Moreira et al., 2018); iv) parasites as biological tags (e.g. Sequeira et al., 2010; Klapper et al., 2016; Irigoitia et al., 2017); and v) fatty acid profiles (e.g. Lança et al., 2014; Shen et al., 2018).

The development of electrophoretic methods to study allozyme frequencies, in the last few decades of the 1900s, shifted the emphasis of stock identification from phenotypic to genotypic methods (Cadrin and Secor, 2009). Since then, several molecular genetic markers have been developed and used to infer on fish stock structure. Some of the most commonly used include: i) mitochondrial DNA (e.g. Damasceno et al., 2015; Vieira et al., 2016; Machado et al., 2017); ii) microsatellites (e.g. Charrier et al., 2007; Beacham et al., 2008; Gilbey et al., 2018); and iii) single nucleotide polymorphisms (e.g. Laconcha et al., 2015; Maroso et al., 2016; Westgaard et al., 2017).

However, both environmental and genetic components affect the stock composition and no single technique is able to discriminate between all fish stocks (Begg et al., 1999a). Multiple approaches to stock identification, both genetic and phenotypic, should be applied, ideally on the same samples, and the results compared to achieve an interdisciplinary perspective and increase the probability of correctly identify the stocks (Cadrin and Secor, 2009).

1.2 Black seabream

Fig. 1.1 *Spondyliosoma cantharus* male, during spawning season.

The black seabream, *Spondyliosoma cantharus* (Fig. 1.1), is a protogynous Sparidae fish, developing first as female and changing to male afterwards. It has a wide geographical range (Fig. 1.2), occurring in all Mediterranean and in the Eastern Atlantic from Scandinavia to Angola (Bauchot and Hureau, 1986). It is a gregarious species that is found over seagrass beds and rocky or sandy bottoms in inshore waters, at depths of < 50 m for younger individuals to depths of up to 300 m for adults (Bauchot and Hureau, 1986). Black seabream is highly valued for its sporting and eating qualities, being exploited by both recreational and commercial fishers (Pinder et al., 2016). It is caught by several gear types and it is consumed fresh, frozen or dried-salted; being also reduced to fishmeal and oil (Carpenter, 2016).

Fig. 1.2 Geographical distribution area of *Spondyliosoma cantharus*

1.2.1 Age and growth

Maximum total length (TL) given for the species is 60 cm (Bauchot and Hureau, 1986) although most published works only sampled fishes below 40 cm (Bradai et al., 1998; Pajuelo and Lourenzo, 1999; Gonçalves and Erzini, 2000; Abecasis et al., 2008; Mouine et al., 2011; Boughamou et al., 2015). Ages estimations varied highly among the published studies, from 7 years for a fish with 40 cm TL for Algerian eastern coasts (Boughamou et al., 2015) to 20 years for fishes with 46 cm TL in Bay of Biscay (Soletchnik, 1982). In Portuguese coast the maximum observed age was 13 years, for a 40 cm TL individual (Abecasis et al., 2008). Although it is known that growth pattern differs by area due to biological and ecological factors, such discrepancies most likely also reflect differences in the methodological age readings of the otoliths.

1.2.2 Reproduction

As already mentioned, the black seabream is a protogynous hermaphrodite which has spawning aggregations (Pinder et al., 2017). It has external insemination and eggs are laid in nests constructed and guarded by the males on exposed gravel or sand, with each nest containing several thousands of eggs (Russell et al., 2014; Pinder et al., 2017). During the spawning season a sexual dimorphism occurs, with males showing a darker colouration and blue marks between their eyes.

Reproductive season is mainly between February and May, with a wider extension in Canary Islands, from November to April (Pajuelo and Lourenzo, 1999) and a smaller period, from May to June, in the colder waters of Bay of Biscay (Perodou and Nedelec, 1980).

Females reach maturity around 17.5 cm TL, in warmer waters (Pajuelo and Lourenzo, 1999; Mouine et al., 2011), while 22 cm TL were estimated for Bay of Biscay (Soletchnik, 1982). Sex change is referred by most of the authors to occur mostly between 20 – 30 cm TL, however Perodou and Nedelec (1980) and Soletchnik (1982) refer most individuals changing sex above 30 cm TL. Unfortunately, there is no recent biological parameters assessment to explore the possibility of a reduction of the length at sex change due to fishing pressure.

In Portuguese waters data is only available for Algarve, where the species reproduces from February to April and females attain sexual maturity at 19.98 cm TL with sexual inversion occurring at 24.5 cm TL (Gonçalves and Erzini, 2000).

Although relevant information has been gathered in several areas for the species, almost none of the studies used histological techniques to assure maturation stages and investigate transitional individuals. The use of such technique is most important for reproduction studies and even more in the presence of a hermaphroditic species (Sadovy and Shapiro, 1987; Alonso-Fernández et al., 2011).

1.2.3 Feeding Ecology

The black seabream is an opportunistic feeder with an omnivorous behaviour, adapting its diet to the availability of prey in the environment. In the Southern Portuguese coast, the species inhabiting rocky and patchy bottoms fed mainly on polychaetes and amphipods (Gonçalves and Erzini, 1998) while individuals caught in a shallow water reservoir in the Ria Formosa preyed mainly on highly mobile epifauna such as mysids, cumaceans, ostracods and tanaids (Pita et al., 2002). Polychaetes and amphipods were also the main preys of specimens from Adriatic Sea (Dulčić et al., 2006), with smaller individuals feeding abundantly of ophiuroids.

In Balearic Islands, Box et al. (2009) found 550 invertebrate preys in black seabream stomachs with caridea representing over half of the prey consumed. The authors also evaluated the occurrence in the diet of the invasive Chlorophyte *Caulerpa racemose*, which has been colonising large areas from 0 to 70 m in depth in the Mediterranean, and found its presence in more than 75% of the stomachs at invaded areas while no presence was detected in control areas. *Caulerpa* species have shown some levels of toxicity (Vidal et al., 1984), and the accumulation of caulerpin on the tissues of black seabream may cause impacts in areas where the species is target for human consumption (Felline et al., 2017).

1.2.4 Black seabream fisheries

The black seabream is commercially caught along the Eastern Atlantic Ocean and in the Mediterranean Sea. Data retrieved from The Global Capture Production of Fishery Statistical Collections from FAO (2010-2018) indicates that, almost 10,000 t per year were landed in the last decade, with a major input from the north and central Eastern Atlantic, with an increasing trend since 1980 (Fig. 1.3).

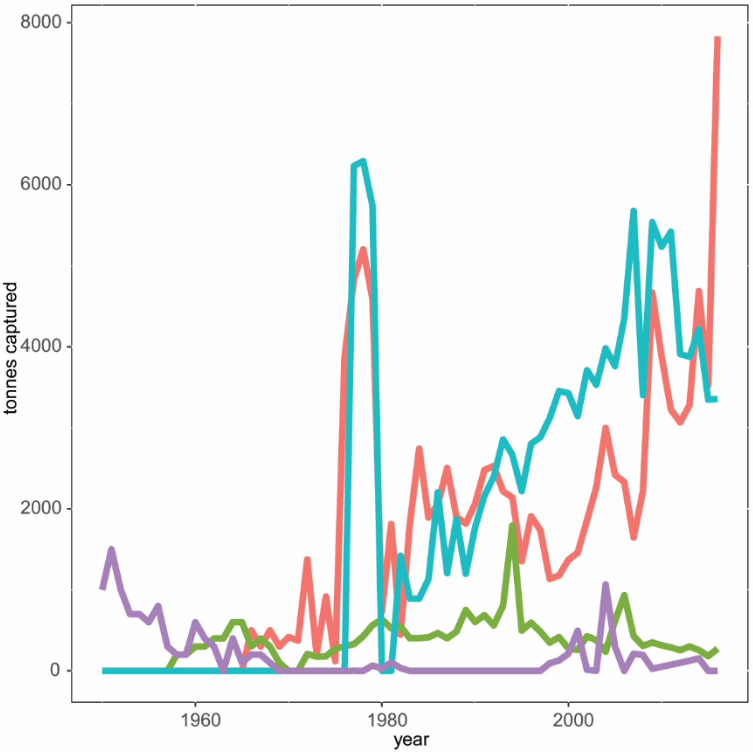

Fig. 1.3 Landings from 1950 to 2015 of the black seabream, *Spondyliosoma cantharus*, for the four regions of its geographical distribution, Northeast Atlantic (blue), Central East Atlantic (red), Southeast Atlantic (purple) and Mediterranean Sea (green). Data retrieved from The Global Capture Production of Fishery Statistical Collections from FAO (2010-2018).

Landings are mainly due to France, with 43%, followed by Senegal with 25%, for the last 35 years.

Portugal has the 6th higher landing values with an average of 170 t in the past two decades. The species is not the main target of fisheries fleets being caught by small-scale fleets which operate in short distances from fishing grounds, usually one day or less. The data provided by the Portuguese authorities (DGRM) shows that landings come from bottom trawl, purse seine and multi-gear fisheries, with the larger values originated by the latter (Fig. 1.4). The black seabream is captured by many multi-gear boats but in small quantities each, whereas trawlers and purse seiners show similar catches of the species.

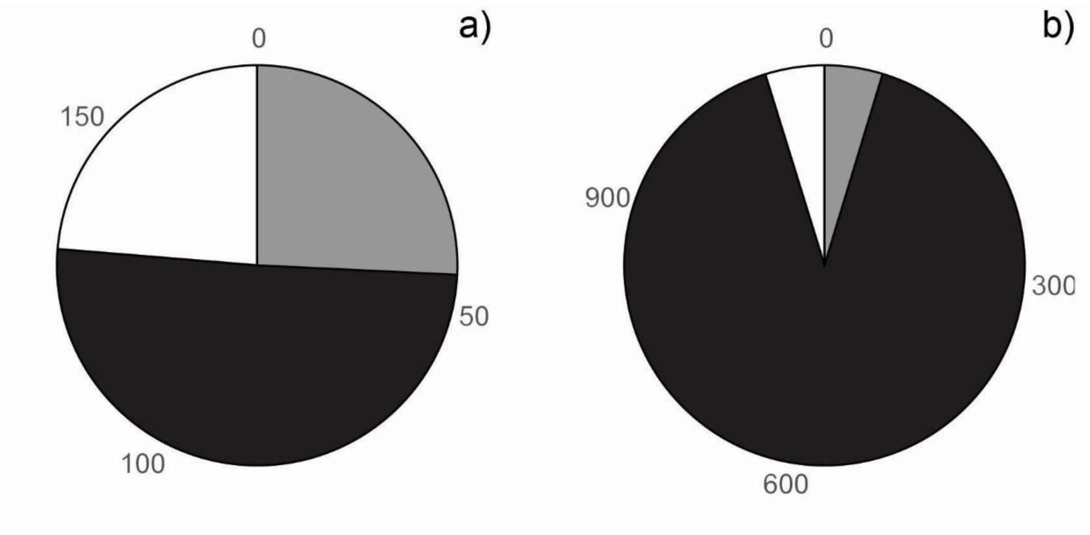

Fig. 1.4 Number of a) tonnes and b) boats, by fishing gear landings in the Continental Portuguese coast for *Spondyliosoma cantharus*. Bottom trawl – white; purse seine – grey; multi gear – black.

The species is landed in all continental Portuguese regions, but with higher values in the centre and south, being Peniche, Quarteira and Setúbal the most representative ports. Landing values show no trend for the last 25 years, with similar values and short oscillations. A larger decrease occurred in the end of the 1990' but returned to the previous values in the beginning of the century (Fig. 1.5). Yearly variability in abundance of captured populations is normal to happen and is influenced by environmental factors such as sea temperature, upwelling and wind regime (e.g. Lloret et al., 2001; Levi et al., 2003; Leitão et al., 2016; Teixeira et al., 2016).

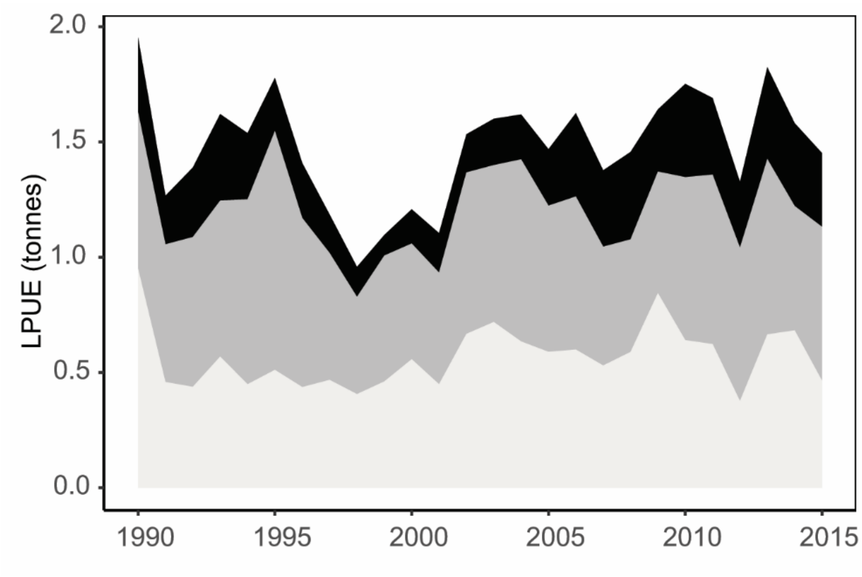

Fig. 1.5 Average landings per month of *Spondyliosoma cantharus* in the North (black), Centre (grey) and South (light grey) Continental Portuguese coast.

1.3 Aims and importance of the thesis

In the present study, data on the black seabream was gathered from several locations to evaluate the stock structure of the species. Stock identification is a central theme in fisheries and should include different methods related to diverse aspects of the stock concept (Coyle, 1998; Cadrin et al., 2014a). Several methodologies were applied to the same individuals, including genetic markers, otolith shape and isotopic ratios and morphometric characters.

Life history parameters information is fundamental to understand and respond to risks in natural populations and ecosystems. Estimations on age and growth, mortality, sex ratio, reproductive season, length at first maturity, length at sex change, fecundity type and annual fecundity were assessed for the Portuguese western coast.

The information gathered with this study intends to improve the data available for commercial fish species and increase the knowledge on species with specific reproduction strategies such as the protogynous hermaphroditism.

1.4 Thesis outline

This thesis is formed by scientific papers published or in revision in peer reviewed international journals, except for the “General Introduction” and “General Conclusion and Final Remarks”. It comprises seven chapters, the first and the last are the General Introduction and General Conclusion and Final Remarks, respectively. Chapters 2, 3 and 4 (section 1) are dedicated to the stock structure of the black seabream along the Eastern

Atlantic; and the chapters 5 and 6 (section 2) address the life history of the species in the Portuguese western coast.

Three papers are present in the section 1. In the first one, differences on the body morphometry along the Eastern Atlantic are explored. The second paper presents the results of otolith shape and stable isotopic ratios analyses regarding the same regions of the first one. Genetic population analysis is discussed in the third paper, with additional data for the Mediterranean Sea and Cape Verde.

The second section is composed by two chapters. In the first information on age, growth and mortality for the western Portuguese coast are compared to previous published data and in the second chapter several aspects of reproduction of the species like the sex-ratio, the reproductive cycle, the length at first maturity and at sex change, the fecundity type and the fecundity estimation, are discussed.

In the last chapter, results for the several techniques are linked and possible conclusions are discussed and the state of the species in the Portuguese waters is also addressed. In final remarks, perspectives on future work on the species are discussed focusing the aspects for which additional information is required.

SECTION 1

Stock Structure

CHAPTER 2

Phenotypic changes in the body of black seabream, *Spondyliosoma cantharus* (Teleostei: Sparidae), along the eastern Atlantic

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Phenotypic changes in the body of black seabream, *Spondyliosoma cantharus* (Teleostei: Sparidae), along the eastern Atlantic

ABSTRACT

Knowledge on population spatial structure is essential in fisheries management. The study of morphological variation patterns in fishes can indicate differences in growth and maturation rates, and thus provide information on group dynamics that should be modelled and managed separately. Identification of intraspecific groups which have different life history features is crucial for understanding population dynamics and achieve a sustainable harvest. The black seabream, *Spondyliosoma cantharus*, has a vast distribution along the eastern Atlantic Ocean and is an important commercial fish, exploited in European waters by recreational and commercial fishers. In this paper, body geometric morphometrics was used in order to discriminate among black seabream geographical populations caught in seven different geographical areas. The results showed the existence of intraspecific groups of black seabream across the eastern Atlantic Ocean, with Canary and Angola having clear distinct morphotypes and at least four populations in the European Coast (English Channel, Galicia, Peniche and Algarve) that present stable body shape differences. These populations should be addressed separately and studies on life history parameters should be enhanced and updated.

Keywords: body shape, eastern Atlantic, geometric morphometrics, hermaphrodite, spatial analysis, *Spondyliosoma cantharus*

2.1 Introduction

The black seabream, *Spondyliosoma cantharus*, is a protogynous Sparidae with a vast distribution along the eastern Atlantic, occurring from Scandinavia to Namibia, around the Madeira, Azores, Cape Verde and the Canary Islands. It is also common in the Mediterranean Sea and the western Black Sea (Bauchot and Hureau, 1986) and is an important commercial fish, exploited in European waters by recreational and commercial fishers (Russell et al., 2014). According to FAO (2010-2017), black seabream landings in Europe have been showing an increasing pattern with an average of nearly 5000 tonnes per year in the past decade. Over 90% of the landings come from France, Spain, United Kingdom and Portugal. Also, in Africa, this species has seen an increase in landings with over 3500 tonnes per year since 2010 (FAO, 2010-2017).

Despite its commercial importance, there is no information on its population structure along the eastern Atlantic. However, the knowledge on population spatial structure is essential in fisheries management and the absence of such data may lead to incorrect assumptions and unexpected risks of overexploitation (Ying et al., 2011).

The morphometric analysis offers a unique perspective for scientists involved in studying marine population structure since it helps to identify intraspecific groups of animals that can be effectively monitored and conserved (Cadrin, 2010). Morphological variation is phenotypic (i.e., influenced both by genetic composition and environmental factors) and the study of morphological variation patterns in fishes can signal possible differences in growth and maturation rates since body form is a product of ontogeny, and groups with different growth or reproductive dynamics should be modelled and managed separately. In this way, geographic patterns in morphology provide a unique perspective on spatial population structure (Cadrin, 2014).

Geometric morphometrics is the statistical analysis of shape variation and its covariation with other variables (Bookstein, 1991). The general geometric morphometrics uses landmark coordinates to extract the shape information and afterwards, patterns of shape changes can be quantified and compared through a standard statistical approach (Adams et al., 2013). One of the key advantages of geometric morphometrics is that shape differences can be visualised directly as graphical representations generated to facilitate biological interpretation (Klingenberg, 2013).

In this paper, geometric morphometrics was used to discriminate among black seabream geographical populations by studying the body morphological characteristics of black seabream caught in seven areas along its eastern Atlantic geographical distribution and thus clarify the population structure by test the hypothesis of the existence of seven different phenotypic populations.

2.2 Material and methods

2.2.1 Fish collection

For this study fishes from seven areas along the eastern Atlantic were collected: English Channel (EN), Bay of Biscay (BI), Galicia, north Spain (GL), Peniche, west coast of Portugal (PN), Algarve, south coast of Portugal (AL), Canary Islands (CN) and Angola (AN) (Fig. 2.1). Specimens from all areas with exception of PN and AL were frozen and sent to the authors in Lisbon by plane through an international shipping service. Portuguese specimens were acquired in the fishing ports and brought to the laboratory where they were frozen.

For all specimens, total length (TL, ±1 mm) and sex were recorded. Average TL was calculated for each area, significant differences were evaluated through the Kruskal-Wallis test and posterior multiple comparisons were evaluated with post-hoc tests according to Dunn. These tests were accomplished with the package PMCMR (Pohlert, 2014) for R (R Core Team, 2015).

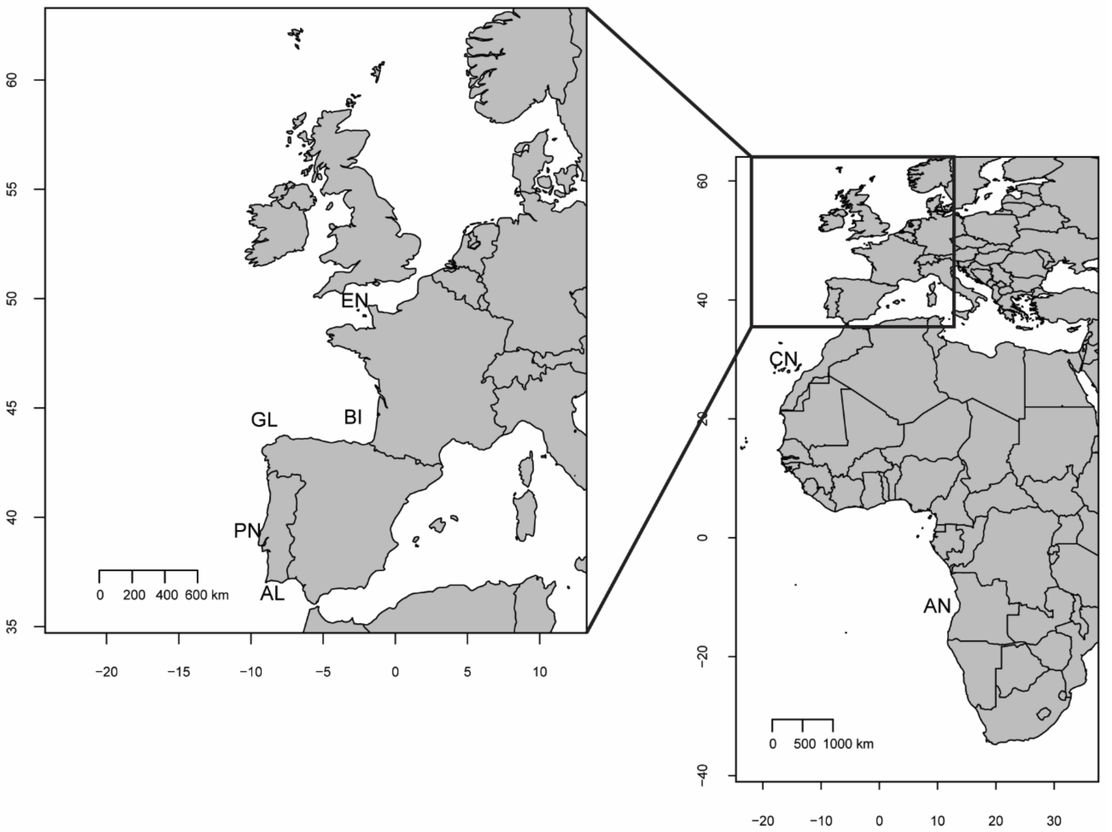

Fig. 2.1 Geographical location of sampling areas of black seabream, *Spondyliosoma cantharus*, along the eastern Atlantic Ocean. (EN – English Chanel; BI – Golf of Biscay; GL – Galicia; PN – Peniche; AL – Algarve; CN – Canary Islands; AN – Angola).

2.2.2 Image Processing

A digital image of each fish was taken using a digital camera (Canon EOS 350D) with a fixed focus 50 mm lens to avoid image distortion. A total of 19 landmarks were selected (Fig. 2.2), 12 of which were marked with entomological pins to aid the accurate location of the points. Each landmark was then recorded for each fish using the software tpsDig version 2.17 (Rohlf, 2013).

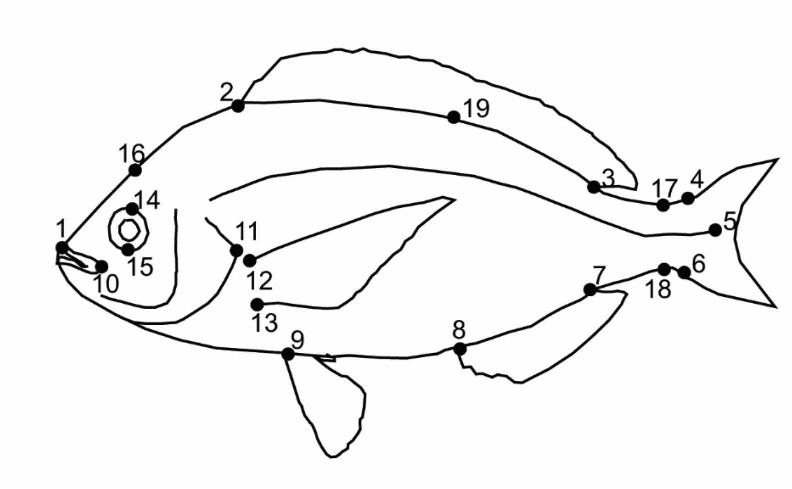

Fig. 2.2 Landmarks used on black seabream, *Spondyliosoma cantharus*, geometric morphometrics analysis. 1 – snout tip; 2 and 3 – anterior and posterior insertion of the dorsal fin; 4 and 6 – superior and inferior insertion of the caudal fin; 5 – end of lateral line; 7 and 8 – posterior and anterior insertion of the anal fin; 9 – insertion of the pelvic fin; 10 – posterior extremity of premaxillar; 11 – point of maximum extension of operculum on the lateral profile; 12 and 13 – superior and inferior insertion of the pectoral fin; 14 and 15 – superior and inferior margin of the eye; 16 – insertion point between vertical axis of eye and superior lateral profile; 17 and 18 – points of maximum curvature of the caudal peduncle; 19 – last spiny ray of the dorsal fin.

2.2.3 Morphometric Analysis

To extract the shape information from the landmarks positions, the unnecessary variation of size, position and orientation was removed using a generalised Procrustes superimposition procedure. The coordinates of landmarks aligned by this procedure should exclusively contain shape variation (Klingenberg, 2010). However, the shape data may still contain a component of size-related shape variation due to the effects of allometry. A multivariate regression using the Procrustes coordinates on centroid size was performed and its significance was evaluated. This procedure divides the variation in the dependent variables into predicted and residual components and since residuals are uncorrelated with the size measure they are the optimal choice to use as size free data (Klingenberg, 2016). These new size free variables were used in the subsequent analyses.

Considering that the black seabream is a hermaphrodite species, being the primary individuals' females and the secondary individuals' males, a preliminary analysis was conducted to verify if the sexual dimorphism within area could be attributed only to allometric effects. Differences between the mean body shapes from a subsample of individuals of both sexes within the same TL range (22cm to 26cm) were evaluated with

Procrustes distances and a permutation test using 10,000 runs to test the null hypothesis of no difference between primary and secondary individuals was performed.

Mean shape differences of black seabream between the seven areas were explored with pairwise comparisons based on Procrustes distances, and the null hypothesis of no difference between areas was tested using a Bonferroni correction for multiple tests for an error rate of 0.05. To promote the interpretation of shape changes of the mean shape variation by area, warped outline drawings were done using the thin-plate spline interpolation function (Bookstein, 1989). These morphometric analyses and visualisations were done in the MorphoJ software package (Klingenberg, 2011).

2.2.4 Multivariate Analysis

Classification of specimens to a particular area was based on Principal Components Analysis (PCA) and Discriminant Analysis (DA). PCA allows reducing the dimensionality of the data since it summarises the major patterns of variation along a few axes that account for as much of the variation in the original data set as possible (Bro and Smilde, 2014). PCA was conducted using the correlation matrix.

Discriminant analysis (DA) was carried out on the PC scores to assess the quality of discrimination among areas. Jackknife cross-validation was used to calculate an unbiased estimation of classification success and the proportion of correctly allocated specimens. The discriminatory effectiveness of the analysis was accessed using the Wilks' lambda (λ) and the Cohen's Kappa statistic (κ), which estimates the correct classification rate adjusted by chance (Titus et al., 1984).

Multivariate analyses were executed with packages MASS (Venables and Ripley, 2002), irr (Gamer et al., 2015) and rrcov (Todorov, 2016) for R (R Core Team, 2015).

2.3 Results

A total of 383 specimens, ranging from 18.1 cm to 38.7 cm were used for the morphometric study. The characterisation of samples from each area is summarised in Table 2.1. Mean TL was similar among areas, however significant differences ($p < 0.0001$) were found between several areas (reported in Table 2.1), and a small (3.2%) but significant ($p = 0.0004$) amount of shape variation was related to size.

Table 2.1 Sample characterisation by geographical area.

Area	N		TL (cm)	
	F	M	mean ± sd	max - min
English Channel (EN)	16	9	24.6 ± 3.9	18.5 – 34.1
Bay of Biscay (BI)	20	7	26.3 ± 4.9 ^{CN}	18.1 – 38.7
Galicia (GL)	38	16	26.5 ± 2.8 $^{AL, CN, PN}$	22.2 – 34.7
Peniche (PN)	54	50	25.3 ± 3.4 $^{CN, GL}$	20.3 – 33.8
Algarve (AL)	15	58	23.9 ± 1.8 $^{AN, GL}$	19.5 – 26.9
Canary Island (CN)	25	31	23.5 ± 1.8 $^{AN, BI, PN, GL}$	20.2 – 28.6
Angola (AN)	13	31	25.6 ± 2.5 $^{AL, CN}$	20.5 – 31.1

Labels are given in brackets next to the area. N – number of individuals sampled (F – female, M – male). Pair comparisons with statistical significant differences in total length composition are given as superscript next to mean and standard deviation (mean ± sd) values.

2.3.1 Morphometric Analysis

No significant differences were detected between the mean body shape for primary and secondary individuals (p value for Procrustes distances > 0.1), and subsequent analyses were performed in the whole sample. The mean body shape of black seabream presented significant differences among the seven areas analysed (Table 2.2).

Table 2.2 Procrustes distances between mean body shapes of black seabream, *Spondyliosoma cantharus*, from the seven study areas.

Area	EN	BI	GL	PN	AL	CN
BI	0.0179 (< 0.0001)					
GL	0.0121 (0.0164)	0.0170 (0.0002)				
PN	0.0129 (< 0.0001)	0.0172 (< 0.0001)	0.0143 (< 0.0001)			
AL	0.0177 (< 0.0001)	0.0137 (< 0.0001)	0.0207 (< 0.0001)	0.0135 (< 0.0001)		
CN	0.0256 (< 0.0001)	0.0268 (< 0.0001)	0.0268 (< 0.0001)	0.0227 (<0.0001)	0.0262 (< 0.0001)	
AN	0.0366 (< 0.0001)	0.0300 (< 0.0001)	0.0381 (< 0.0001)	0.0337 (< 0.0001)	0.0248 (< 0.0001)	0.0470 (< 0.0001)

Areas labels as in Table 1. p-values obtained from permutation tests (10,000 permutation runs) are given between brackets.

The mean body shape of black seabream presented significant differences among the seven areas analysed (Table 2.2). Marked differences from the mean shape were observed for Canary and Angola specimens. The former had a clearly slender body and the later had a forehead strongly arched. The European areas showed smaller differences among them, nevertheless, displacements in the insertion of the dorsal and pelvic fin, snout tip and last spiny ray of the dorsal fin were evident (Fig. 2.3).

