Universitat
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DOCTORAL THESIS
2021

DYNAMICS OF THE DEEP-SEA TELEOST BLACK SCABBARDFISH (*APHANOPUS CARBO* LOWE, 1839) IN THE NORTHEAST ATLANTIC

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Doctor by the Universitat de les Illes Balears

Ackowledgments

My first words and my most special gratitude goes to Dr. Ivone Figueiredo, my thesis tutor at the Portuguese Institute for Sea and Atmosphere (IPMA), not only for helping developing the original idea of this Thesis and for all the support throughout the work, but most especially for walking with me, side-by-side, this long walk.

I am also especially grateful to Dr. Beatriz Morales Nin for accepting the challenge of directing this Thesis, mostly at distance, for always making me feel welcome in Esporles and at IMEDEA, and for not letting me lose the enthusiasm needed to finish this Thesis.

This work was supported by the Portuguese Foundation for Science and Technology (FCT – Fundação para a Ciência e Tecnologia) under the PhD Grant SFRH/BD/64434/2009. I thank Eng. Carlos Vale and Dr. Graça Pestana for accepting to host my PhD project within the scope of the activities of the Portuguese National Institute of Biological Resources/Fisheries and Marine Research Institute (presently, the Portuguese Institute for Sea and Atmosphere, IPMA).

I also thank Dr. Guillem Mateu Vicens y Dr. Gabriel Moyà Alcover, present and former coordinators of UIB's PhD in Marine Ecology, for facilitating my integration and helping me follow the PhD program and everyone at the Doctoral School for always being available to kindly help.

I am forever grateful to Sílvia Pérez Mayol and Miquel Palmer for everything they have taught me and for all the help. To Sílvia, I also thank the long-lasting friendship.

I would not have been able to pursue this challenge without many people with whom I was very fortunate to collaborate. They are, without any calculated order: Paulo B. Oliveira, Irineu Batista, Narcisa Bandarra, Júlia Ferreira, Ana Isabel Janeiro, Adelino Canário, Elsa Couto, Sofia Vieira, Sara Reis, João Delgado, Alicia Cantarero, Francis Neat, Pascal Lorance, Gabriel Martorell, Ana Rita Vieira, Henrique Santos and the fishermen in Varamar, Carlos Macedo and everyone at the fishermen association ArtesanalPesca in Sesimbra. I am also indebted to everyone who so kindly provided samples and data: Ana Ribeiro Santos, Klara Jacobsdóttir, Lise Helen Ofstad, among others.

For helping me keep the enthusiasm, for their friendship, I thank Neide Lagarto (in the office, in the lab, and on board), Teresa Moura, Bárbara Pereira, Catarina Maia, Nuno Veiga, José Lago, and everyone who has been a part of our deep-sea/elasmobranchs group. And Yorgos Stratoudakis for getting to know me and knowing when to say the right thing. I also thank all present and former colleagues at IPMA who participated in this work in any way.

I will always have great memories of all the friends I left in Mallorca, most of whom have flew to all over the world: Maite, Eva, Ceci, Luana, Carlos, Merit, Veri, Cata, Clara, Adri, Morten, JuanMa, Charles, Isis, Isma, Pep, Itzi,… I hope I'm not forgetting anyone.

To my beautiful girls, close to my heart, even when away: Catarina Oliveira and Elisa Barreto, who even gave me shelter and food during my work trips, Sara Cortez, Ana Queirós (brava!), Inês Sequeira, Ana Guerra, and Filipa Gouveia.

My most special gratitude goes to the most important people in my life, my family: my parents, my brother, Duarte and the love of my life, my daughter Beatriz. Thank you for giving me the strength to get here. And because families tend do grow, to Fernanda, Marta, Margarida, Valter, and my beautiful nephews Francisco, Teresa, and Mariana. None of this would have been possible without all of you.

In memory of Alberto Murta

Abstract

Aphanopus carbo (Lowe, 1839), the black scabbardfish, is a benthopelagic teleost with high commercial value in the NE Atlantic, especially in Madeira Archipelago and mainland Portugal. Given its commercial interest and high susceptibility to overfishing, as a consequence of low fecundity and low growth rate, improving the knowledge on its life cycle and population dynamics is of uttermost importance for improving fisheries management and advice. Although some questions persist, the agreed assumption is that there is a unique stock that undergoes a clockwise migration around the NE Atlantic driven by feeding and reproduction. The available information that supports the hypothetical migratory cycle of *A. carbo* was reviewed in this Thesis and different methodological approaches were proposed to explore its life history traits and relate them with the migratory cycle, in order to increase information on the species for a sustainable knowledge-based fishery management, and to assess its population dynamics and spatial pattern in the NE Atlantic. Differences in fatty acids and stable isotopes concentrations in the muscle tissue of specimens from different areas supported the species hypothetical migratory cycle. Mature specimens showed a prevalence of polyunsaturated fatty acids (PUFA) which are related with high energetic demands, supporting the hypothesis that the black scabbardfish continues to feed during the spawning period. PUFA associated with a response to stress were high in specimens from the southernmost areas which might be related with the expected long-distance migrations. The analysis of sex steroids in blood serum confirmed their role as intrinsic triggers for gonadal maturation and spawning in black scabbardfish. Based on estradiol (E_2) and testosterone (T) concentrations, some developing females from Madeira were clustered with females from mainland Portugal, giving evidence that not all developing females will have the ability to mature and reproduce during the current spawning season. The previous methodologies allowed relating biochemical traits with the species life cycle and compare them between geographical areas in a short and recent time-frame. To understand the species taxonomic and species spatial diversity throughout its life cycle, otoliths were used as chemical markers. In a first study, otolith trace element composition (TEC) was shown to be adequate to separate the two *Aphanopus* species that are mixed in landings from the Madeiran longline fleet. It was further demonstrated that quantifying Mg, Cr, Sr, and Ba in the otolith increment that corresponds to age-class 9 can be an effective way to identify the species using LA- ICPMS at relatively reduced costs. This technique could be applied to archived collections of otoliths to perceive the historical presence of *A. intermedius* in landings in Madeira, reconstruct species abundance time-series and infer if the species is moving northwards. Otolith microchemical analysis was also applied to infer the migratory movements of *A. carbo* along the NE Atlantic by quantifying certain trace elements at selected otolith zones that represent different life history stages, namely the core, age three, age five, and the edge. TEC in the otolith edge could discriminate the locations where specimens were caught, separating the northernmost from the southernmost areas. The longitudinal multivariate analyses of TEC also sustained the separation of the otoliths into two groups, but there is high mixing between them, which agrees with the migratory hypothesis. The existence of two natal sources was suggested from otolith core TEC analysis. The acceptance of both southern and northern spawning grounds and of migratory movements along the NE Atlantic in both northward and southward directions implies changes to the current migratory hypothesis that might translate into changes in *A. carbo*'s stock assessment.

Resum

Aphanopus carbo (Lowe, 1839), el peix sable negra, és un teleòste bentopelàgic amb un alt valor comercial a l'NE Atlàntic, especialment a l'arxipèlag de Madeira i a Portugal continental. Atès el seu interès comercial i la seva alta susceptibilitat a la sobrepesca, com a conseqüència de la baixa fecunditat i la baixa taxa de creixement, millorar la informació sobre el seu cicle de vida i la dinàmica de la població és de màxima importància per millorar la gestió i l'assessorament de la pesca. Tot i que hi ha preguntes sobre el seu cicle de vida i la dinàmica de la població que romanen sense resposta, la suposició acordada és que hi ha un estoc únic que experimenta una migració en sentit horari al voltant de l'NE Atlàntic impulsada per l'alimentació i la reproducció. La informació disponible que dóna suport al hipotètic cicle migratori de *A. carbo* es va revisar en aquesta tesi i es van proposar diferents enfocaments metodològics per explorar els trets de la seva història de vida i relacionar-los amb el cicle migratori, per tal d'incrementar la informació sobre la espècie per a una gestió sostenible de la pesca basada en el coneixement de les espècies i la seva dinàmica de població i patró espacial al Nord-Atlàntic. Les diferències en àcids grassos i les concentracions estables d'isòtops en el teixit muscular d'exemplars de diferents àrees van donar suport a l'hipotètic cicle migratori de l'espècie. Els exemplars madurs van mostrar una prevalença d'àcids grassos poliinsaturats (PUFA) que es relacionen amb elevades demandes energètiques, donant suport a la hipòtesi que el sable negre continua alimentant-se durant el període de posta. Els PUFA associats amb una resposta a l'estrès van ser elevats en exemplars de les zones més meridionals, cosa que podria estar relacionada amb les migracions esperades de llarga distància. L'anàlisi d'esteroides sexuals al sèrum sanguini va confirmar el seu paper com a desencadenants intrínsecs per a la maduració gonadal i la posta en sable negre. Basant-se en les concentracions d'estradiol (E_2) i testosterona (T), algunes femelles en desenvolupament de Madeira es van agrupar amb femelles del Portugal continental, donant evidència que no totes les femelles en desenvolupament tindran la capacitat de madurar i reproduir-se durant la temporada actual de posta. Les metodologies anteriors permeten relacionar trets bioquímics amb el cicle de vida de les espècies i comparar-los entre àrees geogràfiques en un període de temps curt i recent. Per entendre diversitat taxonòmica i espacial de l'espècie al llarg del seu cicle de vida, es van utilitzar otòlits com a marcadors químics. En un primer estudi, es va demostrar que la composició d'elements traça d'otòlits (TEC) era adequada per separar les dues espècies d'*Aphanopus* que es barregen en els desembarcaments de la flota de palangre de Madeira. Es va demostrar, a més, que quantificar Mg, Cr, Sr i Ba en l'increment de l'otòlit que correspon a la classe d'edat 9 pot ser una manera eficaç d'identificar les espècies mitjançant LA-ICPMS a costos relativament reduïts. Aquesta tècnica es podria aplicar a col·leccions d'otòlits arxivades per percebre la presència històrica d'*A. intermedius* als desembarcaments a Madeira, reconstruir sèries temporals d'abundància d'espècies i inferir si l'espècie es mou cap al nord. L'anàlisi microquímica d'otolits també es va aplicar per inferir els moviments migratoris d'*A. carbo* al llarg de l'Atlàntic NE quantificant certs oligoelements en zones seleccionades d'otòlits que representen diferents etapes de la història de la vida, és a dir, el nucli, tres anys, cinc anys i la vora. El TEC a la vora de l'otòlit podria discriminar els llocs on es van capturar els exemplars, separant la zona més septentrional de la zona més meridional. Les anàlisis longitudinals multivariants de TEC també van mantenir la separació dels otòlits en dos grups, però hi ha una gran barreja entre ells, cosa que concorda amb la hipòtesi migratòria. L'existència de dues fonts natals es va suggerir a partir de l'anàlisi TEC del nucli d'otòlits. L'acceptació de les zones de posta tant del sud com del nord i dels moviments migratoris al llarg de l'NE Atlàntic en direccions nord i sud implica canvis en la hipòtesi migratòria actual que es podria traduir en canvis en l'avaluació d'estoc d'*A. carbo*.

Resumen

Aphanopus carbo (Lowe, 1839), el sable negro, es una especie bentopelágica con elevado valor comercial en el Atlántico NE, especialmente en Madeira y en Portugal peninsular. Dado su interés comercial y su elevada susceptibilidad a sobrepesca, como consecuencia de baja fecundidad y baja tasa de crecimiento, mejorar el conocimiento sobre su ciclo de vida y dinámica poblacional tiene máxima importancia para la gestión y asesoramiento de las pesquerías. Aunque quedan algunas cuestiones, la suposición acuerdada es que hay un stock único que hace una migración al redor del Atlántico NE en el sentido de las agujas del reloj estimulado por la reproducción y alimentación. La información disponible que sustenta la hipótesis del ciclo migratorio del *A. carbo* fue revisada en esta Tesis y se proponen diferentes abordajes metodológicos para explorar sus características vitales y relacionarlos con el ciclo migratorio, con el objetivo de aumentar la información sobre la especie en el sentido de un asesoramiento de la pesquería basado en el conocimiento, y para investigar su dinámica poblacional y patrón espacial en el Atlántico NE. Diferencias en la concentración de ácidos grasos y de isotopos estables en el tejido muscular de especímenes de diferentes áreas soportan el hipotético ciclo migratorio de la especie. En individuos maduros prevalecieron los ácidos grasos polinsaturados (PUFA) que están relacionados con elevada demanda energética, apoyando la hipótesis de que el sable negro sigue alimentándose durante la época de madurez sexual. Los PUFA, que están relacionados con la respuesta al estrés, alcanzaron niveles más altos en los especímenes de las áreas más a sur, pudendo estar relacionado con las migraciones de larga escala. Los análisis de esteroides sexuales en el suero sanguíneo confirmaron su papel como desencadenantes intrínsecos de la maduración de las gónadas y del desove del sable negro. Con base en la concentración de estradiol (E_2) y de testosterona (T), algunas hembras en maduración capturadas en Madeira se agruparon con las hembras de Portugal peninsular, dando pruebas de que no todas las hembras en maduración tendrán la capacidad de madurar y reproducirse durante la presente época de desove. Las metodologías anteriores permiten relacionar trazos bioquímicos con el ciclo de vida de la especie y compararlos entre áreas geográficas. Para comprender la diversidad taxonómica y espacial de la especie a lo largo del ciclo de vida, los otolitos fueran usados como registradores químicos del ambiente donde el individuo vivió. En un primero estudio, se demostró que la concentración de elementos traza (TEC) de los otolitos es adecuada para separar las dos especies de *Aphanopus* que están mezcladas en los desembarques de la flota de palangre de Madeira. Además, se demostró que es posible separar las dos especies usando el LA-ICPMS con base en la cuantificación de los elementos Mg, Cr, Sr y Ba en el incremento del otolito que corresponde a la clase de edad 9. Esta técnica podrá ser empleada en colecciones archivadas de otolitos para demostrar la presencia histórica de *A. intermedius* en los desembarques en Madeira, reconstruir series temporales de abundancia de las especies y inferir si la especie se está desplazando hacia el norte. El análisis de microquímica de otolitos también fue empleada para inferir los movimientos migratorios de *A. carbo* alrededor del Atlántico NE por cuantificación de determinados elementos traza en zonas seleccionadas del otolito que representan diferentes fases de su historia de vida, a saber, el núcleo, los incrementos correspondientes a la edad tres y la edad cinco y el borde del otolito. Fue posible discriminar las áreas donde los especímenes fueron capturados con base en las TEC, separando las áreas más al norte de las más al sur. El análisis longitudinal multivariado de las TEC también suportó la separación de los otolitos en dos grupos, pero con elevada mezcla entre ellos, lo que corrobora la hipótesis de migración. La existencia de dos zonas de reproducción fue sugerida con base en el análisis de TEC en el núcleo de los otolitos. La aceptación de dos áreas de desove, en el sur y en el norte y de movimientos migratorios en el sentido norte y sur del Atlántico NE implica cambios en la actual hipótesis migratoria que podrán traducirse en cambios en el asesoramiento del stock de *A. carbo*.

List of publications

The present Thesis is a compendium of the following peer-reviewed publications:

Farias, I., Morales-Nin, B., Lorance, P., Figueiredo, I., 2013. Black scabbardfish, Aphanopus carbo, in the northeast Atlantic: distribution and hypothetical migratory cycle. Aquatic Living Resources 26(4), 333-342. https://doi.org/10.1051/alr/2013061

Farias, I, Figueiredo, I., Janeiro, A.I., Bandarra, N., Batista, I., Morales-Nin, B., 2014. Reproductive and feeding spatial dynamics of the black scabbardfish, Aphanopus carbo Lowe, 1839, in NE Atlantic inferred from fatty acid and stable isotope analyses. Deep-Sea Research Part I 89, 84-93. https://doi.org/10.1016/j.dsr.2014.04.010

Farias, I., Couto, E., Lagarto, N., Canário, A.V.M., Figueiredo, I., 2020. Sex steroids of black scabbardfish, Aphanopus carbo, in relation to reproductive and migratory dynamics. Aquaculture and Fisheries. https://doi.org/10.1016/j.aaf.2020.03.006

The original manuscripts are compiled in the Annex.

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Chapter 1: General introduction

1.1. Into the deep: the ecosystem

The deep-sea ecosystem comprises the whole water mass below 200 m, above which is the shallow sea or shelf (Merrett and Haedrich, 1997; Drazen and Haedrich, 2012). The agreed boundary is the epipelagic zone (below 200 m), where sunlight no longer penetrates (Herring, 2002). The deep-sea ecosystem is characterised by low light, low temperature, high pressure, and low dissolved oxygen, which have a major effect on biological aspects, namely low productivity, limited nutrient availability (major source is the seasonal deposition of detrital aggregates), and increased predation and competition (Merrett and Haedrich, 1997).

Regarding fishes dwelling in the deep-sea, in literature, the bathymetric range adopted for deep-sea fishes may not coincide with the range for deep-sea habitat. While some authors situate the deep-sea habitat below 1000 m, including only the bathypelagic species, others consider those living deeper than 400-500 m as well, including many mesopelagic species that have adaptations for occasionally moving or even living deeper (Weitzman, 1997; Clarke et al., 2003). Based on their ecology, marine fishes can be classified as either pelagic, if they live in the water column, or demersal, if they live on the sea floor (benthic) or just above the sea floor (benthopelagic) (Haedrich, 1996; Merrett and Haedrich, 1997).

Deep-sea fish species are representatives of groups that appeared early in the evolution of the modern fishes, reflecting how specifically evolved and adapted they are to the deep-sea and hence they can be classified according to two criteria: evolution and habitat (Haedrich, 1996; Merrett and Haedrich, 1997). In evolutionary terms, two groups of deep-sea ichthyofauna that colonized the depths at different times have been defined: “ancient” and “secondary” (Andriyashev, 1953). Ancient forms moved early into the deep-sea, where they underwent their primary evolution and radiation, whereas secondary forms are considered to have undergone their primary evolution and radiation on the shallow continental shelves, where most are still found today, and moved deeper much later. For that reason, these last do not display marked morphological adaptations to the deep-sea environment. Andriyashev’s (1953) theory came from the observation that relatively warm waters of the Atlantic Ocean, south of the Wyville Thompson Ridge, were populated by the ancient deep-water, which are absent in cold waters to the north where secondary species prevail (Priede and Froese, 2013). In reality, species do not naturally fall into these groups but range along a continuum of life history attributes (Drazen and Haedrich, 2012). White (1987) formulated the “deep allopatry hypothesis” which states that, during periods when the deep-sea was warm, the low oxygen-level in the ocean vertically expanded causing periodic episodes of recurrent regional hypoxia. These episodes, associated with topographic features, such as seamounts, provided opportunities for isolation and speciation which altered the gene flow patterns at slope depths, contributing to the richness of benthic and pelagic deep-sea assemblages. Andriyashev’s (1953) and White’s (1987) theories are complementary (Merrett and Haedrich, 1997).

Deep-sea fishes exhibit morphological adaptations for successfully living in a harsh environment, such as black or red pigmentation for best camouflage (since the red end of the spectrum is most readily absorbed by sea water); large and tubular eyes to compensate for low light; a well-developed lateral line to improve sound localization; teeth and mouth adaptations to optimize predation and compensate for low food availability (Haedrich, 1996; Merrett and Haedrich, 1997). Some species also exhibit elaborate light organ systems that enable interspecific recognition. To facilitate buoyancy, benthopelagic fish typically have a well-developed gas-filled swim bladder, whereas deep pelagic fish show a reduced skeletal and muscular robustness in replacement of the swim bladder. Moreover, the life history traits and physiology of these species, namely growth, reproduction, diet, and energy consumption, are directly dependent on the environmental conditions (Merrett and Haedrich, 1997). The growth of deep-sea species is limited by low temperature, high pressure, and low food availability because they cause a slowdown in the metabolic rate processes (Graham et al., 1985; Merrett and Haedrich, 1997). This constraint has also a direct influence on reproduction since, in many deep-sea species, females become mature only after they reach full size and when somatic growth has slowed down or ceased (late maturity) (Gordon et al., 1995). In general, benthopelagic species show low fecundity, which means they produce fewer and larger eggs than pelagic species and, in some species, semelparity (one single reproductive event before death) is frequent and is possibly depth-related (Elgar, 1990).

Deep-sea fisheries are widely spread to all oceans since the 1960s, but gained a more incisive expression in the late 1990s and early 2000s, when depths below 1000 m were reached, with fleets from different countries targeting on several species with high economic value, such as orange roughy (*Hoplostethus atlanticus*), roundnose grenadier (*Coryphaenoides rupestris*), redfish (*Sebastes* spp.), oreos (Family Oreosomatidae), blue ling (*Molva dypterygia*), shark and ray species, crabs, and shrimps (Gordon, 2001; Morato et al., 2006; Armstrong et al., 2010; Large et al., 2013). With the depth increase of commercial fisheries exploitation, the term ''deep-sea fishery'' became generalized to fisheries conducted below 200 m (Drazen and Haedrich, 2012). Despite this surge, deep-sea species cannot sustain high levels of exploitation and are very susceptible to overfishing as a consequence of the previously described biological characteristics (Clarke et al., 2003; Wells et al., 2003). This sensitivity to exploitation is aggravated by the fact that deep-sea species are mostly data-deficient and, therefore, assessment models ordinarily used for shallow species or stocks are usually not applicable (Heymans et al., 2011).

Regardless of the difficulties in studying and sampling deep-sea species due to the constraints caused by the access to the deep-sea ecosystem and the physiological characteristics of the fishes that impede obtaining live individuals due to barotrauma, a growing interest has instigated the scientific research on this groups of fish. This is expressed in the rising number of publications for Atlantic waters focusing on a wide variety of subjects such as fisheries (Clark, 2001; Gordon, 2001; Gordon et al., 2003; Lorance and Trenkel, 2006; Morato et al., 2006; Armstrong et al., 2010; Lorance et al., 2011; Priede et al., 2011; Villasante et al., 2012; Large et al., 2013; Parra et al., 2017; Vasconcelos et al., 2020b); age and growth (Mauchline, 1988; Gordon and Swan, 1996; Morales-Nin and Sena-Carvalho, 1996; Allain and Lorance, 2000; Morales-Nin et al., 2002; Kelly et al., 2005; Pajuelo et al., 2008; Vieira et al., 2009; Vieira et al., 2013); reproduction (Mauchline, 1988; Figueiredo et al., 2003; Neves et al., 2009; Ribeiro Santos et al., 2013); migration (Arkhipkin and Laptikhovsky, 2010), diet (Mauchline and Gordon, 1986; Santos et al., 2013); distribution (Haedrich and Merrett, 1990; Gordon et al., 1996; Hareide and Garnes, 2001; Bergstad et al., 2012); life histories (Gordon and Duncan, 1987; Drazen and Haedrich, 2012); genetics (Knutsen et al., 2009; White et al., 2010; Longmore et al., 2014); and general biology (Mauchline and Gordon, 1986; Sutton et al., 2008; Bergstad et al., 2012).

1.2. Background on black scabbardfish, *Aphanopus carbo*

Aphanopus carbo Lowe, 1839, the black scabbardfish, is a deep-sea teleost for which there has been an increasing scientific interest and investment in the NE Atlantic, mostly driven by its economic value and knowledge gaps still to fill regarding the species spatial dynamics and population biology (Longmore et al., 2014). It belongs to family Trichiuridae (Class Actinopterygii, Order Perciformes), for which 50 accepted species are presently described, seven of them belonging to the genus *Aphanopus* (Froese and Pauly, 2020). According to Andriyashev's (1953) division of deep-sea fish fauna, order Perciformes belongs to the secondary group, which are the most recent teleost taxa (Priede and Froese, 2013). *A. carbo* was first described over 180 years ago in Madeira Island, by an English naturalist, Reverend Richard Thomas Lowe, who had been living there for 50 years (Maul, 1950). *A. carbo* lives in sympatry with *A. intermedius* in Madeira, the Azores, the Canaries, and the north western coast of Africa (Stefanni and Knutsen, 2007; Knutsen et al., 2009; Stefanni et al., 2009; Biscoito et al., 2011; Delgado et al., 2013). In areas further north, it has been confirmed that only *A. carbo* is present using three different mtDNA molecular markers – the control region (CR) and the cytochrome b (Stefanni and Knutsen, 2007) and the CR and the cytochrome oxidase subunit I (COI) (Biscoito et al., 2011).

A. carbo is widely distributed in the North Atlantic, being more common on the eastern side, where it occurs between the Strait of Denmark (70° N) and the Western Sahara (30° N). The species abundance is higher on the continental slope, seamounts, and ridges, namely south of the Faroe Islands, in the Rockall Trough, along mainland Portugal, and around Madeira and the Canary Archipelagos, also occurring in Iceland, the Mid-Atlantic Ridge and Corner Rise, and the Azores (Martins et al., 1987; Nakamura and Parin, 1993; Parin, 1995; Pajuelo et al., 2008; Machete et al., 2011). Further details on the distribution of *A. carbo* are presented in Chapter 2 of this thesis.

This benthopelagic species has been found between 200 m of depth, west of the British Isles (Nakamura and Parin, 1993; Kelly et al., 1998), and 2300 m around the Canary Islands (Pajuelo

et al., 2008), being more common between 400 and 1800 m (Ehrich, 1983; Martins et al., 1987; Morales-Nin and Sena-Carvalho, 1996; Allain et al., 2003). It is an iteroparous species (multiple spawning events throughout its life) and a total spawner (spawns in one single event) (Pajuelo et al., 2008; Ribeiro Santos et al., 2013) with determinate fecundity (the potential annual fecundity is the number of vitellogenic oocytes minus the number of oocytes reabsorbed on account of atresia) (Neves et al., 2009; Ribeiro Santos et al., 2013). Spawning has only been described for Madeira (Figueiredo et al., 2003; Neves et al., 2009; Ribeiro Santos et al., 2013), the Canaries (Pajuelo et al., 2008), and the northwest coast of Africa (Perera, 2008), in the last quarter of the year. Reproduction was also reported but never confirmed in Porcupine Bank (Ehrich, 1983) and Icelandic waters (Magnússon and Magnússon, 1995). The relative fecundity by weight of female is low (88-323 oocytes.g^{-1} in spawning females) (Neves et al., 2009). The short spawning period, synchronous spawning, and determinate fecundity are the opposite of the most common deep-sea fish reproductive strategies. Ribeiro Santos et al. (2013) suggest this could be a strategy to synchronize the reproductive cycle with the surface primary production, to guarantee that the eggs float upwards and larvae are produced in food-rich waters.

Although eggs and larvae have not been recorded, juveniles are reported to be mesopelagic (Parin, 1986), whilst the adults live deeper but undertake horizontal and vertical movements driven by spawning and by feeding (Zilanov and Shepel, 1975; Clarke and Wagner, 1976; Du Buit, 1978; Ehrich, 1983; Anon., 2000). The smaller individuals reported are two specimens with 10 and 15 cm total length found in the stomach of a longnose lancetfish (*Alepisaurus ferox* Lowe, 1833) (Maul, 1950) and a specimen with approximately 10 cm, identified by DNA barcoding, caught at the Senghor Seamount, off the northeast of Cape Verde (Hanel et al., 2010). Juveniles recruit to the fisheries off the west of the British Isles, documented to be a feeding area, where they remain some time growing (Figueiredo et al., 2003; Santos et al., 2013), and later move to areas off mainland Portugal where caught specimens reach larger sizes and pre-spawning individuals are seldom captured (Figueiredo et al., 2003; Neves et al., 2009). After another period of feeding and growth, *A. carbo* move further south to the spawning areas around Madeira. This is the accepted hypothetical migratory cycle of the species in the NE Atlantic (ICES, 2020), which explains the differences in length distribution among catch locations, but does not clarify why *A. carbo* does not mature and spawn elsewhere other than Madeira and the Canaries.

Differences in maximum length have been found between growth studies with otoliths caught in different areas along the NE Atlantic. A study with whole otoliths from Madeira (length range 90-151 cm) attained a maximum age of 12 years and a fast growth rate (Morales-Nin and Sena-Carvalho, 1996). In sectioned otoliths from the Rockall Trough, with a length range of 37-119 cm, the maximum estimated age was 32 years (Kelly et al., 1998). Morales-Nin et al. (2002) determined a maximum age of 12 years, analysing whole otoliths from fish caught off Madeira and the Rockall Trough (length range 56-144 cm and 82-112 cm, respectively). Pajuelo et al. (2008), in a study with whole burned otoliths, also reported a maximum age of 12 years for specimens caught off the Canary Islands. Vieira et al. (2009) reported a maximum age of 14 years for sectioned otoliths from Madeira (length range 125-148 cm) and 12 years for sectioned otoliths from fish caught off mainland Portugal. Since the length ranges are similar, the differences are most likely explained by external factors (e.g., readers' experience, preparation techniques, and interpretation of growth increments). More detailed information is presented in Chapter 2 of this thesis.

The number of studies on the diet and feeding ecology of *A. carbo* is very scarce because stomach content examination is difficult since most fish are caught with their stomach everted by the abrupt drop in pressure when hauled and the stomach vacuity index is very high. A vacuity index of 66.2 to 94.1 % was found for specimens caught by trawls to the west of the British Isles (Mauchline and Gordon, 1984; Santos et al., 2013). The vacuity index estimated for fish caught off Madeira was higher (93.3-98.3 %) probably because fish can stay some time hooked to the longline before dying (Freitas, 1998; Santos et al., 2013). *A. carbo* is a top predator, which feeds on a wide variety of prey, such as fish, crustaceans, and cephalopods (Zilanov and Shepel, 1975; Nakamura and Parin, 1993; Freitas, 1998; Santos et al., 2013). Santos et al. (2013) observed seasonal changes in its diet in the northern area, which were corroborated by stable isotope analysis, namely a preference for blue whiting, *Micromesistius poutassou*, when this species is readily available, and a change to cephalopods and crustaceans when the former moves to its spawning grounds in the Norwegian Sea.

Total mercury (THg), cadmium (Cd), and lead (Pb) concentrations have been analysed in the muscle, liver, and gonad tissue of *A. carbo* caught off mainland Portugal, Madeira, and the Azores (Costa et al., 2009). The relationship between the metals' concentrations in fish tissues and region was explained by trace metal contents in the water (higher in Madeira and the Azores because of their volcanic origin), species physiology and feeding (larger fish feed upper on the food web, hence, are subject to bioaccumulation). Median THg was above the limit established by the EU (1.0 mg kg-1 ww) in muscle samples from Madeira and in the liver from all areas, and Cd was above the limits in liver and gonads of samples from the three areas, hence the authors advice that the consumption of this fish should be restricted. In a subsequent work, the consumption of *A. carbo* was associated with the probability of exceeding the provisional tolerable weekly intakes (PTWI) of those heavy metals (Cardoso et al., 2010). Following the recommendations of the World Health Organization (WHO) and the Food and Agriculture Organization (FAO) (FAO/WHO, 2003), the intake of Cd and Pb from *A. carbo* posed no serious risk to human health, but the hypothetical intake scenarios estimated for the Portuguese population surpassed the PTWI for methyl-mercury.

In the NE Atlantic, *A. carbo* holds a relatively high commercial value and scientific interest, being caught in three distinct areas: in the Madeira Archipelago, by drifting-bottom longline; off the coast of mainland Portugal, by bottom longline; and around the British Isles and Iceland, mainly by French, Icelandic, and Spanish trawlers (ICES, 2020). The fishery in Madeira is probably one of the world's oldest and longest deep-sea fisheries since it dates back to the 17th century (Merrett and Haedrich, 1997). Like other deep-sea species, *A. carbo* cannot sustain high levels of exploitation and is very susceptible to overfishing (Clarke et al., 2003; Wells et al., 2003) as a consequence of its biology, namely determinate and low fecundity and short spawning period (Neves et al., 2009; Ribeiro Santos et al., 2013) . Moreover, the fact that it is a data-deficient species, limited to fishery-dependent data in some areas, determines that assessment models ordinarily used for pelagic species or stocks are not applicable (Heymans et al., 2011; ICES, 2020). To produce advice for the NE Atlantic (excluding the Macaronesia archipelagos), the International Council for the Exploration of the Sea (ICES), considers two components as a single assessment unit: the Northern Component includes all fisheries around the British Isles and Iceland; the Southern component corresponds mostly to the fishery in mainland Portugal (ICES, 2015). Although the species is not in the condition of overexploitation, in recent years, catches in the northernmost areas have shown a slight decrease, whereas the Southern component has kept a steady stable trend. In 2005, Madeira's fishing fleet started expanding to areas close to and within the Azores and the Canaries EEZ (Exclusive Economic Zone) (Delgado et al., 2018). In this area, standardized CPUE (catch per unit effort in kg.haul^{-1}) was decreasing in the early 2000's, remained low from 2009 to 2014 and has been increasing since 2015 (Vasconcelos et al., 2020a). Considering the migratory hypothesis and the impact of fisheries on this species, understanding how each component relates in terms of biological signals and population dynamics is a key issue for establishing an adequate management system for sustainable fisheries in the NE Atlantic.

1.3. Objectives of the PhD Thesis

The main goal of this thesis is to contribute to fill the gaps on this poorly understood deep-sea species, specifically, to assess the migratory dynamics and spatial pattern of *A. carbo* in the NE Atlantic.

A first objective is to explore life history traits, in order to increase information on the species for a sustainable knowledge-based fishery management. The species feeding ecology will be inferred from the association between stable isotopes and fatty acids and ecological attributes such as diet and length distribution. The reproductive potential of adults or readiness for reproduction will be evaluated from biological/physiological parameters such as sex hormones and fatty acids. Reproductive and biological condition will be compared among regions to understand why spawning is geographically restricted to Macaronesia.

Since the direct observation of migratory movements of species that live deep into the ocean is not possible, chemical and biological markers will be used to identify the ecological niche occupied by *A. carbo* at different life stages. Individual otolith trace element concentrations (TEC) profile and deposition pattern will be used as proxy for the environmental conditions that are expected to differ between geographical areas (if they represent different water masses) to guarantee the discriminatory capacity of otolith microchemical analysis for this species and the water masses (region) it lives in.

1.4. Structure of the PhD Thesis

The present thesis is organized into six chapters. Chapters 2 and 3 are a compilation of three scientific papers that have already been published in peer-reviewed journals. The original content and structure (sections, figures, tables, etc) has been kept, but publishing formatting (letter type and size, line spacing, figures and tables numbering, etc) has been homogenised for better aesthetics and facilitating the reading process. Since this PhD thesis is the result of collaborative research with different institutions and people, the co-authors' names are included in published papers and in those sections that have not been submitted for publication but are intended to. The chapters are in chronological order of publication, rather than of PhD research activities execution, to guarantee consentaneity when relating and comparing their results.

Chapter 1 is the thesis' general introduction where the state-of-the-art and the most significant references on the subject of the thesis are reviewed. This chapter also includes the main objectives of the study and a methodological approach section where theoretical issues regarding each of the followed methodologies are detailed. These were excluded from the manuscripts originating the chapters, because they would be too extensive for publication.

Chapter 2 provides an exhaustive review on the biology, ecology, and distribution of the deep-sea teleost *A. carbo* in the Northeast Atlantic and the analysis of pertinent fisheries and surveys data from different sources. The chapter's main objectives are describing the species hypothetical migratory cycle and suggesting the multidisciplinary approach to confirm the spatiotemporal migration and habitats used by black scabbardfish populations in the NE Atlantic at different life stages, which is the basis of the thesis.

Chapter 3 is divided into two sections, each one corresponding to a published paper. The first section relates the analyses of fatty acids profile and $\delta^{15}N$ and $\delta^{13}C$ stable isotopes in the muscle tissue of *A. carbo* with diet, maturity and migratory dynamics. The second section compares the hormonal status and gonadal development stage of *A. carbo* caught off mainland Portugal and off Madeira and links the species reproductive cycle with the migratory path between the two areas. Additionally, the results of a pilot study using Mozambique tilapia (*Oreochromis mossambicus*) designed to evaluate the effect of blood sample collection at different times after the fish is dead are presented and interpreted.

Chapter 4 explores the applicability of otolith microchemical analysis to separate two sympatric species, *A. carbo* and *A. intermedius*, and to infer the spatial distribution of *A. carbo* along the NE Atlantic, each in a different section. Moreover, the otolith microchemical analyses for comparing the two species served as preliminary test to optimise the technique for posteriorly using otoliths to infer *A. carbo* population structure and life cycle reconstruction.

In Chapter 5, all the methodological approaches and their results and conclusions, presented in the previous chapters, are integrated in a general discussion where biological, physiological and oceanographic data are combined to improve the comprehension of *A. carbo*'s population and migratory dynamics and support the hypothesis of a second spawning ground in the NE Atlantic.

Lastly, Chapter 6 presents the general conclusions of the PhD Thesis.

1.5. Methodological approach

1.5.1. Stable isotopes

Whenever direct observation of feeding or stomach contents is not feasible, the quantification of chemical constituents acquired through diet has been shown to be a good representation of the feeding regimes and trophic relationships (DeNiro and Epstein, 1978; Petursdottir et al., 2008), including for deep-sea fish species (Drazen et al., 2009; Stowasser et al., 2009; Santos et al., 2013). Moreover, the chemical composition of fish muscle reflects the energetic trade-offs between intake, mostly through food, and expenditure with metabolism, migratory swimming, and the reproductive cycle (Petursdóttir et al., 2008; Stowasser et al., 2009).

Stable isotopes (SI) signatures provide a good insight into trophic relationships and food web interactions because they record both source and trophic level information and their patterns reflect the dietary intake over longer time periods than stomach content analyses (Iken et al., 2001; Polunin et al., 2001; Michener and Kaufman, 2007). SI ratios change between diet and consumer due to differential digestion or fractionation during assimilation and metabolic processes (DeNiro and Epstein, 1978, 1981; Tieszen et al., 1983; McCutchan et al., 2003; Michener and Kaufman, 2007). Stable carbon isotope ratio ($\delta^{13}C$) is commonly used to trace the carbon source, the regional origin of the primary producers, and the pathways of organic matter (DeNiro and Epstein, 1978, 1981; Post, 2002; McCutchan et al., 2003; Laakmann and Auel, 2010; Stowasser et al., 2009, 2012). Naturally occurring isotopes of nitrogen ($^{15}N/^{14}N$) show a stepwise enrichment of 2–5% at every step of the food web and, hence, nitrogen isotope ratio ($\delta^{15}N$) can be used as an indicator of the trophic position of organisms (DeNiro and Epstein, 1978, 1981; Post, 2002; Stowasser et al., 2009, 2012). The isotopic trophic discrimination factor between a consumer and its diet is, on average, 0.5-1‰ for $\delta^{13}C$ and 3.4‰ for $\delta^{15}N$ (DeNiro and Epstein, 1978, 1981; Vander Zanden and Rasmussen, 2001; Post, 2002; Michener and Kaufman, 2007; Laakmann and Aul, 2010; MacKenzie et al., 2011). Apart from isotopic fractionation, the relative abundance of SI within the organisms is influenced by external factors of geological, climatic, and ecological origin, as well as by internal factors, such as the synthetic pathways and position in the food web (DeNiro and Epstein, 1978, 1981; McCutchan et al., 2003).

Part of the variability in $\delta^{13}C$ in biological matrices is associated with varying lipid content amongst samples, since lipids are isotopically depleted in ^{13}C relative to proteins and carbohydrates (DeNiro and Epstein, 1977). This means that a tissue with a higher lipid content will have a lower $\delta^{13}C$ value (or greater depletion) compared to a tissue with a lower lipid content independent of diet (Hoffmann and Sutton, 2010). For aquatic organisms, the lipid depletion factor ($\Delta\delta^{13}C_{lipid}$; the isotopic depletion between the protein and lipid components) ranges from -6% to -7% (Schlechtriem et al., 2003; Kiljunen et al., 2006; Logan et al., 2008). To remove variability in $\delta^{13}C$ associated with varying lipid content, lipid extraction is increasingly used during tissue processing (Post, 2002; Sweeting et al., 2006). However, lipid extraction will simultaneously enrich the $\delta^{15}N$ of the sample, which is also undesirable (Hoffman and Sutton, 2010).

Therefore, Hoffman and Sutton (2010) proposed a simple mass balance correction to remove the effect of lipids on $\delta^{13}C$ of untreated tissue:

$$\delta^{13}C_{protein} = \delta^{13}C_{bulk} + \frac{\left(\Delta\delta^{13}C_{lipid} \times \left(C{:}N_{protein} - C{:}N_{bulk}\right)\right)}{C{:}N_{bulk}}.$$

For deep-sea fish, the isotopic depletion factor ($\Delta\delta^{13}C_{lipid}$) is –6.39‰, and the carbon mass balance given that simple lipids do not contain nitrogen is $C{:}N_{protein} = 3.76$ (Hoffman and Sutton, 2010).

1.5.2. Fatty acids

Lipids and the fatty acids (FA) that compose proteins are, along with proteins, the major organic constituents of fish, functioning as the main source of metabolic energy for growth, reproduction, and locomotion, including migration (Tocher, 2003; Parzanini et al., 2018). FA provide a good insight on the dietary intake and food web interactions because they allow the sequestering of lipid reserves over longer time periods than stomach content analyses (Polunin et al., 2001; Dalsgaard et al., 2003; Michener and Kaufman, 2007; Stowasser et al., 2009). Fatty acid trophic markers (FATM) are synthesized by specific primary producers and zooplankton, are transferred relatively unchanged along food webs, and accumulate in grazers and predators over time, allowing regional and temporal variations in plankton dynamics to be tracked down in the marine food webs (Lee et al., 1971; Dalsgaard et al., 2003; Drazen et al., 2009; Letessier et al., 2012).

FA also play a key role in the endocrine control of reproduction since certain polyunsaturated fatty acids (PUFA), such as the arachidonic acid (ARA) and the docosahexaenoic acid (DHA) are precursors of prostaglandins, which have an important role in reproduction (Stacey and Goetz, 1982). ARA is the major precursor of series II prostaglandins (paracrine hormones) that stimulate ovulation and spawning (Stacey and Goetz, 1982; Ruggeri and Thoroughgood, 1985; Sargent et al., 1999;

Bergé and Barnathan, 2005) and is important for the production of viable eggs (Sargent et al., 1999). DHA plays a major role in egg production because it is responsible for maintaining the structure and function of cellular membranes, specifically the ovarian membrane fluidity and stability, through a process called homeoviscous adaptation (Sargent et al., 1999; Tocher, 2003; Mayor et al., 2013).

1.5.3. Sex steroids

Linking gonad development with the levels of sex steroids in blood plasma is a valuable tool to comprehend the endocrine control of reproduction in some deep-sea teleosts, but this field is not fully explored (Pankhurst and Conroy, 1987; Lee and Yang, 2002; Sisneros et al., 2004; Sequeira et al., 2017).

In vertebrates, gonadotropins are the primary hormones to regulate gametogenesis, working through the gonadal biosynthesis of steroid hormones that mediate various stages of gametogenesis (Nagahama, 1994). In teleost females, the follicle stimulating hormone (FSH) stimulates granulosa cells to produce estradiol-17β (E_2), the key hormone that induces the liver to synthesize vitellogenin and egg shell proteins, which are incorporated into the oocyte during vitellogenesis (Lubzens et al., 2010). After the growth phase, a surge of luteinizing hormone (LH) stimulates the follicle to produce the maturation-inducing steroid – either 17,20β-dihydroxypregn-4-en-3-one (17,20β-P) or 17,20β,21-trihydroxypregn-4-en-3-one (17,20β,21-P), depending on the species – that promotes final oocyte maturation (resumption of meiosis) and ovulation (Nagahama and Yamashita, 2008; Lubzens et al., 2010).

In males, FSH regulates Sertoli cell activity to support germ cell development while LH acts on Leydig cells to promote steroidogenesis (Schulz etal., 2010; Chauvigne et al., 2014). The key androgen is 11-ketotestosterone (11-KT), which promotes germ cell proliferation and maturation, as well as the development of secondary sexual characters and the mediation of reproductive behaviours (Borg, 1994; Schulz et al., 2010). Androgen levels are low in spent and regressed fish, increase during gonadal recrudescence and peak prior to the end of spermatogenesis (Nagahama, 1994). 17,20β-P is responsible for endorsing the initiation of meiosis, for stimulating spermiation, and for enhancing sperm motility (by alteration of the pH and fluidity of the seminal fluid) and can act as a pheromone, e.g. in goldfish (Scott et al., 2010). Finally, both male and female gonads produce testosterone (T) which is a precursor of E_2 and 11-KT and feeds back on the pituitary gland to promote the synthesis of gonadotropins, among other functions (Nagahama, 1994; Lubzens et al., 2010; Schulz et al., 2010).

1.5.4. Otolith trace element composition

Otoliths are concretions located in the inner ear of fishes, formed from the crystallisation of calcium carbonate, in the form of aragonite, on an organic matrix composed largely of a keratin-like protein, the otolin (Degens et at., 1969; Watabe et at., 1982; Morales-Nin 1987; Wright et al., 2002). Mineral-rich and matrix-rich (and mineral-deficient) layers are alternatively and periodically deposited by addition of concentric layers (Watabe et al., 1982) with significant physiological regulation (Kalish, 1989).

The otolith formation is an acellular process dependent on the endolymph (the fluid that fills the entire inner ear system), blood plasma, the external medium and the otolith itself (de Pontual and Geffen, 2002; Wright et al., 2002). The endolymph modulates the various signals and regulates the formation of the otolith, the external medium provides the abiotic elements, and the blood plasma responds to the external medium under endogenous variations. The otolith integrates and records a response to all these signals (de Pontual and Geffen, 2002). The deposited material contains trace elements from the surrounding water, reflecting the physical and chemical environment where the fish lives (Fowler et al., 1995; Gallahar and Kingsford, 1996). Since newly deposited material is neither resorbed nor altered and otoliths potentially grow through the whole life of the fish, even when somatic growth has naturally ceased, otoliths are continuous recorders of the physical and chemical environment the fish experiences along its lifetime (Campana and Neilson, 1985; Campana et al. 1997; Wright et al., 2002). That premise is the basis for the application of numerous otolith features, such as increment deposition, morphometry, and chemical composition, for determining age and growth patterns, tracking migratory histories, classifying populations and habitat interactions, and tracing spawning

and nursery grounds (Campana, 1999; Begg et al., 2005; Campana, 2005; Chang and Geffen, 2013). The incorporation of elements from the environment into the otolith matrix is influenced by pH, salinity, temperature as well as concentrations gradients (Mugiya and Tanaka, 1995; Elsdon et al., 2008; Sturrock et al., 2012; Izzo et al., 2015), hence the incorporation of elements into the otolith might not always be proportional to their concentration in the environment (Sturrock et al., 2015).

Within otolith science, elemental composition has been as increasingly common tool for inferring fish stock dynamics, migration patterns, pollution exposure, connectivity between habitats, and natal source (Campana, 1999; Wells et al., 2003; Campana, 2005; Begg et al., 2005; Elsdon et al., 2008; Chang and Geffen, 2013; Longmore et al., 2014; Sturrock et al., 2015; Hüssy et al., 2020). Recent studies are giving more attention to the effect of endogenous and physiological processes in the incorporation of some elements, such as Li, Mg, Mn, Ca, Cu, Zn, Se, Sr, and Ba (Sturrock et al., 2012, 2014, 2015; Chang and Geffen, 2013; Grønkjær, 2016).

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Chapter 2: Distribution and hypothetical migratory cycle

Black scabbardfish, *Aphanopus carbo*, in the northeast Atlantic: distribution and hypothetical migratory cycle

Inês Farias, Beatriz Morales-Nin, Pascal Lorance and Ivone Figueiredo

Abstract
The biology, ecology, and dynamics of the deep-sea teleost black scabbardfish in the northeast Atlantic are reviewed. The black scabbardfish is a commercial bathypelagic species found in the NE Atlantic mostly from Iceland to the Canary Islands and Western Sahara, at depths from 800 to 1300 m. The spatial structure of its population is still uncertain, although the existence of one single stock that migrates around the NE Atlantic driven by feeding and reproduction is the most likely hypothesis consistent with available data. This review is based on data from commercial fisheries off the Faroe Islands, Hatton Bank, the west of the British Isles, and Portugal (mainland, Azores, and Madeira) and from Icelandic and Scottish scientific surveys collected between 1988 and 2012. Spawning of black scabbardfish occurs around Madeira and the Canary Archipelagos during the last quarter of the year. According to the migratory hypothesis, eggs, larvae, and possibly juveniles move north to areas from south of Icelandic and Faroe Islands to the west of the British Isles where they remain for some years to feed and grow. They then move south to the area off mainland Portugal, where they reach the size of first maturity but do not reproduce, and later move further south to the spawning grounds. Further studies are needed to understand which of the environmental conditions prevailing around Madeira and the Canaries, but not elsewhere, allow this species to mature and subsequently reproduce. This review suggests that a multidisciplinary approach is required to confirm the spatiotemporal migration and habitats used by black scabbardfish populations in the NE Atlantic at different life stages. Otolith contour shape and microchemistry, fatty acids, carbon and nitrogen stable isotopes, as well as steroid hormones are proposed as promising alternative tools for responding to this challenge.

Keywords: Deep-water longline fisheries; migration; life cycle; Trichiuridae; *Aphanopus carbo*; North Atlantic

2.1. Introduction

Aphanopus carbo Lowe, 1839 (Actinopterygii: Trichiuridae) occurs throughout the North Atlantic between 30º N and 70º N, from the strait of Denmark to western Sahara, being most abundant to the south of the Faroe Islands, in the Rockall Trough, to the west of mainland Portugal, and around Madeira and the Canary archipelagos, but also occurring in Iceland, the Mid-Atlantic Ridge and Corner Rise, and the Azores (Nakamura and, Parin 1993; Parin, 1995; Pajuelo et al., 2008; Machete et al., 2011). The species has also been reported in the western part of the Atlantic, in Greenland, Canada, and the USA (Templeman and Squires, 1963; Fitch and Gotshall, 1972; Parin and Becker, 1972; Peden, 1974; Clarke and Wagner, 1976; Gorbunova, 1977; Howe et al., 1980; Nakamura, 1984; Borets, 1986; Lauth, 1997). Reports of *A. carbo* occurrence in the eastern Pacific (Clarke and Wagner, 1976; Howe et al., 1980; Pequeño, 1989; McAllister, 1990) and the southern Indian oceans (Piotrovskiy, 1981) were deemed questionable by Nakamura and Parin (1993). The absence of confirmed records in those areas, despite the worldwide development of deep-sea fisheries and surveys since the early 1990s, suggests that *A. carbo* is restricted to the North Atlantic.

A. carbo is a bathypelagic species that has been found at depths from 200 m, in the northern section of the NE Atlantic (Nakamura and Parin, 1993; Kelly et al., 1998), to 2300 m around the Canary Islands (Pajuelo et al., 2008), but is more frequent between 800 and 1800 m in mainland Portugal (Martins et al., 1987), 800 and 1300 m in Madeira (Morales-Nin and Sena-Carvalho, 1996), and 400 and 1400 m off the west of the British Isles (Ehrich, 1983; Allain et al., 2003). Its distribution is mainly associated with steep slopes, underwater rises, and canyons to the west of Portugal and along the gentle sedimentary slope to the west of the British Isles (Martins et al., 1987; ICES, 2012). Information regarding the black scabbardfish available from fisheries and surveys in the NE Atlantic

is summarised in Table 2.1.

Table 2.1. Summary of fisheries and surveys from which data on black scabbardfish were available for this review; total length (TL, cm), total weight (g), maturity stage.

Area	Source	Depth (m)	Gear	Total length (cm) range & mode		Weight range (g)	Maturity stage	Reference
Iceland	Icelandic autumn survey	176-1307	Bottom trawl	26-123	90	572-1955	I-II	K. Jakobsdóttir (pers. comm.)
Faroe Islands	Fishery	*NA*	Traditional bottom trawl; doors did not touch the bottom	65-142	100	1000-4000	*NA*	ICES 2012
Hatton Bank	Fishery	1000-1400	Bottom trawl	71-120	87	*NA*	Immature	ICES 2012
W British Isles	Fishery; Scottish deep water survey	500-1800	Bottom trawl	62-130	90	232-2740	I-II	This study; Ribeiro Santos et al. 2013a
Mainland Portugal	Fishery	800-1450	Bottom longline	53-136	105	137-3650	I-III	This study
Azores	Experi--mental fishery	650-1900	Drifting bottom longline	60-156	120	*NA*	*NA*	Machete et al. 2011
Madeira	Fishery	~1000	Drifting horizontal longline	51-151	117	131-4020	All	This study; Bordalo-Machado et al. 2009

NA: not available.

Although no eggs or larvae of black scabbardfish have been found, juveniles are reported to be mesopelagic (Parin, 1986). Maul (1950) found two small specimens (10 and 15 cm total length) in the stomach of a longnose lancetfish (*Alepisaurus ferox* Lowe, 1833). More recently, a specimen of approximately 10 cm, identified by DNA barcoding, was caught at the Senghor Seamount, off the northeast of Cape Verde (Hanel et al., 2010). There are also records of fish as small as 26 cm from Icelandic surveys (Table 2.1).

In terms of morphology, this species has a narrow elongated body with a pointed head and long dorsal fin, adapted for fast swimming; a large terminal mouth with large fang-like teeth, modified for efficient predation; large eyes (diameter is about a fifth to a sixth of head length) that facilitate sight in low light; and a coppery-black coloration with an iridescent tint to facilitate camouflage (Parin, 1986; Nakamura and Parin, 1993; Merrett and Haedrich, 1997). Life history traits such as diet, growth, reproduction, and energy consumption are directly dependent on physical conditions of the deep sea such as high pressure and low temperature, which hinder the metabolic rate processes of fishes, as well as on predation stress and low food availability (Graham et al., 1985; Merrett and Haedrich, 1997).

Recently it was found that *A. carbo* coexists spatially with *Aphanopus intermedius* Parin, 1983 in the Azores, Madeira, the Canaries, and off the coasts of Morocco and the western Sahara (Nakamura and Parin, 1993; Stefanni and Knutsen, 2007; Stefanni et al., 2009; Biscoito et al., 2011). However, it has

been genetically confirmed that *A. carbo* is the only species that reaches the northernmost latitudes of the NE Atlantic (Stefanni and Knutsen, 2007; Biscoito et al., 2011; Ribeiro Santos et al., 2013a). The two species are morphologically very similar, yet both genetics (Stefanni and Knutsen, 2007; Knutsen et al., 2009; Stefanni et al., 2009) and meristic characteristics (Tuset et al., 2010; Biscoito et al., 2011) have proven suitable for reliable identification. Throughout this manuscript the common names accepted by FAO are used: black scabbardfish for *A. carbo* and intermediate scabbardfish for *A. intermedius*.

How the black scabbardfish completes its life cycle is still in question. The most widespread hypothesis is that one single stock undertakes a large-scale clockwise migration around the NE Atlantic. Under this hypothesis, spawning is restricted to areas off Madeira, the Canary Islands, and possibly further south. Thereafter, juveniles recruit to the fisheries south of Iceland, around the Faroe Islands, and to the west of the British Isles, where they stay to feed and grow for a few years. From there, they first move south towards mainland Portugal and then further south to the spawning areas (Figure 2.1).

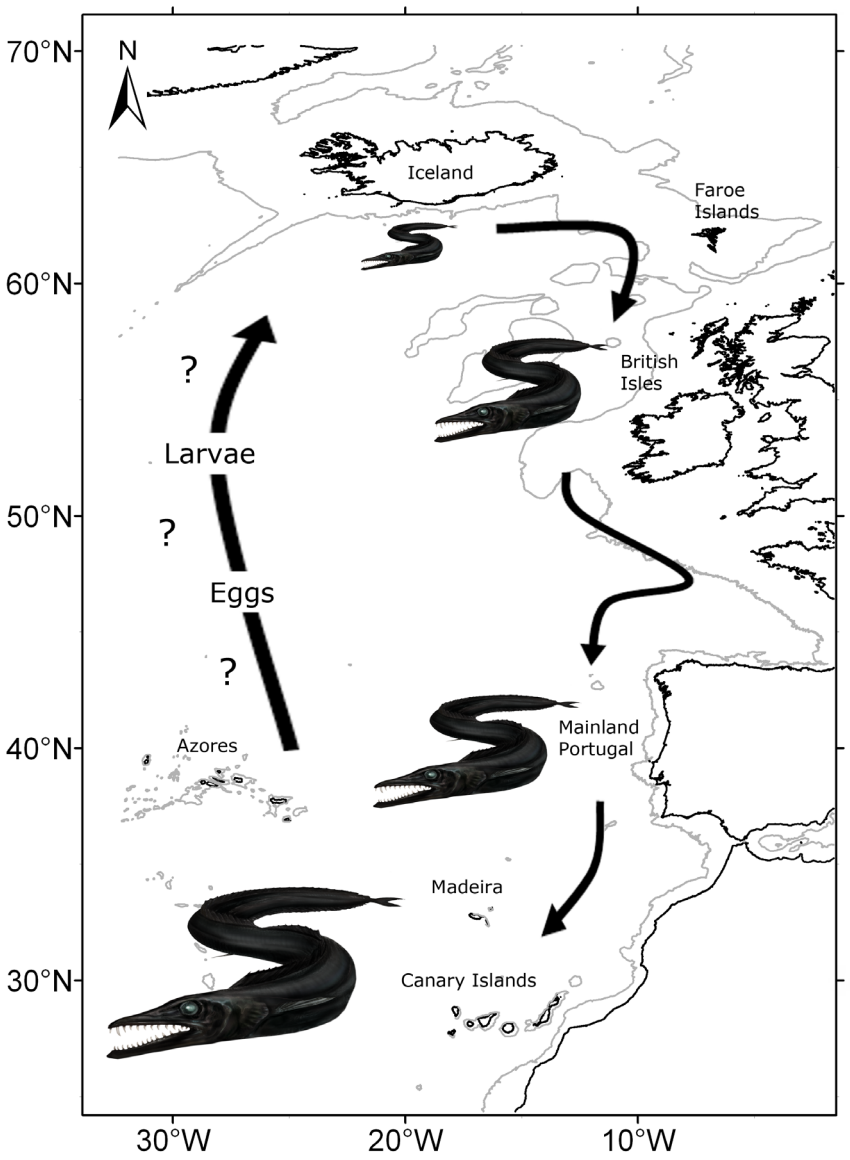

Figure 2.1. Map of the northeast Atlantic Ocean representing the hypothetical migratory cycle of the black scabbardfish. The 1000 m depth contour is shown (drawing of black scabbardfish adapted from MARPROF, www.marprof.org).

