Variability in Life History and Population Structure in a Model Shark Species, *Scyliorhinus canicula* (Linnaeus 1758)

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Dissertação de Mestrado apresentada à Faculdade de Ciências da Universiadade do Porto em Biodiversidade, Genética e Evolução
2014

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Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

Porto, _____/_____/_____

Agradecimentos (Acknowledgements)

É quando fechamos um capítulo que nos dá mais prazer olhar para trás. Quando o faço, apercebo-me de todas as coisas que aprendi e de tantas coisas que me tornam afortunado e pelas quais poderia agradecer toda a vida.

Começo pelos agradecimentos pessoais. Não poderia deixar de mencionar toda a minha família que sempre me apoiou nas minhas decisões ou se assegurou que podia voar alto mantendo os pés sempre bem assentes na terra. Não podia também deixar de expressar a importância que foi o apoio da Márcia, do Sr. João e D. Natália Fernandes; que após todos estes anos são como família para mim. Também um obrigado muito caloroso à Diana por sempre me iluminar pelo melhor e me ter ensinado o valor do trabalho e da amizade. Também ao Tiago agradeço com um abraço amigo pelos conselhos e gargalhadas. A todos os meus amigos de infância pelos quais agradeço todos os dias: mais uma vez – obrigado. Queria desde já deixar aqui uma referência especial à Professora Dra. Maria Susana Jorge Pereira e ao Professor Dr. Paulo José Talhadas dos Santos, que ainda nos anos de licenciatura me guiaram acerca do mestrado que deveria tomar e me incentivaram sempre a seguir em frente. A sua influência no meu trabalho e os seus conselhos, que se transformaram em decisões, fazem hoje parte de mim. Não posso também esquecer o Professor Dr. Paulo Célio Pereira Martins Alves, o Dr. Ricardo Pita da Universidade de Évora, a Dra Joana Paupério, e a Soraia Barbosa pela preciosa ajuda e toda o conhecimento que me transmitiram durante o decorrer do estágio de licenciatura.

No passar destes dois anos de mestrado conheci melhor um mar de gente sempre disponível. Tenho de começar por agradecer à Dra Susana Lopes pelo imenso auxílio prestado, e pela força herculana que encontrava nela mesma em arranjar sempre alguns “5 minutinhos” do seu precioso tempo para ensinar, explicar e verificar diversos resultados. Não tenho como agradecer demais à Patrícia: sempre bem disposta, incansável e profissional. Fazia das tripas coração para tentar corresponder aos pedidos de toda a gente com as “corridas no sequenciador”. E agradeço também pelas vezes em que, havendo “poços livres no sequenciador” era ela quem corria à procura de “voluntários” que quisessem processar mais algumas amostras. Agradeço também à Filipa, Diana e Teresa pelas inúmeras vezes que me corrigiram, ajudaram ou simplesmente disseram “bom dia”. Um obrigado muito especial à Sara por estar sempre

atenta à contabilidade e estar sempre disponível para me “chegar as valiosas *Taq*s”. Quero também agradecer a todos os meus colegas de mestrado que durante estes dois anos me ajudaram. Não tenciono me esquecer de ninguém, mas há pessoas que devem ser mencionados: Jolita pela boa disposição e tão importante vontade de ajudar (tanto no Porto como em Lisboa); Mafalda, pelas dicas com o “R”; Luana, pelo estímulo intelectual e ajudas em alturas de *stress* no laboratório; Carolina, pelas sugestões e auxílio naquelas horas extra (fins-de-semana extra); Sara, pelos grandes momentos de descontração; Mariana, pela ajuda na questão das concentrações; Alvarina, pelos almoços e cafés que tão bem me fazem; etc. Não posso ainda assim, deixar de mencionar a Joana Rocha e Ana Miranda e a sua imprescindível assistência que foi crucial no primeiro ano do mestrado.

The next acknowledgments, I’m going to write them in English, so the people they are addressed to can understand. A special thank you to all the people who helped me to get the samples from far away locations and that made all this work possible: Dr. Joan Navarro (CSIC, Spain); Dr. Cristina Rodriguez-Cabello (IEO, Spain); Dr. Carlotta Mazzoldo & Licia Finotto (UNIPD, Italy); Dr. Peter McCorriston (AFBI, Northern Ireland); Dr. Chrysa Gubilli (Univ. Salford, U.K.). I never really met you, but I’d really like to meet you in person just to thank you properly.

A special thank you to Dr. Andrew Griffiths for his availability either via Skype conferences or by email, thank you for the so much important feedback and annotations. I can’t thank you enough for the much you have taught and inspired me.

Por último, mas não menos importante, um enorme obrigado à minha orientadora Dra Ana Veríssimo. Obrigado pelas imensas horas de trabalho dedicadas a este projecto: o meu projecto de tese. Pelo feedback e pelo estímulo crítico que despertaste em mim que espero que me acompanhe toda a vida. Por me receberes em tua casa em Lisboa, por me receberes em tua casa em Vila do Conde, essa calorosa amizade merece todo o meu agradecimento. E, acima de tudo, obrigado por todo o incentivo, pela força em me pôr a trabalhar e vencer. Obrigado por essas lições.

Finalizo estes agradecimentos com uma citação de Agostinho da Silva numa conversa com Herman José: “A obrigação do Homem é voar alto, mas sem nunca perder de vista a linha de terra”. A todos, muito obrigado por me ajudarem a aprender a voar.

Abstract

The small-spotted catshark, *Scyliorhinus canicula* (Linnaeus, 1758), is one of the most common elasmobranchs in the eastern North Atlantic and the Mediterranean Sea. This small (<100 cm of total length), bottom dwelling oviparous shark ranges from off the British Isles southwards to Senegal, and is also found across the whole Mediterranean basin. In the Atlantic it is often caught as by-catch in demersal fisheries, but its commercial importance is growing through its use as whelk bait. In the Mediterranean it is targeted for consumption, where huge declines from historic levels of abundance have recently been reported. Previous studies have shown that *S. canicula* from different geographical locations along its range have striking differences in reproductive and life history traits. Specifically, individuals inhabiting regions at higher latitudes have a larger maximum total length and reach sexual maturity at larger body sizes than individuals from regions of inferior latitudinal values. Diverse environmental conditions at different locations have been suggested as a plausible explanation for this phenotypic variability. However, this represents a classic paradigm in biology, where environment and genetic composition can contribute to the observed phenotypic variability in reproductive and life history strategies. We studied a set of life history traits, as maximum length and length at which 50% individuals attain sexual maturity, never before described for the west off Portuguese coast and fit the results to the previously described pattern for the species. Additionally, we explored the potential for a genetic basis on the apparent variability of reproductive traits described. We did so by comparing the pattern of genetic differentiation at a 401 bp fragment of the mitochondrial DNA control region and at 11 nuclear microsatellite markers from ten locations along the species' range. Maximum length (644 mm) and length at sexual maturity (532 mm for males and 506 – 564 mm for females) for the northern Portuguese coast are in accordance with the previously described pattern of phenotypic variation for *S. canicula*. The molecular markers detected strong genetic differences between the Mediterranean Sea collections (as off Mallorca, Gulf of Lion, Adriatic Sea and Crete) and those from the Atlantic (North Sea, Irish Sea, British Islands, Cantabrian Sea and off North and South Portugal), in agreement with the major phenotypic differences seen between the two areas in terms of reproductive traits.

Keywords: *Scyliorhinus canicula*, small-spotted catshark, phenotypic variation, life history traits, genetic population structure

Resumo

A pata-roxa, *Scyliorhinus canicula* (Linnaeus, 1758), é um dos elasmobrânquios mais comuns no nordeste do Oceano Atlântico e no mar Mediterrâneo. Este pequeno tubarão (<100 cm de comprimento total) ovíparo e bentónico tem uma distribuição desde as Ilhas Britânicas em direção ao Sul até ao Senegal, e é também encontrado por toda a bacia do Mediterrâneo. No Oceano Atlântico é normalmente capturado acessoriamente por barcos de pesca profunda, mas a sua relevância económica tem vindo a crescer devido ao uso como isco de pesca. No Mediterrâneo é capturado para consumo, e grandes declínios em termos de abundância a nível histórico têm sido documentados recentemente. Estudos anteriores têm revelado que *S. canicula* de pontos geográficos distintos ao longo da sua distribuição apresentam diferenças evidentes em características reprodutivas e de estratégias de vida. Mais especificamente, indivíduos que habitam em maior latitude apresentam valores de comprimento máximo maiores, e atingem a maturidade sexual a maior tamanho. Condições ambientais diversas em locais distintos tem sido sugerido como a principal hipótese por trás desta variabilidade fenotípica. No entanto, este fenómeno representa um paradigma clássico em biologia, nomeadamente, em que medida o ambiente e/ou a componente genética contribuem para uma variabilidade fenotípica em estratégias de vida e reprodutivas. Foi estudado um conjunto de características de estratégias de vida, tais como comprimento máximo e comprimento ao qual 50% dos indivíduos atingem maturação sexual, até agora por descrever para a costa oeste de Portugal e justapor estes valores no padrão anteriormente descrito para a espécie. Seguidamente, explorou-se a hipótese de existir algum sinal genético subjacente à aparente variabilidade das características fenotípicas descritas. Foi utilizado um fragmento da região controlo do ADN mitocondrial com 401 bp e 11 microssatélites de ADN nuclear para dez locais ao longo da distribuição da espécie. O comprimento máximo (644 mm) e comprimento aquando da maturação sexual (532 mm para os machos e 506 – 564 para as fêmeas) para a costa norte de Portugal estão em concordância com o padrão de variabilidade fenotípica anteriormente descrito para *S. canicula*. Os marcadores moleculares detetaram fortes sinais de diferenciação genética entre as coleções do Mediterrâneo (como Mallorca, Golfo de Lion, Mar Adriático e Creta) e as do Oceano Atlântico (Mar do Norte, Mar da Irlanda, Ilhas Britânicas, Mar da Cantábria, e Norte e Sul

de Portugal), em concordância com o principal sinal de variação fenotípica entre as duas áreas em termos de características reprodutivas.

Palavras-chave: *Scyliorhinus canicula*, Pata-roxa, variação fenotípica, características de estratégias de vida, estrutura genética populacional

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List of Acronyms

AMOVA - Analysis Of Molecular Variance

CL - Clasper Length

CR - Control Region

ECL - Egg Capsules Length

EW - Eviscerated Weight

FCA - Factorial Correspondence Analysis

gDNA - Genomic DNA

GSI - Gonadosomatic Index

HIS - Hepatosomatic Index

HWE - Hardy-Weinberg Equilibrium

L50 - Length at which 50% of the individuals attain sexual maturity

L95 - Length at which 95% of the individuals attain sexual maturity

LD - Linkage Disequelibrium

LFD - Largest Folicle Diameter

LW - Liver Weight

MCMC - Markov Chain Monte Carlo

mtDNA - Mitochondrial DNA

NDWD - Nemenyi-Damico-Wolfe-Dunn Test

OGW - Oviducal Gland Weight

PCL - Pre-Caudal Length

QTL - Quantitative Trait Loci

TL - Total Length

TW - Total Body Weight

UPW - Ultra Pure Water

CHAPTER 1

General Introduction

Phenotypic variability within populations and/or within species is crucial for adaptation to ever changing environments (Berry, 2001). One type of phenotypic variability is the described intraspecific variation in life history traits, which include variations in age at maturity, survival rate, size at birth, maximum body size and other aspect of particular behavioural strategies (Roff, 2001). Some extrinsic factors appear to drive the variability in life history traits, including temperature (Harkey and Semlitsch, 1988), population densities (Peacor and Pfister, 2006) and food availability (Anholt *et al.*, 2000). However, such phenotypic variability may be explained by means other than environmental factors alone. Differences in the genetic composition of individuals may result in different phenotypes being expressed regardless of environment.

Cases of intraspecific life history variability are evident in nature. Amphibians are a well acknowledged case of reported variation in life history traits. Individuals at higher latitudes have longer larval periods, have larger body size and reach maturity at older age (Berven and Gill (1983); and reviewed in Morrison and Hero (2003)). One other case regarding a smaller geographic scale, is the altitudinal variation of life history traits for *Sceloporus jarrovi* lizard. Ballinger (1979) reported that *S. jarrovi* individuals from the South-east Arizona inhabiting lower altitudes matured at younger ages than individuals at higher altitudes. In the marine environment, cases of life history variation are well documented. Variations in length, weight, growth, age at maturity and rate of mortality are some of the most used life history indices studied (Anderson and Neumann, 1996). One good example is the one of the red bandfish, *Cepola macrophthalma*, in the Aegean Sea which is known for having larger body sizes and attaining maturity at older age in the northern regions (Stergiou, 1999). Food availability and temperature may be the reasons underlying this variation (Caddy *et al.*, 1995; Stergiou, 1999). Another illustrative case is the study by Ruttenberg *et al.* (2005), where the variation in life history traits of the ringtail damselfish, *Stegastes beebei*, in the Galápagos Islands is described. *S. beebei* individuals from warmer waters revealed to be larger and more long lived than the smaller individuals inhabiting colder waters. Moreover, a correlation between body size and water

temperature was confirmed for the species in the Galápagos islands (Ruttenberg *et al.*, 2005).