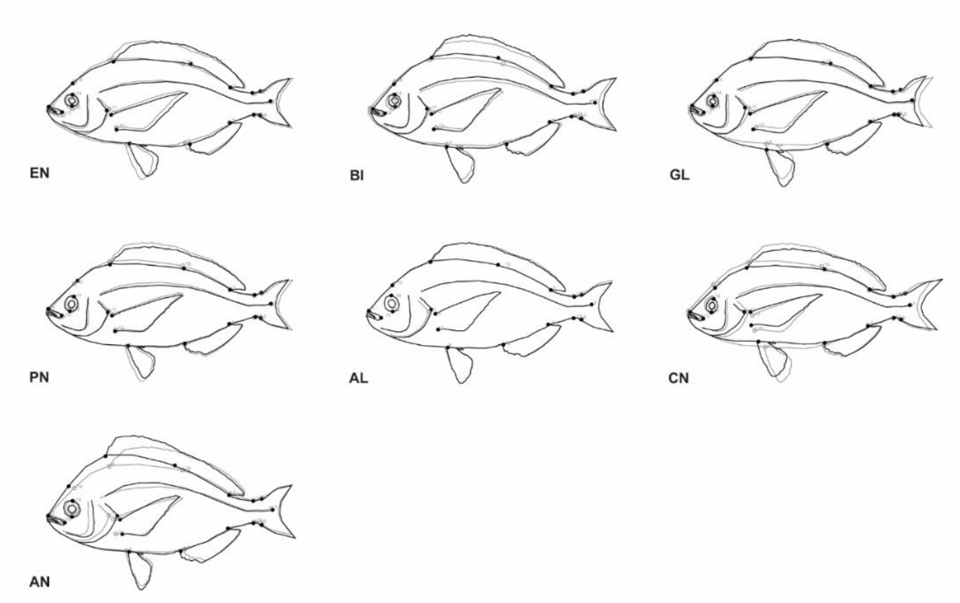

Fig. 2.3 Mean shapes for the black seabream, *Spondyliosoma cantharus*, from different areas (EN – English Channel, BI – Golf of Biscay, GL – Galicia, PN – Peniche, AL – Algarve, CN – Canary Islands, AN – Angola). The figures represent the transformation from the overall mean shape (light grey outline) to the mean shape for each location (black outline). Shape changes from European areas have been exaggerated threefold for better visualisation, while shape changes from Canary Island and Angola areas are onefold exaggerated.

2.3.2 Multivariate Analysis

The first two discriminant functions (DF) explained nearly 75% of the total among-area variance (Fig. 2.4) (Wilks' $\lambda = 0.004$, $p < 0.001$; Cohen's $\kappa = 0.728$, $p < 0.001$). The first DF is related to the height of the body while the second relates to the elongation of the posterior region and displacements in the insertion of the dorsal and

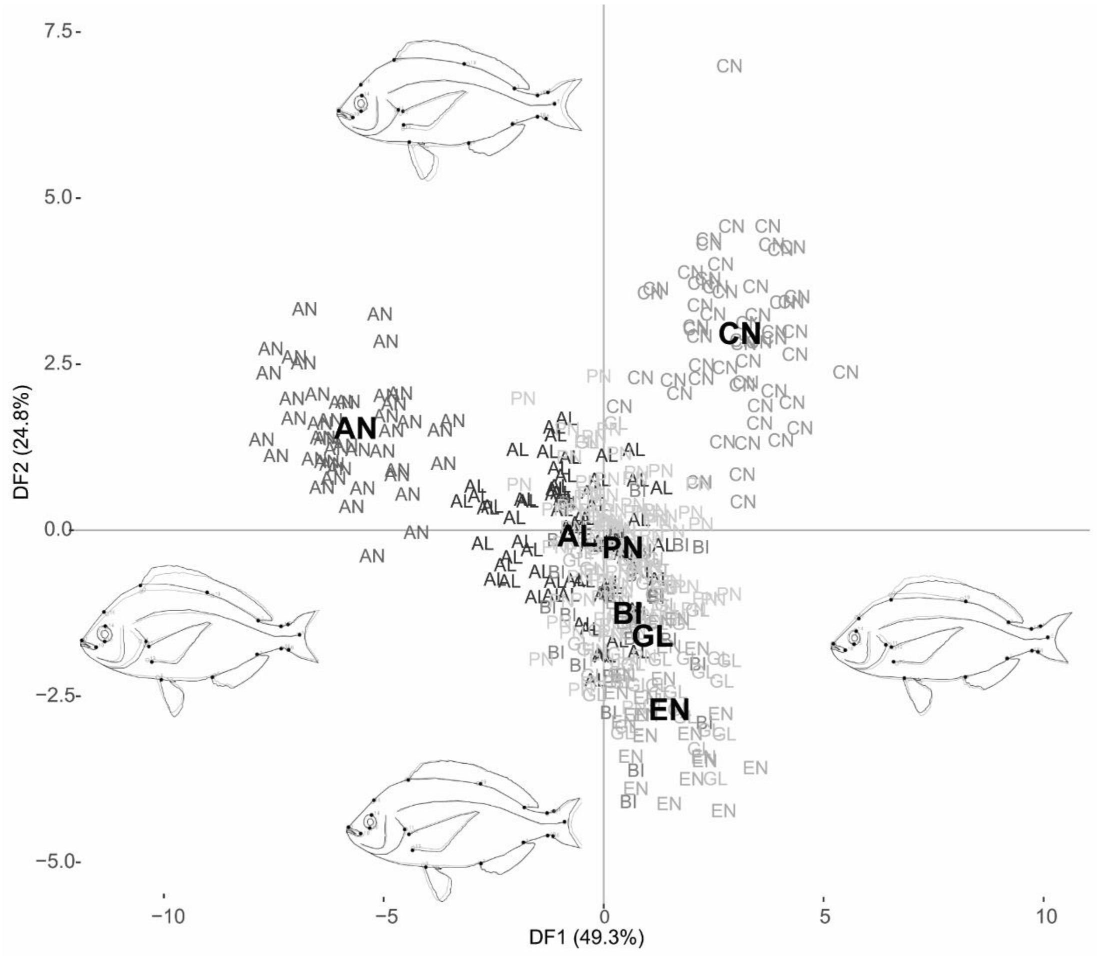

Fig. 2.4 Scatter plot of the scores of black seabream, *Spondyliosoma cantharus*, specimens from the seven locations (EN – English Channel, BI – Golf of Biscay, GL – Galicia, PN – Peniche, AL – Algarve, CN – Canary Islands, AN – Angola) for the first two discriminant functions (DF). Group centroids are marked with bold. Fish images represent extreme shape changes associated with DF1 and DF2.

pelvic fin. Once more clusters from Angola and Canary Island specimens are obvious, while the European areas show some overlap, especially for the first DF. The discriminant analysis assigned 84.9% of the total specimens to the correct geographical area, with very high classification success, above 90%, found for Angola, Canary Island and Peniche (Table 2.3). The Golf of Biscay specimens had the lower classification rate (51.9%), with several individuals assigned to the other European areas, mainly the English Channel and Galicia.

Table 2.3 Jackknifed classification matrix of the discriminant analysis performed on black seabream, *Spondyliosoma cantharus*, specimens from the seven study areas.

Area	EN	BI	GL	PN	AL	CN	AN
EN	**72.0**	0.0	16.0	8.0	4.0	0.0	0.0
BI	11.1	**51.9**	14.8	7.4	14.8	0.0	0.0
GL	5.6	0.0	**77.8**	14.8	1.9	0.0	0.0
PN	1.9	0.0	5.8	**90.4**	1.9	0.0	0.0
AL	2.7	5.5	2.7	5.5	**83.6**	0.0	0.0
CN	0.0	1.8	0.0	3.6	0.0	**94.6**	0.0
AN	0.0	0.0	0.0	0.0	2.3	0.0	**97.7**

Percentages in rows represent the classification into the areas given in columns (correct classification in bold). Overall classification success: 84.9%, Wilks' $\lambda = 0.004$, Cohen's $\kappa = 0.728$. Areas labels as in Table 1.

2.4 Discussion

In the present study, the differences in the body shape of black seabream along the eastern Atlantic were investigated using geometric morphometrics. Morphometric patterns are often associated with geographic differences in growth, maturity, or mortality which are critical to population dynamics (Cadrin, 2010) and if the groups are isolated enough, a structuring pattern of these phenotypic differences can be retrieved. Identification of intraspecific groups which have different life history features is crucial for understanding population dynamics and achieve a sustainable harvest (Cadrin and Silva, 2005). Molecular markers have gained an increased importance in the identification of marine population and fisheries stocks (Valentin et al., 2014), however, the timescale of these markers often fails to reflect the biological characteristics that induce population structure. Phenotypic differences which are temporally stable can be indicative of distinct biological population and offer a practical measure for population discrimination (Cadrin et al., 2010).

A clear isolation for the Canary Island and Angola specimens is reported in this paper, which is expected since they have a high geographic distance from all the other areas. Information on life history parameters also reflect the separation of Canary Islands from European areas, with a reproductive season much wider than for the rest of the areas (Perodou and Nedelec, 1980; Soletchnik, 1983; Pajuelo and Lorenzo, 1999; Gonçalves and Erzini, 2000) and, although the estimated growth rate was similar to those reported by Perodou and Nedelec (1980); Soletchnik (1983) and Abecasis et al. (2008) for Golf of

Biscay, the English Channel and Algarve, respectively, it is higher than the one reported for western Portuguese coast (Neves et al., 2017). Natural mortality rates found for the Canary Islands were also much higher than those reported for the western Portuguese coast (Neves et al., 2017).

Among the European areas, differences in phenotypic patterns were not so evident, although a high percentage of correct assigned specimens was obtained for most of the areas. The exception was the Golf of Biscay that had a large number of individuals assigned to other areas, mainly Galicia and the English Channel, the nearby regions. This can indicate migratory movement among these regions and thus there is no stability of the Golf of Biscay population to achieve a clear phenotypic pattern. The heterogeneity of Golf of Biscay sample, which presented the smallest and biggest specimen caught in this study, could, at a first sight, be an explanation for the low discrimination in this area since during fish growth, from larvae to adulthood, the body relative proportions change as an adaptation to habitat and diet. However, all the fishes sampled had already reached maturity, and therefore there were no ontogenetic shape changes that could influence the morphologic characterisation of this area. The Golf of Biscay has already been reported as a convergent area for Northern and Southern characteristics. Zarraonaindia et al. (2012) found high haplotypic diversity for European anchovy specimens of Golf of Biscay, which presented similar phylogroup frequencies for the English Channel-North Sea group and the East Atlantic Coast group.

Life history information for the black sea bream in the sampled areas is scarce and probably some is already outdated, but the existing data, sort out differences between the reproductive season for the northern areas, English Channel and Golf of Biscay (Perodou and Nedelec, 1980; Soletchnik, 1983) where the sea temperature is colder and the Portuguese coast (Gonçalves and Erzini, 2000; Neves, unpublished data) where the black seabream shows a larger reproductive season. Some differences between the west and the south Portuguese coasts also seem to exist, having the specimens from Algarve a larger size at first maturation (Gonçalves and Erzini, 2000) and a higher growth rate (Abecasis et al., 2008) than those from the west coast (Neves et al., 2017; Neves et al., 2018).

A high correct classification for the black seabream for three very close Portuguese areas was also achieved by Correia et al. (2012) using otolith chemistry. These authors found that even for areas as close as Olhão and Sagres (southern Portuguese coast) a near 100%

of accuracy was achieved, however when the analysis was done on otolith core, the accuracy drop to values below 50%. This points to a stability in the habitat use in juvenile and adult fish, which supports the use of morphometric analysis for population study.

Shape changes among the areas analysed could be visualised by graphical representations, with evident differences in body and head height, fins insertion and snout tip. This easy visualisation in shape changes that enable biological interpretation is one of the main advantages of geometric morphometrics. To improve the interpretability of landmarks relative displacements in the context of their overall arrangement, shape changes should be visualised in conjunction with the original shape (Klingenberg, 2013). This allows a better interpretability of the variation patterns as adaptive significance of morphological features. For example, body form, fin size and fin location are adaptive for movement and manoeuvrability of fishes (Webb, 2006), which indicates differences in the habitat use in the seven areas analysed.

Costa et al. (2010) referred sea temperature and hydrodynamic as some of the environmental forces that can affect fish shape through their effect on growth rate and swimming activity. The sea temperature in the Canary Islands and Angola is much higher than in the European areas, with mean values of 21ºC and 25ºC for the first two and temperatures ranging between 10ºC in the English Channel to 18ºC in Algarve for the European areas (World sea temperature, 2017). If there is a latitudinal gradient in sea temperature that could explain the differences in morphotypes between European and African black seabream specimens and even among the Northern and Southern European areas, hydrodynamics is more difficult to relate to the shape differences found for black seabream populations. A general solution found in fishes for coping with high hydrodynamic systems is a fusiform body shape (Langerhans et al., 2003). Energetic currents and waves, promoting high hydrodynamic habitats, are found in the Canary Islands, West Iberian coast and Golf of Biscay, but only the Canary specimens show a clear fusiform shape, while specimens from the other areas have a deeper body form. Therefore, other issues must be related to these divergent intraspecific adaptations like differences in habitat topography, resources, predation and salinity (Domenici et al., 2008; Langerhans and Reznick, 2010; Antonucci et al., 2012). In volcanic islands, like the Canary Islands, special topographic conditions occur, with narrow shelf and a steep slope, that lead to trophic interactions between various ecological groups of coastal, oceanic, benthic, and benthopelagic organisms (Uiblein and Bordes, 1999). In the Iberian coast, the continental shelf is the narrowest in the Northeast Atlantic margin and is characterized by several extensive submarine canyons, the longest of which is Nazaré Canyon (Aquarone et al., 2008), located about 40 km north of Peniche. A clear marine community boundary located around this Canyon has been reported by Sousa et al. (2005), with distinct fish assemblages for shallow and intermediate depths (< 370m) between north and south of this canyon. The south part has warmer and saltier water, that contrasts with the north coast of Portugal and the Galician Rias where the run-off from several rivers creates, mainly during winter, a low-salinity surface water body (ICES, 2008), the Western Iberia Buoyant Plume (Peliz et al., 2002). These physical, geological and biological differences between Galicia and Peniche habitats, lead to some divergent adaptations enabling a good individualization of the mean body form for each area.

From the results of this and other studies (eg. Cadrin and Silva, 2005; Sequeira et al., 2011; Cronin-Fine et al., 2013) it is clear that the morphometric analysis provides an easy, fast, and economical method to infer on population structure across regions. The results present in this paper show the existence of intraspecific groups of black seabream across the eastern Atlantic Ocean, with at least four populations in the European Coast that present stable shape differences and other two clear groups from the Canary Islands and Angola. These populations should be addressed separately and studies on life history parameters should be enhanced and updated since information on the population structure is essential to accurately model population dynamics and assess and manage fisheries properly.

CHAPTER 3

Otolith shape and isotopic ratio analyses as a tool to study *Spondyliosoma cantharus* population structure

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Ana Neves, Ana Rita Vieira, Vera Sequeira, Rafaela Barros Paiva, Ana Isabel Janeiro, Luis Miguel Gaspar, Leonel Serrano Gordo. *In press.* Marine Environmental Research. DOI: 10.1016/j.marenvres.2018.11.012

Otolith shape and isotopic ratio analyses as a tool to study *Spondyliosoma cantharus* population structure

ABSTRACT

Knowledge on population units is critical for understanding population dynamics and providing an adequate fisheries management. Despite its vast distribution and commercial importance, there is no information on black seabream population structure. Different approaches to otolith analysis were used to elucidate on the stock structure of this species. Seven areas along the eastern Atlantic: English Channel, Bay of Biscay, Galicia (Spain), Peniche (west coast of Portugal), Algarve (south coast of Portugal), the Canary Islands and Angola were compared through elliptical Fourier analysis and oxygen and carbon stable isotopes ratio. Otolith shape analysis data achieved 80% of correct assigned specimens in discriminant analysis while the PERMANOVA conducted on $\delta^{13}C$ and $\delta^{18}O$ values revealed significant differences between all areas, with exception of the two Portuguese areas. However, some mixture in the European areas was observed, especially with isotopes ratio analyses. The results of this study indicate that black seabream probably spends most of its life in the same area and that genetic studies should explore the possibility of some mixture of specimens in the European areas.

Keywords: Sparidae; protogynous; sagittae; shape analysis; spatial distribution; stable isotopes;

3.1 Introduction

The recognition of self-sustaining elements within a natural fish population is a central theme in fisheries science (Cadrin et al., 2014a). The knowledge on stock, and population structure is fundamental for a sustainable management of fisheries since these are the assessment units for which management measures will be applied. It is necessary to accurately identify such structures as failures may lead to incorrect assumptions and unexpected risks of overexploitation (Stephenson, 1999; Hutchinson, 2008). However, determining stock structure is extremely challenging as it requires understanding the demographic consequences of fish movement over potentially large spatial scales across complex oceanographic and inshore environments (Fowler et al., 2017). It is therefore advocated to apply a holistic approach to stock identification so that information on multiple spatial and temporal scales can be integrated (Secor, 2014).

Fish otoliths are acellular and metabolically inert, composed mainly of calcium carbonate (~97%) in a noncollagenous organic matrix, deposited continuously throughout the lifetime of the animal reflecting the aquatic environments that fish experienced during its life (Campana, 1999; Kerr and Campana, 2014). In this way, otolith's shape and stable isotopes ratios are strongly influenced by factors such as diet, growth rates, and ontogenetic and physiological processes (Høie et al., 2003; Cardinale et al., 2004; Mille et al., 2016). The exposure to different patterns of such factors will originate different shape and chemical fingerprints among fish groups (Cardinal et al., 2004; Kerr and Campana, 2014) and as long as populations or stocks of fish inhabit different environments, such signatures can serve as a predictor for stock identity (Campana, 2005). The analysis of otolith morphology, morphometry and chemical composition has already proven to be a useful tool for stocks differentiation of important commercial species such as Atlantic cod (Campana and Casselman, 1993), Mediterranean horse mackerel (Turan, 2006), horse mackerel (Stransky et al., 2008) and European anchovy (Bacha et al., 2014).

The black seabream, *Spondyliosoma cantharus*, is a protogynous Sparidae species with a vast distribution along the eastern Atlantic, occurring from Scandinavia (rare) to Namibia, around the Madeira, Azores, Cape Verde and the Canary Islands. It is also common in the Mediterranean Sea and the western Black Sea (Bauchot and Hureau, 1986) and is an important commercial fish, exploited in European waters by recreational and commercial fishers (Russell et al., 2014). According to FAO (2010-2017), black seabream landings in Europe have been showing an increasing pattern with an average of nearly 5000 tonnes per year in the past decade. Over 90% of the landings come from France, Spain, United Kingdom and Portugal. Also, an increase in the landings from Africa has been noticed, with over 3500 tonnes per year since 2010 (FAO, 2010-2017). The black seabream exhibits life history characteristics that make it particularly vulnerable to local over-exploitation: it is a hermaphrodite slow growing, long-lived, and exhibits habitat specificity during the spawning season, being targeted by fisheries when it forms aggregations around nesting areas (Russel et al., 2014; Pinder et al., 2017). Nurseries of the species are found in shallow waters mainly in lagoon systems and estuaries (Bussotti and Guidetti, 2011; Gonçalves et al., 2013). Despite its commercial importance, it is not subject to ICES stock assessment or any assessment by the General Fisheries Commission for the Mediterranean and there is no information on its population structure along the eastern Atlantic.

In the present study, otoliths collected from seven areas along the black seabream geographical distribution were compared through their shape and carbon and oxygen isotopic ratios, and the population structure of the species in the eastern Atlantic was investigated by combining the results of the two approaches and testing the hypothesis of the existence of seven different phenotypic populations.

3.2 Material and methods

3.2.1 Fish collection

For this study, fishes from seven areas along the eastern Atlantic were collected: English Channel (EN), Bay of Biscay (BI), Galicia, north Spain (GL), Peniche, west coast of Portugal (PN), Algarve, south coast of Portugal (AL), Canary Islands (CN) and Angola (AN) (Fig. 3.1). Specimens from all areas with exception of PN and AL were frozen, placed in boxes and sent by courier, after assuring that it would not be any logistical problems to transport the frozen samples, to the authors in Lisbon. Samples from Portugal were acquired in fishing ports and brought to the laboratory where they were frozen. For all specimens, total length (TL, ± 1 mm) and sex were recorded.

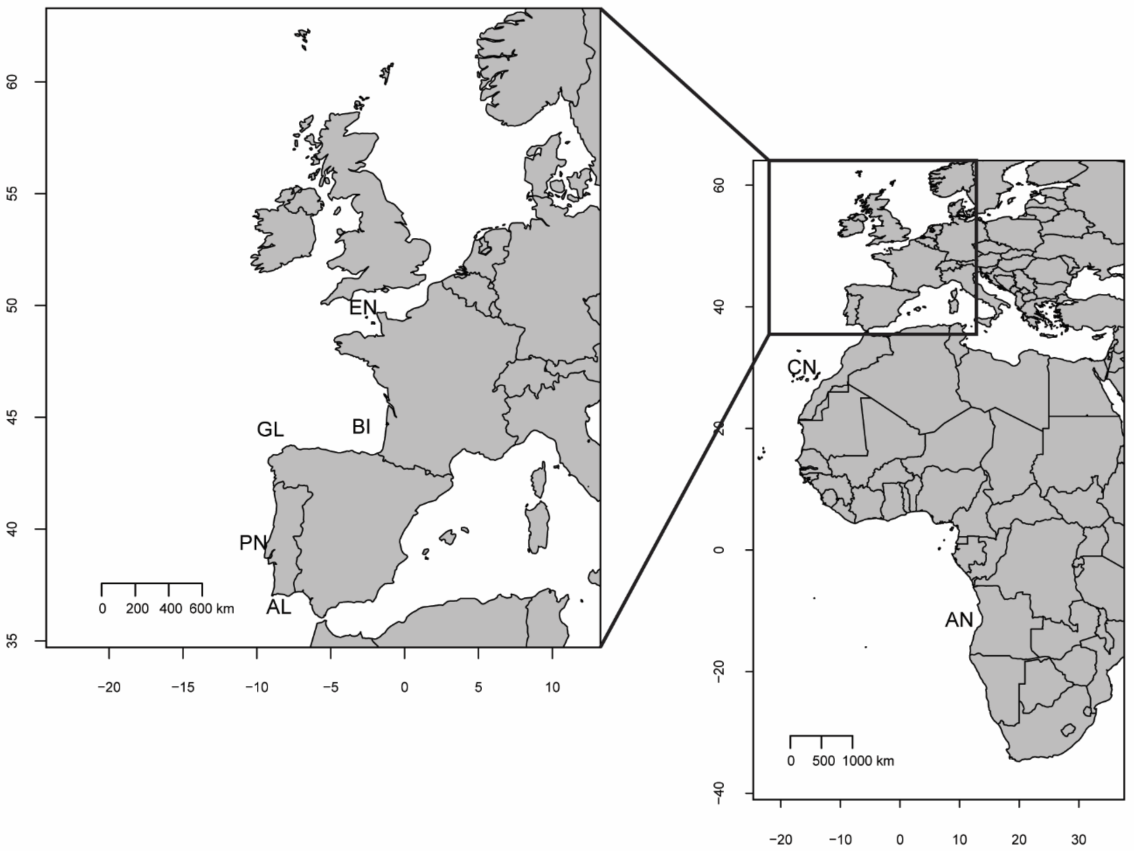

Fig. 3.1 Geographical location of sampling areas of black seabream, *Spondyliosoma cantharus*, along the eastern Atlantic Ocean. (EN – English Chanel; BI – Bay of Biscay; GL – Galicia; PN – Peniche; AL – Algarve; CN – Canary Islands; AN – Angola).

Whenever macroscopic assigned of sex was doubtful, histological preparations were analysed for sex confirmation. Sagittal otoliths (hereafter referred to as otoliths) were removed, rinsed with water, air dried and stored in labelled plastic vials. Ages of individuals were assigned by reading the right otolith immersed in glycerine-ethanol (1 : 1) to improve the clarity of annual increments, with a binocular microscope (magnification: 18×) under reflected light against a dark background according to Neves et al. (2017).

Average TL was calculated for each area and significant differences of the sampled lengths between areas were evaluated through the Kruskal-Wallis test and posterior multiple comparisons were evaluated with post-hoc tests according to Dunn. These tests were accomplished with the package PMCMR (Pohlert, 2014) for R (R Core Team, 2015).

3.2.2 Image and shape analysis

Digitized images for each right otolith were captured using a video camera (Leica DFC290) linked to a Wild stereomicroscope (6× magnification). The contour of the otolith from its digital image was extracted with the ChainCoder package from the SHAPE 1.3 program (Iwata and Ukai, 2002) which stores the relevant information as chain-codes. The files with this information were then imported to Momocs package (Bonhomme et al., 2014) in R (R Core Team, 2015) where the ensuing morphometric analyses were executed.

To select the minimum number of harmonics for the best reconstruction of the otolith outline, the level of 99% accumulated variance was adopted as suggested by Stransky et al. (2008). A random subsample of 10 otoliths for each area was used to define the adequate number of harmonics needed for the analysis. Once the number of needed harmonics was defined, the elliptical Fourier analysis (EFA) was performed and normalization was achieved by using the parameters of the first harmonic so that all coefficients were invariant to size, rotation and starting point of the outline trace (default for Momocs EFA). This resulted in the normalised elliptic Fourier (NEF) coefficients a_n, b_n, c_n and d_n and since the three first coefficients of the first harmonic are consumed by this process they were excluded from all subsequent analyses.

Considering that the black seabream is a hermaphrodite species, being the primary individuals' females and the secondary individuals' males, a preliminary analysis was conducted to verify if the sexual dimorphism within area could be attributed only to allometric effects. Differences between the mean otolith shapes from a subsample of individuals of both sexes within the same TL range (22cm to 26cm) were evaluated with multivariate analysis of variance (MANOVA). Due to the lack of an adequate number of individuals of both sexes within this TL range for the other areas, the analysis was conducted using only PN (46 males and 48 females) and CN (36 males and 29 females) samples.

Discriminant analysis (DA) was carried out to assess the quality of discrimination among areas. Jackknife cross-validation was used to calculate an unbiased estimation of classification success and the proportion of correctly allocated specimens. The discriminatory effectiveness of the analysis was accessed using the Wilks' lambda (λ) and the Cohen's Kappa statistic (κ), which estimates the correct classification rate adjusted by chance (Titus et al., 1984). Multivariate analyses were executed with package MASS (Venables and Ripley, 2002) in R (R Core Team, 2015).

3.2.3 Stable isotope analysis

For each sample area, twelve left otoliths were selected from fish with ages between 4 and 6 years. Although in ideal conditions the stable isotope analysis should be applied to fish with the same age, the lack of enough individuals of the same age to apply this technique force the use of adult fish within a range of ages between 4 and 6 years. To remove the organic matter, the otoliths were soaked in 10% hydrogen peroxide, sonicated, rinsed with ultrapure water and oven dried at 60 ºC for 24 hours. Each otolith was ground with a pestle and mortar into a fine powder. Subsamples of about 50 μg from this homogenised material were used for oxygen and carbon stable isotopes determinations ($\delta^{13}C$ and $\delta^{18}O$) using Dual–Inlet Isotope Ratio Mass Spectrometer hyphenated with a Multiprep carbonate reaction device at Laboratory of Stable Isotopes at Geology Department, Faculty of Sciences, University of Lisbon. The reaction time of the carbonate powder with 100% phosphoric acid was 20 minutes and the temperature was set at 70 ºC. The isotopic values were normalized to the international standard NBS-19 and reported to VPDB (Vienna Pee Dee Belemnite). The precision was better than 0.1 per mil for both carbon and oxygen isotopes (McCrea, 1950; Bastow et al., 2002).

Analysis of covariance (ANCOVA) was used to test for differences among locations (fixed factor), with otolith weight as a covariate. To avoid that differences in otolith mass among populations could confound any stock-specific differences in isotopes composition, the observed values were replaced by the regression residuals when ANCOVA test was significant (Campana et al., 2000).

The relationship between temperature and salinity with $\delta^{18}O$ and $\delta^{13}C$ was investigated by scatterplots visualization and linear correlation (Steer et al., 2010; Correia et al., 2011). Information on sea surface temperature (SST) and salinity (Sal) was achieved using GODAS data provided by the NOAA/OAR/ESRL PSD, Boulder, Colorado, USA, from their website at http://www.esrl.noaa.gov/psd/. The mean monthly values recorded from 2010 to 2015 were used to calculate the values used in the analyses.

The spatial variation in the stable isotopes of black seabream was evaluated with PERMANOVA (Permutational ANOVA, Anderson, 2001). $\delta^{13}C$ and $\delta^{18}O$ values were normalised and the resemblance matrix was based on Euclidean distance. PERMANOVA was based on a single fixed factor (area) and on 1000 permutations. *A posteriori* multiple comparisons of means were made using the pairwise tests code developed by Arbizu (2017). K-nearest neighbour cross-validatory classification (knn.cv) was used to assess the accuracy of specimens' assignment. The knn is a non-parametric method that does not require multi-variate normality of the data or the assumption of equal group variance-covariance matrices. Equal prior probabilities in the group assignment during each analysis were assumed. The performance of models based on the 5, 7, and 9 nearest neighbors was evaluated (Gao et al., 2013). All statistical analyses were performed using R (R Core Team, 2015) with packages vegan (Oksanen et al., 2014), class (Venables and Ripley, 2002) and ggplot2 (Wickham, 2009).

3.3 Results

3.3.1 Shape analysis

No significant differences were detected between the mean otolith shape for primary and secondary individuals (F = 1.23, p = 0.1052), being the subsequent analyses performed in the whole sample.

Table 3.1 Summary table of black seabream, *Spondyliosoma cantharus*, collected along the eastern Atlantic Ocean.

Area	Sample date	N		TL (cm)		Estimated age range (years)	Otolith Mass(mg)
		Shape	Isotopes	Shape	Isotopes	Isotopes	Isotopes
EN	Apr 2014	30	12	$24.0^{\text{GL AN}}$ (4.1)	25.8 (1.2)	4-6	42.93 (6.49)
BI	Nov 2014	26	12	25.9^{CN} (4.3)	24.1 (1.5)	4-6	38.00 (6.00)
GL	Mar 2014	58	12	$26.3^{\text{EN PN AL CN}}$ (2.6)	25.4 (0.6)	5-6	46.56 (8.31)
PN	Mar - Dec 2014	104	12	$25.2^{\text{CN GL}}$ (3.4)	24.4 (0.7)	6	47.01 (8.30)
AL	July 2014	72	12	$23.8^{\text{GL AN}}$ (1.9)	25.0 (0.4)	6	37.64 (4.74)
CN	Mar 2014	91	12	$23.4^{\text{BI GL PN AN}}$ (1.8)	25.2 (0.7)	4-6	40.61 (7.43)
AN	Sep 2015	44	12	$25.6^{\text{EN AL CN}}$ (2.5)	24.7 (1.0)	5-6	36.84 (7.36)

Mean and standard deviation (between brackets) are given for total length (TL) and otolith mass. Significant differences between areas' TL are given as superscript with mean values. EN – English Chanel; BI – Bay of Biscay; GL – Galicia; PN – Peniche; AL – Algarve; CN – Canary Islands; AN – Angola.

A total of 398 specimens, ranging from 18.1 cm to 34.1 cm were used for the shape analysis study. Mean TL varied between 23.4 cm for the CN and 26.3 for Galicia (Table 3.1). Samples were restricted to sexually mature individuals, and although significant differences for TL were observed among some areas (Table 3.1), the length ranged used intended to be the narrower so to reduce variability caused by size-related effects but maximize sample size and minimize differences in TL among areas.

In order to gather 99% of the otolith outline a total of 21 harmonics was needed (Fig. 3.2). Since the first 3 coefficients of the first harmonic became invariant from the normalization process, a total of 81 coefficients were used in the subsequent analyses.