Some specific habitat properties that allow the metabolic processes inherent to reproduction, survival of eggs and larvae or both might exist exclusively around Madeira and the Canaries and hence merit further investigation.

2.2. State of the art

Fisheries

There are three main deep-sea fisheries targeting the black scabbardfish in the NE Atlantic: (i) to the west of the British Isles, fish are mainly exploited by the French deep-sea trawl fishery (Nakamura and Parin, 1993; ICES, 2012); (ii) an artisanal fleet operates with bottom longlines in ICES (International Council for the Exploration of the Seas) Subarea IXa, off mainland Portugal (Bordalo-Machado et al., 2009; ICES, 2012); and (iii) a third important commercial fishery is operated by artisanal horizontal drifting longliners off the Madeira Archipelago, within the CECAF (Fishery Committee for the Eastern Central Atlantic) area (Bordalo-Machado et al. 2009). The fishery in Madeira dates back to the seventeenth century (Merrett and Haedrich, 1997), whereas it started in mainland Portugal in the early 1980s (Martins et al., 1987) and in northern Europe in the early 1990s (Merrett and Haedrich, 1997). In ICES Subarea X, another directed fishery has recently started in Azorean waters (Portugal) (Machete et al., 2011). In Madeira and the Azores, *A. intermedius* might be landed mixed with *A. carbo* (Stefanni and Knutsen, 2007). Additionally, a few smaller, discontinuous fisheries occur in Faroese and Icelandic waters where the species is exploited by both longliners and trawlers (ICES, 2012) and in the Canary Islands, where it is exploited by drifting mid-water longliners (Pajuelo et al., 2008).

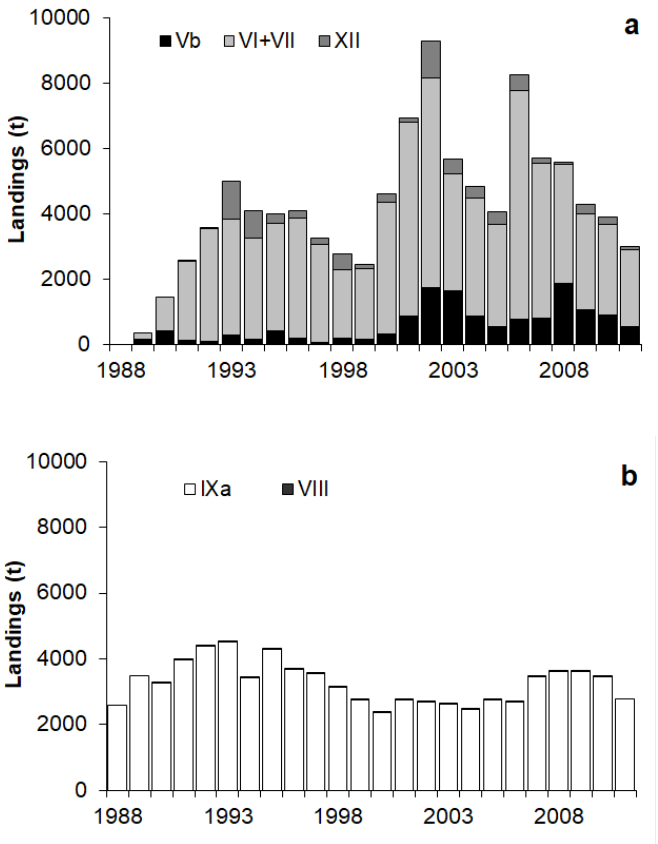

Figure 2.2. Annual black scabbardfish landings from 1988 to 2011 for ICES, (a) northern component: ICES Division Vb (around Faroe Islands), VI+VII (west of the British Isles), and XII (north of the Azores); and (b) southern component: ICES Subarea VIII (Bay of Biscay), and Division IXa (west of mainland Portugal).

Landings data are from ICES (2012). 2011 data are provisional.

The trend in black scabbardfish landings in ICES northern (Subareas Vb, VI, VII, and XII) and southern (Subareas IXa and VIII) components in the last two decades is shown in Figure 2.2. Estimated landings in 2011 were 2357 t for ICES Subareas VI and VII (W British Isles), 2781 t for mainland Portugal (ICES, 2012) and 1941 t for Madeira (Anon., 2012). Regarding the smaller fisheries, landings increased from 139 t in 2011 to 458 t in 2012 in the Azores (M. Ruivo, pers. comm.); reached 109 t in Iceland in 2010 (no data was provided for 2011); and were 111 t caught by Spanish vessels in ICES Division XIV in 2010 (ICES, 2012).

Age, growth, and length structure

Over time, studies on age and growth of the black scabbardfish have led to different conclusions (Table 2.2): initially, the maximum age was estimated to be 8 years for fish from Madeira (Morales-Nin and Sena-Carvalho, 1996) and 32 years for the Rockall Trough (Kelly et al., 1998). Later it was estimated to be 12 years for Madeira and the Canaries (Morales-Nin et al., 2002; Pajuelo et al., 2008). More recently, the maximum age was estimated to be 12 years in mainland Portugal (Vieira et al., 2009) and 14 years in Madeira (Vieira et al., 2009; Delgado et al., 2013). Since the length range used in all studies was similar, the differences might be associated with the age assignment criteria, otolith preparation techniques, quality of the equipment used or experience of the readers.

Table 2.2. Von Bertalanffy growth parameter estimates from different studies carried out in the NE Atlantic, including otolith age reading method and clearing solution. F: females, M: males, SD: standard deviation.

Area	Method	Clearing	Sex	N	Total length range (cm)	Age range (year)	L_{inf} ± SD (cm)	k ($years^{-1}$)	t_0 (year)	Source
W. British Isles	Thin sections in epoxy resin	Alcohol	both	230	75-120	4-32	*NA*	0.1	*NA*	Kelly et al., 1998
Mainland Portugal	Thin sections in epoxy resin	1:1 glycerin-alcohol	F	248	64-131	5-13	135 ± 4	0.2	-2.0	Vieira et al., 2009
			M	206		4-10	124 ± 3	0.2	-1.7	
Madeira	Surface	Glycerol	F	334	58-151	0-8	142	0.3	-2.1	Morales-Nin and Sena-Carvalho, 1996
			M	357	58-132		155	0.2	-3.3	
			both	649	58-151		139	0.3	-2.3	
	Thin sections in epoxy resin	1:1 glycerin-alcohol	F	200	125-148	8-15	159 ± 4	0.1	-2.3	Vieira et al., 2009
			M	163		8-14	146 ± 1	0.1	-1.4	
	Surface	1:1 glycerin-alcohol	F	554	100-140	6-14	136 ± 5	0.2	-4.2	Delgado et al., 2013
			M				132 ± 5	0.2	-3.1	
Canary Islands	Surface, burned	50% glycerol	F	196	100-148	2-12	149 ± 2	0.2	-4.7	Pajuelo et al., 2008
			M	102	104-134	2-8	141 ± 4	0.3	-3.5	
			both	298	100-148	2-12	148 ± 2	0.2	-4.6	

NA: not available.

The maximum age estimated by Morales-Nin and Sena-Carvalho (1996) corresponded to a male of 130 cm and a female of 150 cm total length. These ages were probably underestimated because, when using whole otoliths in larger specimens from this species, the growth increments closer to the border are very difficult to identify (Vieira et al., 2009). On the contrary, the maximum age assigned by Kelly et al. (1998) using thin otolith sections was most likely overestimated since, with this preparation technique, the number of visible rings is very high and the authors reported problems in their interpretation. Regarding age estimations in Madeira and the Canary Islands in studies prior to 2008, when caught specimens started being routinely separated by species, the possible mixing of black and intermediate scabbardfish specimens could also explain the differences found between regions and should be taken into consideration. The maximum ages assigned by Delgado et al. (2013) using whole otoliths were 14 years for black scabbardfish and 15 years for intermediate scabbardfish. Overall, the age estimation of the black scabbardfish is difficult and has not yet been validated. Both the seasonality of the deposition of material at the otolith margin and of daily growth increments would deserve additional studies with standardised methods using material from all areas and seasons.

The growth parameters estimated based on the von Bertalanffy growth equation showed a relatively rapid growth rate for the black scabbardfish (Table 2.2). Figure 2.3 represents the growth curves according to sex for all available studies, restricted to the length range of each fish sample. Growth estimates from Vieira et al. (2009) and Delgado et al. (2013) seem to be in agreement, without any meaningful area effect. Age-at-length from Kelly et al. (1998) was consistently higher than in all the other studies. This implies a low k for fish from the west of the British Isles which is not in agreement with the predominance of young immature specimens in this area.

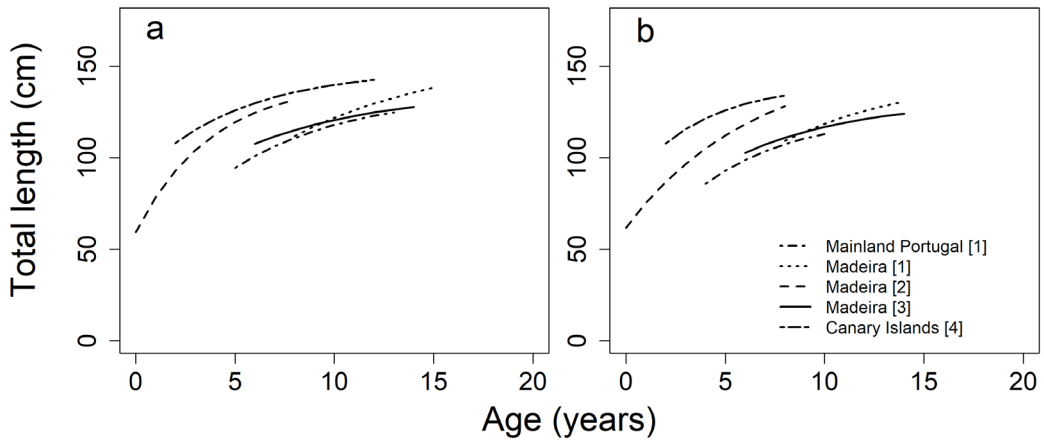

Figure 2.3. Growth curves for black scabbardfish from different studies, areas and methods. (a) females; (b) males. Growth parameters are from [1] Vieira et al. (2009); [2] Morales-Nin and Sena-Carvalho (1996); [3] Delgado et al. (2013); [4] Pajuelo et al. (2013).

Rapid growth of deep-sea juvenile fishes has been shown to be an advantageous strategy in feeding success and predation avoidance (Crabtree and Sulak, 1986). Nonetheless, in the results of Vieira et al. (2009) the absence of small individuals caught off Madeira may have interfered with the accuracy of the growth parameter estimates. In contrast, slow growth rate is observed for adults, as a result of a transfer of energy investment from growth to reproduction (Lika and Nisbet, 2000). This strategy contrasts with that of species whose growth continues after maturation, such as most shelf demersal and pelagic commercial fish.

Size ranges reported for different areas in the NE Atlantic are presented in Table 2.1. The smallest specimens were reported in Iceland, where this species can be caught in relatively shallow waters, whereas the largest fish were caught off the Azores, where this species reaches deeper waters.

Length frequency distributions for different ICES and CECAF management units in 2011 are presented in Figure 2.4. The French fleet operates mainly in ICES Division VIa but also in Vb and VII; the Spanish fleet in Divisions VIb and XIIb; the Portuguese longline fishery in Division IXa; the

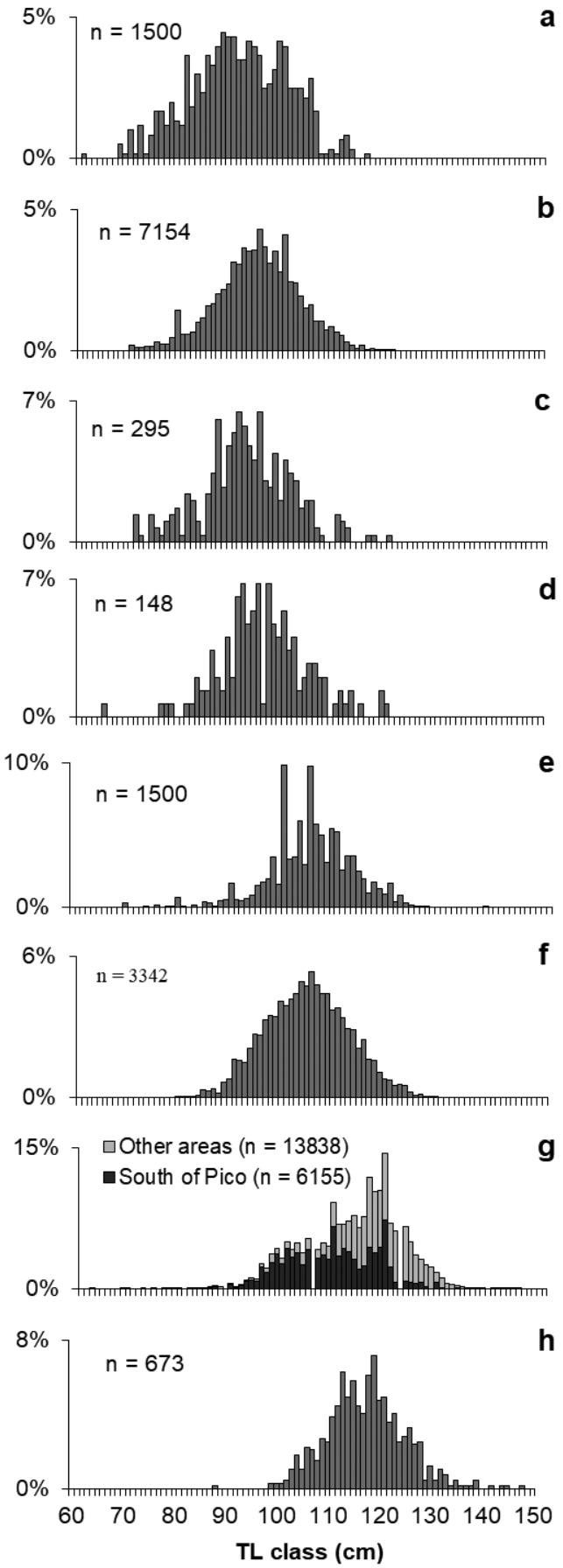

Figure 2.4. Length frequency distribution of black scabbardfish in 2011 from north to south: (a) Icelandic surveys (ICES Division Va); (b) on-board observations of French trawlers (mostly in ICES Division VIa); (c) on-board observations of Spanish trawlers off the west of the British Isles (Division VIb); (d) on-board observations of Spanish trawlers (Subarea XII); (e) self-sampling Faroese exploratory surveys (Subarea X); (f) Portuguese longline fishery off mainland Portugal (Division IXa); (g) experimental fishery in the Azores (data are from 2005); (h) sampling of commercial landings in Madeira. Length frequency data are from [a-f] ICES (2012); [g] Machete et al. (2011); [h] Delgado et al. (2013).

Azores fleet in Subarea X (ICES, 2012); and the Madeira fleet in CECAF division 34.1.2 (Delgado et al., 2013). Moreover, data from Subarea X were collected during Faroese surveys (ICES, 2012). In general, the size distributions move towards higher values from north to south of the NE Atlantic. In the Azores, the bimodal length distribution found to the south of Pico (Figure 4-g) is probably a consequence of mixing between *A. carbo* and *A. intermedius*, since the latter species has been described in this area (Stefanni and Knutsen, 2007).

Diet

The black scabbardfish is a top predator, which feeds on a large food spectrum (Zilanov and Shepel, 1975; Nakamura and Parin, 1993; Ribeiro Santos et al., 2013b). Diet studies based on gut content analyses are difficult in this species because, most of the time, these are either already fully digested or have been regurgitated as a result of hydrostatic decompression, as happens in deep-sea fishes that have a swim bladder (Stowasser et al., 2009). This difficulty is worsened when sampling fish caught by the commercial longlines off Madeira and mainland Portugal because the soaking time can be up to 40 h (Bordalo-Machado et al., 2009), enabling the full digestion of the stomach contents. As a consequence, the vacuity index calculated for fish from Madeira was 93.3 to 98.3% (Freitas, 1998; Ribeiro Santos et al., 2013b), whereas it varied from 66.2 to 94.1% for specimens caught by trawls to the west of the British Isles (Mauchline and Gordon, 1984; Ribeiro Santos et al., 2013b) (Table 2.3).

Table 2.3 summarizes the published information on the diet of the black scabbardfish in the NE Atlantic. To the west of the British Isles, several studies have focused on the diet of this species. Du Buit (1978) identified two fish taxa, Argentina sp. and Gadidae, in eight non-empty stomachs. Mauchline and Gordon (1984) and Ribeiro Santos et al. (2013b) found mostly fish, namely blue whiting (*Micromesistius poutassou*), and a small amount of cephalopods. The differences between these studies may be related to the seasonal changes observed by Ribeiro Santos et al. (2013b) who associated the decrease in blue whiting and consequent increase in cephalopods and crustaceans in the diet of the black scabbardfish with the migration of blue whiting to the Norwegian Sea. In fact, when Howell et al. (2009) combined the diet composition of fish caught by scientific surveys off the Rockall Trough with earlier data from Mauchline and Gordon (1984), the resulting diet was approximately 50% cephalopods and 44% blue whiting.

In fish caught off Madeira, the main prey were the cephalopods *Chiroteuthis* spp., *Mastigoteuthis* sp., *Histioteuthis* sp., and *Taonius* sp., and the fish *Chauliodus* sp., as well as several *Myctophidae* (Freitas, 1998). The differences between the diets of black scabbardfish caught off the British Isles and off the Madeira archipelago may be related to food availability given that, in the latter area, no large stocks of small pelagic and mesopelagic fish, such as blue whiting, can be found.

Reproduction

The black scabbardfish is an iteroparous species, since it can spawn multiple times throughout its life, and is also a total spawner, as it spawns in one single event (Pajuelo et al., 2008; Ribeiro Santos et al., 2013a). Moreover, it has determinate fecundity, which means that the potential annual fecundity corresponds to the number of vitellogenic oocytes minus the number of oocytes reabsorbed on account of atresia (Neves et al., 2009; Ribeiro Santos et al., 2013a). Mature and spawning adults have only been observed in the last quarter of the year in Madeira (Figueiredo et al., 2003; Neves et al., 2009; Ribeiro Santos et al., 2013a), the Canaries (Pajuelo et al., 2008), and the northwest coast of Africa (Perera, 2008). Estimated female length at first maturity (L_{50}) was 103 cm around Madeira (Figueiredo et al., 2003) and 114 cm around the Canary Islands (Pajuelo et al., 2008). Once again, the possible mixture of black and intermediate scabbardfish specimens in the samples may have biased these results. In a more recent work, female L_{50} was estimated to be 111 cm for Madeira and 116 cm when also including specimens from the west of the British Isles (Ribeiro Santos et al., 2013a). These values are probably overestimated because the estimation did not include specimens from Madeira smaller than 92 cm in total length. Despite the available information questions remain on the reproductive dynamics of this species.

Table 2.3. Summary of the diet composition of black scabbardfish caught in the NE Atlantic.

Area	Depth (m)	Total length range (cm)	No. stomachs sampled	No. stomachs with food	Vacuity index (%)	Main prey	Source
W. British Isles	750-1100	80 (a)	NA	8	NA	Fish (*Argentina* sp. and Gadidae)	Du Buit, 1978
	500-1250	77-108	142	52	66.2	Fish (*Micromesistius poutassou* and Scombridae) and cephalopods	Mauchline and Gordon, 1984
	NA	*NA*	*NA*	*NA*	*NA*	Cephalopods, fish (*Micromesistius poutassou*), prawns and shrimp	Howell et al., 2009 (including data from Mauchline and Gordon, 1984)
	500-1800	97 ± 7 (b)	1581	133	91.6	Fish (*Micromesistius poutassou* and mesopelagic species) and cephalopods	Ribeiro Santos et al., 2013b
Madeira	600-1300	~110-150	3688	378	93.3	Cephalopods (*Chiroteuthis* spp., *Mastigoteuthis* sp., *Histioteuthis* sp., and *Taonius* sp.) and fish (*Chauliodus* sp. and *Myctophidae*)	Morales-Nin and Sena-Carvalho, 1996; Freitas, 1998

(a) Mean. (b) Mean ± standard deviation.

First, why does the black scabbardfish not mature and spawn elsewhere than Madeira and the Canaries? In mainland Portugal, vitellogenesis begins at the same time as in those areas and a high proportion of caught individuals (ca. 25 %) is larger than L_{50} (Figueiredo et al., 2003; Neves et al., 2009). Additionally, although reported in the past at Porcupine Bank, to the west of the British Isles (Ehrich, 1983) and in Icelandic waters (Magnússon and Magnússon, 1995), reproduction has not been observed in these locations since. In fact, even though fish attain relatively high total length, only maturity stages I and II have been observed in either of these areas (Table 1). Because gonad macroscopic features are difficult to interpret, which could have led to incorrect assignments of maturity stage in the past, a standardized maturity scale was proposed for the black scabbardfish (Gordo et al., 2000).

In terms of physiological condition indicators, the gonadosomatic index (GSI) is higher around Madeira than off mainland Portugal (Neves et al., 2009) and to the west of the British Isles (Ribeiro Santos et al., 2013a) for the same body length. Furthermore, atresia occurs in stage II ovaries from fish caught off the previous areas before and during the spawning period (Neves et al., 2009; Ribeiro Santos et al., 2013a). The most likely hypothesis is that intrinsic (e.g., energy budget, chemical predisposition) and extrinsic (e.g., water temperature) factors that condition the maturity process are lacking in the previous areas mentioned above. Fish in a poor nutritional state would cease the maturation process and remain in the same location, whereas fish in a better state would migrate to areas that provide the environmental conditions to optimize spawning and the survival of eggs and larvae (Neves et al.,

2009; Ribeiro Santos et al., 2013a). The differences found in GSI are in accordance with the proposed hypothesis for population dynamics, even though no conclusions regarding migratory movements can be drawn from it.

Steroid hormones are responsible for triggering reproductive processes such as gametogenesis, so their levels are expected to change during the reproductive cycle and to be different between fish in distinct maturity stages (Modesto and Canário, 2003). In an on-going study, variations in the level of steroid hormones in the plasma of black scabbardfish caught off Madeira and mainland Portugal are being analysed (Farias et al., unpublished). Further work is needed to assess whether the study of steroid hormones in black scabbardfish in different areas and seasons would help us to understand the relationship between the migratory and reproductive cycles.

A second question is whether females are able to reproduce in consecutive years. Females spawn all oocytes contained in their ovaries in a single event at each reproductive cycle. Therefore, all females larger than the length at first maturity are expected to mature and spawn simultaneously. Assuming that the age at maturity is approximately 6.5 years (after Figueiredo et al. (2003) and Vieira et al. (2009)) and that the oldest specimens found were 14 years old (Vieira et al., 2009; Delgado et al., 2013), females are expected to be able to spawn for a period of 8 years. However, the presence of non-reproductive adults mixed with spawning adults in Madeira during the spawning period suggests that skipped spawning may occur in this species (Neves et al., 2009; Ribeiro Santos et al., 2013a). Skip spawning is an efficient strategy for saving energy that can then be allocated to growth and large scale migration (Ribeiro Santos et al., 2013a) as well as to subsequent reproduction.

Migration

Techniques to study the migration of coastal species, namely mark-recapture, tagging, telemetry, hydroacoustics and diet composition, are difficult to apply to deep-sea species owing to sampling constraints that result from depth and pressure. An innovative combination of tools that may provide complementary information for clarifying the migration pattern, stock structure and spatiotemporal dynamics of this species in the NE Atlantic could include otolith microstructure, otolith shape, fatty acids, and stable isotopes. Multidisciplinary studies carried out so far to uncover these aspects are quickly reviewed below.

In a previous study, black scabbardfish from the three Portuguese directed fisheries (mainland, Madeira, and the Azores) was characterized in terms of reproductive strategy (Neves et al., 2009), growth (Vieira et al., 2009), otolith shape (Farias et al., 2009), parasites (Santos et al., 2009), and contamination (Costa et al., 2009), with the goals of identifying its stock structure and assessing its biochemical composition. The differences found between areas support the migratory hypothesis but do not provide proof because they are mainly dependent on the ontogenetic structure of each sampling location, besides reflecting an unbalanced sampling scheme, given that the sample size collected off the Azores was much smaller than for the other areas.

Otolith contour shape analysis was used to discriminate specimens from mainland Portugal, the Azores, and Madeira (Farias et al., 2009). Differences were found in otolith contour shape between geographical regions, which is in agreement with the hypothesis of fish remaining a few years at each location. In another study, otolith shape supported the existence of a single population of black scabbardfish off Madeira and the Canaries, but the number of specimens was too small to be conclusive (Tuset et al., 2013). This result is very interesting in terms of the migration hypothesis and its relation with the reproductive cycle because it is evidence that fish from Madeira and the Canaries constitute the same population.

Fatty acids

In fishes, fatty acids (FA) can be used as indicative biomarkers for different trophic levels (Kirsch et al., 2000). Preliminary results of analyses performed on black scabbardfish muscle indicate that immature specimens caught off Iceland and the west of the British Isles accumulate mainly oleic acid, which is a precursor of all omega-3 and omega-6 polyunsaturated fatty acids (PUFA) (Dalsgaard

et al., 2003). Additionally, in fishes caught off Madeira, which were mostly mature, and mainland Portugal, which are mostly in the developing and pre-spawning stages, a prevalence of PUFA, namely arachidonic acid and docosapentaenoic acid, has been observed (Farias et al., unpublished). PUFA are precursors of prostaglandins, which have an important role in reproduction, stimulating ovulation and spawning and eliciting female sexual behaviour (Stacey and Goetz, 1982), and their prevalence in specimens from Madeira and mainland Portugal might be related to the predominance of pre-spawning and spawning maturity stages.

Similar results were obtained by Nogueira et al. (2013), who analysed FA in the muscle and liver of black scabbardfish caught off Madeira. The main differences between these two studies are in two monounsaturated fatty acids (MUFA), which were not detected in muscle samples in Nogueira et al. (2013), but were found in muscle samples from all the geographical areas analysed in the aforementioned study (Farias et al., unpublished). These divergences might result from differences in apparatus sensitivity, as well as from the deterioration of the tissues, since the samples analysed by Nogueira et al. (2013) were left in ice for 1–2 days between capture and laboratory analyses.

Stable isotopes

Ribeiro Santos et al. (2013b) analysed the diet of black scabbardfish caught off Madeira and to the west of the British Isles in terms of $\delta^{15}N$ and $\delta^{13}C$ and concluded that this species forms a link between the pelagic and the benthopelagic food webs, reflecting preferred feeding on pelagic fish and cephalopods. Seasonal changes in $\delta^{15}N$ were observed in fish caught off the west of the British Isles, supporting a shift to prey of a lower trophic level during the season when blue whiting migrates northward. The observed depletion in $\delta^{13}C$ in November in fish from Madeira could result from adults moving to shallower waters closer to the shore to spawn, as reported by local fishermen, which would imply a change between very different food sources (DeNiro and Epstein, 1978). $\delta^{13}C$ of black scabbardfish caught in the Bay of Biscay (5 individuals, Chouvelon et al., 2012) was intermediate between values from the west of the British Isles and Madeira from Ribeiro Santos et al. (2013b), whereas $\delta^{15}N$ was lower.

Preliminary results obtained for samples caught off Madeira and the west of the British Isles, as well as mainland Portugal and Iceland, are intermediate between those of previous work (Farias et al., unpublished). Mean $\delta^{15}N$ is lower than the values presented by Ribeiro Santos et al. (2013b) but higher than the estimates obtained for the Bay of Biscay. The mean $\delta^{13}C$ for Iceland and the west of the British Isles is higher than the estimate of Ribeiro Santos et al. (2013b) for the latter area, whereas the values from fish caught in the two southernmost areas are higher than the value for Madeira. The differences between studies suggest annual or seasonal variations associated with the sampling periods, which need to be further analysed. It is worth noting that the similar $\delta^{13}C$ observed in fish caught off Iceland and the west of the British Isles is supported by the geographical proximity between these sampling sites, whereas different mean $\delta^{15}N$ reflects dissimilarities in the diet of black scabbardfish between these regions (Farias et al., unpublished).

2.3. Discussion

The assessment of exploited populations and the subsequent proposal of management measures are focused on stocks. There are several definitions for stock but the most commonly agreed upon is that it is a group of fishes with similar life history characteristics large enough to be self-reproducing (Hilborn and Walters, 1992). Since the stock structure is still unknown for most exploited species, stock units are adopted for management purposes. These units are reasonably heterogeneous for a number of biological, spatial, and temporal dimensions (Hilborn and Walters, 1992). In the case of the black scabbardfish, ICES considers three management units (ICES 2012): “Northern” (Divisions Vb and XIIb and Subareas VI and VII); “Southern” (Subareas VIII and IX); and “Other areas” (Divisions IIIa and Va Subareas I, II, IV, X, and XIV).
Quinta et al. (2004) found genetic evidence of the black scabbardfish population being genetically structured into two groups: those from the eastern Atlantic (mainland Portugal and the Hatton Bank)

and those from around the Madeira archipelago. However, it is now clear that these results were most likely confounded by the occurrence of *A. intermedius* around Madeira (Stefanni and Knutsen, 2007; Knutsen et al., 2009; Stefanni et al., 2009).
The current understanding of the population dynamics of black scabbardfish in the NE Atlantic implies that spawning occurs around Madeira, the Canary Islands, and possibly a few other southern areas, like the NW coast of Africa (Figueiredo et al., 2003; Pajuelo et al., 2008; Perera, 2008; Neves et al., 2009). Juveniles occur mainly in the northernmost areas, namely Iceland, the Faroe Islands, and the west of the British Isles, where small fish of 2–3 years old are caught by fisheries and surveys. The northward migration from the spawning areas to the latter areas might also involve larvae and juveniles up to a length of 60 cm or more. After having grown in northern areas for a few years, these fish move south towards mainland Portugal, where they remain a few more years before migrating further south to the spawning areas. This migratory behaviour is expected to be driven by feeding and reproduction (Zilanov and Shepel, 1975; Anon., 2000; Figueiredo et al., 2003).
The depth and route of these migratory movements, as well as the contribution of active swimming vs. passive drifting, are unknown. It may be that the species migration makes use of poorly known oceanic features, allowing small juvenile black scabbardfish to reach northern areas. Furthermore, the geographical limitation of the known spawning areas for a species that is widespread in the NE Atlantic suggests the occurrence of particular hydrological or trophic features in those areas. The proposed innovative approach can thus yield new knowledge on the biological as well as physicochemical features of the NE Atlantic deep-sea ecosystem.
In conclusion, several methods have already been used to clarify the migration of this species. So far, otolith shape (Farias et al., 2009), oocyte maturity (Ribeiro Santos et al., 2013a), and microchemical analysis of the larval portion of otoliths (Longmore, 2011) support the migratory hypothesis, showing evidence of a single black scabbardfish stock in the NE Atlantic.
Notwithstanding, other techniques have a good potential to provide complementary information. For example, elemental composition of the otolith core could be used to assess whether black scabbardfish from different areas were all born in similar hydrological conditions, i.e., in the same location. Moreover, the combination of fatty acids and stable isotopes provides important information for understanding the structure of this species in the NE Atlantic by elucidating the connection of trophic and reproductive processes with prevailing environmental features in the different areas where the species spends parts of its life cycle.

Acknowledgements

The authors would like to thank Elena Guijarro Garcia (IEO, Spain), Klara Jakobsdóttir (MRI, Iceland), Lise Helen Ofstad (Havstovan, Faroe Islands), Miguel Machete (DOP, Azores), and Sara Reis (DSIP, Madeira) for providing data, and Teresa Moura (IPMA) for helping with the map. This work was supported by the DCF - European Commission Fisheries Data Collection Framework, the EU project *Management and monitoring of deep-sea fisheries and stocks* (DEEPFISHMAN, grant No 227390), the Portuguese *project Certificação MSC em Portugal: obtenção de conhecimento e estudos de viabilidade para candidaturas de pescarias costeiras e artesanais* (CERTIFICA, PROMAR, 2011-2015), and a doctoral fellowship granted to I. Farias by Fundação para a Ciência e Tecnologia (SFRH/BD/64434/2009). They also thank two anonymous referees for their comments and suggestions.

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Chapter 3: Biological processes associated with the species migratory dynamics and population structure in the NE Atlantic

3.1. Reproductive and feeding spatial dynamics of the black scabbardfish, *Aphanopus carbo* Lowe, 1839, in NE Atlantic inferred from fatty acid and stable isotope analyses

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Abstract

The black scabbardfish (*Aphanopus carbo*) is a benthopelagic species widely distributed across the NE Atlantic, where it is admitted to perform a clockwise migration throughout its life cycle stimulated by feeding and reproduction. To overcome the limitations of direct observation of this species, fatty acids profile (FA) and $\delta^{15}N$ and $\delta^{13}C$ stable isotopes (SI) were analysed in the muscle tissue of the black scabbardfish and related with diet and maturity. Specimens were collected in four geographic areas in the NE Atlantic: Iceland, the west of the British Isles, mainland Portugal, and Madeira. For all areas, the FA profile was related with the different phases of the reproductive cycle and with diet, whereas the SI were related with diet, environmental characteristics, such as latitude and depth, and particulate organic matter (POM). Stomach content of black scabbardfish caught off mainland Portugal was analysed and the most frequent prey item identified was the lophogastrid crustacean *Gnathophausia zoea*, followed by the cephalopod *Mastigotheutis* spp. and the teleost *Rouleina maderensis*. For specimens from Iceland and the west of the British Isles, monounsaturated fatty acids (MUFA) were the most important FA, followed by polyunsaturated (PUFA) and saturated FA (SFA), whereas for specimens from mainland Portugal and from Madeira the sequences were PUFA > MUFA > SFA and PUFA > SFA > MUFA, respectively. Immature specimens from the first three areas were found to be accumulating oleic acid which is an intermediate product of the metabolic pathway that transforms SFA to MUFA and these into PUFA. Specimens caught off Madeira were mature and showed a significant prevalence of ARA and DHA which are PUFA with an important role in reproduction. $\delta^{15}N$ was significantly higher in the muscle of black scabbardfish from Madeira, whereas $\delta^{13}C$ was significantly lower in specimens from Iceland. The low isotopic ratios as well as the prevalence of certain fatty acid trophic markers (FATM) connected specimens from Iceland with small prey. Results indicated that the spatial differences in physiological aspects of this species are related with diet and prey availability in Madeira, mainland Portugal, and the west of the British Isles, as well as variations in the baseline values of the primary production that are related with latitude and depth, mainly in Iceland. The allometric effect of each area's size ranges over $\delta^{15}N$ supports the existence of ontogenic differences in the black scabbardfish's diet. This diet is typical of mobile benthopelagic predators that are opportunistic feeders.

Keywords: *Aphanopus carbo*, diet, fatty acids, NE Atlantic, reproductive cycle, stable isotopes.

3.1.1. Introduction

The black scabbardfish (*Aphanopus carbo* Lowe, 1839) is a benthopelagic species widely distributed across the Northeast Atlantic, from Iceland southward to the Canary Islands, including the Mid-Atlantic Ridge and Corner Rise and the Azores (Nakamura and Parin, 1993; Parin, 1995; Pajuelo et al., 2008; Machete et al., 2011). The species is reported to live at depths from 200 m, at the west of the British Isles and around Iceland (Nakamura and Parin, 1993; Kelly et al., 1998), to 2300 m around the Canary Islands (Pajuelo et al., 2008). At its southernmost limit of distribution, namely Azores, Madeira, and the Canary Islands, *A. carbo* coexists with *Aphanopus intermedius* Parin, 1983 (Nakamura and Parin, 1993; Stefanni and Knutsen, 2007; Tuset et al., 2010; Biscoito et al., 2011).

Although there are some unresolved questions regarding the spatial structure of the black scabbardfish population, the existence of one single stock that moves clockwise along the NE Atlantic driven by feeding and reproduction is widely accepted (Zilanov and Shepel, 1975; Figueiredo et al., 2003; Farias et al., 2013). Spawning occurs around Madeira and the Canary Islands (Figueiredo et al., 2003; Pajuelo et al., 2008; Neves et al., 2009; Ribeiro Santos et al., 2013a), where total length ranges from 88 to 151

cm (Farias et al., 2013) and from 99 to 147 cm (Pajuelo et al., 2008), respectively, but has also been reported around the Azores (Vinnichenko, 2002) and the northwest coast of Africa (Perera, 2008). Estimated female length at first maturity (L50) is 103 cm around Madeira (Figueiredo et al., 2003). More recently, L50 was estimated to be 114 cm around the Canary Islands (Pajuelo et al., 2008), 111 cm for females from Madeira and 116 cm for females from Madeira and the west of the British Isles together (Ribeiro Santos et al. 2013a). However, these values are prone to be overestimated because specimens smaller than 99 cm in total length from the Canaries and 92 cm from Madeira were not included in the analyses (Farias et al., 2013). Eggs and larvae have not been reported so far. Some of the juveniles probably move northward to south of Iceland and around the Faroe Islands and west of the British Isles where they remain 4-6 years to feed and grow (ca. 60-120 cm in total length) (Farias et al., 2013). Then they move southward to the area west of mainland Portugal, where specimens are caught with 79 to 136 cm in total length (4-14 years old) (Vieira et al., 2009; Farias et al., 2013), reaching the size of first maturity but not reproducing, and later move further south to the spawning grounds. Although Madeira and the Canary Islands are the only incontrovertible areas where mature specimens are caught, philopatry has not been studied for this species. Moreover, the corroboration of its migration pattern along the NE Atlantic has not been possible on account of constraints driven by depth and pressure that hinder the use of techniques generally applied to coastal species to study migration, such as tagging and mark-recapture methods.

An extensively used alternative to direct observation of migratory movements is the analysis of a species trophic ecology since the prey diversity available for a predator is considered representative of the specific richness of the ecosystem where it lives. However, stomach analyses are difficult in deep-sea fishes because their contents are usually either regurgitated as a result of hydrostatic decompression or fully digested (Stowasser et al., 2009). For those fish, the quantification of chemical constituents acquired through diet has been shown to be a good representation of the feeding regimes and trophic relationships (DeNiro and Epstein, 1978; Petursdottir et al., 2008; Drazen et al., 2009; Stowasser et al., 2009; Ribeiro Santos et al., 2013b). Moreover, the chemical composition of fish muscle reflects the energetic trade-offs between intake, mostly through food, and expenditure with metabolism, migratory swimming, and the reproductive cycle (Petursdottir et al., 2008; Stowasser et al., 2009). Within those chemical constituents, both fatty acid (FA) and stable isotopes (SI) signatures provide a good insight into trophic relationships and food web interactions as their patterns reflect the dietary intake over longer time periods than stomach content analyses.

Lipids and the FA that compose them are, along with proteins, the major organic constituents of fish, functioning as the main source of metabolic energy for growth, reproduction, and locomotion, including migration (Tocher, 2003). Fatty acid trophic markers (FATM) are synthesized by specific primary producers and zooplankton, are transferred relatively unchanged along food webs, and accumulate in grazers and predators over time, allowing regional and temporal variations in plankton dynamics to be tracked down in the marine food webs (Lee et al., 1971; Dalsgaard et al., 2003; Drazen et al., 2009; Letessier et al., 2012).

SI analysis (SIA) has proven its value as a complementary and sometimes preferable tool for food web analyses in marine systems, recording both source and trophic level information (Iken et al., 2001; Polunin et al., 2001; Michener and Kaufman, 2007). Stable carbon and nitrogen isotope ratios ($\delta^{13}C$ and $\delta^{15}N$) are used as trophic markers; the first, to trace the carbon source, the regional origin of the primary producers, and the pathways of organic matter, and the other as an indicator of the trophic position of organisms (DeNiro and Epstein, 1978, 1981; Post, 2002; McCutchan et al., 2003; Laakmann and Auel, 2010; Letessier et al., 2012). SI ratios can change between diet and consumer due to differential digestion or fractionation during assimilation and metabolic processes (DeNiro and Epstein, 1978, 1981; Tieszen et al., 1983; McCutchan et al., 2003; Michener and Kaufman, 2007). The isotopic trophic discrimination factor between a consumer's tissue and its diet is on average 0.5-1‰ for $\delta^{13}C$ and 3.4‰ for $\delta^{15}N$ (DeNiro and Epstein, 1978, 1981; Vander Zanden and Rasmussen, 2001; Post, 2002; Michener and Kaufman, 2007; Laakmann and Aul, 2010; MacKenzie et al., 2011). Moreover, the relative abundance of SI within the organisms is influenced by external factors, namely geological, climatic, and ecological, as well as by internal factors, such as the synthetic pathways and position in the food web (DeNiro and Epstein, 1978, 1981; McCutchan et al., 2003).

The aim of the present study is to assess the feeding ecology and reproductive potential of the black

scabbardfish through the association between chemical biomarkers, specifically fatty acids and stable isotopes, and ecological attributes such as diet, length distribution, and maturity stage. Regional variations on the chemical composition of black scabbardfish in terms of fatty acid profile and stable carbon and nitrogen isotope ratios will be evaluated using specimens caught at four different geographical areas in the NE Atlantic.

3.1.2. Materials and methods

Sampling

Specimens of black scabbardfish were collected in 2010 and 2011 at four different locations in NE Atlantic, namely Iceland, the west of the British Isles, mainland Portugal, and Madeira Island (Figure 3.1.1). Specimens from mainland Portugal and Madeira were caught by commercial longline vessels, whereas specimens from the other locations were collected with bottom trawl during the autumn survey conducted by the Icelandic Marine Research Institute and the deep-water survey held by the Marine Scotland. In Madeira, sampling took place in November to account for the peak in the reproductive period. Specimens from mainland Portugal were collected under the DCF - European Commission Fisheries Data Collection Framework during the last semester to comprise the months sampled in the other locations. Specimens were selected randomly to cover the ranges of total length and of maturity stages available at each sampling area (Table 3.1.1).

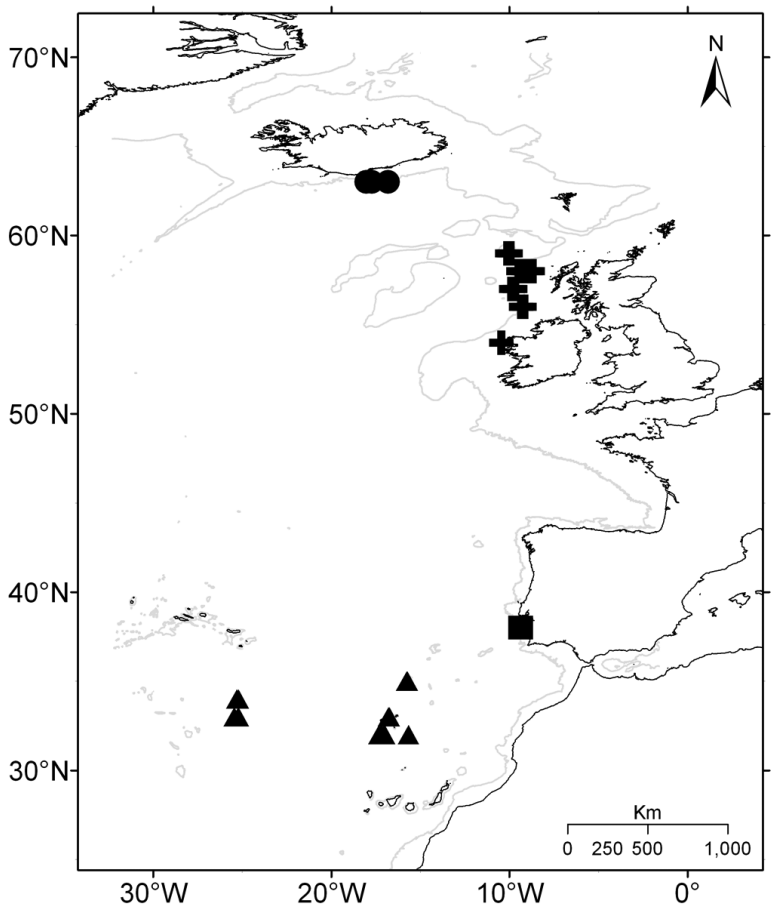

Figure 3.1.1. Map of NE Atlantic with the black scabbardfish sampling areas marked: triangles represent the sampling locations around Iceland; crosses, off west of the British Isles; circles, around Madeira Island; and squares, off mainland Portugal. The 1000 m depth contour is shown.

Table 3.1.1. Summary of the data regarding the black scabbardfish specimens used in FA and SI analyses.

Region	Gear	Depth (m)	Year	Month	Sex	Maturity	TL (mm)	n
Iceland	bottom trawl (survey)	533-585	2010	11	F	1	920-960	4
					M	1	880-1010	6
W British Isles	bottom trawl (survey)	550-1650	2011	9	F	1	890-1080	3
						2	910-1160	11
					M	1	890-1010	2
						2	920-1080	4
Mainland Portugal	bottom longline (commercial)	1100-1650	2010-2011	5-12	F	1	969-1001	3
						2	990-1215	8
						3	1212	1
					M	1	980-1010	3
						2	1007	1
						3	1009-1119	4
Madeira	drifting horizontal longline (commercial)	1200-1500	2010	11	F	2	1071-1164	7
						3	1127-1358	6
						4	1135-1323	2
						5	1260-1345	5

The following information was collected for all specimens: total length (TL, in mm), sex, and maturity stage. Maturity stage was macroscopically assigned according to the scale proposed by Gordo et al. (2000): stage I, immature or resting; stage II, developing; stage III, pre-spawning; stage IV, spawning; and stage V, post-spawning or spent. For further data analyses, maturity stages were grouped as immature, which included stages I and II, and mature, which included stages III to V. Owing to constraints in sample availability, all specimens from Madeira were females.

White muscle samples from all study areas were extracted from the dorso-lateral region behind the head and frozen directly after collection. Afterwards, samples were freeze-dried and milled with a pestle and mortar. White muscle tissue was also collected from all sampled specimens for genetic analysis, being preserved in absolute ethanol at +4 ºC.

In specimens caught off mainland Portugal it was possible to collect 151 stomachs between May and December 2011. Each stomach was weighted and frozen at –20 ºC for further content analysis.

Genetic analysis

Molecular markers were used to guarantee that only specimens of *A. carbo* were considered in this study since misidentification problems with *A. intermedius* exist (Stefanni and Knutsen, 2007). The cytochrome oxidase subunit I (COI) gene from mitochondrial DNA was selected to identify the species. This gene has been shown to be adequate for discriminating between *Aphanopus* species (Stefanni et al., 2009) as well as to identify a wide number of vertebrate and invertebrate taxa (Hebert et al., 2003).

DNA was extracted from muscle tissue following the Qiagen DNeasy Tissue Kit protocol. Amplification of the 5' end of the COI fragment was conducted in 25 µl reaction volumes containing ca. 50 ng of DNA sample, 10× reaction buffer, 1.5 mM $MgCl_2$, 0.2 nM dNTPs, 10 nM of each primer, and 0.1 U of Taq DNA Polymerase. The primers FishF1 (5´TCAACCAACCACAAAGACATTGGCAC3´)

and FishR1 (5´TAGACTTCTGGGTGGCCAAAGAATCA3´), designed by Ward et al. (2005), were used. The PCR thermal cycle consisted of an initial denaturing step of 5 min at 95 ºC, followed by 35 cycles of repeating the sequence 30 s at 95 ºC, 30 s at 50 ºC, and 60 s at 72 ºC, and a final extension step of 10 min at 72 ºC. The PCR products were enzymatically purified following a modification of the Exo-SAP method (Werle et al., 1994) and sequenced using the dye-labelled termination method (BigDye Terminator v3.1, Applied Biosystems, Inc., USA) on an ABI 3730XL sequencer (Macrogen Europe, The Netherlands). Amplicons were sequenced in both forward and reverse directions. Sequences were aligned manually and edited using BioEdit (Hall, 1999).

The number of segregating sites, the number of haplotypes, and the haplotype and the nucleotide diversities (and standard deviation) were estimated using DnaSP 4.20.2 (Rozas et al., 2003). The sequence obtained for each specimen was compared with three sequences from *A. carbo* available in GenBank using the Blast service from the National Centre for Biotechnology Information (NCBI).

Diet analysis

The stomachs from specimens caught off mainland Portugal were defrosted at room temperature and the food categories were identified to the lowest possible taxonomic level, counted, weighted, and measured when possible. Each food category is further designated as prey.

The index of vacuity (%IV) was determined as the percentage of stomachs without food contents in the whole sample of stomachs. A stomach was considered to be without food contents when it was empty, everted, or only contained the bait. In mainland Portugal's longline fishery the bait used was either *Sardina pilchardus* or *Scomber colias*.

The frequency of occurrence (%O, proportion between the number of stomachs containing a food category and the total number of stomachs with food items) was used to evaluate the relative importance of each prey in the diet of the black scabbardfish off mainland Portugal.

Fatty acids analysis

Fatty acid methyl esters (FAME) were prepared according to Bandarra et al. (2009), using 0.3 g of freeze-dried muscle tissue and 5 ml of the acetyl chloride:methanol mixture (1:19, v/v). The transesterification was carried out at 80 ºC for 1 h. After cooling, 1 ml of water and 2 ml of n-heptane were added to the mixture, which was stirred and centrifuged at 2.1×10^4 m/s^2 for 10 min. The organic phase was collected, filtered and dried over anhydrous sodium sulphate. The solvent was removed under nitrogen and the FAME dissolved in 0.1 ml of n-heptane.

The FAME analyses were performed in a Varian CP-3800 (Walnut Creek, CA, USA) gas chromatograph equipped with an auto sampler and fitted with a flame ionization detector (GC-FID). The separation was carried out on an Omegawax (Supelco, USA) capillary column (25 m × 0.25 mm id). Temperature was programmed from 180 °C to 200 °C at 4 °C.min^{-1}, holding for 10 min at 200 °C and heating to 210 °C at 4 °C.min^{-1}, holding at 210 °C for 14.5 min with the injector and detector at 250 °C. Methyl esters were quantified using the Varian software.

Thirty-nine fatty acids comprised the array of identified FA chosen for this study. Values were expressed as percentage of total area of all identified FA (% of total FA).

Stable isotopes analysis

To evaluate the carbon and the nitrogen isotope ratios ($\delta^{13}C$ and $\delta^{15}N$, respectively) approximately 2 mg of powdered lyophilised muscle was weighted into a tin capsule. For quality control purposes, for each specimen two duplicates were prepared, randomly ordered and one standard was analysed at every fifth sample. The isotope ratios $\delta^{13}C$ and $\delta^{15}N$ were determined by continuous-flow isotope ratio mass spectrometry (CF-IRMS) using a Finnigan ConFlo III coupled to a Flash Elemental Analyser 1112 Series (Thermo Electron Corporation, USA). The results were reported in δ notation according to the equation

$$\delta X(‰) = \left[\left(\frac{R_{sample}}{R_{standard}}\right) - 1\right] \times 1000,$$

where X is ^{13}C or ^{15}N and R is the ratio $^{13}C/^{12}C$ or $^{15}N/^{14}N$ in the sample and in the standard. $\delta^{13}C$ and $\delta^{15}N$ were expressed as per mill (‰) relative to Vienna-Pee Dee Belemnite (V-PDB) and atmospheric N_2 (air), respectively. These compounds are defined as the 0‰ point of the δ scale. The precision of the method was inferior to 0.1‰ for both isotope ratios.

Part of the variability in $\delta^{13}C$ in biological matrices is associated with varying lipid content amongst samples, since lipids are isotopically depleted in ^{13}C relative to proteins and carbohydrates (DeNiro and Epstein, 1977). The common approach to correct this effect is removing lipids from biological samples (Post, 2002; Sweeting et al., 2006). However, lipid extraction implies an undesirable and unintended enrichment of the sample in ^{15}N (Hoffman and Sutton, 2010). Therefore, to optimize this analysis in terms of time and costs, the method proposed by Hoffman and Sutton (2010) to correct the effect of lipids on $\delta^{13}C$ of untreated tissue was followed:

$$\delta^{13}C_{protein} = \delta^{13}C_{bulk} + \frac{\left(\Delta\delta^{13}C_{lipid} \times \left(C{:}N_{protein} - C{:}N_{bulk}\right)\right)}{C{:}N_{bulk}}.$$

For deep-sea fish, the isotopic depletion factor is $\Delta\delta^{13}C_{lipid} = -6.39‰$, whereas the ratio $C{:}N_{protein} = 3.76$ reflects the carbon mass balance given that simple lipids do not contain nitrogen (Hoffman and Sutton, 2010).

Data analyses

Canonical cluster analysis (CCA) was used for exploratory analysis of the most abundant fatty acids found in the muscle of black scabbardfish regarding the factors region, sex, maturity stage, and total length. The CCA is a combination of ordination and regression methods that allows extracting the synthetic gradients (ordination axes) that maximise the separation between FA and the levels of the four factors under analysis (ter Braak, 1985).

Posteriorly, the following sums were calculated for each specimen: SFA, as the sum of all saturated fatty acids; MUFA, as the sum of all monounsaturated FA; and PUFA, as the sum of all polyunsaturated FA. The importance of the different FA groups relies on the fact that each can be associated to particular physiological metabolic processes (Bandarra et al., 2009; Huynh and Kitts, 2009). Linear-mixed model (LMM) was fitted to each FA group (SFA, MUFA, and PUFA) by restricted maximum likelihood, considering region, sex, and maturity stage as fixed effects and TL as random effect. The LMM was chosen to allow removing the effect of the different TL range by area.

LMM was also fitted to PUFA with mean values higher than 1.00% of total FA in all areas by restricted maximum likelihood, considering region, sex and maturity stage as fixed effects and TL as random effect.

The correlation between $\delta^{15}N$ and $\delta^{13}C$ and TL by area was estimated through the Pearson's correlation coefficient. LMM was fitted to $\delta^{15}N$ and $\delta^{13}C$ separately by restricted maximum likelihood, considering region, sex and maturity stage as fixed effects and TL as random effect.

For all LMM, the function 'lme' from library 'nlme' version 3.1-113 was used (Pinheiro and Bates, 2000). The following assumptions for LMM were assessed by graphical analyses: (i) the within-group errors are independent and identically distributed, with mean zero and variance σ^2, and are independent of the random effects; (ii) the random effects are normally distributed and are independent for different groups (Pinheiro and Bates, 2000).

All data analyses where performed in R version 3.0.2.

3.1.3. Results

Length composition and maturity stages

The total length (TL) range of specimens used in the present study varied between sampling areas (Table 3.1.1). Moreover there was no overlap in the TL range of specimens from Iceland and from Madeira.

Specimens from Iceland and the west of the British Isles were in maturity stages I and I-II, respectively, hence were all immature, whereas specimens from mainland Portugal were in stages I to III, and specimens from Madeira were females in stages II to V.

Genetic analysis

A COI fragment with 651 bp was sequenced in all specimens caught off the four geographical areas (N = 70). Sequence analysis revealed five segregating sites defining five haplotypes and low haplotype and nucleotide diversities which were estimated as 0.2530 ± 0.1040 and 0.0005 ± 0.0002, respectively. The most common haplotype was shared between most of the specimens. The highest degree of similarity of the analysed specimens was with the sequences of specimens identified as *A. carbo* available in GenBank (99-100%); hence all specimens unambiguously corresponded to *A. carbo*.

Diet

The diet of black scabbardfish caught off mainland Portugal was analysed through stomach content examination. Of the 151 stomachs collected, 15 were everted, 9 contained only bait, 98 were empty, and 29 contained food items. The corresponding vacuity index was 80.8%.

Fish showed the highest occurrence (50% of the stomachs), followed by crustaceans (28%) and cephalopods (22%). In most stomachs with traces of fish, only scales were found, therefore it was not possible to estimate the total number of prey. Nonetheless, two undigested specimens of *Rouleina maderensis* (14 and 24 g total weight) were identified in two stomachs (6%). The vestiges found for crustacean prey were mostly unidentifiable parts of the exoskeleton, but in eight nearly undigested specimens it was possible to go as far as the species and identify the lophogastrid *Gnathophausia zoea*. This was the most frequent prey identified to species level (23%). The remains of cephalopods were counted and identified as *Mastigoteuthis* spp. through their beaks (eight specimens in seven stomachs). Following Clarke's (1986) regressions that relate the lower beak rostral length r with the body wet weight w ($\ln w = 0.184 + 2.88 \ln r$, $n = 45$, r2 = 0.94) and the mantel length l ($l = -1.8 + 29.08r$, $n = 47$ and r2 = 0.91), the mean weight (57 g) and the mean mantel length (108 mm) of *Mastigoteuthis* prey found in the analysed stomachs were estimated.