A few studies with elasmobranchs have also shown cases of phenotypic variability. For instance, in the case of bonnethead shark, *Sphyrna tiburo*, females inhabiting colder waters at higher latitudes reach larger sizes than females from warmer waters at lower latitudes (Parsons, 1993; Carlson and Parsons, 1997). In addition to larger body sizes in northern areas, varying sizes different ages at maturity were also found in *S. tiburo* individuals from different areas (Lombardi-Carlson *et al.*, 2003). Another interesting case is the starspotted dogfish *Mustelus manazo*, in which individuals from off Japan inhabiting colder waters have lower growth rates and attain maturity at a later age than those from off Taiwan occurring in warmer waters (Yamaguchi *et al.*, 2000).

These differences in life history traits of fish, such as size, growth rates and productivity are crucial in determining population dynamics and important cornerstones in fisheries research and management (Anderson and Neumann, 1996). Populations with specific life history traits that differ from one another, may evolve in a way that mating does not occur randomly, and can be defined as different “stocks” (Begg *et al.*, 1999). So, a stock can be identified and distinguished from another by assessing its life history parameters, since they are a phenotypic expression of the interactions between the genotypic and environmental influences (Ihssen *et al.*, 1981; Pawson and Jennings, 1996).

Stock identification is crucial for correct management of fisheries resources (Begg *et al.*, 1999; Pawson and Jennings, 1996). On the one hand, stocks can be identified as strictly demographic, where individuals that share similar life history strategies and behaviour are sorted into a stock unit (Pawson and Jennings, 1996). On the other hand, stocks can be identified by assessing migration patterns, reproductive isolation, diversity and recruitment through the use of genetic tools (reviewed in Carvalho and Hauser, 1995). Therefore, genetic differences among fish populations can help determining their structure and establish independent stocks (Ward, 2000).

The case of defining stocks in elasmobranchs is more complex due to many species being widely distributed and highly migratory, which may lead to individuals forming potentially separate stocks co-occurring in the same area (Cortés, 2004). Although, genetics have proven to be able to discriminate between different stocks (Carvalho and Hauser, 1995; Pawson and Ellis, 2005). Accurate stock identification in elasmobranchs

species is especially important for effective conservation measures since declines in abundance have been reported for the Northwest Atlantic ocean (Baum *et al.*, 2003) and more recently in the Mediterranean (Barausse *et al.*, 2014)

Phenotypic variability in life history traits is also well described for *S. canicula*, where differences in maximum body size and size at sexual maturity between Atlantic and Mediterranean individuals have been reported and confirmed by several studies conducted over the last decades (Capapé *et al.*, 1991; Capapé and Zaouali, 1977; Leloup and Olivereau, 1951; Zupanovic, 1961). Compagno (1984) even suggested the possibility of two different subspecies occurring in the eastern North Atlantic and Mediterranean respectively. Beyond the above differences, a pattern of increasing body size and size at sexual maturity with increasing latitude along the species' Atlantic distribution has also been documented (Bendiab *et al.*, 2012; Capapé *et al.*, 2014, 2008a, 1991; Capapé and Zaouali, 1977; Ellis and Shackley, 1995; Henderson and Casey, 2001; Ivory *et al.*, 2005; Jennings *et al.*, 1999; Kousteni *et al.*, 2010; Leloup and Olivereau, 1951; Mendes *et al.*, 2004; Rodríguez-Cabello *et al.*, 1998; Zupanovic, 1961). This latitudinal variation has been associated with varying water temperatures and its influence in the growth rates of embryos (Thomason *et al.*, 1996).

Despite the many studies conducted on *S. canicula,* detailed knowledge of the life history traits and of the reproductive biology of *S. canicula* for the Portuguese coast is still lacking despite previous attempts at the subjects (e.g. Machado, 1996; Mendes *et al.*, 2004). This shark is a coastal, bottom-dwelling elasmobranchs that is commercially targeted for consumption in some regions of its Mediterranean distribution (Capapé *et al.*, 2008a) or caught as by-catch by many Atlantic coastal fisheries (Philippart, 1998; Rodríguez-Cabello *et al.*, 2005a). Such knowledge is crucial to the adequate management of this fishery resource at the regional level. Moreover, given the location of the Portuguese coast as the lowest latitude of all studies conducted in the eastern North Atlantic thus far, and as the closest Atlantic location to the Mediterranean Sea, it is of relevance to further describe and understand the latitudinal variability in life history traits previously described for the species.

Assessing the distribution of genetic diversity of *S. canicula* throughout its distribution will help to unravel the genetic structure of the species in the eastern North

Atlantic and Mediterranean Sea. This knowledge on population structure of a commercially exploited species as *S. canicula* is of extreme importance for good conservation and management practices (Avise, 1998), and even more so when abundance declines have already been reported for the species (e.g. Barausse *et al.*, 2014). On the other hand, knowledge of the genetic population structure will also provide an opportunity for a comparison of the distribution of genetic diversity at the regional level against the regional differences in life history traits.

The goals of this study are two-fold: 1) to describe the reproductive biology and estimate some life history traits of *S. canicula* from off the coast of Portugal, namely maximum body size, sexual maturity (i.e. length at which 50% of the individuals attain sexual maturity) and reproductive cycle; and 2) to study the genetic population structure of the species along its eastern North Atlantic and Mediterranean range. For this purpose *S. canicula* individuals were collected monthly through an year period from landings of commercial fisheries from North Portuguese fishing ports, namely Póvoa do Varzim and Matosinhos. Male and female individuals were studied separately. For each, macroscopic features, as total length and development of reproductive organs were recorded. Afterwards, a sexual maturity scale was constructed for male and female *S. canicula* off the Portuguese coast; and the results were compared to the already described pattern of phenotypic variability for the species. Additionally, a 401bp sequence of mitochondrial DNA control region and 12 nuclear microsatellite loci were analyzed to assess the population structure for the species from the eastern North Atlantic to the Mediterranean.

CHAPTER 2

REPRODUCTIVE BIOLOGY OF *Scyliorhinus canicula* (Linnaeus, 1758) IN PORTUGUESE WATERS

Introduction

The small-spotted catshark, *Scyliorhinus canicula* (Linnaeus, 1758), is an Elasmobranch that belongs to the Family Scyliorhinidae (Order Carcharhiniformes; Compagno, 1984), one of the largest families of sharks comprising more than 14 genera and over 100 species (Hamlett, 1999). This shark family is known for presenting a stout and cylinder-shaped body, a short snout, triangular-like nasal flaps, eyes dorsolateral on head, an angular arched mouth, and two dorsal fins with the second considerably smaller than the first (Compagno, 1984). The distinctive characters of *S. canicula* include a slender dark-spotted body, a narrow head with elongated eyes and triangular nasal flaps extending to the mouth (Compagno, 1984). An illustrative image of the species morphology is present in Figure 1.

Figure1. Illustrative image of a *S. canicula* specimen (photo by: Hans Hillewaert).

Its maximum recorded total length is 100 cm, although specimens larger than 80 cm are rarely observed (Compagno, 1984; Ivory *et al.*, 2005; Picton and Morrow, 2007). It is

one of the most common elasmobranchs in the East Atlantic and the Mediterranean Sea (Compagno, 1988), ranging from off the British Isles southwards to the coast of Senegal (Ellis and Shackley, 1997; Froese and Pauly, 2013) (Figure 2). Generally, *S. canicula* is found in shallow coastal waters down to 400 m depth (Compagno, 1984; Ellis *et al.*, 2005). As a bottom dwelling shark, *S. canicula* feeds mostly on macrobenthic fauna, mainly molluscs and some crustaceans (Lyle, 1983; Rodríguez-Cabello *et al.*, 2007), although it is known to prey on small bony fishes (Compagno, 1984). *S. canicula* is an oviparous species that usually deposits its eggs on algae substrates (Compagno, 1984).

S. canicula is of little economic value in Atlantic countries, where it is landed as by-catch of bottom trawl fisheries and often discarded or used as bait (Henderson and Casey, 2001; Rodríguez-Cabello *et al.*, 2004). In contrast, the species is targeted for human consumption in the Mediterranean Sea (Capapé *et al.*, 2008b). Lately, a noticeable decline in catches of *S. canicula* off the Tunisian coast and in the Adriatic Sea suggests that increasing interest in the species may put the sustainability of this resource at risk (Barausse *et al.*, 2014; Mnasri, 2008 *in* Capapé *et al.*, 2014).

Figure 2. Geographical distribution of *S. canicula.*

Like all elasmobranch fishes, the small-spotted catshark is considered a *k*-selected species: it has a relatively slow growth rate, late maturation and low fecundity. This latter condition results from it being an oviparous shark that lays two eggs at a time over an extended egg-laying season (Compagno, 1984), and with an estimated fecundity of only 29 to 62 eggs per year (Ellis and Shackley, 1997). These particular biological characteristics make elasmobranch populations in general, and *S. canicula* in particular, at a high risk of depletion under moderate to high exploitation rates. A quantitative approach of life history traits is thus essential when trying to assess elasmobranch species that are harvested (Walker, 2005), as is the case of the small-spotted catshark.

S. canicula is a well-documented species with several life history parameters described from throughout its geographic distribution (see Table 1). Indeed, intraspecific variation in life history traits has been noted for *S. canicula* according to region (see Table 1). Leloup and Olivereau (1951) were the first to observe a clear difference of size at sexual maturity and maximum size between *S. canicula* from the North Atlantic (English Channel) and the Mediterranean (Gulf of Lion). Compagno (1984) even suggested that this regional difference may be evidence of two different subspecies. Later studies have also shown that *S. canicula* from higher latitudes have larger maximum body sizes and a larger size at sexual maturity (Table 1). In general, there seems to be an influence of latitude on growth, maximum size and size at maturity in the species (Capapé *et al.*, 1991; Leloup and Olivereau, 1951; Rodríguez-Cabello *et al.*, 2004).

Even so, a good description for the reproductive biology of *S. canicula* is missing for the Atlantic Ocean off the Portuguese coast, although some work has been done to describe maximum and minimum individual sizes for the region see Machado (1996) and Mendes *et al.* (2004). The lack of information on the reproductive biology makes the stock assessment of exploited species deficient (Walker, 2005). Thus, the aim of this study is to describe the reproductive cycle and estimate important life history parameters of *S. canicula* off the Portuguese coast, namely size at maturity for males and females, and size at maternity for females. Moreover, the results will be compared with the latitudinal pattern previously described for the species in the Atlantic Ocean and Mediterranean Sea.

Table 1. Life history traits of *S. canicula* form different geographic locations.

Location	Reference	Maximum Size (mm)		Size at 50% Maturity (mm)		Maximum GSI[a] (%)		Egg Capsule Length	Egg Laying Period
		Male	Female	Male	Female	Male	Female	(mm)	(months)
North Sea	(Jennings *et al.*, 1999)	880[b]		580		-	-	-	-
West off Ireland	(Henderson and Casey, 2001)	>760	>710	575	581	-	-	-	All year round
Bristol Channel	(Ellis and Shackley, 1997)	662	670	520	550	5.57	6.72	-	Oct to July
Bristol Channel	(Ivory et al., 2005)	710	700	535	570	-	-	-	Oct to July
English Channel	(Leloup and Olivereau, 1951)	680		520-600	520-600	-	-	58	-
Cantabrian Sea	(Rodriguez-Cabello et al., 1998)	700	680	-	542	-	-	-	Jan to August
West off Portugal	(Mendes *et al.*, 2004)	629	626	-	-	-	-	-	-
Algerian Sea	(Bendiab *et al.*, 2012)	500	474	-	410[2]	-	-	-	-
Gulf of Lion	(Leloup and Olivereau, 1951)	485	520	375-445	375-445	-	-	46	-
Gulf of Lion	(Capapé *et al.*, 1991)[1]	550	510	440	410-470	-	-	-	-
Gulf of Lion	(Capapé *et al.*, 2008)	550	510	-	-	6.50[c]	9.00[c]	-	Oct to August
Tunisia Shore	(Capapé and Zaouali, 1977)[1]	530	430	400	350	-	-	-	-
Tunisia Shore	(Capapé *et al.*, 2014)	521	531	400-478	346-471				
Adriatic Sea	(Zupanovic, 1961)[1]	-	-	340	340	-	-	-	-
Adriatic Sea	(Finotto *et al.*, in prep.)	530		370	390	-	-	-	All year round
Aegean Sea	(Kousteni *et al.*, 2010)	488	467	396	399	6.36	9.77	56.7	All year round

a - Gonadosomatic Index

b - Asymptotic Maximum Length

c - Taken from Figure 5 and Figure 6 in Capapé *et al.* (2008)

1 - Cited from Ivory *et al.* (2005); Bendiab *et al.* (2012) Capapé *et al.* (2014)

2- Interpreted from Figure 7 in Bendiab *et al.* (2012)

Material and Methods

Monthly visits from August 2013 to July 2014 were made to two commercial fishing ports in the northern coast of Portugal, namely Póvoa do Varzim and Matosinhos (Figure 3). Visits were often made over several days per month in order to obtain good sample sizes (>50) of small-spotted catshark from fisheries landings. The samples were obtained from landings of bottom trawl and gillnet fisheries operating locally along the coast. Individuals were kept frozen at -20ºC until processed. Body measurements were recorded to the nearest millimetre from all individuals sampled, such as stretched total length (TL) measured from the tip of the snout to the tip of the caudal fin with the tail aligned with the body axis. Total body weight (TW) as well as liver weight (LW) were recorded to the nearest gram.