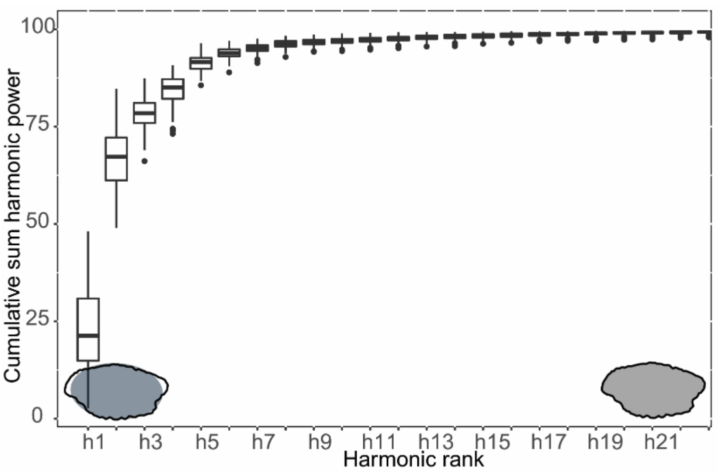

Fig. 3.2 Boxplot (according to momocs R package) of the cumulative variance of harmonics for 70 randomly selected otoliths of *Spondyliosoma cantharus* (10 per area). Grey profile indicates the otolith contour defined by 1 and 21 harmonics.

Black seabream otoliths have an oval shape. EN and BI samples showed a mean otolith shape with a visible *excisura major* and *antirostrum* while for all the other areas these characters were poorly defined (Fig. 3.3). The southernmost samples (CN and AN) showed a mean shape with a more peaked dorsal edge, with a broader and slender shape, respectively (Fig. 3.3).

The discriminant analysis assigned 80% of the total specimens to the correct geographical area with values of classification success above 90% for the CN and AN. GL showed the lower classification success with 67% of correct assigned specimens (Table 3.2).

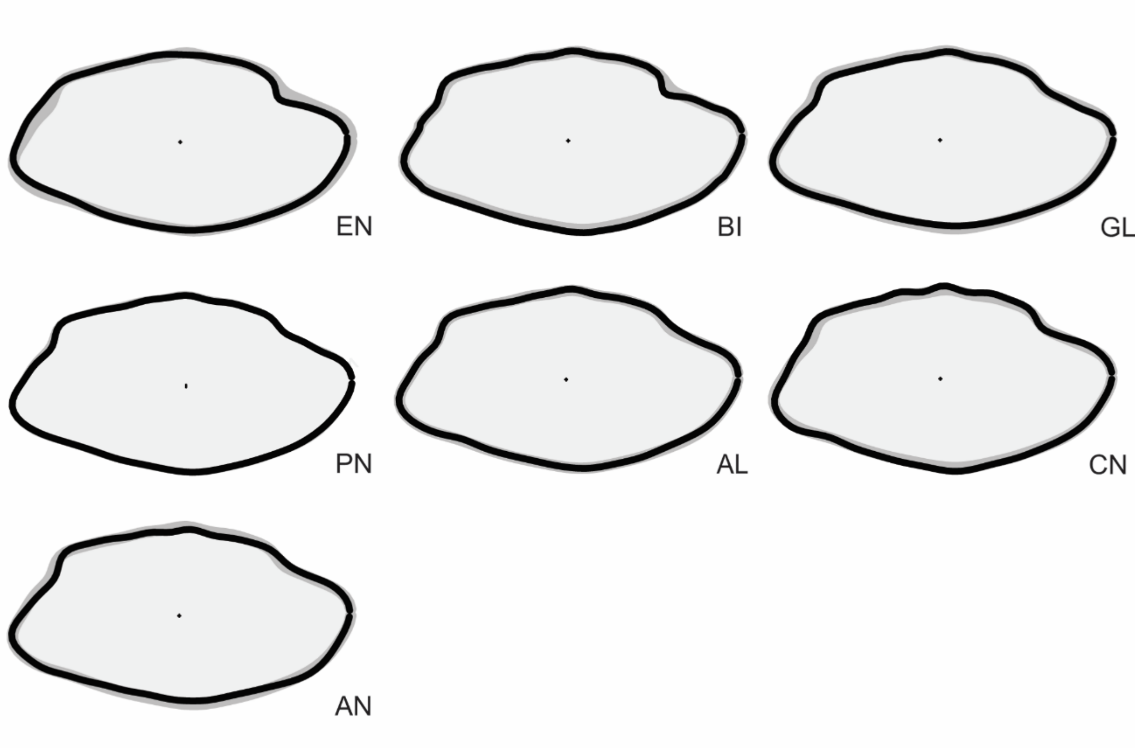

Fig. 3.3 Mean shapes for the black seabream, *Spondyliosoma cantharus*, from different areas (EN – English Channel, BI – Bay of Biscay, GL – Galicia, PN – Peniche, AL – Algarve, CN – Canary Islands, AN – Angola). The figures represent the transformation from the overall mean shape (grey) to the mean shape for each location (black outline).

Table 3.2 Jackknifed classification matrix of the discriminant analysis performed on black seabream, *Spondyliosoma cantharus*, otolith shape data from the seven study areas.

	Predicted						
Original	EN	BI	GL	PN	AL	CN	AN
EN	**77**	3	10	7	3		
BI	4	**75**	4	13	4		
GL	3	7	**67**	16	5		2
PN	1	2	8	**77**	10	1	1
AL			7	11	**78**	3	1
CN				1	7	**92**	
AN			2	5			**93**

Percentages in rows represent the classification into the areas given in columns (correct classification in bold). Overall classification success: 80%, Wilks' λ = 0.020, Cohen's κ = 0.761. EN – English Channel, BI – Bay of Biscay, GL – Galicia, PN – Peniche, AL – Algarve, CN – Canary Islands, AN – Angola.

The first two discriminant functions (DF) explained about 58% of the total among-area variance (Wilks' $\lambda = 0.02$, $p < 0.001$; Cohen's $\kappa = 0.761$, $p < 0.001$). The score plots for the first two discriminant functions showed a clear separation between AN, the CN and EN cluster, with more blending for the other areas (Fig. 3.4).

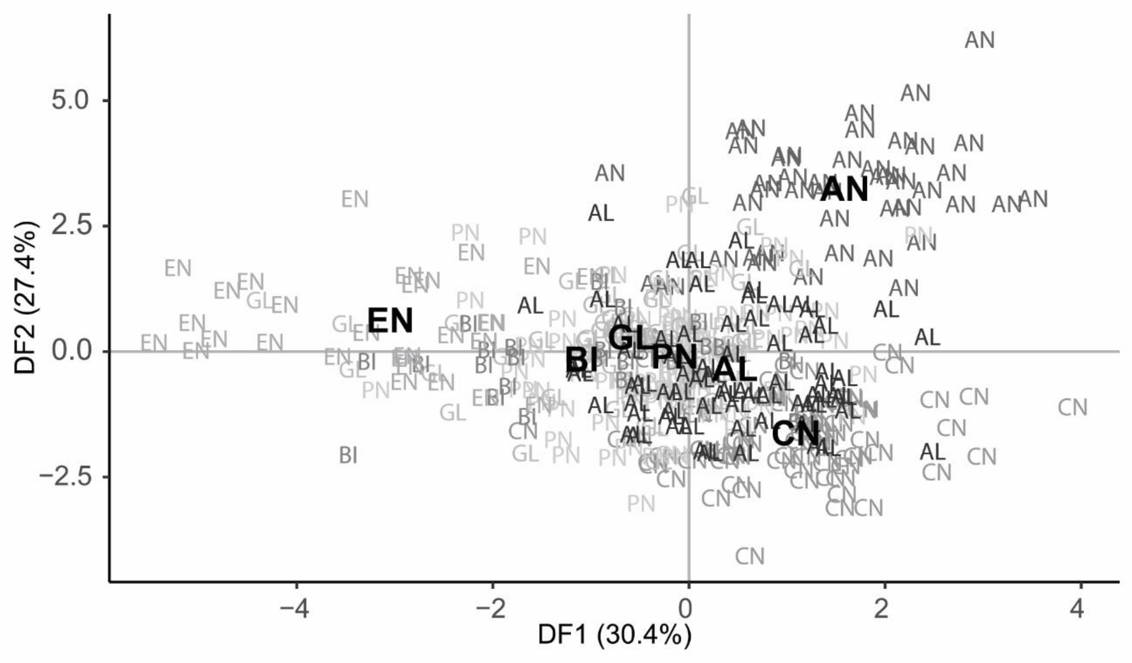

Fig. 3.4 Discriminant analysis function scores (DF) of black seabream, *Spondyliosoma cantharus*, specimens from the seven locations (EN – English Channel, BI – Bay of Biscay, GL – Galicia, PN – Peniche, AL – Algarve, CN – Canary Islands, AN – Angola). Group centroids are marked in bold.

3.3.2 Stable isotope analysis

The values for the black seabream otoliths varied between -0.73‰ and 0.92‰ for $\delta^{18}O$ and between -6.65‰ and -1.20‰ for $\delta^{13}C$. In average, PN (west coast of Portugal) showed the higher values of $\delta^{18}O$ while AN had $\delta^{13}C$ values well above the other areas. ANCOVA yielded significant variation of both $\delta^{18}O$ and $\delta^{13}C$ with otolith mass ($p < 0.001$, Table S3.1), and the subsequent analyses were performed on residuals obtained from the ANCOVA.

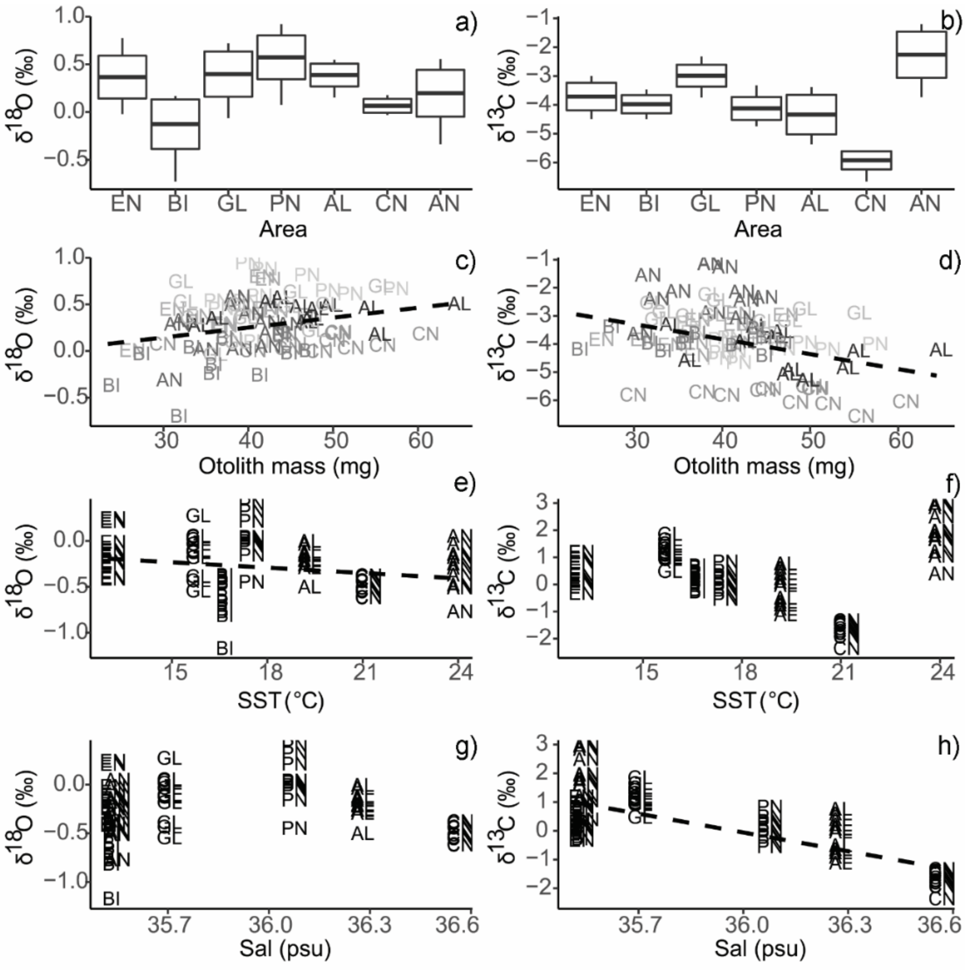

Fig. 3.5 Mean, standard deviation with minimum and maximum values of (a) $\delta^{18}O$ and (b) $\delta^{13}C$ of the whole otolith of black seabream, *Spondyliosoma cantharus*, specimens for each area (EN – English Channel, BI – Bay of Biscay, GL – Galicia, PN – Peniche, AL – Algarve, CN – Canary Islands, AN – Angola). Biplots of $\delta^{18}O$ and $\delta^{13}C$ values against otolith mass (c, d), estimates of sea surface temperatures – SST (e, f) and estimates of sea surface salinity – Sal (g, h) for each of the seven sampled areas.

A weak but significant negative relation between $\delta^{18}O$ and SST was obtained ($r^2 = 0.04$; $p = 0.034$) and $\delta^{13}C$ showed a significant negative relation with salinity ($r^2 = 0.53$; $p < 0.001$), while no significant relation could be established between salinity and SST with $\delta^{18}O$ and $\delta^{13}C$, respectively (Fig. 3.5).

The PERMANOVA, combining both $\delta^{18}O$ and $\delta^{13}C$, showed significant differences between locations (Pseudo-F = 52.84, $p < 0.001$). The *a posteriori* pairwise tests indicated that all locations were significantly different from each other at $p < 0.05$, with the exception of AL and PN (Table 3.3).

Table 3.3 PERMANOVA *a posteriori* pairwise tests performed on black seabream, *Spondyliosoma cantharus*, otolith stable isotopes, $\delta^{18}O$ and $\delta^{13}C$, in relation to the seven study areas.

	Predicted					
Original	BI	GL	PN	AL	CN	AN
EN	0.007	0.003	0.033	0.036	0.001	0.001
BI		0.001	0.001	0.003	0.001	0.001
GL			0.001	0.001	0.001	0.012
PN				0.228	0.001	0.001
AL					0.001	0.001
CN						0.001

EN – English Channel, BI – Bay of Biscay, GL – Galicia, PN – Peniche, AL – Algarve, CN – Canary Islands, AN – Angola.

The combined isotopic relation plot (Fig. 3.6) show clear differences in the otolith signatures from BI, CN and AN samples. The $\delta^{13}C$ values differentiate the AN and CN samples, with much higher and lower values, respectively, than those found in the European areas.

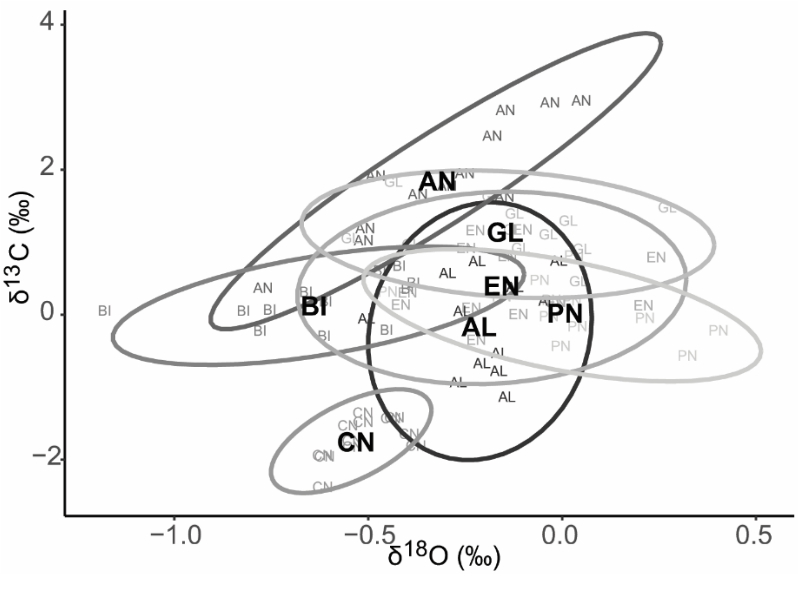

Fig. 3.6 Isotopic results for the whole otolith of black seabream, *Spondyliosoma cantharus*, specimens from the seven locations (EN – English Channel, BI – Bay of Biscay, GL – Galicia, PN – Peniche, AL – Algarve, CN – Canary Islands, AN – Angola). Mean area values are given in bold and ellipses represent the 95% confidence intervals.

The $\delta^{18}O$ values marked a separation of BI samples from the rest of the European areas. For the knn.cv an overall correct assignment of 55%, 58% and 56% was obtained for k = 5, 7 and 9 respectively, with very similar assignment values. Classification set for k=7 is showed in Table 3.4. As noticed on the combined isotopic relation plot, CN, BI and AN showed high correct assignment values, while AL, PN and GL had values around 50%, with EN showing very poor correct assignment below the aleatorily.

Table 3.4 Classification matrix for the k-sample nearest neighbour discriminant analysis (k = 7) performed on otolith isotopes ratio data of black seabream, *Spondyliosoma cantharus*, from the seven study areas.

	Predicted						
Original	EN	BI	GL	PN	AL	CN	AN
EN	**8**	17	33	33	8		
BI	8	**83**		8			
GL	17		**50**	8			25
PN	25	8		**50**	17		
AL	17	17		25	**33**	8	
CN						**100**	
AN	8	8	17				**67**

Percentages in rows represent the classification into the areas given in columns (correct classification in bold). Overall classification success: 58%. EN – English Channel, BI – Bay of Biscay, GL – Galicia, PN – Peniche, AL – Algarve, CN – Canary Islands, AN – Angola.

3.4 Discussion

Traditionally otolith shape analysis has been used for species stock discrimination (e.g. Campana and Casselman, 1993; Stransky, 2005; Stransky et al., 2008; Neves et al., 2011; Vieira et al., 2014), however is recommended the use of a holistic approach for more reliable results when such an investigation is set (Begg and Waldman, 1999). In the present study, a clear discrimination based on the Fourier analysis of otolith shape was observed for all the seven analysed areas. As expected the two samples originated from the two remote areas, CN and AN, had a very typical average outline with a very high correct assemblage of specimens. The European areas also showed distinct outlines through which a high discrimination could be achieved by this method. Still a subsample from each area was analysed for their stable isotopic composition, in order to support the results found in shape analysis. The findings for the isotopic relationship among areas reinforced the pattern found with the shape results, although most European areas showed lower discrimination for the isotopic ratio analysis.

Otolith shape is known to depend on a combination of genetic and environmental factors (Cardinale et al., 2004). Differences in depth, temperature and food availability can change fish physiology, metabolism rate, growth and otolith deposition pattern, and these changes will be recorded in the otolith shape and chemical composition (Gauldie and Nelson, 1990; Campana and Casselman, 1993; Begg and Weidman, 2001; Bang and Grønkjær, 2005; Lombarte et al., 2010; Vignon and Morat, 2010).

Physical conditions vary greatly along the eastern Atlantic, a clear increase in sea temperature from North to South is observed, and basic salinity values are highest for the North Atlantic and lowest for the South Atlantic, due to influence of high-salinity water from the Mediterranean that outflow to the Atlantic Ocean (Bozec et al., 2011). Several currents and different topography are also present along the geographic distribution of the black seabream. In Iberian Peninsula it is known that the north coast is characterised by the run-off of several rivers that creates, mainly during winter, a low-salinity surface water body (ICES, 2008), while the south coast has warmer and saltier water. In oceanic areas, like the Canary Islands, topographic conditions lead to complex trophic interactions between various ecological groups of coastal, oceanic, benthic, and benthopelagic organisms (Uiblein and Bordes, 1999). These factors promote divergences in the communities inhabiting the different sample areas of this study.

It is known that otolith shape is influenced by the growth rate that a population presents and thus, increasing differences in growth rate will potentially increase discrimination power between stocks (Campana and Cassleman, 1993). Diverse growth rates for the species have been mentioned in literature for different geographical areas: English Channel and Golf of Biscay (Perodou and Nedelec, 1980; Soletchnik, 1983), Canary Islands (Pajuelo and Lorenzo, 1999), south Portuguese coast (Abecasis et al., 2008) and central Portuguese coast (Neves et al., 2017), influencing also the otolith growth rate and ultimately their shape.

In the present study European regions showed some level of morphotypes mixture since the misclassified individuals were found in every area. This was even more pronounced for the stable isotopes results. The fact that analyses were performed in the whole otolith can increase the variability in the values obtained, since $\delta^{18}O$ and $\delta^{13}C$ incorporated into sagittal otolith carbonate reflect the environmental conditions that an individual fish encounters during its entire life (Gao and Beamish, 1999). There is no knowledge of long migrations by the black seabream, the species undergoes migrations from more coastal spawning areas to deeper, around 100 m, wintering areas and juveniles stay in coastal or inshore waters for 2 to 3 years when they migrate to the wintering areas (Perodou and Nedelec, 1980). However, the species presents a long larval phase duration, 38 days (Raventós and Macpherson, 2001) which may enable a possible higher dispersion of individuals from different nursery grounds. In fact, Correia et al. (2012) using otolith chemistry found that even for areas as close as Olhão and Sagres (southern Portuguese coast) the black seabream showed near 100% of discrimination accuracy. However, when the analysis was done on otolith core, the accuracy dropped to values below 50%.

The overlap obtained in the isotopic ratios for the European areas is more likely due to a convergent adaptation to different environments than rather an effective migration between areas. In fact, EN has the most heterogeneous group, with individuals falling within four of the European areas while the nearest area, BI, has a distinct isotopic ratio signature. The otolith $\delta^{13}C$ clearly distinguished the 3 main geographical areas, with AN presenting the higher values and CN the lower ones, while the European areas showed more similar values. The ambient water can represent a main source for the otolith carbonate (Tohse and Mugiya, 2008), and although no relationship has ever been established between $\delta^{13}C$ and the physical parameters, a significant negative relationship between water salinity and $\delta^{13}C$ was observed in the present study. Nevertheless, it is known that $\delta^{13}C$ is dependent on a variety of exogenous and endogenous factors (Høie et al., 2003; Correia et al., 2011; Geffen, 2012), mostly related to the metabolic rate and fish diet. Being an opportunistic feeder (Gonçalves and Erzini, 1998) the prey availability determines the black seabreams' type of diet and differences in activity level and metabolic activity influence otolith $\delta^{13}C$ values (Sherwood and Rose, 2003) which should be reflected in the otolith $\delta^{13}C$ signals. The lower values found for the Canary Islands can be justified by the low productive typically exhibit open ocean waters (Martins et al., 2007) which probably will be reflected in the low carbon isotope content in the otoliths (Moreira et al., 2018).

Otolith $\delta^{18}O$ show a negative relationship with temperature for several species (e.g. Gao, 1999; Høie et al., 2004; Dorval et al., 2011). The values obtained in the present study showed a significant but weak relationship. The fact that was not possible to conduct the isotopic ratio analyses in otoliths with exactly the same age can increased variability to the results masking this relationship. Other factors should also be influencing the results found for the black seabream samples. Different physiological adaptations to various environmental conditions might influence the isotope value of otoliths (Høie et al., 2004), and specimens with such diverse origin have most likely divergent environmental adjustments. Being the black seabream a protogynous species, transition from female to male may also influence the deposition rate of the isotopes in the otolith and therefore special attention to the sex composition of the samples should be taken in account. The unavailable number of specimens from all sampled areas to account for homogeneity of age and sex, are probably influencing the results. Despite this, clear isotopic signatures were still obtained for some areas, and only EN showed very poor correct assignment.

Concluding, the results obtained in this study show a clear mean otolith shape for each sampled area. This indicates that the black seabream should spend most of its life in the same area and that studies on life history parameters should be addressed separately in order to conduct an effective management of the species. However, isotopic ratio analyses showed a higher mixture in the isotopic signature for several European areas. Due to the possible influence that some sample heterogeneity may have in the results, these should be regarded with attention, but the possibility of some mixture of specimens, in the European areas, with different nurseries grounds should be explored by other methodologies, including genetic studies. Mixed stocks, considering them as a result of incomplete stock separation where groups overlap in their spatial range for a certain period of time, constitute great challenge for fishery scientists in order to gather the means to properly assess them and provide future management scenarios.

CHAPTER 4

Highly regional population structure of *Spondyliosoma cantharus* across the Eastern Atlantic and the Mediterranean Sea using nuclear and mitochondrial DNA data

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Highly regional population structure of *Spondyliosoma cantharus* across the Eastern Atlantic and the Mediterranean Sea using nuclear and mitochondrial DNA data

ABSTRACT

Understanding a species population structure is fundamental for an efficient fisheries management, and perceiving the genetic differences among geographical separated populations have proved to be an useful tool to infer stock structure in fisheries. In the present study, the degree of geographic population differentiation for the protogynous sparid *Spondyliosoma cantharus*, in the East Atlantic and the Mediterranean Sea, was investigated with nuclear (S7) and mitochondrial (cytochrome *b*) DNA markers. A significant stock structure at regional scale was observed for both markers, with marked genetic divergence between the North Eastern Atlantic and the Mediterranean Sea. The two most southern populations analysed (Cape Verde and Angola) had no shared haplotypes, with very low levels of diversity in Cape Verde samples. Demographic expansion of the species seems to have occurred during the Pleistocene. No population structuring within each of the North Atlantic and Mediterranean regions was detected. However high levels of private haplotypes for all population were observed, which can indicate that not enough dispersal/gene flow exists to homogenize derived and more recent mutations at the ecological timescale. Although these results must be considered together with other methodologies, stock management for *S. cantharus* should probably be assessed independently in most of the analysed areas.

Keywords: Phylogeography; Protogynous fish; Sparidae; Cytochrome *b*; S7 ribosomal protein gene intron 1

4.1 Introduction

Fisheries management goal is to ensure that fishing is environmentally, economically and socially sustainable (Casey et al., 2016). Fish stocks, in fish management, represent demographically cohesive groups of individuals from one species with the number of effectives defined mainly by the local birth and death rates and not by immigration and emigration, representing natural management units since it is possible to establish a relationship between productivity and harvest rates (Carvalho and Hauser, 1995; Ovenden et al., 2015).

Molecular markers have been used for a long time to infer stock structure in fish and current genetic offers a diverse collection of useful tools that can be used to inform fisheries managers about issues that have a biological basis (Ovenden et al., 2015). Molecular genetic methods use inherited stable markers to identify genotypes that characterize populations and test whether the fish samples are drawn from a large, randomly mating population with equilibrium genotype frequencies, or from an assemblage of genetically distinct units (Coyle, 1998). Most species are composed of populations, also called genetic stocks, between which limited gene flow occurs, and each population maintains distinct genetic characteristics due to genetic variation within the species (Gopalakrishnan et al., 2017). Migration, mutation, selection, and genetic drift forces, which act on the species/population during its evolution, promote this differentiation (Gopalakrishnan et al., 2017). Overfishing such units can lead to a collapse of the fishery since a recover of population sizes from migration is unlikely to occur and with the loss of a genetic stock, a species also loses the individuals that are adapted to a particular habitat through evolution (Gopalakrishnan et al., 2017).

The black seabream, *Spondyliosoma cantharus*, is a protogynous Sparidae species that shows a wide distribution along the eastern Atlantic, occurring from Scandinavia to Namibia, and around the Madeira, Cape Verde and the Canary Islands (Bauchot and Hureau, 1986). It is also common in the Mediterranean Sea and the western Black Sea. It is an important commercial fish, exploited in European waters by recreational and commercial fishers (Russell et al., 2014). In Portuguese waters, it is caught by several fishing gears, with mean annual landings of 180 ton in the last decade (DGRM, unpublish data). Life history characteristics of *S. cantharus* make it particularly vulnerable to local over-exploitation since it is a hermaphrodite slow-growing, long-lived, showing habitat specificity during the spawning season, with spawning aggregations and male nest guarding behaviours (Russel et al., 2014; Pinder et al., 2017).

A sustainable exploitation and conservation depend on solid understanding of connectivity and population differentiation, allowing the identification of discrete populations that represent demographically independent stocks (Begg et al., 1999). Despite the wide geographical distribution of *S. cantharus*, no information on stock structure is available for the species. Assessing the genetic structure of a species population can help to discern its phylogeographic history and thus assess the fisheries stocks (Antoniou and Magoulas, 2014). Mitochondrial DNA (mtDNA) has proven to be a powerful tool in species and stock identification as a population-genetic, phylogeographic, and phylogenetic marker, but for a proper use in stock identification, the data interpretation should take into account additional information from other features as nuclear DNA (Antoniou and Magoulas, 2014). The aim of this study is to explore the phylogeography and population structure of *S. cantharus* across the East Atlantic and Mediterranean Sea using nuclear and mitochondrial DNA markers.

4.2 Material and Methods

4.2.1 Sampling

A total of 263 *S. cantharus* samples were collected from 9 locations along the Eastern Atlantic and 5 locations in the Mediterranean Sea (Fig. 4.1, Table 4.1). Individuals from English Channel (EN), Bay of Biscay (BI), Galicia (GL), Peniche (PN), Algarve (AL), Canary Island (CN) and Angola (AN) were frozen and sent to Lisbon. After arrival, a piece of fin was removed and preserved in 96% ethanol. Samples from all the other locations, Belgium (BG), Cape Verde (CV), Murcia (MU), Valencia (VL), Balearic Islands (BL), Corsica (CO) and Croatia (CR), were taken from fresh fish, stored in 96% ethanol and shipped to Lisbon.

4.2.2 DNA extraction, amplification, sequencing and alignment

Genomic DNA was extracted from fin samples with the REDExtract-N-Amp kit (Sigma-Aldrich) following the manufacturer's instructions. Mitochondrial DNA cytochrome *b* (cyt*b*) and nuclear DNA first intron of the S7 ribosomal protein gene (S7) were amplified by polymerase chain reaction (PCR). Amplification of the region of interest was obtained

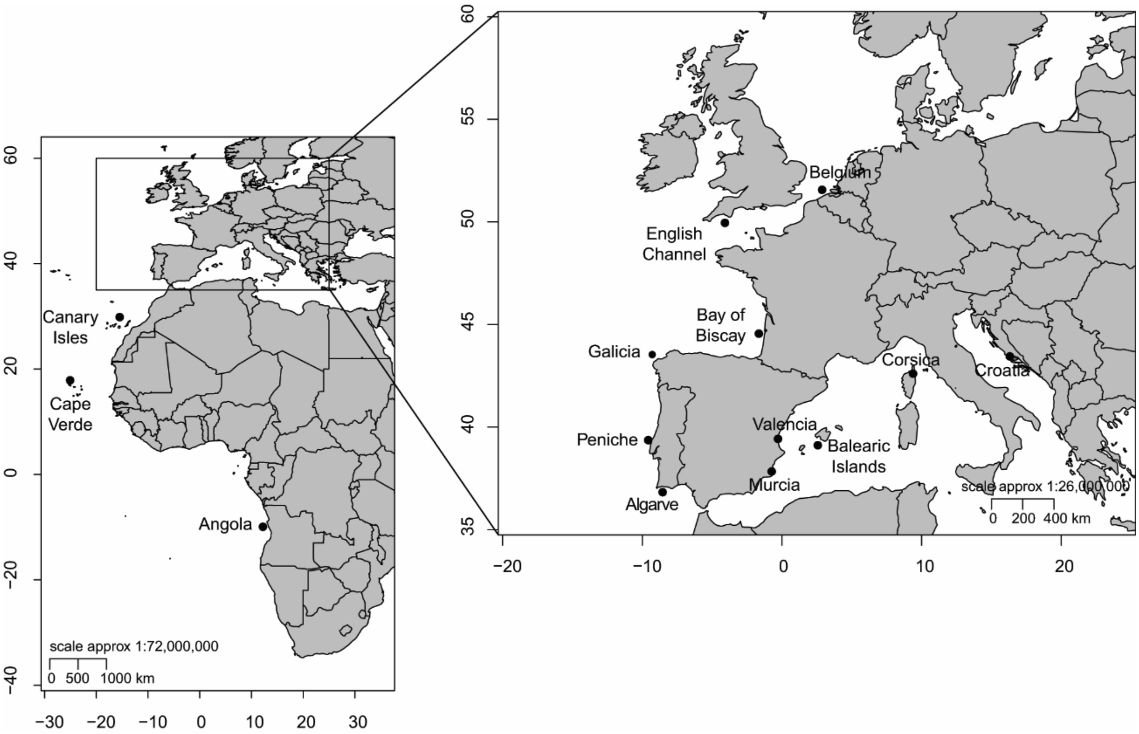

Fig. 4.1 Sampling locations of *Spondyliosoma cantharus* in the Eastern Atlantic Ocean and the Mediterranean Sea. The position of samples with black dots is indicative.

with pair of primers purposely designed by the authors for cyt*b*, based on 7 aligned cyt*b* complete sequences of *S. cantharus* obtained from GenBank (http://www.ncbi.nlm.nih.gov/genbank) (accession numbers: DQ198007, EU036508, EU036509, EF439601, EF439237, EF427608 and EF427607): BRBcytbF (forward) 5'-GCTGACTCATCCGAAATCTT-3' and BRBcytbR (reverse) 5'-ATGTAGGGGTCTTCAACTGG-3'. S7 amplifications were performed with the following pair of primers: S7RPEX1F (forward) 5'-TGGCCTCTTCCTTGGCCGTC-3' and S7RPEX2R (reverse) 5'-AACTCGTCTGGCTTTTCGCC-3'(Chow and Hazama, 1998). PCR amplification reactions were performed in a 20 µl total-reaction volume with 10 µl of REDExtract-N-ampl PCR reaction mix (Sigma–Aldrich), 0.8 µl of each primer (10 mM), 4.4 µl of bidistilled water and 4 µl of template DNA. An initial denaturation at 94 ºC for 5/3 min was followed by 30 cycles (denaturation at 94 ºC for 45 s, annealing at 56 ºC for 30/60 s, and extension at 72 ºC for 1 minute) and a final extension at 72 ºC for 10 minutes on a BioRad Mycycler thermal cycler (values cyt*b*/S7, respectively). PCR products were purified with the SureClean kit (Bioline) following the manufacturer's protocol, and the same primers were used for the sequencing reaction provided by

Macrogen (http://www.macrogen.com, 716 samples) and STABVIDA (http://www.stabvida.net/, 108 samples).