Fatty acids

The most abundant FA were: the SFA myristic acid (14:0), palmitic acid (16:0), and stearic acid (18:0); the MUFA palmitoleic acid (16:1n-7), oleic acid (18:1n-9), vaccenic acid (18:1n-7), eicosenoic acids (20:1n-11 and 20:1n-9), and the cetoleic acid (22:1n-11); the PUFA arachidonic acid (ARA or 20:4n-6), eicosapentaenoic acid (EPA or 20:5n-3), docosapentaenoic acid (DPA or 22:5n-3), and docosahexaenoic acid (DHA or 22:6n-3) (Table 3.1.2). Within these FA, the highest mean percentages were reported for DHA, oleic acid, and palmitic acid. Specimens from Iceland showed the highest mean percentage for myristic acid, palmitoleic acid, vaccenic acid, the eicosenoic acid 20:1n-11, and EPA; specimens from the west of the British Isles presented the highest means for oleic acid, the eicosenoic acid 20:1n-9, and cetoleic acid; whereas specimens from Madeira presented the highest means for palmitic acid, stearic acid, ARA, DPA, and DHA.

The CCA (Figure 3.1.2) reflected the importance of the selected FA in relation to each factor. The first two canonical axes explained approximately 92% of the cumulative percentage of discriminant

data. In this analysis, factors region and TL were highly significant (p=0.005 after 199 permutations of residuals under the reduced model). Region Madeira was positively related with the selected PUFA, the highest TL, and the mature specimens, and negatively related with MUFA. Oppositely, the west of the British Isles was positively related with MUFA (mainly 18:1n-9) and negatively related with PUFA. Mainland Portugal was related with the second axis but there was no clear relation with any FA.

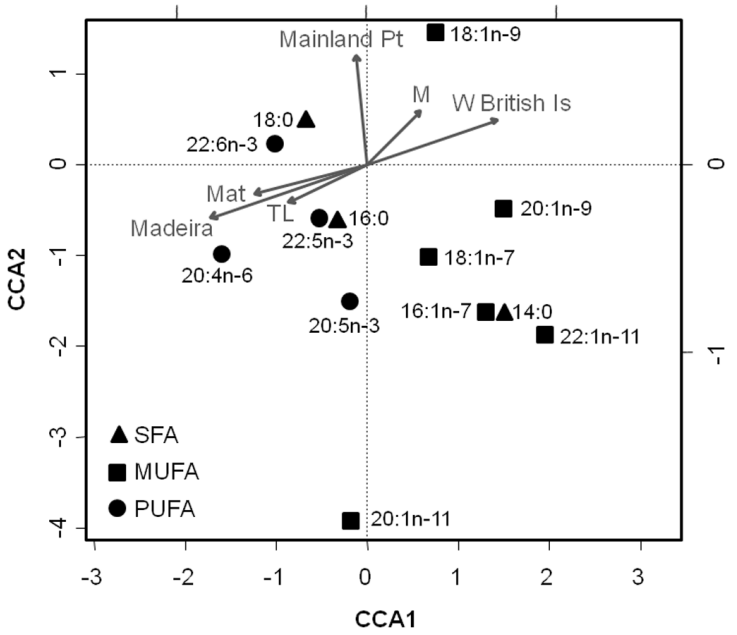

Figure 3.1.2. Canonical cluster analysis (CCA) ordination diagram of most representative fatty acids for black scabbardfish caught in the NE Atlantic. Sampling variables are represented by arrows. SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; Mainland Pt, mainland Portugal; W British Is, west of the British Isles; M, males; Mat, mature.

The pattern for the main FA groups was similar for specimens from Iceland and the west of the British Isles (MUFA > PUFA > SFA), differing from the pattern for both mainland Portugal (PUFA > MUFA > SFA) and Madeira (PUFA > SFA > MUFA) (Table 3.1.2).

Table 3.1.2. Summary of fatty acids (% total FA) in the muscle of black scabbardfish (mean ± standard deviation).

Fatty acid	Iceland	W British Isles	Mainland Portugal	Madeira
14:0 (myristic ac.)*	2.48 ± 1.16	2.24 ± 0.78	1.07 ± 0.82	0.49 ± 0.15
15:0	0.31 ± 0.10	0.23 ± 0.04	0.20 ± 0.07	0.21 ± 0.08
16:0 (palmitic ac.)*	17.99 ± 3.34	15.16 ± 1.66	16.37 ± 2.16	18.92 ± 2.85
17:0	0.26 ± 0.04	0.20 ± 0.04	0.27 ± 0.12	0.38 ± 0.18
18:0 (stearic ac.)*	5.15 ± 1.02	5.04 ± 0.72	6.34 ± 1.20	6.82 ± 0.75
19:0	0.12 ± 0.01	0.09 ± 0.02	0.10 ± 0.06	0.13 ± 0.09
20:0	0.12 ± 0.06	0.14 ± 0.03	0.11 ± 0.07	0.05 ± 0.05
22:0	0.01 ± 0.02	0.02 ± 0.02	0.01 ± 0.04	<0.01 ± 0.01
SFA	**27.22 ± 4.26**	**23.73 ± 1.85**	**25.28 ± 2.66**	**28.15 ± 3.62**
16:1n-9	0.16 ± 0.08	0.37 ± 0.24	0.25 ± 0.16	0.20 ± 0.12
16:1n-7 (palmitoleic ac.)*	2.85 ± 1.07	2.67 ± 0.85	1.48 ± 0.94	0.83 ± 0.32
17:1	0.36 ± 0.08	0.21 ± 0.05	1.22 ± 1.53	1.25 ± 1.26
18:1n-9 (oleic ac.)*	17.95 ± 5.27	25.18 ± 9.19	19.73 ± 13.10	9.48 ± 2.43
18:1n-7 (vaccenic ac.)*	2.99 ± 0.62	2.77 ± 0.56	2.10 ± 0.92	1.63 ± 0.32
18:1n-5	0.30 ± 0.04	0.27 ± 0.05	0.12 ± 0.08	0.11 ± 0.06
20:1n-11 (eicosenoic ac.)*	1.27 ± 1.02	0.18 ± 0.34	0.65 ± 0.65	0.47 ± 1.16
20:1n-9 (eicosenoic ac.)*	6.08 ± 2.70	8.21 ± 2.53	4.32 ± 2.58	2.38 ± 1.44
20:1n-7	0.30 ± 0.11	0.30 ± 0.12	0.27 ± 0.17	0.14 ± 0.12
22:1n-11 (cetoleic ac.)*	6.40 ± 3.02	6.82 ± 3.22	3.08 ± 3.46	0.89 ± 1.19
22:1n-9	0.97 ± 0.45	1.05 ± 0.48	0.77 ± 0.74	0.27 ± 0.33
24:1n-9	0.41 ± 0.51	0.26 ± 1.16	0.73 ± 0.84	0.30 ± 0.50
MUFA	**40.05 ± 11.05**	**48.30 ± 11.74**	**34.72 ± 16.64**	**17.98 ± 4.94**
16:2n-4	0.40 ± 0.06	0.39 ± 0.08	0.25 ± 0.10	0.28 ± 0.13
16:3n-4	0.38 ± 0.10	0.33 ± 0.07	0.35 ± 0.18	0.38 ± 0.47
16:3n-3	0.22 ± 0.15	0.13 ± 0.13	0.62 ± 0.67	0.85 ± 0.81
16:4n-3	0.81 ± 0.60	0.55 ± 0.48	1.49 ± 1.07	2.90 ± 1.14
18:2n-6	0.86 ± 0.14	0.71 ± 0.10	0.56 ± 0.13	0.46 ± 0.18
18:3n-6	0.05 ± 0.03	0.06 ± 0.03	0.05 ± 0.06	0.09 ± 0.08
18:3n-4	0.11 ± 0.03	0.11 ± 0.07	0.17 ± 0.11	0.09 ± 0.08
18:3n-3	0.31 ± 0.12	0.24 ± 0.08	0.52 ± 0.70	0.50 ± 0.66
18:4n-3	0.45 ± 0.25	0.25 ± 0.13	2.17 ± 3.44	2.25 ± 3.35
20:2n-6	0.23 ± 0.05	0.24 ± 0.05	0.27 ± 0.12	0.21 ± 0.14
20:3n-3	0.14 ± 0.04	0.11 ± 0.05	0.08 ± 0.10	0.07 ± 0.08
20:4n-6 (ARA) *	1.90 ± 0.98	1.54 ± 0.89	3.21 ± 1.60	5.87 ± 1.56
20:4n-3	0.66 ± 0.12	0.60 ± 0.14	0.46 ± 0.65	0.41 ± 0.93
20:5n-3 (EPA) *	4.43 ± 0.80	3.28 ± 1.00	2.88 ± 1.12	4.00 ± 1.18
21:5n-3	0.16 ± 0.19	0.23 ± 0.13	0.93 ± 1.59	1.88 ± 2.53
22:4n-6	0.17 ± 0.15	0.25 ± 0.09	0.71 ± 2.04	0.33 ± 0.29
22:5n-6	0.36 ± 0.20	0.34 ± 0.21	0.58 ± 0.30	1.02 ± 0.46
22:5n-3 (DPA) *	1.32 ± 0.32	1.40 ± 0.32	1.13 ± 0.35	1.66 ± 0.30
22:6n-3 (DHA) *	18.21 ± 7.54	15.98 ± 8.35	21.92 ± 9.73	28.95 ± 4.67
PUFA	31.17 ± 9.65	26.73 ± 10.63	38.34 ± 15.4	52.17 ± 6.37

SFA: sum of saturated fatty acids; MUFA: sum of monounsaturated fatty acids; PUFA: sum of polyunsaturated fatty acids.
* FA that were selected for CCA.

The LMM assumptions were met in all the models. According to LMM (Table 3.1.3), SFA was significantly lower in specimens from the west of the British Isles ($t_{0.05(2),\ 13}$ = -3.080; p = 0.009); MUFA was significantly lower in Madeira ($t_{0.05(2),\ 13}$ = -4.333; p < 0.001); and PUFA was significantly

higher in mainland Portugal ($t_{0.05(2),\,13} = 2.428$; $p = 0.031$) and Madeira ($t_{0.05(2),\,13} = 4.774$; $p < 0.001$). In general, there was no significant effect of sex or maturity stage.

Table 3.1.3. Summary of LMM fitted to the sums of FA for black scabbardfish in NE Atlantic.

	Effects	Value	S.E.	d.f.	t-value	p-value
SFA [a]	(Intercept)	27.56	1.09	51	25.40	< 0.001 *
	Region (W British Isles)	-3.66	1.19	13	-3.08	0.009 *
	Region (Mainland Portugal)	-2.05	1.17	13	-1.75	0.104
	Region (Madeira)	-0.40	1.37	13	-0.29	0.775
	Sex (male)	-0.57	0.89	13	-0.64	0.530
	Maturity (mature)	2.81	1.40	51	2.00	0.050
MUFA [b]	(Intercept)	42.51	4.40	51	9.67	< 0.001 *
	Region (W British Isles)	5.18	4.53	13	1.14	0.274
	Region (Mainland Portugal)	-9.00	4.89	13	-1.84	0.089
	Region (Madeira)	-23.93	5.53	13	-4.33	< 0.001 *
	Sex (male)	1.61	3.24	13	0.50	0.629
	Maturity (mature)	-1.73	5.78	51	-0.30	0.767
PUFA [c]	(Intercept)	28.58	4.07	51	7.02	< 0.001 *
	Region (W British Isles)	-1.48	4.15	13	-0.36	0.727
	Region (Mainland Portugal)	11.04	4.55	13	2.43	0.031 *
	Region (Madeira)	24.52	5.14	13	4.77	< 0.001 *
	Sex (male)	-1.55	2.97	13	-0.52	0.611
	Maturity (mature)	-2.84	5.44	51	-0.52	0.604

AIC = [a] 353.13, [b] 530.40, [c] 521.49.
* Significant *p-value*s for $\alpha = 0.05$.

PUFA with mean values higher than 1.00% of total FA in all areas were EPA, ARA, DHA, and DPA (Table 3.1.2). According to LMM (Table 3.1.4), all regions but Madeira and maturity were significant for EPA. Specimens from mainland Portugal showed significantly higher values for ARA. Both ARA and DHA were significantly higher in specimens from Madeira. For DPA, no factor was significant. Maturity was important for explaining the differences in EPA and ARA.

Table 3.1.4. Summary of LMM fitted to PUFA with mean values higher than 1.00% of total FA for black scabbardfish in NE Atlantic.

	Effects	Value	S.E.	d.f.	t-value	p-value
EPA [a]	(Intercept)	4.29	0.37	51	11.66	< 0.001 *
	Region (W British Isles)	-1.00	0.39	13	-2.60	0.022 *
	Region (Mainland Portugal)	-1.48	0.41	13	-3.64	0.003 *
	Region (Madeira)	-0.86	0.46	13	-1.87	0.084
	Sex (male)	0.16	0.28	13	0.59	0.564
	Maturity (mature)	1.53	0.48	51	3.21	0.002 *
ARA [b]	(Intercept)	1.67	0.41	51	4.08	< 0.001 *
	Region (W British Isles)	-0.05	0.40	13	-0.14	0.893
	Region (Mainland Portugal)	1.75	0.46	13	3.79	0.002 *
	Region (Madeira)	3.28	0.52	13	6.29	< 0.001 *
	Sex (male)	-0.29	0.29	13	-1.00	0.335
	Maturity (mature)	2.56	0.57	51	4.46	< 0.001 *
DHA [c]	(Intercept)	16.86	2.85	51	5.92	< 0.001 *
	Region (W British Isles)	-0.33	2.92	13	-0.11	0.912
	Region (Mainland Portugal)	6.30	3.17	13	1.99	0.069
	Region (Madeira)	12.99	3.59	13	3.62	0.003 *
	Sex (male)	-2.04	2.09	13	-0.98	0.347
	Maturity (mature)	-2.41	3.77	51	-0.64	0.526
DPA [d]	(Intercept)	1.36	0.12	51	11.57	< 0.001 *
	Region (W British Isles)	0.07	0.13	13	0.52	0.610
	Region (Mainland Portugal)	-0.21	0.13	13	-1.62	0.129
	Region (Madeira)	0.23	0.15	13	1.54	0.148
	Sex (male)	-0.07	0.09	13	-0.70	0.498
	Maturity (mature)	0.19	0.15	51	1.27	0.208

AIC = [a] 212.09, [b] 230.76, [c] 475.21, [d] 67.67.
* Significant p-values for $\alpha = 0.05$.

Stable isotopes

Since there were no significant differences in $\delta^{13}C$ and $\delta^{15}N$ between the two duplicates analysed for each specimen, the mean value between each pair of values was used in the subsequent analyses. Overall, mean $\delta^{13}C$ and mean $\delta^{15}N$ increased southwardly from Iceland to Madeira, with very similar values detected in specimens from mainland Portugal and Madeira (Table 3.1.5 and Figure 3.1.3). TL was positively correlated with $\delta^{15}N$ ($\rho = 0.55$; $t = 5.37$; $df = 68$; $p < 0.001$), but there was no correlation with $\delta^{13}C$ ($\rho = 0.19$; $t = 1.59$; $df = 68$; $p = 0.117$) (Figure 3.1.4).

Table 3.1.5. Summary of $\delta^{15}N$ and $\delta^{13}C$ (‰) in the muscle of black scabbardfish (mean ± standard deviation).

SI ratio	Iceland	W British Isles	Mainland Portugal	Madeira
$\delta^{15}N$ (‰)	12.54 ± 0.47	13.1 ± 0.39	13.33 ± 0.52	13.35 ± 0.55
$\delta^{13}C$ (‰)	-18.65 ± 0.27	-18.64 ± 0.31	-18.32 ± 0.76	-18.19 ± 0.46

According to LMM (Table 6), $\delta^{15}N$ was significantly higher in mainland Portugal ($t_{0.05(2),\,13} = 3.11$; $p = 0.008$), in Madeira ($t_{0.05(2),\,13} = 2.23$; $p = 0.044$) and in mature specimens ($t0.05(2),\,51 = 2.12$; $p = 0.039$), whereas for $\delta^{13}C$ only mature specimens were significant ($t_{0.05(2),\,51} = 2.19$; $p = 0.033$).

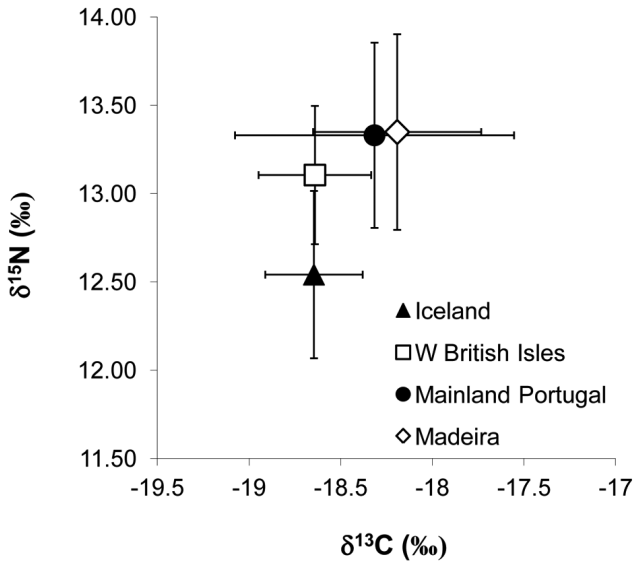

Figure 3.1.3. Bivariate plot of $\delta^{13}C$ and $\delta^{15}N$ for black scabbardfish caught in the NE Atlantic (mean ± standard deviation).

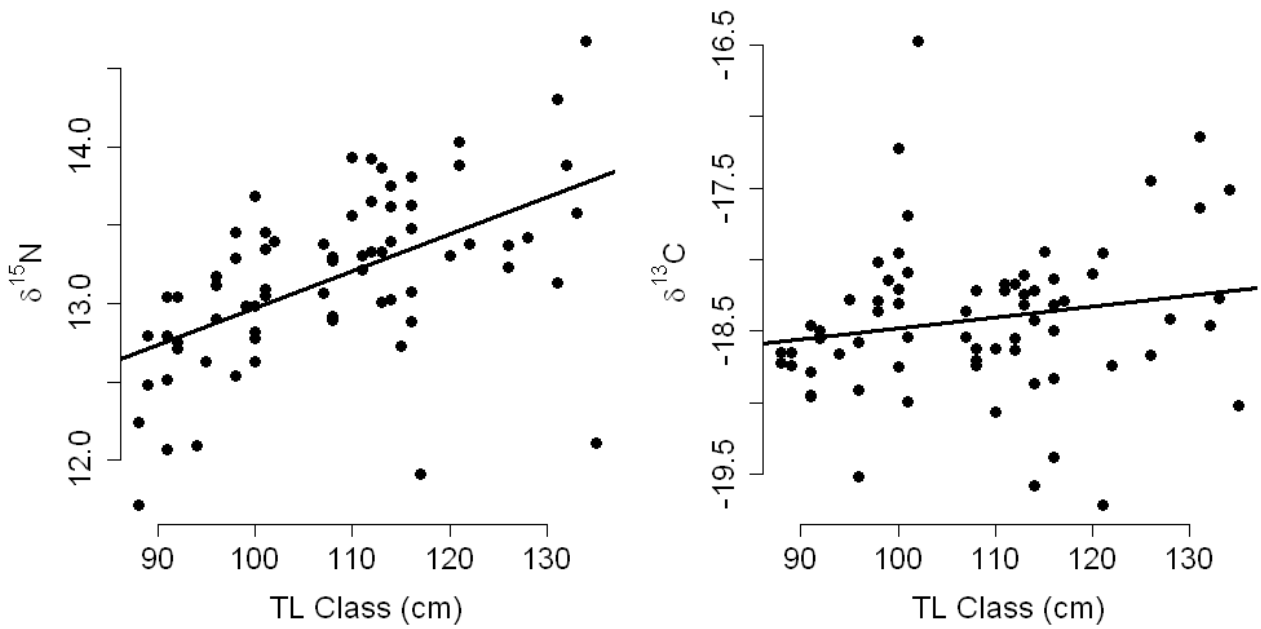

Figure 3.1.4. Plots of $\delta^{13}C$ and $\delta^{15}N$ against total length class (in cm) and fitted regression lines for black scabbardfish caught in the NE Atlantic.

Table 3.1.6. Summary of LMM fitted to $\delta^{15}N$ and $\delta^{13}C$ (‰) for black scabbardfish in NE Atlantic.

	Effects	Value	S.E.	d.f.	t-value	p-value
$\delta^{15}N$ [a]	(Intercept)	12.73	0.16	51	80.08	< 0.001 *
	Region (W British Isles)	0.28	0.15	13	1.90	0.080
	Region (Mainland Portugal)	0.56	0.18	13	3.11	0.008 *
	Region (Madeira)	0.46	0.21	13	2.23	0.044 *
	Sex (male)	0.05	0.11	13	0.44	0.666
	Maturity (mature)	0.50	0.24	51	2.12	0.039 *
$\delta^{13}C$ [b]	(Intercept)	-18.59	0.17	51	-107.52	< 0.001 *
	Region (W British Isles)	-0.12	0.17	13	-0.69	0.500
	Region (Mainland Portugal)	0.23	0.19	13	1.17	0.263
	Region (Madeira)	0.23	0.22	13	1.03	0.321
	Sex (male)	0.11	0.12	13	0.91	0.381
	Maturity (mature)	0.53	0.24	51	2.19	0.033 *

AIC = [a]120.02, [b]114.08.
* Significant *p-value*s for $\alpha = 0.05$.

3.1.4. Discussion

The life cycle of deep-sea fish like the black scabbardfish is difficult to study owing to monitoring problems that hinder having a good temporal and spatial coverage. For this species, information is mostly collected from existing fisheries off the west of the British Isles, mainland Portugal, and Madeira. However, the wide geographical distribution of the black scabbardfish comprises areas without commercial exploitation.

Another problem is the high level of stomach vacuity found for this species which limits stomach content analyses and, consequently, studies on trophic dynamics. For specimens from mainland Portugal the vacuity index (80.8%) was a consequence of barotrauma or total food digestion. That index was similar to what had already been reported for the other areas: from 93.3 to 98.3% for specimens from Madeiran longliners (Freitas, 1998; Ribeiro Santos et al., 2013b) and from 66.2 to 94.1% for specimens caught by trawls to the west of the British Isles (Mauchline and Gordon, 1984; Ribeiro Santos et al., 2013b). Madeira and mainland Portugal showed the highest vacuity indices because the collection of specimens was restricted to commercial landings from the longline fisheries in which the mean soaking time is about 24 h (Bordalo-Machado et al., 2009), favouring the total digestion of food.

To overcome these sampling difficulties, specimens from both commercial fisheries and scientific surveys were used in a combination of biochemical methods that allowed inferring about the diet and the reproductive cycle of the black scabbardfish. The present study covered a wider area of distribution of the black scabbardfish in the NE Atlantic than any other previously published study and combined FA and SI analyses for this species for the first time. Additionally, it was proved through genetic analyses that this study was conducted solely on *A. carbo* without mixing with the cryptic species *A. intermedius*.

Within the sampling areas, Madeira is the only one where spawning of this species occurs. Specimens from Madeira were caught at the peak of the spawning season (Figueiredo et al., 2003; Neves et al., 2009; Ribeiro Santos et al., 2013a) and were mostly mature females. The higher proportion of PUFA and the stronger relationship with ARA and DHA than in the other areas reflected the connection between these FA and the species maturity and spawning since they are precursors of biomolecules involved in reproduction (Stacey and Goetz, 1982; Ruggeri and Thoroughgood, 1985; Tocher, 2003),

therefore highly required by mature specimens. Particularly, ARA is the major precursor of series II prostaglandins (paracrine hormones) that stimulate ovulation and spawning (Stacey and Goetz, 1982; Ruggeri and Thoroughgood, 1985; Sargent et al., 1999; Bergé and Barnathan, 2005) and is important for the production of viable eggs (Sargent et al., 1999). DHA plays a major role in egg production because it is responsible for maintaining the structure and function of cellular membranes, specifically the ovarian membrane fluidity and stability, through a process called homeoviscous adaptation (Sargent et al., 1999; Tocher, 2003; Mayor et al., 2013). In deep-sea organisms, such as the black scabbardfish, the structure of membranes is particularly important to minimize the effects of the exposure to high pressure and low temperatures (Stowasser et al., 2009).

The high percentage of PUFA found in specimens caught off Madeira in the peak of spawning also supports the hypothesis that the black scabbardfish continues to feed during the spawning period since PUFA are obtained through the diet (Sargent et al., 1999; Tocher, 2003). Results from both the FA and the SI analyses are in accordance with the diet composition described by Freitas (1998), who stated that the black scabbardfish caught off Madeira occupies a high trophic level. In that study, squids of genera *Chiroteuthis*, *Mastigoteuthis*, *Histioteuthis*, and *Taonius*, as well as the viperfish *Chauliodus* sp. and several lanternfishes from family *Myctophidae* were the most important prey reported.

In the stomachs of specimens caught off mainland Portugal, the most frequent prey were teleost fish, followed by the lophogastrid crustacean *Gnathophausia zoea* and cephalopods from genera *Mastigotheutis*. The importance of teleosts explains the high values of $\delta^{15}N$ and $\delta^{13}C$ found in specimens from this area, since, in general, teleosts are isotopically enriched in relation to crustaceans and cephalopods (Iken et al., 2001; Polunin et al., 2001; Bergé and Barnathan, 2005; Cherel and Hobson, 2005; Reid et al., 2013). On another hand, the relative importance of PUFA, MUFA, and SFA in those specimens was the same as in *Scomber colias* (Bandarra et al., 2004), which is commonly used as bait in the longline fishery. Regarding the importance of fish, attention must be taken when assuming that fish are the preferred prey in this area because only two specimens were clearly identified as *Rouleina maderensis* and the scales found in most stomachs could belong to fish used as bait (*Sardina pilchardus*). 18:1(n-9) values were similar to those reported for the prey *Gnathophausia zoea* (Letessier et al., 2012). High 18:1(n-9) reflects high physiological requirements (Mayor et al., 2013), which for these specimens can either be for reaching the pre-spawning maturity stage or for migration. The significantly higher proportion of ARA found in specimens from mainland Portugal comparing with those from the northernmost areas explains why the former reach a more advanced maturity stage.

The high levels of ARA in specimens from the southernmost areas can also support the occurrence of migratory movements between them, since a high demand for ARA is associated with stressful periods, such as long distance migrations (Sargent et al., 1999).

Qualitative differences in the diet composition between specimens from mainland Portugal and specimens from Madeira are reflected in their FA signatures and could explain why in the former area specimens that attain the length at first maturity (103 cm) do not proceed with maturation, rather stopping in the pre-spawning stage (Figueiredo et al., 2003; Neves et al., 2009). Maturation would therefore be triggered by feeding and the energetic content of available prey, an exogenous factor, rather than by an endogenous factor. Although the relative importance of FA groups was different from results previously reported for black scabbardfish from mainland Portugal (MUFA > SFA > PUFA; Bandarra et al., 2009) and Madeira (MUFA > PUFA > SFA; Nogueira et al., 2013), those differences cannot be interpreted because sampling season, length range, and the number of specimens were not provided in those studies.

To the west of the British Isles, black scabbardfish were all immature or developing. The most important prey that have been previously identified were fish, namely Argentina sp. and *Scomber scombrus* on one occasion (Du Buit, 1978), but mostly blue whiting (*Micromesistius poutassou*), with a minor contribution of cephalopods (Mauchline and Gordon, 1984; Ribeiro Santos et al., 2013b). Combining data from Mauchline and Gordon (1984) with the diet composition of specimens caught off the Rockall Trough, Howell et al. (2009) found approximately 50% of cephalopods and 44% of blue whiting. Ribeiro Santos et al. (2013b) explained these differences with seasonal changes in prey availability: the increase in cephalopods and crustaceans in the diet of the black scabbardfish coincided with blue whiting migrating to the Norwegian Sea for spawning, between late April and the end of

the year (Bailey, 1982; Was et al., 2008). In the present study, SI ratios in specimens from the west of the British Isles were lower than in Madeira and mainland Portugal because they were caught in September, when blue whiting is not available as food source and when Ribeiro Santos et al. (2013b) reported a predominance of crustaceans in their stomachs. The consequent lower food availability can also explain the high retention or synthesis of MUFA found in specimens from that area because these FA have the capacity to provide energy stores in deep-sea fish (Stowasser et al., 2009). The pattern MUFA > PUFA > SFA had already been described for a specimen of black scabbardfish caught in the eastern slopes of the Rockall Trough (Dunne et al., 2010). The differences in SIA between the present study and Ribeiro Santos et al. (2013b) show that temporal variations constrained by the sampling periods need to be further analysed, extending the temporal and the specimens length ranges of all the different areas.

Specimens caught off Iceland and off the west of the British Isles were geographically close and, as expected, their FA pattern and $\delta^{13}C$ values were similar and different from those of specimens from Madeira and mainland Portugal. Differences in baseline $\delta^{13}C$ values can be explained by both abiotic factors, such as latitude and depth (DeNiro and Epstein, 1978; Laakman and Auel, 2010) and biotic factors like particulate organic matter (POM) (Letessier et al., 2012). There is a poleward depletion in ^{13}C because the decrease in temperature increases the CO_2 solubility (Rau et al., 1989; Michener and Kaufman, 2007; Laakmann and Auel, 2010); there is a progressive enrichment in ^{13}C with increasing habitat depth (Hoffman and Sutton, 2010); and POM $\delta^{13}C$ increases southwardly in NE Atlantic (Letessier et al, 2012). Although around Iceland diet data are not available for the black scabbardfish, the depletion in ^{15}N in comparison with the other three areas is likely to be a consequence of differences in the phytoplankton baseline $\delta^{15}N$ because POM $\delta^{15}N$ also increases southwardly (Letessier et al, 2012) and in the feeding regimes. Since, in this area, the species reaches smaller sizes it is restricted to a lower trophic level and to a narrower variety of prey both in size and mobility.

Overall, the allometric effect of each area's size ranges over $\delta^{15}N$ supports the existence of ontogenic differences in the black scabbardfish's diet, which is common in fish (Drazen et al., 2001; Dalsgaard et al., 2003; Stowasser et al., 2009; Mayor et al., 2013; Reid et al., 2013). In the present study, those changes were expressed not only in terms of $\delta^{15}N$ and $\delta^{13}C$ but also in terms of FATM. Specimens caught off Iceland showed higher values for the diatoms' FATM EPA and, together with specimens from the west of the British Isles, relatively higher percentages of 20:1n-9 and 22:1n-11, whose primary producers are the copepods *Calanus* spp., and of 16:1n-7 and 18:1n-7, which are related to benthic organisms (Dalsgaard et al., 2003; Stowasser et al., 2009). However, specimens caught off mainland Portugal and Madeira fed on bathypelagic fish, cephalopods, and crustaceans that are at a higher trophic level. In general, the diet of black scabbardfish is typical of mobile benthopelagic predators that are opportunistic feeders (Iken et al., 2001) and the change from prey that feed on pelagic zooplankton to bathypelagic prey reflects an improvement in its predatory capacity.

3.1.5. Conclusion

The biochemical analyses performed over the muscle of black scabbardfish were in accordance with the species' spatial distribution in terms of the reproductive cycle and the size distribution pattern, drawing close together specimens from Iceland and the west of the British Isles, and specimens from mainland Portugal and Madeira. This study has proposed a relationship between the reproductive cycle of the black scabbardfish in the NE Atlantic and its fatty acid profile: mature specimens have higher energetic demands and show a prevalence of PUFA, whereas SFA are more representative in immature specimens. Furthermore, FA and SI biomarkers were successfully combined in the interpretation of this species' spatial and size-based feeding variability.

Acknowledgements

This work was supported by the European Commission Fisheries Data Collection Framework, the EU-FP7 project DEEPFISHMAN (Grant agreement no. 227390), the Portuguese project CERTIFICA (PROMAR, 2011-2015), and a doctoral fellowship granted to I. Farias by Fundação para a Ciência e Tecnologia (SFRH/BD/64434/2009). The authors thank Teresa Moura (IPMA) for helping with the genetic analysis and for drawing the map; Ana Rita Vieira, José Lago, and Neide Lagarto (IPMA),

Klara Jakobsdottir (MRI, Iceland), João Delgado, Sara Reis, Sofia Vieira, and the technical staff in Madeira (DSIP), Francis Neat and the scientific team at MRV Scotia (Marine Scotland) for helping in sample collection; Júlia Ferreira (IPMA) for helping with the FA analysis; and Gabriel Martorell (UIB) and Marta Nogueira (IPMA) for helping with the SI analysis. We are also grateful to Joel C. Hoffman (EPA, USA), Monty Priede (DSR I's Associate Editor), and two anonymous reviewers for their pertinent comments and suggestions.

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3.2. Sex steroids of black scabbardfish, *Aphanopus carbo*, in relation to reproductive and migratory dynamics

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Abstract

Black scabbardfish, *Aphanopus carbo*, is a commercially important species that takes distant migrations throughout its life cycle. Sex steroids were measured by radioimmunoassay in the blood plasma of specimens caught off the Madeira Archipelago and mainland Portugal to link this species migratory path with its reproductive cycle. Furthermore, a pilot study using Mozambique tilapia (*Oreochromis mossambicus*) was designed to evaluate the effect of sample freshness on steroid levels because black scabbardfish blood was collected at separate times after specimens were caught. The changes in T and 11-KT concentrations between the time of blood extraction and the time after preservation did not statistically differ among the different methods applied. Therefore, measured black scabbardfish steroid concentrations were directly used in the subsequent data analyses. In females, E_2 and in T concentrations peaked at a late stage of vitellogenesis. E_2 concentration was significantly different between females caught off each area. Clustering E_2 and T concentrations from all developing females resulted in the separation of two distinct groups, independently of their geographical area. In males, T and 11-KT were not significantly different between maturity stages. The hepatosomatic index of males caught off mainland Portugal was relatively high. This may reflect a mechanism for storing energy that will later be consumed during migration to the spawning grounds. The trend of sex steroids concentrations throughout the sexual maturation of the species is consistent with the morphological indicators and shows evidence of the reproductive and migratory pattern hypothesised for the black scabbardfish in NE Atlantic.

Keywords

Black scabbardfish; Deep-sea; Madeira; Mainland Portugal; Migration; Sex steroids

3.2.1. Introduction

The black scabbardfish, *Aphanopus carbo* Lowe, 1839, is an important commercial deep-sea species caught in Portuguese waters. This benthopelagic teleost fish is widely distributed along the NE Atlantic (Ehrich, 1983; Allain et al., 2003; Bordalo-Machado et al., 2009), where it undergoes large-scale migrations: the smallest specimens are reported further north and the largest specimens at the southernmost limit of distribution (Farias et al., 2013; Ribeiro Santos et al., 2013). The only known spawning grounds are located off Madeira, the Canary Islands, and possibly Morocco (Figueiredo et al., 2003; Pajuelo et al., 2008; Perera, 2008). Juveniles recruit to the fisheries off the west of the British Isles, documented to be a feeding area, where they remain some time growing (Figueiredo et al., 2003; Ribeiro Santos et al., 2013). Black scabbardfish subsequently moves to areas off mainland Portugal where caught specimens attain larger sizes and pre-spawning individuals are seldom captured (Figueiredo et al., 2003; Neves et al., 2009). After spending some time here to feed and grow, juveniles and pre-adults move further south to the spawning areas around Madeira (Farias et al., 2013). A key question is why fishes off mainland Portugal do not develop beyond the pre-spawning stage, despite attaining sizes larger than the estimated length at first maturity (L_{50} = 102.8 cm) (Figueiredo et al., 2003).

Fish diet, growth, reproduction, and energy consumption are closely dependent on environmental conditions, which highly constrain metabolic rate processes in the deep-sea (Merrett and Haedrich, 1997). Understanding the role of hormonal regulation of deep-sea species' vital processes will contribute to a better knowledge of their spatial dynamics. In this context, associating alterations in gonad development with the levels of sex steroids in blood plasma has proven to be a valuable tool to comprehend the endocrine control of reproduction in some deep-sea teleosts although this is a field not fully explored (Pankhurst and Conroy, 1987; Lee and Yang, 2002; Sisneros et al., 2004; Sequeira et al., 2017).

In teleost fish females, the follicle stimulating hormone (FSH) is responsible for the stimulation of granulosa cells to produce estradiol-17β (E_2), the key hormone that induces the liver to synthesize vitellogenin and egg shell proteins, which are incorporated into the oocyte during vitellogenesis (Lubzens et al., 2010). After the growth phase, a surge of luteinizing hormone (LH) stimulates the follicle to produce the maturation-inducing steroid - either 17,20β-dihydroxypregn-4-en-3-one (17,20β-P) or 17,20β,21-trihydroxypregn-4-en-3-one (17,20β,21-P), depending on the species – that promotes final oocyte maturation (resumption of meiosis) and ovulation (Nagahama and Yamashita, 2008; Lubzens et al., 2010).

In males, FSH regulates Sertoli cell activity to support germ cell development while LH acts on Leydig cells to promote steroidogenesis (Schulz et al., 2010; Chauvigne et al., 2014). The key androgen in males is 11-ketotestosterone (11-KT) which promotes germ cell proliferation and maturation, as well as the development of secondary sexual characters and the mediation of reproductive behaviours (Borg, 1994; Schulz et al., 2010). In males, 17,20β-P is responsible for endorsing the initiation of meiosis, for stimulating spermiation, and for enhancing sperm motility (by alteration of the pH and fluidity of the seminal fluid) and can act as a pheromone, e.g. in goldfish (Scott et al., 2010). Finally, both male and female gonads produce testosterone (T) which is a precursor of E_2 and 11-KT and feeds back on the pituitary gland to promote the synthesis of gonadotrophins, among other functions (Nagahama, 1994; Lubzens et al., 2010; Schulz et al., 2010).

The main objective of this study was to assess and compare the hormonal status and gonadal development stage of specimens caught off mainland Portugal and off Madeira. Black scabbardfish specimens caught by commercial fisheries are already dead when pulled on board and it was only possible to obtain blood from specimens several hours after capture or thawed. To establish how these conditions may affect hormone levels, a pilot study tested the effect of sample age and freezing on the sex steroids of Mozambique tilapia, *Oreochromis mossambicus* (Peters, 1852).

3.2.2. Material and methods

Pilot study

To assess the effect of the time lag between death (capture) and blood collection and the effect of freezing (specimen storage) on sex steroid levels, Mozambique tilapia (*Oreochromis mossambicus*) male specimens were used. The Mozambique tilapia is a freshwater cichlid that can live at temperatures ranging from 8º to 42º C and can be reared in hypersaline conditions (Froese and Pauly, 2019). Despite the biological and ecological differences between the Mozambique tilapia and the black scabbardfish, the former was used in this pilot study because specimens were readily available from a captive stock, raised from fertilised eggs at the University of Algarve and maintained in freshwater under natural annual conditions of photoperiod and water temperature (26 ºC) prior to these experiment. Males were chosen over females because they are expected to show less hormonal variation owing to a simpler reproductive physiology.

Fish care and experimentation complied with the national legislation for the use of laboratory animals under a Group-1 license issued by the Portuguese National Authority for Animal Health. Fish were stunned and killed by immersion in iced water and total length (TL, cm) and total weight (TW, g) were measured. Blood samples were collected in heparinised syringes from the caudal peduncle in larger specimens or from the heart in smaller specimens.

After a first sample of blood was collected from 29 specimens (control, t0), fish were randomly separated into two groups: 18 fish were stored on ice in a refrigerator (temperature 4 ºC) and the remaining 11 fish were frozen (temperature -20 ºC). A second blood sample was collected from each fish at the distinct times that defined the treatments (Table 3.2.1). Frozen fish were thawed at room temperature prior to blood collection. Blood samples were centrifuged at 4 ºC and separated plasma was stored at -20 ºC until assay.

Table 3.2.1. Experimental design and summary of Mozambique tilapia samples used in the pilot study. T, storage temperature; n, sample size; TL, total length range; TW, total weight range.

Treatment	T (ºC)	Time (days)	n	TL (cm)	TW (g)
t1	4	1	6	15.2-18.9	61.74-103.62
t2	4	2	5	15.8-17.3	62.09-75.65
t4	4	4	6	16.5-18.9	66.4-105.21
t15	-20	15	6	15.1-19.3	53.7-103.52
t30	-20	30	5	14.6-17.2	50.68-81.64

Black scabbardfish samples

Black scabbardfish (*Aphanopus carbo*) specimens were collected at irregular times between 2010 and 2012 from commercial longline vessels operating off Madeira Archipelago and off mainland Portugal (Table 3.2.2). Since *Aphanopus intermedius* Parin, 1983 (intermediate scabbardfish) specimens are mixed with *A. carbo* in Madeira's commercial landings (Stefanni and Knutsen, 2007), specimens caught in this region were assigned to species following the morphological criteria defined by Biscoito et al. (2011). In the present study, only *A. carbo* specimens were used.

Table 3.2.2. Summary of black scabbardfish samples used for measuring sex steroids estradiol-17β, testosterone, and 11-ketotestosterone. Values are total length range (mm) and sample size (parenthesis) by sex and maturity stage (1-5).

		Region	
Sex	**Maturity**	**Madeira**	**Mainland Portugal**
Females	1	-	890-1081 (13)
	2	1087-1506 (16)	1041-1178 (6)
	3	1141-1254 (4)	-
	4	1135-1323 (7)	-
	5	1141-1291 (4)	-
Males	1	-	874-1003 (10)
	2	1117-1179 (2)	1045 (1)
	3	1115-1177 (4)	-
	4	1122-1233 (7)	-
	5	1040-1244 (4)	-

In Madeira, specimens were sampled between October and December from commercial port landings. This month range was selected to encompass the reproductive season. In this fishery, black scabbardfish are caught by mid-water horizontal drifting longline set below 1000 m deep and the soaking time lasts from two to four days (Bordalo-Machado et al., 2009). Specimens from mainland Portugal were collected throughout the year on board commercial vessels or from commercial port landings. In this fishery, the fishing gear is a horizontal bottom longline and the soaking time lasts from one to two days (Bordalo-Machado et al., 2009).

For each specimen, the following information was collected: total length (TL, mm), total weight (TW, g), gutted weight (UW, g), liver weight (LW, g), gonad weight (GW, g), sex, and maturity stage. Maturity stage was macroscopically assigned following the scale proposed by Gordo et al. (2000): stage 1, immature or resting; stage 2, developing; stage 3, pre-spawning; stage 4, spawning; and stage 5, post-spawning or spent.

The gonadosomatic index (GSI) was calculated as the gonad weight as a proportion of the gutted weight; the hepatosomatic index (HSI) was calculated as the liver weight as a proportion of the gutted weight; and Fulton's condition factor (K) was calculated as the ratio between the gutted weight

and the cube of the total length. To calculate the previous reproductive indicators, whole data sets collected between 2010 and 2012 from specimens caught off Madeira (n = 1976) and off mainland Portugal (n = 692) were used.

Blood samples were collected in heparinised syringes from the specimen's caudal vessel, which was exposed by removing the lateral musculature close to the caudal peduncle. To minimize the effect of metabolism and degradation, the collection of blood samples took place as soon after hauling as possible. In specimens caught off Madeira, blood was extracted in the laboratory from fish that had been dead and remained hooked to the longline for one to four days and was kept on ice after hauling for less than 24 hours (n = 48). The exact individual time of death or hauling is not known since each vessel deploys the fishing gear more than once during each fishing trip and the fish is kept on board all together. Specimens caught off mainland Portugal had been dead and remained hooked to the longline for up to one day. Blood samples from these specimens were collected (i) on board from fresh fish immediately after hauling (n = 12); (ii) in the laboratory from fish kept on ice after hauling for less than 24 hours (n = 14); or (iii) in the laboratory from thawed fish that was stored in a freezing room at -20 ºC, less than one day after hauling, for 30 days (n = 4).

Blood samples were centrifuged at 4 ºC to separate the plasma, which was stored at -20 ºC until assay.

Steroid analysis

Blood plasma (100 µl) was extracted twice with 3 ml of diethyl ether to obtain the free steroids. Extracts were dried on a dry bath at 40 ºC under nitrogen gas and suspended in 1 ml of assay buffer (0.5 M phosphate–gelatine buffer, pH 7.6). Free steroids were measured by radioimmunoassay (RIA) following the methodology described by Scott et al. (1982). Individual plasma samples were mixed with 100 µl of distilled water and extracted twice with 4 ml of diethyl ether to obtain free steroids. Extracts were dried under nitrogen, reconstituted in 0.5 M phosphate–gelatine buffer, pH 7.6 and steroids were measured by RIA.

The estradiol-17β (E_2) antiserum was purchased from Research Diagnostics (USA) and the cross-reactions (%) have been reported as follows: <0.2% for 4-pregnene-3,20-dione; <0.2% for 11β,17,21-trihydroxy-4-pregnene-3,20-dione; <0.2% for 4-androstene-3,17-dione; <0.2% for 17β-hydroxy-4-androsten-3-one; <0.2% for 3β-hydroxy-5-pregnen-20-one; <0.2% for 3β-hydroxy-5-androsten-17-one; 15% for 3β-hydroxy-1,3,5(10)-estratrien-17-one; 8% for 3,17β-dihydroxy-1,3,5(10)-estratrien-16-one; 0.7% for 3,16α,17β-trihydroxy-1,3,5(10)-estratrien-3-one; <0.2% for 3,16α-dihydroxy-1,3,5(10)-estratrien-17-one (Guerreiro et al., 2002). The testosterone antiserum was kindly donated by Dr. David Kime (University of Sheffield, UK). The testosterone (T) antiserum cross-reactions were 63% for androstenedione, 35% for 11-ketotestosterone, 55% for 11-hydroxytestosterone, 40% for 5-androstan-17-ol-3-one, 31% for 5-androstan-17-ol-3-one, 12% for 5-androstane-3,17-diol, 25% for 5-androstane-3,17-diol. The 11-ketotestosterone (11-KT) antiserum cross-reactions were 20.1% for 11-hydroxytestosterone, 20.6% for testosterone, 76.9% for androstenedione, 30.1% for 11-hydroxyandrostenedione, 52% for dihydrotestosterone, 3.3% for cortisol, and 1.3% for cortisone (Kime and Manning, 1982). All samples were assayed in duplicate in a single assay. The intra-assay and inter-assay coefficients of variation were, respectively: 6.6% and 14.2% for E_2; 5.0% and 8.2% for T; and 8.2% and 11.6% for 11-KT. The limits of detection were between 10 (E_2) and 100 (T and 11-KT) pg ml^{-1}.

Statistical analyses

In the Mozambique tilapia pilot study, one-way ANOVA was applied for comparing T and 11-KT concentrations between t0 (control) and the time of each treatment. The variation in T and 11-KT concentration in Mozambique tilapia was estimated as the difference between the value at time t0 (control) and at the time of each treatment. One-way ANOVA was applied for comparing the variation in T and in 11-KT between treatments. Whenever necessary, data were log10-transformed to meet the ANOVA assumptions. If ANOVA assumptions were not met after data transformation, nonparametric Kruskal–Wallis test by ranks was used instead. When there were statistically significant differences between treatments, Wilcoxon signed-rank test was applied to compare each pair of treatments.

In black scabbardfish blood plasma, one-way ANOVA was used to investigate the association between E_2, T, and 11-KT concentrations and the way the specimens are preserved prior to blood collection for each sex separately. Whenever necessary, data were log_{10}-transformed to meet the ANOVA assumptions. If assumptions were not met after data transformation, nonparametric Wilcoxon signed-rank test was used instead. When there were statistically significant differences between preservation methods, Wilcoxon test was applied to compare each pair of maturity stages.

Two-way fixed effects ANOVA was applied for analysing the effects of geographical region and maturity stage on GSI, HSI, K, and on E_2, T, and 11-KT concentrations for each sex separately. Whenever necessary, data were log10-transformed to meet the ANOVA assumptions. If assumptions were not met after data transformation, nonparametric Wilcoxon signed-rank test was used instead. When there were statistically significant differences between maturity stages, Wilcoxon test was applied to compare each pair of maturity stages.

A k-means clustering method was applied to steroid (E_2 and T) profile data using developing (stage 2) females both from Madeira and mainland Portugal using the R package *cluster* (Maechler et al., 2018).

The R software (R Core Team, 2018) was used for all statistical analyses and 5% significance level was adopted. Graphics were built with the R package *ggplot2* (Wickham, 2009).

3.2.3. Results

Pilot study

In Mozambique tilapia, no significant differences in T concentration ($\chi2$ (4, n = 28) = 3.918, *p-value* = 0.417) and in 11-KT concentration ($\chi2$ (4, n = 28) = 1.601, *p-value* = 0.809) were found between samples collected initially and samples collected after treatment (Figure 3.2.1).

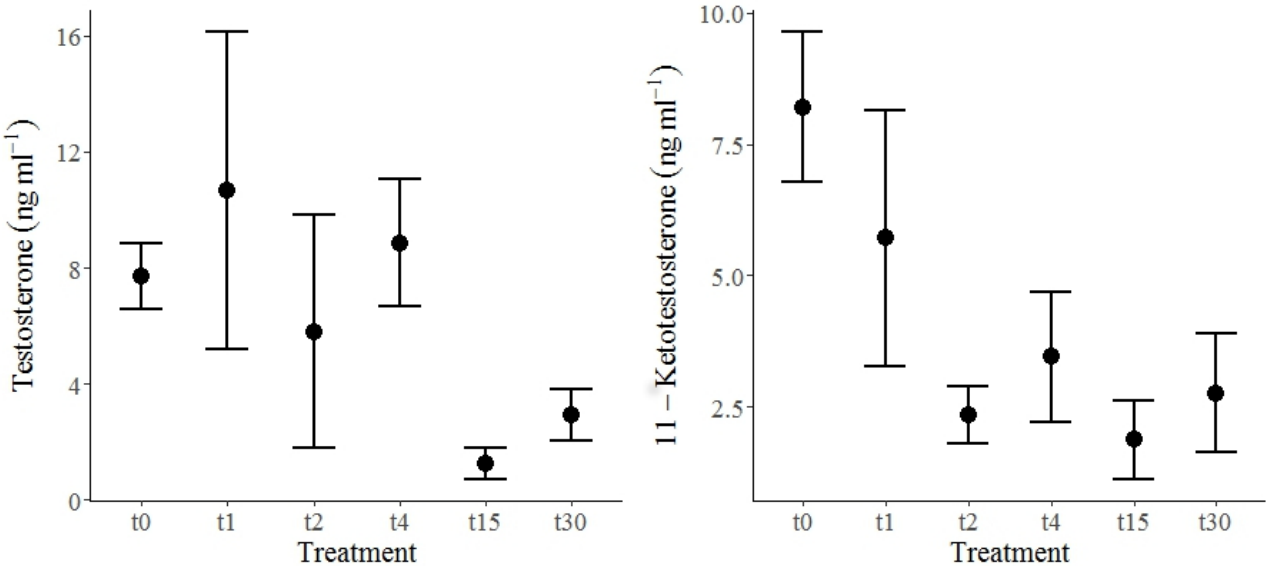

Figure 3.2.1. Testosterone (ng ml^{-1}) (left) and 11-ketotestosterone (ng ml^{-1}) (right) concentration (mean ± SE) in Mozambique tilapia males by treatment.

T and 11-KT concentrations varied between the control (t0) and each treatment but the variations were not significantly different between treatments ($\chi2$ (4, n = 28) = 3.918, *p-value* = 0.417 and $\chi2$ (4, n = 28) = 1.601, *p-value* = 0.809, respectively) (Figure 3.2.2).

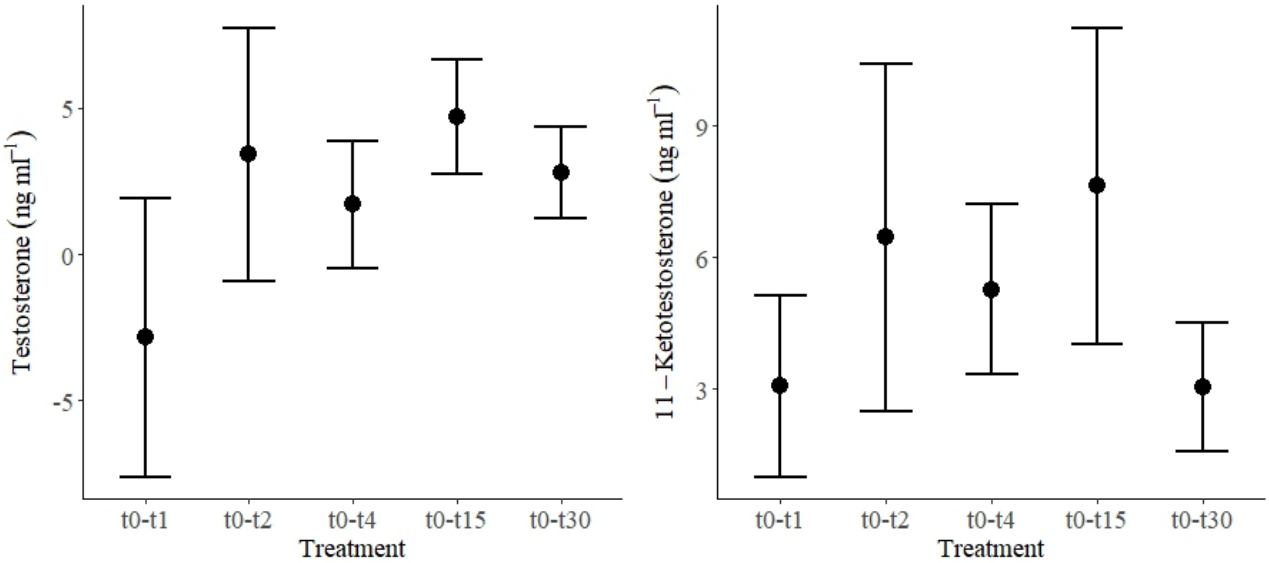

Figure 3.2.2. Change in testosterone (ng ml^{-1}) (left) and 11-ketotestosterone (ng ml^{-1}) (right) concentration (mean ± SE) in Mozambique tilapia males between time of blood collection and the time of the second collection defined by the treatment.

Reproductive indicators in black scabbardfish

Black scabbardfish specimens caught off mainland Portugal, between 2010 and 2012, were in maturity stages 1 to 3 and 5 (Table 3.2.2). Immature or resting females and males (maturity stage 1) were not found among fish sampled in Madeira.

In females caught off mainland Portugal, mean GSI significantly increased between stage 1 and stage 2 ($W = 3544$, *p-value* < 0.001). However, the increase between stage 2 and stage 3 was not statistically significant ($W = 164$, *p-value* = 0.358) (Figure 3.2.3). The mean GSI of females caught off Madeira significantly increased from stage 2 to stage 3 ($W = 1866$, *p-value* < 0.001) and from stage 3 to stage 4 ($W = 1015$, *p-value* < 0.001), and significantly decreased from stage 4 to stage 5 ($W = 6983$, *p-value* < 0.001). Concerning males, mean GSI significantly increased from stage 1 to stage 2 ($W = 488$, *p-value* < 0.001) and from stage 2 to stage 3 ($W = 845$, *p-value* < 0.001) in specimens caught off mainland Portugal, and from stage 2 to stage 3 ($W = 17335$, *p-value* < 0.001) and from stage 3 to stage 4 ($W = 2494$, *p-value* < 0.001) in specimens caught off Madeira, whereas the decrease from stage 4 to stage 5 was statistically significant ($W = 8213$, *p-value* < 0.001) in specimens from Madeira. The mean GSI was significantly higher in specimens caught off Madeira than off mainland Portugal for stage 2 ($W = 23842$, *p-value* < 2.2×10-16) and stage 3 females ($W = 7$, *p-value* = 0.004) and for stage 2 ($W = 11430$, *p-value* = 0.008) and stage 3 males ($W = 8147$, *p-value* = 0.033).

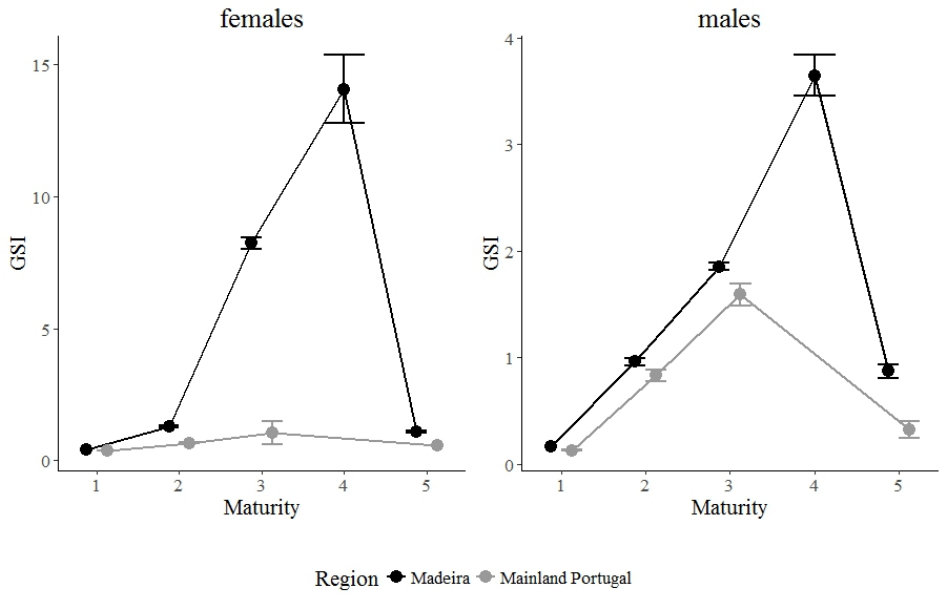

Figure 3.2.3. Black scabbardfish gonadosomatic index (GSI) (mean ± SE) of females (left) and males (right) caught off Madeira and mainland Portugal by maturity stage.

Mean HSI was significantly higher in stage 2 females caught off Madeira than off mainland Portugal ($W = 33667$, *p-value* = 0.020) (Figure 3.2.4). Mean HSI significantly increased from stage 1 to stage 2 females caught off mainland Portugal ($W = 10395$, *p-value* < 0.001) and from stage 2 to stage 3 females caught off Madeira ($W = 13287$, *p-value* < 0.001), whereas it significantly decreased from stage 4 to stage 5 females caught off Madeira ($W = 5690$, *p-value* < 0.001).