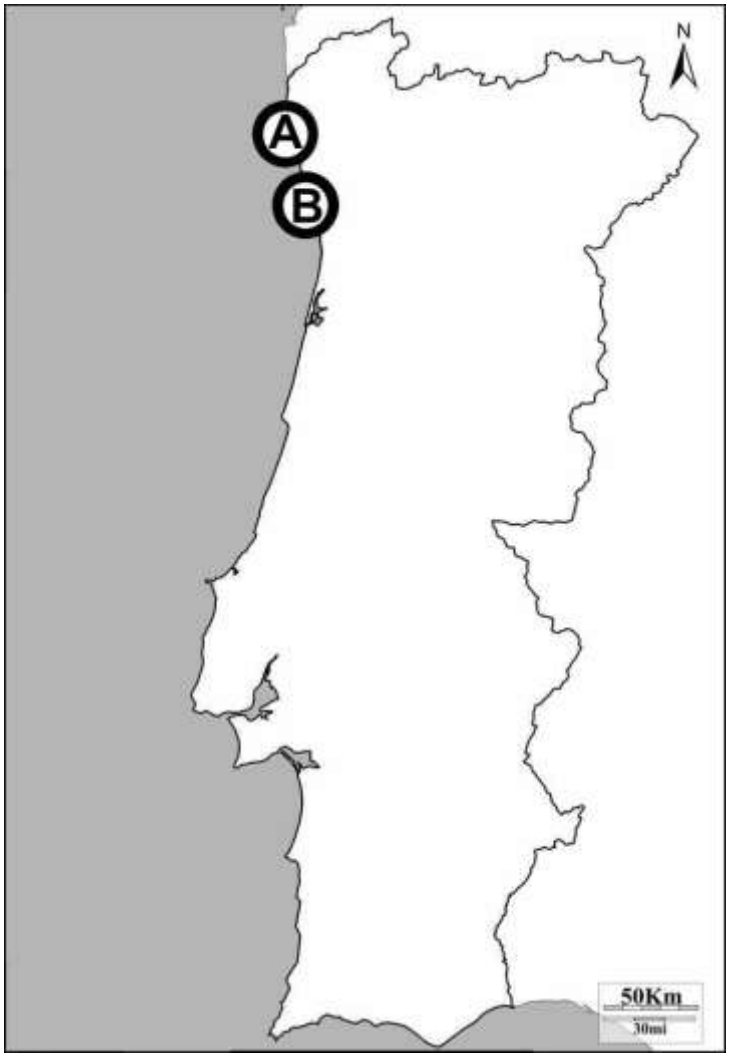


Figure 3. Fishing ports from where *S. canicula* were sampled. Póvoa do Varzim – A; Matosinhos – B.

To assess the sexual maturity stages and describe the reproductive cycle of *S. canicula* off northern Portugal, several additional variables were recorded from the different reproductive organs of both males and females. An overall view of *S. canicula* internal organs and gender differences are illustrated in Figure 4. In the case of males, claspers length (CL) was measured to the nearest millimetre from the claspers' tip to the anterior edge of the cloaca, and the level of calcification of the claspers (i.e. flexible, partly calcified, or fully calcified), as well as the presence/absence of sperm at the claspers' tip and the ability of the clasper hook to rotate (i.e. rotating/non-rotating) were also noted. Testes weight was recorded to the nearest gram, and the gonad morphology was noted (i.e. straight, partly coiled, or fully coiled). Likewise, the external morphology of the epididymis (i.e. straight, partly coiled, or fully coiled) and of the seminal vesicle (i.e. flat or enlarged) was also recorded, as was the presence/absence of sperm in the latter.

In the case of the females, the ovaries were weighted to the nearest gram and the type and number of follicles (i.e. yolked or non-yolked) were recorded. The largest follicle diameter (LFD) as well as the width of the left oviducal gland (OGW) and of the left uterus were measured to the nearest millimetre, and the presence/absence of sperm in either organ was noted. Egg capsule length (ECL) was recorded following Concha *et al.* (2010).

Clasper length (mm), testes weight (g), relative frequency of males with sperm present in the claspers, as well as ovary weight (g), LFD (mm), OGW (mm) and uterus width (mm) was plotted against TL (mm) to infer the ontogenetic changes in the reproductive organs of *S. canicula*. Based on the above results, a sexual maturity scale was constructed for the species from off the northern Portuguese coast.

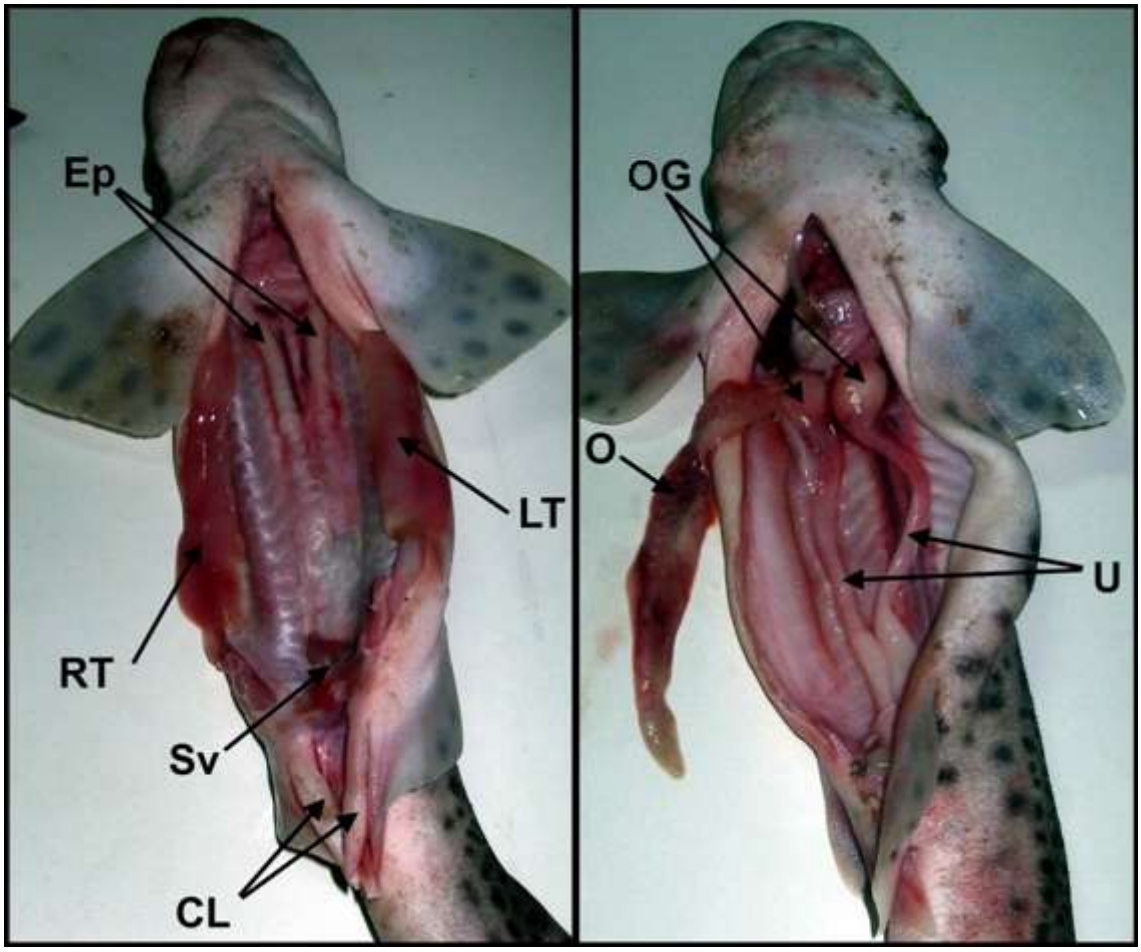


Figure 4. Anatomy of the reproductive organs of adult males (left) and females (right) of *S. canicula*. Ep - epididymis; RT - right testis; LT - left testis; Sv - seminal vesicle; CL - claspers; OG - oviducal glands; O - right ovary; U - uteri.

Hepatosomatic (HSI) and gonadosomatic indices (GSI) were calculated for males and females separately, using the following formulas: HSI = (LW/TW) x 100, GSI = (GW/TW) x 100, where GW equals gonad weight (testes and ovary weight in males and females, respectively). The distribution of HSI and GSI values were plotted against maturity stage to allow the inference of the relative amount of energy investment throughout sexual maturation. Differences in HSI and GSI among sexes of the same maturity stage were tested using an unequal variance Welch's *t*-test. Differences in HSI and GSI among maturity stages were tested using a Kruskal-Wallis one-way analysis of variance. *Post-hoc* analyses of the differences were conducted using Nemenyi-Damico-Wolfe-Dunn (NDWD) tests. All the above analyses were conducted in R software (R Core Team, 2014) using the coin package (Hothorn *et al.*, 2006; Zeileis *et al.*, 2008).

To assess the size at which 50% of the population is sexually mature (L_{50} at maturity), individuals were sorted into two categories - Immature (Juvenile and Maturing) and Mature (Adult) - according to the sexual maturity scale. The value of L_{50}

was estimated for males and females separately, following the method of Walker (2005) in R, and using 10 000 bootstrap replicates. Briefly, the logistic regression model used was

$$P(l) = P_{MAX} \times \left(1 + e^{-\ln(19)\times\left(\frac{1-\beta_1}{\beta_2-\beta_1}\right)}\right)^{-1}$$

where $P(l)$ is the proportion of the population that is sexually mature at size l, β_1 and β_2 are fitted parameters corresponding to L_{50} and to L_{95} (i.e. size at which 95% of the individuals are sexually mature) respectively, and P_{MAX} is the asymptotic value. The size at which 50% of the females are in maternity condition (L_{50} at maternity), i.e. carrying egg capsules in their uteri or showing evidence of recent egg-release (e.g. extended, flaccid uteri but no egg capsule) was also estimated with the above method.

A description of the reproductive cycle of *S. canicula* along a 12-month period was also made by comparing the variation in the relative frequency of adult males and females in the different maturity stages throughout the year. Adult individuals were considered sexually active based on the presence of sperm at the claspers' tips in males, and on the presence of large yolked follicles in the ovaries of adult females. Also, LFD of adult females was plotted against sampling season to infer the cycle of follicle maturation throughout the year. Finally, the egg-laying season was estimated based on the presence of egg capsules in the uteri of the females throughout the year.

To investigate the variability in life history parameters of *S. canicula* along the species distribution, linear regressions between geographical coordinates and two different variables, i.e. size at L_{50} at maturity for females and female maximum observed size (maximum TL), were adjusted to the data using the MASS package (Venables and Ripley, 2002) in R. Data for this analysis were obtained from this study as well as from the literature, and for different regions along the species' range, namely: North Sea (Jennings *et al.*, 1999), West off Ireland (Henderson and Casey, 2001), Bristol Channel (Ivory *et al.*, 2005), English Channel (Leloup and Olivereau, 1951), Cantabrian Sea (Rodríguez-Cabello *et al.*, 1998), Gulf of Lion (Capapé *et al.*, 2008b, 1991), Tunisian Shore (Capapé *et al.*, 2014), Adriatic Sea (Finotto *et al.*, in prep.) and Aegean Sea (Kousteni *et al.*, 2010). In a first approach, all regions' variables were plotted against their latitudinal values. Afterwards and for a more detailed view, eastern North Atlantic and Mediterranean regions' variables separately were plotted against their latitudinal and longitudinal values respectively. Data for the Algerian Sea

published by Bendiab *et al.* (2012) referred to the L_{50} at maternity, therefore it was not included here.

Results

A total of 476 small-spotted catsharks were sampled from August 2013 to May 2014 (Table 2), including 256 males, 219 females and one undetermined individual (see details bellow). From those, 56 males and 25 females were obtained at Póvoa de Varzim fishing port (17% of the total samples) while the remaining individuals were sampled at Matosinhos. No small-spotted catshark samples were obtained in some months (e.g. October 2013, February, April, June and July of 2014) due to bad weather conditions not allowing commercial fisheries to operate safely, as well as to changes in the target species at different times in the year.

A total of 153 individuals were captured during the Summer (from June 22st to September 23st 2013), 114 during the Fall (from September 24th to December 21st 2013), 103 during the Winter (from December 22nd 2013 to March 21st 2014) and 105 during the Spring (March 22nd to June 21st 2014).

Table 2. Samples of *S. canicula*. N – number of individuals; TL – Total Length.

Sampling Date	Season	N	Sex Ratio (F/F+M)	TL Range (mm)	Fishing Port
06/08/2013	**Summer**	81	0,31	436-644	Póvoa do Varzim
23/09/2013	**Summer**	72	0,07	491-610	Matosinhos
04/11/2013	**Fall**	16	0,56	471-571	Matosinhos
25/11/2013	**Fall**	65	0,52	477-620	Matosinhos
18/12/2013	**Fall**	33	0,85	445-575	Matosinhos
23/01/2014	**Winter**	81	0,48	468-620	Matosinhos
10/03/2014	**Winter**	22	0,64	470-578	Matosinhos
31/03/2014	**Spring**	54	0,65	459-575	Matosinhos
28/05/2014	**Spring**	51	0,59	485-591	Matosinhos

The length frequency distributions of the sampled catsharks showed a unimodal distribution for females (modal value: 530 mm) and a bi-modal distribution for males (modal values: 520 and 570 mm) (Figure 5). Males attained larger sizes compared to females, with total lengths ranging from 449 to 644 mm in males and from 436 to 595 mm in females.