Sequences were aligned using Clustal W (Thompson et al., 1994; Larkin et al., 2007) using BioEdit (Hall, 1999). S7 haplotypes of length-variant heterozygotes were determined using Mixed Sequence Reader (Chang et al., 2012), and manual adjustments were made whenever needed. Allelic states of S7 sequences were estimated using the Bayesian programme PHASE 2.1 (Stephens et al., 2001; Stephens and Donnelly, 2003; Stephens and Scheet, 2005). Five runs with different seeds for the random number generator and 500 iterations as burn-in, 500 main iterations and a thinning interval = 1 were performed to check for consistency across results. All runs returned consistent allele identities.

4.2.3 Haplotype networks

The relationship between haplotypes was analysed by haplotype networks built with the software PopART (Leigh and Bryant, 2015) using a TCS network (Clement et al., 2002). For cyt*b* network some additional haplotypes from Madeira (MD, GenBank accession no. EF439237) and Greece (GR, GenBank accession nos. EU036508–9) available in GenBank database were added to extend the sampling area.

4.2.4 Population structure

Hierarchical analysis of molecular variance (AMOVA; Excoffier et al., 1992) was used to estimate genetic structure among and between regions, on three different populations assemblages. Estimates of genetic divergence and the number of migrants among regions and among populations within region were calculated with the fixation index Φ_{ST} (Excoffier et al., 1992) as implemented in ARLEQUIN V3.5 (Excoffier and Lischer, 2010), with p-values being corrected for multi comparisons by Benjamini-Hochberg (BH) false discovery rate method (Benjamini and Hochberg, 1995).

The correlation between geographical distance, measured along the coastline, and Φ_{ST} was computed with the Mantel test (Mantel, 1967; Smouse et al., 1986), also in ARLEQUIN with 100,000 permutations.

Diversity indexes (number of haplotypes, haplotype and nucleotide diversities, mean pairwise differences and % of private haplotypes) were estimated for all populations using ARLEQUIN V3.5 (Excoffier and Lischer, 2010).

4.2.5 Demographic analysis

Demography was investigated in ARLEQUIN with neutrality tests by region (Tajima's D and Fu's F_S), and the mismatch distribution analysis was also performed for each region and compared to the distribution expected in populations affected by sudden expansion (100,000 replicates), under the assumption of selective neutrality, in which a unimodal distribution is expected (Rogers and Harpending, 1992). The sum of squared deviation (SSD) and raggedness index (Hri) were used to detect departure between observed and expected distributions. If evidence of expansion was found (p-values > 0.05) the τ parameter of demographic and spatial expansions was used to estimate the time since the expansion (t, in years), using the equation $t = (\tau \times n) / (2 \times \mu \times k)$, μ is the nucleotide mutation rate, k is the sequenced number of nucleotides and n is the generation time (equal to age for sexual maturity, Yang et al., 2016). Since there is no information on mutation rate of cyt*b* and S7 for the black seabream, the values used in the present study were 2% per nucleotide per Myr for cyt*b* based on Bargelloni et al. (2003) and 0.46% per nucleotide per Myr for S7 based on Henriques et al. (2016). The generation time used was 3.8 years, according to Neves et al. (2018).

4.3 Results

A total of 263 cytb sequences were obtained, aligned, and trimmed to 663 bp, with 63 distinct haplotypes identified (GenBank accession nos. MH545766 – MH545828) showing 99 polymorphic sites yielding 81 transitions and 26 transversions. For S7, 366 sequences (corresponding to 183 individuals) with 483 bp were analysed and 85 haplotypes were identified (GenBank accession nos. MH545829 – MH545911), with 90 polymorphic sites representing 44 transitions, 34 transversions and 17 indels.

4.3.1 Haplotype networks

The haplotype network for the cyt*b* of *S. cantharus* revealed four distinct groups (Fig. 4.2). The first group included mainly sequences from the North Eastern Atlantic (NEAT) areas (BG, EN, BI, GL, PN, AL and CN), with one haplotype shared between 61 individuals, three of them sampled in the Mediterranean Sea (MEDS), one from MU and two from VL. This was the inferred ancestral haplotype with an outgroup weight of 0.127. A second group, represented by MEDS sequences, showed two main haplotypes shared by 31 and 26 individuals from all the MEDS sampled areas. The two other groups were composed exclusively by private haplotypes, 4 for Cape Verde (West African Transition

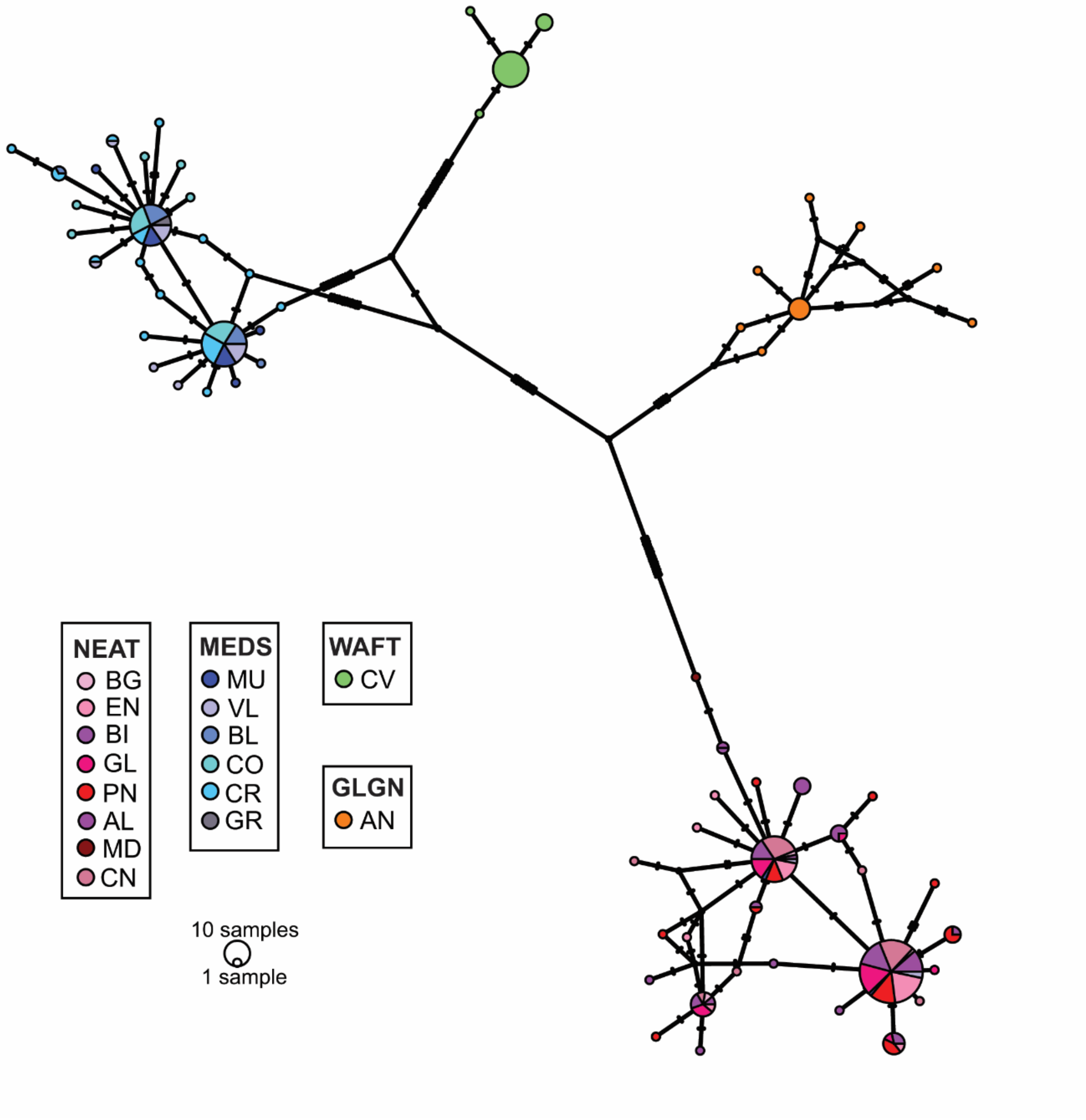

Fig. 4.2 Parsimony network for *Spondyliosoma cantharus* calculated with mtDNA cytochrome *b* sequences (663 bp). The area of the circles is proportional to each haplotype frequency. Colours refer to the region in which haplotypes were found. Acronyms for populations are given in sampling section.

Table 4.1 Diversity measures for the sample areas and population groups of *Spondyliosoma cantharus* for cyt*b* and S7.

	cyt*b*						**S7**					
Area	N	Nh	Hp	Hd	π	PD	N	Nh	Hp	Hd	π	PD
NEAT	**137**	**26**	**24 (92%)**	**0.768**	**0.002**	**1.525**	**170**	**52**	**48 (92%)**	**0.848**	**0.005**	**2.443**
BG	3	3	0 (0%)	1.000	0.003	2.000	4	4	2 (50%)	1.000	0.008	4.000
EN	22	7	3 (43%)	0.671	0.002	1.134	10	9	7 (78%)	0.978	0.010	4.800
BI	21	7	1 (14%)	0.771	0.002	1.324	24	9	4 (44%)	0.706	0.003	1.565
GL	21	6	1 (16%)	0.723	0.002	1.314	10	6	2 (33%)	0.778	0.004	1.956
PN	24	10	5 (50%)	0.855	0.003	1.931	42	21	9 (43%)	0.905	0.005	2.541
AL	21	10	4 (40%)	0.857	0.003	2.181	28	16	8 (50%)	0.907	0.006	2.817
CN	25	7	4 (57%)	0.697	0.002	1.307	52	13	7 (54%)	0.780	0.004	1.968
WAFT	**25**	**4**	**4 (100%)**	**0.410**	**0.001**	**0.440**	**42**	**7**	**7 (100%)**	**0.512**	**0.001**	**0.678**
CV	25	4	4 (100%)	0.410	0.001	0.440	42	7	7 (100%)	0.512	0.001	0.678
GLGN	**14**	**8**	**8 (100%)**	**0.769**	**0.005**	**3.000**	-	-	-	-	-	-
AN	14	8	8 (100%)	0.769	0.005	3.000	-	-	-	-	-	-
MEDS	**87**	**27**	**25 (93%)**	**0.800**	**0.007**	**4.917**	**154**	**30**	**26 (87%)**	**0.842**	**0.004**	**1.948**
MEDS **	**82**	**25**	**25 (100%)**	**0.775**	**0.002**	**1.207**						
MU	14	7	3 (43%)	0.824	0.016	10.033	28	8	2 (25%)	0.741	0.002	1.185
*MU ***	*12*	*5*	*3 (60%)*	*0.758*	*0.002*	*1.030*						
VL	16	8	2 (25%)	0.858	0.019	12.150	18	10	3 (30%)	0.889	0.004	2.033
*VL ***	*13*	*6*	*2 (33%)*	*0.795*	*0.002*	*1.154*						
BL	13	4	1 (25%)	0.679	0.001	0.846	26	10	0 (0%)	0.871	0.005	2.308
CO	20	7	5 (71%)	0.742	0.001	1.005	32	11	2 (18%)	0.839	0.004	1.970
CR	24	14	9 (64%)	0.884	0.003	1.732	50	17	7 (41%)	0.861	0.004	2.105

Number of sequences (N), number of haplotypes (Nh), private haplotypes (Hp), haplotype diversity (h), nucleotide diversity (π) and mean number of pairwise differences (PD). NEAT – North Eastern Atlantic; WAFT – West African Transition; GLGN – Gulf of Guinea; MEDS – Mediterranean Sea. Acronyms for populations are given in sampling section region, WAFT) and 8 for Angola (Gulf of Guinea region, GLGN). A similar pattern was present for the S7 haplotype network (Fig. 4.3), although not so distinct, since one haplotype occurred 110 times and was shared by all sampled areas from the NEAT and MEDS, which was inferred as the ancestral haplotype with an outgroup weight of 0.095.

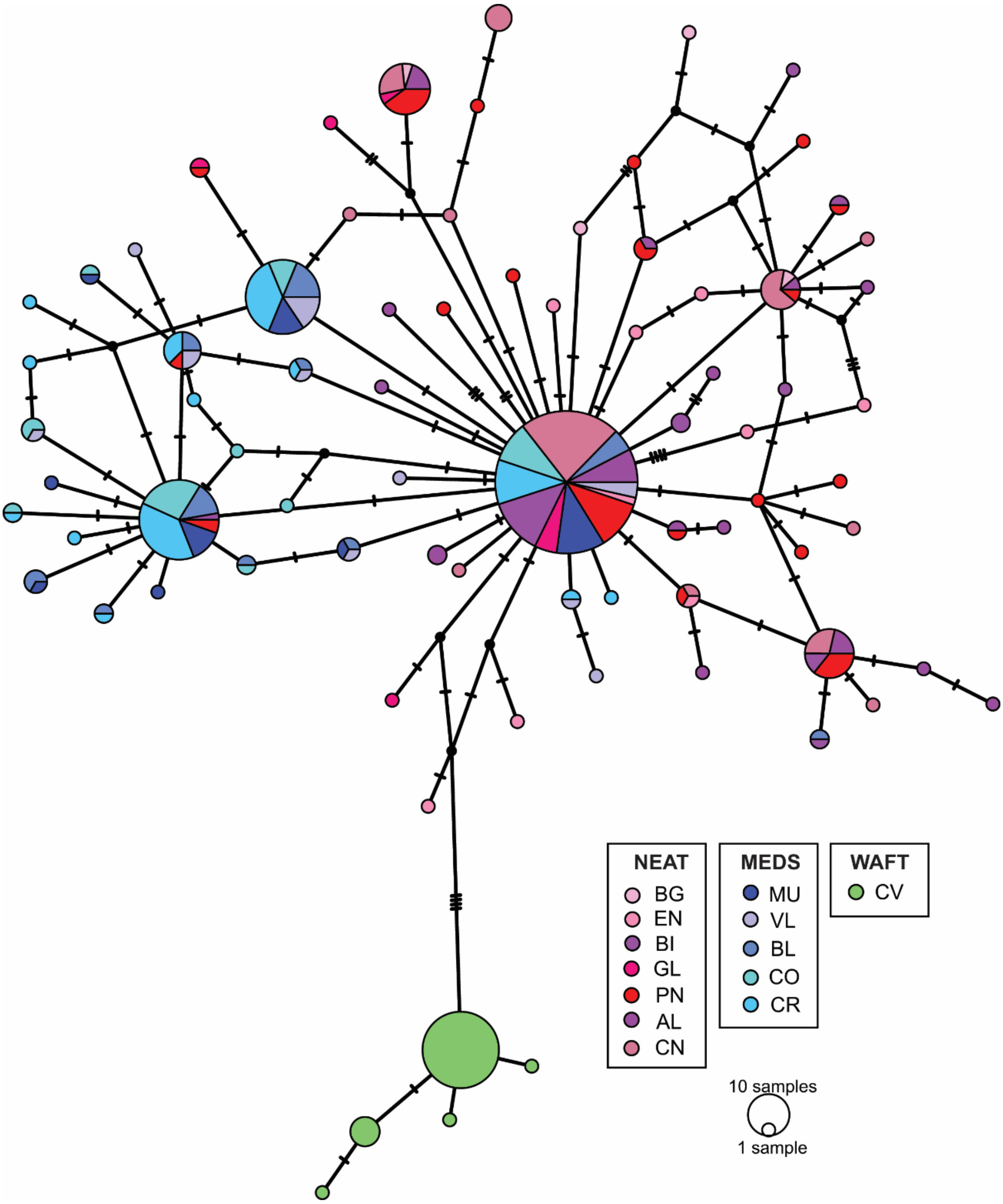

Fig. 4.3 Parsimony network for *Spondyliosoma cantharus* calculated with nDNA first intron S7 sequences (483 bp). The area of the circles is proportional to each haplotype frequency. Colours refer to the region in which haplotypes were found. Acronyms for populations are given in sampling section.

However, only three more haplotypes were shared between NEAT and MEDS areas (Fig. 4.3, Table 4.1) allowing a separation from haplotypes occurring in NEAT individuals from MEDS individuals. The Cape Verde samples showed, again, only private haplotypes. None of the DNA samples from AN could be amplified for the S7 region and therefore AN was excluded from S7 results.

4.3.2 Population structure

The AMOVA results for all the region structures assigned the main portion of molecular variance to the "among regions" level. For cyt*b*, the 4 and 5 regions structure (Table 4.2) showed very similar values (92.44% and 92.50% respectively) and the 4 regions structure (NEAT, MEDS, WAFT, GLGN) was chosen for the follow up analyses. For S7 values the 3 regions structure (NEAT, MEDS, WAFT) maximize the variance explained among regions with 60% (Table 4.3). A significant population structuring for both cyt*b* (FST = 0.928, p<0.000) and the S7 (FST = 0.618, p<0.000) was defined.

Table 4.2 Hierarchical analyses of molecular variance (AMOVA) considering three hierarchical levels in three different region structures for cyt*b* sequences of *Spondyliosoma cantharus*.

	Fixation Indices	P value	Percentage
a) 3 groups: CV vs AN vs rest of populations			
Among groups	Φ_{CT} 0.514	0.0215	51.40
Among populations within groups	Φ_{SC} 0.863	< 0.0001	41.95
Within populations	Φ_{ST} 0.934	<0.0001	6.64
b) 4 groups: CV vs AN vs NEAT vs MEDS			
Among groups	Φ_{CT} 0.924	< 0.0001	92.44
Among populations within groups	Φ_{SC} 0.048	0.0450	0.36
Within populations	Φ_{ST} 0.928	<0.0001	7.19
c) 5 groups: CV vs AN vs NEAT vs (BL, CO, CR) vs (MU, VL)			
Among groups	Φ_{CT} 0.925	< 0.0001	92.50
Among populations within groups	Φ_{SC} 0.029	0.9502	0.22
Within populations	Φ_{ST} 0.923	<0.0001	7.71

Probability values were obtained after a permutation test with 99,999 replicates.

Pairwise Φ_{ST} values showed a significant genetic structure between all regions, with migration values below 1 for all pairs with exception of NEAT and MEDS for S7 marker (Table 4.4). When analysing the populations within each region (Table S1 supplementary material), no structuration among the populations is perceptible with nearly all Φ_{ST} values below 0.1 and large estimated migration values obtained.

Table 4.3 Hierarchical analyses of molecular variance (AMOVA) considering three hierarchical levels in three different region structures for S7 sequences of *Spondyliosoma cantharus*.

	Fixation Indices	P value	Percentage of variation
a) 2 groups: CV vs rest of populations			
Among groups	Φ_{CT} 0.796	0.0779	79.58
Among populations within groups	Φ_{SC} 0.095	< 0.0001	1.93
Within populations	Φ_{ST} 0.815	< 0.0001	18.49
b) 3 groups: NEAT vs MEDS vs CV			
Among groups	Φ_{CT} 0.608	0.0119	60.81
Among populations within groups	Φ_{SC} 0.025	0.0048	0.98
Within populations	Φ_{ST} 0.618	< 0.0001	38.21
c) 4 groups: NEAT vs CV vs (BL, CO, CR) vs (MU, VL)			
Among groups	Φ_{CT} 0.580	0.0264	57.97
Among populations within groups	Φ_{SC} 0.025	0.0076	1.06
Within populations	Φ_{ST} 0.590	< 0.0001	40.97

Probability values were obtained after a permutation test with 99,999 replicates

The Mantel test showed significant correlations between genetic and geographical distances for cyt*b* ($r = 0.50$, $p < 0.001$) but not for S7 ($r = 0.51$, $p = 0.083$). However, when the Mantel test was carried out for each region independently, isolation by distance was not found for either cyt*b* ($r = 0.06$, $p=0.370$ and $r = 0.14$, $p = 0.298$ for NEAT and MEDS, respectively) or S7 ($r = 0.56$, $p = 0.098$ and $r = -0.23$, $p = 0.623$, NEAT and MEDS, respectively).

Table 4.4 Gene flow among collecting sites of *Spondyliosoma cantharus* represented by FST (below diagonal) and Nm (number of migrants; above diagonal).

	cyt*b*				S7		
	NEAT	MEDS	WAFT	GLGN	NEAT	MEDS	WAFT
NEAT		0.047	0.016	0.032		3.792	0.121
MEDS	**0.914**		0.059	0.081	**0.117**		0.094
WAFT	**0.970**	**0.895**		0.016	**0.805**	**0.842**	
GLGN	**0.940**	**0.861**	**0.969**				

Significant values of probability assessed by permutation test with 99,999 replicates are shown in bold. Acronyms for populations are in sampling section.

4.3.3 Diversity analysis

A large proportion of singleton haplotypes were obtained both for cyt*b* (73%) and S7 (62%). Haplotype diversity was low for WAFT with estimated values around 0.5, while all the other groups showed similar values. The number of private haplotypes was high for both cyt*b* (50 out of 63) and S7 (58 out of 83), occurring in all locations (with exception of BL S7) and suggesting the presence of genetic structuring in the study area. Cape Verde had the lowest values of nucleotide diversity for both DNA markers. The cyt*b* nucleotide diversity was higher for AN and MEDS, being the values inflated for this last region by MU and VL populations (Table 4.1). Five individuals from these populations had shared haplotypes with the NEAT region, which highly increased the nucleotide diversity of these populations. When these individuals are removed from the analyses, the diversity values from NEAT and MEDS are similar, as those from all the within region populations (Table 4.1, lines flagged with asterisks).

4.3.4 Demographic analysis

Neutrality results suggest demographic expansion for NEAT and MEDS regions with significant negative values estimated for both DNA regions. For WAFT and GLGN regions the estimated values were also negative but not significant, except for the significant Fu's F_S yielded for WAFT with S7 (Table 4.5). With exception of MEDS for cyt*b*, the analyses of the mismatch distributions for both cyt*b* and S7 were compatible with the models of demographic and spatial expansion for all regions (Fig. 4.4, Table 4.5).

Table 4.5 Demographic parameters of *Spondyliosoma cantharus* based on cyt*b* and S7.

	cyt*b*				**S7**		
	NEAT	MEDS	WAFT	GLGN	NEAT	MEDS	WAFT
neutrality tests							
Tajima's D	**-1.912**	**-1.694**	-1.108	-1.484	**-2.382**	**-1.803**	-1.360
Fs	**-22.014**	**-8.188**	-1.653	-1.936	**-26.760**	**-23.324**	**-3.677**
mismatch distribution							
demographic expansion							
t (ky)	143		71	1318	2394	1539	598
	(64 – 454)		(0 – 170)	(19 – 11463)	(668 – 4117)	(1227 – 1914)	(232 – 1104)
SSD	0.0013	**0.0011**	0.0074	0.0199	0.0039	0.0008	0.0006
Hri	0.0413	**0.0888**	0.1675	0.0494	0.0275	0.0462	0.0808
spatial expansion							
t (ky)	140		75	974	2223	1453	598
	(78 – 396)		(14 – 220)	(27 – 13982)	(562 – 3395)	(904 – 1942)	(229 – 1141)
SSD	0.0013	**0.1073**	0.0073	0.0181	0.0022	0.0008	0.0006
Hri	0.0413	**0.0888**	0.1675	0.0494	0.02752	0.0462	0.0808

Neutrality tests: Fs (Fu's), D (Tajima's). Mismatch distributions: t (time in thousand years), SSD (sum of square deviation) and Hri (Harpending's Raggedness index). Significant values of probability assessed by a permutation test with 99,999 replicates are shown in bold and 95% CI are given in parenthesis

The average time estimated for demographic and spatial expansion showed similar values, with cyt*b* and S7 reflecting different expansion periods, indicating that might have been an early expansion period ranging from 2.4 in NEAT to 0.6 Myr in WAFT, from late Pliocene to Pleistocene and a recent one, from 143 Kyr in NEAT to 71 Kyr in WAFT during the last glacial period. For GLGN region it seems to have only occurred an early expansion since time estimation for cyt*b* gives values closer to those found for the other regions with S7.

4.4 Discussion

Sparid family has a wide distribution and, contrary to other percoid families, have great diversity in Eastern Atlantic Ocean (Hanel and Tsigenopoulos, 2011). Most species have high commercial value and the population trend is unknown or decreasing (IUCN, 2017).

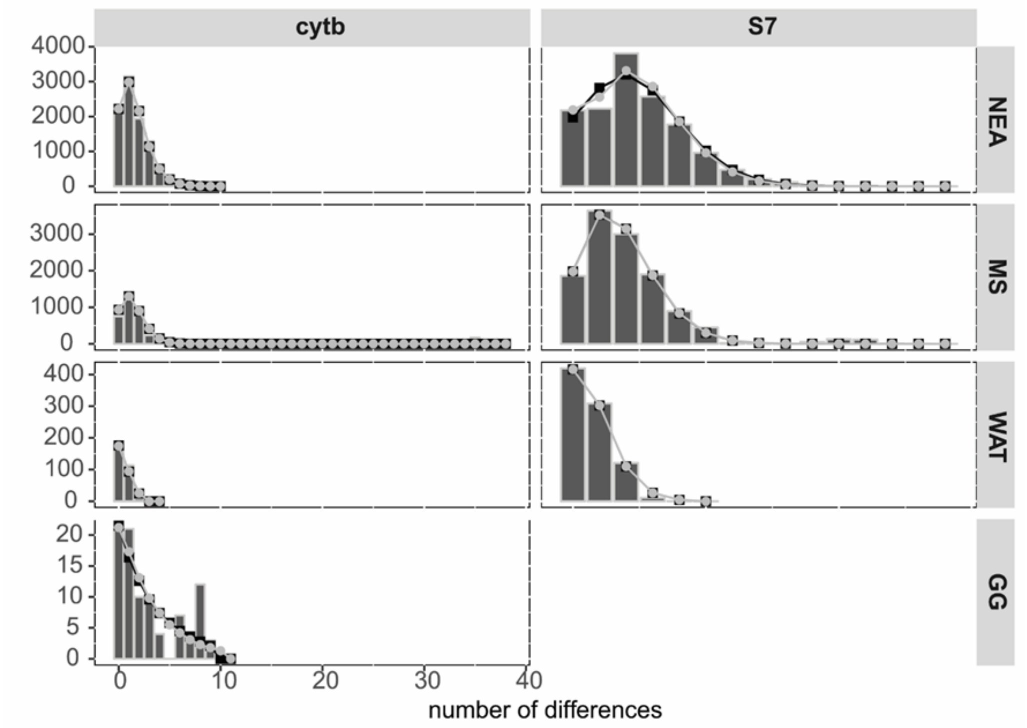

Fig. 4.4 Mismatch distributions for the mtDNA cyt*b* and nDNA S7 of *Spondyliosoma cantharus* in the four regions identified. NEAT: North East Atlantic; MEDS: Mediterranean Sea; WAFT: West African Transition; GLGN: Gulf of Guinea. Bars represent data observed, grey dots represent simulated spatial expansion and black squares represent simulated demographic expansion.

In the present study the genetic structure of *S. cantharus* was analysed with nuclear and mitochondrial DNA markers in 14 sampling areas along the geographical distribution of the species. The results revealed high population structure, with four clear groups in the analysed area: the North Eastern Atlantic, the Mediterranean Sea, the Western African Transition (represented by Cape Verde) and the Gulf of Guinea (represented by Angola). Pairwise Φ_{ST} values clearly showed this structuration among these regions, with a significant isolation by distance showed for cyt*b* marker but not for the S7. The lack of significance in S7 marker is probably due to one haplotype highly shared by both North Eastern Atlantic and Mediterranean Sea populations. Within each region, no population structuration was found, with very low pairwise Φ_{ST} values and isolation by distance absent in NEAT and MEDS for both DNA markers.

The two southern regions (GLGN and WAFT) showed no shared haplotypes with any other analysed population. This is not totally unexpected since these are the two distant regions and this species has been described as mostly sedentary with small scale migrations from deeper, around 100 m, wintering areas to more coastal spawning areas (Perodou and Nedelec, 1980). NEAT and MEDS shared a small number of haplotypes. From the results retrieved by the nuclear marker, four haplotypes were common to both regions; one, as mentioned above, was shared by all populations of both region while the others were shared by a small number of individuals. For the mitochondrial marker, only two haplotypes were shared, but only two of the MEDS populations, which are nearer to the Atlantic, present these haplotypes which occur in all populations from NEAT. This points for a secondary contact with unidirectional gene flow from the Atlantic to the Mediterranean.

Gulf of Guinea

Despite efforts to gather samples for multiple areas within the Gulf of Guinea, only one population, Angola, was analysed. Also, no data for the S7 could be obtained, since it was not possible to amplify this DNA region for the samples in this population. This did not allow to explore the possibility of the existence of shared haplotypes with the northern regions, as described for other species such *Pomatomus saltatrix* (Reid et al., 2016).

For the mitochondrial marker, AN showed haplotypic diversity values similar to the NEAT and MEDS regions and higher values of nucleotide diversity, despite the lower number of individuals analysed. This can be related to the probability that several populations exist along the African coast and that migration between these areas occurs. Also, populations from GLGN region are likely to have expanded from a refugial tropical area where the mild conditions allowed to maintain a high number of breeders, justifying the high diversity found in the small number of individuals analysed in this area.

West African Transition

The haplotype and nucleotide diversity found for CV population were much lower than the ones estimated for the other areas. Two hypotheses can be suggested. The low genetic diversity could have been caused by bottleneck that led to a drastic loss of DNA diversity and a recolonization from a single strong founder effect still persists. Other explanation can be a high level of inbreeding and/or population decline. The protogynous reproductive strategy of the species leads to skewed sex ratios which can decrease the effective population size and consequently increase the susceptibility to genetic drift (Chopelet et al., 2009). In a region where the strong influence of large-scale oceanic circulation and dominant currents are likely to promote a physical barrier to larval drifting across the archipelago (Cunha et al., 2011) this can even be more pronounced. Also, the increase in fishing from 2005 (FAO, 2005-2018) can also be diminishing the genetic pool available in the archipelago.