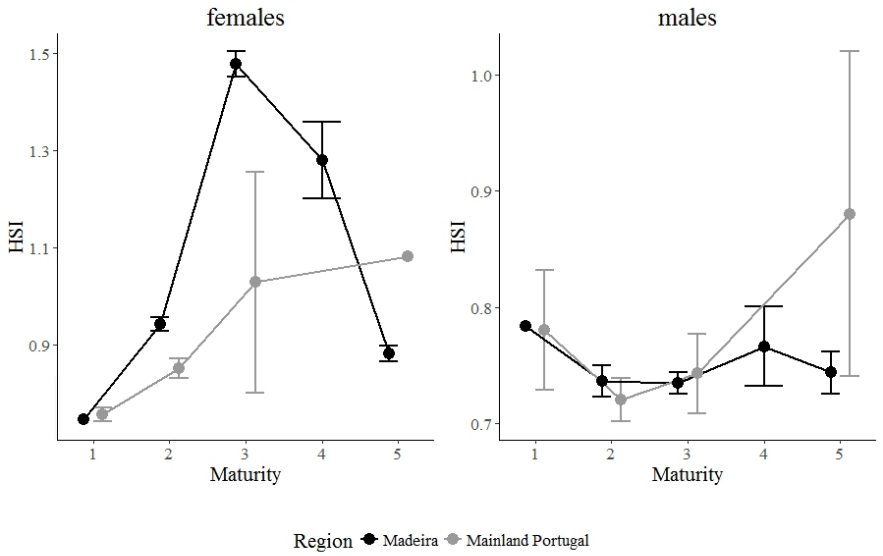

Figure 3.2.4. Black scabbardfish hepatosomatic index (HSI) (mean ± SE) of females (left) and males (right) caught off Madeira and mainland Portugal by maturity stage.

Mean K significantly increased between stage 1 and stage 2 females ($W = 9041$, *p-value* < 0.001) and males ($W = 7505.5$, *p-value* = 0.002) caught off mainland Portugal, and from stage 3 to stage 4 males ($W = 7946$, *p-value* < 0.001) caught off Madeira, whereas it significantly decreased from stage 4 to stage 5 males ($W = 5828$, *p-value* < 0.001) (Figure 3.2.5). Mean K was significantly higher in stage 2 females ($W = 57444$, *p-value* < 0.001), in stage 2 males ($W = 18945$, *p-value* < 0.001), and in stage 3 males ($W = 15444$, *p-value* < 0.001) caught off Madeira than in the same sex and same maturity stage

specimens caught off mainland Portugal.

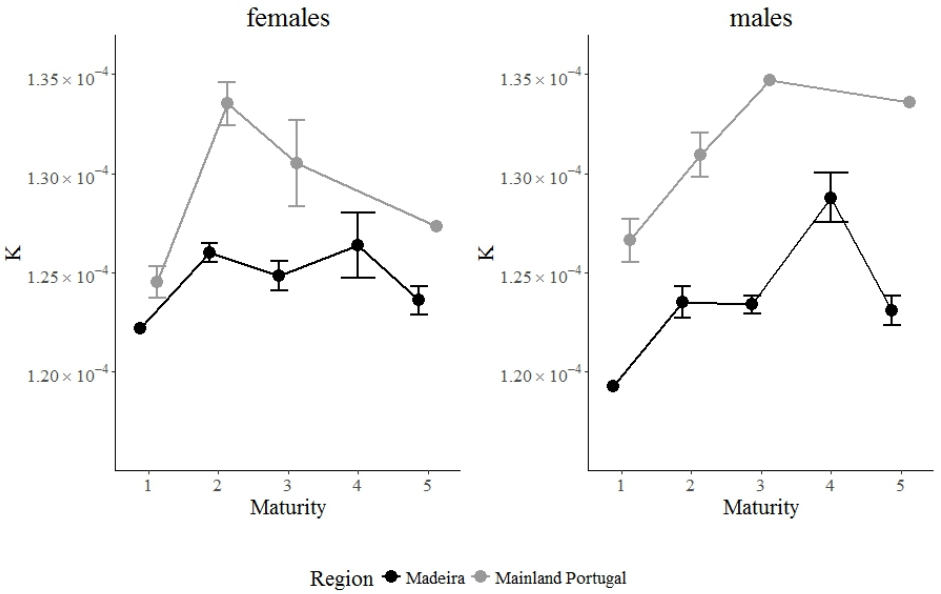

Figure 3.2.5. Black scabbardfish Fulton's condition factor (K) (mean ± SE) of females (left) and males (right) caught off Madeira and mainland Portugal by maturity stage.

Sex steroids in black scabbardfish

The changes of both T and 11-KT concentrations in Mozambique tilapia between the time of blood extraction and the time of measurement after preservation did not statistically differ among the different treatments applied. Therefore, the sex steroid concentrations quantified in the black scabbardfish samples were directly used in the data analyses.

In black scabbardfish specimens, no significant differences were found between the specimens preservation methods for all three analysed sex steroids: E_2 ($F_{2,41} = 2.851$, *p-value* = 0.069); T ($F_{2,64} = 1.789$, *p-value* = 0.175); and 11-KT ($F_{1,26} = 0.239$, *p-value* = 0.629).

In black scabbardfish females, T was not significantly different between regions ($F_{1,36} = 1.470$, *p-value* = 0.233) nor between maturity stages ($F_{1,36} = 0.830$, *p-value* = 0.368) neither was the interaction between region and maturity stage statistically significant ($F_{1,36} = 1.349$, *p-value* = 0.253) (Figure 3.2.6). E_2 was significantly different between regions ($F_{1,40} = 5.717$, *p-value* = 0.022), but no significant differences were found between maturity stages ($F_{1,40} = 2.215$, *p-value* = 0.145) neither was the interaction between region and maturity stage statistically significant ($F_{1,40} = 2.954$, *p-value* = 0.093).

11-KT was significantly different between males caught off Madeira and males caught off mainland Portugal ($F_{1,24} = 14.364$, *p-value* = 0.001), but no significant differences were found between maturity stages ($F_{1,24} = 0.465$, *p-value* = 0.502) neither was the interaction between region and maturity stage statistically significant ($F_{1,24} = 0.389$, *p-value* = 0.539) (Figure 3.2.5). T in males was not significantly different between regions ($F_{1,24} = 2.228$, *p-value* = 0.149) and no significant differences were found between maturity stages ($F_{1,24} = 0.008$, *p-value* = 0.928). The interaction between factors was not analysed because factors were not crossed.

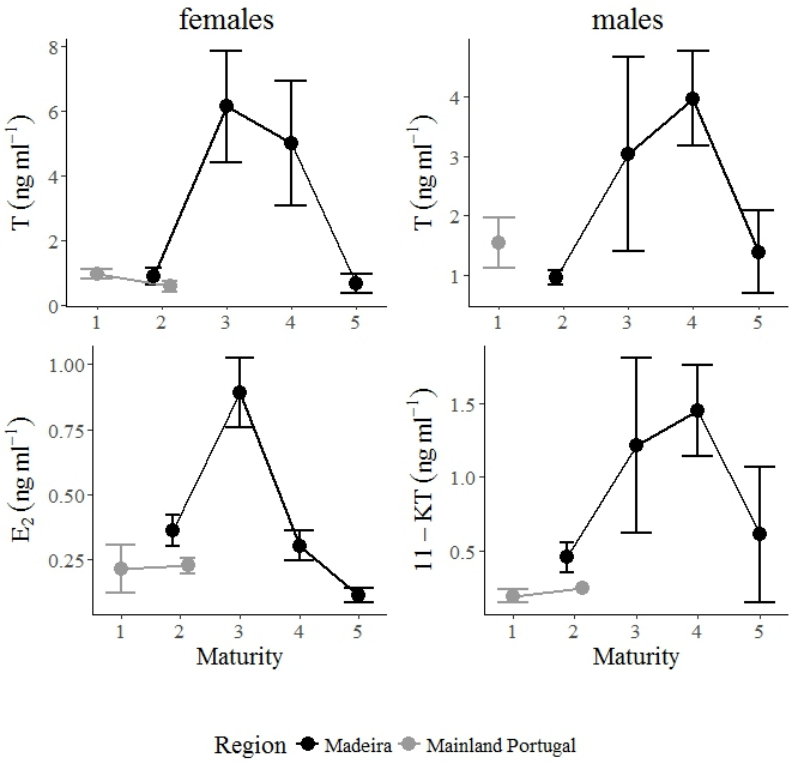

Figure 3.2.6. Sex steroids concentration (mean ± SE) in the blood of black scabbardfish females (left column) and males (right column) caught off Madeira and mainland Portugal by maturity stage. T is testosterone in ng ml^{-1}; E_2 is estradiol in ng ml^{-1}; 11-KT is 11-ketotestosterone in ng ml^{-1}.

The only maturity stage within the same sex for which blood was collected in specimens caught off both Madeira and mainland Portugal areas was stage 2 females. Considering T and E_2 concentrations together, stage 2 female samples were grouped into two clusters that were statistically different ($F_{1,16}$ = 6.793, *p-value* = 0.019). T and E_2 concentrations did not show evidence of geographic differentiation as each cluster included specimens from both regions (Figure 3.2.7).

3.2.4. Discussion

The pilot study with the Mozambique tilapia showed that it is possible to obtain meaningful measures of sex steroids in blood collected from fish that has been preserved refrigerated or frozen for a relatively prolonged period. Furthermore, it was assumed that the general behaviour of the hormonal steroids during refrigeration and the degradation mechanisms would follow similar patterns, although this is an area that requires more in-depth studies. In a previous study with plainfin midshipman fish, *Porichthys notatus*, blood plasma, no significant differences in E_2, T, and 11-KT concentrations were found between samples collected at different times after capture (within one or four hours) in either offshore or intertidal zones for both males and females (Sisneros et al., 2004). Black scabbardfish blood samples were collected up to 24 hours or 30 days after capture and steroid levels were not corrected. Nonetheless, black scabbardfish sex steroid levels should be regarded as relative and not absolute values. In addition to the time between death and blood collection and the above-mentioned factors that influence hormone levels, the stress of capture, which may vary with time and method of capture, can also lower sex steroid levels (Pankhurst and Conroy, 1988; Clearwater and Pankhurst, 1997; Cleary et al., 2002).

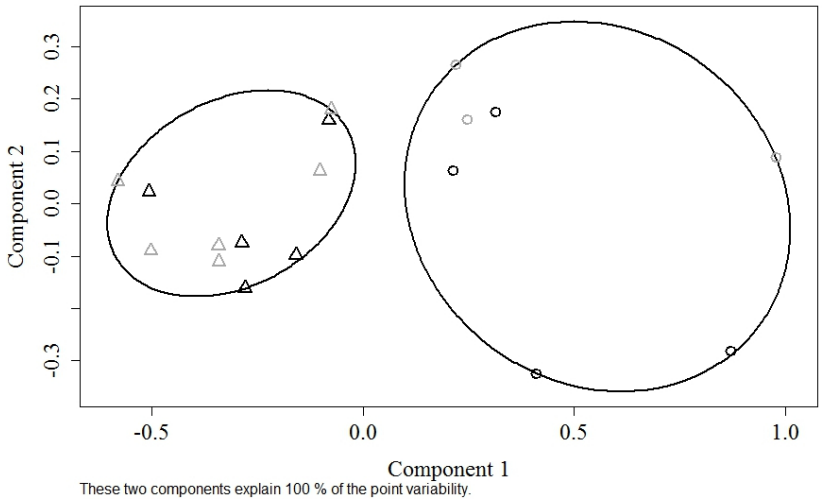

Figure 3.2.7. Representation of k-means clustering applied to steroid (E_2 and T) profile data using developing (stage 2) females caught off Madeira and mainland Portugal.

E_2 and T increased during vitellogenesis (stages 1 to 3), peaked at the pre-spawning stage (stage 3), and decreased from stage 3 onwards. The same steroid profile was observed for GSI in females. Although the increase in GSI between stages 1 and 2 was not statistically significant, the concomitance between the highest levels of E_2 and T and the peak in vitellogenesis (stage 3 in this species) has also been described for other teleosts (e.g. Prat et al., 1990; Frisch et al., 2007; Hachero-Cruzado et al., 2007; Li et al., 2007). Furthermore, the same profile has been observed in the HSI of female black scabbardfish, demonstrating that the hepatic reserves are being consumed during the maturation process (Domínguez-Petit and Saborido-Rey, 2010; Ribeiro Santos et al., 2013).

In fact, E_2 and T are connected since T is a precursor of E_2, such as other steroids (Pankhurst and Conroy, 1987), and E_2 is crucial for the start of vitellogenesis because it promotes the synthesis of hepatic yolk precursors (vitellogenin) in a variety of teleost species and provides negative feedback to LH secretion (Tyler and Sumpter, 1996; Sisneros et al., 2004; Lubzens et al., 2010). Chemical and physiological changes occurring throughout female developing stage are of uttermost importance for comprehending the reproductive cycle of black scabbardfish and the differences between fish living off Madeira and fish living off the Portuguese mainland coast. To disentangle this moment in the species' life cycle, E_2 and T concentrations in developing females were analysed together through cluster analysis. The mixing of females from both areas within each cluster shows that the steroid profiles are similar between areas. Madeiran females were sampled at the spawning season and during this period it is likely that females between an early and a late development stage may concur. Fish from the group with the lowest steroid concentrations represent an earlier development stage. These fish will not have the time to mature and reach the spawning stage and will fail to spawn in the current season. It is not a case of skipped spawning, which refers to a mature fish that fails to spawn at a given year (Rideout and Tomkiewicz, 2011), but the drivers of both processes are similar. In black scabbardfish, the developing stage during early vitellogenesis proved to be the critical window for the decision to spawn or not to spawn (Skjæraasen et al., 2010; Neves et al., 2009). The energy saved not developing into mature stages is invested in growth and in increased fecundity, similar to what happens when fish skip spawning (Jørgensen et al., 2006). The persistence of a high proportion of relatively immature individuals in a plentiful environment may allow them to grow fast before the next reproductive cycle drives energy towards the gonads (Roff, 1983; Alonso-Fernández and Saborido-Rey, 2012; Folkvord et al., 2014). Moreover, the simultaneous occurrence of two groups of developing females with similar length, steroid levels, and reproductive indicators points to a long duration of this maturity stage.

Stage 2 females caught off mainland Portugal during the spawning period show a high degree of follicular atresia in the ovaries and do not develop into mature stages (Neves et al., 2009). The

abidance of fish at the same location after the maturation process is interrupted explains why ca. 25% of individuals off mainland Portugal are larger than L50 but immature (Figueiredo et al., 2003; Neves et al., 2009; Farias et al., 2013). This phenomenon was also observed off the west of the British Isles (Ribeiro Santos, Minto, et al., 2013). The capability of specimens to migrate to the spawning grounds will depend on the completion of high energy reserves (Merret and Haedrich, 1997; Ribeiro Santos et al., 2013). In fact, the relatively high HSI observed in immature males caught off mainland Portugal suggests that energy is being stored in the liver to be spent when moving to the spawning grounds. Black scabbardfish's readiness for migration was also supported by the high content of arachidonic acid (ARA), which is demanded for long distance movements (Sargent et al., 1999; Tocher, 2003), that was found in the muscle of specimens caught off mainland Portugal (Farias et al., 2014). Off the west of the British Isles, the species prepares for migration towards the south, between January and April, through an intense feeding activity on blue whiting (Ribeiro Santos et al., 2013).

Androgen (T and 11-KT) levels did not significantly differ among reproductive stages. Nevertheless, in males caught off Madeira, the two steroids were low in spent and regressed fish, increased during gonadal recrudescence, and peaked at the end of spermatogenesis as described in several studies (Pankhurst and Conroy, 1988; Prat et al., 1990). The significant differences between regions are expected to be a consequence of the unbalanced sampling amongst maturity stages.

The present work supports the role of sex steroids as intrinsic triggers for gonadal maturation and spawning in black scabbardfish. It also shows that it is possible to measure sex steroids in blood plasma that was collected late after death and relate the values with the dynamics of the species reproduction, overcoming some sampling constraints of deep-sea species. Given the fact that specimens used in this study were collected from commercial fisheries with a soak time greater than one day, the number of blood samples were limited because, as the time passes after capture, the blood extraction is more difficult. Moreover, the depth at which black scabbardfish specimens were collected implies that fish are boarded already dead and, subsequently, the collection of an adequate volume of blood is problematic. To overcome these difficulties, dedicated surveys are required, which in the case of deep-sea fishes are costly and do not take place in Portuguese waters. Nonetheless, the present results put in evidence that the RIA method is appropriate for the quantification of sex steroid concentration even with small blood volumes. The metabolization of sex steroids after capture was considered to be negligible or to have occurred equally in all individuals, as the relationships between hormones and reproductive stages were maintained.

Since spawning occurs during a relatively short period, for future work it would be important to have a monthly or more frequent coverage in the two regions to test if there are differences between maturity stages related with the time of the year when specimens are caught.

Acknowledgements

The authors are especially grateful to the master, Henrique Santos, and crew of the longliner Varamar for hosting researchers on board to collect samples off mainland Portugal, and to Sofia Vieira, Sara Reis, and the technical staff from DSI-DRP for their collaboration in collecting the samples in Madeira. We also thank Pedro Guerreiro (CCMAR-CIMAR) for support in the tilapia experiment. This work was supported by the Portuguese Foundation for Science and Technology (FCT – Fundação para a Ciência e Tecnologia) under the PhD Grant SFRH/BD/64434/2009 to I. Fa. and the project UID/Multi/04326/2013 to A.V.M.C.; the Portuguese Government Development Investments and Expenses Program (PIDDAC – Programa de Investimentos e Despesas de Desenvolvimento da Administração Central) under the EU/DG Fisheries' Data Collection Framework (DCF); the Portuguese Fisheries Operational Program (2007-2013) (PROMAR – Programa Operacional Pescas), co-financed by the European Fisheries Fund (EFF) (2007-2013), under project CERTIFICA (2011-2015), MSC Certification in Portugal - Acquiring the knowledge and studying the viability for its application to coastal and artisanal fisheries.

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Chapter 4: Taxonomic and spatial diversity in the NE Atlantic inferred from otolith microchemical analysis

4.1. Separation of *Aphanopus carbo* and *Aphanopus intermedius* using otolith microchemistry and shape analysis

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Abstract

Aphanopus represents one of the most important commercial deep-sea teleosts in the NE Atlantic with annual landings varying around 2-3 tonnes since 2008. Two species of the genus *Aphanopus*, the black scabbardfish, *Aphanopus carbo*, and the intermediate scabbardfish, *A. intermedius*, have been recently reported. Genetic studies indicated that the two species coexist in Madeira, Azores, and Canaries Archipelagos. In Madeira, these species are landed as a single commercial category, "black scabbardfish", because it is not possible to distinguish them by external visual examination. New tools to identify the species based on otoliths are proposed and tested as a solution to analyse their proportion in historical data. Otolith elemental composition, and otolith contour shape specimens of *A. carbo* and *A. intermedius* caught off Madeira were compared. Otolith trace element composition was significantly different at age 9. No significant differences on otolith contour shape were found between the two species, since intraspecific variability was too high. This study has demonstrated that otoliths are an efficient tool to distinguish between *A. carbo* and *A. intermedius*, particularly using trace element composition. Genetic studies indicated that the two species coexist in Madeira, Azores and Canaries Archipelagos. Based on the present results, otolith microchemistry and otolith ageing can be combined to estimate the proportion of each *Aphanopus* species in historical collections of otoliths to reconstruct species abundance time-series.

Keywords: NE Atlantic, Madeira, deep-sea teleost, scabbardfish, Fourier analysis, trace elements.

4.1.1. Introduction

In the NE Atlantic, the genus *Aphanopus*, known as scabbardfish, has an important commercial value, specifically in Madeira, which is included in CECAF area 34.1.2, where it represents the most important fish landed both in weight and in value (Bordalo-Machado et al., 2009; Delgado et al., 2018). In this area, there is a long-standing fishery targeting the scabbardfish, which has expanded geographically from the Madeira Archipelago until reaching as far as the Southern Azores Seamount Chain and the Canaries' Economic Exclusive Zone (EEZ) from 2005 onwards (Delgado et al., 2018). Fish are caught by small vessels operating with a horizontal drifting longline that is set at depths of 1000 to 1200 m in fishing grounds with depths varying between 1200 and 3000 m (Bordalo-Machado et al., 2009). In 2008, scabbardfish landings in Madeira reached a peak of 3100 t, but have been below 2500 t since 2009 (Vasconcelos et al., 2020a).

Based on genetic and meristic identification criteria, two species of the genus *Aphanopus* have been shown to coexist in some areas of the NE Atlantic: the black scabbardfish, *Aphanopus carbo* Lowe, 1839, and the intermediate scabbardfish, *A. intermedius* Parin, 1983 (Parin, 1983; Stefanni and Knutsen, 2007; Biscoito et al., 2011; Delgado et al., 2013). The two species are benthopelagic: *A. carbo* lives between 400 and 1800 m and *A. intermedius* from 700 to 1350 m (Parin, 1983; Pajuelo et al., 2008). *A. carbo* is widely distributed across the NE Atlantic, from the Strait of Denmark to off the Western Sahara coast, whereas *A. intermedius* occurs in the Azores, Madeira, the Canaries, and off the coasts of Morocco and the Western Sahara (Ehrich 1983; Nakamura and Parin, 1993; Stefanni and Knutsen, 2007; Biscoito et al., 2011; Delgado et al., 2013). *A. intermedius* attains larger sizes but at a slower growth rate and the mean size by age is significantly higher in *A. intermedius* than in *A. carbo* from age 11 onwards (Delgado et al., 2013; Vieira et al., 2009). The spawning period of both species overlaps: *A. carbo* ripening and spawning females are caught between September and December (Figueiredo et al., 2003; Neves et al. 2009) and *A. intermedius* ripening females were more frequent between September and November (Delgado et al., 2013).

Aphanopus landings in Madeira include both *A. carbo* and *A. intermedius*, in a proportion of 1:5, which are registered as a single commercial category named "espada" (the local Portuguese name

for black scabbardfish) (Delgado et al., 2013; Delgado et al., 2018). In 2008, Madeira's Regional Fisheries Directorate initiated a program for species assignment based on the combination of several meristic characters, namely the number of dorsal-fin spines, the number of dorsal-fin rays, the number of dorsal-fin elements, the number of pre-caudal vertebrae, the number of caudal vertebrae, and the number of total vertebrae (Biscoito et al., 2011).

The fishery in Madeiran waters has changed over time both in terms of effort (number of vessels and number of fishing days) and technical characteristics (e.g. number of hooks), but also in terms of geographical coverage, since more distant fishing grounds are presently being exploited (Bordalo-Machado et al., 2009; Delgado et al., 2018; Vasconcelos et al., 2020a,b). To understand if these changes have had a reflection on the presence of *A. intermedius* in the landings it would be important to quantify the occurrence of this species over time. Although, *Aphanopus* species are not separated in historical data collected from landings, port length sampling, or biological sampling, prior to 2008, time series of otolith collections for ageing have been routinely collected since the 1980´s (Morales-Nin and Sena-Carvalho, 1996), constituting valuable material that might provide information on both species.

Otoliths are calcified structures on the inner ear composed mainly of $CaCO_3$ (aragonite) precipitated on a protein matrix (Chang and Geffen, 2013). They are formed through an acellular process that involves the incorporation of external elements from different sources: (i) the external medium, where variations in abiotic factors occur; (ii) the blood plasma, which responds to the external medium and exhibits endogenous variations; (iii) the endolymph, which modulates the various signals and regulates the formation of the otolith; and (iv) the otolith itself, which integrates and records a response to all these signals (de Pontual and Geffen, 2002). Otoliths have species-specific shape (Lombarte and Castellón, 1991; Stransky and McLellan, 2005) and encode in their chemical composition the fish physiology and the environmental conditions of their habitat (Campana, 1999; Begg et al., 2005; Campana, 2005a; Sturrock et al., 2014, 2015). Based on these premises, fish from distinct species that coexist are expected to exhibit differences in terms of otolith composition and formation.

Many studies have proven the importance of otoliths for fisheries science, specifically in areas such as age determination, larval fish ecology, tracing spawning and nursery grounds, environmental reconstructions, population dynamics, and species identification (Meunier and Panfili, 2002; Pontual and Geffen, 2002; Begg et al., 2005; Campana, 2005a, 2005b; Geffen and Chang, 2013). In mixed-species fisheries, species assignment is a key issue because the differences in stock status and distribution might demand different management measures. Not accounting for the mixed-species aspect of the Madeira *Aphanopus* fishery might result in the collapse of *A. intermedius* because management measures set for the fishery are based only on the biology and resilience of *A. carbo*.

The present study combines different otolith traits to discriminate the two scabbardfish species landed in Madeira, namely (i) quantification and qualification of otolith elemental composition; and (ii) analysis of otolith contour shape. Our aim is to provide a tool to determine the relative abundance of both species throughout time for a better understanding and management of the Madeiran fisheries.

4.1.2. Material and methods

Sampling

Aphanopus carbo and *A. intermedius* specimens were collected from commercial landings in Funchal, Madeira Archipelago. All fish were caught by artisanal drifting longline vessels at a depth of around 1000 m. The taxonomic criterium described in Biscoito et al. (2011) was used to identify the species. This criterium combines the following meristic traits: number of dorsal-fin spines; number of dorsal-fin rays; number of dorsal-fin elements; number of pre-caudal vertebrae; number of caudal vertebrae; and number of total vertebrae.

Total length (TL) and sex were registered between October 2008 and July 2009 and a subsample of otoliths was randomly selected to cover the length distribution of *Aphanopus* spp. (Table 4.1.1). Additionally, length frequency distributions by sex and by species collected between 2010 and 2012 were analysed (Figure 4.1.2).

Table 4.1.1. Summary of black scabbardfish specimens used in each analysis. TL, total length; n, number; F, females, M, males.

		Length frequency		Microchemistry		Contour shape	
Species	**Sex**	**TL range (cm)**	**n**	**TL range (cm)**	**n**	**TL range (cm)**	**n**
A. carbo	F	101-148	938	-	-	126-135	106
	M	97-136	857	107-125	15	107-130	24
A. intermedius	F	96-149	246	-	-	126-135	55
	M	93-144	182	127-129	9	126-135	50
Total			2223		24		235

Right and left sagitta otoliths were extracted from each specimen, rinsed with distilled water, air dried, and stored in labelled plastic vials.

Trace element analysis

i. Otolith preparation

Right sagitta otoliths from adult males were prepared for laser ablation inductive coupled plasma mass spectrometry (LA-ICPMS). Males were used to avoid the effect of physiological and biochemical variations related with the reproductive cycle, namely gonad maturation and spawning. Otoliths were individually washed in ultrapure water type-I (MilliQ water) in an ultrasonic bath for 5 min, immersed in 2% HNO_3 (Suprapur quality) for 15 s, rinsed with ultrapure water type-I, and air dried for 24 h over lab paper. Clean otoliths were embedded in a thin layer of resin (Buehler® Epo-Thin Low Viscosity Epoxy) over a glass slide with the *sulcus acusticus* facing up. Four otoliths with similar size were mounted on each slide. Otoliths were ground and polished with an automatic polisher Struers Rotopol, using MilliQ water as lubricant. First, abrasive papers of decreasing grain (FEPA P1200, P2400, and P4000) were used to expose the cores and roughly polish the surfaces. Later, decreasing grain size diamond suspensions (3 μm and 1 μm) were applied with microcloth papers to polish the otolith until its surface showed a mirrored appearance. In the end, each slide was individually washed in MilliQ water in an ultrasonic bath for 5 min, immersed in 2% HNO_3 (Suprapur quality) for 15 s, three times rinsed with MilliQ water, air dried in a laminar flow hood and stored in a double zip bag until further analysis.

ii. LA-ICPMS analysis

Otolith chemical composition was determined using a NewWave UP-213nm Nd:YAG Laser Ablation System coupled to a Thermo Finnigan Element2 inductively coupled plasma mass spectrometer (LA-ICPMS; Resonetics, Resolution M50). High resolution photographs of the otoliths were compared with a video image of the ablation chamber to locate the defined ablation sites.

First, three laser ablation (LA) spots with 40 μm diameter were positioned in the otolith core. Then, spots with 40 μm diameter were located at 80 μm intervals (from centre to centre) along a linear transect from the otolith core to the ventral-posterior edge, following the otolith growth axis (Figure 1). The following isotopes were measured at each LA spot: ^{23}Na, ^{24}Mg, ^{39}K, ^{43}Ca, ^{44}Ca, ^{52}Cr, ^{55}Mn, ^{56}Fe, ^{63}Cu, ^{66}Zn, ^{85}Rb, ^{88}Sr, ^{112}Cd, ^{138}Ba, ^{202}Hg, and ^{208}Pb. Two fish otolith powder reference materials pressed into pellets, FEBS-1 (Sturgeon et al., 2005) and NIES No. 22 (Yoshinaga et al., 2000), and two glass reference materials, NIST 610 and NIST 612 (Pearce et al., 1997), were used for calibration and drift correction. All the reference materials were shot at the beginning of each slide, every 20 otolith spots, and at the end of the slide.

The raw LA-ICPMS output was corrected for any instrumental drift with the signal integration software Glitter (GEMOC, Macquarie University). Trace element concentrations (TEC) were calculated as $\mu g.g^{-1}$ using ^{43}Ca as internal standard and NIES certified values were selected for the calibration of the element concentrations since results on this standard followed the requirements on accuracy and precision described in Geffen et al. (2013). Minimum detection limits (MDL) for each spot and element were calculated with Glitter software as MDL = 2.3 $\sqrt{2}$B (where B is the number of total counts in the background interval).

Digital images of each otolith were taken after the LA-ICPMS. The position of each laser spot was expressed in terms of distance from the core and the corresponding annual age group was assigned based on otolith increment analysis, following the assumptions previously adopted for *A. carbo*. The first assumption is that an annual growth increment corresponds to the succession of an opaque and a translucent band (Morales-Nin et al., 2002; Vieira et al., 2009). Increments are accepted to be annual if they can be followed all the way around the otolith (ICES, 2013). Therefore, age can be assigned by counting only the translucent bands. The second assumption is that the birth date is the 1st of January since *A. carbo* spawning season is between September and December (Figueiredo et al., 2003; Neves et al., 2009; Ribeiro Santos et al., 2013) and *A. intermedius* mature females are more frequent in November (Delgado et al., 2013). The number of translucent increments is counted from the otolith core to the edge and the edge is classified as opaque or translucent (ICES, 2013). The assigned age (or annual age group) corresponds to the total number of translucent increments. The only exception is when the specimen is caught between January 1st and June 30th and the otolith has an opaque border, in which case the assigned age corresponds to the number of translucent increments plus one (ICES, 2013).

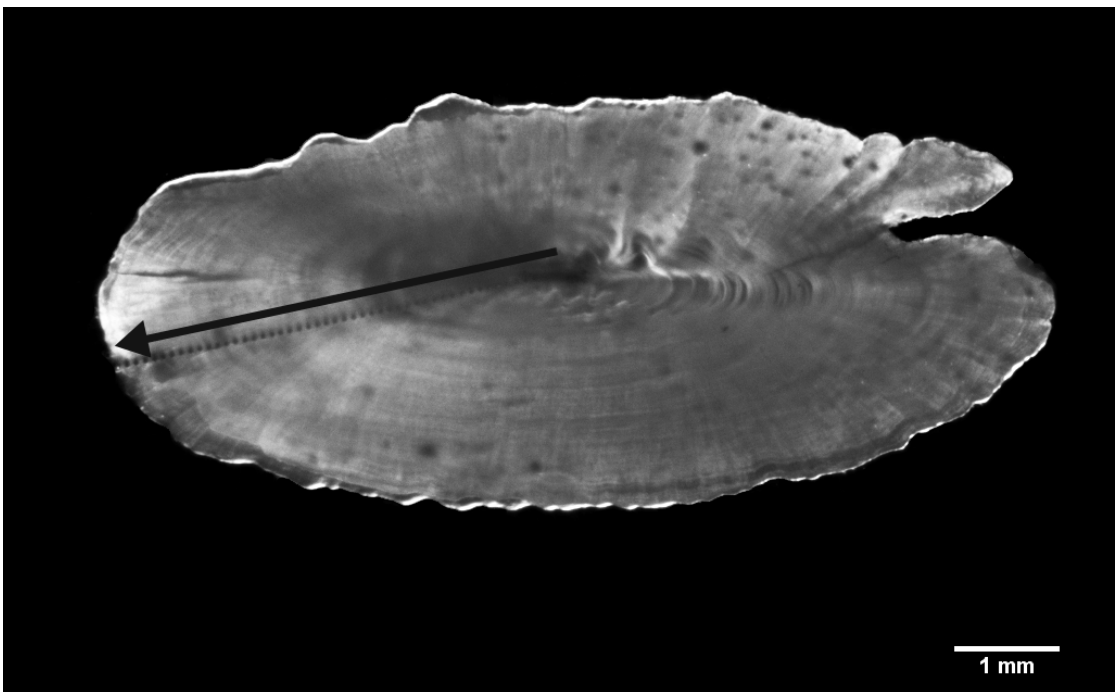

Figure 4.1.1. *Aphanopus carbo* right sagitta otolith, polished until the core was exposed, with the transect of laser ablation spots marked between the core and the ventral-posterior edge (black arrow). This specimen is a male with 117 cm total length.

iii. Data analyses

Otoliths for which there was a clear contamination from resin after polishing (visible on the otolith and/or detected through outlier TEC values) were excluded from the analyses. Moreover, for each otolith, all spots with missing values were deleted. Trace elements excluded from the proceeding analyses because either the values on the otoliths were below the minimum detection limits (MDL) or they were not detected at the Certified Reference Materials (CRMs) were: ^{7}Li. ^{55}Mn, ^{56}Fe, ^{63}Cu,

^{85}Rb, ^{88}Sr, ^{202}Hg, and ^{208}Pb.

Univariate ANOVA and non-parametric Kruskall-Wallis tests were applied to each TEC (^{23}Na, ^{24}Mg, ^{39}K, ^{52}Cr, ^{66}Zn, ^{88}Sr, ^{112}Cd, and ^{138}Ba) to test the equality between the two species, using the functions *aov* and *kruskal.test* from the *stats* library in R software (R Core Team, 2020).

Linear discriminant analysis (LDA) was applied to compare trace element composition between species at each age group, using the function *lda* from the *MASS* library in R. For each age, a principal component analysis (PCA) was used to identify and eliminate potential outliers using the *rda* function from vegan library version 2.0-10 (Oksanen et al., 2013) in R software.

Otolith contour shape

i. Otolith preparation

Digital images of left sagitta otoliths (n=235, Table 4.1.1) were acquired with the Noesis® TNPC 4.1 image analysis software using a Sony® DFW-SX910 digital camera linked to an Olympus® SZX9 stereomicroscope. Otoliths were photographed using the same magnification and ensuring that otoliths collected from fish with different total lengths fit the image. Otoliths were placed with the *sulcus acusticus* facing down and the rostrum towards the left side of the image and the images were acquired using a black background and reflected light, enhancing the colour contrast between the otolith and the background. Malformed and damaged otoliths were not included in the analysis.

ii. Contour extraction

A MATLAB script developed by Palmer et al. (2010) was used to automatically extract the otolith contour, using a threshold-based method. For each otolith, the starting point was marked at the tip of the rostrum and a random point in the middle of the otolith was set as reference. The extracted contour was then adjusted to an elliptic Fourier transformation adapted from Claude (2008) and formulated by Rohlf (1993). The elliptic Fourier transformation decomposes a closed contour, in this case the otolith contour shape, into a series of harmonics, called elliptic Fourier descriptors (EFDs), which are a series of sine and cosine curves that are generated by taking a Fourier expansion of radius vectors drawn from the centroid of the object as a function of the phase angle (Younker and Ehrlich, 1977; Bird et al., 1986). The EFDs are normalised in relation to the first harmonic (represented by an offset circle and with almost no information on the contour shape), and consequently they become invariant to size, rotation and starting point or location (Kuhl and Giardina, 1982).

iii. Data analyses

Multivariate analysis of variance (MANOVA) was applied to test the equality of the normalized elliptic Fourier descriptors (NEFDs) between the two species, using the *rda* function from *vegan* library (Oksanen et al., 2013) in R software.

After removing the effect of otolith size, discriminant analysis was adjusted to the NEFDs using a PCA and Random Forests (RF) conditioning the analysis to the effect of sex, using the *vegan* (Oksanen et al., 2013) and the *randomForest* (Breiman, 2001) libraries, respectively, in R software.

4.1.3. Results

Length distribution

The total length distribution of specimens collected at Funchal landing port (Madeira) between 2010 and 2012 (1795 *A. carbo* and 428 *A. intermedius*) was analysed (Figure 4.1.2). For both species, the smaller individuals sampled were females and the larger specimens were males. Nonetheless, the distribution medians and modes were higher for females than for males in both species.

Otolith elemental composition

Three specimens were excluded from the otolith elemental composition data analyses because it was visible that the laser had perforated the otolith and hit the resin. This happened because the otolith became too thin after grinding and polishing. Mn, Fe, Cu, and Hg, were excluded from the analysis because results were below the MDL. The highest TEC found in the otoliths of *A. carbo* were, in descending order, Sr, Na, and K, whereas in the otoliths of *A. intermedius* were Na, Sr, and K (Table 4.1.2). Cd and Zn were the elements with the lowest concentrations in both species. Cr, Sr, and Ba concentrations were significantly different between *A. carbo* and *A. intermedius* at a significance level of 0.05, with both ANOVA and Kruskall-Wallis test.

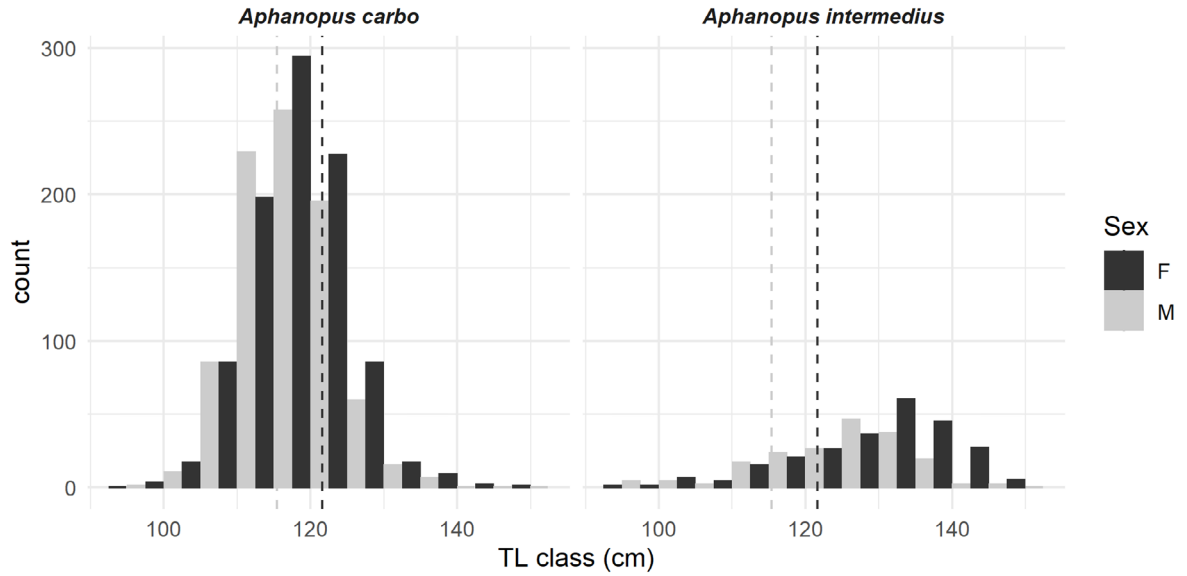

Figure 4.1.2. Total length distribution of *Aphanopus carbo* (N = 1795) and *A. intermedius* (N = 428) specimens collected between 2010 and 2012 off Madeira. The dashed lines are the medians of the distributions.

Table 4.1.2. Mean concentration (± standard deviation) of trace elements detected in the otoliths by LA-ICPMS ($\mu g.g^{-1}$) and results of ANOVA and Kruskall-Wallis tests investigating differences between *A. carbo* and *A. intermedius*.

Trace element	A. carbo		A. intermedius		d.f.	ANOVA		Kruskall-Wallis	
	Mean	SD	Mean	SD		F	p	$\chi 2$	p
^{23}Na	$2.30x10^{3}$	$3.41x10^{2}$	$2.51x10^{3}$	$3.26x10^{2}$	1	2.26	0.150	2.15	0.143
^{24}Mg	$1.37x10^{1}$	3.06	9.85	1.11	1	13.51	**0.002**	3.72	0.054
^{39}K	$2.66x10^{2}$	$4.54x10^{1}$	$2.74x10^{2}$	$6.04x10^{1}$	1	0.16	0.692	0.86	0.355
^{52}Cr	5.41	1.50	4.35	$5.69x10^{-1}$	1	4.44	**0.049**	5.72	0.017
^{66}Zn	$1.37x10^{-1}$	$1.09x10^{-1}$	$1.03x10^{-1}$	$1.41x10^{-1}$	1	1.13	0.301	1.72	0.190
^{88}Sr	$2.44x10^{3}$	$4.54x10^{2}$	$1.98x10^{3}$	$2.64x10^{2}$	1	21.79	**< 0.001**	11.52	**< 0.001**
^{112}Cd	$2.50x10^{-3}$	$4.17x10^{-4}$	$2.27x10^{-3}$	$4.74x10^{-4}$	1	1.71	0.207	1.52	0.217
^{138}Ba	$1.25x10^{1}$	$2.73x10^{-1}$	$8.69x10^{-1}$	$1.93x10^{-1}$	1	45.31	**< 0.001**	13.71	**< 0.001**

A. intermedius presented lower concentrations of Mg, Cr, Sr, and Ba but with some specimens overlapped with *A. carbo* in Cr and Mg (Figure 4.1.3). Element variation along the entire lifespan was similar for both species: Mg and Ba presented higher concentrations at birth, strongly decreasing at

age 1; Sr followed an increasing trend from birth to catch date showing higher variability from age 4 hereafter; Cr presented a similar trend with age in both species but with high intraspecific variability, specially

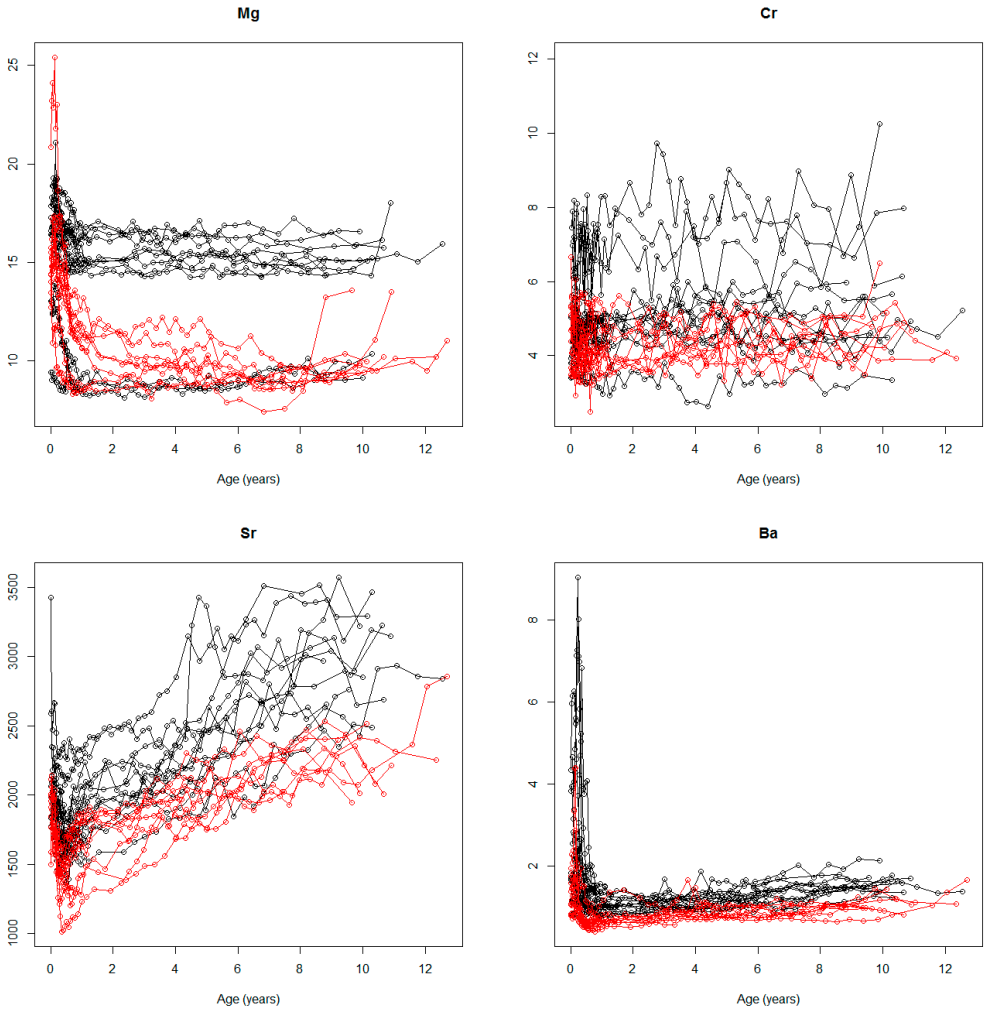

Figure 4.1.3. Trace element concentration (µg.g^{-1}) profiles of ^{24}Mg, ^{52}Cr, ^{88}Sr, and ^{138}Ba detected in the otoliths of *Aphanopus carbo* (black) and *A. intermedius* (red) as a function of fish age.

LDA revealed that the best ages to separate *A. carbo* and *A. intermedius* based on trace element composition were ages 6 (K = 0.89), 9 (K = 0.89), and 10 (K = 0.88), where the kappa index k is a measure of classification success used for unbalanced samples (Fielding and Bell, 1997; Jones et al., 2017). The trace element composition was significantly different between species at those ages (Table 4.1.3). At age 9, there was a clear separation between the two species in terms of otolith trace element composition (Figure 4.1.4).

Table 4.1.3. Summary of ANOVA for LDA models adjusted to otolith TEC on selected ages.

Age	Model	d.f.	var	F	No. Perm.	p-value
6	Factor Species Residual	1 18	1.1817 5.8183	3.6559	999	0.001
9	Factor Species Residual	1 44	1.4743 5.5257	11.74	999	< 0.001
10	Factor Species Residual	1 25	1.3296 5.6704	5.8619	999	< 0.001

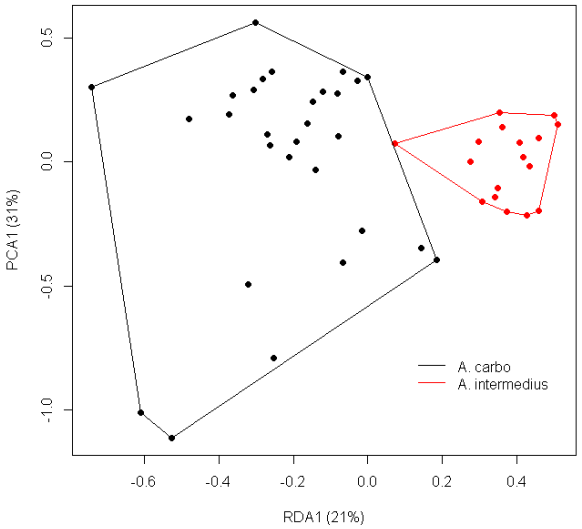

Figure 4.1.4. PCA for trace element composition at age-class 9.

Otolith contour shape

NEFDs were not significantly different between *A. carbo* and *A. intermedius* (for 999 permutations, F = 1.28; d.f. = 1; p = 0.232). Both PCA and RF could correctly classify more than 50% of specimens from both species (Table 4.1.4). PCA's discriminant power was 59%, whereas with the RF the classification agreement was 47%. RF proved to be a better classification method for these species than the PCA although the classification success was poor for both methods.

Table 4.1.4. Classification success of PCA and RF adjusted to otolith contour shape of *A. carbo* and *A. intermedius*.

	PCA		**RF**	
	A. carbo	*A. intermedius*	*A. carbo*	*A. intermedius*
A. carbo	62%	38%	85%	15%
A. intermedius	45%	55%	39%	61%

4.1.4. Discussion

The chemical signatures of sagitta otoliths of *A. carbo* from six different locations on the NE Atlantic had previously been compared through solution-based ICP-MS (Swan et al., 2003). More recently, Longmore et al. (2014) found significant heterogeneity in *A. carbo* otolith chemical signatures between life stages, but no genetic structure nor significant differences in otolith element composition between the sampled areas. The strong overlap in otolith microchemistry across areas could result from overall panmixia or physicochemical similarities between the environments. Although genetic divergence in deep-sea fishes is generally low or nearly absent, when it occurs it may reveal cryptic patterns (Longmore et al., 2014).

In the present study, trace element composition was determined for *A. carbo* and *A. intermedius* by age class throughout their lifetime. Although age 6 presented the best classification index, it should not be used for species identification because it is close to the age at first maturity in A. carbo (Neves et al., 2009). During maturation, the incorporation of the protein and calcium carbonate layers in the otolith matrix is affected by physiological changes, namely the production of proteins for oogenesis, specially the calcium-binding protein vitellogenin (Geffen et al., 2002). Kalish (1991) observed

significant changes in the level of free Ca in the blood plasma and endolymph associated with the increase in the gonadosomatic index. This relationship between Ca available for otolith growth and for maturation is the reason why only male fish were used for microchemical analysis in the present study. The present results suggest that quantifying Mg, Cr, Sr, and Ba in the otolith increment that corresponds to age-class 9 can be an effective way to identify the species using LA- ICPMS at reduced costs comparing to quantifying TEC along a transect. Since the otolith increments in these species are very thin at higher ages, most of the times only one laser spot fits in each age-class. A solution to minimize the intraspecific variability that results from having one single value per age-class is additionally sampling age-class 10 because it also presented a good discriminating ability.

The mechanisms controlling uptake and incorporation of elements into the otolith are mediated by both exogenous (e.g. temperature and salinity) and endogenous (e.g. growth rate or stress) factors and their interaction (Walther et al., 2010). According to Thorrold et al. (1997), the differences in Mg and Ba, two elements that contributed to separate the *Aphanopus* species, reflect environmental variability between locations, and Ba replicates the nutrients' distribution. In samples from the Madeira fishery, it is not possible to determine if specimens collected from the same fishing trip were caught in the same haul, hence, at the same location and depth. Therefore, the differences found between species might result from different vertical or horizontal distribution of the two *Aphanopus* species. Tuset et al. (2010) have suggested that *A. carbo* and *A. intermedius* live in different spatial niches and that *A. carbo* inhabits deeper waters based on differences in eye diameter.

On the other hand, Sturrock et al. (2014) demonstrated that for the thiophilic elements (Mn, Cu, Zn, Se, and Pb) and the quasi-conservative elements (Sr and Ca) physiological influences could outweigh environmental signals, whereas hard acid metal ions like Li^{+}, Mg^{2+}, K^{+}, Rb^{+}, and Ba^{2+} were less affected by physiology, with reduced temporal variation. Variations in Mg values were best explained by salinity but higher uptake rates were also linked to increased metabolic activity (Sturrock et al., 2014), hence the present results might also support physiological differences between the two *Aphanopus* species.

Another interesting result is the absence of mercury (Hg) in the trace element analysis. Previous studies in Portuguese waters have shown that *Aphanopus* accumulates large amounts of total Hg in soft tissues like muscle, gonads, and liver (Afonso et al., 2007, 2008; Bebiano et al., 2007; Costa et al., 2009; Cardoso et al., 2010). Hg has also been detected in the endolymph of *Acanthopagrus butcheri* inner ear (Thomas et al., 2017). To reach the endolymph and crystallize on the surface of the otolith, ions must pass through two membranes (Campana, 1999) by a passive process (de Pontual and Geffen, 2002). The fact that Hg could not be detected in the otoliths of both *A. carbo* and *A. intermedius* suggests that either the concentration was too low to be detectable by LA-ICP-MS or that the otolith membranes are somewhat permeable to this element. Geffen et al. (1998) have suggested, from experiments with reared plaice and sole specimens, that Hg moves easily into the otolith some period after the initial exposure and its availability for deposition depends on the concentrations in the body. However, they have also seen that different species have distinct physiological mechanisms that can detoxify Hg in the body and excrete it.

A. carbo and *A. intermedius* have been successfully separated based on morphological and meristic traits, namely the relationship between cephalic length and eye diameter; otolith height; otolith weight; and sulcus acusticus area; and the relationship between otolith weight and otolith length and otolith height (Tuset et al., 2010). Those differences were thought to be dependent on the depth distribution of the species: *A. carbo* was assumed to inhabit deeper waters than *A. intermedius* because it presented larger eye diameter, larger otolith weight and height, and larger *sulcus acusticus* area. In a previous study, otolith contour shape was effective in separating *A. carbo* caught off different geographical locations in the southern NE Atlantic, namely mainland Portugal, Madeira, and the Azores (Farias et al., 2009). Using otolith mass and Fourier descriptors, Tuset et al. (2013) found significant differences between *A. carbo* and *A. intermedius* and between specimens of the same species caught off different locations, but the separation was not clear when species and location were combined (Tuset et al., 2013). In that study, the number of specimens (6-25 specimens per area and species) was too small and variable to eliminate the effect of the interaction between species and location and make robust inferences based only on morphometric traits. These results might indicate that environmental factors have a stronger effect than genetic factors in the shape of *Aphanopus* otoliths. To test the previous

hypothesis, it would be important to apply otolith shape analysis to larger samples of both species from different locations than what was used in Tuset et al. (2013) to eliminate the possible effect of intraspecific and intraregional variability.

In the present study, the Fourier shape analysis was not conclusive for species separation. There were two problems with the samples used in this analysis. First, the wide TL range could be responsible for the confounding effect of allometric growth on otolith shape (Cardinale et al., 2004). Secondly, the intraspecific variability was higher than the interspecific variability because the sample size was small. Additionally, the fact that Fourier shape analysis could not detect differences between the two species using specimens caught in the same area may be an evidence of a higher dependency of their otoliths' contour shape on environmental variables rather than on genotype. Although it is generally recognised that otolith growth and shape are genetically regulated, there is a strong variability related to environmental factors (Vignon and Morat, 2010; Morat et al., 2012) but it is unclear to what extent and how each of those aspects affect otolith shape (Begg and Brown, 2000; Simoneau et al., 2000). While otolith shape is genetically constrained, species specific, and reflects phylogenetic relationships (Lombarte and Castellón, 1991; Lombarte and Lleonart, 1993), it is also subject to intraspecific variation, mainly influenced by sex, age, year class, and stock (Castonguay et al., 1991; Begg and Brown, 2000; Monteiro et al., 2005), as well as by local environmental conditions such as depth, water temperature, substrate type, and feeding (Lombarte and Lleonart, 1993; Gagliano and McCormick, 2004; Mérigot et al., 2007; Hüssy, 2008). However, intraspecific genetic variations due to long-time separation only affect otolith shape locally, mainly in the rostrum and antirostrum (Vignon and Morat, 2010).

This and previous studies (Biscoito et al., 2011; Longmore et al., 2014; Delgado et al., 2018) have demonstrated that otoliths are an efficient tool to separate *A. carbo* and *A. intermedius*. Given that the species only started being separated in landings from Madeira in 2008 and that the last only represents 20% of the landings (Delgado et al., 2013; Delgado et al., 2018), it was difficult to obtain otolith samples that were balanced and large enough to make robust inferences. Nonetheless, otolith trace element composition can be applied to archived collections of otoliths to estimate the historical frequency of occurrence of *A. intermedius* in landings, not only in Madeira but also in the Azores and the Canaries, where genetic studies have detected that both species coexist (Stefanni and Knutsen, 2007) and where vessels from Madeira operate (Delgado et al., 2018). The proportion of each species in total catches throughout time could further be related with fluctuations in catches, with fishing effort data (fishing grounds, number of vessels, number of hooks, depth inferred from the length of the gear), and management or regulatory measures that have changed throughout time. Another interesting approach would be to use rapid low-cost DNA extraction methods, which can lead to over 80% success of microsatellite amplification in otoliths using both contemporary and archived samples, proving to be suitable for conservation genetic studies (Schaerlaekens et al., 2011; Meissner et al., 2013). Analysing DNA from historical collections has provided new knowledge about demographic parameters and population structure in wild populations, as well as new insights into the effects of over-harvesting, habitat degradation and stocking, which were used to make significant changes in management practices (Nielsen and Hansen, 2008). Perceiving the historical presence of *A. intermedius* in commercial landings in Madeira might contribute to establishing better management measures, species-specific if necessary (as suggested in Vasconcelos et al., 2020b), and inferring if the species is moving northwards and since when or if it has always been in the vicinities of Madeira but the frequency of occurrence increased with the geographical expansion of the area covered by the fishing fleet.

Acknowledgements

The authors would like to thank João Delgado and the technical staff in Madeira (DSIDP) for the otolith samples and Alicia Cantarero (University of A Coruña) for helping with the LA-ICPMS analyses. I. Farias was supported by a doctoral fellowship granted by Fundação para a Ciência e Tecnologia (SFRH/BD/64434/2009).