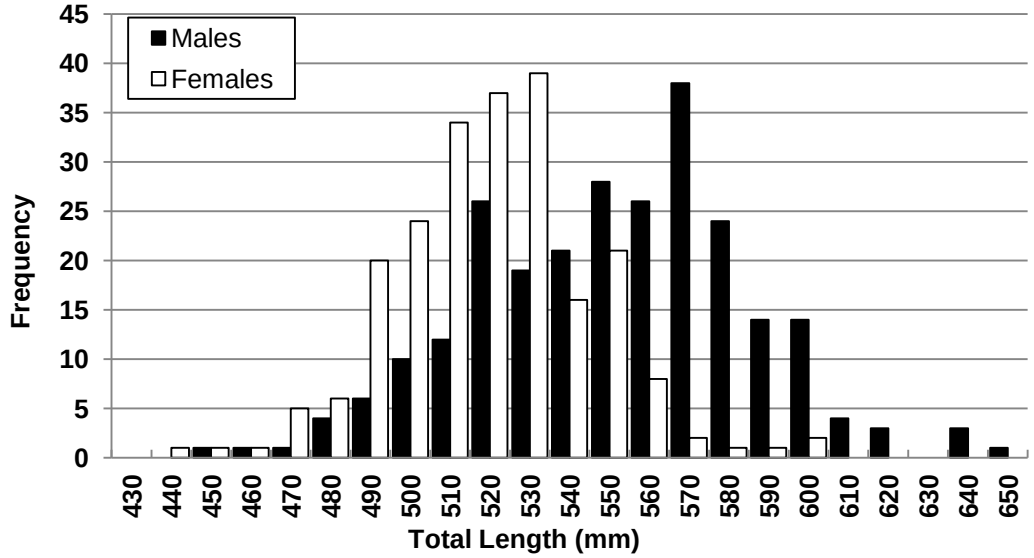


Figure 5. Total length distribution of male and female *S. canicula* sampled off northern Portugal.

Description of the reproductive organs

Males

All male *S. canicula* had two functional testes, which undergo changes in external morphology with increasing body size. Macroscopic examination of the gonads allowed the differentiation of three stages of testes development: undeveloped testes were flat, narrow and little vascularised (T1; Table 3), increasing in width and level of vascularisation with individual growth and becoming partly coiled at an intermediate state (T2; Table 3), while fully developed testes were enlarged, fully coiled and highly vascularised (T3; Table 3). Males always had both testes at the same stage of development. Moreover, gonad weight, vascularisation and coiling increased with TL (Figure 6).

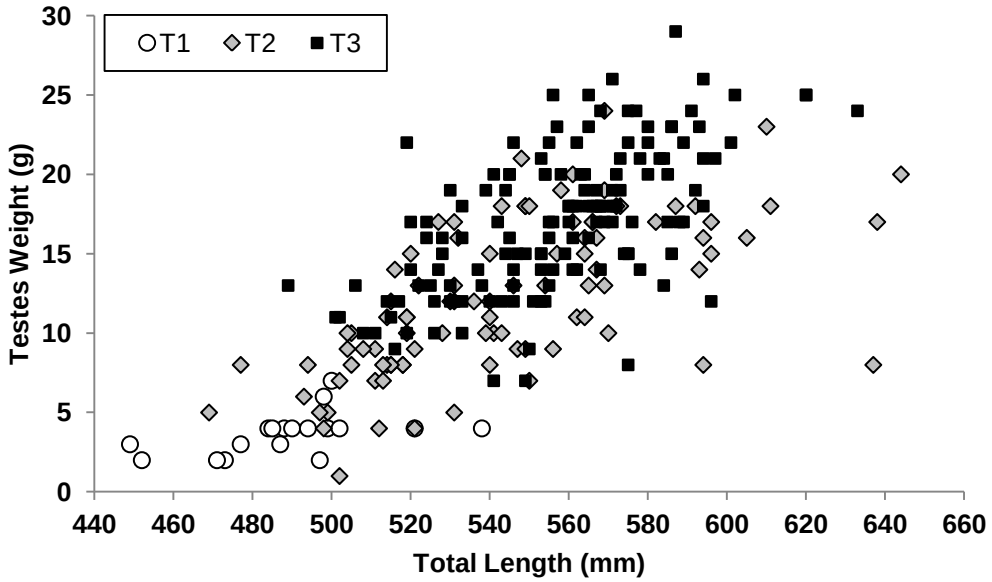


Figure 6. Relationship between gonad weight and total length in male *S. canicula* off northern Portugal.

Observations of the seminal vesicle morphology also indicated changes with growth and led to the discrimination of two different developmental stages: flat, narrow vesicle (V1; Table 3), and enlarged vesicle (V2; Table 3). The epididymis also changed with body growth, becoming increasingly coiled and enlarged with increasing total length (Table 3).

The claspers also undergo changes in the level of calcification and size with body growth (Figure 7). Two different stages of clasper development were recognised: undeveloped claspers were flexible (i.e. easily bent) and pale in colour (C1; Table 3), while fully developed claspers were calcified, reddish in colour and with a rotating hook (C2; Table 3). The slow progression from soft, flexible claspers to fully calcified ones makes it hard to distinguish between the two stages in some individuals. Clasper length increased steadily with TL, although claspers at stage C2 were observed in a wide range of body sizes (Figure 7). The smallest male in which sperm was observed at the claspers' tip was 501 mm TL while males larger than 597 mm TL always had sperm present (Figure 7).

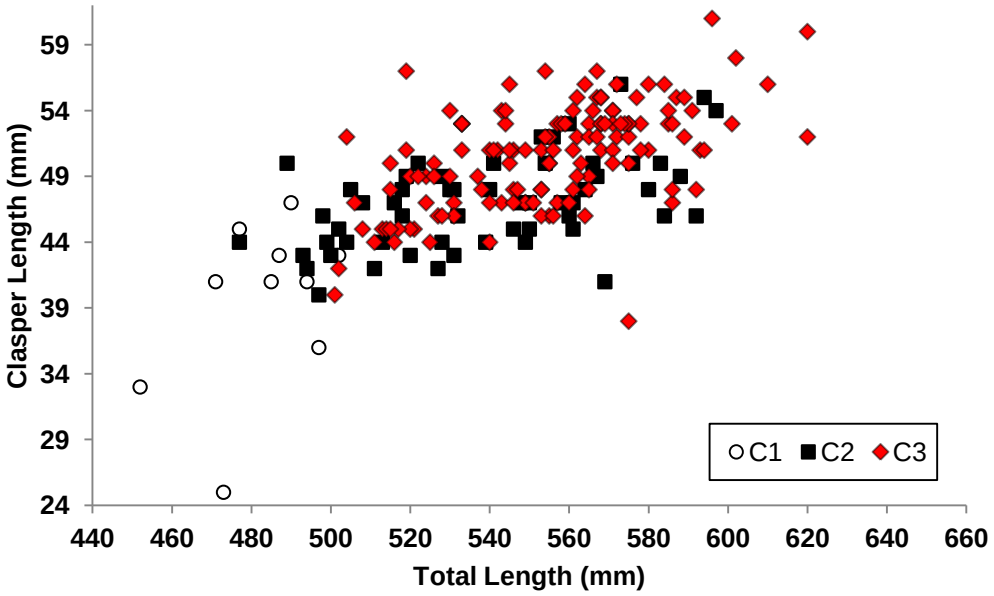


Figure 7. Relationship between clasper length and stretched total length in male *S. canicula* off northern Portugal.

Females

All female *S. canicula* had a single functional ovary placed on the right side of the abdominal cavity, and had two functional uteri. Macroscopic examination of the ovaries showed a progression from narrow, membranous structures with little vascularisation and bearing small non-yolked follicles in the undeveloped stage (O1; Table 3), to an intermediate stage forming a pouch-like structure of increased size and bearing larger follicles, both yolked and non-yolked (O2; Table 3). Well-developed ovaries were largest and harboured several large yolked follicles (O3; Table 3). Every stage of ovary development was observed in a wide range of body sizes, and gonad weight showed little association with TL (Figure 8). On the other hand, large yolked follicles were found only in females larger than 533 mm TL, whilst females smaller than 479 mm TL only had small non-yolked follicles (Figure 9). In addition, bigger LFD values were found in heavier ovaries (Figure 10).

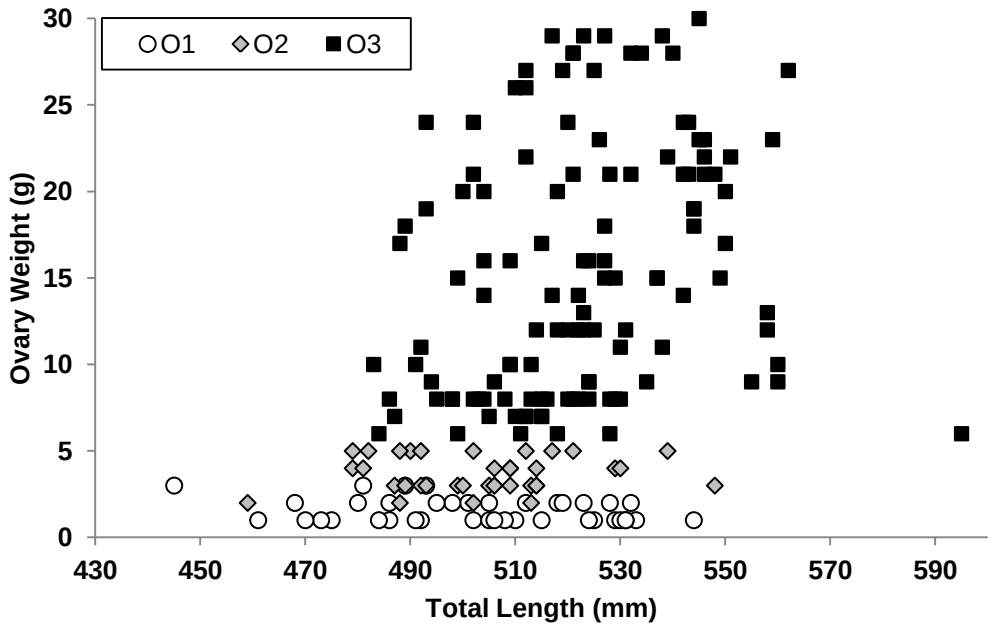


Figure 8. Relationship between ovary weight and total length in female *S. canicula* off northern Portugal.

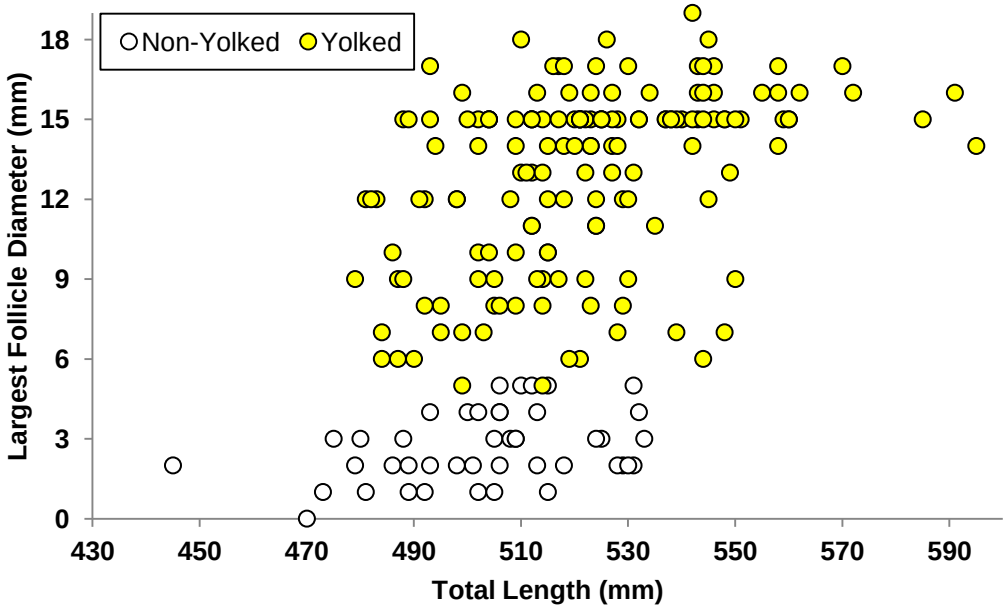


Figure 9. Relationship between largest follicle diameter and total length for female *S. canicula* off northern Portugal.