The estimated time of expansion for Cape Verde was much recent than for the other regions. This support the first hypothesis, where the population had still no time to highly increase diversity. However, it is probable that the second hypothesis is also contributing for the low levels of diversity.

Mediterranean Sea

The MEDS showed the highest haplotype and nucleotide diversity for cyt*b*, however these values were strongly skewed due to only five samples from MU and VL populations. In fact, if these samples were removed from the analyses the haplotype diversity was still the highest but with values very similar to those found for NEAT and GLGN, while nucleotide diversity drops to values equal to NEAT and lower than GLGN. For the S7, the diversity values estimated were similar but somewhat lower than those from the NEAT. At population level, values of diversity for cyt*b* were mostly similar, being higher in CR and lower in BL, while for S7, the estimated values were lower in MU and similar for all the other populations.

In MEDS different barriers to gene flow have been identified, such as the Almeria-Oran front, the Siculo-Tunisian Strait, which divides the eastern and western Mediterranean basins, and the hydrographic isolation of the Aegean, Ionian and Adriatic Seas (Bahri-Sfar et al., 2000; Mattiangeli et al., 2003; Pérez-Losada et al., 2007). From the results of population comparisons, it seems that these barriers are not influencing the gene flow of *S. cantharus* within the Mediterranean, since shared mitochondrial and nuclear haplotypes are present in all populations. The larval 38-days phase duration (Raventós and Macpherson, 2001) of *S. cantharus* can be promoting the gene flow. Nevertheless, the presence of several private haplotypes in all populations, especially in CO and CR, points to some isolation that the protogynous strategy of the species may tend to fade, since all breeders contribute to the mitochondrial gene pool of the species. The mtDNA genomic compartment in this species is therefore ½ and not ¼ as for most species, because the same individual will mature as female and change sex along its life and consequently, "all" individuals will function as females first, transmitting a copy of mtDNA into the population.

North Eastern Atlantic

The Atlantic Ocean and the Mediterranean Sea are connected by the Straits of Gibraltar with 12.9 km wide and 286 m deep. They have been fully separated during the Messinian salinity crisis which occurred in the late Miocene (Krijgsman et al., 1999) and by recurrent short periods of separation during the Quaternary (Patarnello et al., 2007). The Strait of Gibraltar can represent a phylogeographical break, but no obvious relationship has been established between species dispersal ability or life history, and the observed patterns of genetic isolation between Atlantic and Mediterranean populations (Patarnello et al., 2007). In the Sparidae family, divergent patterns have been observed for species with similar biology. A clear differentiation from Atlantic and Mediterranean populations have been found for species such as *Lithognathus mormyrus*, *Dentex dentex* (Bargelloni et al., 2003), *Diplodus puntazzo* (Bargelloni et al., 2005) but not for others like *Pagrus pagrus* (Ball et al., 2007; Patarnello et al., 2007), *Pagellus bogaraveo* (Bargelloni et al., 2003), *P. erythrinus* (Angiulli et al., 2016), *Diplodus sargus* (Bargelloni et al., 2005; González-Wangüemert et al., 2011). For *S. cantharus*, Bargelloni et al. (2003) found different patterns when using different methodologies, with a clear differentiation obtained with the mitochondrial DNA marker, and no differentiation found with allozymes.

In the present study, one haplotype of the nuclear marker was shared by all NEAT and MEDS populations revealing incomplete lineage sorting from a common ancestor of both regions. Although other scenarios can be placed, the one that seems most likely to have occurred is a southern refugia that expanded to the Mediterranean Sea and to the North-eastern Atlantic and originated two separated populations. Although the estimated expansion time from the mismatch analyses must be looked with caution, since no molecular clock calibration is available for the species and the one used might not be the most accurate, the values of expansion for MEDS and NEAT are within similar range, mainly the early/middle Pleistocene, which is probably the time since when the two populations have been evolving separately.

No genetic divergence was found for NEAT populations. Nucleotide diversity showed no defined geographical trend, with similar values for most of the populations, and with curiously, the high nucleotide diversity for S7 marker found in the northern regions (BG and EN). Three cyt*b* haplotypes, which represented more than 75% of haplotype frequency for this region, were shared by all the populations, with 18 haplotypes appearing only in one population. It is expected for a species with a reproduction strategy such as *S. cantharus*, with spawning aggregations and male nest guarding behaviours (Russel et al., 2014; Pinder et al., 2017), that a small number of effectives contribute highly for the genetic pool of the population, since older and larger males are likely to monopolize access to females (Chopelet et al., 2009) and females that don't change sex will produce much more offspring's than smaller females (Hislop, 1988). This reduction in the number of possible genotypic combinations available and skewed sex ratio is predictable to reduce effective population size, increasing genetic drift and hence present a higher potential for exhibiting more spatially structured populations, although no clear support for these theoretical expectations still exists (Chopelet et al., 2009).

S. cantharus populations within each of the analysed regions show no evidence of such structure, with very low $\Phi_{ST.}$ However, the presence of several private haplotypes for each population might suggest some population segregation. Information available on the species also points for this segregation, with clear morphological shape of body and otoliths varying across the sampled areas (Neves et al., submitted). Low values of Φ_{ST} can be due to just enough gene flow existing to homogenize neutral markers despite extremely limited exchange of individuals on average among sites (Conover et al., 2006), and significant differences between the populations still exist for other biological characteristics that are changeable on a shorter timescale. The same authors refer much higher values of divergence for quantitative trace loci between populations than those obtained with neutral markers (Φ_{ST}). In fact, several authors have already detected, for other species, no population structure with molecular markers but differentiation with phenotypical traits (eg. Abaunza et al., 2008; Marin et al., 2016; Vieira et al., 2016), pointing to the importance from a fisheries management point of view that other methodologies in addition to genetic analyses should be gathered to establishing an effective knowledge of the species population structure.

Conclusion

The obtained results reveal a large scale structured population for *S. cantharus*, alerting for the fact that drastic decreases in any of the analysed regions are unlikely to be overcome by migration from one other region. Despite that only a single location has been sampled in Cape Verde, the low genetic diversity estimated for the region should be taken in account and considered in future studies for the area.

Regardless of the low differentiation between the populations within NEAT and MEDS regions, the presence of a high rate of private haplotypes in every location and the data available from phenotypic traits which show significant differences within NEAT population, indicates that, although some gene flow exists among locations there is still sufficient isolation to account some differentiation. Such pattern is also the most likely to occurs in MEDS populations although no phenotypic data is available for this region.

For fisheries propose, data presented in this study suggest a significant stock structure at regional scale for *S. cantharus,* and that, some population segregation at smaller spatial scale shouldn't be excluded. Thus, stock assessment for the species should be done independently in most of the analysed areas, and life history parameters should be updated for each area. This would help to understand how the gene flow detected in the present study, influences the life traits of the species.

SECTION 2

Life History Parameters

CHAPTER 5

Modelling the growth of a protogynous sparid species, *Spondyliosoma cantharus*

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Modelling the growth of a protogynous sparid species, *Spondyliosoma cantharus*

ABSTRACT

Age determination of fish species is a key process for fisheries management and the accuracy of assessments is highly dependent on age estimates. To model complex life histories such as protogynous hermaphroditism, the growth curve to fit the age-length data should be carefully chosen. For the first time, the first annual growth increment of *Spondyliosoma cantharus* was validated and several growth functions were applied, in order to find the best growth model. *S. cantharus* specimens ranged from 2.1 to 38 cm total length and were aged from 0 to 17 years. For the growth functions applied, the hyperbolic modifications of von Bertalanffy curve showed the best fit to the data. Under this model, a change in growth occurs at 8 years, which corresponds to the average age for sex reversal in the species. Estimated total mortality was similar for the two years studied, varying between 0.65 yr^{-1} and 0.69 yr^{-1}. Natural mortality was estimated by the updated $Hoenig_{nls}$ t_{max}-based estimator and the $Pauly_{nls-T}$ estimator, ranged between 0.26 yr^{-1} and 0.37 yr^{-1}. Fishing mortality (0.28 yr^{-1} – 0.43 yr^{-1}) and exploitation rate (0.43 – 0.62) were relatively high, indicating that although the species is not a main target its management needs careful attention.

Keywords: growth modelling; hermaphroditism; mortality; von Bertalanffy curve modifications

5.1 Introduction

Sustainability of marine resources relies on a secure stock assessment of marine fisheries. Age studies are very important when addressing a fish stock's population dynamics, as this information is critical for studying population characteristics such as growth, recruitment, mortality, and reproduction, and it is necessary for detailed studies on life history strategies and ecology can be carried out (Labropoulou and Papaconstantinou, 2000).

Most age studies involve the count of growth increments in hard body parts. Otolith formation involves seasonal variations in the deposition of organic matrix and carbonate crystals, resulting in the formation of macroscopic opaque and translucent zones (Morales-Nin, 2001), which promote the wide use of these structures for age assignment studies.

The mostused model for describing fish growth is the von Bertalanffy model (von Bertalanffy, 1938), which presuppose an equal growth parameter throughout life, and therefore does not take in account differences in energy allocation for reproduction and somatic activities at different life stages (Charnov, 2008). In sequential hermaphrodites, energy invested from maturing one type of gonads, sex transition and maturation of the second set of gonads will probably undergo several shifts. Also, for territorial protogynous species, size will be an advantage for territory protection and therefore an increased investment in growth will precedes the investment in reproductive output (Hoffman et al., 1985).

To model such complex life histories, the ideal curve to fit should be carefully chosen. Over the years several authors have proposed modifications of the basic von Bertalanffy model, in order to accommodate smooth transitions between growth rates at a given moment of life, where the surplus energy is partially allocated to reproduction and partially to growth (Minte-Vera et al., 2016). To overcome possible ineffective growth modelling of species, different methods available on the literature should be tested and their adjustment to the data compared and the best model should be considered as an enhancement to parameters estimation.

Black seabream, *Spondyliosoma cantharus*, is a sparid species with a distribution from Scandinavia to Angola, occurring in Madeira, Cape Verde, Canary Islands and in

Mediterranean Sea. It is a gregarious species found over seagrass beds both in rocky and sandy bottoms, from depths < 50 m up to 300 m (Bauchot and Hureau, 1986). Black seabream is a protogynous hermaphrodite, with sex change from female to male occurring at various ages (Pajuelo and Lorenzo, 1999). Despite the large distribution range, little information exists on its biology, and no information on the stock structure is available. Information on the growth of the species is scarce and mainly available for the Tunisian coast (Mouine-Oueslati et al., 2015), Adriatic Sea (Dulčić and Kraljević, 1996), Canary Islands (Lorenzo and Pajuelo, 1997), and southern Portuguese coast (Abecasis et al., 2008). These studies have only applied the traditional version of the von Bertalanffy model, and no validation studies were ever attempted (even indirectly) on deposition of the first annulus. Mortality parameters for the species are only available for the Canary Islands (Pajuelo and Lorenzo, 1999).

In order to gather information on *S. cantharus* for the Portuguese western coast, this study has 4 main goals: 1) estimate the size of the species at the end of the first year of life and validate the first annual growth increment; 2) validate the assigned ages; 3) model the growth of the species; and 4) estimate natural and total mortality, in order to obtain an initial approximation of the exploitation level for this species.

5.2 Material and Methods

5.2.1 Sampling

Monthly samples were collected between April 2014 and June 2015 from commercial landings at Peniche, one of the main commercial ports on the Portuguese mainland (Fig. 5.1). Samples were taken from several gears: trawl, trammel net, traps, hooks and lines. A summary of sampling methodology is given in Table 5.1.

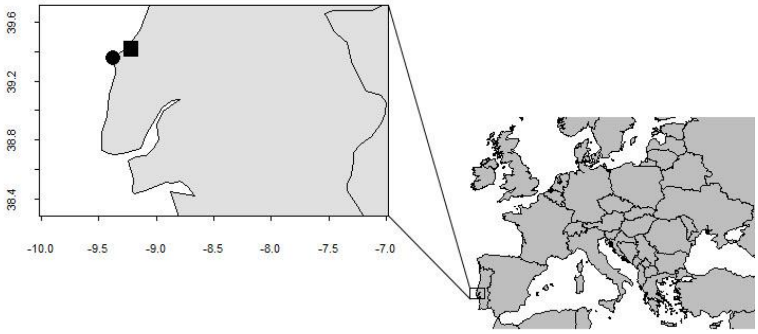

Fig. 5.1 Map of sampling sites location on the Portuguese coast. Black circle indicates Peniche and black square indicates Óbidos Lagoon.

In order to follow the first year cohort growth and establish the first annual growth increment, additional samples from Óbidos Lagoon (Fig. 5.1) were collected from April to December 2014, using a beach seine. This lagoon, located on the West coast of Portugal ca 20 km from Peniche, is a shallow estuarine system, with a mean depth of 1.5 – 2 m (Gordo and Cabral, 2001) and is considered the largest coastal lagoon on the Portuguese coast.

Table 5.1 Sampling strategy summary.

		Sampling Duration		Number of individuals sampled		Fishing gear
		2014	2015	2014	2015	
Ageing	Peniche and Obidos Lagoon	Mar - Dec	Jan - Dec	535	213	Tr; TN; Tp; H; L
Validation of first age growth increment	Obidos Lagoon	Apr - Dec		1342		BS
Length-Weight relationship	Peniche and Obidos Lagoon	Mar - Dec	Jan - Dec	547	213	Tr; TN; Tp; H; L
Mortality	Peniche	May - Dec	Jan - Dec	1749	1714	Tr

Tr: trawl; TN: trammel net; Tp: traps; H: hooks; L: lines; BS: beach seine

5.2.2 Length–weight relationship

The relationship between TL (cm) and EW (g) was calculated using a power function: $EW = a \times TL^b$

where a is the intercept and b is the allometric exponent. Allometry in growth was investigated by the Student’s t-test (Zar, 1996).

5.2.3 First annual growth increment validation

Length-frequency data obtained from Óbidos Lagoon samples were grouped into 1 cm intervals, from 2 to 13 cm, and evaluated by modal progression analysis (MPA). MPA infers growth from the apparent shift of the modes or means in a time series of length-frequency samples (Gayanilo et al., 2005). This analysis was performed with the Bhattacharya’s method using the routine in FAO-ICLARM Fisheries Assessment Tools (FiSAT II version 1.2.2) software (Gayanilo et al., 2005). The Bhattacharya method is based on approximating the assumed normal curve of a length–frequency distribution as a parabola, which is then converted to a straight line with the form:

$$dt(\ln[N]) = a + b(L),$$

where dt(ln[N]) is the difference between the natural logarithms of the number in one length class and the number in the preceding length class, and L is the upper limit of the preceding length class. The Bhattacharya plot is a graphical method of separating a length–frequency distribution into a series of normal distributions or pseudo-cohorts (King, 2007).

An image from the right otolith of 230 individuals, ranging from 2.6 to 13.0 cm TL, was captured at 40× magnification using a visual image analysis system (Leica DFC 290). The radius of each otolith was recorded using ImageJ software 1.48v (available at http://imagej.nih.gov/ij/, Rasband, 1997-2016), measured from the nucleus towards the edge of the posterior region. The relationship between fish TL and otolith radius was investigated, in order to estimate the width of the first annual growth increment.

5.2.4 Ageing methodology, precision and bias

Right otoliths were read immersed in 1 : 1 glycerine-ethanol blend, under reflected light against a dark background to enhance the differentiation of annual increments, with a 18× magnification. The annual periodicity of annuli formation was validated through Marginal Increment Ratio (MIR) (Samamé, 1977). If the increments are annual, the mean width of the latest increment (normalised by the otolith radius) will follow a periodic trend across the year (Panfili and Morales-Nin, 2002).

Ageing was performed with the *sulcus acusticus* side down and towards the posterior region of otoliths. A subsample of 186 otoliths (10 by 1 cm length classes, whenever possible), was read by two of the authors to find an interpretation pattern and ensure consistency (Campana, 2001). Readings were compared through the average percentage error (APE) (Beamish and Fournier, 1981), the coefficient of variation (CV) (Chang, 1982), and the percentage of perfect agreement (PAgree). Bias was evaluated based on age bias plots (Campana et al., 1995), which allow an insight on departures from the 1 : 1 equivalence line, and systematic differences on assigned ages between readers were assessed with the Bowker-type test for symmetry (Hoenig et al., 1995). Since the achieved agreement between readers was good, the remaining otoliths were read twice by the first author. A third reading was performed for all divergent readings and if this differed from the previous two, the otolith was discarded.

5.2.5 Growth models

Beside the typical von Bertalanffy curve, biphasic models and the five parameter von Bertalanffy model were fitted to length-at-age data for *S. cantharus* (Table 5.2). Biphasic models include a hyperbolic function that modifies either L_∞ or K as age increases (Soriano et al., 1992); the five parameter von Bertalanffy model allows a modification on growth rate at some moment of the lifespan (Alós et al., 2010). Models were implemented in R (cran.r-project.org), using the packages FSA (Ogle, 2016) and nlstools (Baty et al., 2015), and were compared using the Akaike Information Criterion (AIC, Akaike, 1973).

Table 5.2 Models fitted to length-at-age data for *Spondyliosoma cantharus*.

Model	NP	Equation
VBGF	3	$\mathrm{TL} = \mathrm{L}_\infty \times \left(1\text{-}\exp\left(\text{-}\mathrm{K}\times(\mathrm{Age}\text{-}\mathrm{t}_0)\right)\right)$
VB-hyperL_∞	5	$\mathrm{TL}=\mathrm{L}_\infty\times\left(1\text{-}\left(\frac{\mathrm{h}}{((\mathrm{Age}\text{-}\mathrm{t_h})^2+1)}\right)\right)\times\left(1\text{-}\exp\left(\text{-}\mathrm{K}\times(\mathrm{Age}\text{-}\mathrm{t}_0)\right)\right)$
VB-hyperK	5	$\mathrm{TL}=\mathrm{L}_\infty\times\left(1\text{-}\exp\left(\text{-}\mathrm{K}\times\left(1\text{-}\left(\frac{\mathrm{h}}{((\mathrm{Age}\text{-}\mathrm{t_h})^2+1)}\right)\right)\times(\mathrm{Age}\text{-}\mathrm{t}_0)\right)\right)$
5 VBGF	5	$\mathrm{TL}=\begin{cases}\mathrm{L}_\infty\times\left(1\text{-}\exp\left(\text{-}\mathrm{K}_0\times(\mathrm{Age}\text{-}\mathrm{t}_0)\right)\right) & \text{if Age}<\mathrm{t}_1\\ \mathrm{L}_\infty\times\left(1\text{-}\exp\left(\text{-}\mathrm{K}_0\times(\mathrm{Age}\text{-}\mathrm{t}_0)\text{-}\mathrm{K}_1\times(\mathrm{Age}\text{-}\mathrm{t}_1)\right)\right) & \text{if Age}\geq\mathrm{t}_1\end{cases}$

VBGF: von Bertalanffy growth function; VB-hyperL_∞: hyperbolic modification of von Bertalanffy growth function for L_∞; VB-hyperK: hyperbolic modification of von Bertalanffy growth function for K; 5 VBGF: five parameter von Bertalanffy growth function. NP: number of parameters estimated. L_∞ is the maximum asymptotic length; K is the relative growth rate; t_0 is the theoretical age for time at which length is zero; h is an expression of the difference between each phase in the growth curve; t_h and t_1 are the age at which the nature of the curve changes; K_1 is the relative growth rate for the second phase of life.

5.2.6 Mortality

Total instantaneous mortality rate, *Z*, was estimated using the Chapman–Robson mortality estimator (Chapman and Robson, 1960), with the first age group used being 1 year older than the age of peak abundance. According to Smith et al. (2012), this is the preferred estimator for age-frequency simulation data. Only data from trawl landings were used (1749 records from 2014 and 1714 from 2015), since this gear represents more than 60% of total landings. To estimate the instantaneous natural mortality rate (*M*), the updated Hoenig$_{nls}$ t$_{max}$-based estimator, $M = 4.899 \times t_{max}^{-0.916}$ and the Pauly$_{nls\text{-}T}$ estimator, $M = 4.118 \times K^{0.73} \times L_{\infty}^{-0.33}$, were used (Then et al., 2015). Temperature was not included in Paulys' equation since it did not appear to bring any information to the model (Gislason et al., 2010; Then et al., 2015). Fishing mortality (*F*) and exploitation rate (*E*) were estimated as $F = Z - M$ and $E = F / Z$. Mortalities were estimated using the R package FSA (Ogle, 2016).

5.3 Results

5.3.1 Length–weight relationship

Among the 758 individuals examined, 174 were immature, 312 were female, 3 were bisexual and 268 were male. Total length ranged between 2.6 – 13.1 cm (mean 8.2 ± 1.8 cm), 9.1 – 33.2 cm (mean 22.6 ± 3.3 cm), 20.9 – 23.9 cm (mean 22.8 ± 1.7 cm), and 20.0 – 38.0 cm (mean 27.6 ± 3.5 cm), respectively. Eviscerated weight varied between 0.19 – 33.2 g (mean 8.0 ± 5.7 g), 9.67 – 504 g (mean 164.3 ± 74.4 g), 140 – 210 g (mean 177.0 ± 35.0 g), and 115 – 841g (mean 309.9 ± 131.6 g), respectively.

Black seabream showed a strong relationship between TL and EW ($r^2 = 99.7$) expressed by the equation: $EW = 0.0073 \times TL^{3.1944}$. This species shows a positive allometric growth (t-test = 10.3, df = 758, $P < 0.001$).

5.3.2 First annual growth increment validation

A total of 1342 individuals, ranging from 2.1 to 13.6 cm TL, were collected during the sampling period. MPA detected three cohorts in Óbidos Lagoon (Fig. 5.2a). Larger individuals were sporadic (only 2% above 10 cm), suggesting that individuals stay in the lagoon mainly for the two first years of life. On average, black seabream individuals had reached 7.8 ± 0.9 cm TL by the end of their first year of life.

Body and otolith size followed a positive linear relationship (Fig. 5.2b, $r^2 = 94.6$) which allowed the estimation of the first annual growth increment width at 1.65 ± 0.16 mm.

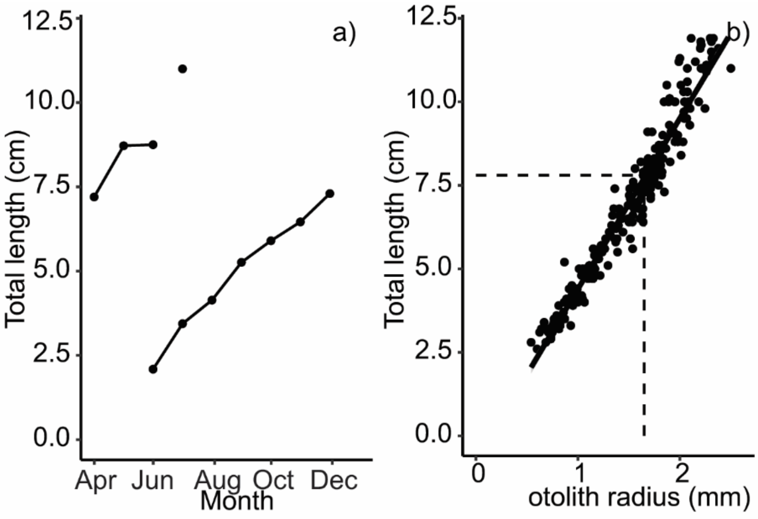

Fig. 5.2 (a) Bhattacharya plot for length-frequency data of black seabream, *Spondyliosoma cantharus*, obtained from Óbidos Lagoon and (b) relationship between total length (TL) and otolith radius (OR) from black seabream, *S. cantharus*, sampled in Óbidos Lagoon. Solid line is the linear regression, dashed lines correspond to values of TL and OR for the 1st year of life.

5.3.3 Ageing methodology, precision and bias

The deposition pattern of growth increments varied with otolith size: typically the first three increments were wider, becoming thinner and closer together with age. For some individuals, the first growth increment was not clearly marked. MIR analyses showed an annual pattern in growth increment formation, with an increasing trend from January to July of the marginal increment followed by a decreasing trend (Fig. 5.3). The highest values of MIR occurred between May and September, which points this as the period when new increments are formed.

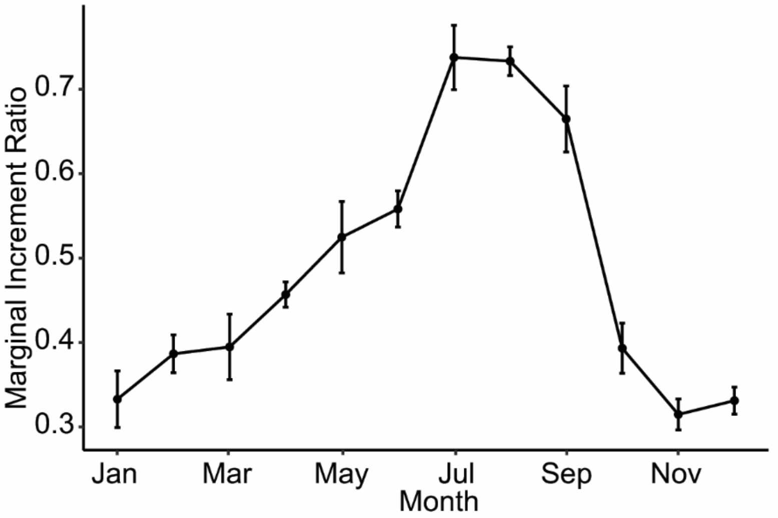

Fig. 5.3 Monthly evolution of marginal increment ratio (MIR) in otoliths of black seabream, *Spondyliosoma cantharus*, from the Portuguese continental waters. Dots are the mean values and whiskers are ± standard error.

Indices of precision within and between readers (Table 5.3) revealed a fair precision of age readings. Age-bias plots (Fig. 5.4) and the test of symmetry (χ^2_{R1} = 20.1, df = 18, P = 0.3248; χ^2_{R1vsR2} = 21.2, df = 16, P = 0.1709) showed a random error pattern for age disagreement between readers.

Table 5.3 Indices of precision for age readings of black seabream, *Spondyliosoma cantharus* within and between readers.

Index	Index comparison (%)	
	Reader 1	Between Readers
APE	1.89	2.14
CV	2.67	3.03
PAgree	75.6	77.3

APE is the mean percentage error, CV is the mean coefficient of variation and PAgree is the percentage of perfectly agreement

5.3.4 Growth models

For age estimation, 188 immature individuals (2.8 – 12.8cm TL), 301 females (9.1 – 33.2 cm TL), 3 bisexuals (20.9 – 23.9 cm TL) and 256 males (19.6 – 38 cm TL) were used. Only 2.2% individuals showed non-interpretable otoliths. Age assignments varied from 0 to 17 years. The frequency composition of TL and age are shown in Fig. 5.5.

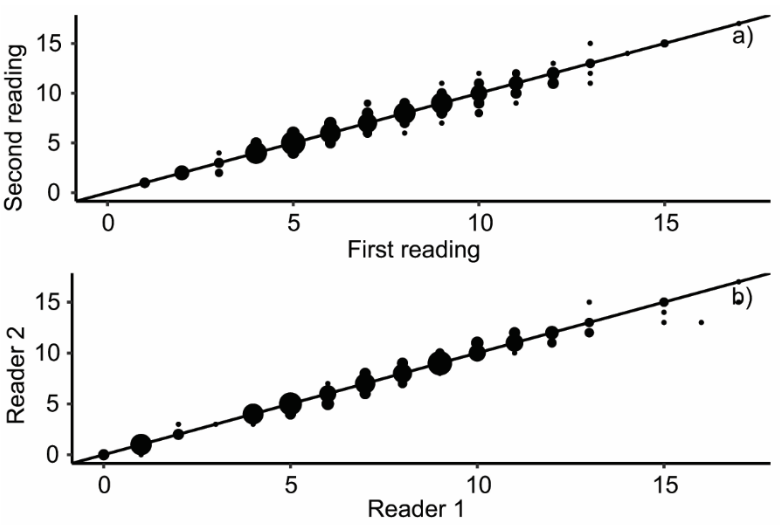

Fig. 5.4 Age bias plots for the readings comparisons within (a) and between (b) readers for black seabream, *Spondyliosoma cantharus*. The 45º line represents 100% agreement and dot size represents number of observations.

Uniphasic and biphasic versions of von Bertalanffy growth function were fitted to length-at-age data for *S. cantharus*. Biphasic models provided better fits to the data than the uniphasic model, as they presented lower AIC (Table 5.4). The best models were the hyperbolic modifications of von Bertalanffy curve, both with very similar estimates for the parameters and pointing for a change in growth strategy to occur during the eighth year (t_h = 7.46 – 7.51), corresponding to a TL around 24.5cm. The five parameter von Bertalanffy curve provided an unrealistic estimate for L_∞, since it was lower than the largest individual sampled. Moreover, the age estimated for the change of growth pattern appears to be overestimated by this model.

Table 5.4 Summary of von Bertalanffy growth parameters resulting from the four models fitted to black seabream, *Spondyliosoma cantharus*, data.

Parameters	VBGF	VB-hyperL_∞	VB-hyperK	5 VBGF
L_∞ (cm)	41.19 (0.7383)	40.66 (0.6325)	40.49 (0.6352)	36.16 (0.8441)
K (years^{-1})	0.11 (0.0040)	0.12 (0.0040)	0.12 (0.0041)	0.14 (0.0064)
t_0 (years)	-1.41 (0.0512)	-1.26 (0.0399)	-1.27 (0.0465)	-1.25 (0.0502)
h	na	0.07 (0.0077)	0.12 (0.0130)	na
t_h (years)	na	7.46 (0.0871)	7.51 (0.0854)	na
K_1 (years^{-1})	na	na	na	0.13 (0.0385)
t_1 (years)	na	na	na	8.73 (0.1827)
AIC	2638.94	2557.74	2560.91	2600.74
Δ AIC	81.20	0.00	3.17	43.00

Standard error of parameters is given in parenthesis. AIC is the Akaike Information Criterion; Δ AIC is the rescaled information criterion. Δ AIC valued as zero indicates the most appropriate model.

5.3.5 Mortality

Total mortality was estimated for two years: Z_{2014} = 0.65 ± 0.06 yr^{-1} and Z_{2015} = 0.69 ± 0.05 yr^{-1}. Estimated natural mortality ranged between 0.26 yr^{-1} (Pauly$_{nls\text{-}T}$ estimator) and 0.37 yr^{-1} (Hoenig$_{nls}$ t_{max}-based estimator). Estimates of fishing mortality varied between 0.28 – 0.39 yr^{-1} for 2014 and 0.32 – 0.43 yr^{-1} for 2015. The exploitation rate was very similar for both years, with values between 0.43 – 0.60 and 0.46 – 0.62 for 2014 and 2015, respectively.