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4.2. Ontogenic spatial dynamics of the deep-sea teleost *Aphanopus carbo* in the NE Atlantic according to otolith geochemistry

Inês Farias, Sílvia Pérez-Mayol, Sofia Vieira, Paulo B. Oliveira, Ivone Figueiredo and Beatriz Morales-Nin

Abstract

The distribution of deep-sea fish is related to major water masses or regional circulatory features and reflects differences in food-web structure and productivity. *A. carbo* is a benthopelagic species that undergoes horizontal and vertical migrations driven by spawning and by feeding, and for which a hypothetical large-scale clockwise migration around the NE Atlantic is accepted. The adequacy of otolith microchemical composition in discriminating *A. carbo* specimens caught at different areas was analysed. Furthermore, potential birth areas and spatial pattern throughout the species life cycle were investigated. Trace element concentrations (TEC) in the otolith edge could discriminate the locations where specimens were caught and supported the discrimination between the northern and the southern areas. The longitudinal multivariate analyses of TEC also sustained the separation of the otoliths into two groups, but there is high mixing between them, supporting the migratory hypothesis. The existence of two natal sources was suggested from otolith core TEC analysis. The acceptance of both southern and northern spawning grounds and of migratory movements along the NE Atlantic in both northward and southward directions implies changes to the current migratory hypothesis that might translate into changes in *A. carbo* stock assessment.

Keywords: Black scabbardfish, Northeast Atlantic, Otolith microchemistry, LA-ICP-MS, Trace elements

4.2.1. Introduction

The deep-sea is the region below the epipelagic zone, where sunlight no longer penetrates and whose upper boundary is generally set at 200 m depth, being characterized by high pressure, low temperature, low food availability, and harsh predation (Merrett and Haedrich, 1997; Herring, 2002). Some researchers support that the deep-sea is bounded by the thermocline, the distinct layer that marks a sharp temperature decrease at the base of the surface mixed layer where waters are generally uniform in temperature. Species living just above and in straight connection with the sea floor are benthopelagic species, usually covering depths between 200 and 1000 m (Haedrich, 1996). However, they are adapted to scatter in shallower depths throughout their lifetime – e.g. floating eggs, larvae feeding in the surface layers, vertically diel migrations of adults to feed (Priede, 2017). The distribution patterns of deep-water demersal fish are related to major water masses or regional circulatory features, also reflecting differences in food-web structure and productivity (Haedrich and Merrett, 1988, 1990; Koslow, 1993; Bergstad et al., 2012).

The Northeast Atlantic Deep Water (NADW) is formed by the mixing of four major local deep-water sources: (1) the Iceland–Scotland Overflow Water (ISOW), (2) the Lower Deep Water (LDW), (3) the Labrador Sea Water (LSW), and (4) the Mediterranean Sea Water (MSW) (van Aken, 2000). The ISOW originates from the Norwegian Sea, on the north side of the Reykjanes Ridge, between Greenland, Iceland, and Scotland, where the surface ocean is cooled and flows to the eastern subpolar gyre mainly through the Faroe Bank Channel and the Faroe-Shetland Channel, and can be traced southwards as far as the Madeira Abyssal Plain (van Aken and De Boer, 1995; van Aken, 2000; Eldevik et al., 2009; Zou et al., 2017). The LDW is the dominant water type near the bottom layer, characterized by low salinity and low dissolved oxygen content (van Aken, 2000). The LSW are cold and less saline waters that enter the eastern North Atlantic basins near the Charlie-Gibbs Fracture Zone. The MSW is the shallow high-salinity water that originates from the overflow of sub-surface water from the Mediterranean Sea near Gibraltar.

These water masses are connected by major water flows and currents along the eastern side of the Mid-Atlantic Ridge (MAR), which are part of a global-scale system of ocean currents known as the Thermohaline Circulation or the Meridional Overturning Circulation (MOC) which is driven by

differences in temperature and salinity. At Central Water depths, the North Atlantic Current (NAC) and the Azorean Current (AC) may be regarded as two branches of the Gulf Stream that diverge around the Great Banks, the first going north along the eastern slope of the Grand Banks and the second going east and crossing the MAR (Daniault et al., 2016). The Canary Current (CC) is a cold current that integrates the Atlantic Subtropical Gyre at about 500 m deep (Wooster et al., 1976). At intermediate levels (800-1400 m), the Mediterranean outflow water (MOW) originates as a bottom outflow of dense, cold (12.9 °C), and saline (38.45 ‰) water that runs through the Strait of Gibraltar accompanied by a surface inflow of the warmer (16.6-22.6 °C) and less saline (36.5 ‰) Atlantic Inflow (AI) Water (Price et al., 1993; Sánchez-Leal et al., 2017).

Aphanopus carbo, black scabbardfish, is a benthopelagic species that undergoes horizontal and vertical movements driven by spawning and by feeding (Zilanov and Shepel, 1975; Clarke and Wagner, 1976; Du Buit, 1978; Ehrich, 1983; Anon., 2000). The agreed migratory hypothesis states that this species undergoes a large-scale clockwise migration around the NE Atlantic (Figueiredo et al., 2003; Farias et al., 2013; Ribeiro Santos et al., 2013; Santos et al., 2013). Spawning adults occur around Macaronesia (Figueiredo et al., 2003; Ribeiro Santos et al., 2013) but juveniles are caught as far north as Iceland and the Faroe Islands (Ehrich and Cornus, 1979; ICES, 2020). Young immature adults are found west of the British Isles, where they remain some years feeding and growing, and off mainland Portugal, where the species reaches larger sizes and, occasionally, pre-spawning adults are found (Farias et al., 2013; Ribeiro Santos et al., 2013). Posteriorly, adults move from those locations to the spawning areas. So far, the location of eggs and larvae is the missing link in the species life cycle – the smallest specimens (ca. 10 cm) have been reported off Madeira (Maul, 1950) and the northeast of Cape Verde (Hanel et al., 2010), and there are records of specimens with 26 cm caught off Iceland (Farias et al., 2013; ICES, 2020). This migratory hypothesis is substantiated by differences found in the size distribution, maturity, and chemical markers, such as stable isotopes, fatty acids, and otolith chemical composition (Figueiredo et al., 2003; Farias et al., 2014; Santos et al., 2013; Longmore et al., 2014).

Otoliths are concretions located in the inner ear of fishes, formed from the crystallisation of calcium carbonate, in the form of aragonite, on an organic matrix composed largely of a keratin-like protein, the otolin (Degens et at., 1969; Watabe et at., 1982; Morales-Nin, 1987; Wright et al., 2002). Because the otolith is metabolically inert, the continuous deposition of trace elements from the environment creates an elemental pattern or chemical signature that is preserved throughout the life of the fish, providing a natural tag of the environment where it the fish has lived (Gillanders and Kingsford, 1996; Campana, 1999; Campana et al., 2000; Campana and Thorrold, 2001; Bailey et al., 2015).

Otolith chemical composition has been successfully used for predicting migratory movements whenever tagging and tracking are not feasible (Elsdon and Gillanders, 2003; Campana, 2005; Sturrock et al., 2012), for comparing population structure or define stock delimitation (Ashford et al., 2006; Higgins et al., 2013; Reis-Santos et al., 2018; Wright et al., 2018), for investigating connectivity during ontogenetic development (Campana et al., 1994; Longmore et al., 2014; Morales-Nin et al., 2014; Régnier et al., 2017; Rogers et al., 2019), for tracing natal origin and nursery grounds (Thorisson et al., 2011; Guidetti et al., 2013), for determining age and growth patterns (Hüssy et al., 2015; Siskey et al., 2016), and for reconstructing environmental exposure histories (Thorrold et al., 1997; Stanley et al., 2015).

The present study aims to clarify *A. carbo* population dynamics and structure in the NE Atlantic using otolith chemistry, namely (i) assessing the species otolith trace element signature around its distribution area, from Iceland to the Canary Islands; (ii) evaluating the adequacy of otolith trace element composition to discriminate specimens caught at different locations; (iii) investigating the species potential birth areas; and (iv) inferring the species spatial pattern throughout its life cycle. All this information will contribute to determine the life history of a deep-sea species with a wide distribution and which represents a relevant fisheries resource that has been intensively exploited.

4.2.2. Material and methods

Sampling

A. carbo specimens were collected between 2006 and 2014 at six different locations in NE Atlantic: Iceland, the Faroe Islands, west of the British Isles, mainland Portugal, Madeira Archipelago, and Canary Islands (Figure 4.2.1, Table 4.2.1). Samples from the northern locations (Iceland, Faroes, and west of the British Isles) were caught by bottom trawling in scientific surveys. Specimens from mainland Portugal were collected by a single fishing vessel that operated at the same fishing ground over the years. Fish samples were requested to the skipper prior to the fishing trip because black scabbardfish for auction are eviscerated onboard. Each fishing trip lasts around 24 h and a single horizontal bottom longline is deployed in each trip and hauled at the next trip. The coordinates and depth were an approximation based on data records from inquiries. Specimens from Madeira were landed whole and the samples were bought at the auction. Here, a fishing trip lasts from 15 to 20 days, during which vessels move around different fishing grounds deploying a horizontal mid-water drifting longline, hence, it is not possible to know the exact location where a specimen was caught. In this case, the coordinates and depth of a theoretical sampling site were calculated as the mean of the haul coordinates recorded in trips from the same vessel sampled in 2010. Samples from the Canary Islands were collected from an experimental fishery using a horizontal mid-water drifting longline (for more information, see Pajuelo et al., 2008).

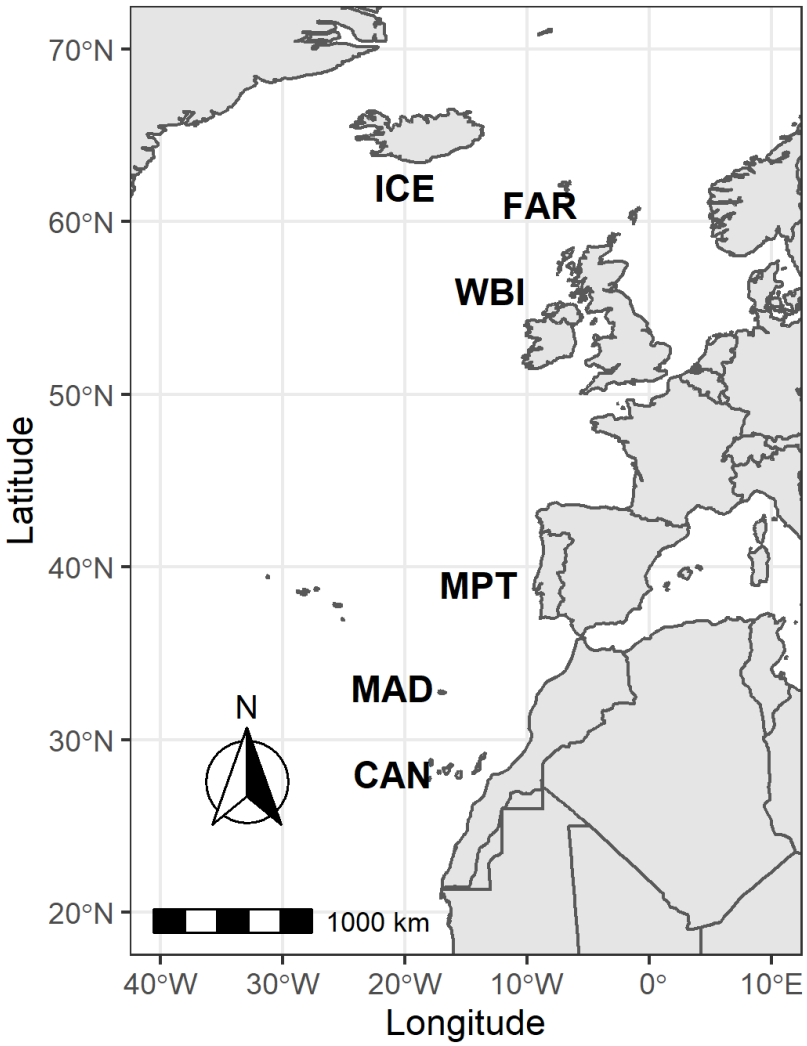

Figure 4.2.1. Map of the Northeast Atlantic showing *Aphanopus carbo* sampling locations. ICE, Iceland; FAR, Faroe Islands; WBI, West British Isles; MPT, Mainland Portugal; MAD, Madeira; CAN, Canaries.

For each sampled specimen, total length and sex were recorded and *sagitta* otoliths were extracted. For each location, male specimens were randomly selected among the collected samples and their right *sagitta* otolith was used for further analyses.

Table 4.2.1. Summary of *Aphanopus carbo* samples. TL is total length range in cm; n is number of specimens.

Location	Year	Month	Depth	TL (cm)	N
Iceland	2010	11	530-590	72-104	41
	2014	10	510-770		
Faroes	2014	09	580-830	76-116	30
W British Isles	2009	12	620-1250	71-108	44
	2011	09	400-1800		
Mainland Portugal	2008, 2010-2014	01-02, 04-12	600-800	71-122	44
Madeira	2010-2011	01-05, 07-12	1200-1500	88-142	45
Canaries[1]	2006	09	800-2300	107-119	11

[1] Source: Pajuelo et al. (2008)

Otolith preparation

All laboratory material was acid-cleaned prior to use by soaking in 4% HNO_3 (PP and Teflon coated tweezers) or 10% HNO_3 (glass material, such as Petri dishes, slides, beakers) for a minimum of one day and rinsing under running Milli-Q water.

Otoliths were individually washed in an ultrasonic bath with Milli-Q water for 5 min, immersed in 2% HNO_3 for 15 s, rinsed with Milli-Q water, and air dried for 24 h over lab paper. Each cleaned otolith was imbedded in a small block of resin (Buehler® Epo-Thin Low Viscosity Epoxy) and a 0.5 mm transverse section enclosing the primordium was cut using a Buehler® IsoMet® Low Speed Saw.

Otolith sections were individually mounted on a coverslip with Crystalbond® and fixed to a glass slide. Each section was polished manually using lapping papers of decreasing grain (1200, 2400, 4000) and Milli-Q as lubricant, to roughly polish the otolith section. Decreasing grain size diamond suspensions (3 μm and 1 μm) were then applied with a polishing cloth until the surface of the otolith section showed a mirrored appearance. Finally, each glass slide was individually washed in Milli-Q water in an ultrasonic bath for 5 min, immersed in 2% HNO_3 for 15 s, rinsed with Milli-Q water, and air dried.

Otolith sections were photographed with a Sony® DFW-SX910 camera coupled to an Olympus® SZX9 stereomicroscope, using the image analysis software Noesis® TNPC 4 (developed with Ifremer), which is based on the Visilog 6™ platform. Annual growth increments were assigned for each otolith following the assumptions adopted for *A. carbo*: i) an annual growth increment corresponds to the succession of an opaque and a translucent band (Morales-Nin et al., 2002; Vieira et al., 2009); ii) the birth date is 1st January since its spawning season is between September and December (Figueiredo et al., 2003; Neves et al., 2009; Ribeiro Santos et al., 2013). In each picture, the growth axis and the annual growth increments were marked on the ventral side of the otolith section.

The embedded otolith sections were detached from the glass slides and glued with Henkel® Loctite to a petrographic glass slide in random order to avoid sample bias. Each slide was then sonicated in Milli-Q water and cleaned in acetic acid ultrapure 5% and running Milli-Q water.

Otolith elemental analysis

Otolith chemical composition was determined using a NewWave UP-213nm Nd:YAG Laser Ablation System coupled to a Thermo Finnigan Element2 inductively coupled plasma mass spectrometer (LA-

ICPMS; Resonetics, Resolution M50). High resolution photographs of the otolith sections with the growth axis and increments previously marked were compared with the live video image of the ablation chamber to locate the ablation sites (Ferguson et al., 2011). The laser conditions were set at 10 Hz repetition rate, 60% output and 49 s dwell time. The laser was programmed to run ablation lines with 147 µm of length, 55 µm of width, and 15.7 µm of depth along the otolith growth axis. Ablation lines were used instead of spots to maximise the otolith material available for chemical analysis. Each otolith was sampled with an ablation line positioned at the opaque growth increment in four distinct otolith zones (Figure 4.2.2) that corresponded to the following life-history stages:

i. Core, which represents the time of birth;

ii. Age 3, which is a clear and wide increment in this species;

iii. Age 5, which corresponds to the age right before 50% of the population are mature (L_{50},) and was also the TL mode in otoliths from Iceland (88 cm TL);

iv. Edge, which represents the end of the specimens' life time.

The core was ablated close to the edge to avoid maternal effect.

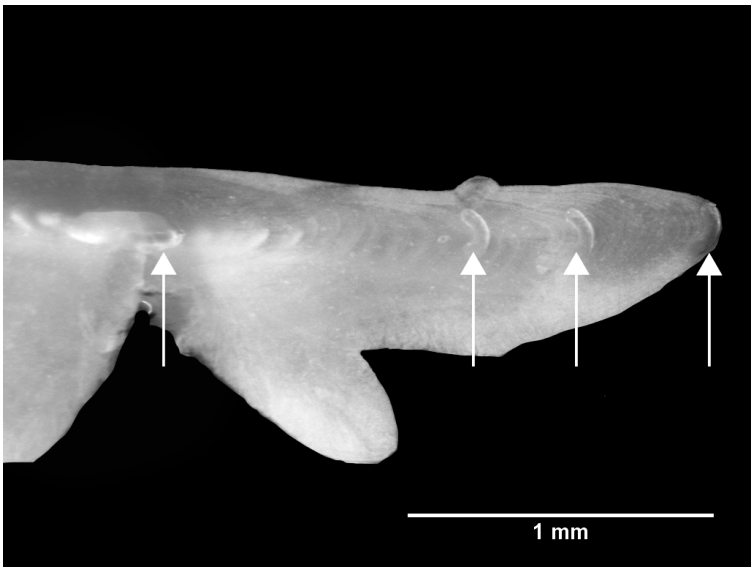

Figure 4.2.2. *Aphanopus carbo* right sagitta otolith section, with the ablation lines marked (white arrows). This specimen is a male with 110 cm total length.

The following isotopes were measured at each ablated line in the otolith: ^{7}Li, ^{23}Na, ^{24}Mg, ^{43}Ca, ^{44}Ca, ^{55}Mn, ^{63}Cu, ^{65}Cu, ^{66}Zn, ^{137}Ba, and ^{138}Ba. Three reference materials were employed, FEBS-1 (Sturgeon et al., 2005) NIES No. 22 (Yoshinaga et al., 2000), and NIST 614 (Kurosawa et al. 2002). The reference materials were used for calibration and drift correction and were measured at the beginning and end of each slide and every twenty ablations over the otoliths. The raw LA-ICPMS output was corrected for any instrumental drift with the signal integration software Glitter (GEMOC, Macquarie University). Trace Element Concentrations (TEC) were calculated as $\mu g.g^{-1}$ based on natural isotope ratios. Values below the limit of detection (LOD) were set to LOD/ $\sqrt{2}$ (Geffen et al., 2011). TEC were standardized against ^{44}Ca to correct for sample-to-sample variation in laser energy and ablated mass (Campana et al., 1994), being expressed as $\mu mol\ TEC.mol^{-1}$ Ca, and are hereafter referred to as Li, Na, Cu, Mn, Mg, Zn, and Ba. Due to the high correlation with their isotopes and since the interferences are always additive (De Pontual et al., 2000), ^{63}Cu and ^{138}Ba were excluded and the isotopes with the lowest natural abundance were selected: ^{65}Cu and ^{137}Ba.

Data analyses

Correlations among TEC were tested with a Pearson rank correlation test, combining data from all otolith zones, using the function *chart.Correlation* of the library *PerformanceAnalytics* (Peterson and

Carl, 2020) in R software (R Core Team, 2020).

Univariate analysis of variance (ANOVA) was performed to test which TEC could be used to discriminate between sampling locations for each otolith zone using the function *aov* from the *stats* library in R (R Core Team, 2020). ANOVA assumptions of normality and homogeneity of variances (homoscedasticity) were tested using the Shapiro-Wilk's test (function *shapiro.test* from the *stats* library in R) and the Levene's test (function *leveneTest* from the *car* library in R (Fox and Weisber, 2019)), respectively. When normality was not met, data were transformed with the Box-Cox formulae (Jones et al., 2017) using the function *boxcox* from the *MASS* library in R (Venables and Ripley, 2002). Tukey's HSD (honestly significant difference) test (function *TukeyHSD* from the *stats* library in R) was used for pairwise comparisons of means among regions. TEC that did not meet the assumptions or were found not to significantly differ between sampling locations (at a significance level of 0.05) were removed from further analyses.

Quadratic Discriminant Analysis (QDA) was applied to predict the membership of specimens to the location where they were caught, at each of the life stages represented by the different otolith zones, using the function *qda* from the *MASS* library in R. Only TEC that met the assumptions of normality and that significantly differed between regions were considered for QDA. The kappa index, k, was used as a measure of the classification success because it is unbiased when the number of samples differs between groups (Fielding and Bell, 1997; Jones at al., 2017). Landis and Koch (1977) have suggested the following ranges of agreement for k: poor if $k < 0.4$; good if $0.4 < k < 0.75$; and excellent if $k > 0.75$.

Robust clustering was adjusted to otolith core and edge TEC to investigate the number of natal sources of *A. carbo* in the NE Atlantic and to evaluate the adequacy of otolith trace element composition to discriminate the sampling locations, using the *tclust* library in R (Fritz et al., 2012). This package implements robust non-hierarchical clustering algorithms to detect homogeneous clusters with large heterogeneity among them, applying trimming to the data to remove the influence of outlying observations and identify potentially interesting anomalous observations (Fritz et al., 2012). The number of clusters, k, and the trimming rate, $\alpha \in]0, 1[$, must be chosen beforehand (Ruwet et al. 2012). To choose k and α, the classification trimmed likelihoods were approached by successfully applying the *tclust* function to a sequence of values of k and α, using the function *ctlcurves* from *tclust* library (Fritz et al., 2012). To avoid finding "spurious" clusters, function *tclust* has to be constrained by a restriction factor and the strength of the constraint will control the group size and/or sphericity (shape) of the clusters. The larger the restriction factor, the more heterogeneity is allowed among clusters. The default restriction factor of 50 was considered, since increasing the value did not return different cluster scatters or sizes.

Finally, a cluster analysis was adjusted to the TEC profiles extracted from the whole otoliths using *mixAK* library in R (Komárek, 2009) to perform a multivariate longitudinal analysis. This library implements routines that allow performing cluster analysis based on multivariate continuous and discrete longitudinal data (Komárek and Kormáková, 2013, 2014). It begins with a set of routines for Bayesian estimation of finite mixture models that is followed by clustering based on possibly censored data. The mixture of multivariate generalized linear mixed models provides some features that allow (i) using irregularly sampled longitudinal data (the time corresponding to the "edge" zone is not the same for all specimens, since they were captured at different ages) and (ii) not necessarily having to assume that multivariate responses are independent for one subject. The Bayesian approach is based on the output from the Markov chain Monte Carlo (MCMC) simulation, which is prospected to infer the unknown model parameter vector, θ, and to perform the clustering. The MCMC algorithm was ran for (500 x 10) burn-in and (5000 x 10) subsequent iterations with 1:10 thinning to get two samples of length M = 1000 from the joint posterior distribution obtained by starting the MCMC from two different sets of initial values. Mg, Mn, Cu, Zn, and Ba were log10-transformed to meet linear mixed-effects model assumptions. The MCMC was based on longitudinal measurements of the TEC/Ca ratios with assumed Gaussian distribution. Na and Mn were excluded from the MCMC because, on a preliminary run assuming two clusters, the two estimated cluster specific mean longitudinal profiles behaved almost equally, nearly overlapping. The number of mixture components (clusters) was fixed between one and four. All other values of prior hyperparameters were selected automatically to achieve a weakly informative prior distribution according to Komárek and Komárková (2013). The function

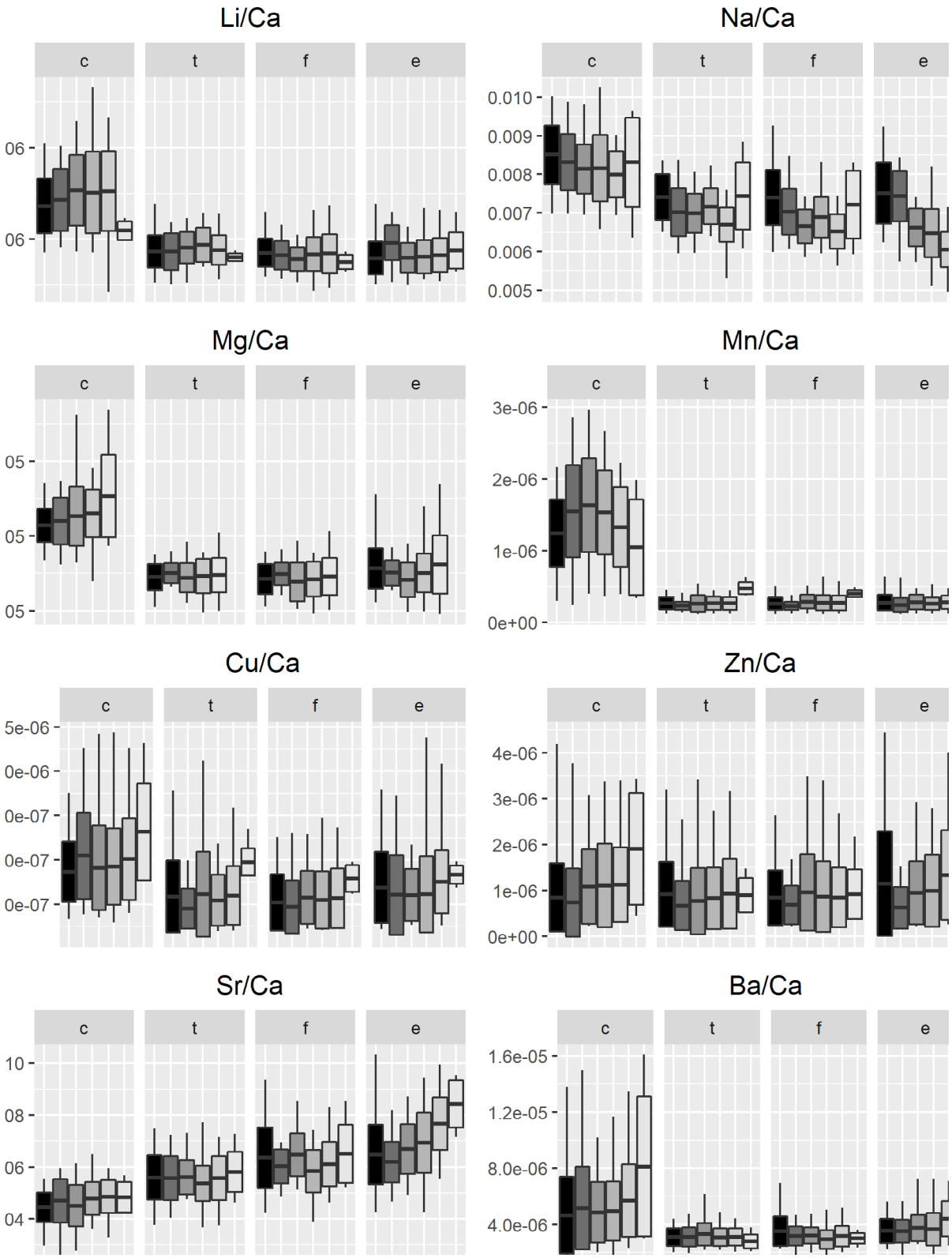

Figure 4.2.3. *Aphanopus carbo* untransformed otolith trace element concentrations ratioed to Ca (µmol TEC. mol^{-1} Ca) by otolith zone (c = core; t = age 3; f = age 5; e= edge) and sampling region (Iceland, Faroes, West British Iles, Mainland Portugal, Madeira, and and Canaries). In the Mg/Ca plot, values from the Canaries were hidden because its high dispersion was concealing the values from the other regions. Summary statistics are mean and standard deviation; the whiskers are the minimum and maximum values.

GLMM_MCMC automatically generates the reasonable initial values to start the MCMC simulation. Stephens' (2000) relabelling algorithm was applied to minimize the posterior expected loss, hence resolving the problem arising from the model likelihood invariance (Komárek and Komárková, 2014). The number of mixture components, K, was selected according to two approaches included in *mixAK* library. First, the penalized expected deviance (PED), derived from cross-validation arguments, was used as a measure of the model complexity and fit (Komárek and Komárková 2014). The second approach was based on the posterior distribution of the deviances. Considering two models M_j and M_k, a likelihood ratio L_{jk} of 9 is equivalent to a deviance difference $D_{jk} = D_j - D_k$ of $-2 \log 9 = -4.4$; hence, there is a high posterior probability of quite strong evidence for M_j against M_k when the empirical $P[D_{jk} < -4.4|data] > 0.9$ (Atkin et al., 2009). The posterior cumulative distribution functions (CDF) were plotted to perform an overall comparison of the posterior distributions of deviances for $K = 1, ..., 4$ and select the best model (Aitkin, 2010).

4.2.3. Results

Sr and Na were the elements with the highest mean concentrations. Na values decreased throughout the life of the fish, from the core to the edge of the otolith, whereas Sr showed a pronounced ontogenic increase, from the core to the edge. Li, Mg, Mn, Cu, and Ba values were higher in the otolith core than on the other otolith zones for all regions (Figure 4.2.3). Within otolith zones, apparent differences between regions were more noticeable in the otolith core and edge, especially for the element ratios Na, Mn, Sr, and Ba. TEC/Ca dispersion was higher in the otolith core, especially in Li, Na, Mg, Mn, and Ba.

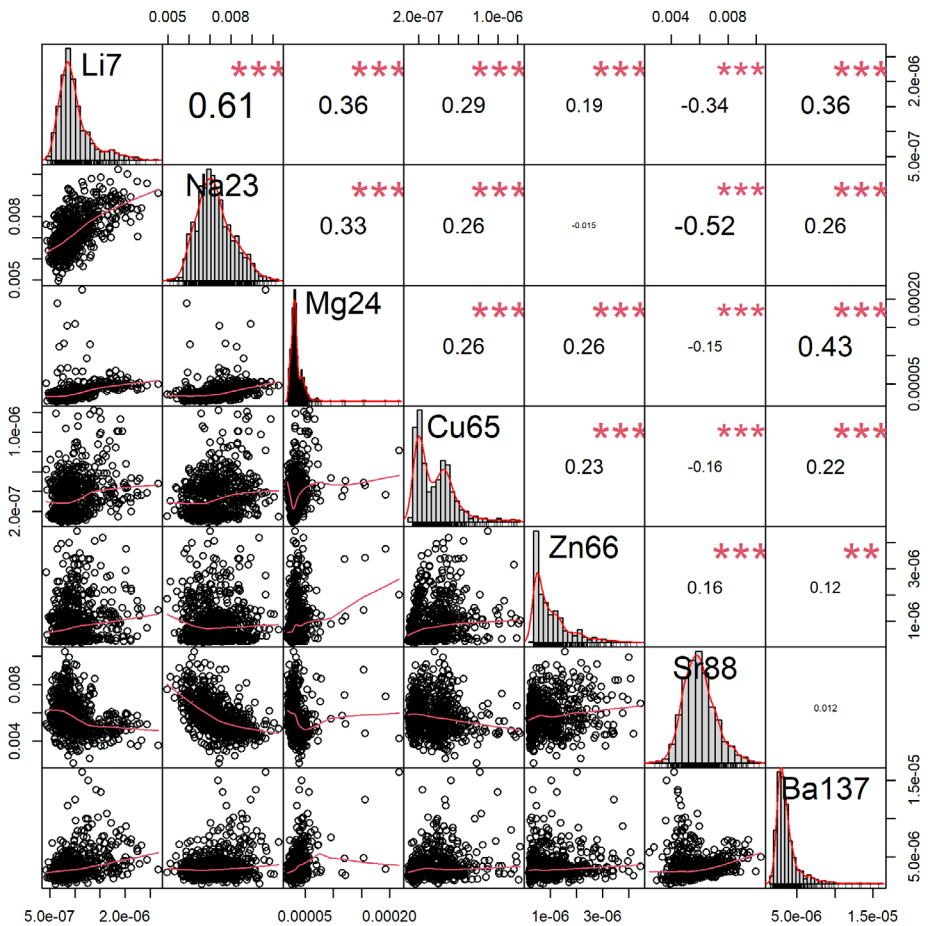

Figure 4.2.4. Pearson rank test correlation matrix among untransformed trace element concentrations ratioed to Ca (µmol TEC.mol-1 Ca). *** p = 0.001; ** p = 0.01.

Li and Na were found to be positively correlated with each other (Pearson's r = 0.61), whereas Na and Sr were found to be negatively correlated (r = -0.52), at a significance level of 0.001 (Figure 4.2.4).

In the otolith core, Na (Shapiro-Wilk's $W = 0.991$, $p = 0.372$), Mn ($W = 0.987$, $p = 0.166$), and Sr ($W = 0.990$, $p = 0.362$), and Box-Cox transformed Li ($W = 0.987$, $p = 0.130$), Cu ($W = 0.986$, $p = 0.120$), and Ba ($W = 0.995$, $p = 0.838$) met the assumption of normality. However, these TEC were not found to significantly differ among regions ($p > 0.05$) considering a significance level of 95%, hence, were excluded from further analyses. In the otolith zone corresponding to age 3, Li ($W = 0.987$, $p = 0.062$), Na ($W = 0.993$, $p = 0.513$) and Box-Cox transformed Ba ($W = 0.994$, $p = 0.608$) met the assumption of normality, but only Na was statistically different between regions at a confidence level of 95% and was, hence, retained for QDA. In the otolith zone that represents age 5, Box-Cox transformed Li ($W = 0.996$, $p = 0.898$, Na ($W = 0.995$, $p = 0.835$), Sr ($W = 0.996$, $p = 0.927$), and Ba ($W = 0.991$, $p = 0.073$) met the assumption of normality, and the former three TEC were retained for QDA for being significantly different among regions. Regarding the otolith edge, Sr ($W = 0.987$, $p = 0.096$) and Box-Cox transformed Li ($W = 0.990$, $p = 0.301$), Na ($W = 0.995$, $p = 0.806$), and Ba ($W = 0.989$, $p = 192$) met the assumption of normality and were significantly different among regions, being retained for QDA.

QDA fitted to the selected TEC, performed very poorly for age-3 zone ($K = 0.12$, 28 % accuracy), and age-5 zone ($K = 0.10$, 26 % accuracy). For edge TEC, QDA performed better than in the previous otolith zones ($K = 0.20$, 35 % accuracy), although k was below the range assigned as "good" according to Landis and Koch (1977). The percentage of correct classification was higher than 50 % for all regions except mainland Portugal (Table 4.2.2).

Table 4.2.2. Classification summary for the quadratic discriminant analysis (QDA) based on *Aphanopus carbo* otolith edge TEC. The overall classification success was $K = 0.20$. n is number of samples.

	Predicted group membership (%)						
True group	**Iceland**	**Faroes**	**W British Isles**	**Mainland Portugal**	**Madeira**	**Canaries**	**n**
Iceland	59	10	21	7	3	0	29
Faroes	12	62	19	8	0	0	26
W British Isles	5	14	51	16	14	0	37
Mainland Portugal	3	9	43	14	23	9	35
Madeira	0	0	15	12	74	0	34
Canaries	14	0	0	14	14	57	7

Based on the classification trimmed likelihoods (Figure 4.2.5-a), otolith core TEC were clustered into two groups, selecting parameters $K = 2$ groups and data trimming proportion $\alpha = 0.2$ (Figure 4.2.5-b). To get a clear separation between the groups, the trimming proportion was set at a relatively high value ($\alpha = 0.2$), hence, 32 otoliths were excluded from any of the groups. The clusters had similar sizes (64 and 63 elements) even though the default option "false" for the *tclust* argument that searches clusters with equal sizes was used. Clusters 1 and 2 presented similar distribution and mean of discriminant factors in the silhouette plot (Figure 4.2.5-c) and only nine observations were doubtfully assigned (Figure 4.2.5-d), hence, the clusters were assumed to be well determined.

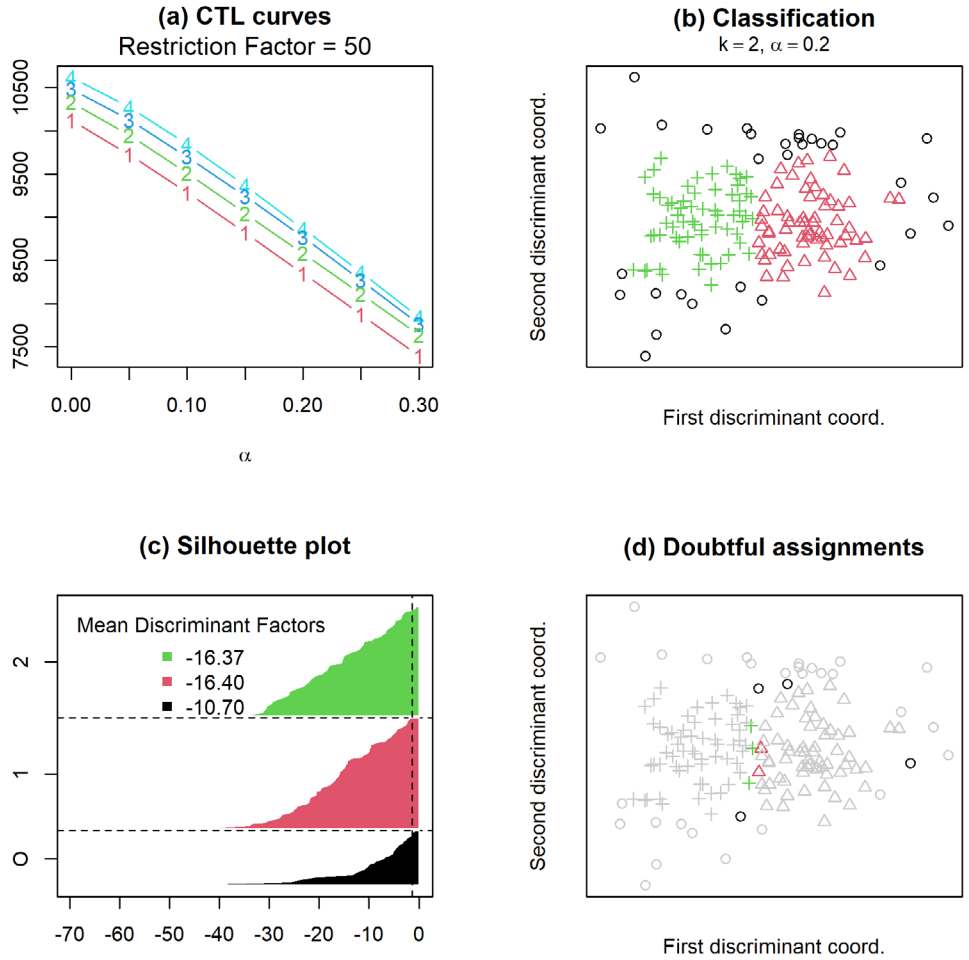

Figure 4.2.5. *Aphanopus carbo* robust clustering analysis for otolith core TEC: (a) Classification trimmed likelihoods (CTL) with $K = 1, ..., 4$, $\alpha = 0, ..., 0.30$, and restriction factor = 50; (b) Trimmed k-means with $K = 2$, $\alpha = 0.20$ and restriction factor = 50. Open circles represent the trimmed observations. The two first Fisher's canonical coordinates derived from the final cluster assignments are displayed. (c) Silhouette plot and (d) doubtful assignments (coloured marks) based on the discriminant factor values for the cluster classification with $K = 2$, $\alpha = 0.20$, and restriction factor = 50.

Otoliths caught in the Faroes, W British Isles, and Madeira were equally distributed between the two groups, whereas otoliths from specimens caught off Iceland and off the Canaries were mostly assigned to one group, and otoliths caught off mainland Portugal were assigned to the other group (Table 4.2.3).

Table 4.2.3. *Aphanopus carbo* estimated group membership by region based on otolith core TEC.

	Group 1	**Group 2**
Iceland	17	8
Faroes	10	9
W British Isles	14	13
Mainland Portugal	9	18
Madeira	10	14
Canaries	4	1

Considering otolith edge TEC and based on the classification trimmed likelihoods (Figure 4.2.6-a), two main groups were discriminated, with parameters k = 2 groups and data trimming proportion α = 0.1 (Figure 4.2.6-b). The clusters had very different sizes (100 and 59 elements). Clusters 1 and 2 presented similar distributions in the silhouette plot (Figure 4.2.6-c) and only five observations were found to be doubtfully assigned (Figure 4.2.6-d), hence, the clusters were considered to be well determined.

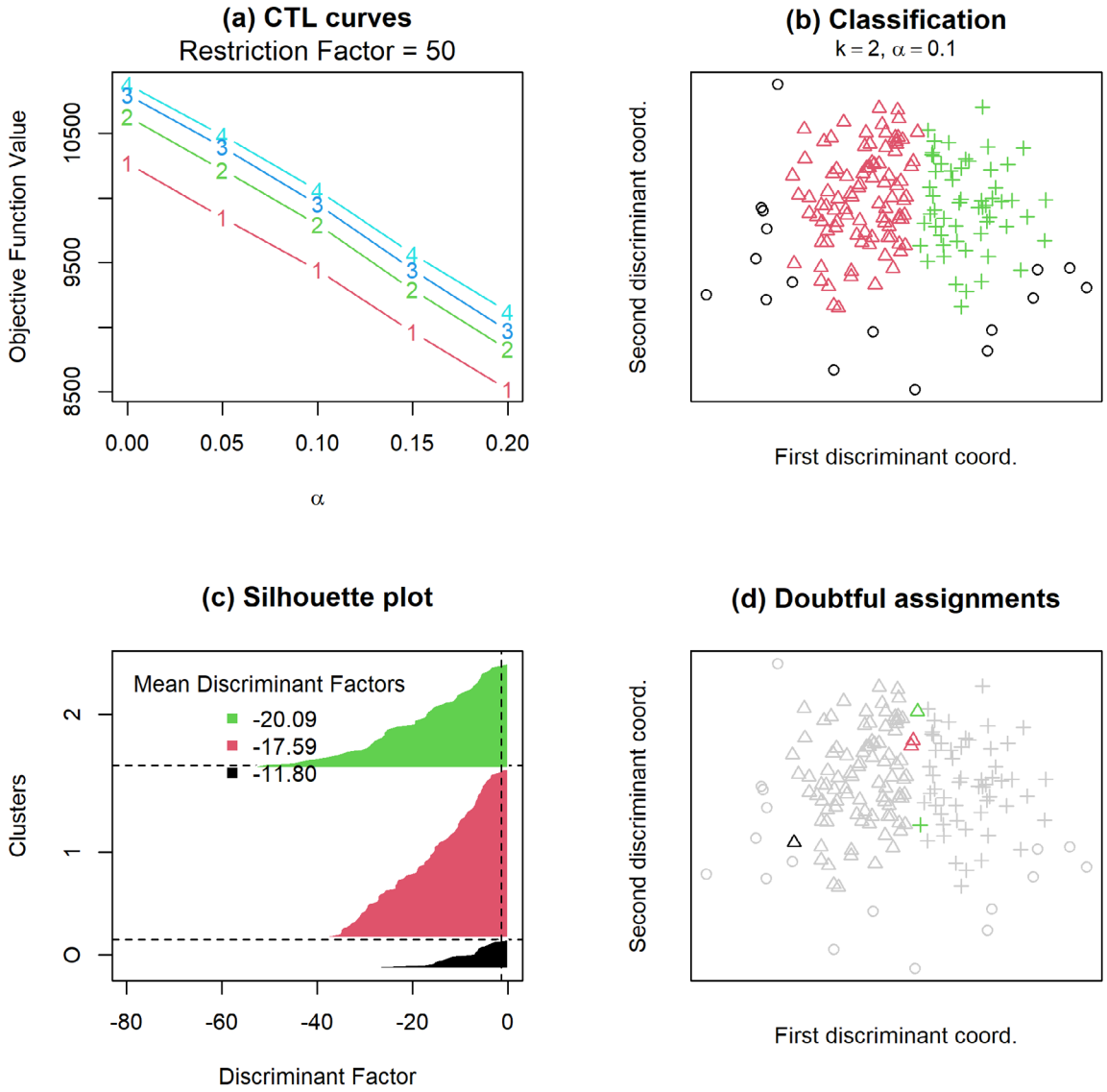

Figure 4.2.6. *Aphanopus carbo* robust clustering analysis for otolith core TEC: (a) Classification trimmed likelihoods (CTL) with $K = 1, \ldots, 4$, $\alpha = 0, \ldots, 0.30$, and restriction factor = 50; (b) Trimmed k-means with $K = 2$, $\alpha = 0.10$ and restriction factor = 50. Open circles represent the trimmed observations. The two first Fisher's canonical coordinates derived from the final cluster assignments are displayed. (c) Silhouette plot and (d) doubtful assignments (coloured marks) based on the discriminant factor values for the cluster classification with $K = 2$, $\alpha = 0.10$, and restriction factor = 50.

For the otolith edge TEC data, the estimated group membership of specimens from all regions was clearer than what was found for the otolith core data (Table 4.2.4). The first group was composed of specimens caught off the northernmost locations (83 % of otoliths from the Faroes, Iceland, and the west of the British Isles) and the second group was represented by specimens from Madeira and the Canaries (74 % of specimens from these locations). Specimens caught off mainland Portugal were represented in both groups in similar proportions.

Table 4.2.4. *Aphanopus carbo* estimated group membership by region based on otolith edge TEC.

	Group 1	Group 2
Iceland	21	2
Faroes	21	1
W British Isles	26	11
Mainland Portugal	18	14
Madeira	9	24
Canaries	0	2

The TEC longitudinal profiles varied among otoliths (Figure 4.2.7). Li, Na, and Mg showed a clear decrease from core to edge, whereas Sr presented a marked linear increase.

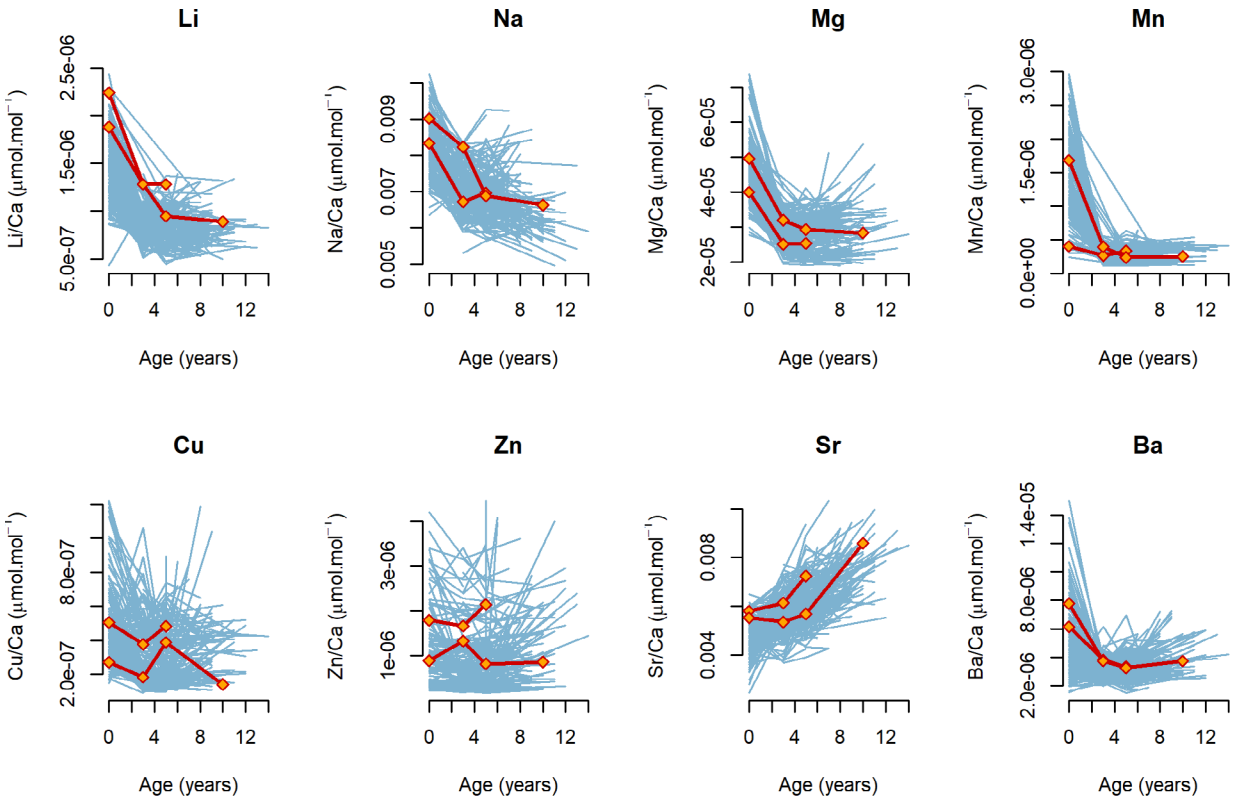

Figure 4.2.7. *Aphanopus carbo* otolith TEC (µmol TEC.mol^{-1} Ca) longitudinal profiles with two random otoliths highlighted.

Observing the estimated cluster specific mean profiles (Figure 4.2.8), the first cluster (black) is characterized by lower values of all TEC than the second cluster throughout time. This difference is not clear for Cu and Sr. With respect to the longitudinal evolution of the otolith TEC, both groups behave almost equally on average, with the exception of Zn. This was the element for which the differences between clusters were more marked, with the first cluster being characterized by a decrease from core (age 0) to the end of the fish lifetime and the second cluster showing an increase.

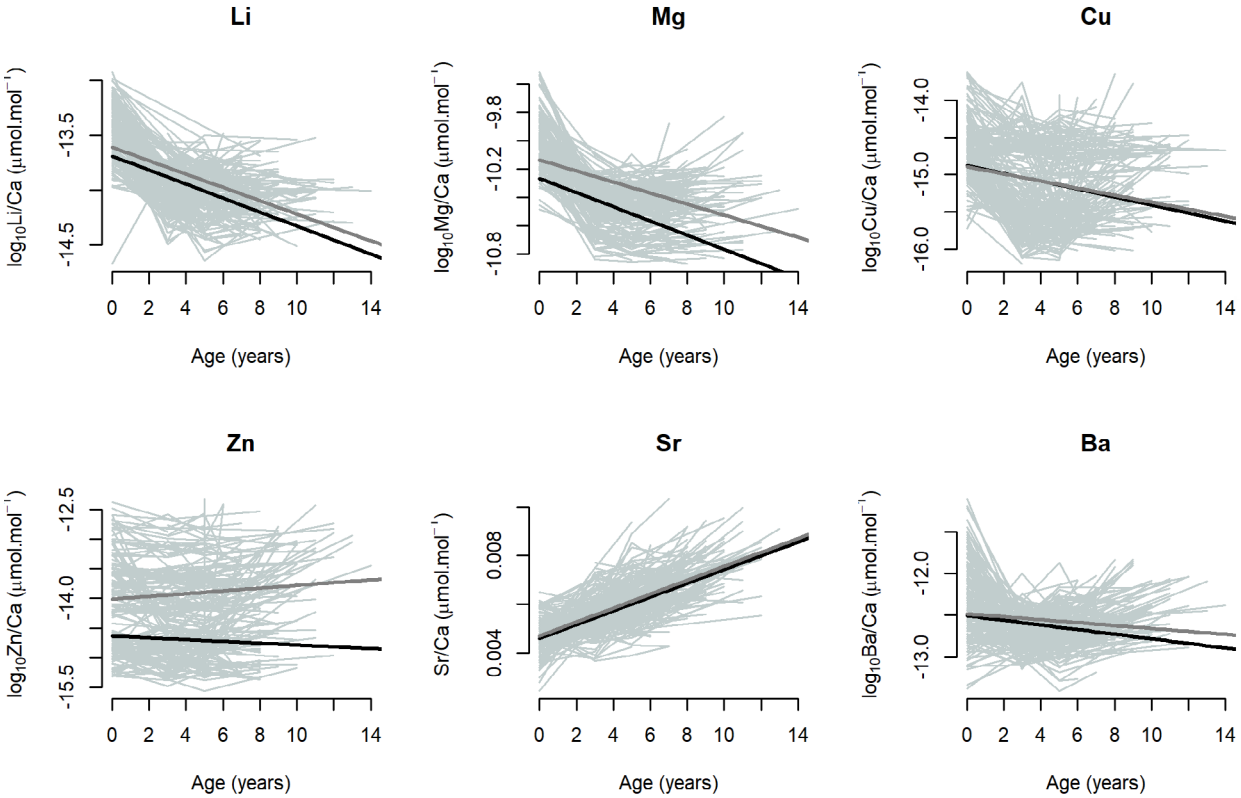

Figure 4.2.8. *Aphanopus carbo* observed longitudinal profiles of selected TEC (µmol TEC.mol-1 Ca) along the lifetime together with the estimated cluster specific mean profiles (cluster 1 in black, cluster 2 in grey).

Classification based on posterior means of the individual component probabilities, lead to 69 otoliths being classified in group 1 and 126 otoliths being classified in group 2. Using the posterior median, the classification of one subject has changed but both the posterior mean and the posterior median are close to the classification threshold of 0.5. The ultimate classification could not be determined for 40 otoliths because their observed longitudinal TEC did not provide enough certainty for classification into any of the two considered groups. Among these unclassified otoliths, 20 were originally classified in the first and 20 in the second group.

According to the penalized expected deviance (PED) values for the four models (Table 4.2.5), the number of mixture components (clusters) that best fit the otolith TEC longitudinal profiles is that with K = 1 since the best model is the one with the lowest PED.

Table 4.2.5. Penalized expected deviance and the related quantities of the models fitted to otolith TEC longitudinal profiles with the posterior MCMC simulation for models with *K* = 1, 2, 3, 4 clusters, according to Komárek and Kormáková (2014). D.expect = estimated expected deviance, where the estimate is based on two parallel chains; popt = estimated penalty, where the estimate is based on simple MCMC average based on two parallel chains; PED = estimated penalized expected deviance (D.expect + popt); wpopt = estimated penalty, where the estimate is based on weighted MCMC average (through importance sampling) based on two parallel chains; wPED = estimated penalized expected deviance (D.expect + wpopt).

K	D.expect	p(opt)	PED	wp(opt)	wPED
1	-5626.92	126.28	-5500.64	127.826	-5499.1
2	-5669.33	274.428	-5394.9	274.813	-5394.52
3	-4540.32	729.988	-3810.33	722.074	-3818.24
4	-4885.67	820.179	-4065.49	816.387	-4069.28

However, following the approach of Aitkin et al. (2009), the model with $K = 2$ components should be selected because it is better than a model with $K = 1$ components since the posterior probability P(diff < -4.4) is higher than 0.9 (Table 7).

Table 4.2.6. Posterior summary statistics for the difference between the deviances of two models with $K = 1$, 2, 3, 4 mixture components, according to Atkin et al. (2009).

	P(diff < -4.4)	**P(diff < 0)**	**Mean**	**2.50%**	**50%**	**97.50%**
2 - 1	0.97	0.98	-42.41	-83.48	-42.58	-1.99
3 - 1	0	0	1086.61	1058.43	1086.99	1114.75
4 - 1	0	0	741.25	703.25	741.15	779.97
3 - 2	0	0	1129.02	1093.87	1129.08	1161.87
4 - 2	0	0	783.66	741.03	783.15	832.26
4 - 3	1	1	-345.35	-376.96	-345.94	-310.38

This result is supported by the graphical comparison of the posterior cumulative distribution functions of the deviances of the competing models suggested by Aitkin (2010) (Figure 4.2.9). This plot shows that, even though the variability of the posterior distribution of the deviance in a model with $K = 2$ components is practically the same as with $K = 1$, the model deviance decreases when moving from the $K = 1$ model to the $K = 2$ model.

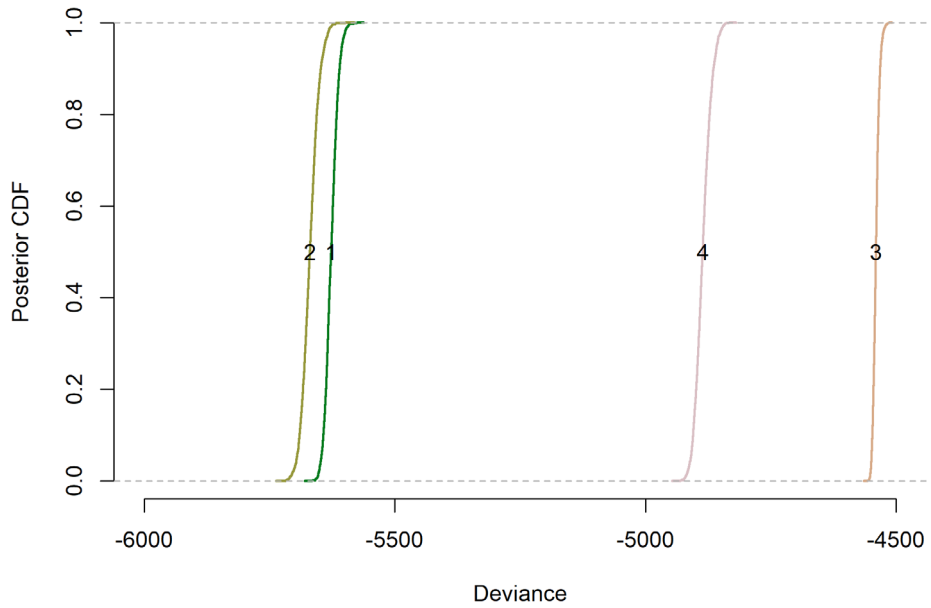

Figure 4.2.9. Posterior cumulative distribution functions (CDF) of the otolith TEC longitudinal profiles deviances for models with $K = 1, 2, 3, 4$, according to Atkin (2010).

Classification based on posterior medians of the individual component probabilities of otolith TEC longitudinal profiles grouped the otoliths from all locations in the two estimated groups and most specimens from the different locations were preferably in the same group (Table 4.2.7).

Table 4.2.7. *Aphanopus carbo* estimated group membership by region based on the posterior medians of the individual component probabilities of otolith TEC profiles.