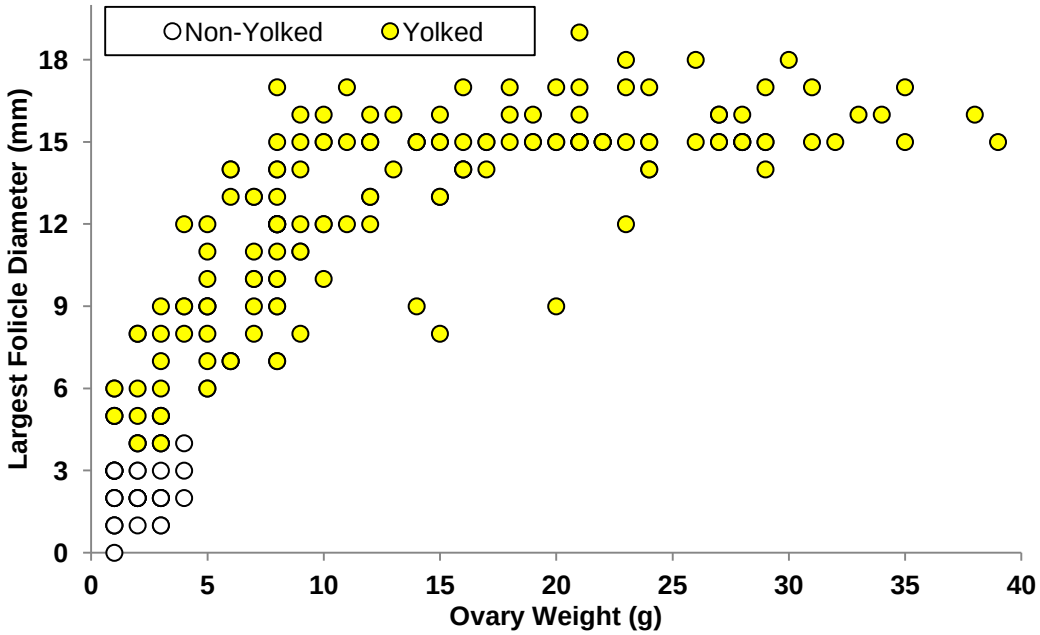


Figure 10. Relationship between largest follicle diameter and ovary weight for female *S. canicula* off northern Portugal.

Three different stages of oviducal gland development were also observed. In the first stage, undeveloped oviducal glands were indistinguishable from the uteri (OG1; Table 3) but in the intermediate stage they could always be distinguished and began to enlarge with growth of the individuals (OG2; Table 3). Well-developed oviducal glands were wider than the uteri and yellowish in colour (OG3; Table 3). Oviducal glands with developing egg cases inside were observed in some females, and were always larger than 22 mm in width (Figure 11).

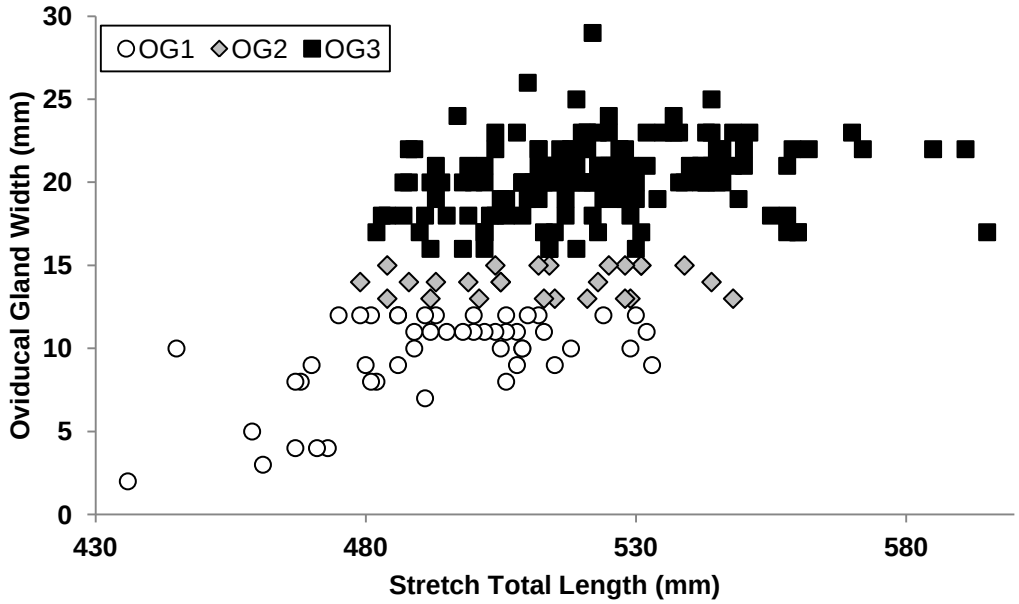


Figure 11. Relationship between oviducal gland width and total length for female *S. canicula* off northern Portugal.

Macroscopic examinations of the uteri showed three distinct development stages. Undeveloped uteri appeared as narrow tubular structures (U1; Table 3), while fully developed uteri were wide tubular structures with thickened walls (U2; Table 3). Some uteri were further enlarged due to the presence of egg capsules (U3; Table 3). Uterine width increased with body size up to around 10-13 mm, with a pronounced step in widening due to the presence of egg capsules in the uteri (Figure 12). Usually, egg-carrying females had egg capsules in both uteri, ranging from 40 to 56 mm in length. There are two exceptions to the above described pattern: one female had wide uterine walls but no egg cases (likely the result of recent egg release), while another had only one egg case in the right uterus while the left uterus was empty (only the left uterus was measured).

One individual of 586 mm TL was classified as “undetermined” since it had no claspers, no oviducts and the ovary-like structure was a membranous sac with no visible follicles.

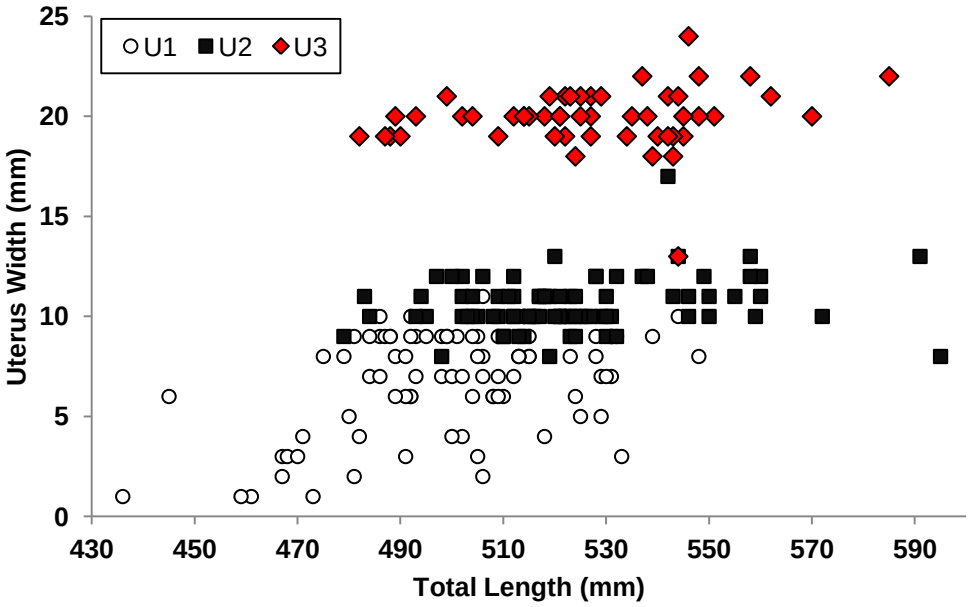


Figure 12. Relationship between uterus width and total length for female *S. canicula* off northern Portugal.

Table 3. Indices used to characterize the development stages of reproductive organs of *S. canicula* (modified from Walker, 2005).

MALE		
Claspers		
	C1	No calcification and whitish
	C2	Calcified, reddish and w/ flexible hook
	C3	Calcified, reddish w/ flexible hook and sperm present in claspers' tip
Testis		
	T1	Flat and narrow
	T2	Beginning to enlarge and coil
	T3	Enlarged, extremely coiled and vascularised
Seminal Vesicle		
	V1	Flat and narrow
	V2	Coiled and enlarged
FEMALE		
Ovary		
	O1	Membranous; largest follicle not yolked and < 5mm in diameter
	O2	Becoming a pouch-like structure, largest follicle yolked and < 8mm in diameter
	O3	Large, pouch-like structure; largest follicle yolked and > 8mm in diameter
Oviducal Gland		
	OG1	Indistinct < 13mm width
	OG2	Distinctive and enlarging < 16mm width
	OG3	Enlarged and yellowish > 16mm width
Uterus		
	U1	Enlarging tubular structure < 8mm width
	U2	Enlarged and thickened structure > 8mm width
	U3	Enlarged, egg cases present

Assessment of sexual maturity

Based on the developmental stages of the reproductive organs described above, a sexual maturity scale was constructed for males and females of *S. canicula* from off the northern Portuguese coast. Three maturity stages were distinguished for each sex: juvenile, maturing and adult. A detailed description as well as representative photographs of each maturity stage are presented in Table 4 and 5, and shown in Figures 13 to 18, respectively.

Table 4. Description of the morphological characters defining the different maturity stages for male *S. canicula* from off northern Portugal.

STAGE	MATURITY	DESCRIPTION
IMMATURE	Juvenile	Short, flexible claspers; absence of sperm; small, straight testes, not vascularised. Figure 13.
	Maturing	Partly calcified claspers, sometimes with sperm present; testes becoming enlarged and vascularised, partly coiled. Figure 14.
MATURE	Adult	Calcified claspers with rotating hook, usually with sperm present; testes large, highly vascularised and coiled. Figure 15.

Table 5. Description of the morphological characters defining the different maturity stages for female *S. canicula* from off northern Portugal.

STAGE	MATURITY	DESCRIPTION
IMMATURE	Juvenile	Small, narrow ovary with non-yolked follicles; narrow uteri with indistinguishable oviducal glands (< 10 mm in width). Figure 16.
	Maturing	Enlarged ovary with small yolked and non-yolked follicles (< 8 mm in diameter); uteri wider with thicker walls and distinguishable oviducal glands. Figure 17.
MATURE	Ripe	Large ovary occupying great part of the abdominal cavity, with large yolked follicles (8 to 19 mm in diameter); uteri wider and conspicuous, but with no egg cases; oviducal glands enlarged and yellowish.
	Egg-laying	Large ovary occupying great part of the abdominal cavity, with large yolked follicles (8 to 19 mm in diameter); uteri expanded with egg cases present in the uteri or uteri wide but flaccid from recent egg release. Figure 18.

Figure 13. Juvenile Male

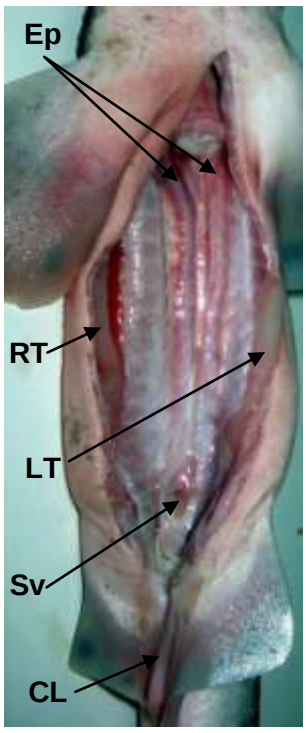


Figure 14. Maturing Male

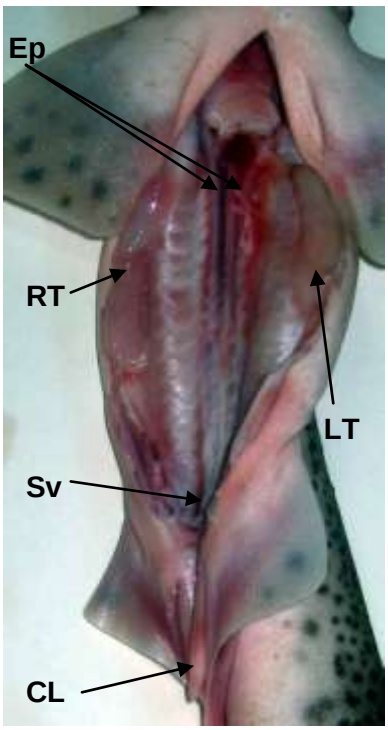
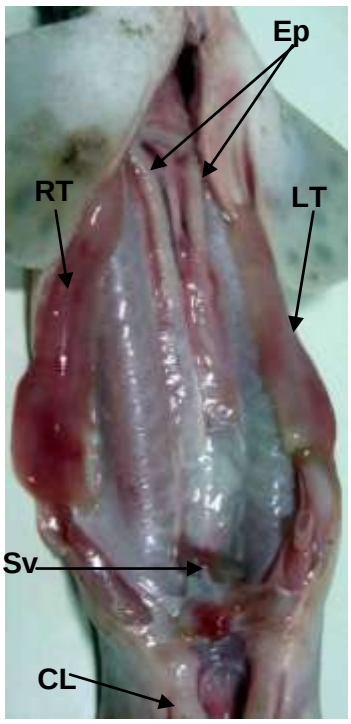


Figure 15. Adult Male



Ep - epididymis; **RT** - right testis; **LT** - left testis; **Sv** - seminal vesicle; **CL** - claspers

Figure 16. Juvenile Female

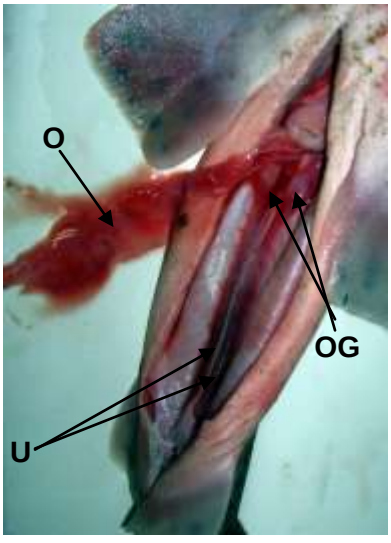


Figure 17. Maturing Female

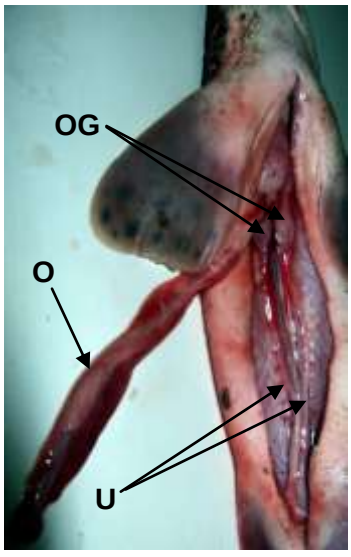
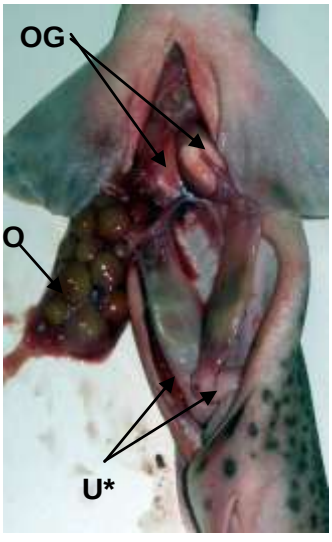


Figure 18. Adult Female



OG - oviducal glands; **O** - right ovary; **U** - uteri; **U*** - uteri with egg capsules

Maturity Ogives

A total of 13 males were classified as juveniles, 61 as maturing and 126 as adults. Males smaller than 489 mm were juveniles, while those larger than 592 mm were adults. The largest juvenile male observed measured 592 mm TL while, the smallest adult male measured 489 mm TL. The maturity ogive for males estimated the L_{50} at maturity at 532 mm TL (Figure 19b).