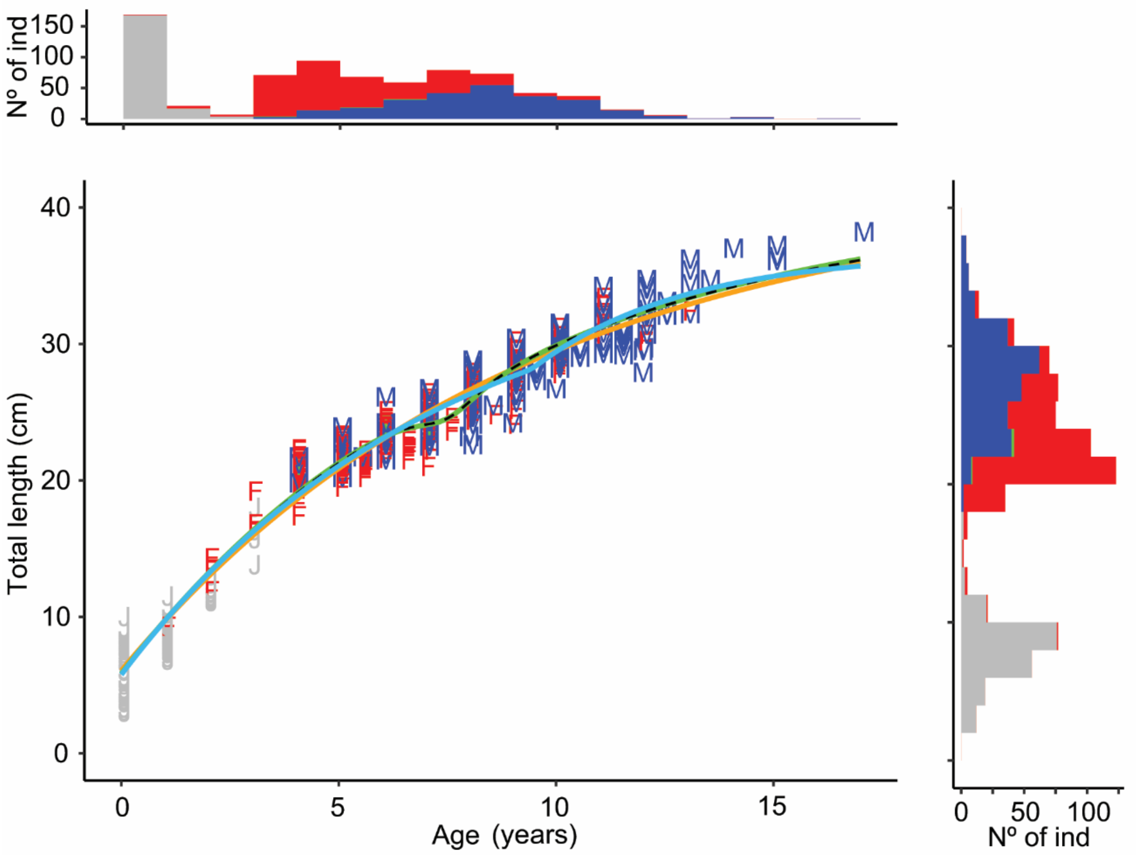

Fig. 5.5 Frequency distribution of the observed TL and age and fit of the four growth models to the black seabream, *Spondyliosoma cantharus,* data. Grey / J are juveniles, red / F are females, blue / M are males and green / T are transitional individuals. Orange line is the typical von Bertalanffy curve; green solid line is L_∞ hyperbolic biphasic model; black dashed line is K hyperbolic biphasic model and light blue solide line is the five parameter von Bertalanffy model.

5.4 Discussion

Spodyliosoma cantharus is a protogynous hermaphrodite species and all analyses were conducted over the entire sample, without sex sorting. However, it is likely that some individuals spent most of their life with a single sex, since males of 20 cm were found and the females sampled reached almost 34 cm.

This species showed a positive allometric growth on the Portuguese western coast, as already noticed for the Canary Islands (Pajuelo and Lorenzo, 1999), Algeria (Derbal et al., 2010; Boughamou et al., 2015), Tunisia (Mouine-Oueslati et al., 2015), and the Adriatic Sea (Dulčić and Kraljević, 1996). Bilge et al. (2014) found a negative allometry for the black seabream population in Aegean Sea, but the small sample (79 individuals) and narrow length range analysed (8.6 to 18.7 cm TL) might justify the opposite result for this area.

The age estimation of fish species is fundamental for the accuracy of assessments in fisheries management. It is therefore essential that age estimates will be as accurate as possible. One important component of any age validation study is the identification of the first annual growth increment (Campana, 2001). From the several age validation methods only a subsample possess, in practice, the necessary precision to be used in the identification and validation of the first annual growth increment (Campana, 2001). Release of known age or chemically-marked young-of-the-year fish, modal progression and daily increment counts are the most suited for this type of application (Campana, 2001). In this study the modal progression was used to validate the first annual growth increment by following the first year cohort in Óbidos Lagoon. Individuals reached their first year of life with a mean size of 7.8 ± 0.9 cm TL and an otolith radius of 1.65 ± 0.16 mm. The validation of first annual growth increment is important to understand the pattern of deposition of growth increments, especially since this first growth increment was not always well defined, possibly due to the longer or shorter permanence of the fish in the lagoon.

Otolith edge analysis demonstrated that black seabream deposited one annulus per year, showing a clear larger increment during the summer months. There was a small number of rejected otoliths and the precision obtained both within and between readers were much better than the reference values (*CV* < 7.6% and *APE* < 5.5%) suggested by Campana (2001). Hence, the otolith structure of the black seabream allows an adequate identification of its growth pattern. Nevertheless, the discrepancies found in the literature, with assigned ages varying between 7 years for a 35 cm specimen (Boughamou et al., 2015) and 17 years for a 38 cm specimen (this study), point to a need to improve age reading methods and age determination between different research groups.

For the first time, several growth models were applied to *S. cantharus* age-length data. Ontogenetic transitions such as sex change can disrupt energy allocation, such that the period before and after the change become identifiable as distinct life phases (Higgins et al., 2015). It is therefore important to search alternative methodologies to better understand growth in such species. In the present study, the hyperbolic modifications of von Bertalanffy curve showed the better fit to the data. The estimated von Bertalanffy K, 0.12 years^{-1}, was lower than those reported for the species in the Canary Islands (0.23 yr^{-1}; Lorenzo and Pajuelo, 1997), southern Portugal (0.21 yr^{-1} and 0.26 yr^{-1}, estimated for scales and otoliths readings, respectively; Abecasis et al., 2008), Algerian eastern coast (0.52 yr^{-1}; Boughamou et al., 2015), and Adriatic sea (0.18 yr^{-1}; Dulčić and Kraljević, 1996), but similar to those reported for Tunisian coasts (0.10 - 0.14 yr^{-1}; Bradai et al., 1998, Mouine-Oueslati et al., 2015). Although it is natural for species to show different growth patterns for each region, due to environmental/habitat characteristics, the differences reported between geographic areas may also reflect divergences in the species ageing.

The estimated age at which the curve changes shape corresponds to the average time at which black seabream undergoes sex reversal (Neves, unpublished data). This observed deceleration in growth is probably caused by the allocation of energy towards the development and maturation of new gonadal tissue.

In stock assessment models, the mean length and variation of length in particular for the oldest ages are highly influential on the estimated fishing mortality and abundance levels (Maunder and Piner, 2015). Therefore, it is important to improve the performance of growth models and get the growth curve correct for the greater ages (Minte-Vera et al., 2016).

Most studies on mortality estimation use catch-curve analysis, which assumes: a) constant recruitment and mortality after fish enter the fishing area (recruitment age), being the specimens then equally vulnerable to fishing and sampling; and b) no age-estimation errors (Ricker, 1975). These assumptions are unlikely to be met. Some attempts have been made to verify the performance of catch curve mortality estimators in response to several error types in simulated scenarios, and in most scenarios the better performance was achieved by Chapman–Robson estimator (eg. Murphy, 1997; Dunn et al., 2002; Smith et al., 2012). In the present study we assume that the black seabream population is in equilibrium with no significant variation in recruitment or mortality across time, and we attempt to minimise age estimation errors by following precision and bias methodology. *S. cantharus* is caught by multiple fishing gears, but only trawl, the most representative one, was used for the mortality study, since the number of individuals caught by other gears was low and variable along the year. Trawl selectivity is generally characterised by logistically shaped curves (e.g. Huse et al., 1999; Zuur et al., 2001), but a knife-edge selectivity was assumed, according to the above-mentioned assumptions. The estimated *Z* values were very similar for the two years analysed, whereas *M* presented some variation depending on the method used. This was expected since according to Then et al. (2015), the Hoenig$_{nls}$ model gives generally higher *M* estimates than the Pauly$_{nls\text{-}T}$ method for stocks that experience *M* rates higher than 0.2 yr^{-1}. Although several authors defend that estimates of *M* should take variations over age and size into account (eg. Gilslason et al., 2010; Johnson et al., 2015), a single value for *M* can provide a useful representation of mortality and the assumption of a constant *M* in stock assessments is still very useful (Then et al., 2015).

The estimated values for *Z* and *M* in this study were much lower than the ones reported for the Canary Islands (Pajuelo and Lorenzo, 1999), even though both areas presented high exploitation rates. Although this species is not a main target for a specific fishery, it is caught by several fishing gears that operate near the coast. These non-selective gears catch individuals of different populations from the ecosystem and may lead to an overfishing of some species.

This species has a 23 cm (TL) minimum legal size ruled in the Portuguese fishery (DGRM, 2016), which is above the estimate of size at 50% maturity for the species females for the Portuguese coast (Gonçalves and Erzini, 2000). However, 24% of the specimens landed in Peniche were smaller than the minimum legal size. As mentioned above, most landings of black seabream come from trawl fisheries, which have as target species mainly the horse mackerel, *Trachurus trachurus*, Atlantic chub mackerel, *Scomber colias*, Atlantic mackerel, *Scomber scombrus*, pouting, *Trisopterus luscus* and European hake, *Merluccius merluccius* (Campos et al., 2007). These species have smaller minimum landing sizes, varying from 15 cm to 20 cm (DGRM, 2016), which explains the large number of small black seabream landed.

In protogynous species such as *S. cantharus*, the overfishing scenario can be even more problematic since the catch can truncate size distributions and at an extreme, virtually remove males from the population and thus lead to the population collapse by sperm limitation (Alonzo and Mangel, 2004). In the Portuguese coast, it seems that smaller individuals, consisting mostly of females, are the most vulnerable to the fishery. The overexploitation of these smaller individuals will affect the reproductive potential, with a negative impact for the stock. As referred by Gonçalves and Erzini (2000) more than a change in the legal minimum fish length, there needs to be a reinforcement of the actual legislation, in order to obtain a sustainable management of this species. Studies on short-term hooking mortality showed a low percentage of dead after release for black seabream juvenile individuals (Veiga et al., 2011). This suggests that if individuals under the minimum legal size were released, the rate of survival would probably be high. Enhanced fishermen awareness on this subject is called for, to better preserve this species.

CHAPTER 6

Insight on reproductive strategy in Portuguese waters of a commercial protogynous species, the black seabream *Spondyliosoma cantharus* (Sparidae)

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Insight on reproductive strategy in Portuguese waters of a commercial protogynous species, the black seabream *Spondyliosoma cantharus* (Sparidae)

ABSTRACT

Information on fish reproductive strategy is essential to understand population dynamics. Samples of black seabream, *Spondyliosoma cantharus*, were collected from the western Portuguese continental coast to investigate the reproductive cycle, the timing of spawning, length at maturity and sex change, fecundity type and fecundity. This species is a protogynous hermaphrodite, showing a sharped biased sex ratio towards females for lengths smaller than 25 cm, and significantly biased towards males above this value, with no females occurring above 35 cm. Development of secondary growth follicles was asynchronous, and it was estimated that each female spawns 27 batches during the spawning season, which takes place from February to May. Half of the females' population was mature at 18.41 cm and have changed sex at 25.62 cm. Sex change takes only a brief period of time, as transitional individuals were scarce and most showed oocytes regressing into cystic structures. The species presents a clear indeterminate fecundity type with massive atresia happening at the end of the spawning season. Mean values of 203 oocytes and 5431 oocytes by gram of eviscerated female were estimated for relative batch fecundity and relative annual fecundity, respectively.

Keywords: cysts; fecundity; length at first maturity; reproduction; sex-change; spawning season

6.1 Introduction

Portugal has the highest seafood consumption per capita in European Union and one of the largest in the world (FAO, 2010), and although approximately 370 fish species are commercially explored in its waters (Leitão et al., 2014), only a small group is assessed for management purposes. The small-scale fisheries have a considerable socioeconomic importance for the local population and represent 80% of the national fleet (Gaspar et al., 2014) playing a decisive role in exploring a large group of coastal species. But despite its importance, the management of these fisheries is still inconsequent or inexistent due to the lack of biological information (Gaspar et al., 2014).

Sparids are one of the most targeted fish families in Portuguese coastal fisheries (Erzini et al., 1996; Cabral et al, 2003; Leitão et al., 2016). The black seabream, *Spondyliosoma cantharus,* is a protogynous Sparidae species with a vast distribution along the eastern Atlantic, occurring from Scandinavia to Namibia, around the Madeira, Cape Verde and the Canary Islands. It is also common in the Mediterranean Sea and the western Black Sea (Bauchot and Hureau, 1986) and is an important commercial fish, exploited in European waters by recreational and commercial fishers (Russell et al., 2014). In Portuguese waters it is caught by several different fishing gears, with mean annual landings of 180 ton in the last decade (DGRM, unpublish data).

Knowledge on the reproductive strategy of a fish species is essential for an effective population management. In order to understand this strategy, information on reproductive cycle, the timing of spawning and follicle development should be gathered (Alonso-Fernández et al., 2011; Lowerre-Barbieri et al., 2011). Sex changing species need a special attention to their reproductive strategy since sequentially hermaphroditic fish present different social organizations and reproductive modes according to their life-history system (Benvenuto et al., 2017).

S. cantharus exhibits life history characteristics that make it particularly vulnerable to local over-exploitation since it is a hermaphrodite slow-growing, long-lived, and exhibits habitat specificity during the spawning season, when it shows spawning aggregations and male nest guarding behaviours (Russel et al., 2014; Pinder et al., 2017). Information of spawning season, length at first maturity and sex change for the species is available for the English Channel and Bay of Biscay (Perodou and Nedelec, 1980; Soletchnik, 1982), South Coast of Portugal (Gonçalves and Erzini, 2000), Canary Islands (Pajuelo and Lorenzo, 1999), Saharan bank (Balguerías, 1995) and Mediterranean (Mouine et al., 2007; Mouine et al., 2011; Boughamou et al., 2015), fecundity data is also reported on Soletchnik (1982), Balguerías (1995), Gonçalves and Erzini (2000) and Dulčić et al. (1998) for the eastern middle Adriatic. However, none of these studies present a thorough histological analysis and fecundity type study for the species.

Despite the particular life history strategy and commercial importance of *S. cantharus*, no data is available for the reproductive strategy of the species for the western Portuguese coast. The present study intends to enlighten diverse aspects of the reproduction of *S. cantharus* for this area. Sex transition aspects are investigated, and reproductive cycle is examined. Ovary organisation and oocyte development are studied over the reproductive cycle. Fecundity type is defined and according to the results, batch and annual fecundity are estimated.

6.2 Material and Methods

6.2.1 Sampling

Monthly samples were acquired between April 2014 and June 2015 from commercial vessels operating off mainland Portugal (Peniche, Fig. 6.1). During the spawning season sampling had a fortnightly basis. Additional samples using a beach seine were carried on Óbidos lagoon, near Peniche, from April to December 2014, to obtain juvenile individuals since in commercial landings only mature individuals are caught.

Samples were processed in fresh, and total length (TL, to the nearest 0.1 cm), eviscerated weight (EW, to the nearest 0.01 g), gonad and liver weights (GW and LW, respectively, to the nearest 0.01 g), and sex were recorded. Gonads were fixed in 4% buffered formalin for posterior histological analysis.

Sex ratio was established for classes of 5 cm interval and significant differences from 1/1 ratio were evaluated with Pearson's Chi-squared test in R studio version 1.0.143 (RStudio Team, 2016).

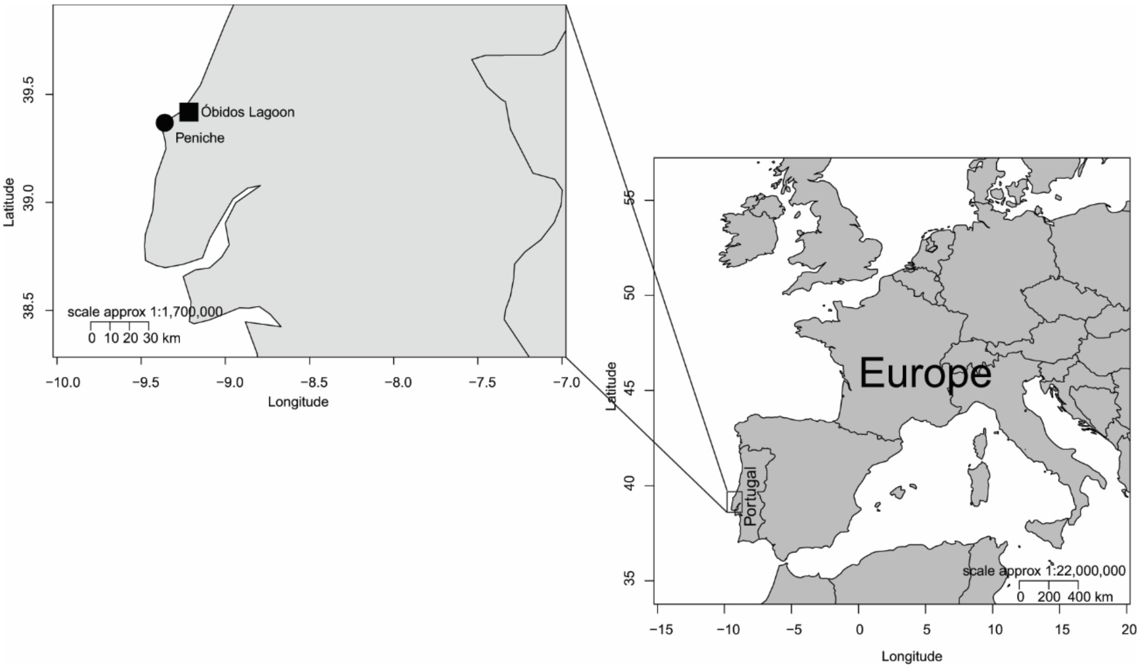

Fig. 6.1 Map of sampling sites location on the Portuguese coast. Black circle indicates Peniche and black square indicates Óbidos Lagoon.

6.2.2 Ovary and test organisation

A total of 1530 individuals were sampled, from these, 773 gonads were analysed histologically. All female from December to July were histologically processed, while for the other months a subsample of 5 females with macroscopically equal gonads were histologically analysed in order to assure that maturation stage was the same. The same criteria of 5 samples per macroscopically equal gonads was defined for clear male gonads, while gonads that showed any possible presence of different tissues were processed histologically. For the juvenile individuals caught in Óbidos Lagoon, 30 gonads were analysed histologically to verify if the female tissue has already started to develop. Each gonad portion was dehydrated with ethanol, embedded in methacrylate, sectioned at 3 µm and stained with toluidine blue. The spawning season of *S. cantharus* was defined by the occurrence of the first and last actively spawning females during the sampling period.

Oogenesis (Table 6.1) and spermatogenesis (Table 6.2) stages were classified based on Wallace and Selman (1981) and Grier and Uribe-Aranzábal (2009) respectively, and gonads were histologically classified based on the most advanced cellular type development stage, and maturity phases were assigned according to the standardized terminology proposed by Brown-Peterson et al. (2011): immature (I), developing (D), spawning capable (SC), actively spawning subphase (AS), regressing (RG) and regenerating (RN). Transition individuals were defined as those with both oocytes and spermatogenic tissue, with clear degeneration of the former and proliferation of the later (Sadovy and Shapiro, 1987; Sadovy and Liu, 2008). The presence of atresia along the spawning season was also assessed from the histological sections, with only α and β stages identified based on Hunter and Macewicz (1985). Presence and intensity of cystic structures, which are regressing oocytes which aggregate and form a thick brown structure (Dominguez-Petit and Saborido-Rey, 2005; Dominguez-Petit et al., 2011), was evaluated by month and sex.

Table 6.1 Summary of oocyte growth stages defined for *Spondyliosoma cantharus* females caught in western Portuguese continental coast during 2014 and 2015.

Oocyte development stage	Description	Diameter (μm)	N
Primary growth (PG)	These oocytes possess intensely basophilic ooplasm, with high intensity staining. Several nucleoli are normally seen with spherical or flattened shape	13.2–133.8 (64.4 ± 24.9)	354
Cortical alveolar (CA)	Cortical alveoli start to develop in the peripheral ooplasm and oil droplets can also be seen	111.2–204.3 (158.7 ± 17.6)	192
Early vitellogenesis (EVtg)	The cortical ooplasm contains both cortical alveoli and yolk globules	162.8–385.7 (220.6 ± 33.0)	192
Advanced vitellogenesis (AVtg)	Large yolk globules and oil droplets fill the cytoplasm.	253.5–547.6 (375.6 ± 64.0)	142
Oocyte maturation (OM)	The first indicator of maturation is the coalescence of numerous oil droplets to form fewer, larger oil globules. As maturation progresses hydrating yolk globules coalesce. When the germinal vesicle is at the animal pole of the oocyte, its membrane breaks down and the germinal vesicle is no longer visible.	433.0–898.1 (689.2 ± 110.4)	158
Hydrated oocytes (H)	Germinal vesicle breaks down with completely cleared fluid yolk and a single oil globule.	712.6–1039.9 (842.4 ± 104.8)	18

Values of minimum and maximum diameter for each stage is given, with mean and standard deviation between brackets. N – number of oocytes measured.

To characterise each stage of development only oocytes with a visible nucleus were measured for maximum and minimum diameter and averaged afterwards, to minimize the error from the loss of spherical shape caused by the histological processing. Measurements were performed on 25 females and 5 males from all maturity stages and a total of 1056 oocytes and 809 male cells were analysed using the software package ImageJ (http://imagej.nih.gov/ij/).

Table 6.2 Summary of male cells stages defined for *Spondyliosoma cantharus* males caught in western Portuguese continental coast during 2014 and 2015.

Male cells stage	Description	Diameter (µm)	N
Spermatogonia (Sg)	Appear single or in small groups and have round shape with a conspicuous cytoplasm and a prominent central nucleus.	5.89-11.03 (7.99 ± 0.95)	118
Primary spermatocysts (Sc1)	Spherical cells, smaller than Sg, enclosed in cysts.	3.27-4.96 (4.08 ± 0.32)	203
Secondary spermatocysts (Sc2)	Similar to Sc1, but smaller.	2.19-3.71 (3.08 ± 0.26)	147
Spermatids (St)	Even smaller than Sc2 but still found enclosed in cysts.	1.75-3.17 (2.39 ± 0.24)	162
Spermatozoids (Sz)	Similar to St but with cilia; they can appear enclosed in cysts or free in the lumen.	1.50-2.54 (1.95 ± 0.18)	179

Values of minimum and maximum diameter for each stage is given, with mean and standard deviation between brackets. N – number of cells measured.

6.2.3 Sexual cycle

The monthly evolution of the gonadosomatic index (GSI = 100 × GW / EW) was estimated to further corroborate the spawning season period. The fish condition was evaluated with two bioenergetic indices, the hepatosomatic index (HSI = 100 × LW / EW) and Fulton's condition factor ($K = 100 \times EW / TL^3$). Significant differences over the year were investigated with Kruskal-Wallis rank sum test and post-hoc tests according to Nemenyi for pairwise multiple comparisons of the ranked data, using the R package PMCMR version 4.1 (Pohlert, 2014). Correlation between GSI and HSI, K and sea surface temperature (SST) was evaluated with Spearman rank correlation coefficient. SST mean values for the sampled months in the Portuguese western coast were obtained using ICOADS data provided by the NOAA-ESRL Physical Sciences Division, Boulder

Colorado from their Web site at http://www.esrl.noaa.gov/psd/. These analyses were only performed on females and males that had already reached maturity.

6.2.4 Length at first maturity and sex change

Maturity ogive was constructed using a generalised linear model (GLM), as implemented in R environment, with a binomial error distribution and a logit link function to fit the logistic curve, considering exclusively the initial sex (females), when maturation takes place at the first time. The sex change ogive was estimated using the equivalent to calculate maturity ogive but replacing the proportion of mature individuals by the proportion of males (Domínguez-Petit et al., 2017).

6.2.5 Oocyte size frequency

Oocyte size distribution was estimated with the gravimetric method following the methodology proposed by Ganias et al. (2014), where the number of the target oocytes is counted in each subsample and the total number of oocytes in the whole ovary is subsequently calculated by multiplying the sum of the number of oocytes in the subsamples divided by the sum of the subsample weights by the weight of the ovaries. Three subsamples of ca 0.25 g for each female were flushed with a jet water spray into a sieve of 125 μm (cortical alveoli stage threshold, see Table 6.1). Afterwards, samples were placed on a watch glass dish and additional separation of oocytes with needles and forceps was done when required. Digital images were obtained under a stereomicroscope and oocytes were automatically measured and counted using ImageJ software with the ObjectJ plugin (https://sils.fnwi.uva.nl/bcb/objectj/). Manual correction was applied, when needed, in each processed image.

In order to check the homogeneous distribution of oocytes in the ovary, samples from posterior, middle and anterior zones of the right ovary lobe of 5 SC females were compared according to the frequency of oocytes above 125 μm, in 50 μm interval classes. The values estimated for each zone and female were compared with a beta regression model for proportions using the R package betareg version 3.1-0 (Cribari-Neto and Zeileis, 2010).

Oocyte size-frequency distribution for maturation stage was characterised using 5 females by phase. Immature and RN females were not analysed since the sieve used did not retain primary growth oocytes.

6.2.6 Fecundity type

Fecundity type of *S. cantharus* was investigated with the four lines of evidence suggested by Hunter et al. (1992), Greer-Walker et al. (1994) and Murua and Saborido-Rey (2003): (1) the presence or absence of an hiatus between pre-vitellogenic and vitellogenic oocytes; (2) the mean diameter of the advanced vitellogenic oocytes in the standing stock over the spawning season; (3) the number of vitellogenic oocytes in the ovary during the spawning season; and (4) the prevalence of atresia over the spawning season. For the first three lines of evidence, 34 females in SC phase, sampled from February to May, were analysed. Trend of oocyte diameter and number along spawning season was investigated with a linear regression, and differences among sampling dates were analysed with Kruskal-Wallis rank sum test and with a beta regression model for proportions, respectively. The prevalence of atresia by month, quantified as percentage of ovaries showing atresia, was investigated by inspection of histologic preparation of all females sampled in the spawning season period.

6.2.7 Fecundity estimation

Since only a small number of females showed hydrated oocytes, it was not possible to use the “hydrated oocyte method” for batch fecundity estimation. As a consequence, other developmental stages had to be used for batch measurements, and the “most advanced mode of oocytes” was the method selected for this estimation (Ganias et al., 2004). Only females with no signs of new post-ovulatory follicles (POF, Hunter and Macewicz, 1985) in the histological sections were used. Size frequency distribution from 12 AS females’ ovaries were analysed to identify the size at which the spawning batch separates from the remaining batches and the critical size at which the gap is established (Ganias et al., 2014). The least and most advanced modes were also determined by using the Bhattacharya’s based Modal Progression Analysis (MPA) using the available routine in FAO-ICLARM Fisheries Assessment Tools (FiSAT II version 1.2.2) software (Gayanilo et al., 2005). Batch estimation values obtained either counting all size oocytes above the estimated gap or using a combination of the Bhattacharya’s method and the NORMSEP module inside the FiSAT II software (Plaza et al., 2002) were compared by a chi-square test.

The spawning frequency (number of spawning events per unit time) was estimated as the inverse of spawning fraction (Murua et al., 2003), and the POF method proposed by

Hunter and Goldberg (1980) was used to estimate the daily proportion of spawning females by sample date. POF staging was achieved based on Hunter and Goldberg (1980) and Ganias et al. (2014) description of POF degradation. A total of 231 histological slides were inspected for presence and ageing of POFs. POFs were staged as day 0, day 1 and day >1. Only one stage was evident in each ovary, and 48 ovaries showed day 0 POFs and 45 ovaries showed day 1 POFs. Spawning season duration was estimated as the number of days between the first and last occurrence of AS individuals.

Relationship between absolute batch fecundity and total number of advanced vitellogenic oocytes with TL and EW was investigated with linear regression model. Model assumptions were investigated through the analyses of residual plots and normality and heteroscedasticity were further analysed with Shapiro-Wilk and chi^2 score tests, respectively, using the R package olsrr version 0.5.0 (Hebbali, 2018).

Relative annual fecundity estimates were obtained as the product of relative batch fecundity (the number of oocytes per gram of eviscerated female) by spawning fraction by the duration of spawning season.

All graphics were done with the R package ggplot2 version 2.2.1 (Wickham, 2009).

6.3 Results

6.3.1 Sampling

Over the sampling period a total of 431 juvenile (2.1–13.6 cm TL), 658 females (9.1–33.2 cm TL), 45 transition (20.1–32.0 cm TL) and 396 males (20.0–38.0 cm TL) (Fig. 6.2) were analysed for characterisation of maturity phases and 22, 460, 45, 262 samples from each group respectively, were used for gonad structure analyses. Sex ratio was significantly biased towards females ($p<0.001$) in classes 15-20 cm (35/1) and 20-25 cm (5/1), while in larger classes males were significantly numerous ($p<0.001$) with sex ratio of 0.3/1, 0.2/1 and 0/1 in 25-30 cm, 30-35 cm and 35-40 cm classes, respectively.

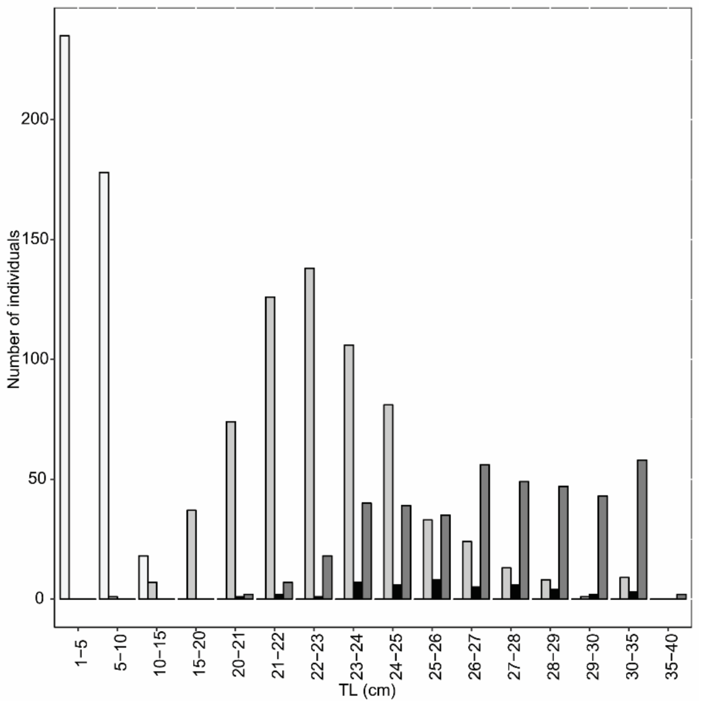

Fig. 6.2 Number of immature (white), females (light grey), transitional (black) and males (dark grey) by total length (TL) class used in the present study. Classes of 5 cm interval were used for specimens bellow 20 cm and above 30 cm and classes of 1 cm interval were used for the lengths in between.

6.3.2 Ovary and test organisation

Juvenile gonads showed an ovarian lumen with primary growth stage oocytes and spermatogenic tissue (Fig. 6.3a). A small portion of spermatogenic tissue could be observed in most females, mainly in the posterior ovarian zone. All gonads where both sex tissues were present showed a complete separation between the testicular and ovarian components by a thin layer of connective tissue. No female tissue remained in the developed adult males.

S. cantharus shows an asynchronous oocyte development, with a continuous oocyte diameter distribution and all oocyte stages occurring simultaneously in the mature phase (Fig. 6.3b, 6.3c; Table 6.1).