	Group 1	**Group 2**
Iceland	11	27
Faroes	13	17
W British Isles	17	23
Mainland Portugal	17	23
Madeira	10	33
Canaries	2	3

4.2.4. Discussion

The present study covered the whole latitudinal range of distribution of *A. carbo* in the NE Atlantic, from Iceland to the Canaries, widening the area covered in previous studies focusing on the species migratory dynamics and habitat use (Neves et al., 2009; Santos et al., 2013; Longmore et al., 2014). The sample was representative of the known ontogenic distribution of the species, with the smallest individuals caught in the northernmost locations, whereas the largest specimens were caught in the southernmost areas (Farias et al., 2013). It is important to highlight that the species length distribution may be limited by the sampling method because different fishing gears are used throughout the NE Atlantic. Specifically, the longlines used in mainland Portugal, Madeira, and the Canaries are very selective in terms of fish size because of the size of the hooks (Pajuelo et al., 2008; Bordalo-Machado et al., 2009) whilst bottom trawl is less selective but larger specimens might escape the haul as a result of faster swimming ability and longer endurance (Winger et al., 2010). Despite this possible bias in the distribution and migratory hypothesis, otolith microchemical analysis provided independent information that could enlighten the behaviour of *A. carbo*.

The present multivariate approach had two singular features. First, a vector of all quantified TEC was associated to each zone of the otolith. The objective was to analyse the trace elements together, knowing that the incorporation of each selected element in the otolith is differently influenced by the environmental and physiological factors and that this influence is generally addressed element by element (Tanner et al., 2011; Longmore et al., 2014; Sturrock et al., 2015). Secondly, a vector of all the TEC quantified in four different zones of the otolith was used for a temporal analysis of the otolith elemental signature. The most routinely used trace elements to infer past location in fishes were selected (Li, Mg, Mn, Cu, Zn, Sr, and Ba). These elements are preferably used for otolith microchemical analyses because of their environmental heterogeneity and because their concentration in the otolith is often above their analytical detection limits (Sturrock et al., 2012).

The incorporation of elements from the environment into the otolith's matrix is physiologically regulated, being mostly influenced by sex and maturation (Kalish, 1989; Farrell and Campana, 1996; Morales-Nin et al., 2012; Sturrock et al., 2014; Thomas and Swearer, 2019). Longmore et al. (2014) found clear differences between sexes on the Mg profile of *A. carbo* specimens caught off Madeira. Therefore, to minimize the effect of physiological regulation, all *A. carbo* specimens used in the present study were males. Additionally, the possibility of incorrect age assignment was minimised by selecting for TEC analysis the growth increment corresponding to age 3, which is clear in *A. carbo* otoliths, and was present in all the sampled otoliths. Moreover, since age at first maturity of *A. carbo* was estimated at 6 years (Figueiredo et al., 2003; Vieira et al., 2009), the trace element pattern at age 3 is not expected to manifest any effect of physiological changes driven by the maturation process.

Geographical natal origin

In the present study, core trace element profile supports the existence of two natal sources. Specimens caught off Iceland were mainly allocated to one group, specimens caught off Madeira and mainland

Portugal were primarily allocated to the other group, and specimens caught off the Faroes were equally allocated to the two hypothetical spawning grounds. The existence of a northern spawning location is concomitant with the known southernmost spawning grounds that have been previously reported around Madeira and the Canaries and with the hypothesis that eggs and larvae disperse from the southern areas (Figueiredo et al., 2003; Neves et al., 2009; Pajuelo et al., 2008; Farias et al., 2013; Ribeiro Santos et al., 2013). Mature and spent specimens had previously been reported in the Rockall Trough (Zilanov and Shepel, 1975), the Porcupine Bank (Ehrich, 1983), and off Iceland (Magnússon and Magnússon, 1995). More recently, Longmore et al. (2014) have also suggested the existence of *A. carbo* spawning aggregations other than Madeira based on the wide-ranging concentrations of Mn and Mg in the larval to early juvenile portion of the otoliths.

Geographical clustering based on otolith edge composition

Although otoliths from the Canaries seem to be separated from the other regions when analysing individual TEC distributions, they are grouped with the northernmost locations when considering the core TEC and with Madeira when considering the edge TEC. Given their geographical proximity, otoliths from the Canaries were expected to cluster with otoliths from Madeira in their edges and possibly in their cores. The fact that otolith cores from specimens caught off the Canaries present similarities with cores from otoliths caught off Iceland and the Faroes suggests a common natal origin and supports the migratory hypothesis. In the future, it will be important to consolidate these results with a larger number of samples caught off the Canaries to exclude any effect of intragroup variability because of the small sample size.

The separation of the sampling locations into two groups based on otolith edge chemical composition, which represents the recent otolith growth, suggests there are differences between the northern and southernmost water masses and that otolith TEC is adequate to discriminate specimens from those two areas. In Madeira, *A. carbo* occurs typically at depths between 700 and 1300 m where the influence of the Mediterranean outflow water (Leite, 1989; Morales-Nin and Sena-Carvalho, 1996; Swan et al., 2003) and the Canary Current are also felt. Moreover, the group dominated by fish from Madeira also presented fish from mainland Portugal (43 %) and from the west of the British Isles (30 %), whereas specimens caught off Madeira were also grouped with the northernmost samples (27 %). The allocation of fish caught in other places to Madeira implies that their otoliths exhibit a chemical signature that characterizes Madeiran waters. Longmore et al. (2014) also found a mix of fish from different origins were located off Madeira based on adult fish otolith trace element composition. Additionally, otolith oxygen isotope composition evidenced the occurrence of ontogenetic migrations of *A. carbo* from cooler waters during larval growth to relatively warm water conditions, such as those found off Madeira (Longmore et al., 2014). The allocation of fish caught off Madeira to areas further north at their younger ages can be explained by a northward migration during their first years of life. This migration can be driven by the northward water flow that results from the merging of the upper portion of the MOW merging with a poleward eastern boundary undercurrent towards the Norwegian Sea, just west of Ireland (Postel and Du Buit, 1965; Ehrich, 1983; Price et al., 1993). The connection between NE Atlantic northern and southern water masses has been previously supported by the presence of Madero-Mediterranean species, namely *Malacocephalus laevis*, *Haloporphyrus güntheri*, *Aphanopus carbo*, *Coelorinchus caelorhincus*, *Gaidropsarus vulgaris*, *Phycis blennoides*, *Hoplostethus mediterraneus*, *Epigonus telescopus*, as northerly as the Wyville-Thompson Ridge (Postel and Du Buit, 1965), by physical oceanographic traits (Tait, 1957), and by plankton distribution (Fraser 1961). The range of temperatures (8-10 °C) recorded between Gibraltar and the Wyville-Thomson Ridge, at 200 to 800 m depth, can justify the absence of a biogeographical barrier between these slopes and the Mediterranean deep-water homothermic layers (12-13 °C) (Postel and Du Buit, 1965; Miller et al., 1970).

On the northernmost part of the study area, specimens caught off the Faroes and specimens caught off Iceland were classified in a common group, which is expected to be environmentally homogeneous given the geographical proximity and similar depth of capture. Specimens caught off the west of the British Isles were more strongly connected with this group than with the southern group. Longmore et al. (2014) found a strong correct classification in edge elemental composition of specimens caught off the Rockall Trough. As previously described, the NAC and the MOW take water with relatively

high salinity into the Norwegian Sea, consequently increasing the density of the very cold waters that are produced there during the winter (Postel and Du Buit, 1965; Price et al., 1993). While smaller *A. carbo* are found at lesser depths in Iceland and the Faroes (500-830 m), larger fish that live deeper might find, in the deep-water overflows occurring in the vicinities, a barrier that hinders them to move northwards, remaining off the west of the British Isles for feeding and growing as previously reported (Zilanov and Shepel, 1975; Du Buit, 1978; Ehrich, 1983; Santos et al., 2013). Here, *A. carbo* concentrate in the sections of the banks and rises where there is an increased biological productivity induced by the mixing of deep and surface waters (Zilanov and Shepel, 1975).

Otoliths from specimens caught off mainland Portugal relate almost equally to both the northernmost area (dominated by the Faroes, Iceland, and the west of the British Isles) and Madeira when considering the edge trace element profile. The occurrence of two different chemical compositions might be explained by the confluence of the Portugal Current, that runs southwards off the Iberian Peninsula, with the spreading of the upper portion of the MOW, that runs northwards and influences the Madeiran waters, as previously described. The absence of a biogeographic barrier off the continental shelf between the Gibraltar Strait and the Wyville Thomson Threshold (Postel and Du Buit, 1965) implies that the otolith trace element composition is related with the species' depth distribution and, consequently, with physical factors such as temperature and salinity.

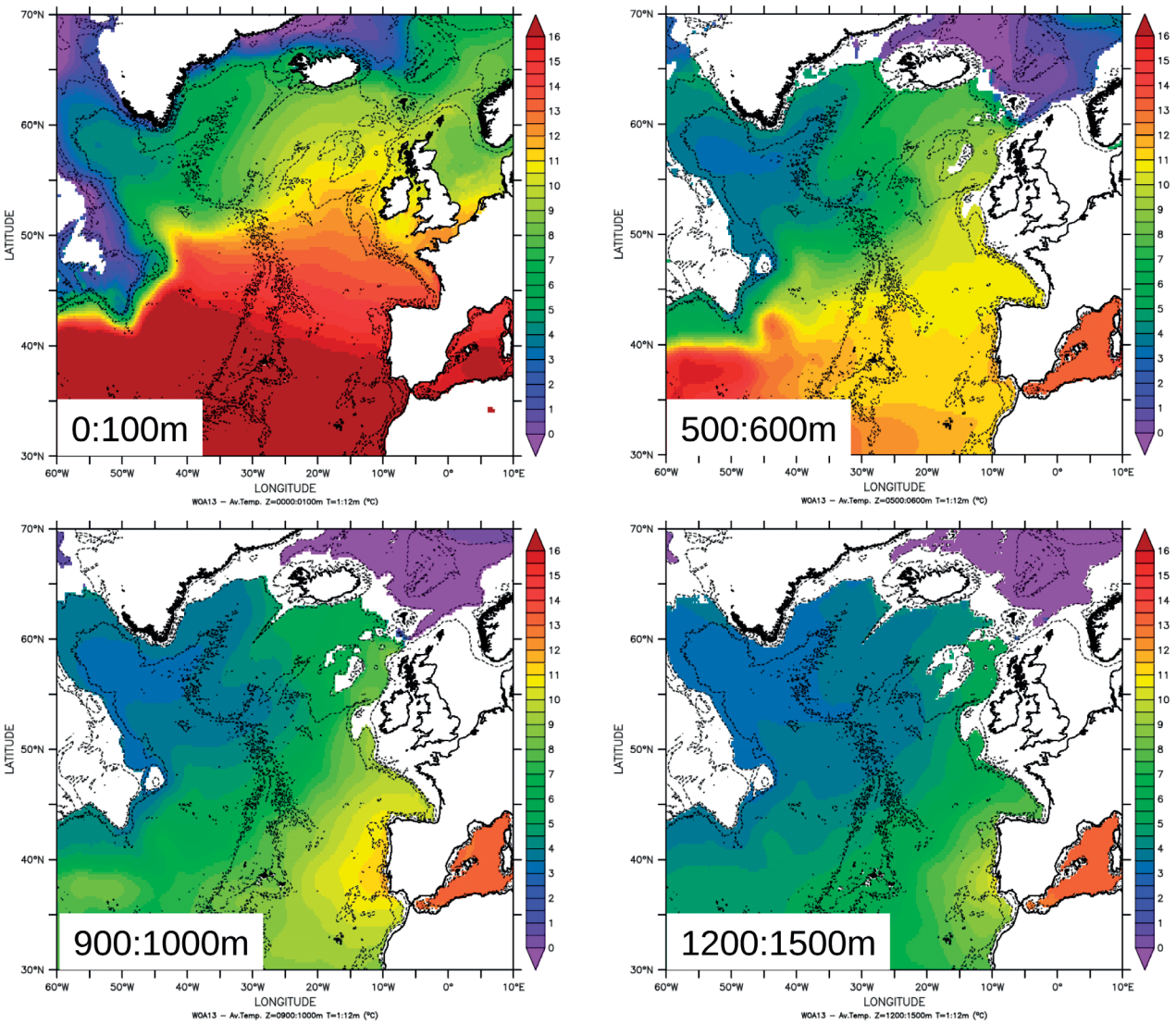

Figure 4.2.10. Mean annual temperature by depth (0-100 m, 500-600 m, 900-1000 m, 1200-1500 m) in the NE Atlantic, computed from World Ocean Atlas 13 (Locarnini et al., 2013) mean averaged decades monthly fields at 1/4° degree resolution.

Off Madeira and off the Canary Islands the species occurs at deeper waters (800-1300 m and 800-2300 m, respectively) (Le Gall, 1972; Morales-Nin and Sena-Carvalho, 1996; Pajuelo et al., 2008), where the temperature varies from 11.1 to 8.6 °C and the salinity is around 35.5 ‰ (Locarnini et al., 2013). In the present study, specimens assigned to the northernmost group were caught at 500-800 m, where recent records of temperature are between 7 and 8 °C and salinity is 35.1-35.2 ‰, whereas fish from off the west of the British Isles were caught at 1000-1100 m depth, where temperatures is 8-9 °C and salinity is 35.2-35.3 ‰ (Figure 4.2.10) (Locarnini et al., 2013). The species ontogenetic distribution appears to be highly dependent on the relationship between depth and temperature.

Element variation throughout the life history

In general, Na and Mn decreased whereas Sr increased from the core to the edge. Mg, Cu, and Ba decreased between the core and age 3 but increased from here towards the otolith edge. These results suggest there is an ontogenic effect on trace element incorporation in *A. carbo* otoliths. The influence of ontogeny occurs because older fish frequently have a greater relative investment in reproduction compared with juveniles. As fish mature, the level of Ca-binding proteins, such as albumin and vitellogenin, increases in the blood, reducing the free plasma Ca (Kalish, 1989). The concentration of free Ca in plasma is directly proportional to Ca in the endolymph that involves the otolith and from where Ca is precipitated (Mugiya, 1966; Mugiya and Takahashi, 1985; de Pontual and Geffen, 2002; Wright et al., 2002). The reduction of plasma Ca will induce a reduction of Ca in the endolymph and a consequent increase of the TEC/Ca ratio, which will finally be manifested as a higher TEC/Ca in the otolith (Kalish, 1989). This ontogenic effect was evident for Sr in *A. carbo* otoliths in the present and in a previous study (Longmore et al., 2014), as well as in other species, such as the Australian salmon (Kalish, 1989), sole (de Pontual et al., 2003), and European hake (Morales-Nin et al., 2014).

Updated migratory hypothesis

The acceptance of both southern and northern spawning locations and of migratory movements along the NE Atlantic in northward and southward directions implies changes to the current migratory hypothesis. The existence of two distinct groups is here hypothesized based on the trace element profile throughout the species lifetime. Most specimens caught off the Faroes, Iceland, and the west of the British Isles (83 %) and off mainland Portugal (56 %), belong to the same group estimated from otolith edge TEC. Specimens caught off Madeira were classified into the two groups (27 % and 73 %), which implies that this area is receiving specimens from the two expected spawning grounds. The portion of specimens that compose the second group might be the resident part of the Madeiran population that does not supply individuals to other locations but rather receives input from the other locations, mostly mainland Portugal and the west of the British Isles.

The chemical signatures of *A. carbo* otoliths had previously been compared between six distinct locations on the NE Atlantic through solution-based ICP-MS (Swan et al., 2003). The high percentage of correct classification among regions was consistent with the single stock hypothesis (Farias et al., 2013) but using dissolved whole otoliths masked the TEC variability throughout the specimens' lifetime. The assumption was that all fish derived from the same spawning area, hence, the greater part of the otolith that represented the first years of life would not show differences in TEC and the differences found in total TEC would represent a later migration to different areas. Longmore et al. (2014) found significant ontogenic changes in *A. carbo* otolith chemical composition but could not detect significant differences between the areas sampled. They justified the strong overlap across areas with overall panmixia or physicochemical similarities between the environments. The present study proved that differences in water masses between geographical areas could be discriminated based on otolith edge TEC and that geographical locations could be compared through otolith microchemical composition following a multivariate approach. Also, ablating precise zones in the otolith is more sensitive than analysing dissolved whole otolith.

Swan et al. (2003) had acknowledged the importance of otolith microchemical analysis for comparing *A. carbo* from different locations, stressing out the need to conjugate it with other stock identification methods, such as morphometrics, otolith shape analysis, and genetics. However, microsatellite DNA has suggested the absence of genetic structure among the Rockall, the western Irish Slope, the

Hebridean Shelf, the Bay of Biscay, Madeira, the Azores, and mainland Portugal (Longmore et al., 2014). Genetic divergence in deep-sea fishes is generally low or nearly absent and when it occurs it may be an evidence for cryptic species (Stefanni et al., 2009; Longmore et al., 2014). Since low genetic divergence implies a higher investment in sample numbers with consequent increased costs to achieve conclusive results, otolith trace element analyses have a better cost-benefit ratio for studying the population dynamics of deep-sea fish.

Although, otolith microchemistry provided evidence of the existence of two reproductive stocks in the NE Atlantic and changes to the migratory pathways considered up to now were proposed, there is some mixing between the two reproductive stocks and both are contributing to the recruitment to the most important fisheries. The existence of a second spawning ground implies the population occurring west of the British Isles and off mainland Portugal would be also receiving recruits from the northernmost areas of occurrence (Iceland and the Faroes). The species assessment model assumes that the transition between adjacent areas takes around three years (age corresponding to smaller individuals occurring in W British Isles), hence strong fluctuations in biomass in the spawning grounds are expected to have an effect in the biomass in the later area with a three-year time-lag. Abundance trends from the last decade show a strong decrease in the catches-per-unit-effort (CPUE) in Madeira and in the biomass indices from the Icelandic survey, in 2012, and in the French fishery in 2015 (ICES, 2020). Although the influence of the northernmost spawning ground is not clear on *A. carbo* abundance fluctuations between areas, the results from the otolith microchemical analyses support the importance of incorporating the Madeiran data into the stock assessment model, which only considers a Northern (west of the British Isles) and a Southern (mainland Portugal and Bay of Biscay) component (ICES, 2020).

Acknowledgments

We are indebted to all scientific and technical staff who provided samples, namely: Ana Ribeiro Santos (Marine Institute, Ireland); Klara B. Jakobsdóttir (Marine and Freshwater Research Institute, Iceland); Lise Helen Ofstad (Havstovan Faroe Marine Research Institute); Víctor M. Tuset (University of Las Palmas de Gran Canaria, Canary Islands). We also express our gratitude to Ana Rita Vieira and Neide Lagarto for participating in the sample collection in mainland Portugal (IPMA), and to Francis Neat and the technical staff on Marine Scotland Science's deep-sea survey and to João Delgado, Sara Reis, and the technical staff from Regional Fisheries Directorate, Research Service (Madeira) for facilitating in situ sample collection. The authors are grateful to Alicia Cantarero (University of A Coruña) for the technical support with LA-ICPMS analyses and to Miquel Palmer (IMEDEA) for statistical support. We also express our gratitude to Audrey Geffen and Steven S. Campana for the insightful discussions and suggestions on a preliminary phase of the data analyses.

This work was supported by the Portuguese Foundation for Science and Technology (FCT – Fundação para a Ciência e Tecnologia) under the PhD Grant SFRH/BD/64434/2009 to I. Farias; the Portuguese Fisheries Operational Program (2007-2013) (PROMAR – Programa Operacional Pescas), co-financed by the European Fisheries Fund (EFF) (2007-2013), under project CERTIFICA (2011-2015), MSC Certification in Portugal - Acquiring the knowledge and studying the viability for its application to coastal and artisanal fisheries; and the Portuguese Government Development Investments and Expenses Program (PIDDAC – Programa de Investimentos e Despesas de Desenvolvimento da Administração Central) under the EU/DG Fisheries' Data Collection Framework (DCF).

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Chapter 5. General discussion

This PhD Thesis aims to bring a better understanding on the dynamics of the deep-sea teleost *Aphanopus carbo* in the NE Atlantic. *A. carbo* is a commercially important species in the NE Atlantic, being the most important deep-sea exploited teleost in Portugal, in terms of value and landed weight, both continentally and in Madeira Archipelago (Bordalo-Machado et al., 2009). Besides the Portuguese fisheries, that are operated by artisanal longliners, there is a third target fishery to the west of the British Isles, where fish are mainly exploited by the French deep-sea trawl fishery (Nakamura and Parin, 1993; ICES, 2012; ICES, 2020).

In the Macaronesia region, the cryptic species *Aphanopus intermedius* has been shown recently to coexist with *A. carbo*, with a mixing rate of 1:5 in commercial landings in Madeira (Stefanni et al., 2009; Biscoito et al., 2011). To guarantee that only *A. carbo* specimens were being considered in the present study, samples from Madeira and the Canaries were identified to species levels through a combination of meristic and morphometric measures following the morphological criteria defined by Biscoito et al. (2011). At the start of our study (Chapter 3, section 3.1), species assignment was validated through sequencing of the cytochrome oxidase subunit I (COI) gene from mitochondrial DNA for each specimen and comparison of each sequence with three sequences from *A. carbo* available in GenBank using the Blast service from the National Centre for Biotechnology Information (NCBI). In samples from Iceland, the west of the British Isles, mainland Portugal, and Madeira, only *A. carbo* were present. Among specimens from Madeira and the Canaries analysed throughout this work, only *A. carbo* were selected based on validated meristic and morphometric traits (Biscoito et al., 2011).

A. carbo stock structure in the whole NE Atlantic is still uncertain, but scientific evidences support the assumption of a single stock that undergoes a clockwise migration throughout its life cycle (ICES, 2020). These evidences are taken into consideration in the model ICES adopted to monitor the stock dynamics (ICES, 2020). The existence of a seasonal trend in abundance, estimated from fisheries-dependent information, is assumed to be associated with the migratory processes (ICES, 2015). Lower abundances are usually registered from March to August, whereas higher abundances are observed between September and February. To incorporate these fluctuations in abundance, the time unit adopted to analyse the stock dynamics is the semester as follows: the 1st semester includes months from March to August; the 2nd semester conjugates the months from September of one year to February of the succeeding year.

The commercial importance of the species, its vulnerability, and the fact that the available information is scarce have boosted the scientific interest in this species, which is reflected in the number of peer-reviewed publications focusing on *A. carbo*. Over 70 scientific papers have been published since the year 2000, 80% of which since 2009, covering the whole NE Atlantic, with special incidence in the areas with target fisheries: Madeira, mainland Portugal, and the west of the British Isles.

The main reason why there are still uncertainties regarding the biology, the life cycle, and the dynamics of *A. carbo* is its benthopelagic ecology. In general, deep-sea fish are difficult to study because they are not easily accessible to fishing and because they are subject to barotrauma upon capture. The abrupt difference in pressure when fish are hauled causes, for one side, the eversion of stomachs, making it impossible to analyse their diet directly from stomach content; on another side, fish die during the ascent because their gas bladder inflates or explodes, which makes tagging and mark-recapture methods impossible to apply and might interfere with the quantification of biochemical parameters in live tissues, such as fatty acids and stable isotopes in muscle and steroids in blood. To analyse the impact of time after death and the effect of preserving the samples frozen, an innovative pilot study with Mozambique tilapia (*Oreochromis mossambicus*) was designed and is presented in Chapter 4. Moreover, information is mostly fisheries-dependent and limited to areas where the species is exploited because the number of scientific surveys covering the species depth range and wide horizontal distribution is low. In the present Thesis, thanks to international collaboration with Iceland, the Faroes, Scotland, and Ireland, complementary data have been gathered on research cruises.

In this Thesis, a multidisciplinary approach was followed, using several methodologies, associating biochemical markers that reflect biological processes, such as reproduction and feeding, and investigating taxonomic and spatial diversity in the NE Atlantic based on otolith shape and microchemical composition. The aim was to validate the migratory hypothesis (Chapter 2) currently accepted of a single stock with a reproduction area in the Macaronesian Islands. The factors that

determine the migratory behaviour of fishes are complex and different hypotheses have been proposed: productivity, energy, environmental heterogeneity, and genetic predisposition, driving the migratory behaviour of fishes (Alò et al., 2021). Several of these aspects have been envisaged in the present work.

Understanding the feeding and reproductive biology of *A. carbo* is important to interpret and infer the ecological connections and ontogenic variations between areas of occurrence of the species and related them to its hypothetical migratory movements (Chapter 3). The effect of barotrauma was evident when *A. carbo* stomach contents were analysed. Another consequence of sample collection being limited to commercial fisheries in mainland Portugal and Madeira was the total food digestion during the time fish remained hooked to the fishing gear before dying. The vacuity index was 80.8% for specimens caught off mainland Portugal, which was similar to values reported for Madeira (93.3%, Freitas, 1998; 98.3%, Santos et al., 2013) and for specimens caught by trawls off the west of the British Isles (66.2%, Mauchline and Gordon, 1984; 94.1%, Santos et al., 2013). Nevertheless, new information was added to the feeding ecology of the species. It was possible to identify the presence of the lophogastrid crustacean *Gnathophausia zoea*, followed by the cephalopod *Mastigotheutis* spp. and the teleost *Rouleina maderensis*, in descending order of frequency, in stomachs of *A. carbo* caught off mainland Portugal. This was the first description of the species stomach content for this area.

To overcome the previous sampling limitations and understand the feeding ecology of the species, stable isotopes (SI) and fatty acids (FA) were quantified in the muscle of *A. carbo*, covering a wider area of distribution (Iceland, the west of the British Isles, mainland Portugal, and Madeira) than other previously published work. Moreover, FA and SI analyses were combined for this species for the first time. The results from the analysis of stomach content analysis were sustained by the fact that the observed level of oleic acid, 18:1(n-9), in the muscle of *A. carbo* from mainland Portugal was similar to the value reported for its preferential prey, *Gnathophausia zoea* (Letessier et al., 2012). High levels of oleic acid reflect high physiological requirements (Mayor et al., 2013), which for these specimens can either be for gonadal development (reaching the pre-spawning maturity stage) or for migration. The significantly higher proportion of arachidonic acid (ARA), which has an important role in reproduction, in specimens from mainland Portugal comparing with those from the northernmost areas explains why the former reach a more advanced maturity stage.

The differences in the relative importance of the three groups of FA are in accordance with the maturity stages found in each region, hence with the hypothetical migratory cycle. Immature specimens from the three northernmost areas (Iceland, Faroes, and W British Isles) were found to be accumulating oleic acid which is an intermediate product of the metabolic pathway that transforms saturated fatty acids (SFA) to monosaturated fatty acids (MUFA) and these into polyunsaturated fatty acids (PUFA), hence is at the base of this metabolic process. The significant prevalence of ARA and docosahexaenoic acid (DHA) in the specimens caught off Madeira, that were mostly mature, was expected given the important role of those PUFA in reproduction, but also indicates that the species continues to feed during the spawning period since PUFA are obtained through the diet (Sargent et al., 1999; Tocher, 2003). The high levels of ARA, in particular, in specimens from the southernmost areas can also support the occurrence of migratory movements between them, since a high demand for ARA is associated with stressful periods, such as long-distance migrations (Sargent et al., 1999).

The trophic position inferred from the relationship between $\delta^{15}N$ and $\delta^{13}C$ and the allometric effect of each location's size range on $\delta^{15}N$ indicated that the ontogenic and spatial differences in physiological aspects of this species in Madeira, mainland Portugal, and the west of the British Isles are related with diet and prey availability, as well as with variations in the baseline values of the primary production,. The later are dependent on latitude and depth, which is more evident in Iceland. On a general overview, *A. carbo* is an opportunistic feeder, which is typical of mobile benthopelagic predators. The change from prey that feed on pelagic zooplankton to bathypelagic prey reflects an ontogenic improvement in its predatory capacity.

SI and FA analyses brought together specimens from Madeira and specimens from mainland Portugal in terms of trophic level and needs, which is easily justifiable by the overlap in size ranges. However, results were not so clear in terms of reproductive potential. In specimens from both areas, there was a prevalence of PUFA over the other FA. If PUFA play such an important role in reproduction as precursors of essential biomolecules (Tocher, 2003), what impedes *A. carbo* to complete the

gonadal maturation and reproduce in mainland Portugal? Apparently, some endogenous factor might be detaining gonad maturation in favour of accumulating energy for fish to migrate to the known spawning grounds. One obvious hypothesis for the endogenous factor controlling maturation is the endocrine control of reproduction.

Although the field is not fully explored, because, once more, sampling his highly constrained by depth, several studies have proven that associating alterations in gonad development with the levels of sex steroids in blood plasma is a valuable tool to comprehend the endocrine control of reproduction in some deep-sea teleosts (e.g., Pankhurst and Conroy, 1987; Sequeira et al., 2017). Three sex hormones, estradiol (E_2), testosterone (T), and 11-ketotestosterone (11-KT) were quantified in *A. carbo* blood serum from specimens caught off Madeira and mainland Portugal (Chapter 3). Although there were unavoidable limitations related with sampling because blood was collected from fish that had already been dead for some time, the results bespoke the suitability of the RIA method to quantify sex steroid concentration even with small blood volumes. Moreover, the effect of metabolization of sex steroids after capture was considered to be negligible or to have occurred equally in all individuals since the relationships between hormones and reproductive stages were maintained. In specimens from Madeira, the sex steroids and GSI followed the same profile along the maturation process, which was equivalent to what has been observed for other teleosts. However, it was only possible to compare females from both areas at the developing stage, and only E_2 was significantly different between the two regions. The developing stage, during early vitellogenesis, proved to be the critical window for the decision to spawn or not to spawn (Skjæraasen et al., 2010; Neves et al., 2009). The most interesting result was that developing females from Madeira could be separated into two groups. The group that had lower steroid concentrations are those in an earlier development stage that will not have the time to mature and reach the spawning stage and, hence, will fail to spawn in the current season. The energy saved not maturing is invested in growth and in increased fecundity in the next spawning season, similar to what happens when fish skip spawning (Jørgensen et al., 2006). The persistence of a high proportion of relatively immature individuals in a plentiful environment indicates there will be a high investment in growth before the next reproductive cycle drives energy towards the gonads (Folkvord et al., 2014). Moreover, the simultaneous occurrence of two groups of developing females with similar length, steroid levels, and reproductive indicators points to an extension of this maturity stage.

The previous methodologies allowed relating biochemical traits with the species life cycle and compare them between geographical areas in a short and recent time-frame. To understand the species taxonomic diversity and species spatial diversity throughout its life cycle, otoliths were used as chemical markers (Chapter 4). On a first study, otolith trace element composition (TEC) and otolith contour shape analysis were applied to compare the two sympatric species that are caught in Macaronesia, *A. carbo* and *A. intermedius*.

Fourier shape analysis was not conclusive for species separation, probably because the wide total length range was responsible for a confounding effect of allometric growth on otolith shape and because the small sample size made the intraspecific variability being higher than the interspecific variability. However, the fact that otolith contour shape had been effective for separating *A. carbo* caught off different geographical locations in the southern NE Atlantic, namely mainland Portugal, Madeira, and the Azores (Farias et al., 2009) and for separating the two species combining specimens from different locations (Tuset et al., 2013), might indicate that environmental factors have a stronger effect than genetic and physiological factors in the shape of *Aphanopus* otoliths.

In the present study, TEC was shown to be adequate to separate the two *Aphanopus* species and it was further demonstrated that quantifying Mg, Cr, Sr, and Ba in the otolith increment that corresponds to age-class 9 can be an effective way to identify the species using LA-ICPMS at relatively reduced costs because the trace element composition in that age was significantly different between the two species according to linear discriminant analysis (LDA). This result is very important for fisheries management, since this technique could be applied to archived collections of otoliths to perceive the historical presence of *A. intermedius* in landings in Madeira, reconstruct species abundance time-series and infer if the species is moving northwards or if its presence in landings is a result of the geographical expansion of the fishery.

The applicability of the former two techniques has to be interpreted in two different time scales.

Otolith contour shape represents a large time-scale since it is the result of the accumulated otolith growth reflecting the individual life at a certain moment but influenced both by present and previous environmental and physiological factors. Otolith microchemical composition analysed by LA-ICPMS reflects the specific time in the specimens' life at which the otolith is sampled. When the two species were compared by shape analysis, only specimens from landings in Madeira were used, hence environmental differences were probably negligible, since the two species depth ranges overlap (Nakamura and Parin, 1993; Stefanni and Knutsen, 2007). As previously suggested, the physiological and genetic factors acting on both species might not be sufficiently different to be reflected in differences on otolith shape. When analysing the transect along the otolith growth axis, the whole individual life-history is being compared, hence, if the species are born in different places or live at different depths or in different areas sometime in their life, those differences will be marked on the otolith's chemical composition. In the present study, it was possible to quantify differences in the chemical composition at specific times in the specimens life-span, namely age-classes 6, 9 and 10, albeit *A. carbo* and *A .intermedius* live in overlaping environments and share some physiological and biological characteristics (e.g., spawning occurs in the last quarter, Delgado et al., 2013).

Otolith microchemical analysis was also applied to infer the migratory movements of *A. carbo* along the NE Atlantic by quantifying certain trace elements at selected otolith zones that represent different life history stages, namely the core, age three, age five, and the edge, which correspond respectively to the time of birth, a clear and wide increment in this species, the age right before 50% of the population are mature (L_{50},) and the total length mode in otoliths from Iceland (88 cm), and the end of the specimens' lifetime. The previous work was very important for planning this one, mostly for tuning the otolith preparation techniques (using otolith sections instead of whole otoliths polished until reaching the core) and for selecting the trace elements.

TEC in the otolith edge could discriminate the locations where specimens were caught, separating the northernmost from the southernmost areas. The longitudinal multivariate analysis allowed to associate all TEC to each otolith zone representing a certain time in each specimen's life and simultaneously make a temporal analysis of the otolith elemental signature. In this analysis, TEC also sustained the separation of the otoliths into two groups, but there was evidence of high mixing between them, which agrees with the migratory hypothesis. The existence of two natal sources was suggested from otolith core TEC analysis. The acceptance of both southern and northern spawning grounds and of migratory movements along the NE Atlantic in northward and southward directions implies changes to the current migratory hypothesis that might translate into changes in *A. carbo*'s stock assessment.

An ontogenic effect in the incorporation of Sr, Mg, Cu, and Ba was observed in *A. carbo* otoliths, which is a consequence of the greater relative investment in reproduction of adults compared with juveniles. As fish mature, the level of Ca-binding proteins, such as albumin and vitellogenin, increases in the blood, reducing the free plasma Ca (Kalish, 1989).

Sampling locations were separated into two groups based on otolith edge chemical composition, which represents the recent otolith growth, suggesting there are differences between the northern and southernmost water masses and that otolith TEC is adequate to discriminate specimens from those two areas. However, the allocation of fish caught in other places to Madeira implies that their otoliths exhibit a chemical signature that characterizes Madeiran waters. This could mean those young fish have recently left Madeira and migrated northwards driven by the northward water flow that results from the merging of the upper portion of the MOW with a poleward eastern boundary undercurrent towards the Norwegian Sea, just west of Ireland (Postel and Du Buit, 1965; Price et al., 1993). Longmore et al. (2014) also found a mix of fish from different origins were located off Madeira based on adult fish otolith trace element composition. The connection between NE Atlantic northern and southern water masses has been previously supported by the presence of Madero-Mediterranean species northwards and by the absence of a geographical barrier (Postel and Du Buit, 1965). The existence of two concomitant natal sources was suggested based on otolith core TEC, one around Madeira and the Canaries, which is already known, and a second one, probably at the northernmost areas, supporting observations previously reported (Zilanov and Shepel, 1975; Ehrich, 1983; Magnússon and Magnússon, 1995; Longmore et al., 2014).

The assessment model for *A. carbo* in the NE Atlantic, accepted by ICES, consists of two state-space

models for geographically distinct parts of the population, one part occurring west of the British Isles (commercially exploited by the French deep-sea trawl fishery) and another part in mainland Portugal (exploited by the Portuguese continental deep-water longline fishery) (ICES, 2020). In each case, four groups (states) are defined according to two criteria: length – specimens from 70 to 103 cm TL (total length) and specimens larger than 103 cm TL, which is the length at first maturity (Figueiredo et al., 2003) – and fishing status (fished or not fished). In the first case, the model assumes four subprocesses: survival to natural mortality, transition from one length group to the other, displacement by migration (recruits entering the W British Isles from the southern spawning areas), and survival to fishery, whereas five subprocesses occur in the second case (immigration from W British Isles, survival to natural mortality, transition from one length group to the other, emigration to Madeira (southernmost areas), and survival to fishery (Stock Annex, ICES, 2020) .

The existence of a second spawning ground implies the population occurring west of the British Isles and off mainland Portugal would be also receiving recruits from the northernmost areas of occurrence (Iceland and the Faroes). Assuming that the transition between adjacent areas takes around three years (age corresponding to smaller individuals occurring W British Isles), strong fluctuations in biomass in the spawning grounds are expected to have an effect in the biomass in the later area with a three-year time-lag. Abundance trends from the last decade show a strong decrease in the catch-per-unit-effort (CPUE) in Madeira and in the biomass indices from the Icelandic survey in 2012, and in the French fishery in 2015 (ICES, 2020). Contrariwise, the CPUE in mainland Portugal has been slightly increasing since 2014. Although the influence of a second source of recruitment from the proposed northernmost spawning ground on the abundance of *A. carbo* is not clear in the previous abundance trends, the aggregation of the sampling locations into two groups, one of which is dominated by Madeiran specimens, based on the microchemical composition of otoliths supports the importance of incorporating data from Madeira, the primary natal area, into the assessment model.

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Chapter 6. Conclusions

1. Specimens from the northernmost areas (Iceland, Faroes, and the west of the British Isles) and specimens from the southernmost areas (mainland Portugal and Madeira) were separated based on FA, SI and otolith microchemical analysis.

2. A high index of vacuity was observed in the stomachs of specimens caught off mainland Portugal; but the following prey were identified in stomachs from this area for the first time: the lophogastrid crustacean *Gnathophausia zoea*, followed by the cephalopod *Mastigotheutis* spp. and the teleost *Rouleina maderensis*, in descending order of frequency.

3. A relationship between the reproductive cycle of *A. carbo* in the NE Atlantic and its fatty acid profile was clarified: mature specimens have higher energetic demands and show a prevalence of PUFA, whereas SFA are more representative in immature specimens.

4. The significant prevalence of PUFA in specimens caught off Madeira indicates that the species continues to feed during the spawning period.

5. The significantly higher proportion of arachidonic acid (ARA), which has an important role in reproduction, in specimens from mainland Portugal comparing with those from the northernmost areas explains why the former reach a more advanced maturity stage.

6. The trophic position inferred from the relationship between $\delta^{15}N$ and $\delta^{13}C$ and the allometric effect of each area's size range on $\delta^{15}N$ indicated that ontogenic and spatial differences in physiological aspects are related with diet and prey availability in Madeira, mainland Portugal, and the west of the British Isles, as well as with variations in the baseline values of the primary production.

7. The relationship between the baseline values of the primary production and latitude and depth are more evident in Icelandbecause specimens are smaller and, hence, positioned in a lower trophic level.

8. Developing females from Madeira were separated into two groups based on sex steroids: females from one group will mature and spawn; females from the second groups will not have the time to mature and reach the spawning stage and, hence, will fail to spawn in the current season.

9. TEC in the otolith edge could discriminate the locations where specimens were caught, separating the northernmost from the southernmost areas.

10. The existence of two natal sources was suggested from otolith core TEC analysis.

11. The acceptance of both southern and northern spawning locations and of migratory movements along the NE Atlantic in northward and southward directions implies changes to the current migratory hypothesis.

12. The existance of two natal sources reinforces the importance of including the fishery in Madeira in the stock assessment model, because recruitment from one spawning area might be compensating for biomass fluctuations in the other.

Annex: Publications

Aquat. Living Resour. 26, 333–342 (2013)

DOI: 10.1051/alr/2013061
www.alr-journal.org

Aquatic Living Resources

Review

Black scabbardfish, *Aphanopus carbo*, in the northeast Atlantic: distribution and hypothetical migratory cycle

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Received 7 February 2013; Accepted 24 July 2013

Abstract – The biology, ecology, and dynamics of the deep-sea teleost black scabbardfish in the northeast Atlantic are reviewed. The black scabbardfish is a commercial bathypelagic species found in the NE Atlantic mostly from Iceland to the Canary Islands and Western Sahara, at depths from 800 to 1300 m. The spatial structure of its population is still uncertain, although the existence of one single stock that migrates around the NE Atlantic driven by feeding and reproduction is the most likely hypothesis consistent with available data. This review is based on data from commercial fisheries off the Faroe Islands, Hatton Bank, the west of the British Isles, and Portugal (mainland, Azores, and Madeira) and from Icelandic and Scottish scientific surveys collected between 1988 and 2012. Spawning of black scabbardfish occurs around Madeira and the Canary Archipelagos during the last quarter of the year. According to the migratory hypothesis, eggs, larvae, and possibly juveniles move north to areas from south of Icelandic and Faroe Islands to the west of the British Isles where they remain for some years to feed and grow. Then, they move south to the area off mainland Portugal, where they reach the size of first maturity but do not reproduce, and later move further south to the spawning grounds. Further studies are needed to understand which of the environmental conditions prevailing around Madeira and the Canaries, but not elsewhere, allow this species to mature and subsequently reproduce. This review suggests that a multidisciplinary approach is required to confirm the spatiotemporal migration and habitats used by black scabbardfish populations in the NE Atlantic at different life stages. Otolith contour shape and microchemistry, fatty acids, carbon and nitrogen stable isotopes, as well as steroid hormones are proposed as promising alternative tools for responding to this challenge.

Keywords: Deep-water longline fisheries / Migration / Life cycle / Trichiuridae / *Aphanopus carbo* / North Atlantic Ocean

1 Introduction

Aphanopus carbo Lowe, 1839 (Actinopterygii: Trichiuridae) occurs throughout the North Atlantic between 30° N and 70° N, from the strait of Denmark to western Sahara, being most abundant to the south of the Faroe Islands, in the Rockall Trough, to the west of mainland Portugal, and around Madeira and the Canary archipelagos, but also occurring in Iceland, the Mid-Atlantic Ridge and Corner Rise, and the Azores (Nakamura and Parin 1993; Parin 1995; Pajuelo et al. 2008; Machete et al. 2011). The species has also been reported in the western part of the Atlantic, in Greenland, Canada, and the USA (Templeman and Squires 1963; Fitch and Gotshall 1972; Parin and Becker 1972; Peden 1974; Clarke and Wagner 1976; Gorbunova 1977; Howe et al. 1980; Nakamura 1984; Borets 1986; Lauth 1997). Reports of *A. carbo* occurrence in the eastern Pacific (Clarke and Wagner 1976; Howe et al. 1980; Pequeño 1989; McAllister 1990) and the southern Indian oceans (Piotrovskiy 1981) were deemed questionable by Nakamura and Parin (1993). The absence of confirmed records in those areas, despite the worldwide development of deep-sea fisheries and surveys since the early 1990s, suggests that *A. carbo* is restricted to the North Atlantic.

A. carbo is a bathypelagic species that has been found at depths from 200 m, in the northern section of the NE Atlantic (Nakamura and Parin 1993; Kelly et al. 1998), to 2300 m around the Canary Islands (Pajuelo et al. 2008), but is more frequent between 800 and 1800 m in mainland Portugal (Martins et al. 1987), 800 and 1300 m in Madeira (Morales-Nin and Sena-Carvalho 1996), and 400 and 1400 m off the west of the British Isles (Ehrich 1983; Allain et al. 2003). Its distribution is mainly associated with steep slopes, underwater rises, and canyons to the west of Portugal and along the gentle

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Article published by EDP Sciences

Table 1. Summary of fisheries and surveys from which data on black scabbardfish were available for this review; total length (TL, cm), total weight (g), maturity stage.

Area	Source	Depth (m)	Gear	Total length (cm) range & mode		Weight range (g)	Maturity stage	Reference
Iceland	Icelandic autumn survey	176–1307	Bottom trawl	26–123	90	572–1960	I-II	K. Jakobsdóttir (pers. comm.)
Faroe Islands	Fishery	*NA*	Traditional bottom trawl; doors did not touch the bottom	65–142	100	1000–4000	*NA*	ICES 2012
Hatton Bank	Fishery	1000–1400	Bottom trawl	71–120	87	*NA*	Immature	ICES 2012
West British Isles	Fishery; Scottish deep water survey	500–1800	Bottom trawl	62–130	90	232–2740	I-II	This study; Ribeiro Santos et al. 2013a
Mainland Portugal	Fishery	800–1450	Bottom longline	53–136	105	137–3650	I-III	This study
Azores	Experimental fishery	650–1900	Drifting bottom longline	60–156	120	*NA*	*NA*	Machete et al. 2011
Madeira	Fishery	~1000	Drifting horizontal longline	51–151	117	131–4020	All	This study; Bordalo-Machado et al. 2009

NA: not available.

sedimentary slope to the west of the British Isles (Martins et al. 1987; ICES 2012). Information regarding the black scabbardfish available from fisheries and surveys in the NE Atlantic is summarised in Table 1.

Although no eggs or larvae of black scabbardfish have been found, juveniles are reported to be mesopelagic (Parin 1986). Maul (1950) found two small specimens (10 and 15 cm total length) in the stomach of a long snouted lancetfish (*Alepisaurus ferox* Lowe, 1833). More recently, a specimen of approximately 10 cm, identified by DNA barcoding, was caught at the Senghor Seamount, off the northeast of Cape Verde (Hanel et al. 2010). There are also records of fish as small as 26 cm from Icelandic surveys (Table 1).

In terms of morphology, this species has a narrow elongated body with a pointed head and long dorsal fin, adapted for fast swimming; a large terminal mouth with large fang-like teeth, modified for efficient predation; large eyes (diameter is about a fifth to a sixth of head length) that facilitate sight in low light; and a coppery-black coloration with an iridescent tint to facilitate camouflage (Parin 1986; Nakamura and Parin 1993; Merrett and Haedrich 1997). Life history traits such as diet, growth, reproduction, and energy consumption are directly dependent on physical conditions of the deep sea such as high pressure and low temperature, which hinder the metabolic rate processes of fishes, as well as on predation stress and low food availability (Graham et al. 1985; Merrett and Haedrich 1997).

Recently, it was found that *A. carbo* coexists spatially with *Aphanopus intermedius* Parin, 1983 in the Azores, Madeira, the Canaries, and off the coasts of Morocco and the western Sahara (Nakamura and Parin 1993; Stefanni and Knutsen 2007; Stefanni et al. 2009; Biscoito et al. 2011). However, it has been genetically confirmed that *A. carbo* is the only species that reaches the northernmost latitudes of the NE Atlantic (Stefanni and Knutsen 2007; Biscoito et al. 2011; Ribeiro Santos et al. 2013a). The two species are morphologically very similar, yet both genetics (Stefanni and Knutsen 2007; Knutsen et al. 2009; Stefanni et al. 2009) and meristic characteristics (Tuset et al. 2010; Biscoito et al. 2011) have proven suitable for reliable identification. Throughout this manuscript the common names accepted by FAO are used: black scabbardfish for *A. carbo* and intermediate scabbardfish for *A. intermedius*.

How the black scabbardfish completes its life cycle is still in question. The most widespread hypothesis is that one single stock undertakes a large-scale clockwise migration around the NE Atlantic. Under this hypothesis, spawning is restricted to areas off Madeira, the Canary Islands, and possibly further south. Thereafter, juveniles recruit to the fisheries south of Iceland, around the Faroe Islands, and to the west of the British Isles, where they stay to feed and grow for a few years. From there, they first move south towards mainland Portugal and then further south to the spawning areas (Fig. 1).

Some specific habitat properties that allow the metabolic processes inherent to reproduction, survival of eggs and larvae or both might exist exclusively around Madeira and the Canaries and hence merit further investigation.

2 State-of-the-art

2.1 Fisheries

There are three main deep-sea fisheries targeting the black scabbardfish in the NE Atlantic: (i) to the west of the British Isles, fish are mainly exploited by the French deep-sea trawl fishery (Nakamura and Parin 1993; ICES 2012); (ii) an artisanal fleet operates with bottom longlines in ICES (International Council for the Exploration of the Seas) Subarea IXa, off mainland Portugal (Bordalo-Machado et al. 2009; ICES 2012); and (iii) a third important commercial fishery is operated by artisanal horizontal drifting longliners off the Madeira Archipelago, within the CECAF (Fishery Committee for the Eastern Central Atlantic) area (Bordalo-Machado et al. 2009). The fishery in Madeira dates back to the seventeenth century (Merrett and Haedrich 1997), whereas it started in mainland

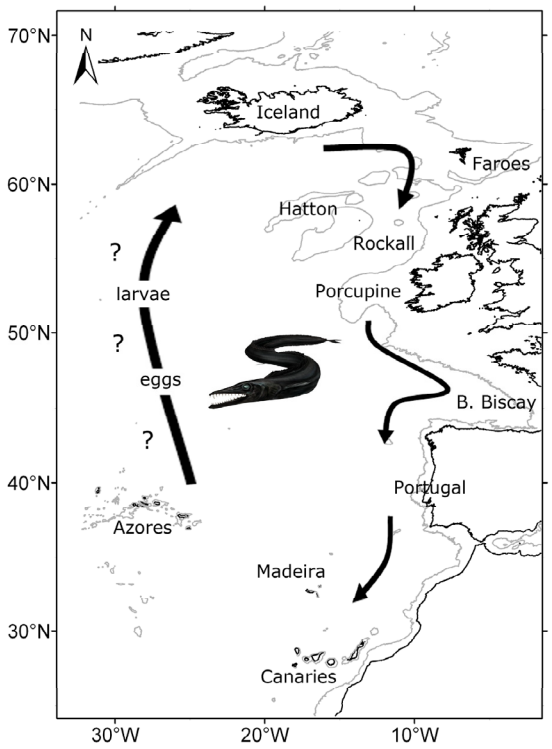

Fig. 1. Map of the northeast Atlantic Ocean representing the hypothetical migratory cycle of the black scabbardfish. The 1000 m depth contour is shown (drawing of black scabbardfish adapted from MARPROF, www.marprof.org).

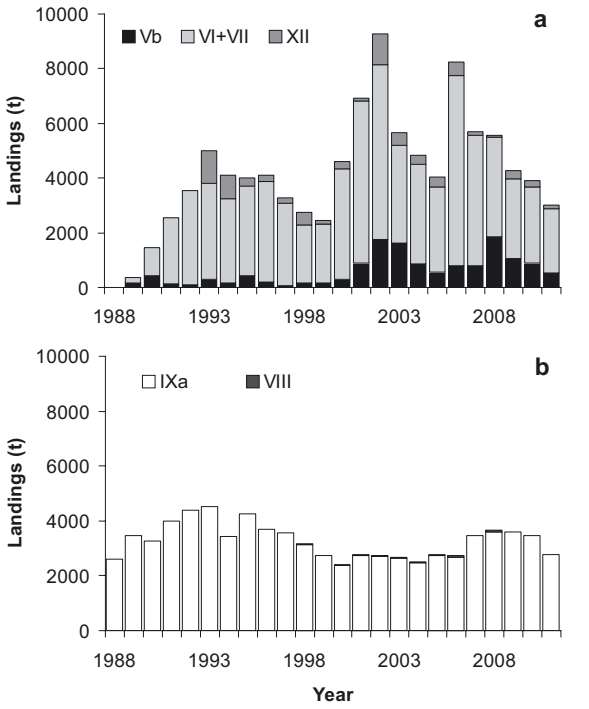

Fig. 2. Annual black scabbardfish landings from 1988 to 2011 for ICES, (a) northern component: ICES Division Vb (around Faroe Islands), VI+VII (west of the British Isles), and XII (north of the Azores); and (b) southern component: ICES Subarea VIII (Bay of Biscay), and Division IXa (west of mainland Portugal). Landings data are from ICES (2012). 2011 data are provisional.

Portugal in the early 1980s (Martins et al. 1987) and in northern Europe in the early 1990s (Merrett and Haedrich 1997). In ICES Subarea X, another directed fishery has recently started in Azorean waters (Portugal) (Machete et al. 2011). In Madeira and the Azores, *A. intermedius* might be landed mixed with *A. carbo* (Stefanni and Knutsen 2007). Additionally, a few smaller and interrupted fisheries occur in Faroese and Icelandic waters where the species is exploited by both longliners and trawlers (ICES 2012) and in the Canary Islands, where it is exploited by drifting mid-water longliners (Pajuelo et al. 2008).

The trend in black scabbardfish landings in ICES northern (Subareas Vb, VI, VII and XII) and southern (Subareas IXa and VIII) components in the last two decades is shown in Figure 2. Estimated landings in 2011 were 2357 t for ICES Subareas VI and VII (W British Isles), 2781 t for mainland Portugal (ICES 2012) and 1941 t for Madeira (Anon. 2012). Regarding the smaller fisheries, landings increased from 139 t in 2011 to 458 t in 2012 in the Azores (M. Ruivo, pers. comm.); reached 109 t in Iceland in 2010 (no data was provided for 2011); and were 111 t caught by Spanish vessels in SE Greenland, ICES Division XIV in 2010 (ICES 2012).

2.2 Age, growth, and length structure

Over time, studies on age and growth of the black scabbardfish have led to different conclusions (Table 2): initially, the maximum age was estimated to be 8 years for fish from Madeira (Morales-Nin and Sena-Carvalho 1996) and 32 years for the Rockall Trough (Kelly et al. 1998). Later, it was determined to be 12 years for Madeira and the Canaries (Morales-Nin et al. 2002; Pajuelo et al. 2008). More recently, the maximum age was estimated to be 12 years in mainland Portugal (Vieira et al. 2009) and 14 years in Madeira (Vieira et al. 2009; Delgado et al. 2013). Since the length range used in all studies was similar, the differences might be associated with the age assignment criteria, otolith preparation techniques, quality of the equipment used or experience of the readers.

The maximum age estimated by Morales-Nin and Sena-Carvalho (1996) corresponded to a male of 130 cm and a female of 150 cm total length. These ages were probably underestimated because, when using whole otoliths in larger specimens from this species, the growth increments closer to the border are very difficult to identify (Vieira et al. 2009). On the contrary, the maximum age assigned by Kelly et al. (1998) using thin otolith sections was most likely overestimated since, with this preparation technique, the number of visible rings is very high and the authors reported problems in their interpretation. Regarding age estimations in Madeira and the Canary Islands in studies prior to 2008, when caught specimens started being routinely separated by species, the possible mixing of black and intermediate scabbardfish specimens could also explain the differences found between regions and should be

Table 2. Von Bertalanffy growth parameter estimates from different studies carried out in the NE Atlantic, including otolith age reading method and clearing solution. F: female, M: male, SD: standard deviation.

Area	Method	Clearing	Sex	*N*	Total length range (cm)	Age range (year)	L_{inf} ± SD (cm)	*k* (year^{-1})	t_0 (year)	Source
W. British Isles	Thin sections in epoxy resin	Alcohol	both	230	75–120	4–32	*NA*	0.1	*NA*	Kelly et al. 1998
Mainland Portugal	Thin sections in epoxy resin	1:1 glycerin-alcohol	F	248	64–131	5–13	135 ± 4	0.2	–2.0	Vieira et al. 2009
			M	206		4–10	124 ± 3	0.2	–1.7	
Madeira	Surface	Glycerol	F	334	58–151	0–8	142	0.3	–2.1	Morales-Nin and Sena-Carvalho 1996
			M	357	58–132		155	0.2	–3.3	
			both	649	58–151		139	0.3	–2.3	
	Thin sections in epoxy resin	1:1 glycerin-alcohol	F	200	125–148	8–15	159 ± 4	0.1	–2.3	Vieira et al. 2009
			M	163		8–14	146 ± 1	0.1	–1.4	
	Surface	1:1 glycerin-alcohol	F	554	100–140	6–14	136 ± 5	0.2	–4.2	Delgado et al. 2013
			M				132 ± 5	0.2	–3.1	
Canary Islands	Surface, burned	50% glycerol	F	196	100–148	2–12	149 ± 2	0.2	–4.7	Pajuelo et al. 2008
			M	102	104–134	2–8	141 ± 4	0.3	–3.5	
			both	298	100–148	2–12	148 ± 2	0.2	–4.6	

NA: not available.

taken into consideration. The maximum ages assigned by Delgado et al. (2013) using whole otoliths were 14 years for black scabbardfish and 15 years for intermediate scabbardfish. Overall, the age estimation of the black scabbardfish is difficult and has not yet been validated. Both the seasonality of the deposition of material at the otolith margin and of daily growth increments would deserve additional studies with standardised methods using material from all areas and seasons.

The growth parameters estimated based on the von Bertalanffy growth equation showed a relatively rapid growth rate for the black scabbardfish (Table 2). Figure 3 represents the growth curves according to sex for all available studies, restricted to the length range of each fish sample. Growth estimates from Vieira et al. (2009) and Delgado et al. (2013) seem to be in agreement, without any meaningful area effect. Age-at-length from Kelly et al. (1998) was consistently higher than in all the other studies. This implies a low k for fish from the west of the British Isles which is not in agreement with the predominance of young immature specimens in this area.

Rapid growth of deep-sea juvenile fishes has been shown to be an advantageous strategy in feeding success and predation avoidance (Crabtree and Sulak 1986). Nonetheless, in the results of Vieira et al. (2009) the absence of small individuals caught off Madeira may have interfered with the accuracy of the growth parameter estimates. In contrast, slow growth rate is observed for adults, as a result of a transfer of energy investment from growth to reproduction (Lika and Nisbet 2000). This strategy contrasts with that of species whose growth continues after maturation, such as most shelf demersal and pelagic commercial fish.