A total of 21 females were classified as juveniles, 79 as maturing and 120 as adults. Females smaller than 482 mm were juveniles and those larger than 547 mm were adults. The largest juvenile female observed measured 548 mm TL; on the other hand, the smallest adult female measured 482 mm TL. The maturity ogives for females estimated the L_{50} at maturity at 506 mm TL (Figure 20b). Furthermore, the L_{50} at maternity was estimated at 564 mm TL (Figure 20c).

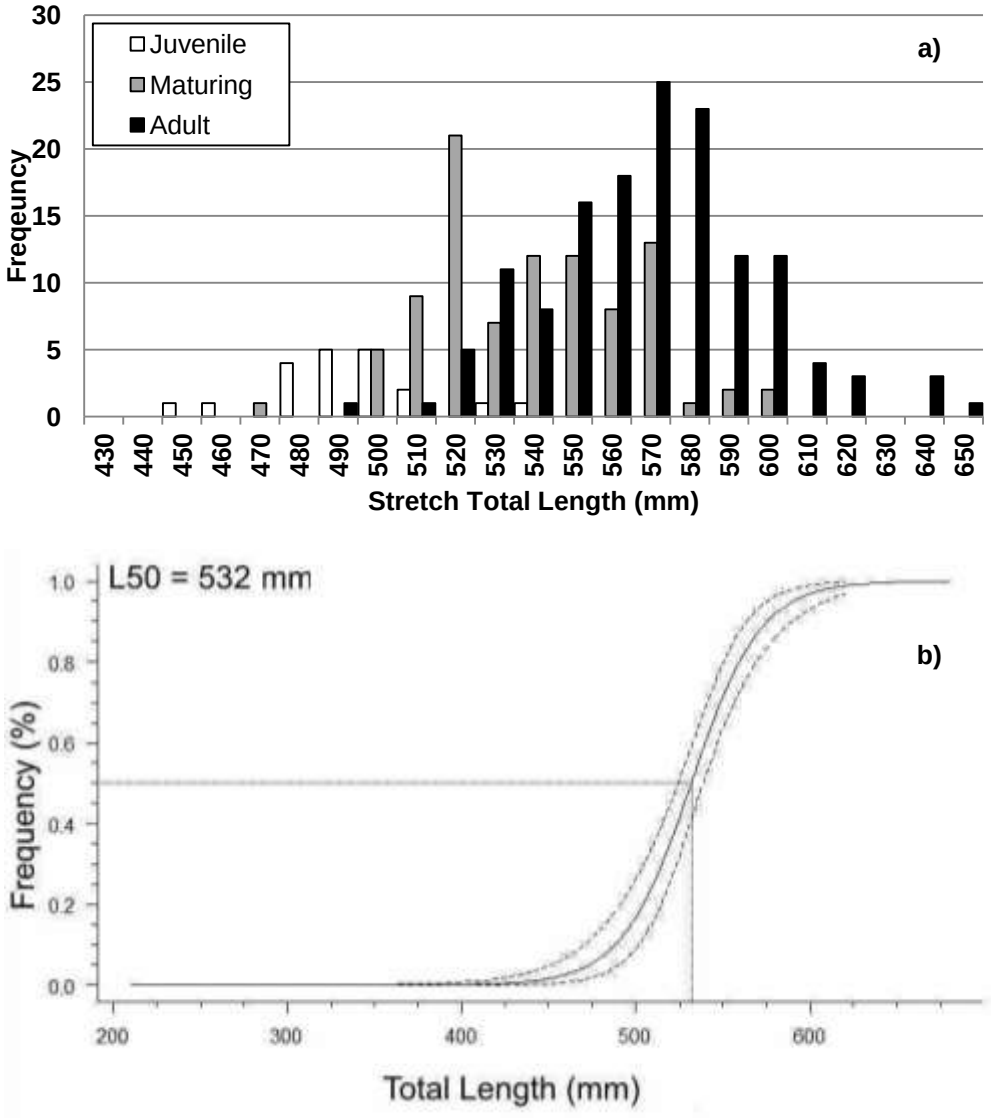


Figure 19. Length frequency distribution of maturity stages (a) and maturity ogive with respective L_{50} at maturity value (b) for *S. canicula* males from off northern Portugal.

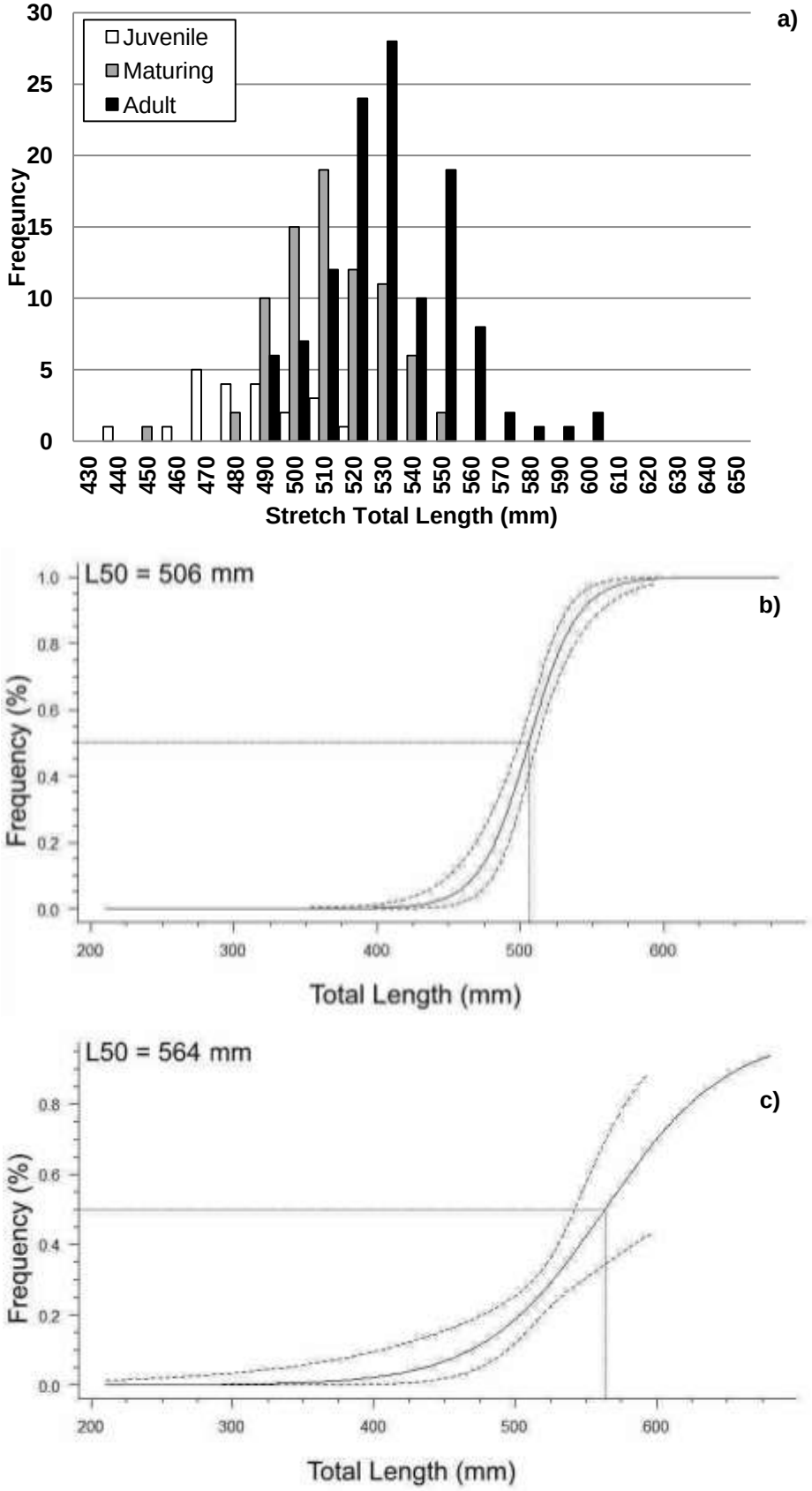


Figure 20. Length frequency distribution of maturity stages (a), maturity ogive with respective L_{50} at maturity value (b) and L_{50} at maternity ogive (c) for *S. canicula* females from off northern Portugal.

Hepatosomatic and Gonadosomatic Indices

HSI reached larger values in females than in males (females ranged from 2.84 to 15.88, and males ranged from 2.01 to 12.40; Figure 21), and showed significant differences with maturity stage in both sexes (males: Kruskal-Wallis X^2 = 8.676, p = 0.013; females: Kruskal-Wallis X^2 = 16.057, p = 3.26×10^{-4}). In males, maturing individuals showed the lowest HSI values, which were significantly lower than those of adult individuals ($p < 0.05$). In contrast, maturing females showed significantly larger HSI values than both juveniles and adults ($p < 0.05$) (Figure 21b). HSI reached larger values in maturing and adult females than in males of the same maturity stages (maturing: p-value < 2.2×10^{-16}; adult: p-value < 2.2×10^{-16}), but showed no significant differences between juvenile individuals of different sexes (p-value = 0.132) (Figure 21).

GSI also reached larger values in female *S. canicula* compared to males (females ranged from 0.17 to 5.98, and males ranged from 0.51 to 4.25; Figure 22), and showed significant differences with maturity stage regardless of gender (males: Kruskal-Wallis X^2 = 44.352, p = 2.34×10^{-10}; females: Kruskal-Wallis X^2 = 130.979, p = 2.2×10^{-16}). In male *S. canicula*, GSI values were statistically different among all maturity stages ($p < 0.05$), with adults and juveniles attaining the largest and lowest GSI values, respectively. In the case of female *S. canicula*, only the adults had significantly larger GSI values compared to juvenile and maturing females ($p < 0.05$) (Figure 22). GSI reached larger values in juvenile and maturing males compared to females of the same maturity stages (juvenile: p-value = 1.67×10^{-3}; maturing: p-value < 2.2×10^{-16}), but showed no significant differences between adult individuals of different sexes (p-value = 0.225).