Sex transition in this species appears to be brief since a small number of adult individuals (4%) presented both sex tissues in transition (Fig. 6.3d, 6.3e). Several specimens showed cystic formation in the gonads (14%), which in some cases occupied more than half of the gonad diameter (Fig. 6.3h). Although these structures appeared over the year, in both male (Fig. 6.3h) and females (Fig. 6.3i) in all development stages and in transition individuals (Fig. 6.3j), they showed lower incidence in the spawning season months and in females (Fig. 6.4). Whereas only 5% of females showed cysts, they appear in almost 60% of transitional individuals and 22% of males, showing a potential relation of these

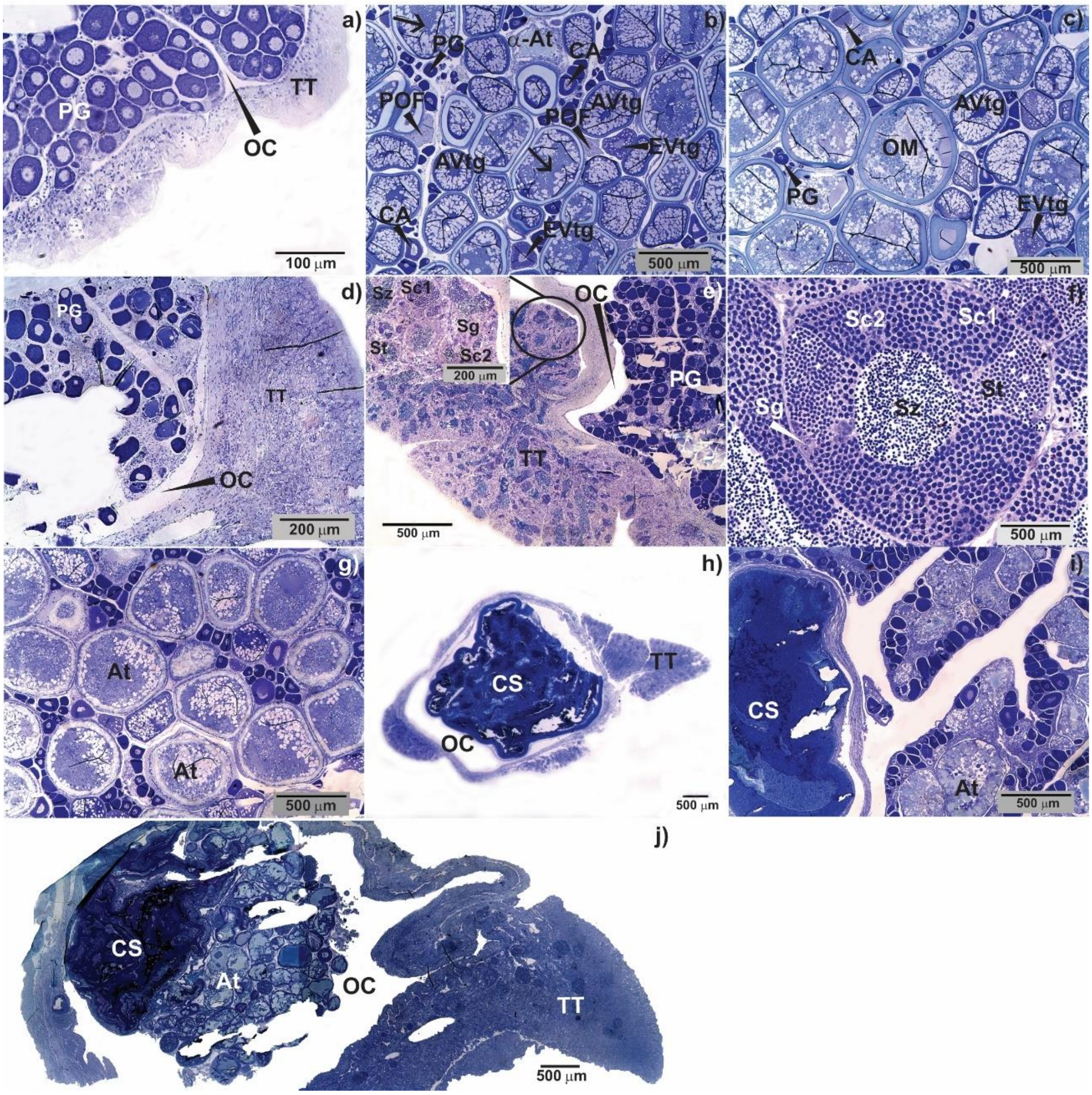

Fig. 6.3 Transverse sections of gonads of *Spondyliosoma cantharus*. a) juvenile specimen with both female and male tissues inactive (13.3 cm TL); b) spawning-capable female (22.9 cm TL); c) actively spawning female (23.4 cm TL); d) transitional specimen (23.4 cm TL); e) developing male with inactive female tissue (20.9 cm TL); f) spawning-capable male (26.4 cm TL); g) regressing female at the beginning of mass atresia (23.7cm TL); h) transitional specimen (29.4 cm TL); i) early developing male (30.5 cm TL); j) regressing female (31.9 cm TL). α-At – α atresia; At – atresia; AVtg – advanced vitelogenic oocyte; CA – cortical alveoli oocyte; CS – cystic structure; EVtg – early vitelogenic oocyte; OC – ovarian cavity; OM – mature oocyte; PG – primary growth oocytes; POF – post-ovulatory follicle; Sc1– primary spermatocyte; Sc2 – secondary spermatocyte; Sg – spermatogonia; St – spermatid; Sz – spermatozoid; TT– testicular tissue; arrow – advance vitelogenic oocyte going to germinal vesicular migration.

structures with sex change. Seasonally, almost 23% of the individuals showed cysts out of the reproductive season, dropping this value to 11% during the spawning season.

The testicular tissue of *S. cantharus* is organized into lobules consisting of many seminiferous tubules containing cysts. Each cyst is formed by spermatogenic cells in the same stage of development and limited by a thin layer of connective tissue. Following the terminology of Grier and Uribe-Aranzábal (2009), five stages of cells could be observed in the cross-section of the testis (Fig. 6.3e, 6.3f; Table 6.2).

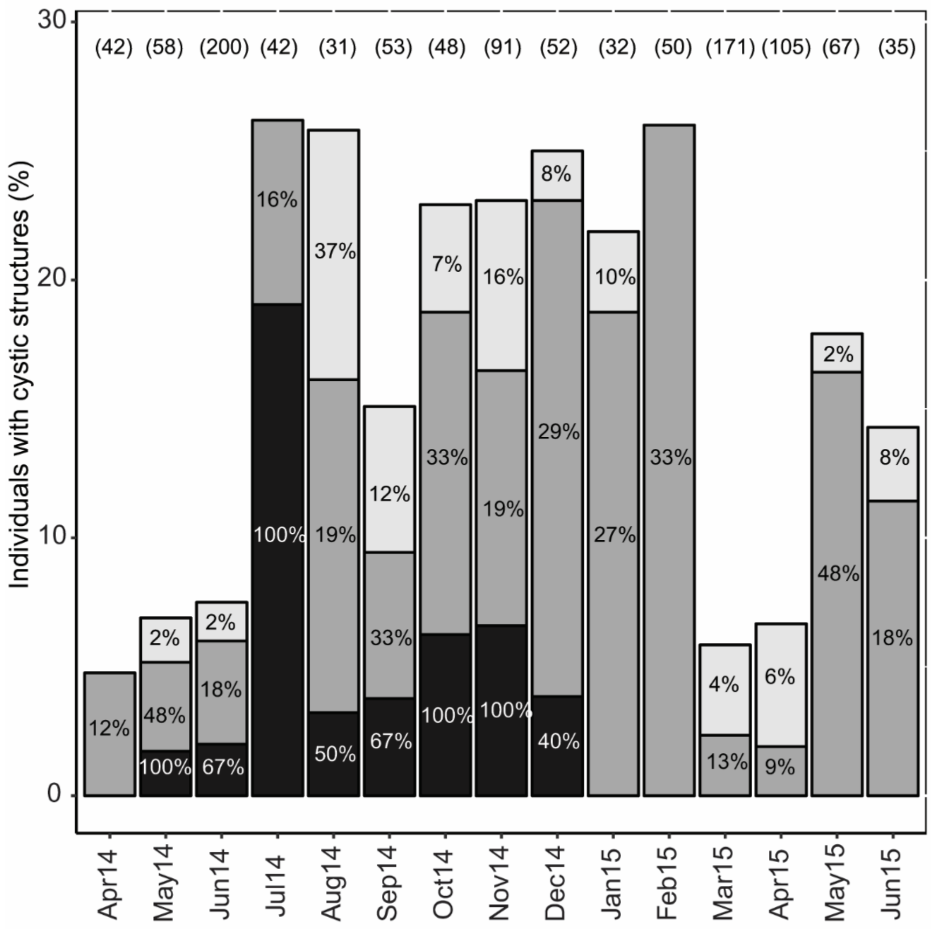

Fig. 6.4 Percentage of individuals of *Spondyliosoma cantharus* with cystic structures over the sampled months. Relative percentage for each sex is given in bars. Females – light grey; Transitional – black; Male – grey. Total number of individuals sampled by month is given between brackets above bars.

6.3.3 Sexual cycle

Females and males presented a similar pattern of maturation stages along the year (Fig 6.5). Developing individuals occurred mainly in October, November, December and January, while February until May were characterised by SC and AS individuals. RS individuals were predominant during June, and RN showed the higher values from July to September. GSI presented significant differences among months, with the period from February to May showing significantly higher values than the rest of the year ($p<0.01$), for both sexes (Fig. 6.6). This corroborated the maturation stages frequency results, allowing to define the duration of spawning season from February to May. Female GSI showed a significant positive correlation with HSI ($r = 0.45$, $p<0.001$) and a low but significant negative correlation with K ($r = -0.27$, $p<0.001$). For males the correlation between GSI and HSI, although significant, was very low ($r = 0.15$, $p = 0.004$) and no correlation existed with K ($p = 0.187$). SST had a significant negative correlation with GSI for both female ($r = -0.65$, $p<0.001$) and male ($r = -0.49$, $p<0.001$), with the higher values of GSI obtained for the months where SST was lower.

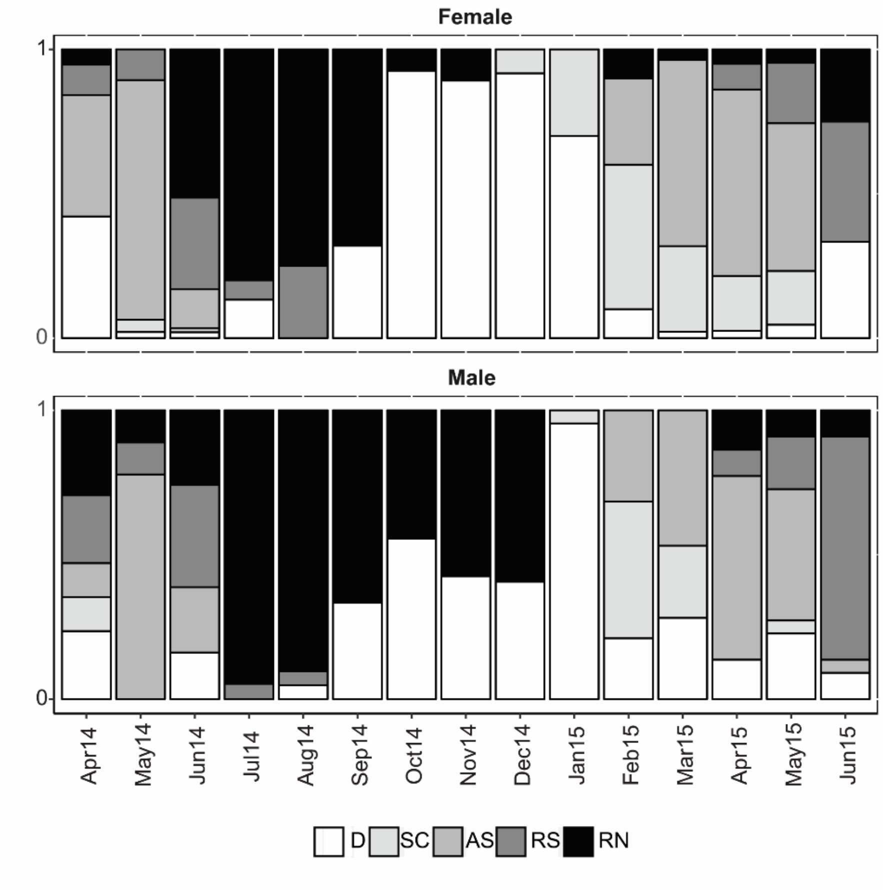

Fig. 6.5 Maturity stage frequency by month for females and males of *Spondyliosoma cantharus* caught in western Portuguese coast. D – developing; SC – spawning capable; AS – actively spawning; RS – regressing; RN – regenerating.

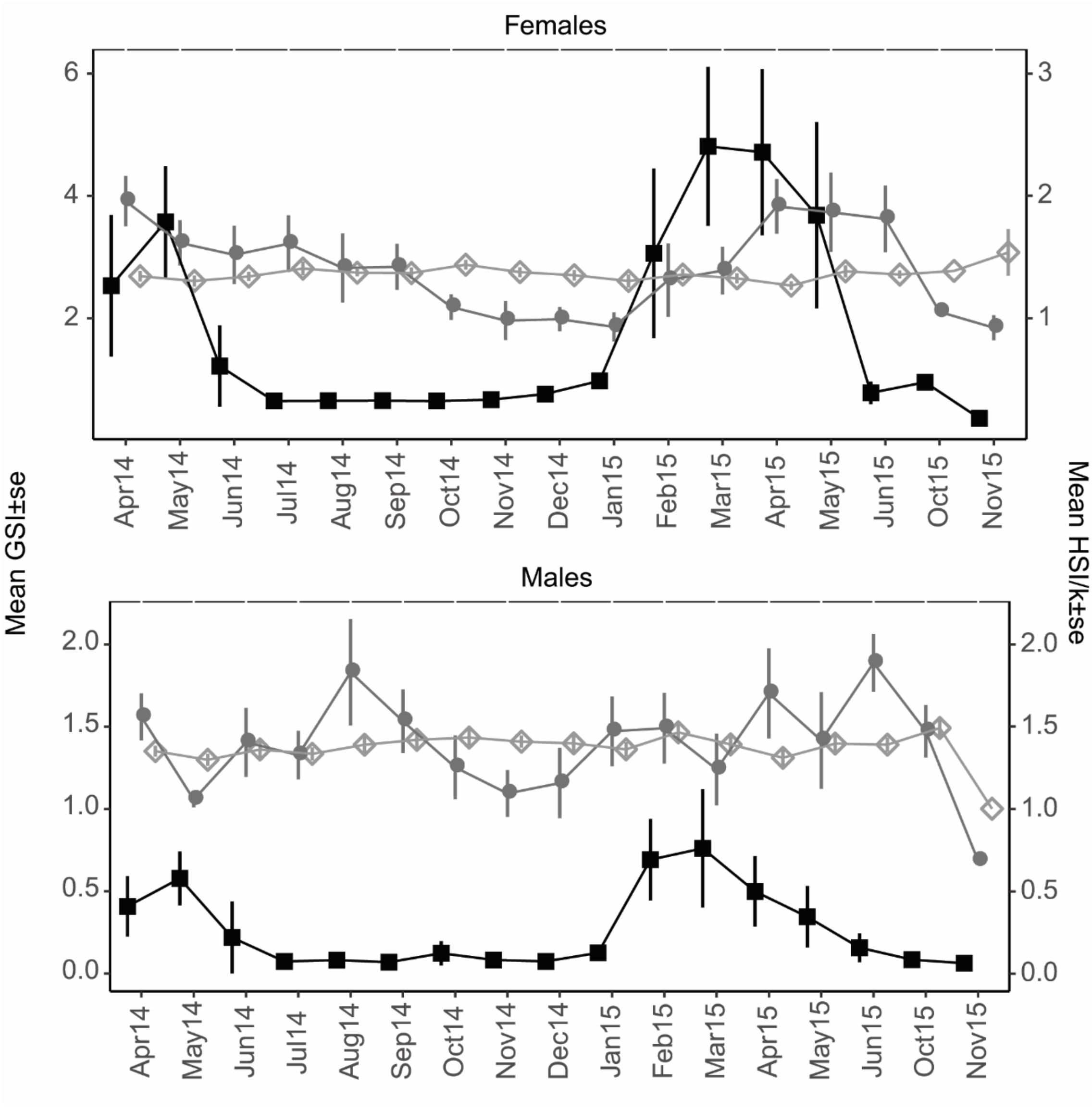

Fig. 6.6 Monthly variation for females and males of the mean and standard error of gonadosomatic index (GSI, black squares), hepatosomatic index (HSI, grey circles), and Fulton's condition factor (K, open diamonds) for *Spondyliosoma cantharus* caught in western Portuguese coast.

6.3.4 Length at first maturity and sex change

The smallest mature female measured 18.1 cm TL and the estimated mean length at first maturity was 18.41 ± 0.54 cm TL. The smallest male found during the sampling period had 20 cm TL, while the estimated mean length of sex change was 25.62 ± 0.09 cm TL (Fig. 6.7, Table 6.3). There was no defined season for sex changing since transitional individuals appeared nearly every month, but with lower incidence during the spawning season.

Table 6.3 Summary table with the fit for the generalised linear model applied to maturity and sex change data.

Response variable	Effect	Estimate	SE	Pr (>|z|)	Res Dev (df)	AIC
Maturity	Intercept	-33.7075	7.4217	5.37e-06	59.197 (1286)	54.197
	TL	1.8306	0.3762	1.15e-06		
Sex change	Intercept	-15.0854	0.9050	< 2e-16	903.37 (1286)	907.37
	TL	0.5889	0.0364	< 2e-16		

SE – standard error; Res Dev – residual deviance; df – degrees of freedom; AIC - Akaike's Information Criterion

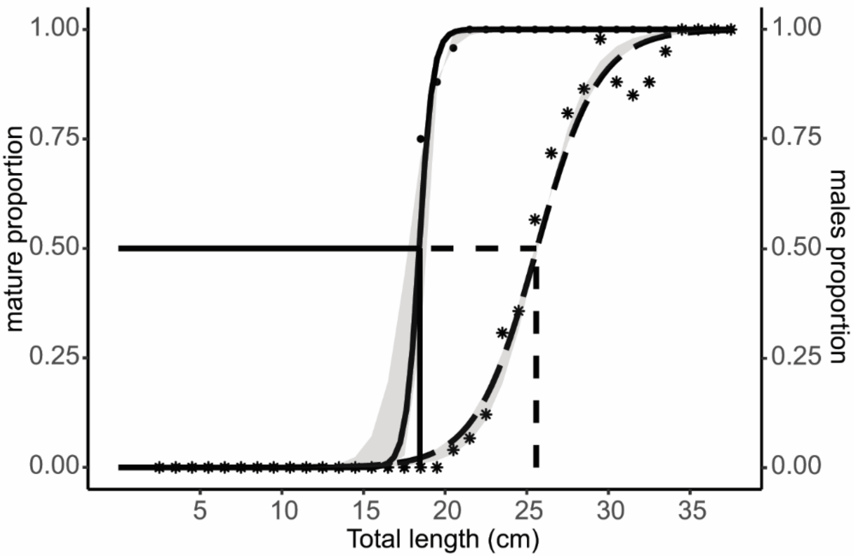

Fig. 6.7 Maturity (black circles and solid line) and sex change (asterisks and dashed line) ogives for *Spondyliosama cantharus* caught in western Portuguese coast. Vertical lines indicate the length at which 50% of the individuals are mature (solid) and change sex (dashed). 95% confidence interval for the model is given by the grey area.

6.3.5 Oocyte size frequency

Oocyte distribution showed no significant differences between zones (p > 0.35) neither between the two lobes of the ovary (p = 0.68). The subsequent analyses were performed on random samples from the right ovary lobe.

The oocyte size frequency distribution for D to RS phase is characterised in Fig. 6.8. In D phase, the size of oocytes was small, with a limited number above 225 µm. During the next month, the vitellogenesis process continued and oocyte size grew in the SC phase. It was clear the start of this phase in January with a small number of advanced vitellogenic oocytes, which increased in the next month remaining similar for the rest of the spawning season. In the AS sub-phase a gap was formed between the spawning batch and the remaining standing stock of oocytes. This gap became wider as the oocyte size increased to hydration stage. Some ovaries in the regressing phase had still larger oocytes in maturation stage, although most of them had already entered follicular atresia (Fig. 6.3g), and as the season proceeded these oocytes regressed and only small oocytes could be seen.

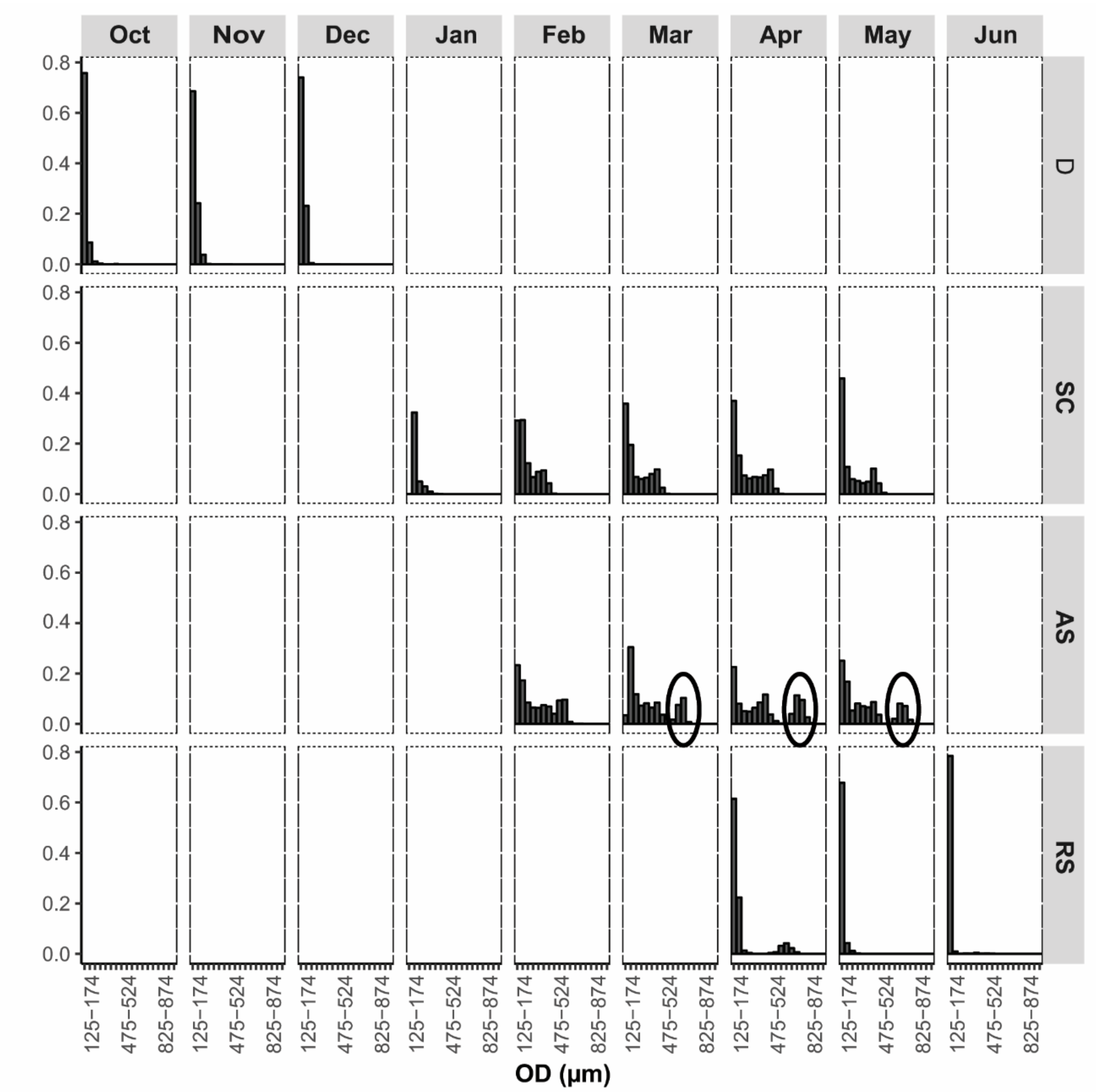

Fig. 6.8 Oocyte size frequency distribution for the developing (D), spawning capable (SC), actively spawning (AS) and regressing (RS) females of *Spondyliosoma cantharus* caught in western portuguese coast from October 2014 to June 2015. The most advance oocyte cohort in AS phase is highlighted by a circle.

6.3.6 Fecundity type

During the spawning season, bimonthly samples were collected, with exception of February with only one sample acquired.

From the gravimetric analysis of the SC females, a continuous oocyte size frequency distribution was clear with no gap between pre-vitellogenic (< 204 µm) and vitellogenic oocytes (Fig. 6.9). The estimated mean diameter of advance vitellogenic oocytes from February to May varied between 370.2 µm to 403.3 µm. No significant trend for the mean diameter over the spawning season was observed ($r^2 = 0.09$, $p = 0.07$), and no significant differences among mean diameter by month (KW = 10.8, $p = 0.09$) were detected. The monthly progress of the number of developing (cortical alveoli and early vitellogenic oocytes) and advanced vitellogenic oocytes over the spawning season present no significant trend ($r^2 < 0.07$, $p > 0.09$). No significant differences in the number frequency between months were observed ($p > 0.18$) with only the interception being significant in the regression model. Atresia prevalence over the spawning season varied between 25.0% in February and 46.6% in May, with a mean of 29.4%, but a massive atresia in the end of spawning season was present.

All the results obtained from the studied parameters indicate that *S. cantharus* has an indeterminate fecundity strategy.

6.3.7 Fecundity estimation

According to the fecundity type presented by the species, the batch frequency and the number of oocytes spawn by batch were estimated.

Size frequency distribution of 12 AS females analysed for establishing the oocyte cohort to be estimated for batch fecundity, showed a clear gap formed around 500 µm, which was also corroborated by Bhattacharya's MPA results where two clear modes could be observed, one placed around 420 µm, corresponding to the advanced vitellogenic oocytes and a most advanced mode varying from 570 µm to 880 µm according to the stage progress of the spawning batch (Fig. 6.8, AS phase). Since no significant differences between the values estimated with the two methodologies were obtained ($\chi^2 = 12.1$, $p = 0.15$), the fecundity batch for the remaining females was estimated by counting the oocytes above 500 µm.

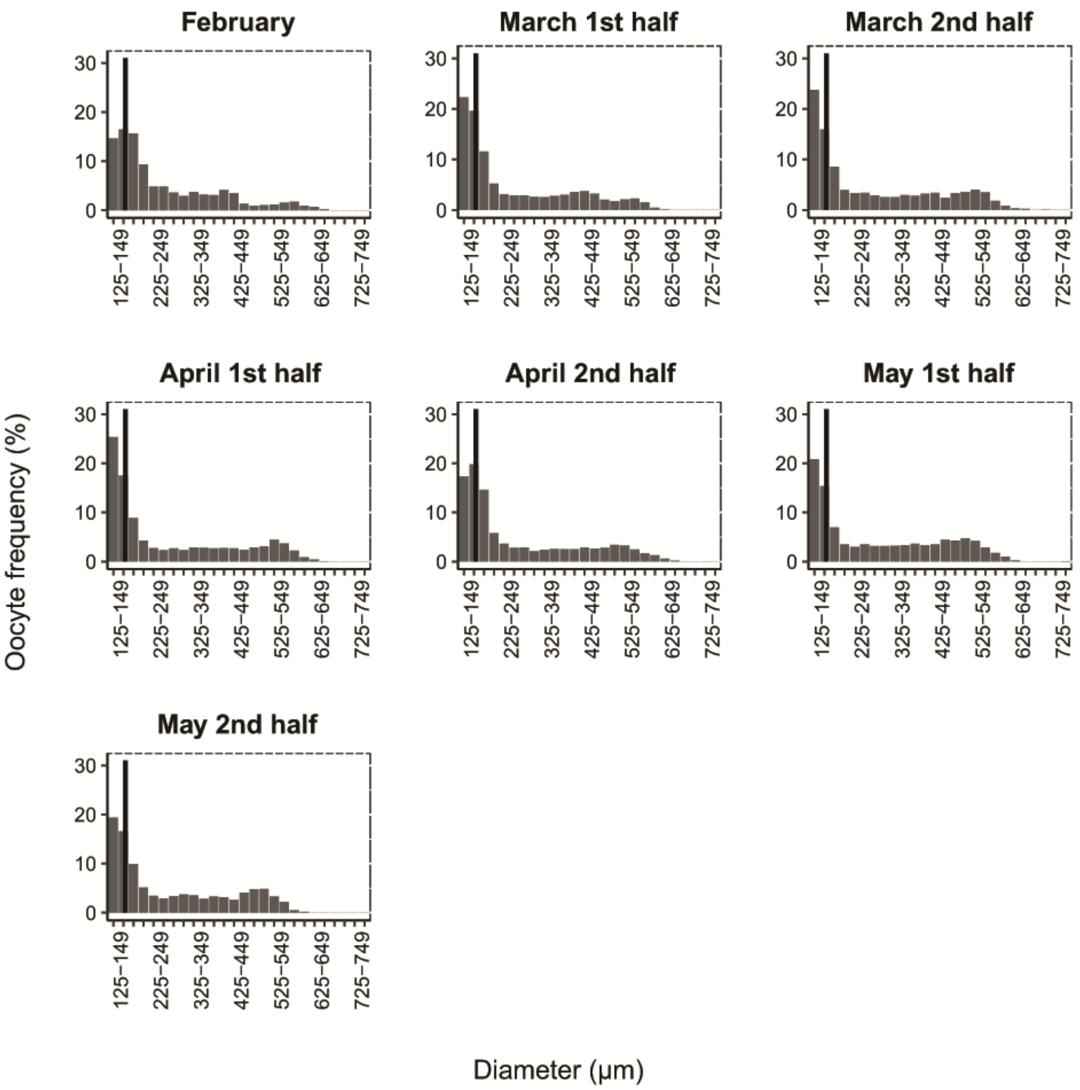

Fig. 6.9 Oocyte size frequency variation over the spawning season for *Spondyliosoma cantharus* spawning capable females caught in western Portuguese coast. Vertical lines indicate the transition from pre-vitellogenic to vitellogenic oocytes.

For monthly batch estimation 31 females were used. In February, only one female matched all criteria to be used for batch estimation, for the remaining spawning season months a total of respectively 11, 10 and 9 females were analysed. Absolute batch fecundity estimation ranged from 5041 to 95398 oocytes with a mean of 42513 oocytes. A significant positive relation between both absolute batch fecundity (-87964.8 + 5270.9 × TL, $r^2 = 0.6116$, $p < 0.0001$; -3225.27 + 224.64 × EW, $r^2 = 0.6215$, $p < 0.0001$) and number of advanced vitellogenic oocytes (-180822 + 9910 × TL, $r^2 = 0.5661$, $p < 0.0001$;

-16283.29 + 376.76 × EW, $r^2 = 0.7276$, $p < 0.0001$) with total length and eviscerated weight (Fig. 6.10) was obtained. Values of advanced vitellogenic oocytes were about 2 times superior to those of batch fecundity, however larger females showed larger differences and with the values from these females no relation between the two slopes could be established. Residual analyses showed that assumption for normality was met for all regressions but revealed that some heteroscedasticity was present mainly in the residuals from TL regression with advanced vitellogenic oocytes (see supplementary material: Table S6.1, Fig. S6.1). Relative batch fecundity varied between 28 to 322 oocytes×g^{-1} EW with a mean of 203 oocytes×g^{-1} EW.