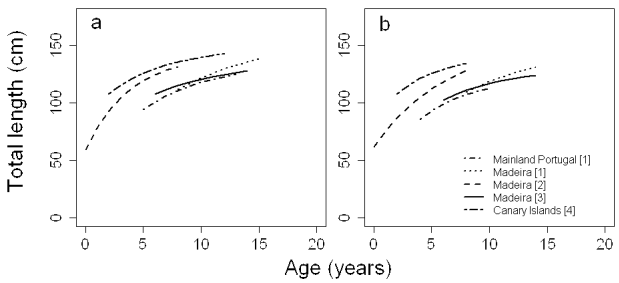

Fig. 3. Growth curves for black scabbardfish from different studies, areas and methods. (a) females; (b) males. Growth parameters are from [1] Vieira et al. (2009); [2] Morales-Nin and Sena-Carvalho (1996); [3] Delgado et al. (2013); [4] Pajuelo et al. (2013).

Size ranges reported for different areas in the NE Atlantic are presented in Table 1. The smallest specimens were reported in Iceland, where this species can be caught in relatively shallow waters, whereas the largest fish were caught off the Azores, where this species reaches deeper waters.

Length frequency distributions for different ICES and CECAF management units in 2011 are presented in Figure 4. The French fleet operates mainly in ICES Division VIa but also in Vb and VII; the Spanish fleet in Divisions VIb and XIIb; the Portuguese longline fishery in Division IXa; the Azores fleet in Subarea X (ICES 2012); and the Madeira fleet in CECAF division 34.1.2 (Delgado et al. 2013). Moreover, data from Subarea X were collected during Faroese surveys

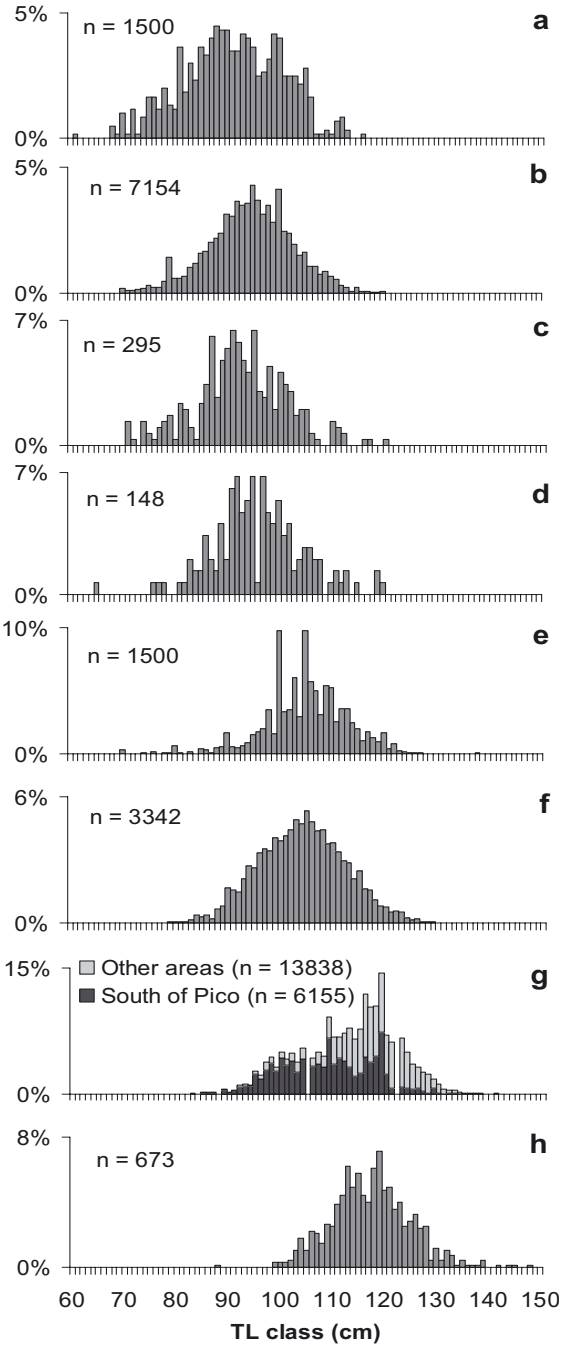

Fig. 4. Length frequency distribution of black scabbardfish in 2011 from north to south: (a) Icelandic surveys (ICES Division Va); (b) on-board observations of French trawlers (mostly in ICES Division VIa); (c) on-board observations of Spanish trawlers off the west of the British Isles (VIb); (d) on-board observations of Spanish trawlers (Subarea XII); (e) self-sampling Faroese exploratory surveys (Subarea X); (f) Portuguese longline fishery off mainland Portugal (IXa); (g) experimental fishery in the Azores (data are from 2005); (h) sampling of commercial landings in Madeira. Length frequency data are from [a-f] ICES (2012); [g] Machete et al. (2011); [h] Delgado et al. (2013).

(ICES 2012). In general, the size distributions move towards higher values from north to south of the NE Atlantic. In the Azores, the bimodal length distribution found to the south of Pico (Fig. 4g) is probably a consequence of mixing between *A. carbo* and *A. intermedius*, since the latter species has been described in this area (Stefanni and Knutsen 2007).

2.3 Diet

The black scabbardfish is a top predator, which feeds on a large food spectrum (Zilanov and Shepel 1975; Nakamura and Parin 1993; Ribeiro Santos et al. 2013b). Diet studies based on gut content analyses are difficult in this species because, most of the time, these are either already fully digested or have been regurgitated as a result of hydrostatic decompression, as happens in deep-sea fishes that have a swim bladder (Stowasser et al. 2009). This difficulty is worsened when sampling fish are caught by the commercial longlines off Madeira and mainland Portugal because the soaking time can be up to 40 h (Bordalo-Machado et al. 2009), enabling the full digestion of the stomach contents. As a consequence, the vacuity index calculated for fish from Madeira was 93 to 98% (Freitas 1998; Ribeiro Santos et al. 2013b), whereas it varied from 66 to 94% for specimens caught by trawls to the west of the British Isles (Mauchline and Gordon 1984; Ribeiro Santos et al. 2013b) (Table 3).

Table 3 summarizes the published information on the diet of the black scabbardfish in the NE Atlantic. To the west of the British Isles, several studies have focused on the diet of this species. Du Buit (1978) identified two fish taxa, *Argentina* sp. (Osmeriformes) and Gadidae, in eight non-empty stomachs. Mauchline and Gordon (1984) and Ribeiro Santos et al. (2013b) found mostly fish, namely blue whiting (*Micromesistius poutassou*), and a small amount of cephalopods. The differences between these studies may be related to the seasonal changes observed by Ribeiro Santos et al. (2013b) who associated the decrease in blue whiting and consequent increase in cephalopods and crustaceans in the diet of the black scabbardfish with the migration of blue whiting to the Norwegian Sea. In fact, when Howell et al. (2009) combined the diet composition of fish caught by scientific surveys off the Rockall Trough with earlier data from Mauchline and Gordon (1984), the resulting diet was approximately 50% cephalopods and 44% blue whiting.

In fish caught off Madeira, the main prey were the cephalopods *Chiroteuthis* spp., *Mastigoteuthis* sp., *Histioteuthis* sp., and *Taonius* sp., and the fish *Chauliodus* sp., as well as several Myctophidae (Freitas 1998). The differences between the diets of black scabbardfish caught off the British Isles and off the Madeira archipelago may be related to food availability given that, in the latter area, no large stocks of small pelagic and mesopelagic fish, such as blue whiting, can be found.

2.4 Reproduction

The black scabbardfish is an iteroparous species, since it can spawn multiple times throughout its life, and is also a total spawner, as it spawns in one single event (Pajuelo et al. 2008; Ribeiro Santos et al. 2013a). Moreover, it has determinate

Table 3. Summary of the diet composition of black scabbardfish caught in the NE Atlantic.

Area	Depth (m)	Total length range (cm)	No. stomachs sampled	No. stomachs with food	Vacuity index (%)	Main prey	Source
West British Isles	750–1100	80 (a)	*NA*	8	*NA*	Fish (*Argentina* sp. and Gadidae)	Du Buit 1978
	500–1250	77–108	142	52	66.2	Fish (*Micromesistius poutassou* and Scombridae) and cephalopods	Mauchline and Gordon 1984
	NA	*NA*	*NA*	*NA*	*NA*	Cephalopods, fish (*Micromesistius poutassou*), prawns and shrimps	Howell et al. 2009 (including data from Mauchline and Gordon 1984)
	500–1800	97 ± 7 (b)	1581	133	91.6	Fish (*Micromesistius poutassou* and mesopelagic species) and cephalopods	Ribeiro Santos et al. 2013b
Madeira	600–1300	~110–150	3688	378	93.3	Cephalopods (*Chiroteuthis* spp., *Mastigoteuthis* sp., *Histioteuthis* sp., and *Taonius* sp.) and fish *Chauliodus* sp. and Myctophidae)	Morales-Nin and Sena-Carvalho 1996; Freitas 1998

(a) Mean. (b) Mean ± standard deviation.

fecundity, which means that the potential annual fecundity corresponds to the number of vitellogenic oocytes minus the number of oocytes reabsorbed on account of atresia (Neves et al. 2009; Ribeiro Santos et al. 2013a). Mature and spawning adults have only been observed in the last quarter of the year in Madeira (Figueiredo et al. 2003; Neves et al. 2009; Ribeiro Santos et al. 2013a), the Canaries (Pajuelo et al. 2008), and the northwest coast of Africa (Perera 2008). Estimated female length at first maturity (L_{50}) was 103 cm around Madeira (Figueiredo et al. 2003) and 114 cm around the Canary Islands (Pajuelo et al. 2008). Once again, the possible mixture of black and intermediate scabbardfish specimens in the samples may have biased these results. In a more recent work, female L_{50} was estimated to be 111 cm for Madeira and 116 cm when also including specimens from the west of the British Isles (Ribeiro Santos et al. 2013a). These values are probably overestimated because the estimation did not include specimens from Madeira smaller than 92 cm in total length. Despite the available information questions remain on the reproductive dynamics of this species.

First, why does the black scabbardfish not mature and spawn elsewhere than Madeira and the Canaries? In mainland Portugal, vitellogenesis begins at the same time as in those areas and a high proportion of caught individuals (ca. 25%) is larger than L_{50} (Figueiredo et al. 2003; Neves et al. 2009). Additionally, although reported in the past at Porcupine Bank, to the west of the British Isles (Ehrich 1983) and in Icelandic waters (Magnússon and Magnússon 1995), reproduction has not been observed in these locations since. In fact, even though fish attain relatively high total length, only maturity stages I and II have been observed in either of these areas (Table 1). Because gonad macroscopic features are difficult to interpret, which could have led to incorrect assignments of maturity stage in the past, a standardized maturity scale was proposed for the black scabbardfish (Gordo et al. 2000).

In terms of physiological condition indicators, the gonadosomatic index (GSI) is higher around Madeira than off mainland Portugal (Neves et al. 2009) and to the west of the British Isles (Ribeiro Santos et al. 2013a) for the same body length. Furthermore, atresia occurs in stage II ovaries from fish caught off the previous areas before and during the spawning period (Neves et al. 2009; Ribeiro Santos et al. 2013a). The most likely hypothesis is that intrinsic (e.g., energy budget, chemical predisposition) and extrinsic (e.g., water temperature) factors that condition the maturity process are lacking in the previous areas mentioned above. Fish in a poor nutritional state would cease the maturation process and remain in the same location, whereas fish in a better state would migrate to areas that provide the environmental conditions to optimize spawning and the survival of eggs and larvae (Neves et al. 2009; Ribeiro Santos et al. 2013a). The differences found in GSI are in accordance with the proposed hypothesis for population dynamics, even though no conclusions regarding migratory movements can be drawn from it.

Steroid hormones are responsible for triggering reproductive processes such as gametogenesis, so their levels are expected to change during the reproductive cycle and to be different between fish in distinct maturity stages (Modesto and Canário 2003). In an on-going study, variations in the level of steroid hormones in the plasma of black scabbardfish caught

off Madeira and mainland Portugal are being analysed (Farias et al., unpublished). Further work is needed to assess whether the study of steroid hormones in black scabbardfish in different areas and seasons would help us to understand the relationship between the migratory and reproductive cycles.

A second question is whether females are able to reproduce in consecutive years. Females spawn all oocytes contained in their ovaries in a single event at each reproductive cycle. Therefore, all females larger than the length at first maturity are expected to mature and spawn simultaneously. Assuming that the age at maturity is approximately 6.5 years (after Figueiredo et al. 2003 and Vieira et al. 2009) and that the oldest specimens found were 14 years old (Vieira et al. 2009; Delgado et al. 2013), females are expected to be able to spawn for a period of 8 years. However, the presence of non-reproductive adults mixed with spawning adults in Madeira during the spawning period suggests that skipped spawning may occur in this species (Neves et al. 2009; Ribeiro Santos et al. 2013a). Skip spawning is an efficient strategy for saving energy that can then be allocated to growth and large scale migration (Ribeiro Santos et al. 2013a), as well as to subsequent reproduction.

2.5 Migration

Techniques to study the migration of coastal species, namely mark-recapture, tagging, telemetry, hydroacoustics and diet composition, are difficult to apply to deep-sea species owing to sampling constraints that result from depth and pressure. An innovative combination of tools that may provide complementary information for clarifying the migration pattern, stock structure and spatiotemporal dynamics of this species in the NE Atlantic could include otolith microstructure, otolith shape, fatty acids, and stable isotopes. Multidisciplinary studies carried out so far to uncover these aspects are quickly reviewed below.

In a previous study, black scabbardfish from the three Portuguese directed fisheries (mainland, Madeira, and the Azores) was characterized in terms of reproductive strategy (Neves et al. 2009), growth (Vieira et al. 2009), otolith shape (Farias et al. 2009), parasites (Santos et al. 2009), and contamination (Costa et al. 2009), with the goals of identifying its stock structure and assessing its biochemical composition. The differences found between areas support the migratory hypothesis but do not provide proof because they are mainly dependent on the ontogenetic structure of each sampling location, besides reflecting an unbalanced sampling scheme, given that the sample size collected off the Azores was much smaller than for the other areas.

Otolith contour shape analysis was used to discriminate specimens from mainland Portugal, the Azores, and Madeira (Farias et al. 2009). Differences were found in otolith contour shape between geographical regions, which is in agreement with the hypothesis of fish remaining a few years at each location. In another study, otolith shape supported the existence of a single population of black scabbardfish off Madeira and the Canaries, but the number of specimens was too small to be conclusive (Tuset et al. 2013). This result is very interesting in terms of the migration hypothesis and its relation with the reproductive cycle because it is evidence that fish from Madeira and the Canaries constitute the same population.

2.5.1 Fatty acids

In fishes, fatty acids (FA) can be used as indicative biomarkers for different trophic levels (Kirsch et al. 2000). Preliminary results of analyses performed on black scabbardfish muscle indicate that immature specimens caught off Iceland and the west of the British Isles accumulate mainly oleic acid, which is a precursor of all omega-3 and omega-6 polyunsaturated fatty acids (PUFA) (Dalsgaard et al. 2003). Additionally, in fishes caught off Madeira, which were mostly mature, and mainland Portugal, which are mostly in the developing and pre-spawning stages, a prevalence of PUFA, namely arachidonic acid and docosapentaenoic acid, has been observed (Farias et al., unpublished). PUFA are precursors of prostaglandins, which have an important role in reproduction, stimulating ovulation and spawning and eliciting female sexual behaviour (Stacey and Goetz 1982), and their prevalence in specimens from Madeira and mainland Portugal might be related to the predominance of pre-spawning and spawning maturity stages.

Similar results were obtained by Nogueira et al. (2013), who analysed FA in the muscle and liver of black scabbardfish caught off Madeira. The main differences between these two studies are in two monounsaturated fatty acids (MUFA), which were not detected in muscle samples in Nogueira et al. (2013), but were found in muscle samples from all the geographical areas analysed in the aforementioned study (Farias et al., unpublished). These divergences might result from differences in apparatus sensitivity, as well as from the deterioration of the tissues, since the samples analysed by Nogueira et al. (2013) were left in ice for 1–2 days between capture and laboratory analyses.

2.5.2 Stable isotopes

Ribeiro Santos et al. (2013b) analysed the diet of black scabbardfish caught off Madeira and to the west of the British Isles in terms of δ^{15}N and δ^{13}C and concluded that this species forms a link between the pelagic and the benthopelagic food webs, reflecting preferred feeding on pelagic fish and cephalopods. Seasonal changes in δ^{15}N were observed in fish caught off the west of the British Isles, supporting a shift to prey of a lower trophic level during the season when blue whiting migrates northward. The observed depletion in δ^{13}C in November in fish from Madeira could result from adults moving to shallower waters closer to the shore to spawn, as reported by local fishermen, which would imply a change between very different food sources (DeNiro and Epstein 1978). δ^{13}C of black scabbardfish caught in the Bay of Biscay (5 individuals, Chouvelon et al. 2012) was intermediate between values from the west of the British Isles and Madeira from Ribeiro Santos et al. (2013b), whereas δ^{15}N was lower.

Preliminary results obtained for samples caught off Madeira and the west of the British Isles, as well as mainland

Portugal and Iceland, are intermediate between those of previous work (Farias et al., unpublished). Mean δ^{15}N is lower than the values presented by Ribeiro Santos et al. (2013b) but higher than the estimates obtained for the Bay of Biscay. The mean δ^{13}C for Iceland and the west of the British Isles is higher than the estimate of Ribeiro Santos et al. (2013b) for the latter area, whereas the values from fish caught in the two southernmost areas are higher than the value for Madeira. The differences between studies suggest annual or seasonal variations associated with the sampling periods, which need to be further analysed. It is worth noting that the similar δ^{13}C observed in fish caught off Iceland and the west of the British Isles is supported by the geographical proximity between these sampling sites, whereas different mean δ^{15}N reflects dissimilarities in the diet of black scabbardfish between these regions (Farias et al., unpublished).

3 Discussion

The assessment of exploited populations and the subsequent proposal of management measures are focused on stocks. There are several definitions for stock but the most commonly agreed upon is that it is a group of fishes with similar life history characteristics large enough to be self-reproducing (Hilborn and Walters 1992). Since the stock structure is still unknown for most exploited species, stock units are adopted for management purposes. These units are reasonably heterogeneous for a number of biological, spatial, and temporal dimensions (Hilborn and Walters 1992). In the case of the black scabbardfish, ICES considers three management units (ICES 2012): "Northern" (Divisions Vb and XIIb and Subareas VI and VII); "Southern" (Subareas VIII and IX); and "Other areas" (Divisions IIIa and Va Subareas I, II, IV, X, and XIV).

Quinta et al. (2004) found genetic evidence of the black scabbardfish population being genetically structured into two groups: those from the eastern Atlantic (mainland Portugal and the Hatton Bank) and those from around the Madeira archipelago. However, it is now clear that these results were most likely confounded by the occurrence of *A. intermedius* around Madeira (Stefanni and Knutsen 2007; Knutsen et al. 2009; Stefanni et al. 2009).

The current understanding of the population dynamics of black scabbardfish in the NE Atlantic implies that spawning occurs around Madeira, the Canary Islands, and possibly a few other southern areas, like the NW coast of Africa (Figueiredo et al. 2003; Pajuelo et al. 2008; Perera 2008; Neves et al. 2009). Juveniles occur mainly in the northernmost areas, namely Iceland, the Faroe Islands, and the west of the British Isles, where small fish of 2–3 years old are caught by fisheries and surveys. The northward migration from the spawning areas to the latter areas might also involve larvae and juveniles up to a length of 60 cm or more. After having grown in northern areas for a few years, these fish move south towards mainland Portugal, where they remain a few more years before migrating further south to the spawning areas. This migratory behaviour is expected to be driven by feeding and reproduction (Zilanov and Shepel 1975; Anon. 2000; Figueiredo et al. 2003).

The depth and route of these migratory movements, as well as the contribution of active swimming vs. passive drifting, are unknown. It may be that the species migration makes use of poorly known oceanic features, allowing small juvenile black scabbardfish to reach northern areas. Furthermore, the geographical limitation of the known spawning areas for a species that is widespread in the NE Atlantic suggests the occurrence of particular hydrological or trophic features in those areas. The proposed innovative approach can thus yield new knowledge on the biological as well as physicochemical features of the NE Atlantic deep-sea ecosystem.

In conclusion, several methods have already been used to clarify the migration of this species. So far, otolith shape (Farias et al. 2009), oocyte maturity (Ribeiro Santos et al. 2013a), and microchemical analysis of the larval portion of otoliths (Longmore 2011) support the migratory hypothesis, showing evidence of a single black scabbardfish stock in the NE Atlantic.

Notwithstanding, other techniques have a good potential to provide complementary information. For example, elemental composition of the otolith core could be used to assess whether black scabbardfish from different areas were all born in similar hydrological conditions, i.e., in the same location. Moreover, the combination of fatty acids and stable isotopes provides important information for understanding the structure of this species in the NE Atlantic by elucidating the connection of trophic and reproductive processes with prevailing environmental features in the different areas where the species spends parts of its life cycle.

Acknowledgements. The authors would like to thank Elena Guijarro Garcia (IEO, Spain), Klara Jakobsdóttir (MRI, Iceland), Lise Helen Ofstad (Havstovan, Faroe Islands), Miguel Machete (DOP, Azores), and Sara Reis (DSIP, Madeira) for providing data, and Teresa Moura (IPMA) for helping with the map. This work was supported by the DCF – European Commission Fisheries Data Collection Framework, the EU project *Management and monitoring of deep-sea fisheries and stocks* (DEEPFISHMAN, Grant No. 227390), the Portuguese project *Certificação MSC em Portugal: obtenção de conhecimento e estudos de viabilidade para candidaturas de pescarias costeiras e artesanais* (CERTIFICA, PROMAR, 2011-2015), and a doctoral fellowship granted to I. Farias by Fundação para a Ciência e Tecnologia (SFRH/BD/64434/2009). They also thank two anonymous referees for their comments and suggestions.

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Deep-Sea Research I 89 (2014) 84–93

Contents lists available at ScienceDirect

Deep-Sea Research I

journal homepage: www.elsevier.com/locate/dsri

Reproductive and feeding spatial dynamics of the black scabbardfish, *Aphanopus carbo* Lowe, 1839, in NE Atlantic inferred from fatty acid and stable isotope analyses

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ARTICLE INFO

Article history:
Received 11 November 2013
Received in revised form
16 April 2014
Accepted 22 April 2014
Available online 9 May 2014

Keywords:
Aphanopus carbo
Diet
Fatty acids
NE Atlantic
Reproductive cycle
Stable isotopes

ABSTRACT

The black scabbardfish (*Aphanopus carbo*) is a benthopelagic species widely distributed across the NE Atlantic, where it is admitted to perform a clockwise migration throughout its life cycle stimulated by feeding and reproduction. To overcome the limitations of direct observation of this species, fatty acids profile (FA) and δ^{15}N and δ^{13}C stable isotopes (SI) were analyzed in the muscle tissue of the black scabbardfish and related with diet and maturity. Specimens were collected in four geographic areas in the NE Atlantic: Iceland, the west of the British Isles, mainland Portugal, and Madeira. For all areas, the FA profile was related with the different phases of the reproductive cycle and with diet, whereas the SI were related with diet, environmental characteristics, such as latitude and depth, and particulate organic matter (POM). Stomach content of black scabbardfish caught off mainland Portugal was analyzed and the most frequent prey item identified was the lophogastrid crustacean *Gnathophausia zoea*, followed by the cephalopod *Mastigotheutis* spp. and the teleost *Rouleina maderensis*. For specimens from Iceland and the west of the British Isles, monounsaturated fatty acids (MUFA) were the most important FA, followed by polyunsaturated (PUFA) and saturated FA (SFA), whereas for specimens from mainland Portugal and from Madeira the sequences were PUFA > MUFA > SFA and PUFA > SFA > MUFA, respectively. Immature specimens from the first three areas were found to be accumulating oleic acid which is an intermediate product of the metabolic pathway that transforms SFA to MUFA and these into PUFA. Specimens caught off Madeira were mature and showed a significant prevalence of ARA and DHA which are PUFA with an important role in reproduction. δ^{15}N was significantly higher in the muscle of black scabbardfish from Madeira, whereas δ^{13}C was significantly lower in specimens from Iceland. The low isotopic ratios as well as the prevalence of certain fatty acid trophic markers (FATM) connected specimens from Iceland with small prey. Results indicated that the spatial differences in physiological aspects of this species are related with diet and prey availability in Madeira, mainland Portugal, and the west of the British Isles, as well as variations in the baseline values of the primary production that are related with latitude and depth, mainly in Iceland. The allometric effect of each area's size ranges over δ^{15}N supports the existence of ontogenic differences in the black scabbardfish's diet. This diet is typical of mobile benthopelagic predators that are opportunistic feeders.

1. Introduction

The black scabbardfish (*Aphanopus carbo* Lowe, 1839) is a benthopelagic species widely distributed across the Northeast Atlantic, from Iceland southward to the Canary Islands, including the Mid-Atlantic Ridge and Corner Rise and the Azores (Nakamura and Parin, 1993; Parin, 1995; Pajuelo et al., 2008; Machete et al., 2011). The species is reported to live at depths from 200 m, at the west of the British Isles and around Iceland (Nakamura and Parin, 1993; Kelly et al., 1998), to 2300 m around the Canary Islands (Pajuelo et al., 2008). At its southernmost limit of distribution, namely Azores, Madeira, and the Canary Islands, *A. carbo* coexists with *Aphanopus intermedius* Parin, 1983 (Nakamura and Parin, 1993; Stefanni and Knutsen, 2007; Tuset et al., 2010; Biscoito et al., 2011).

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http://dx.doi.org/10.1016/j.dsr.2014.04.010
0967-0637/

Although there are some unresolved questions regarding the spatial structure of the black scabbardfish population, the existence of one single stock that moves clockwise along the NE Atlantic driven by feeding and reproduction is widely accepted (Zilanov and Shepel, 1975; Figueiredo et al., 2003; Farias et al., 2013). Spawning occurs around Madeira and the Canary Islands (Figueiredo et al., 2003; Pajuelo et al., 2008; Neves et al., 2009; Ribeiro Santos et al., 2013a), where total length ranges from 88 to 151 cm (Farias et al., 2013) and from 99 to 147 cm (Pajuelo et al., 2008), respectively, but has also been reported around the Azores (Vinnichenko, 2002) and the northwest coast of Africa (Perera, 2008). Estimated female length at first maturity (L_{50}) is 103 cm around Madeira (Figueiredo et al., 2003). More recently, L_{50} was estimated to be 114 cm around the Canary Islands (Pajuelo et al., 2008), 111 cm for females from Madeira and 116 cm for females from Madeira and the west of the British Isles together (Ribeiro Santos et al., 2013a). However, these values are prone to be overestimated because specimens smaller than 99 cm in total length from the Canaries and 92 cm from Madeira were not included in the analyses (Farias et al., 2013). Eggs and larvae have not been reported so far. Some of the juveniles probably move northward to south of Iceland and around the Faroe Islands and west of the British Isles where they remain 4–6 years to feed and grow (ca. 60–120 cm in total length) (Farias et al., 2013). Then they move southward to the area west of mainland Portugal, where specimens are caught with 79–136 cm in total length (4–14 years old) (Vieira et al., 2009; Farias et al., 2013), reaching the size of first maturity but not reproducing, and later move further south to the spawning grounds. Although Madeira and the Canary Islands are the only incontrovertible areas where mature specimens are caught, philopatry has not been studied for this species. Moreover, the corroboration of its migration pattern along the NE Atlantic has not been possible on account of constraints driven by depth and pressure that hinder the use of techniques generally applied to coastal species to study migration, such as tagging and mark-recapture methods.

An extensively used alternative to direct observation of migratory movements is the analysis of a species trophic ecology since the prey diversity available for a predator is considered representative of the specific richness of the ecosystem where it lives. However, stomach analyses are difficult in deep-sea fishes because their contents are usually either regurgitated as a result of hydrostatic decompression or fully digested (Stowasser et al., 2009). For those fish, the quantification of chemical constituents acquired through diet has been shown to be a good representation of the feeding regimes and trophic relationships (DeNiro and Epstein, 1978; Petursdottir et al., 2008; Drazen et al., 2009; Stowasser et al., 2009; Ribeiro Santos et al., 2013b). Moreover, the chemical composition of fish muscle reflects the energetic trade-offs between intake, mostly through food, and expenditure with metabolism, migratory swimming, and the reproductive cycle (Petursdottir et al., 2008; Stowasser et al., 2009). Within those chemical constituents, both fatty acid (FA) and stable isotopes (SI) signatures provide a good insight into trophic relationships and food web interactions as their patterns reflect the dietary intake over longer time periods than stomach content analyses.

Lipids and the FA that compose them are, along with proteins, the major organic constituents of fish, functioning as the main source of metabolic energy for growth, reproduction, and locomotion, including migration (Tocher, 2003). Fatty acid trophic markers (FATM) are synthesized by specific primary producers and zooplankton, are transferred relatively unchanged along food webs, and accumulate in grazers and predators over time, allowing regional and temporal variations in plankton dynamics to be tracked down in the marine food webs (Lee et al., 1971; Dalsgaard et al., 2003; Drazen et al., 2009; Letessier et al., 2012).

SI analysis (SIA) has proven its value as a complementary and sometimes preferable tool for food web analyses in marine systems, recording both source and trophic level information (Iken et al., 2001; Polunin et al., 2001; Michener and Kaufman, 2007). Stable carbon and nitrogen isotope ratios ($\delta^{13}C$ and $\delta^{15}N$) are used as trophic markers; the first, to trace the carbon source, the regional origin of the primary producers, and the pathways of organic matter, and the other as an indicator of the trophic position of organisms (DeNiro and Epstein, 1978, 1981; Post, 2002; McCutchan et al., 2003; Laakmann and Auel, 2010; Letessier et al., 2012). SI ratios can change between diet and consumer due to differential digestion or fractionation during assimilation and metabolic processes (DeNiro and Epstein, 1978, 1981; Tieszen et al., 1983; McCutchan et al., 2003; Michener and Kaufman, 2007). The isotopic trophic discrimination factor between a consumer's tissue and its diet is on average 0.5–1‰ for $\delta^{13}C$ and 3.4‰ for $\delta^{15}N$ (DeNiro and Epstein, 1978, 1981; Vander Zanden and Rasmussen, 2001; Post, 2002; Michener and Kaufman, 2007; Laakmann and Auel, 2010; MacKenzie et al., 2011). Moreover, the relative abundance of SI within the organisms is influenced by external factors, namely geological, climatic, and ecological, as well as by internal factors, such as the synthetic pathways and position in the food web (DeNiro and Epstein, 1978, 1981; McCutchan et al., 2003).

The aim of the present study is to assess the feeding ecology and reproductive potential of the black scabbardfish through the association between chemical biomarkers, specifically fatty acids and stable isotopes, and ecological attributes such as diet, length distribution, and maturity stage. Regional variations on the chemical composition of black scabbardfish in terms of fatty acid profile and stable carbon and nitrogen isotope ratios will be evaluated using specimens caught at four different geographical areas in the NE Atlantic.

2. Materials and methods

2.1. Sampling

Specimens of black scabbardfish were collected in 2010 and 2011 at four different locations in NE Atlantic, namely Iceland, the west of the British Isles, mainland Portugal, and Madeira Island (Fig. 1). Specimens from mainland Portugal and Madeira were caught by commercial longline vessels, whereas specimens from the other locations were collected with bottom trawl during the autumn survey conducted by the Icelandic Marine Research Institute and the deep-water survey held by the Marine Scotland. In Madeira, sampling took place in November to account for the peak in the reproductive period. Specimens from mainland Portugal were collected under the DCF – European Commission Fisheries Data Collection Framework during the last semester to comprise the months sampled in the other locations. Specimens were selected randomly to cover the ranges of total length and of maturity stages available at each sampling area (Table 1).

The following information was collected for all specimens: total length (TL, in mm), sex, and maturity stage. Maturity stage was macroscopically assigned according to the scale proposed by Gordo et al. (2000): stage I, immature or resting; stage II, developing; stage III, pre-spawning; stage IV, spawning; and stage V, post-spawning or spent. For further data analyses, maturity stages were grouped as immature, which included stages I and II, and mature, which included stages III to V. Owing to constraints in sample availability, all specimens from Madeira were females.

White muscle samples from all study areas were extracted from the dorso-lateral region behind the head and frozen directly after

collection. Afterwards, samples were freeze-dried and milled with a pestle and mortar. White muscle tissue was also collected from all sampled specimens for genetic analysis, being preserved in absolute ethanol at +4 °C.

In specimens caught off mainland Portugal it was possible to collect 151 stomachs between May and December 2011. Each stomach was weighted and frozen at −20 °C for further content analysis.

2.2. Genetic analysis

Molecular markers were used to guarantee that only specimens of *A. carbo* were considered in this study since misidentification problems with *A. intermedius* exist (Stefanni and Knutsen, 2007). The cytochrome oxidase subunit I (COI) gene from mitochondrial DNA was selected to identify the species. This gene has been shown to be adequate for discriminating between *Aphanopus* species (Stefanni et al., 2009) as well as to identify a wide number of vertebrate and invertebrate taxa (Hebert et al., 2003).

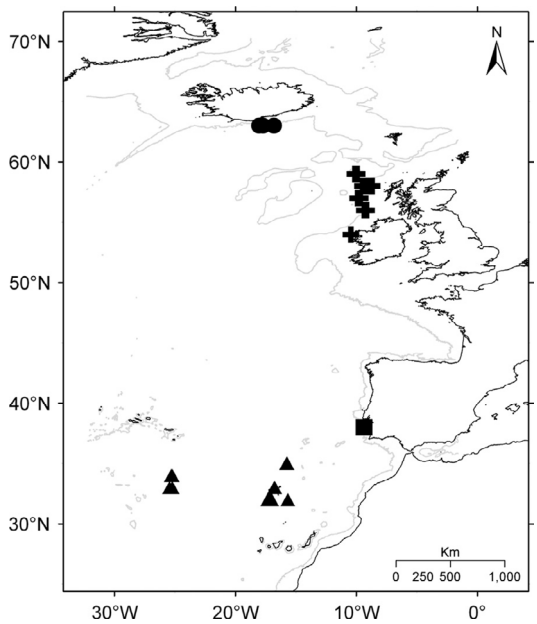

Fig. 1. Map of NE Atlantic with the black scabbardfish sampling areas marked: triangles represent the sampling locations around Iceland; crosses, off west of the British Isles; circles, around Madeira Island; and squares, off mainland Portugal. The 1000 m depth contour is shown.

DNA was extracted from muscle tissue following the Qiagen DNeasy Tissue Kit protocol. Amplification of the 5′ end of the COI fragment was conducted in 25 μl reaction volumes containing ca. 50 ng of DNA sample, 10 × reaction buffer, 1.5 mM $MgCl_2$, 0.2 nM dNTPs, 10 nM of each primer, and 0.1 U of Taq DNA Polymerase. The primers FishF1 (5′TCAACCAACCACAAAGACATTGGCAC3′) and FishR1 (5′TAGACTTCTGGGTGGCCAAAGAATCA3′), designed by Ward et al. (2005), were used. The PCR thermal cycle consisted of an initial denaturing step of 5 min at 95 °C, followed by 35 cycles of repeating the sequence 30 s at 95 °C, 30 s at 50 °C, and 60 s at 72 °C, and a final extension step of 10 min at 72 °C. The PCR products were enzymatically purified following a modification of the Exo-SAP method (Werle et al., 1994) and sequenced using the dye-labelled termination method (BigDye Terminator v3.1, Applied Biosystems, Inc., USA) on an ABI 3730XL sequencer (Macrogen Europe, The Netherlands). Amplicons were sequenced in both forward and reverse directions. Sequences were aligned manually and edited using BioEdit (Hall, 1999).

The number of segregating sites, the number of haplotypes, and the haplotype and the nucleotide diversities (and standard deviation) were estimated using DnaSP 4.20.2 (Rozas et al., 2003). The sequence obtained for each specimen was compared with three sequences from *A. carbo* available in GenBank using the Blast service from the National Centre for Biotechnology Information (NCBI).

2.3. Diet analysis

The stomachs from specimens caught off mainland Portugal were defrosted at room temperature and the food categories were identified to the lowest possible taxonomic level, counted, weighted, and measured when possible. Each food category is further designated as prey.

The index of vacuity (%IV) was determined as the percentage of stomachs without food contents in the whole sample of stomachs. A stomach was considered to be without food contents when it was empty, everted, or only contained the bait. In mainland Portugal's longline fishery the bait used was either *Sardina pilchardus* or *Scomber colias*.

The frequency of occurrence (%O, proportion between the number of stomachs containing a food category and the total number of stomachs with food items) was used to evaluate the relative importance of each prey in the diet of the black scabbardfish off mainland Portugal.

Table 1
Summary of the data regarding the black scabbardfish specimens used in FA and SI analyses.

Region	Gear	Depth (m)	Year	Month	Sex	Maturity	TL (mm)	*n*
Iceland	Bottom trawl (survey)	533–585	2010	11	F	1	920–960	4
					M	1	880–1010	6
W British Isles	Bottom trawl (survey)	550–1650	2011	9	F	1	890–1080	3
						2	910–1160	11
					M	1	890–1010	2
						2	920–1080	4
Mainland Portugal	Bottom longline (commercial)	1100–1650	2010–2011	5–12	F	1	969–1001	3
						2	990–1215	8
						3	1212	1
					M	1	980–1010	3
						2	1007	1
						3	1009–1119	4
Madeira	Drifting horizontal longline (commercial)	1200–1500	2010	11	F	2	1071–1164	7
						3	1127–1358	6
						4	1135–1323	2
						5	1260–1345	5

2.4. Fatty acids analysis

Fatty acid methyl esters (FAME) were prepared according to Bandarra et al. (2009), using 0.3 g of freeze-dried muscle tissue and 5 ml of the acetyl chloride:methanol mixture (1:19, v/v). The transesterification was carried out at 80 °C for 1 h. After cooling, 1 ml of water and 2 ml of n-heptane were added to the mixture, which was stirred and centrifuged at 2.1×10^4 m/s^2 for 10 min. The organic phase was collected, filtered and dried over anhydrous sodium sulfate. The solvent was removed under nitrogen and the FAME dissolved in 0.1 ml of n-heptane.

The FAME analyses were performed in a Varian CP-3800 (Walnut Creek, CA, USA) gas chromatograph equipped with an auto sampler and fitted with a flame ionization detector (GC-FID). The separation was carried out on an Omegawax (Supelco, USA) capillary column (25 m × 0.25 mm id). Temperature was programmed from 180 °C to 200 °C at 4 °C min^{-1}, holding for 10 min at 200 °C and heating to 210 °C at 4 °C min^{-1}, holding at 210 °C for 14.5 min with the injector and detector at 250 °C. Methyl esters were quantified using the Varian software.

Thirty-nine fatty acids comprised the array of identified FA chosen for this study. Values were expressed as percentage of total area of all identified FA (% of total FA).

2.5. Stable isotopes analysis

To evaluate the carbon and the nitrogen isotope ratios (δ^{13}C and δ^{15}N, respectively) approximately 2 mg of powdered lyophilized muscle was weighted into a tin capsule. For quality control purposes, for each specimen two duplicates were prepared, randomly ordered and one standard was analyzed at every fifth sample. The isotope ratios δ^{13}C and δ^{15}N were determined by continuous-flow isotope ratio mass spectrometry (CF-IRMS) using a Finnigan ConFlo III coupled to a Flash Elemental Analyzer 1112 Series (Thermo Electron Corporation, USA). The results were reported in δ notation according to the equation $\delta X(‰) = [(R_{sample}/R_{standard}) - 1] \times 1000$, where X is ^{13}C or ^{15}N and R is the ratio ^{13}C/^{12}C or ^{15}N/^{14}N in the sample and in the standard. δ^{13}C and δ^{15}N were expressed as per mill (‰) relative to Vienna-Pee Dee Belemnite (V-PDB) and atmospheric N_2 (air), respectively. These compounds are defined as the 0‰ point of the δ scale. The precision of the method was inferior to 0.1‰ for both isotope ratios.

Part of the variability in δ^{13}C in biological matrices is associated with varying lipid content amongst samples, since lipids are isotopically depleted in ^{13}C relative to proteins and carbohydrates (DeNiro and Epstein, 1977). The common approach to correct this effect is removing lipids from biological samples (Post, 2002; Sweeting et al., 2006). However, lipid extraction implies an undesirable and unintended enrichment of the sample in ^{15}N (Hoffman and Sutton, 2010). Therefore, to optimize this analysis in terms of time and costs, the method proposed by Hoffman and Sutton (2010) to correct the effect of lipids on δ^{13}C of untreated tissue was followed:

$$\delta^{13}C_{protein} = \delta^{13}C_{bulk} + (\Delta\delta^{13}C_{lipid} \times (C : N_{protein} - C : N_{bulk}))/C : N_{bulk}.$$

For deep-sea fish, the isotopic depletion factor is $\Delta\delta^{13}C_{lipid} = -6.39‰$, whereas the ratio $C{:}N_{protein} = 3.76$ reflects the carbon mass balance given that simple lipids do not contain nitrogen (Hoffman and Sutton, 2010).

2.6. Data analyses

Canonical cluster analysis (CCA) was used for exploratory analysis of the most abundant fatty acids found in the muscle of black scabbardfish regarding the factors region, sex, maturity stage, and total length. The CCA is a combination of ordination and regression methods that allows extracting the synthetic gradients (ordination axes) that maximize the separation between FA and the levels of the four factors under analysis (ter Braak, 1985).

Posteriorly, the following sums were calculated for each specimen: SFA, as the sum of all saturated fatty acids; MUFA, as the sum of all monounsaturated FA; and PUFA, as the sum of all polyunsaturated FA. The importance of the different FA groups relies on the fact that each can be associated to particular physiological metabolic processes (Bandarra et al., 2009; Huynh and Kitts, 2009). Linear-mixed model (LMM) was fitted to each FA group (SFA, MUFA, and PUFA) by restricted maximum likelihood, considering region, sex, and maturity stage as fixed effects and TL as random effect. The LMM was chosen to allow removing the effect of the different TL range by area.

LMM was also fitted to PUFA with mean values higher than 1.00% of total FA in all areas by restricted maximum likelihood, considering region, sex and maturity stage as fixed effects and TL as random effect.

The correlation between δ^{15}N and δ^{13}C and TL by area was estimated through the Pearson's correlation coefficient. LMM was fitted to δ^{15}N and δ^{13}C separately by restricted maximum likelihood, considering region, sex and maturity stage as fixed effects and TL as random effect.

For all LMM, the function 'lme' from library 'nlme' version 3.1-113 was used (Pinheiro and Bates, 2000). The following assumptions for LMM were assessed by graphical analyses: (i) the within-group errors are independent and identically distributed, with mean zero and variance σ^2, and are independent of the random effects; (ii) the random effects are normally distributed and are independent for different groups (Pinheiro and Bates, 2000).

All data analyses where performed in *R* version 3.0.2.

3. Results

3.1. Length composition and maturity stages

The total length (TL) range of specimens used in the present study varied between sampling areas (Table 1). Moreover there was no overlap in the TL range of specimens from Iceland and from Madeira.

Specimens from Iceland and the west of the British Isles were in maturity stages I and I–II, respectively, hence were all immature, whereas specimens from mainland Portugal were in stages I–III, and specimens from Madeira were females in stages II–V.

3.2. Genetic analysis

A COI fragment with 651 bp was sequenced in all specimens caught off the four geographical areas ($N = 70$). Sequence analysis revealed five segregating sites defining five haplotypes and low haplotype and nucleotide diversities which were estimated as 0.2530 ± 0.1040 and 0.0005 ± 0.0002, respectively. The most common haplotype was shared between most of the specimens. The highest degree of similarity of the analyzed specimens was with the sequences of specimens identified as *A. carbo* available in GenBank (99–100%); hence all specimens unambiguously corresponded to *A. carbo*.

3.3. Diet

The diet of black scabbardfish caught off mainland Portugal was analyzed through stomach content examination. Of the 151 stomachs collected, 15 were everted, 9 contained only bait, 98

were empty, and 29 contained food items. The corresponding vacuity index was 80.8%.

Fish showed the highest occurrence (50% of the stomachs), followed by crustaceans (28%) and cephalopods (22%). In most stomachs with traces of fish, only scales were found, therefore it was not possible to estimate the total number of prey. Nonetheless, two undigested specimens of *Rouleina maderensis* (14 and 24 g total weight) were identified in two stomachs (6%). The vestiges found for crustacean prey were mostly unidentifiable parts of the exoskeleton, but in eight nearly undigested specimens it was possible to go as far as the species and identify the lophogastrid *Gnathophausia zoea*. This was the most frequent prey identified to species level (23%). The remains of cephalopods were counted and identified as *Mastigoteuthis* spp. through their beaks (eight specimens in seven stomachs). Following Clarke's (1986) regressions that relate the lower beak rostral length r with the body wet weight w ($\ln w = 0.184 + 2.88 \ln r$, $n=45$, $r^2=0.94$) and the mantel length l ($l = -1.8 + 29.08r$, $n=47$ and $r^2=0.91$), the mean weight (57 g) and the mean mantel length (108 mm) of *Mastigoteuthis* prey found in the analyzed stomachs were estimated.

3.4. Fatty acids

The most abundant FA were: the SFA myristic acid (14:0), palmitic acid (16:0), and stearic acid (18:0); the MUFA palmitoleic acid (16:1n-7), oleic acid (18:1n-9), vaccenic acid (18:1n-7), eicosenoic acids (20:1n-11 and 20:1n-9), and the cetoleic acid (22:1n-11); the PUFA arachidonic acid (ARA or 20:4n-6), eicosapentaenoic acid (EPA or 20:5n-3), docosapentaenoic acid (DPA or 22:5n-3), and docosahexaenoic acid (DHA or 22:6n-3) (Table 2). Within these FA, the highest mean percentages were reported for DHA, oleic acid, and palmitic acid. Specimens from Iceland showed the highest mean percentage for myristic acid, palmitoleic acid, vaccenic acid, the eicosenoic acid 20:1n-11, and EPA; specimens from the west of the British Isles presented the highest means for oleic acid, the eicosenoic acid 20:1n-9, and cetoleic acid; whereas specimens from Madeira presented the highest means for palmitic acid, stearic acid, ARA, DPA, and DHA.

The CCA (Fig. 2) reflected the importance of the selected FA in relation to each factor. The first two canonical axes explained approximately 92% of the cumulative percentage of discriminant data. In this analysis, factors region and TL were highly significant ($p=0.005$ after 199 permutations of residuals under the reduced model). Region Madeira was positively related with the selected PUFA, the highest TL, and the mature specimens, and negatively related with MUFA. Oppositely, the west of the British Isles was positively related with MUFA (mainly 18:1n-9) and negatively related with PUFA. Mainland Portugal was related with the second axis but there was no clear relation with any FA.

The pattern for the main FA groups was similar for specimens from Iceland and the west of the British Isles (MUFA > PUFA > SFA), differing from the pattern for both mainland Portugal (PUFA > MUFA > SFA) and Madeira (PUFA > SFA > MUFA) (Table 2).

The LMM assumptions were met in all the models. According to LMM (Table 3), SFA was significantly lower in specimens from the west of the British Isles ($t_{0.05(2),\ 13} = -3.080$; $p=0.009$); MUFA was significantly lower in Madeira ($t_{0.05(2),\ 13} = -4.333$; $p < 0.001$); and PUFA was significantly higher in mainland Portugal ($t_{0.05(2),\ 13} = 2.428$; $p=0.031$) and Madeira ($t_{0.05(2),\ 13} = 4.774$; $p < 0.001$). In general, there was no significant effect of sex or maturity stage.

PUFA with mean values higher than 1.00% of total FA in all areas were EPA, ARA, DHA, and DPA (Table 2). According to LMM (Table 4), all regions but Madeira and maturity were significant for EPA. Specimens from mainland Portugal showed significantly higher values for ARA. Both ARA and DHA were significantly higher in specimens from Madeira. For DPA, no factor was significant. Maturity was important for explaining the differences in EPA and ARA.

3.5. Stable isotopes

Since there were no significant differences in $\delta^{13}C$ and $\delta^{15}N$ between the two duplicates analyzed for each specimen, the mean value between each pair of values was used in the subsequent analyses.

Overall, mean $\delta^{13}C$ and mean $\delta^{15}N$ increased southwardly from Iceland to Madeira, with very similar values detected in specimens from mainland Portugal and Madeira (Table 5 and Fig. 3). TL was positively correlated with $\delta^{15}N$ ($\rho=0.55$; $t=5.37$; df=68; $p < 0.001$), but there was no correlation with $\delta^{13}C$ ($\rho=0.19$; $t=1.59$; df=68; $p=0.117$) (Fig. 4).

According to LMM (Table 6), $\delta^{15}N$ was significantly higher in mainland Portugal ($t_{0.05(2),\ 13} = 3.11$; $p=0.008$), in Madeira ($t_{0.05(2),\ 13} = 2.23$; $p=0.044$) and in mature specimens ($t_{0.05(2),\ 51} = 2.12$; $p=0.039$), whereas for $\delta^{13}C$ only mature specimens were significant ($t_{0.05(2),\ 51} = 2.19$; $p=0.033$).

4. Discussion

The life cycle of deep-sea fish like the black scabbardfish is difficult to study owing to monitoring problems that hinder having a good temporal and spatial coverage. For this species, information is mostly collected from existing fisheries off the west of the British Isles, mainland Portugal, and Madeira. However, the wide geographical distribution of the black scabbardfish comprises areas without commercial exploitation.

Another problem is the high level of stomach vacuity found for this species which limits stomach content analyses and, consequently, studies on trophic dynamics. For specimens from mainland Portugal the vacuity index (80.8%) was a consequence of barotrauma or total food digestion. That index was similar to what had already been reported for the other areas: from 93.3 to 98.3% for specimens from Madeiran longliners (Freitas, 1998; Ribeiro Santos et al., 2013b) and from 66.2 to 94.1% for specimens caught by trawls to the west of the British Isles (Mauchline and Gordon, 1984; Ribeiro Santos et al., 2013b). Madeira and mainland Portugal showed the highest vacuity indices because the collection of specimens was restricted to commercial landings from the longline fisheries in which the mean soaking time is about 24 h (Bordalo-Machado et al., 2009), favouring the total digestion of food.

To overcome these sampling difficulties, specimens from both commercial fisheries and scientific surveys were used in a combination of biochemical methods that allowed inferring about the diet and the reproductive cycle of the black scabbardfish. The present study covered a wider area of distribution of the black scabbardfish in the NE Atlantic than any other previously published study and combined FA and SI analyses for this species for the first time. Additionally, it was proved through genetic analyses that this study was conducted solely on *A. carbo* without mixing with the cryptic species *A. intermedius*.

Within the sampling areas, Madeira is the only one where spawning of this species occurs. Specimens from Madeira were caught at the peak of the spawning season (Figueiredo et al., 2003; Neves et al., 2009; Ribeiro Santos et al., 2013a) and were mostly mature females. The higher proportion of PUFA and the stronger relationship with ARA and DHA than in the other areas reflected the connection between these FA and the species maturity and spawning since they are precursors of biomolecules involved in reproduction (Stacey and Goetz, 1982; Ruggeri and Thoroughgood,

Table 2
Summary of fatty acids (% total FA) in the muscle of black scabbardfish (mean ± standard deviation).

Fatty acid	Iceland	W British Isles	Mainland Portugal	Madeira
14:0 (myristic ac.)[a]	2.48 ± 1.16	2.24 ± 0.78	1.07 ± 0.82	0.49 ± 0.15
15:0	0.31 ± 0.10	0.23 ± 0.04	0.20 ± 0.07	0.21 ± 0.08
16:0 (palmitic ac.)[a]	17.99 ± 3.34	15.16 ± 1.66	16.37 ± 2.16	18.92 ± 2.85
17:0	0.26 ± 0.04	0.20 ± 0.04	0.27 ± 0.12	0.38 ± 0.18
18:0 (stearic ac.)[a]	5.15 ± 1.02	5.04 ± 0.72	6.34 ± 1.20	6.82 ± 0.75
19:0	0.12 ± 0.01	0.09 ± 0.02	0.10 ± 0.06	0.13 ± 0.09
20:0	0.12 ± 0.06	0.14 ± 0.03	0.11 ± 0.07	0.05 ± 0.05
22:0	0.01 ± 0.02	0.02 ± 0.02	0.01 ± 0.04	< 0.01 ± 0.01
SFA	**27.22 ± 4.26**	**23.73 ± 1.85**	**25.28 ± 2.66**	**28.15 ± 3.62**
16:1n-9	0.16 ± 0.08	0.37 ± 0.24	0.25 ± 0.16	0.20 ± 0.12
16:1n-7 (palmitoleic ac.)[a]	2.85 ± 1.07	2.67 ± 0.85	1.48 ± 0.94	0.83 ± 0.32
17:1	0.36 ± 0.08	0.21 ± 0.05	1.22 ± 1.53	1.25 ± 1.26
18:1n-9 (oleic ac.)[a]	17.95 ± 5.27	25.18 ± 9.19	19.73 ± 13.10	9.48 ± 2.43
18:1n-7 (vaccenic ac.)[a]	2.99 ± 0.62	2.77 ± 0.56	2.10 ± 0.92	1.63 ± 0.32
18:1n-5	0.30 ± 0.04	0.27 ± 0.05	0.12 ± 0.08	0.11 ± 0.06
20:1n-11 (eicosenoic ac.)[a]	1.27 ± 1.02	0.18 ± 0.34	0.65 ± 0.65	0.47 ± 1.16
20:1n-9 (eicosenoic ac.)[a]	6.08 ± 2.70	8.21 ± 2.53	4.32 ± 2.58	2.38 ± 1.44
20:1n-7	0.30 ± 0.11	0.30 ± 0.12	0.27 ± 0.17	0.14 ± 0.12
22:1n-11 (cetoleic ac.)[a]	6.40 ± 3.02	6.82 ± 3.22	3.08 ± 3.46	0.89 ± 1.19
22:1n-9	0.97 ± 0.45	1.05 ± 0.48	0.77 ± 0.74	0.27 ± 0.33
24:1n-9	0.41 ± 0.51	0.26 ± 1.16	0.73 ± 0.84	0.30 ± 0.50
MUFA	**40.05 ± 11.05**	**48.30 ± 11.74**	**34.72 ± 16.64**	**17.98 ± 4.94**
16:2n-4	0.40 ± 0.06	0.39 ± 0.08	0.25 ± 0.10	0.28 ± 0.13
16:3n-4	0.38 ± 0.10	0.33 ± 0.07	0.35 ± 0.18	0.38 ± 0.47
16:3n-3	0.22 ± 0.15	0.13 ± 0.13	0.62 ± 0.67	0.85 ± 0.81
16:4n-3	0.81 ± 0.60	0.55 ± 0.48	1.49 ± 1.07	2.90 ± 1.14
18:2n-6	0.86 ± 0.14	0.71 ± 0.10	0.56 ± 0.13	0.46 ± 0.18
18:3n-6	0.05 ± 0.03	0.06 ± 0.03	0.05 ± 0.06	0.09 ± 0.08
18:3n-4	0.11 ± 0.03	0.11 ± 0.07	0.17 ± 0.11	0.09 ± 0.08
18:3n-3	0.31 ± 0.12	0.24 ± 0.08	0.52 ± 0.70	0.50 ± 0.66
18:4n-3	0.45 ± 0.25	0.25 ± 0.13	2.17 ± 3.44	2.25 ± 3.35
20:2n-6	0.23 ± 0.05	0.24 ± 0.05	0.27 ± 0.12	0.21 ± 0.14
20:3n-3	0.14 ± 0.04	0.11 ± 0.05	0.08 ± 0.10	0.07 ± 0.08
20:4n-6 (ARA) [a]	1.90 ± 0.98	1.54 ± 0.89	3.21 ± 1.60	5.87 ± 1.56
20:4n-3	0.66 ± 0.12	0.60 ± 0.14	0.46 ± 0.65	0.41 ± 0.93
20:5n-3 (EPA) [a]	4.43 ± 0.80	3.28 ± 1.00	2.88 ± 1.12	4.00 ± 1.18
21:5n-3	0.16 ± 0.19	0.23 ± 0.13	0.93 ± 1.59	1.88 ± 2.53
22:4n-6	0.17 ± 0.15	0.25 ± 0.09	0.71 ± 2.04	0.33 ± 0.29
22:5n-6	0.36 ± 0.20	0.34 ± 0.21	0.58 ± 0.30	1.02 ± 0.46
22:5n-3 (DPA) [a]	1.32 ± 0.32	1.40 ± 0.32	1.13 ± 0.35	1.66 ± 0.30
22:6n-3 (DHA) [a]	18.21 ± 7.54	15.98 ± 8.35	21.92 ± 9.73	28.95 ± 4.67
PUFA	**31.17 ± 9.65**	**26.73 ± 10.63**	**38.34 ± 15.4**	**52.17 ± 6.37**

SFA: sum of saturated fatty acids; MUFA: sum of monounsaturated fatty acids; PUFA: sum of polyunsaturated fatty acids.

[a] FA that were selected for CCA.

1985; Tocher, 2003), therefore highly required by mature specimens. Particularly, ARA is the major precursor of series II prostaglandins (paracrine hormones) that stimulate ovulation and spawning (Stacey and Goetz, 1982; Ruggeri and Thoroughgood, 1985; Sargent et al., 1999; Bergé and Barnathan, 2005) and is important for the production of viable eggs (Sargent et al., 1999). DHA plays a major role in egg production because it is responsible for maintaining the structure and function of cellular membranes, specifically the ovarian membrane fluidity and stability, through a process called homeoviscous adaptation (Sargent et al., 1999; Tocher, 2003; Mayor et al., 2013). In deep-sea organisms, such as the black scabbardfish, the structure of membranes is particularly important to minimize the effects of the exposure to high pressure and low temperatures (Stowasser et al., 2009).