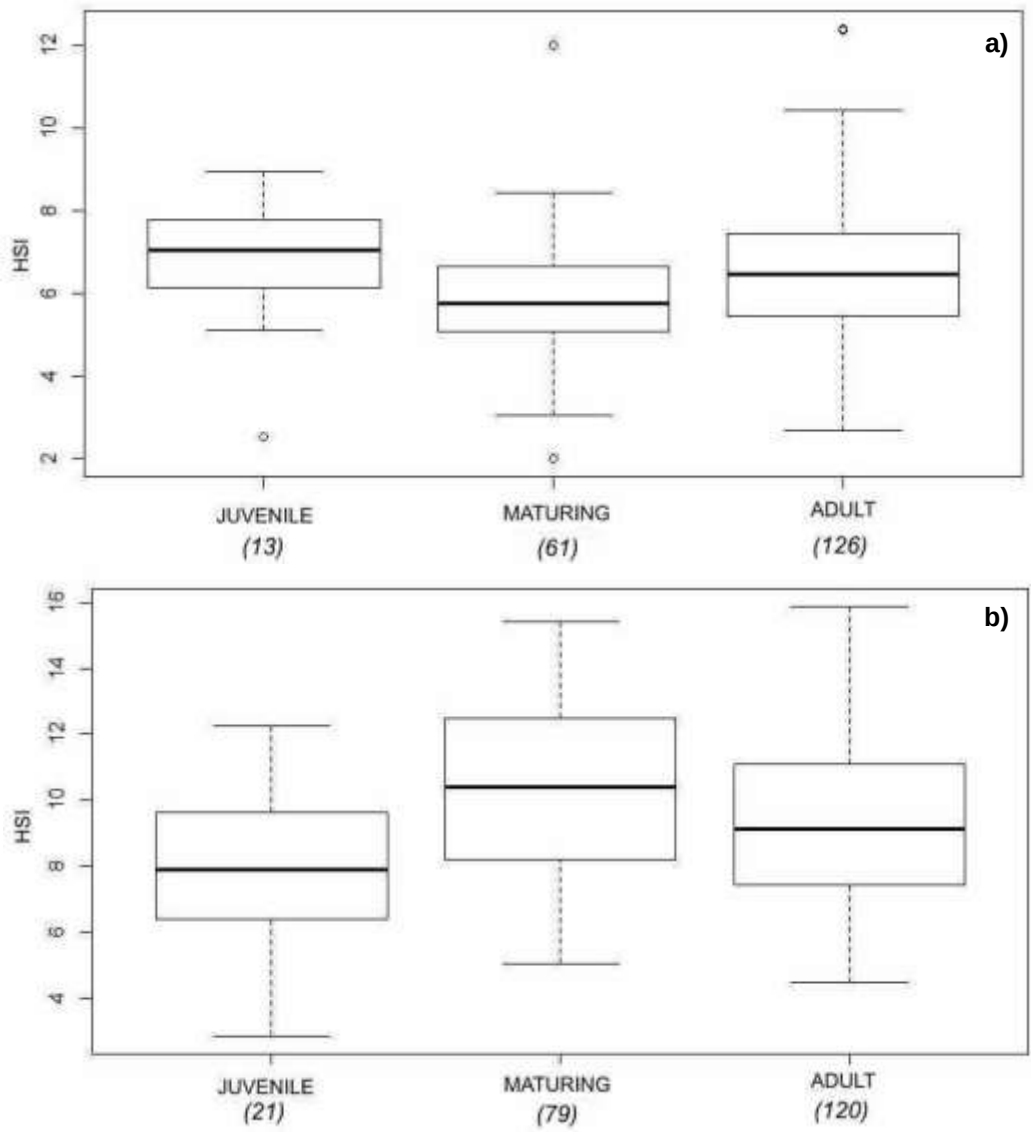


Figure 21. Hepatosomatic indices (HSI) for male (a) and female (b) *S. canicula* according to maturity stage**.** Sample size in parentheses.

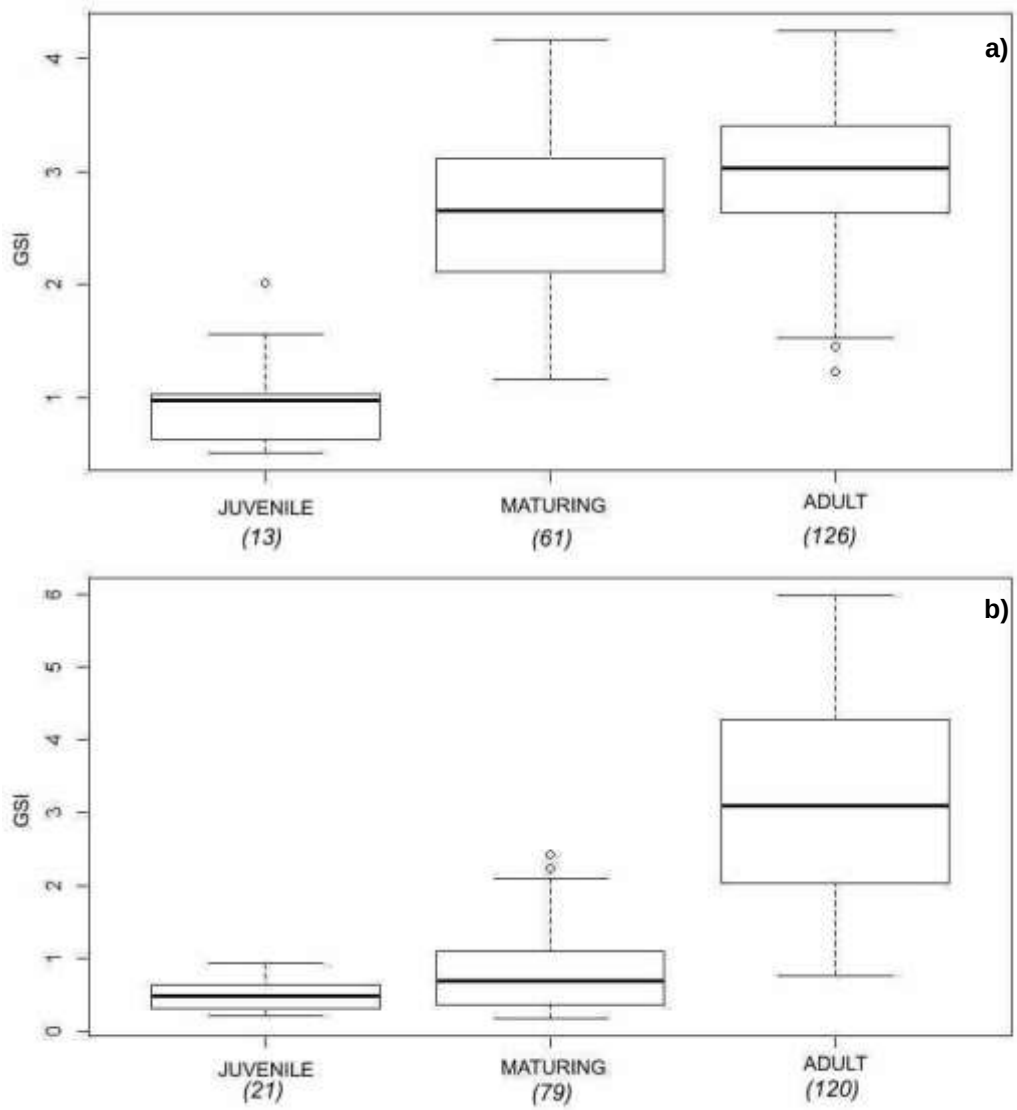


Figure 22. Gonadosomatic indices (GSI) for male (a) and female (b) *S. canicula* according to maturity stage**.** Sample size in parentheses.

Description of the reproductive cycle

Adult male *S. canicula* with sperm present at the claspers' tip were found all year round, although they occurred in much lower frequency in the Summer compared to the remaining seasons (Figure 23). In addition, most adult females had sperm present in their uteri regardless of season, although in lower frequency during Summer (Figure 24).

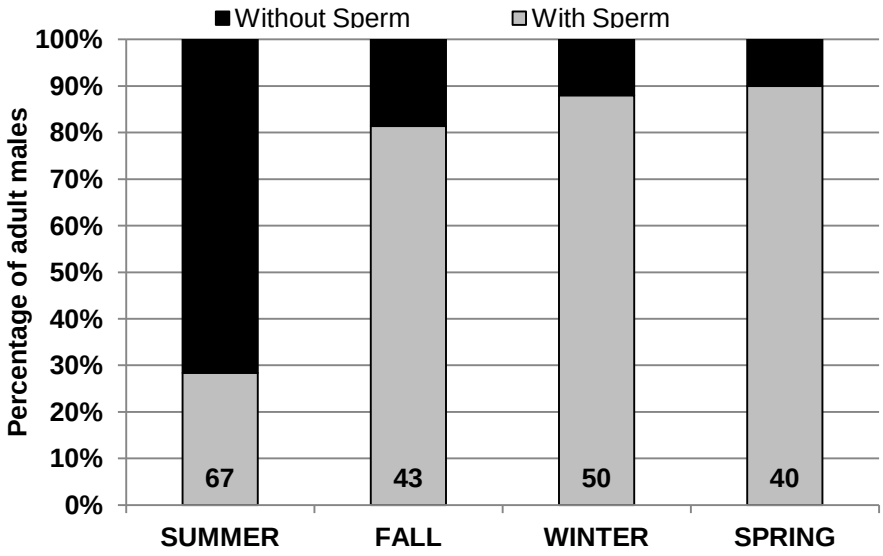


Figure 23. Percentage of adult male *S. canicula* with sperm present on their claspers' tips throughout the year. Sample size at the bottom of each bar.

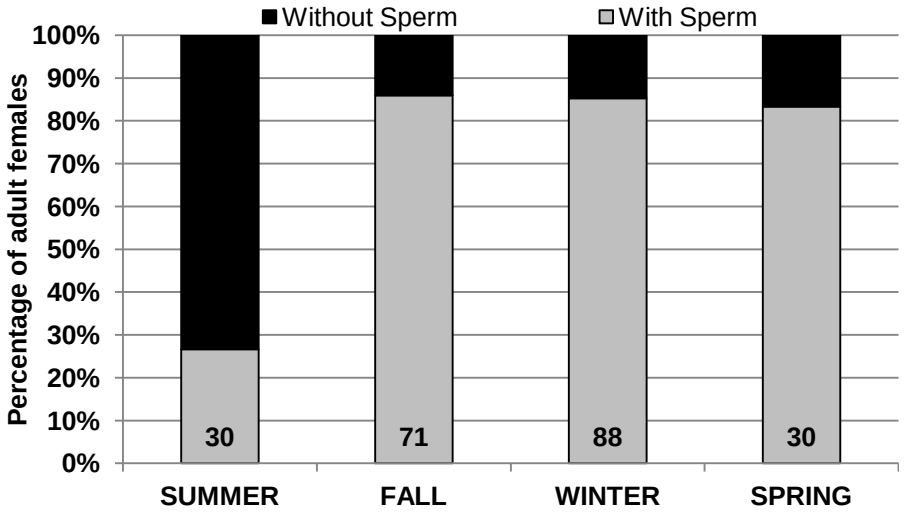


Figure 24. Percentage of adult female *S. canicula* with sperm present in their uteri throughout the year. Sample size at the bottom of each bar.

Adult females showed maximum LFD between the Fall and the Spring, while only LFD smaller than 11 mm were found in the Summer (Figure 25).

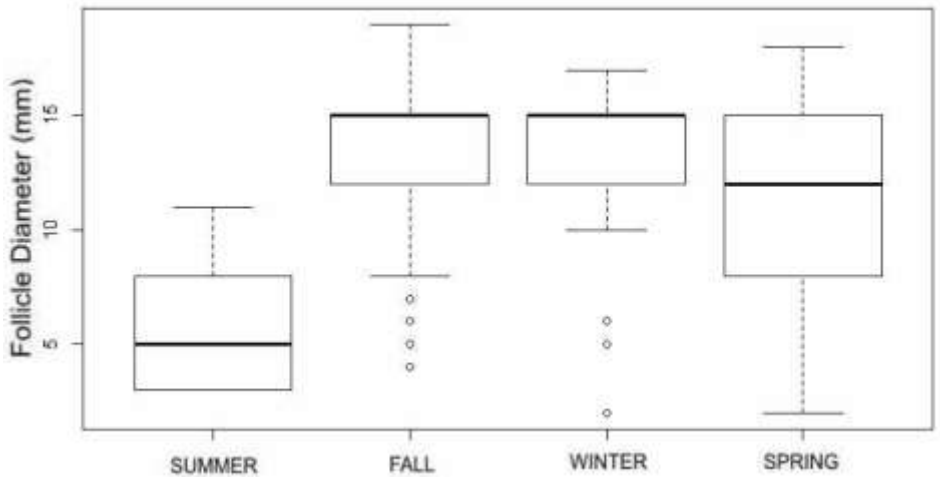


Figure 25. Largest follicle diameter in female *S. canicula* individuals with U3 (enlarged and >8 mm width) throughout the year. Bold horizontal line: median, white box: 50% of the data values, vertical lines: minimum and maximum, white dots: outlines.

On the other hand, adult females carrying egg capsules in their uteri were found year round, although they were in lower frequency during Winter (i.e. 25%) than during Fall and Spring (Figure 26). Only one adult female was sampled during the Summer, which carried egg cases in the uteri.

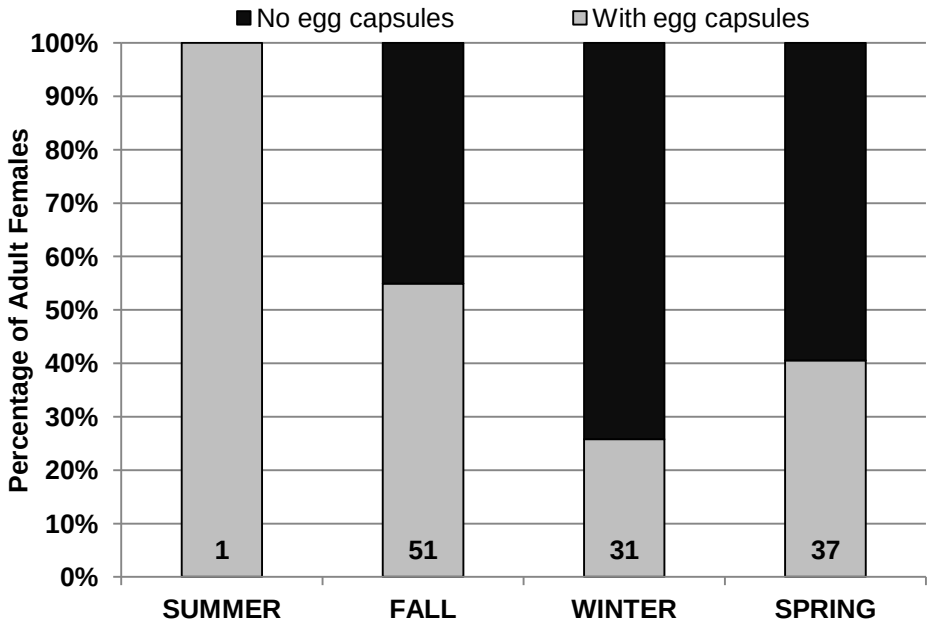


Figure 26. Percentage of adult female *S. canicula* carrying egg capsules on their uteri throughout the year. Sample size at the bottom of each bar.