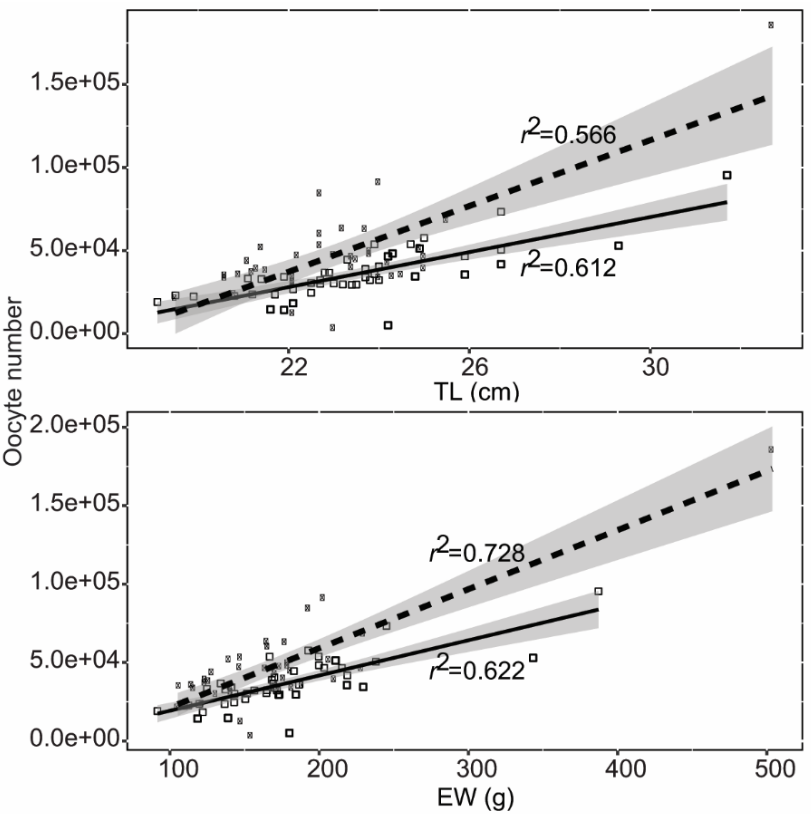

Fig. 6.10 Linear relation between absolute batch fecundity (open squares, solid line) and number of advanced vitellogenic oocytes (crossed squares, dashed line) with total length (TL) and eviscerated weight (EW) for *Spondyliosoma cantharus* females caught in western Portuguese coast. 95% confidence interval for the model is given by the grey area. Correlation of each model is given next to the regression line.

Spawning fraction was estimated with the two POFs daily classes and values varied between 0.25 and 0.20 for class day 0 and class day 1 respectively, with an estimated number of 27 batches by female along the spawning season. According to the various parameters estimated, the relative annual fecundity for *S. cantharus* varied between 748 and 8602 oocytes per g of EW, with a mean of 5431 oocytes×g^{-1} EW.

6.4 Discussion

Knowledge of a species life history, namely reproductive biology, is crucial to understand fish population dynamics. In protogynous fish species the assessment is particularly challenging because the same fish function as a male or female during different life stages and information to describe their life history strategies becomes even more essential (Shepherd et al., 2013). The Sparidae family shows a wide range of reproduction patterns with the occurrence of both gonochorism and sex reversal, and within the latter, protandrous, protogynous, simultaneous and nonfunctional hermaphroditism have all been reported (Buxton and Garratt, 1990). *S. cantharus* shows a protogynous strategy, with females appearing mainly until 25 cm and male prevail above that length. Transitional specimens occur mainly between 23 cm and 28 cm. The species seems to be monandry, although the presence of small male individuals within the 20 cm may indicate a diandry form, but the scarce number of specimens caught within the 16 – 20 cm TL range does not allow a reliable inference that males may develop directly from the juvenile phase. Such possibility has already been hypothesised by Mouine et al. (2011), considering the sexual dimorphism that a large number of males, but not all, show in the spawning season with a darker colouration and a blue mark near the eyes. This dual male colouration has been reported by Gonçalves and Erzini (2000) and was also noticeable in the present study, although it was not numerically characterised. The dichromatism shown by males has been reported for several protogynous species but a clear relation between diandry and this dichromatism has not been established (Chan and Yeung, 1983; Sakai et al., 2007).

The small number of transitional specimens found, indicates a rather brief sex change phase for each individual, with no clear seasonality, since the transitional individuals appear in nearly every month. Other authors also refer a low number of transitional individuals (Pajuelo and Lorenzo, 1999; Gonçalves and Erzini, 2000; Mouine et al., 2011), but since these studies are based mainly on macroscopic classification, the transitional individuals would be difficult to detect. The high percentage of cystic structures found mostly in transitional but also in male specimens suggests that these structures are involved in the sex change process and might be a good criterion to detect this process. Paiva et al. (2014) refer that such cystic structures seem to be easy to release from the gonad leaving a wider space for feasible cells. Forming such cysts can be the way that *S. cantharus* developed for an expedite sex transition whenever the transitional process starts with the presence of larger oocytes. However, these structures need further investigation in order to clarify their function in the reproductive strategy of the species.

The spawning season of *S. cantharus* has been reported mainly in Winter and Spring months, with the wider season from November to April with a peak in January-February for Canary Islands (Pajuelo and Lorenzo, 1999) and the smaller season reported for the Bay of Biscay (Perodou and Nedelec, 1980) taking place from May to June. The spawning season in the western Portuguese continental coast extends from February to May with the peak of GSI values occurring during March and April. Spawning season is related to sea temperature, taking place on the colder months. Wilson (1958) has reported that *S. cantharus* spawns at temperatures from 12 ºC to 14 ºC in captivity. SST from western Portuguese continental coast ranged from 13.6 ºC (March) to 16.6 ºC (May) during the spawning season, with the decrease of SST from 16.6 ºC in December to 14.6 ºC in January marking the start of advanced vitellogenic oocytes. The upwelling regime that occurs seasonally in the west Iberian coast in Spring and Summer, which brings new nutrients into the photic zone allowing phytoplankton growth on the inshore side of the upwelling front (Ambar and Dias, 2008), has a positive impact on recruitment due to the favourable juvenile feeding conditions, as already confirmed for *Sardina pilchardus* and *Trachurus trachurus* by Santos et al. (2001).

Once reaching maturity, individuals have larger energetic demands since a surplus energy is needed to complete a clutch of eggs, as well as for accessory activities such as migration to spawning grounds, courtship and parental care (McBride et al., 2015). In general, there are two forms for fishes to manage this extra energy: i) by storing energy prior to spawning season and drawing it later for reproduction, known as capital breeding; or ii) acquiring energy locally throughout the spawning season and allocating it directly to reproduction, the income breeding (McBride et al., 2015). Despite these two extremes, many species exhibit mixed capital and income breeding patterns (McBride et al., 2015). Females of *S. cantharus* showed a positive correlation between GSI and HSI and a negative correlation between GSI and K. Such correlations point for the possibility that the species stores some energy reserves for reproduction in muscle but not in the liver. Nevertheless, the fact that there was no reduction in the feeding activity in spawning months (Soletchnik, 1982; Neves, pers. obs.) points to a mixed income–capital breeder pattern (McBride et al., 2015) displayed by the species. This is also corroborated by the reproductive strategies that *S. cantharus* shows, the extension of spawning season, the asynchronous oocyte development and the batch-spawning frequency indicate that females must rely mostly on income. Males are predicted to spend fewer resources than females in gamete production (Trivers, 1972), which is reflected in the much smaller values of GSI found for males. However, the energy required in reproductive effort in males is also affected by the reproductive behaviour. Males of this species are nest builders, with territorial defence, egg protection and nest maintenance (Wilson, 1958). Energetic cost of parental care varies greatly and depends on abiotic and biotic factors demands (Steinhart et al., 2005) and thus such variability can mask any possible patterns of energetic stores depletion in males.

The reproductive behaviour exhibit by the species is favoured by a protogynous strategy since large dominant males are more fit to control the reproductive access to females and ensure the successful offspring (Benvenuto et al., 2017). Factors such as inexperience, territoriality and female mate selection depress male fecundity values at early ages, and therefore the reproductive potential is enhanced by a convergence of males to older ages (Warner, 1975).

The estimated mean length at first maturity of females of *S. cantharus* for the western Portuguese continental coast was 18.41 cm TL which correspond to an age of 3.8 years (Neves et al., 2017). The estimated mean length of sex change for this area was 25.62 cm TL corresponding to 7.94 years (Neves et al., 2017). The values estimated for length at first maturity in this study are in the range of those found in other areas. Values of 17.3 cm were found for Canary Islands (Pajuelo and Lorenzo, 1999) and 17.8 cm for Golf of Tunis (Mouine et al., 2011). Larger lengths are reported for Algeria, 19.3 cm (Boughamou et al., 2015), Algarve, 19.98 cm (Gonçalves and Erzini, 2000), Bay of Biscay, 20 cm (Perodou and Nedelec, 1980), and the English Channel, 22 cm (Soletchnik, 1982). Lower values of length at first maturity found in the Canary Islands and the Mediterranean Sea can be justified by the warmer waters that can induce an early maturation of the species. Lower sea temperature in the northern area can also prompt the later maturation described for the Bay of Biscay and the English Channel. The range size used by Gonçalves and Erzini (2000) with all individuals above 19 cm, limits the accuracy of the length at first maturity reported by the authors. The fact that most studies only use macroscopic information and include data outside the spawning season for the analysis mould the reliability of the stated results, and comparisons should be drawn with attention.

Reproductive studies of hermaphroditic fish species should be addressed with histology since many details crucial for internal gonad morphology description are only visible when histological techniques are used (Sadovy and Shapiro, 1987; Alonso-Fernández et al., 2011).

Regarding the mean length of sex change the values publish for the species vary greatly, from 18.8 cm reported for Golf of Tunis (Mouine et al., 2011) to 35 cm in Bay of Biscay (Perodou and Nedelec, 1980). Boughamou et al. (2015) and Gonçalves and Erzini (2000) reported values similar to those estimated in the present work, 24.3 cm for Algerian eastern coasts and 24.5 cm for South Portugal. In the present study, a different methodology was used for the sex change mean length estimates making the comparisons to other studies unfeasible. The species has no clear seasonality for sex change, and different patterns have been reported in the literature. Gonçalves and Erzini (2000) sampled hermaphrodite specimens mainly from September to November in South of Portugal, while Mouine et al. (2011) observed hermaphroditic fish primarily in the spawning months. In the west coast of Portugal, such specimens occurred almost every month but very sparingly in the spawning season. From the available data it seems that at least for the Portuguese coast sex change has a very little expression in spawning females, which might indicate that the number of males in the population is sufficient to assure reproductive success.

Fecundity measurements are of particular importance for understanding reproductive dynamics and spawning energetics of a fish stock. They permit the estimation of annual reproductive output and explore the relation to recruitment, which is a prominent information for fishery biology (Ganias et al., 2014).

Histological and gravimetric analyses of *S. cantharus* female gonads confirmed the simultaneous occurrence of pre-vitellogenic, vitellogenic and maturation oocytes in AS ovaries. The presence of oocytes from all developmental stages in AS females may point to an indeterminate fecundity type. For accurately assess whether *S. cantharus* shows a determinate or indeterminate fecundity type for the Portuguese coast, the four lines of evidence provided by Hunter et al. (1992), Greer-Walker et al. (1994) and Murua and Saborido-Rey (2003) were investigated. It is increasingly known that the analysis of these criteria should be looked with some caution since several species present contradictory results for the different lines (eg. *Sarpa salpa* (Paiva et al., 2018), *Trisopterus luscus* (Alonso-Fernández et al., 2008), *Trachurus trachurus* (Ganias et al., 2017)).

However, in the present study, the four lines of evidence point out to the same conclusion as follows: i) no hiatus separating the yolked oocyte stock from the reservoir of unyolked oocytes, indicating a continuous recruitment of unyolked oocytes; ii) no trend for number and iii) diameter of advanced oocytes during the spawning season denoting the lack of oocytes loss and increasing mean size; iv) and the presence of massive atresia by the end of spawning season clearly shows that *S. cantharus* has an indeterminate fecundity for the western Portuguese continental coast.

It is known that species with boreal geographic distributions tend to have determinate fecundity whilst temperate and tropical species are mostly indeterminate spawners and that stocks residing at intermediate climatic zones or having wide geographic coverage can switch from one fecundity type to the other (Ganias et al., 2015). *S. cantharus* shows both a distribution in intermediate climatic zones and a wide geographic coverage, but the lack of studies on the fecundity of the species do not allow to explore a possible mixing behaviour. The few fecundity studies available for the species (Soletchnik, 1982; Dulčić et al., 1998; Gonçalves and Erzini, 2000) lack a thorough investigation of fecundity type, and therefore it is not possible to compare the results of these studies to those found for the western Portuguese continental coast.

From the samples analysed in this study there was a deficit of females with hydrated oocytes. The reproductive behaviour of the species, where males dig a hole in the sand for females deposit their eggs and then protect them until the hatching (Wilson, 1958) may enclose imminent spawning females in areas where the commercial fleet is less operational. Unlikely of what is reported for Southern England (Pinder et al., 2017), it seems that in Portuguese waters, fishing is not a main disturbance for the spawning grounds of *S. cantharus*. The hydrated oocyte method for batch fecundity estimation was not possible to use. However, during the development of a batch of oocytes is common to occur the formation of a gap in the oocyte size distribution, which usually starts when the oocytes enter the germinal vesicle migration stage and it is completed at hydration (Hunter et al., 1992). In the case of *S. cantharus*, the consistency of the gap formed around the 500 µm was conducted by graphical examination and Bhattacharya's MPA and the spawning batch could be clearly identified prior to hydration. Batch fecundity estimates ranged between 5041 (24.2 cm) at the end of spawning season to 95398 (31.7 cm) in April, the peak of spawning season. Relative annual fecundity estimated mean was 5431 oocytes×g^{-1} EW. Fecundity data reported for the species is available for Croatia and South Portugal, however both studies estimate fecundity only at the beginning of the spawning season. Mean relative fecundity reported for these areas were 646 oocytes×g^{-1} EW for Croatia using females with an average TL of 26.4 cm and 346 oocytes×g^{-1} EW for South Portugal in females with an average of 23.3 cm TL. These values are, as expected, higher than those of relative batch fecundity found in the present study (203 oocytes×g^{-1} EW), but much lower than the relative annual fecundity estimated for the species (5431 oocytes×g^{-1} EW). The fact that these authors did not consider the fecundity type of the species and estimate the number of yolked oocytes in a single point in time justify the differences found.

Soletchnik (1982) referred that in the English Channel *S. cantharus* should spawn two times during the spawning season and reported mean values of 706 oocytes×g^{-1} W of relative annual fecundity for an average female of 33.3 cm TL. These values are lower than those found in the western Portuguese continental coast, where near 30 spawning events were estimated, with females spawning in average every 4.5 days. The sea conditions found in the Portuguese coast may justify the fast oocyte development for continuous batch recruitment. Nevertheless, it is likely that for the relative long spawning period present by *S. cantharus* for western Portuguese continental coast, not all females remain active during the whole season, as evidenced by the presence of regressing females during the spawning season months, and therefore the annual fecundity might be slightly over-estimated.

From the data gathered in this study there is no evidence of stock depletion in the western Portuguese continental coast. However, there is still a shortage of information on the species and over time data is fundamental to thoroughly understand the species life history and determined the possible variation of mean length of first maturity and sex change and perceive possible impacts of fisheries and ecosystem changes on *S. cantharus*.

CHAPTER 7

General Conclusion and Final Remarks

General Conclusion

In the first section of this study, the hypothesis of existing distinct stock units of *Spondyliosoma cantharus* along the Eastern Atlantic was investigated. Samples from five areas along the European waters (English Channel, Bay of Biscay, Galicia, West and South Portuguese Coast) and two areas from African coast (Canary Islands and Angola), were gathered and from the many techniques that can be applied to identify fish stocks, four were used: body morphometry, otolith shape and stable isotopes ratio and genetics, using mitochondrial and nuclear markers. All techniques were applied on the same individuals but with different samples sizes, according to the methodology used.

For the North-eastern Atlantic the 6 stock units hypothesis is supported by the results of most techniques used. Integration of results from a holistic perspective should take in account the sensitivity and the sample suitability of each specific technique for detecting population structure (Cadrin et al., 2014b). Morphometric patterns are often associated with geographic differences in growth, maturity, or mortality which are critical to population dynamics (Cadrin, 2010) and when the groups are isolated enough, a structuring pattern of these phenotypic differences can be retrieved. Morphometrics analyses results, from both body and otolith, showed main differences for CN and AN areas and good differentiation within European areas, with exception of Bay of Biscay (Body morphometrics) where the heterogeneity of the sampled individuals, despite the allometric correction, might have influenced the results.

The environmental variation recorded in fish otoliths reflect the environmental conditions that an individual encounter during its entire life (Gao and Beamish, 1999), and stability within a group of fish indicates that they shared the same environment for most of their life. Homogeneity of age in samples would grant a more reliable interpretation of results; however, this assumption could not be held for some areas in the present study. Nevertheless, three areas showed clear stable isotopic ratios fingerprints: Angola, Canary Islands and Bay of Biscay, whereas Algarve and the English Channel showed poor or absent pattern.

Molecular genetic methods are used to test whether the fish samples are drawn from a large, randomly mating population with equilibrium genotype frequencies, or from an assemblage of genetically distinct units (Coyle, 1998); however the timescale of these markers often fails to reflect the biological characteristics that induce population structure. The two DNA markers used in this study showed a strong large-scale structure for *S. cantharus*, with Mediterranean region markedly differentiated from North-eastern Atlantic, and an absence of haplotypes shared with the southern region of Cape Verde and Angola. In a finer scale the results of population analyses show no structuration, with no divergence between Canary Islands and any of the North-eastern Atlantic European areas. However, a careful analysis of summary statistics denotes a larger number of private haplotypes in each analysed area which can only occur due to some level of isolation among populations. These results, from a fisheries perspective, correspond to different stock units to be managed.

How to merge the information obtained from different techniques in a holistic perspective? Although there is an increase number of studies on stock structure, using various methods on the same samples (e.g. Abaunza et al., 2008; Gordo et al., 2009; De Celles and Cadrin, 2011), interpreting the heterogeneous data from dissimilar analyses is mainly descriptive since there is still no statistical tool appropriate to analyse such diverse data (Cadrin et al., 2014b; Welch et al., 2015). The Pairwise comparison is an approach to easily visualise the similarities between techniques and regions (Table 7.1).

Table 7.1 Pairwise comparison results for the techniques used in this study. Only significant differences are indicated.

	EN	BI	GL	PN	AL	CN
BI	BM, OS, OI					
GL	BM, OS, OI	BM, OS, OI				
PN	BM, OS, OI	BM, OS, OI	BM, OS, OI			
AL	BM, OS, OI	BM, OS, OI	BM, OS, OI	BM, OS		
CN	BM, OS, OI	BM, OS, OI	BM, OS, OI	BM, OS, OI	BM, OS, OI	
AN	BM, OS, OI, G	BM, OS, OI, G	BM, OS, OI, G	BM, OS, OI, G	BM, OS, OI, G	BM, OS, OI, G

BM – body morphometry, OS – otolith shape, OI – otolith stable isotopes, G - genetics.

Genetic values of Φ_{ST} between these areas only showed differentiation for the Angola area. However, despite the absence of significant differences, this technique does not denote a panmictic population in the North-eastern Atlantic. The presence of several private haplotypes infers that ecological isolation is present and areas as close as Central

Western Portugal (Peniche) and North Western Spain (Galicia) do not exchange breeders in a regular basis.

Table 7.2 shows the validation of stock units according to each technique and the level of certainty in this determination. From all the data gathered, the most probable scenario is that *S. cantharus* shows multiple stocks along its geographical distribution.

Table 7.2 Separate stocks identified based on each individual technique and the defined management units.

Body morphometrics	Otolith Shape	Otolith stable Isotope ratio	Genetics	Management units
EN	EN		EN	**EN**
BI	BI	BI	*BI*	***BI***
GL	GL	*GL*	*GL*	**GL**
PN	PN	*PN*	PN	**PN**
AL	AL		AL	**AL**
CN	CN	CN	CN	**CN**
AN	AN	AN	AN	**AN**

Lower values of certainty in the evaluation are given in italics.

Despite this species do not bear a targeted fishery, it is caught by several fishing gears, mainly by the multi-gear (artisanal) fishing sector, which has considerable socioeconomic importance for local populations and shows an intensification along the coast over the last decades (Leitão et al., 2016). Therefore, knowledge on its status is most important for the preservation of viable fisheries.

The species is also important in other countries fisheries and information on life history parameters is scarce and mostly outdated although such information is directly related to fishery productivity and essential for stock assessment (McBride, 2014). For hermaphrodite species, such as *S. cantharus,* a regular monitoring of life history data is most vital since such species can have different responses to high rates of exploitation. High catches can promote a truncate size distribution that at an extreme will virtually remove males from the population and thus lead to the population collapse by sperm limitation (Alonzo and Mangel, 2004) or the populations may remain relatively stable due to plasticity in their age of sex change (Alonzo and Mangel, 2005), although with high variation in traits as age at sex change and total length (Robinson et al., 2017).

In the second section of this study the life history parameters of *S. cantharus* for the Western Portuguese coast were assessed.

Due to the special life condition of the species, the protogyny, several growth models were tested to evaluate their adequacy to age-at-length data. The L_∞ hyperbolic modification of von Bertalanffy curve showed the better fit, with a change in growth strategy around 7.46 years which corresponds roughly to the estimated age at sex change. A validation of the first annual growth increment was accomplished by monthly samples in a nursery area (the Óbidos Lagoon), allowing to understand the pattern of growth increments deposition, which was especially important since this first growth increment was not always well defined. An evidence of the difficulty to interpret the deposition pattern is the inconsistency of age estimation among the different growth studies on the species (eg. Dulčić and Kraljević, 1996; Lorenzo and Pajuelo, 1997; Abecasis et al., 2008; Boughamou et al., 2015; Mouine-Oueslati et al., 2015).

Exploitation rates estimated for 2014 and 2015 were similar for both years, with relatively high values, between 0.43 – 0.60 and 0.46 – 0.62, respectively. Since the species is caught by most of the fishing gears operating near the coast, these high exploitation rates are not totally unexpected.

Knowledge of the reproductive strategy of a fish species is essential for an effective population management and sequentially hermaphroditic fish need a special attention to their reproductive strategy since they present different social organisations and reproductive modes according to their life-history system (Benvenuto et al., 2017).

In this study a thorough analysis of the reproductive strategy of *S. cantharus* for Western Portuguese coast was conducted. Sex transition and reproductive cycle were examined. Ovary organisation and oocyte development were histologically studied over the reproductive cycle, the fecundity type was defined, and fecundity was estimated according to the results.

The species shows highly skewed sex ratio towards females, especially for the smaller classes (below 25 cm TL), which is common in protogynous species. Sex change process could not be totally understood since the low frequency of transitional individuals prevented a more thorough analysis of sex change. The high cystic masses found mainly in transitional and male individuals are likely to be a part of this process but further research on these issues should be carried out to elucidate sex change in the species. The presence of small males and large females, although sporadic, indicates that some individuals probably do not change sex, which should also be further explored, for a full understanding of the species strategy.

Annual fecundity values calculated for the species are relatively high, with an average of 5431 oocytes×g^{-1} EW and a clear linear relation between female size and fecundity exists.

The nearly absence of hydrated females in the samples gathered for all the spawning period indicates that such females are not targeted by fishing. The reproductive behaviour of the species, with habitat specificity during the spawning season, where males dig a hole in the sand for females depositing their eggs (Wilson, 1958), seems to protect the species from commercial fishing during spawning events, unlikely to what is reported for Southern England with recreational fishing (Pinder et al., 2017).

Data from recreational fishing in Portugal were not analysed and therefore the impact of this sector in the species cannot be discussed. However, it is probable that the high recreational fishing pressure reported for the species in England (Pinder et al., 2017), is much lower in the Portuguese scenario.

From the data gathered in this study, it is likely that fishing pressure on *S. cantharus* in Portuguese coast, is within suitable limits for the present time.

Final Remarks

For management proposes only the main target species are subject to detailed assessment, but they represent only a fraction of all species caught. In fisheries with non-selective gears, a mixture of many other species contributes to the catch, and although less abundant, they may comprise an important component of the catch value (Cook and Heath, 2018). The assessment of such species is often absent or limited, and as a result, their status is often unknown (Cook and Heath, 2018). *S. cantharus* is one example of such species that, despite its relevance in commercial and recreational fishing across the Eastern Atlantic and Mediterranean, is not subject to any stock assessment.

The information assembled by this study provides a basis for the assessment of the species' status in the Portuguese coast. Within a national tradition where the fishing sector is socially and culturally important (Failler, 2007), and resources for fisheries assessment are limited, such studies should be considered a valuable asset for fisheries managers, since they offer detailed information on species that would not be evaluated otherwise.

An improvement for the fisheries management will be certainly achieved if data available in the academy and research organisations would be shared and applied by the governmental institutes.

Supplementary Material

Table S3.1 ANCOVA for $\delta^{13}C$ and $\delta^{18}O$ values in otoliths

	Source	Df	Sum sq	Mean sq	F value	P value
$\delta^{18}O$	area	6	3.743	0.5781	15.51	0.0003
	Otolith mass	1	0.578	0.6239	14.37	< 0.0001
	Residuals	76	3.058	0.0402		
$\delta^{13}C$	area	6	80.42	13.403	50.87	< 0.0001
	Otolith mass	1	14.49	14.492	55.00	< 0.0001
	Residuals	76	20.02	0.263		

Table S4.1 Gene flow among collecting sites of *Spondyliosoma cantharus*, within the North-eastern Atlantic (NEAT) and Mediterranean Sea (MEDS) regions, represented by FST (below diagonal) and Nm (number of migrants; above diagonal).

	NEAT							MEDS				
Cyt*b*	EN	BI	GL	PN	AL	CN		MU	VL	BL	CO	CR
EN		inf	inf	inf	20.279	inf	MU		inf	8.789	5.251	4.961
BI	-0.032		inf	inf	47.122	inf	VL	-0.061		4.818	3.359	3.139
GL	-0.028	-0.039		inf	1284.5	inf	BL	0.054	0.094		inf	inf
PN	-0.012	-0.017	-0.010		17.711	inf	CO	0.087	0.130	-0.035		60.17
AL	0.024	0.011	0.000	0.027		136.0	CR	0.092	0.137	-0.030	0.008	
CN	-0.024	-0.024	-0.027	-0.002	0.004							
S7	EN	BI	GL	PN	AL	CN		MU	VL	BL	CO	CR
EN		2.581	5.205	2.575	3.75	2.239	MU		20.207	inf	9.218	74.09
BI	**0.088**		7.016	1288	inf	73.41	VL	0.012		209.5	4.113	18.99
GL	0.046	0.034		inf	31.38	11.76	BL	-0.011	0.001		14.90	inf
PN	**0.088**	0.000	-0.004		inf	24.63	CO	0.026	**0.057**	0.017		302.6
AL	**0.062**	-0.003	0.008	-0.007		30.69	CR	0.003	0.013	-0.012	0.001	
CN	**0.100**	0.003	0.021	0.010	0.008							

Significant values of probability are shown in bold and were assessed by permutation test with 99,000 replicates and corrected for multi comparisons by the Benjamini-Hochberg (BH) false discovery rate method.

Table S6.1 Linear model residuals normality results.

Model	Shapiro-Wilk test Residuals Normality	Correlation between Obs Res and Exp Res	Chi^2 Score Test for Heteroskedasticity
BF~TL	0.9581 (0.1032)	0.9739	3.1564 (0.0756)
BF~EW	0.9574 (0.0969)	0.9733	4.4654 (0.0346)
NDO~TL	0.974 (0.5260)	0.9864	9.1716 (0.0025)
NDO~EW	0.9775 (0.6432)	0.9876	0.5423 (0.4614)

p-values for the Shapiro-Wilk test and Chi^2 Score Test for Heteroskedasticity are given between brackets. BF – absolute batch fecundity; NDO - number of advanced vitellogenic oocytes; TL – total length; EW – eviscerated weight; Obs Res – observed residuals; Exp Res – expected residuals under normality.

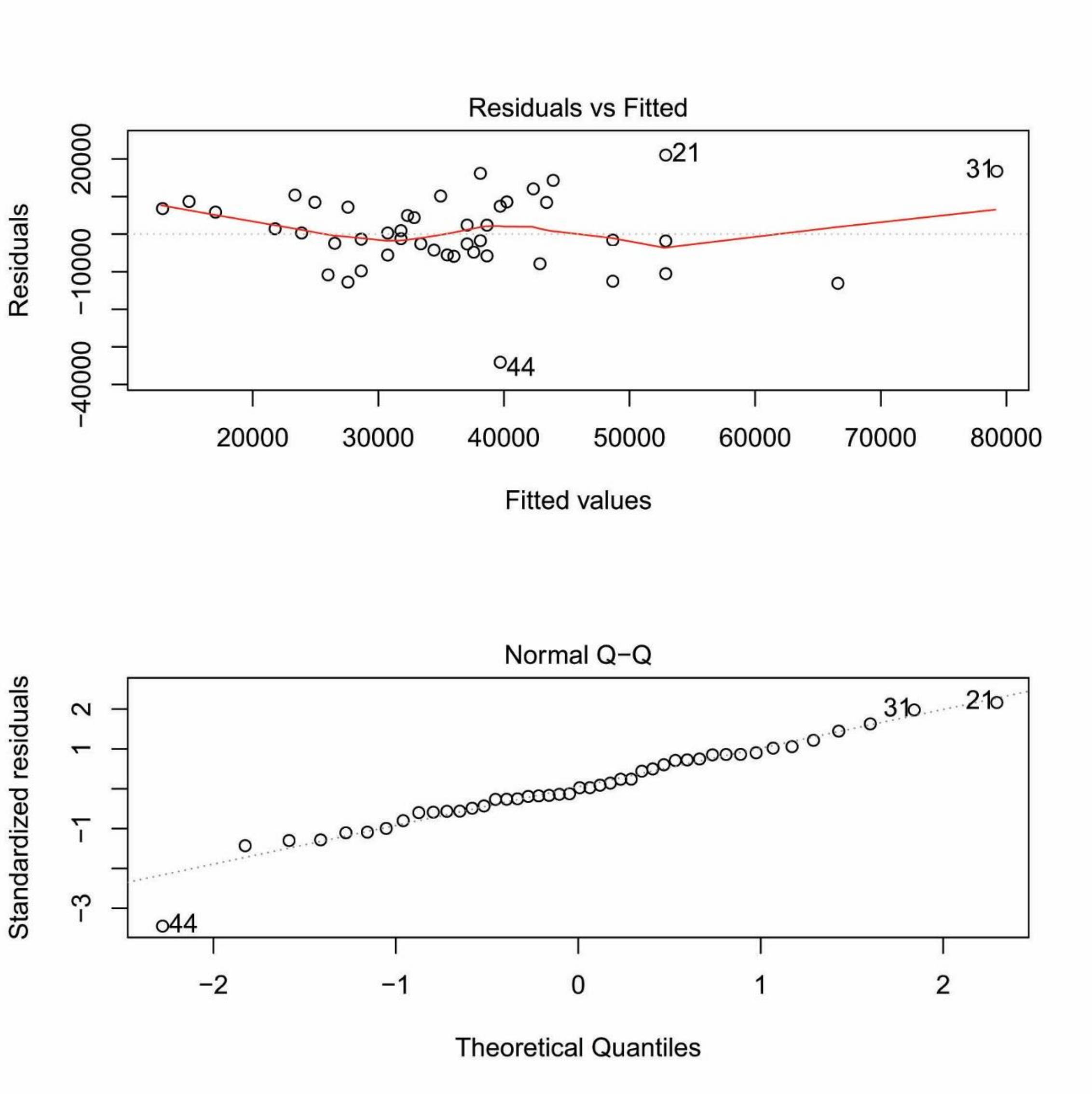

Fig. S6.1 Residuals plot for the linear model fitted for absolute batch fecundity and total length.

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