The high percentage of PUFA found in specimens caught off Madeira in the peak of spawning also supports the hypothesis that the black scabbardfish continues to feed during the spawning period since PUFA are obtained through the diet (Sargent et al., 1999; Tocher, 2003). Results from both the FA and the SI analyses are in accordance with the diet composition described by Freitas (1998), who stated that the black scabbardfish caught off Madeira occupies a high trophic level. In that study, squids of genera *Chiroteuthis*, *Mastigoteuthis*, *Histioteuthis*, and *Taonius*, as well as the viperfish *Chauliodus* sp. and several lanternfishes from family *Myctophidae* were the most important prey reported.

In the stomachs of specimens caught off mainland Portugal, the most frequent prey were teleost fish, followed by the lophogastrid crustacean *G. zoea* and cephalopods from genera *Mastigotheutis*. The importance of teleosts explains the high values of δ^{15}N and δ^{13}C found in specimens from this area, since, in general, teleosts are isotopically enriched in relation to crustaceans and cephalopods (Iken et al., 2001; Polunin et al., 2001; Bergé and Barnathan, 2005; Cherel and Hobson, 2005; Reid et al., 2013). On another hand, the relative importance of PUFA, MUFA, and SFA in those specimens was the same as in *S. colias* (Bandarra et al., 2004), which is commonly used as bait in the longline fishery. Regarding the importance of fish, attention must be taken when assuming that fish are the preferred prey in this area because only two specimens were clearly identified as *R. maderensis* and the scales found in most stomachs could belong to fish used as bait (*S.*

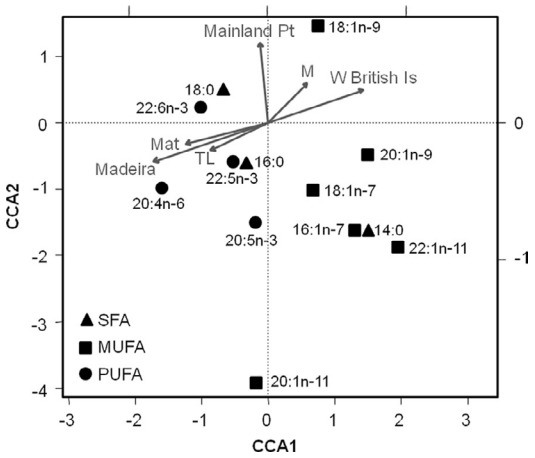

Fig. 2. Canonical cluster analysis (CCA) ordination diagram of most representative fatty acids for black scabbardfish caught in the NE Atlantic. Sampling variables are represented by arrows. SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; Mainland Pt, mainland Portugal; W British Is, west of the British Isles; M, males; Mat, mature.

Table 3
Summary of LMM fitted to the sums of FA for black scabbardfish in NE Atlantic.

	Effects	Value	S.E.	d.f.	*t*-Value	*p*-Value
SFA[a]	Intercept	27.56	1.09	51	25.40	< 0.001*
	Region (W British Isles)	−3.66	1.19	13	−3.08	0.009*
	Region (Mainland Portugal)	−2.05	1.17	13	−1.75	0.104
	Region (Madeira)	−0.40	1.37	13	−0.29	0.775
	Sex (male)	−0.57	0.89	13	−0.64	0.530
	Maturity (mature)	2.81	1.40	51	2.00	0.050
MUFA[b]	Intercept	42.51	4.40	51	9.67	< 0.001*
	Region (W British Isles)	5.18	4.53	13	1.14	0.274
	Region (Mainland Portugal)	−9.00	4.89	13	−1.84	0.089
	Region (Madeira)	−23.93	5.53	13	−4.33	< 0.001*
	Sex (male)	1.61	3.24	13	0.50	0.629
	Maturity (mature)	−1.73	5.78	51	−0.30	0.767
PUFA[c]	Intercept	28.58	4.07	51	7.02	< 0.001*
	Region (W British Isles)	−1.48	4.15	13	−0.36	0.727
	Region (Mainland Portugal)	11.04	4.55	13	2.43	0.031 *
	Region (Madeira)	24.52	5.14	13	4.77	< 0.001*
	Sex (male)	−1.55	2.97	13	−0.52	0.611
	Maturity (mature)	−2.84	5.44	51	−0.52	0.604

[a] AIC = 353.13.
[b] AIC = 530.40.
[c] AIC = 521.49.
* Significant *p*-values for $\alpha = 0.05$.

Table 4
Summary of LMM fitted to PUFA with mean values higher than 1.00% of total FA for black scabbardfish in NE Atlantic.

	Effects	Value	S.E.	d.f.	*t*-Value	*p*-Value
EPA[a]	Intercept	4.29	0.37	51	11.66	< 0.001*
	Region (W British Isles)	−1.00	0.39	13	−2.60	0.022*
	Region (Mainland Portugal)	−1.48	0.41	13	−3.64	0.003*
	Region (Madeira)	−0.86	0.46	13	−1.87	0.084
	Sex (male)	0.16	0.28	13	0.59	0.564
	Maturity (mature)	1.53	0.48	51	3.21	0.002*
ARA[b]	Intercept	1.67	0.41	51	4.08	< 0.001*
	Region (W British Isles)	−0.05	0.40	13	−0.14	0.893
	Region (Mainland Portugal)	1.75	0.46	13	3.79	0.002*
	Region (Madeira)	3.28	0.52	13	6.29	< 0.001*
	Sex (male)	−0.29	0.29	13	−1.00	0.335
	Maturity (mature)	2.56	0.57	51	4.46	< 0.001*
DHA[c]	Intercept	16.86	2.85	51	5.92	< 0.001*
	Region (W British Isles)	−0.33	2.92	13	−0.11	0.912
	Region (Mainland Portugal)	6.30	3.17	13	1.99	0.069
	Region (Madeira)	12.99	3.59	13	3.62	0.003*
	Sex (male)	−2.04	2.09	13	−0.98	0.347
	Maturity (mature)	−2.41	3.77	51	−0.64	0.526
DPA[d]	Intercept	1.36	0.12	51	11.57	< 0.001*
	Region (W British Isles)	0.07	0.13	13	0.52	0.610
	Region (Mainland Portugal)	−0.21	0.13	13	−1.62	0.129
	Region (Madeira)	0.23	0.15	13	1.54	0.148
	Sex (male)	−0.07	0.09	13	−0.70	0.498
	Maturity (mature)	0.19	0.15	51	1.27	0.208

[a] AIC = 212.09.
[b] AIC = 230.76.
[c] AIC = 475.21.
[d] AIC = 67.67.
* Significant *p*-values for $\alpha = 0.05$.

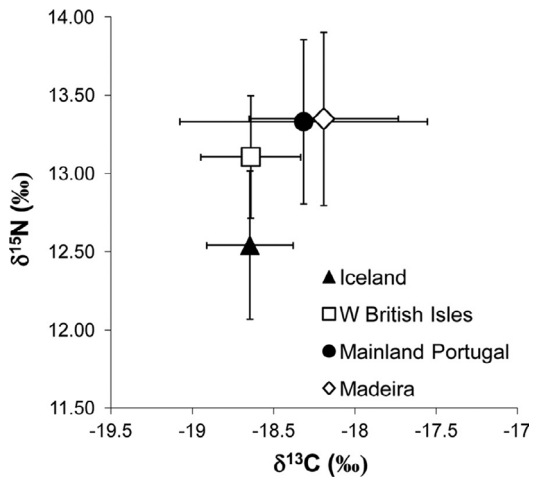

Fig. 3. Bivariate plot of $\delta^{13}C$ and $\delta^{15}N$ (‰) for black scabbardfish caught in the NE Atlantic (mean ± standard deviation).

pilchardus). 18:1(n-9) values were similar to those reported for the prey *G. zoea* (Letessier et al., 2012). High 18:1(n-9) reflects high physiological requirements (Mayor et al., 2013), which for these specimens can either be for reaching the pre-spawning maturity stage or for migration. The significantly higher proportion of ARA found in specimens from mainland Portugal comparing with those from the northernmost areas explains why the former reach a more advanced maturity stage.

The high levels of ARA in specimens from the southernmost areas can also support the occurrence of migratory movements between them, since a high demand for ARA is associated with stressful periods, such as long distance migrations (Sargent et al., 1999).

Qualitative differences in the diet composition between specimens from mainland Portugal and specimens from Madeira are reflected in their FA signatures and could explain why in the former area specimens that attain the length at first maturity (103 cm) do not proceed with maturation, rather stopping in the pre-spawning stage (Figueiredo et al., 2003; Neves et al., 2009). Maturation would therefore be triggered by feeding and the energetic content of available prey, an exogenous factor, rather than by an endogenous factor. Although the relative importance of FA groups was different from results previously reported for black scabbardfish from mainland Portugal (MUFA > SFA > PUFA; Bandarra et al., 2009) and Madeira (MUFA > PUFA > SFA; Nogueira et al., 2013), those differences cannot be interpreted because sampling season, length range, and the number of specimens were not provided in those studies.

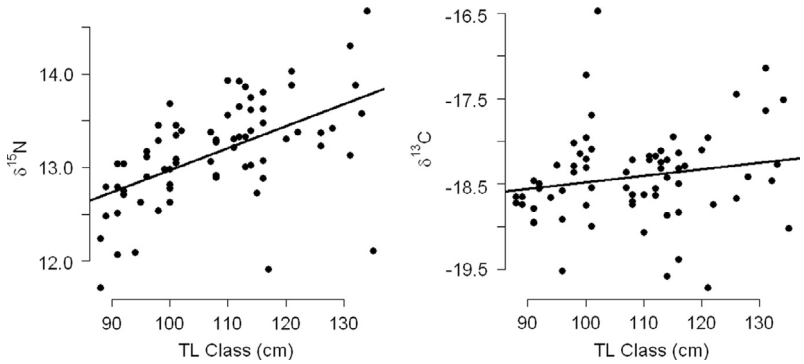

Fig. 4. Plots of δ^{13}C and δ^{15}N (‰) against total length class (in cm) and fitted regression lines for black scabbardfish caught in the NE Atlantic.

Table 5
Summary of δ^{15}N and δ^{13}C (‰) in the muscle of black scabbardfish (mean ± standard deviation).

SI ratio	Iceland	W British Isles	Mainland Portugal	Madeira
δ^{15}N (‰)	12.54 ± 0.47	13.1 ± 0.39	13.33 ± 0.52	13.35 ± 0.55
δ^{13}C (‰)	−18.65 ± 0.27	−18.64 ± 0.31	−18.32 ± 0.76	−18.19 ± 0.46

Table 6
Summary of LMM fitted to δ^{15}N and δ^{13}C (‰) for black scabbardfish in NE Atlantic.

	Effects	Value	S.E.	df	*t*-Value	*p*-Value
δ^{15}N[a]	Intercept	12.73	0.16	51	80.08	< 0.001*
	Region (W British Isles)	0.28	0.15	13	1.90	0.080
	Region (Mainland Portugal)	0.56	0.18	13	3.11	0.008*
	Region (Madeira)	0.46	0.21	13	2.23	0.044*
	Sex (male)	0.05	0.11	13	0.44	0.666
	Maturity (mature)	0.50	0.24	51	2.12	0.039*
δ^{13}C[b]	Intercept	−18.59	0.17	51	−107.52	< 0.001*
	Region (W British Isles)	−0.12	0.17	13	−0.69	0.500
	Region (Mainland Portugal)	0.23	0.19	13	1.17	0.263
	Region (Madeira)	0.23	0.22	13	1.03	0.321
	Sex (male)	0.11	0.12	13	0.91	0.381
	Maturity (mature)	0.53	0.24	51	2.19	0.033*

[a] AIC = 120.02.
[b] AIC = 114.08.
* Significant *p*-values for α = 0.05.

To the west of the British Isles, black scabbardfish were all immature or developing. The most important prey that have been previously identified were fish, namely *Argentina* sp. and *Scomber scombrus* on one occasion (Du Buit, 1978), but mostly blue whiting (*Micromesistius poutassou*), with a minor contribution of cephalopods (Mauchline and Gordon, 1984; Ribeiro Santos et al., 2013b). Combining data from Mauchline and Gordon (1984) with the diet composition of specimens caught off the Rockall Trough, Howell et al. (2009) found approximately 50% of cephalopods and 44% of blue whiting. Ribeiro Santos et al. (2013b) explained these differences with seasonal changes in prey availability: the increase in cephalopods and crustaceans in the diet of the black scabbardfish coincided with blue whiting migrating to the Norwegian Sea for spawning, between late April and the end of the year (Bailey, 1982; Was et al., 2008). In the present study, SI ratios in specimens from the west of the British Isles were lower than in Madeira and mainland Portugal because they were caught in September, when blue whiting is not available as food source and when Ribeiro Santos et al. (2013b) reported a predominance of crustaceans in their stomachs. The consequent lower food availability can also explain the high retention or synthesis of MUFA found in specimens from that area because these FA have the capacity to provide energy stores in deep-sea fish (Stowasser et al., 2009). The pattern MUFA > PUFA > SFA had already been described for a specimen of black scabbardfish caught in the eastern slopes of the Rockall Trough (Dunne et al., 2010). The differences in SIA between the present study and Ribeiro Santos et al. (2013b) show that temporal variations constrained by the sampling periods need to be further analyzed, extending the temporal and the specimens length ranges of all the different areas.

Specimens caught off Iceland and off the west of the British Isles were geographically close and, as expected, their FA pattern and δ^{13}C values were similar and different from those of specimens from Madeira and mainland Portugal. Differences in baseline δ^{13}C values can be explained by both abiotic factors, such as latitude and depth (DeNiro and Epstein, 1978; Laakmann and Auel, 2010) and biotic factors like particulate organic matter (POM) (Letessier et al., 2012). There is a poleward depletion in ^{13}C because the decrease in temperature increases the CO_2 solubility (Rau et al., 1989; Michener and Kaufman, 2007; Laakmann and Auel, 2010); there is a progressive enrichment in ^{13}C with increasing habitat depth (Hoffman and Sutton, 2010); and POM δ^{13}C increases southwardly in NE Atlantic (Letessier et al., 2012). Although around Iceland diet data are not available for the black scabbardfish, the depletion in ^{15}N in comparison with the other three areas is likely to be a consequence of differences in the phytoplankton baseline δ^{15}N because POM δ^{15}N also increases southwardly (Letessier et al., 2012) and in the feeding regimes. Since, in this area, the species reaches smaller sizes it is restricted to a lower trophic level and to a narrower variety of prey both in size and mobility.

Overall, the allometric effect of each area's size ranges over δ^{15}N supports the existence of ontogenic differences in the black scabbardfish's diet, which is common in fish (Drazen et al., 2001; Dalsgaard et al., 2003; Stowasser et al., 2009; Mayor et al., 2013; Reid et al., 2013). In the present study, those changes were expressed not only in terms of δ^{15}N and δ^{13}C but also in terms of FATM. Specimens caught off Iceland showed higher values for the diatoms' FATM EPA and, together with specimens from the west of the British Isles, relatively higher percentages of 20:1n-9 and 22:1n-11, whose primary producers are the copepods *Calanus* spp., and of 16:1n-7 and 18:1n-7, which are related to benthic organisms (Dalsgaard et al., 2003; Stowasser et al., 2009). However, specimens caught off mainland Portugal and Madeira fed on bathypelagic fish, cephalopods, and crustaceans that are at a higher trophic level. In general, the diet of black scabbardfish is typical of mobile benthopelagic predators that are opportunistic feeders (Iken et al., 2001) and the change from prey that feed on pelagic zooplankton to bathypelagic prey reflects an improvement in its predatory capacity.

5. Conclusion

The biochemical analyses performed over the muscle of black scabbardfish were in accordance with the species' spatial distribution in terms of the reproductive cycle and the size distribution pattern, drawing close together specimens from Iceland and the west of the British Isles, and specimens from mainland Portugal and Madeira. This study has proposed a relationship between the reproductive cycle of the black scabbardfish in the NE Atlantic and its fatty acid profile: mature specimens have higher energetic demands and show a prevalence of PUFA, whereas SFA are more representative in immature specimens. Furthermore, FA and SI biomarkers were successfully combined in the interpretation of this species' spatial and size-based feeding variability.

Acknowledgments

This work was supported by the European Commission Fisheries Data Collection Framework, the EU-FP7 project DEEPFISHMAN (Grant agreement no. 227390), the Portuguese project CERTIFICA (PROMAR, 2011–2015), and a doctoral fellowship granted to I. Farias by Fundação para a Ciência e Tecnologia (SFRH/BD/64434/2009). The authors thank Teresa Moura (IPMA) for helping with the genetic analysis and for drawing the map; Ana Rita Vieira, José Lago, and Neide Lagarto (IPMA), Klara Jakobsdottir (MRI, Iceland), João Delgado, Sara Reis, Sofia Vieira, and the technical staff in Madeira (DSIDP-DRPM), Francis Neat and the scientific team at MRV Scotia (Marine Scotland) for helping in sample collection; Júlia Ferreira (IPMA) for helping with the FA analysis; and Gabriel Martorell (UIB) and Marta Nogueira (IPMA) for helping with the SI analysis. We are also grateful to Joel C. Hoffman (EPA, USA), Monty Priede (DSR I's Associate Editor), and two anonymous reviewers for their pertinent comments and suggestions.

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Aquaculture and Fisheries xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Aquaculture and Fisheries

journal homepage: http://www.keaipublishing.com/en/journals/aquaculture-and-fisheries

Sex steroids of black scabbardfish, *Aphanopus carbo*, in relation to reproductive and migratory dynamics

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ARTICLE INFO

Keywords:
Black scabbardfish
Deep-sea
Madeira
Mainland Portugal
Migration
Sex steroids

ABSTRACT

Black scabbardfish, *Aphanopus carbo*, is a commercially important species that takes distant migrations throughout its life cycle. Sex steroids were measured by radioimmunoassay in the blood plasma of specimens caught off the Madeira Archipelago and mainland Portugal to link this species migratory path with its reproductive cycle. Furthermore, a pilot study using Mozambique tilapia (*Oreochromis mossambicus*) was designed to evaluate the effect of sample freshness on steroid levels because black scabbardfish blood was collected at separate times after specimens were caught. The changes in T and 11-KT concentrations between the time of blood extraction and the time after preservation did not statistically differ among the different methods applied. Therefore, measured black scabbardfish steroid concentrations were directly used in the subsequent data analyses. In females, E2 and in T concentrations peaked at a late stage of vitellogenesis. E2 concentration was significantly different between females caught off each area. Clustering E2 and T concentrations from all developing females resulted in the separation of two distinct groups, independently of their geographical area. In males, T and 11-KT were not significantly different between maturity stages. The hepatosomatic index of males caught off mainland Portugal was relatively high. This may reflect a mechanism for storing energy that will later be consumed during migration to the spawning grounds. The trend of sex steroids concentrations throughout the sexual maturation of the species is consistent with the morphological indicators and shows evidence of the reproductive and migratory pattern hypothesised for the black scabbardfish in NE Atlantic.

1. Introduction

The black scabbardfish, *Aphanopus carbo* Lowe, 1839, is an important commercial deep-sea species caught in Portuguese waters. This benthopelagic teleost fish is widely distributed along the NE Atlantic (Allain, Biseau, & Kergoat, 2003; Bordalo-Machado et al., 2009; Ehrich, 1983), where it undergoes large-scale migrations: the smallest specimens are reported further north and the largest specimens at the southernmost limit of distribution (Farias, Morales-Nin, Lorance, & Figueiredo, 2013; Ribeiro Santos, Minto, Connolly, & Rogan, 2013). The only known spawning grounds are located off Madeira, the Canary Islands, and possibly Morocco (Figueiredo et al., 2003; Pajuelo et al., 2008; Perera, 2008, p. 71). Juveniles recruit to the fisheries off the west of the British Isles, documented to be a feeding area, where they remain some time growing (Figueiredo et al., 2003; Ribeiro Santos, Minto, et al., 2013). Black scabbardfish subsequently moves to areas off mainland Portugal where caught specimens attain larger sizes and pre-spawning individuals are seldom captured (Figueiredo et al., 2003; Neves et al., 2009). After spending some time here to feed and grow, juveniles and pre-adults move further south to the spawning areas around Madeira (Farias et al., 2013). A key question is why fishes off mainland Portugal do not develop beyond the pre-spawning stage, despite attaining sizes larger than the estimated length at first maturity (L_{50} = 102.8 cm) (Figueiredo et al., 2003).

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https://doi.org/10.1016/j.aaf.2020.03.006
Received 23 July 2019; Received in revised form 10 January 2020; Accepted 15 March 2020

Fish diet, growth, reproduction, and energy consumption are closely dependent on environmental conditions, which highly constrain metabolic rate processes in the deep-sea (Merrett & Haedrich, 1997, p. 282). Understanding the role of hormonal regulation of deep-sea species' vital processes will contribute to a better knowledge of their spatial dynamics. In this context, associating alterations in gonad development with the levels of sex steroids in blood plasma has proven to be a valuable tool to comprehend the endocrine control of reproduction in some deep-sea teleosts although this is a field not fully explored (Lee & Yang, 2002; Pankhurst & Conroy, 1987; Sequeira et al., 2017; Sisneros, Forlano, Knapp, & Bass, 2004).

In teleost fish females, the follicle stimulating hormone (FSH) is responsible for the stimulation of granulosa cells to produce estradiol-17β (E_2), the key hormone that induces the liver to synthesize vitellogenin and egg shell proteins, which are incorporated into the oocyte during vitellogenesis (Lubzens, Young, Bobe, & Cerdà, 2010). After the growth phase, a surge of luteinizing hormone (LH) stimulates the follicle to produce the maturation-inducing steroid – either 17,20β-dihydroxypregn-4-en-3-one (17,20β-P) or 17,20β,21-trihydroxypregn-4-en-3-one (17,20β,21-P), depending on the species – that promotes final oocyte maturation (resumption of meiosis) and ovulation (Lubzens et al., 2010; Nagahama & Yamashita, 2008).

In males, FSH regulates Sertoli cell activity to support germ cell development while LH acts on Leydig cells to promote steroidogenesis (Chauvigne, Zapater, Gasol, & Cerdà, 2014; Schulz, Sumpter, & Stacey, 2010). The key androgen in males is 11-ketotestosterone (11-KT) which promotes germ cell proliferation and maturation, as well as the development of secondary sexual characters and the mediation of reproductive behaviours (Borg, 1994; Schulz et al., 2010). In males, 17,20β-P is responsible for endorsing the initiation of meiosis, for stimulating spermiation, and for enhancing sperm motility (by alteration of the pH and fluidity of the seminal fluid) and can act as a pheromone, e.g. in goldfish (Scott, Sumpter, & Stacey, 2010). Finally, both male and female gonads produce testosterone (T) which is a precursor of E_2 and 11-KT and feeds back on the pituitary gland to promote the synthesis of gonadotrophins, among other functions (Lubzens et al., 2010; Nagahama, 1994; Schulz et al., 2010).

The main objective of this study was to assess and compare the hormonal status and gonadal development stage of specimens caught off mainland Portugal and off Madeira. Black scabbardfish specimens caught by commercial fisheries are already dead when pulled on board and it was only possible to obtain blood from specimens several hours after capture or thawed. To establish how these conditions may affect hormone levels, a pilot study tested the effect of sample age and freezing on the sex steroids of Mozambique tilapia, *Oreochromis mossambicus* (Peters, 1852).

2. Material and methods

2.1. Pilot study

To assess the effect of the time lag between death (capture) and blood collection and the effect of freezing (specimen storage) on sex steroid levels, Mozambique tilapia (*Oreochromis mossambicus*) male specimens were used. The Mozambique tilapia is a freshwater cichlid that can live at temperatures ranging from 8° to 42 °C and can be reared in hypersaline conditions (Froese & Pauly, 2019). Despite the biological and ecological differences between the Mozambique tilapia and the black scabbardfish, the former was used in this pilot study because specimens were readily available from a captive stock, raised from fertilised eggs at the University of Algarve and maintained in freshwater under natural annual conditions of photoperiod and water temperature (26 °C) prior to these experiment. Males were chosen over females because they are expected to show less hormonal variation owing to a simpler reproductive physiology.

Fish care and experimentation complied with the national legislation for the use of laboratory animals under a Group-1 license issued by the Portuguese National Authority for Animal Health. Fish were stunned and killed by immersion in iced water and total length (TL, cm) and total weight (TW, g) were measured. Blood samples were collected in heparinised syringes from the caudal peduncle in larger specimens or from the heart in smaller specimens.

Table 1
Experimental design and summary of Mozambique tilapia samples used in the pilot study. T, storage temperature; *n*, sample size; TL, total length range; TW, total weight range.

Treatment	T (°C)	Time (days)	*n*	TL (cm)	TW (g)
t1	4	1	6	15.2–18.9	61.74–103.62
t2	4	2	5	15.8–17.3	62.09–75.65
t4	4	4	6	16.5–18.9	66.4–105.21
t15	−20	15	6	15.1–19.3	53.7–103.52
t30	−20	30	5	14.6–17.2	50.68–81.64

After a first sample of blood was collected from 29 specimens (control, t0), fish were randomly separated into two groups: 18 fish were stored on ice in a refrigerator (temperature 4 °C) and the remaining 11 fish were frozen (temperature −20 °C). A second blood sample was collected from each fish at the distinct times that defined the treatments (Table 1). Frozen fish were thawed at room temperature prior to blood collection. Blood samples were centrifuged at 4 °C and separated plasma was stored at −20 °C until assay.

2.2. Black scabbardfish samples

Black scabbardfish (*Aphanopus carbo)* specimens were collected at irregular times between 2010 and 2012 from commercial longline vessels operating off Madeira Archipelago and off mainland Portugal (Table 2). Since *Aphanopus intermedius* Parin, 1983 (intermediate scabbardfish) specimens are mixed with *A. carbo* in Madeira's commercial landings (Stefanni & Knutsen, 2007), specimens caught in this region were assigned to species following the morphological criteria defined by Biscoito et al. (2011). In the present study, only *A. carbo* specimens were used.

In Madeira, specimens were sampled between October and December from commercial port landings. This month range was selected to encompass the reproductive season. In this fishery, black scabbardfish are caught by mid-water horizontal drifting longline set below 1000 m deep and the soaking time lasts from two to four days (Bordalo-Machado et al., 2009). Specimens from mainland Portugal were collected throughout the year on board commercial vessels or from commercial port landings. In this fishery, the fishing gear is a horizontal bottom longline and the soaking time lasts from one to two

Table 2
Summary of black scabbardfish samples used for measuring sex steroids estradiol-17β, testosterone, and 11-ketotestosterone. Values are total length range (mm) and sample size (parenthesis) by sex and maturity stage (1–5).

		Region	
Sex	Maturity	Madeira	Mainland Portugal
Females	1	-	890-1081 (13)
	2	1087-1506 (16)	1041-1178 (6)
	3	1141-1254 (4)	-
	4	1135-1323 (7)	-
	5	1141-1291 (4)	-
Males	1	-	874-1003 (10)
	2	1117-1179 (2)	1045 (1)
	3	1115-1177 (4)	-
	4	1122-1233 (7)	-
	5	1040-1244 (4)	-

days (Bordalo-Machado et al., 2009).

For each specimen, the following information was collected: total length (TL, mm), total weight (TW, g), gutted weight (UW, g), liver weight (LW, g), gonad weight (GW, g), sex, and maturity stage. Maturity stage was macroscopically assigned following the scale proposed by Gordo et al. (2000, p. 35): stage 1, immature or resting; stage 2, developing; stage 3, pre-spawning; stage 4, spawning; and stage 5, post-spawning or spent.

The gonadosomatic index (GSI) was calculated as the gonad weight as a proportion of the gutted weight; the hepatosomatic index (HSI) was calculated as the liver weight as a proportion of the gutted weight; and Fulton's condition factor (K) was calculated as the ratio between the gutted weight and the cube of the total length. To calculate the previous reproductive indicators, whole data sets collected between 2010 and 2012 from specimens caught off Madeira ($n = 1976$) and off mainland Portugal ($n = 692$) were used.

Blood samples were collected in heparinised syringes from the specimen's caudal vessel, which was exposed by removing the lateral musculature close to the caudal peduncle. To minimize the effect of metabolism and degradation, the collection of blood samples took place as soon after hauling as possible. In specimens caught off Madeira, blood was extracted in the laboratory from fish that had been dead and remained hooked to the longline for one to four days and was kept on ice after hauling for less than 24 h ($n = 48$). The exact individual time of death or hauling is not known since each vessel deploys the fishing gear more than once during each fishing trip and the fish is kept on board all together. Specimens caught off mainland Portugal had been dead and remained hooked to the longline for up to one day. Blood samples from these specimens were collected (i) on board from fresh fish immediately after hauling ($n = 12$); (ii) in the laboratory from fish kept on ice after hauling for less than 24 h ($n = 14$); or (iii) in the laboratory from thawed fish that was stored in a freezing room at −20 °C, less than one day after hauling, for 30 days ($n = 4$).

Blood samples were centrifuged at 4 °C to separate the plasma, which was stored at −20 °C until assay.

2.3. Steroid analysis

Blood plasma (100 μL) was extracted twice with 3 ml of diethyl ether to obtain the free steroids. Extracts were dried on a dry bath at 40 °C under nitrogen gas and suspended in 1 ml of assay buffer (0.5 M phosphate–gelatine buffer, pH 7.6). Free steroids were measured by radioimmunoassay (RIA) following the methodology described by Scott, Sheldrick, and Flint (1982). Individual plasma samples were mixed with 100 μL of distilled water and extracted twice with 4 mL of diethyl ether to obtain free steroids. Extracts were dried under nitrogen, reconstituted in 0.5 M phosphate–gelatine buffer, pH 7.6 and steroids were measured by RIA.

The estradiol-17β (E_2) antiserum was purchased from Research Diagnostics (USA) and the cross-reactions (%) have been reported as follows: < 0.2% for 4-pregnene-3,20-dione; < 0.2% for 11β,17,21-trihydroxy-4-pregnene-3,20-dione; < 0.2% for 4-androstene-3,17-dione; < 0.2% for 17β-hydroxy-4-androsten-3-one; < 0.2% for 3β-hydroxy-5-pregnen-20-one; < 0.2% for 3β-hydroxy-5-androsten-17-one; 15% for 3β-hydroxy-1,3,5(10)-estratrien-17-one; 8% for 3,17β-dihydroxy-1,3,5(10)-estratrien-16-one; 0.7% for 3,16α,17β-trihydroxy-1,3,5(10)-estratrien-3-one; < 0.2% for 3,16α-dihydroxy-1,3,5(10)-estratrien-17-one (Guerreiro, Fuentes, Canario, & Power, 2002). The testosterone antiserum was kindly donated by Dr. David Kime (University of Sheffield, UK). The testosterone (T) antiserum cross-reactions were 63% for androstenedione, 35% for 11-ketotestosterone, 55% for 11-hydroxytestosterone, 40% for 5-androstan-17-ol-3-one, 31% for 5-androstan-17-ol-3-one, 12% for 5-androstane-3,17-diol, 25% for 5-androstane-3,17-diol. The 11-ketotestosterone (11-KT) antiserum cross-reactions were 20.1% for 11-hydroxytestosterone, 20.6% for testosterone, 76.9% for androstenedione, 30.1% for 11-hydroxyandrostenedione, 52% for dihydrotestosterone, 3.3% for cortisol, and 1.3% for cortisone (Kime & Manning, 1982). All samples were assayed in duplicate in a single assay. The intra-assay and inter-assay coefficients of variation were, respectively: 6.6% and 14.2% for E_2; 5.0% and 8.2% for T; and 8.2% and 11.6% for 11-KT. The limits of detection were between 10 (E_2) and 100 (T and 11-KT) pg/mL.

2.4. Statistical analyses

In the Mozambique tilapia pilot study, one-way ANOVA was applied for comparing T and 11-KT concentrations between t0 (control) and the time of each treatment. The variation in T and 11-KT concentration in Mozambique tilapia was estimated as the difference between the value at time t0 (control) and at the time of each treatment. One-Way ANOVA was applied for comparing the variation in T and in 11-KT between treatments. Whenever necessary, data were $\log_{10}$-transformed to meet the ANOVA assumptions. If ANOVA assumptions were not met after data transformation, nonparametric Kruskal–Wallis test by ranks was used instead. When there were statistically significant differences between treatments, Wilcoxon signed-rank test was applied to compare each pair of treatments.

In black scabbardfish blood plasma, one-way ANOVA was used to investigate the association between E_2, T, and 11-KT concentrations and the way the specimens are preserved prior to blood collection for each sex separately. Whenever necessary, data were $\log_{10}$-transformed to meet the ANOVA assumptions. If assumptions were not met after data transformation, nonparametric Wilcoxon signed-rank test was used instead. When there were statistically significant differences between preservation methods, Wilcoxon test was applied to compare each pair of maturity stages.

Two-way fixed effects ANOVA was applied for analysing the effects of geographical region and maturity stage on GSI, HSI, K, and on E_2, T, and 11-KT concentrations for each sex separately. Whenever necessary, data were $\log_{10}$-transformed to meet the ANOVA assumptions. If assumptions were not met after data transformation, nonparametric Wilcoxon signed-rank test was used instead. When there were statistically significant differences between maturity stages, Wilcoxon test was applied to compare each pair of maturity stages.

A k-means clustering method was applied to steroid (E_2 and T) profile data using developing (stage 2) females both from Madeira and mainland Portugal using the R package *cluster* (Maechler, Rousseeuw, Struyf, Hubert, & Hornik, 2018, pp. 7–1).

The R software (R Core Team, 2018) was used for all statistical analyses and 5% significance level was adopted. Graphics were built with the R package *ggplot2* (Wickham, 2009).

3. Results

3.1. Pilot study

In Mozambique tilapia, no significant differences in T concentration (χ^2 (4, $n = 28$) = 3.918, *p-value* = 0.417) and in 11-KT concentration (χ^2 (4, $n = 28$) = 1.601, *p-value* = 0.809) were found between samples collected initially and samples collected after treatment (Fig. 1).

T and 11-KT concentrations varied between the control (t0) and each treatment but the variations were not significantly different between treatments (χ^2 (4, $n = 28$) = 3.918, *p-value* = 0.417 and χ^2 (4, $n = 28$) = 1.601, *p-value* = 0.809, respectively) (Fig. 2).

3.2. Reproductive indicators in black scabbardfish

Black scabbardfish specimens caught off mainland Portugal, between 2010 and 2012, were in maturity stages 1–3 and 5 (Table 2). Immature or resting females and males (maturity stage 1) were not found among fish sampled in Madeira.

In females caught off mainland Portugal, mean GSI significantly

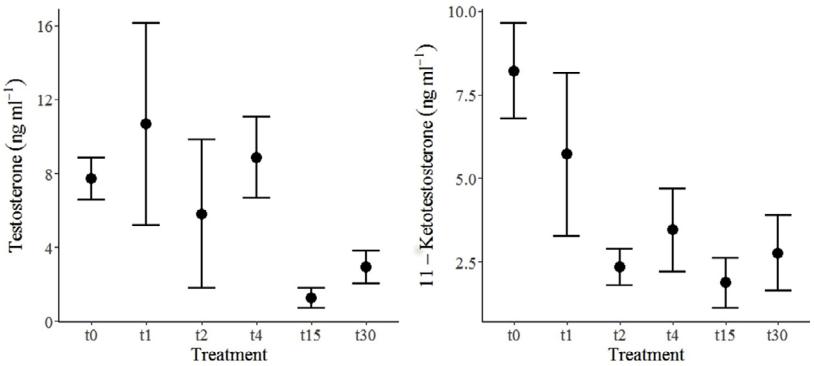

Fig. 1. Testosterone (ng ml^{-1}) (left) and 11-ketotestosterone (ng ml^{-1}) (right) concentration (mean ± SE) in Mozambique tilapia males by treatment.

increased between stage 1 and stage 2 (W = 3544, *p-value* < 0.001). However, the increase between stage 2 and stage 3 was not statistically significant (W = 164, *p-value* = 0.358) (Fig. 3). The mean GSI of females caught off Madeira significantly increased from stage 2 to stage 3 (W = 1866, *p-value* < 0.001) and from stage 3 to stage 4 (W = 1015, *p-value* < 0.001), and significantly decreased from stage 4 to stage 5 (W = 6983, *p-value* < 0.001). Concerning males, mean GSI significantly increased from stage 1 to stage 2 (W = 488, *p-value* < 0.001) and from stage 2 to stage 3 (W = 845, *p-value* < 0.001) in specimens caught off mainland Portugal, and from stage 2 to stage 3 (W = 17,335, *p-value* < 0.001) and from stage 3 to stage 4 (W = 2494, *p-value* < 0.001) in specimens caught off Madeira, whereas the decrease from stage 4 to stage 5 was statistically significant (W = 8213, *p-value* < 0.001) in specimens from Madeira. The mean GSI was significantly higher in specimens caught off Madeira than off mainland Portugal for stage 2 (W = 23,842, *p-value* < 2.2×10^{-16}) and stage 3 females (W = 7, *p-value* = 0.004) and for stage 2 (W = 11,430, *p-value* = 0.008) and stage 3 males (W = 8147, *p-value* = 0.033).

Mean HSI was significantly higher in stage 2 females caught off Madeira than off mainland Portugal (W = 33,667, *p-value* = 0.020) (Fig. 4). Mean HSI significantly increased from stage 1 to stage 2 females caught off mainland Portugal (W = 10,395, *p-value* < 0.001) and from stage 2 to stage 3 females caught off Madeira (W = 13,287, *p-value* < 0.001), whereas it significantly decreased from stage 4 to stage 5 females caught off Madeira (W = 5690, *p-value* < 0.001).

Mean K significantly increased between stage 1 and stage 2 females (W = 9041, *p-value* < 0.001) and males (W = 7505.5, *p-value* = 0.002) caught off mainland Portugal, and from stage 3 to stage 4 males (W = 7946, *p-value* < 0.001) caught off Madeira, whereas it significantly decreased from stage 4 to stage 5 males (W = 5828, *p-value* < 0.001) (Fig. 5). Mean K was significantly higher in stage 2 females (W = 57,444, *p-value* < 0.001), in stage 2 males (W = 18,945, *p-value* < 0.001), and in stage 3 males (W = 15,444, *p-value* < 0.001) caught off Madeira than in the same sex and same maturity stage specimens caught off mainland Portugal.

3.3. Sex steroids in black scabbardfish

The changes of both T and 11-KT concentrations in Mozambique tilapia between the time of blood extraction and the time of measurement after preservation did not statistically differ among the different treatments applied. Therefore, the sex steroid concentrations quantified in the black scabbardfish samples were directly used in the data analyses.

In black scabbardfish specimens, no significant differences were found between the specimens preservation methods for all three analysed sex steroids: E_2 ($F_{2,41}$ = 2.851, *p-value* = 0.069); T ($F_{2,64}$ = 1.789, *p-value* = 0.175); and 11-KT ($F_{1,26}$ = 0.239, *p-value* = 0.629).

In black scabbardfish females, T was not significantly different between regions ($F_{1,36}$ = 1.470, *p-value* = 0.233) nor between maturity stages ($F_{1,36}$ = 0.830, *p-value* = 0.368) neither was the interaction between region and maturity stage statistically significant ($F_{1,36}$ = 1.349, *p-value* = 0.253) (Fig. 6). E_2 was significantly different between regions ($F_{1,40}$ = 5.717, *p-value* = 0.022), but no significant

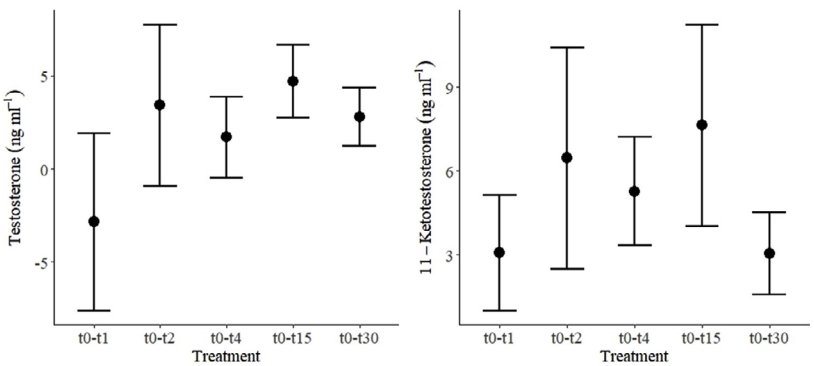

Fig. 2. Change in testosterone (ng/mL) (left) and 11-ketotestosterone (ng/mL) (right) concentration (mean ± SE) in Mozambique tilapia males between time of blood collection and the time of the second collection defined by the treatment.

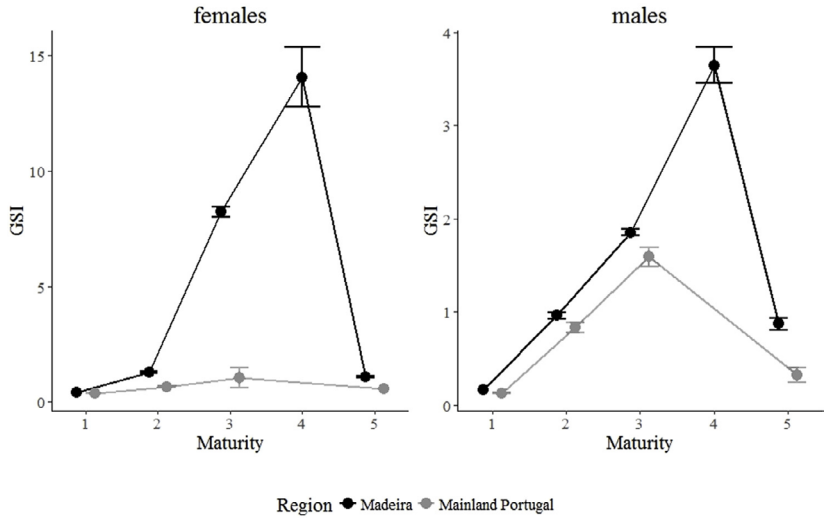

Fig. 3. Black scabbardfish gonadosomatic index (GSI) (mean ± SE) of females (left) and males (right) caught off Madeira and mainland Portugal by maturity stage.

differences were found between maturity stages ($F_{1,40}$ = 2.215, *p-value* = 0.145) neither was the interaction between region and maturity stage statistically significant ($F_{1,40}$ = 2.954, *p-value* = 0.093).

11-KT was significantly different between males caught off Madeira and males caught off mainland Portugal ($F_{1,24}$ = 14.364, *p-value* = 0.001), but no significant differences were found between maturity stages ($F_{1,24}$ = 0.465, *p-value* = 0.502) neither was the interaction between region and maturity stage statistically significant ($F_{1,24}$ = 0.389, *p-value* = 0.539) (Fig. 5). T in males was not significantly different between regions ($F_{1,24}$ = 2.228, *p-value* = 0.149) and no significant differences were found between maturity stages ($F_{1,24}$ = 0.008, *p-value* = 0.928). The interaction between factors was not analysed because factors were not crossed.

The only maturity stage within the same sex for which blood was collected in specimens caught off both Madeira and mainland Portugal areas was stage 2 females. Considering T and E_2 concentrations together, stage 2 female samples were grouped into two clusters that were statistically different ($F_{1,16}$ = 6.793, *p-value* = 0.019). T and E_2 concentrations did not show evidence of geographic differentiation as each cluster included specimens from both regions (Fig. 7).

4. Discussion

The pilot study with the Mozambique tilapia showed that it is possible to obtain meaningful measures of sex steroids in blood collected from fish that has been preserved refrigerated or frozen for a relatively prolonged period. Furthermore, it was assumed that the general behaviour of the hormonal steroids during refrigeration and the degradation mechanisms would follow similar patterns, although this is an area that requires more in-depth studies. In a previous study with plainfin midshipman fish, *Porichthys notatus*, blood plasma, no significant differences in E2, T, and 11-KT concentrations were found between samples collected at different times after capture (within one or 4 h) in either offshore or intertidal zones for both males and females

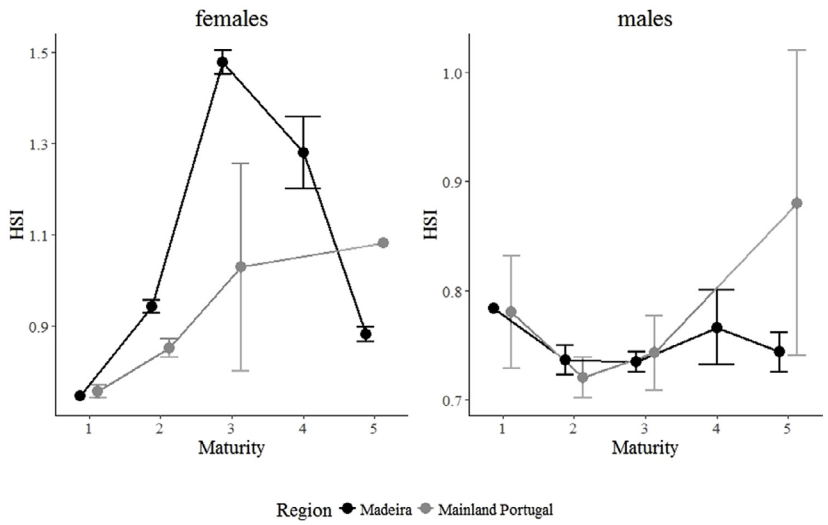

Fig. 4. Black scabbardfish hepatosomatic index (HSI) (mean ± SE) of females (left) and males (right) caught off Madeira and mainland Portugal by maturity stage.

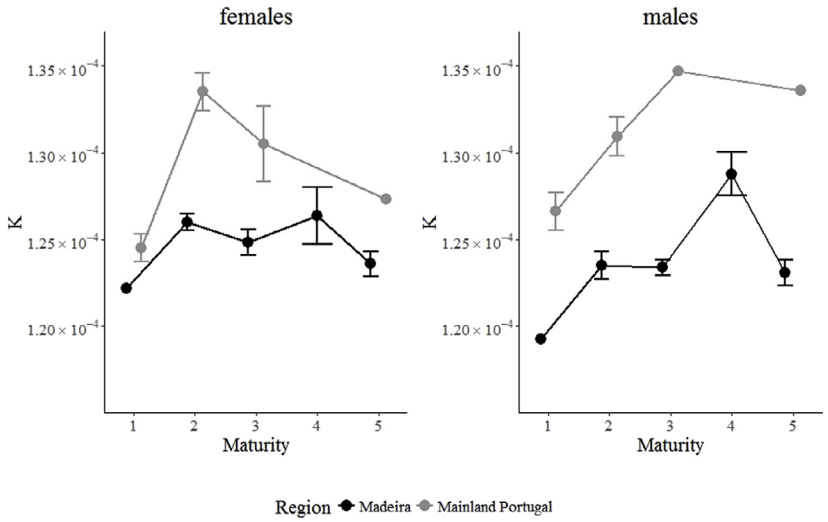

Fig. 5. Black scabbardfish Fulton's condition factor (K) (mean ± SE) of females (left) and males (right) caught off Madeira and mainland Portugal by maturity stage.

(Sisneros et al., 2004). Black scabbardfish blood samples were collected up to 24 h or 30 days after capture and steroid levels were not corrected. Nonetheless, black scabbardfish sex steroid levels should be regarded as relative and not absolute values. In addition to the time between death and blood collection and the above-mentioned factors that influence hormone levels, the stress of capture, which may vary with time and method of capture, can also lower sex steroid levels (Clearwater & Pankhurst, 1997; Cleary, Battaglene, & Pankhurst, 2002; Pankhurst & Conroy, 1988).

E_2 and T increased during vitellogenesis (stages 1–3), peaked at the

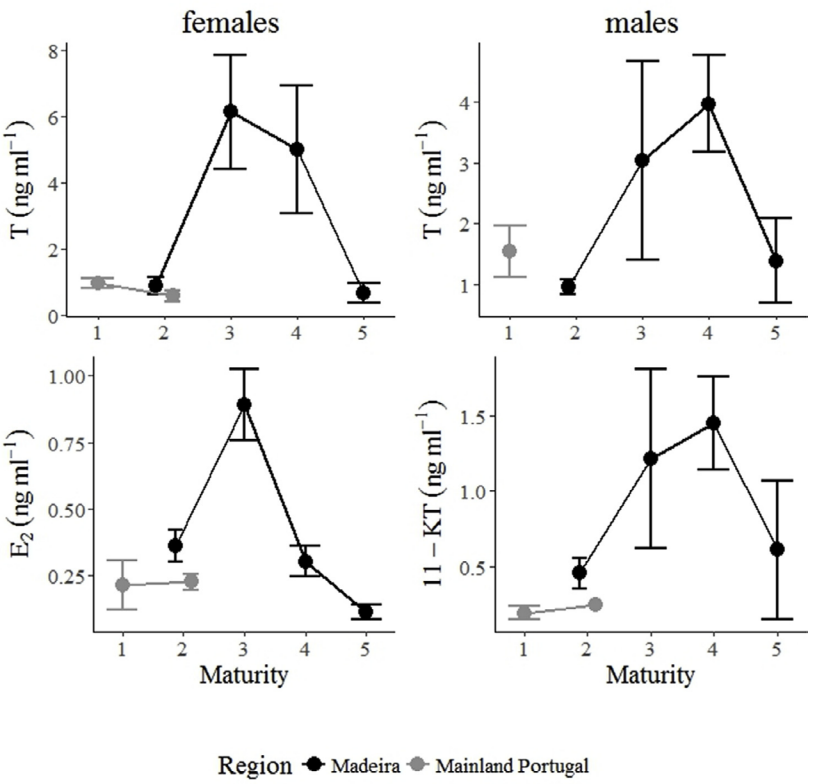

Fig. 6. Sex steroids concentration (mean ± SE) in the blood of black scabbardfish females (left column) and males (right column) caught off Madeira and mainland Portugal by maturity stage. T is testosterone in ng/mL; E_2 is estradiol in ng/mL; 11-KT is 11-ketotestosterone in ng/mL.

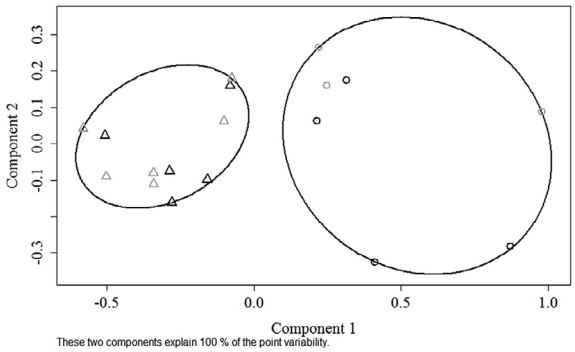

Fig. 7. Representation of k-means clustering applied to steroid (E_2 and T) profile data using developing (stage 2) females caught off Madeira and mainland Portugal.

pre-spawning stage (stage 3), and decreased from stage 3 onwards. The same steroid profile was observed for GSI in females. Although the increase in GSI between stages 1 and 2 was not statistically significant, the concomitance between the highest levels of E_2 and T and the peak in vitellogenesis (stage 3 in this species) has also been described for other teleosts (e.g. Frisch, McCormick, & Pankhurst, 2007; Hachero-Cruzado et al., 2007; Li, Liu, & Lin, 2007; Prat, Zanuy, Carrillo, de Mones, & Fostier, 1990). Furthermore, the same profile has been observed in the HSI of female black scabbardfish, demonstrating that the hepatic reserves are being consumed during the maturation process (Domínguez-Petit & Saborido-Rey, 2010; Ribeiro Santos, Minto, et al., 2013).

In fact, E_2 and T are connected since T is a precursor of E_2, such as other steroids (Pankhurst & Conroy, 1987), and E_2 is crucial for the start of vitellogenesis because it promotes the synthesis of hepatic yolk precursors (vitellogenin) in a variety of teleost species and provides negative feedback to LH secretion (Lubzens et al., 2010; Sisneros et al., 2004; Tyler & Sumpter, 1996). Chemical and physiological changes occurring throughout female developing stage are of uttermost importance for comprehending the reproductive cycle of black scabbardfish and the differences between fish living off Madeira and fish living off the Portuguese mainland coast. To disentangle this moment in the species' life cycle, E_2 and T concentrations in developing females were analysed together through cluster analysis. The mixing of females from both areas within each cluster shows that the steroid profiles are similar between areas. Madeiran females were sampled at the spawning season and during this period it is likely that females between an early and a late development stage may concur. Fish from the group with the lowest steroid concentrations represent an earlier development stage. These fish will not have the time to mature and reach the spawning stage and will fail to spawn in the current season. It is not a case of skipped spawning, which refers to a mature fish that fails to spawn at a given year (Rideout & Tomkiewicz, 2011), but the drivers of both processes are similar. In black scabbardfish, the developing stage during early vitellogenesis proved to be the critical window for the decision to spawn or not to spawn (Neves et al., 2009; Skjæraasen et al., 2010). The energy saved not developing into mature stages is invested in growth and in increased fecundity, similar to what happens when fish skip spawning (Jørgensen, Ernande, Øyvind, & Dieckmann, 2006). The persistence of a high proportion of relatively immature individuals in a plentiful environment may allow them to grow fast before the next reproductive cycle drives energy towards the gonads (Alonso-Fernández & Saborido-Rey, 2012; Folkvord et al., 2014; Roff, 1983). Moreover, the simultaneous occurrence of two groups of developing females with similar length, steroid levels, and reproductive indicators points to a long duration of this maturity stage.

Stage 2 females caught off mainland Portugal during the spawning period show a high degree of follicular atresia in the ovaries and do not develop into mature stages (Neves et al., 2009). The abidance of fish at the same location after the maturation process is interrupted explains why ca. 25% of individuals off mainland Portugal are larger than L_{50} but immature (Figueiredo et al., 2003; Neves et al., 2009; Farias et al., 2013). This phenomenon was also observed off the west of the British Isles (Ribeiro Santos, Minto, et al., 2013). The capability of specimens to migrate to the spawning grounds will depend on the completion of high energy reserves (Merrett & Haedrich, 1997, p. 282; Ribeiro Santos, Minto, et al., 2013). In fact, the relatively high HSI observed in immature males caught off mainland Portugal suggests that energy is being stored in the liver to be spent when moving to the spawning grounds. Black scabbardfish's readiness for migration was also supported by the high content of arachidonic acid (ARA), which is demanded for long distance movements (Sargent, Bell, McEvoy, Tocher, & Estevez, 1999; Tocher, 2003), that was found in the muscle of specimens caught off mainland Portugal (Farias et al., 2014). Off the west of the British Isles, the species prepares for migration towards the south, between January and April, through an intense feeding activity on blue whiting (Ribeiro Santos, Trueman, Connolly, & Rogan, 2013).

Androgen (T and 11-KT) levels did not significantly differ among reproductive stages. Nevertheless, in males caught off Madeira, the two steroids were low in spent and regressed fish, increased during gonadal recrudescence, and peaked at the end of spermatogenesis as described in several studies (Pankhurst & Conroy, 1988; Prat et al., 1990). The significant differences between regions are expected to be a consequence of the unbalanced sampling amongst maturity stages.

The present work supports the role of sex steroids as intrinsic triggers for gonadal maturation and spawning in black scabbardfish. It also shows that it is possible to measure sex steroids in blood plasma that was collected late after death and relate the values with the dynamics of the species reproduction, overcoming some sampling constraints of deep-sea species. Given the fact that specimens used in this study were collected from commercial fisheries with a soak time greater than one day, the number of blood samples were limited because, as the time passes after capture, the blood extraction is more difficult. Moreover, the depth at which black scabbardfish specimens were collected implies that fish are boarded already dead and, subsequently, the collection of an adequate volume of blood is problematic. To overcome these difficulties, dedicated surveys are required, which in the case of deep-sea fishes are costly and do not take place in Portuguese waters. Nonetheless, the present results put in evidence that the RIA method is appropriate for the quantification of sex steroid concentration even with small blood volumes. The metabolization of sex steroids after capture was considered to be negligible or to have occurred equally in all individuals, as the relationships between hormones and reproductive stages were maintained.

Since spawning occurs during a relatively short period, for future work it would be important to have a monthly or more frequent coverage in the two regions to test if there are differences between maturity stages related with the time of the year when specimens are caught.

Author statement

Inês Farias performed conceptualization, methodology, investigation, formal analysis, writing - original draft, writing - review and editing, and visualization. Elsa Couto, Neide Lagarto, and João Delgado performed investigation. Adelino V.M. Canário performed conceptualization, methodology, formal analysis, writing - review and editing, and supervision. Ivone Figueiredo performed conceptualization, methodology, formal analysis, writing - review and editing, supervision, and project administration.

Declaration of competing interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome. All financial support came from public funding.

Acknowledgements

The authors are especially grateful to the master, Henrique Santos, and crew of the longliner Varamar for hosting researchers on board to collect samples off mainland Portugal, and to Sofia Vieira, Sara Reis, and the technical staff from DSI-DRP for their collaboration in collecting the samples in Madeira. We also thank Pedro Guerreiro (CCMAR-CIMAR) for support in the tilapia experiment. This work was supported by the Portuguese Foundation for Science and Technology (FCT – Fundação para a Ciência e Tecnologia) under the PhD Grant SFRH/BD/64434/2009 to I. Fa. and the project UID/Multi/04326/2013 to A.V.M.C.; the Portuguese Government Development Investments and Expenses Program (PIDDAC – Programa de Investimentos e Despesas de Desenvolvimento da Administração Central) under the EU/DG Fisheries' Data Collection Framework (DCF); the Portuguese Fisheries Operational Program (2007–2013) (PROMAR – Programa Operacional Pescas), co-financed by the European Fisheries Fund (EFF) (2007–2013), under project CERTIFICA (2011–2015), MSC Certification in Portugal - Acquiring the knowledge and studying the viability for its application to coastal and artisanal fisheries.

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