Geographic variability in life history parameters

The maximum TL values for the small-spotted catshark from different geographical locations throughout its distribution in the eastern North Atlantic and Mediterranean Sea were positively correlated with latitude (R^2 = 0.802, p = 0.00028)

(Figure 27a). Likewise, the L_{50} at maturity in females was also positively correlated with latitude (R^2 = 0.595, p = 0.0054) (Figure 27b).

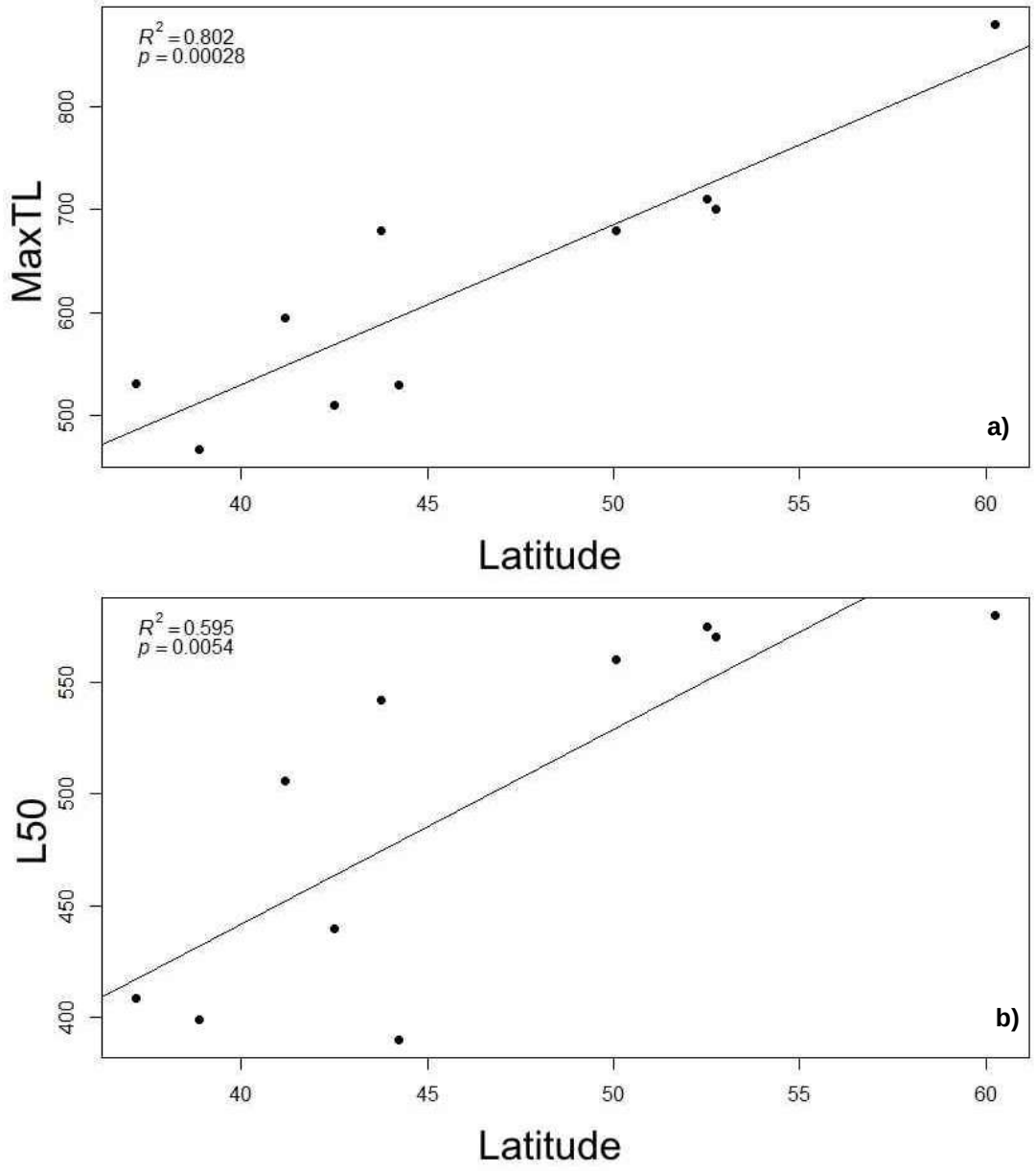


Figure 27. Relationship between latitude and maximum TL (a) and size at which 50% of the females attain sexual maturity (L_{50}) (b) for *S. canicula* from different locations along its distribution in the eastern North Atlantic Ocean and Mediterranean Sea.

When considering locations in the eastern North Atlantic only, maximum TL and L_{50} at maturity in female *S. canicula* were also strongly correlated with latitude (R^2 = 0.775, p = 0.013; and R^2 = 0.774, p = 0.013, respectively), showing increased values northwards (Figure 28).

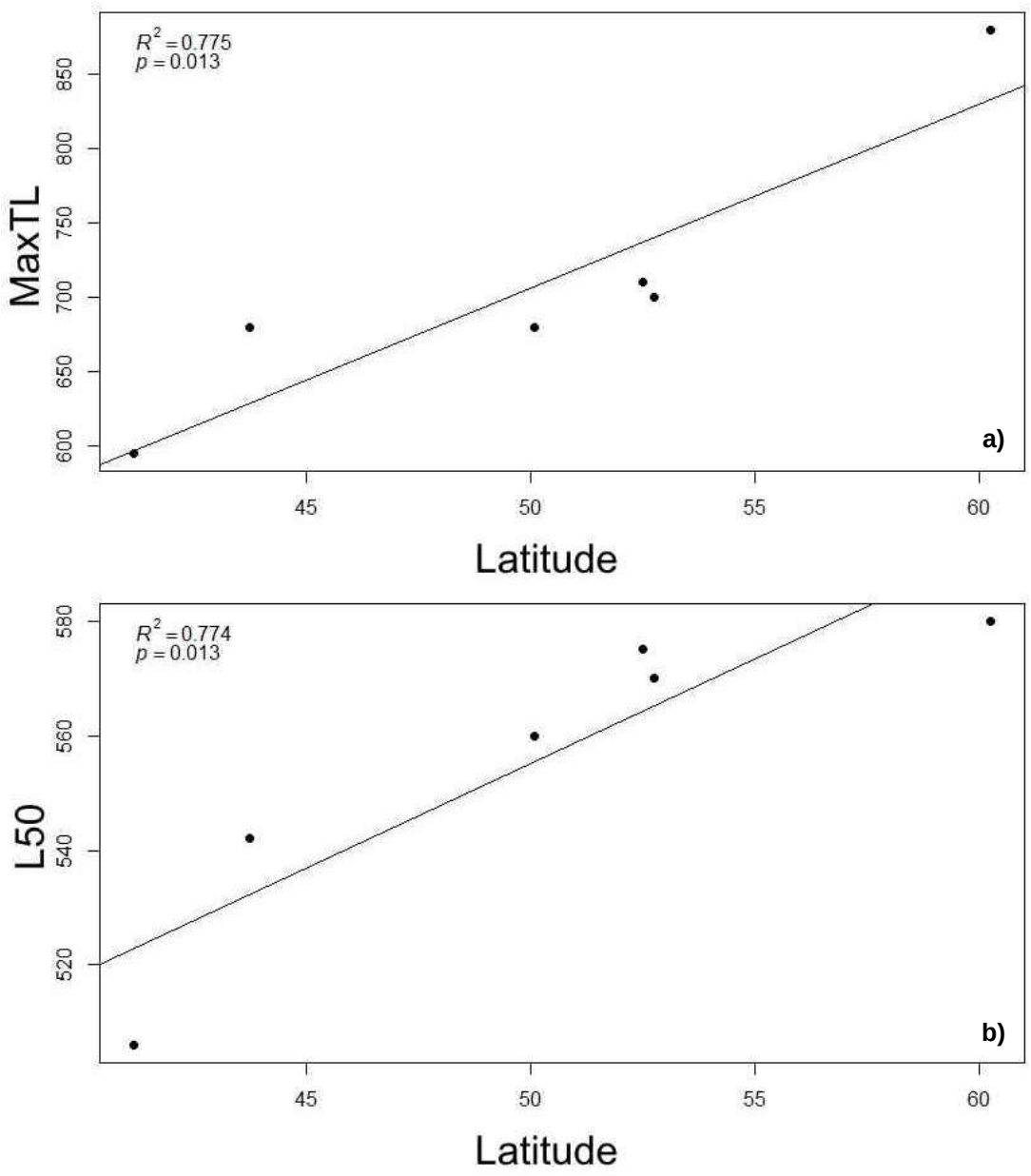


Figure 28. Relationship between latitude and maximum TL (a) and and size at which 50% of the females attain sexual maturity (L_{50}) (b) for *S. canicula* from different locations along its distribution in the eastern North Atlantic Ocean.

In the Mediterranean Sea, maximum TL values for small-spotted catsharks and L_{50} at maturity for females vary according to region, but no correlation with longitude was observed ($R^2 = 0.289$, $p = 0.77$; and $R^2 = 0.221$, $p = 0.31$).

Discussion

The main goal of the present work was to describe the reproductive biology of the small-spotted catshark, *S. canicula*, off the northern Portuguese coast, and to estimate relevant life history parameters, like size at maturity and maternity. For this purpose, sexual maturation was studied in great detail and a sexual maturity scale was developed for the species.

Sexual maturation is a continuous process in which several physiological changes occur and morphological changes happen gradually. Therefore, body size alone may be a poor indicator of sexual maturity (Capapé *et al.*, 2008b), and several morphological parameters should be taken into account when defining sexual maturity stages. When evaluating sexual maturity in male *S. canicula*, studying the development of the testes, seminal vesicle, epididymis and claspers is crucial. For instance, testes weight increased with body size although small individuals may also exhibit large testes, e.g. males with T2 and T3 have broadly overlapping TL. However, an increase in testes weight associated with increased vascularization and coiling is diagnostic of sexually mature males. The presence of sperm in the seminal vesicle also indicates that a male is sexually mature and ready to copulate (Capapé *et al.*, 2014).

Clasper length also tends to increase with increasing body size and its development and level of calcification have been used recurrently to assess sexual maturity in male *S. canicula* (Bendiab *et al.*, 2012; Capapé *et al.*, 2014, 2008; Rodríguez-Cabello *et al.*, 2007). However, clasper calcification alone may also be limited in sorting between maturing and adult male *S. canicula* as the progression from a partly calcified clasper to a fully calcified one is gradual. Additionally, sperm was detected in males with either fully or partly calcified claspers, indicating that males in both conditions were sexually mature. On the other hand, absence of sperm in the claspers does not necessarily indicate that sexual maturity is not complete since sample handling and storage prior to processing may remove any evidence of sperm around the claspers. The presence of sperm at the claspers' tip and a rotating hook are good complementary characters to confirm sexual maturation in males. Hook rotation in elasmobranchs allows males to hold females during copulation in order to copulate effectively (Leigh-Sharpe, 1926). Thus, the presence of a rotating hook is a good indicator of sexual maturation in male *S. canicula*.

Sexual maturity in female *S. canicula* is easier to ascertain than in males. One clearly diagnostic character is the presence of egg cases in the uteri. However, when this condition is not observed, the state and morphological development of the different reproductive organs must be considered (Carrier *et al.*, 2004). Oviducal gland development is intimately associated with sexual maturation since this organ is responsible for egg case formation and maturation. OGW increases with increasing body size until a plateau is reached that usually coincides with the phase of egg case development. Uteri enlargement and thickening of the uterine walls are good indicators of sexual maturity, since the uteri must be ready to receive the eggs.

Regarding the reproductive cycle of *S. canicula* in northern Portuguese coastal waters, it appears that follicle development occurs throughout the year but follicle maturation occurs mainly outside the Summer season, while mating may occur throughout the year but also with lower intensity during the Summer. This findings are in concordance with those of Ellis and Shackley (1997) and Ivory *et al.* (2005), who also reported an interruption in egg laying during the Summer. Fewer males were found with sperm present at the claspers' tip and fewer females had sperm inside their uteri during that season. Additionally, females carrying egg capsules in their uteri were found all year round, suggesting that egg release occurs throughout the year. Such findings are in agreement with those from other regions of the species' distribution (Gulf of Lion: Capapé *et al.* (1991); Leloup and Olivereau (1951); Aegean Sea: Kousteni *et al.* (2010); English Channel: Leloup and Olivereau (1951) and by Compagno (1984). However, the results presented here must be considered with some caution. Sampling and processing of the individuals started during the Summer and the lack of experience by the observer may have led to inaccurate records of sperm presence at the claspers or inside the uteri.

GSI values varied according to sexual maturity since gonad weight tends to increase with body size and sexual maturation. Males revealed greater GSI values than females during the juvenile and maturing stages, while adult males and females GSI did not reveal any significant difference. Most likely, this is a result of the rapid enlargement of the ovary weight, from maturing to adult females, due to the maturation of the follicles. While males testes enlargement is a more gradual process, until maximum weight is attained in the adult stage. The variation in GSI with sexual maturity stage is well described in *S. canicula*, and the results presented here are in line with previous findings (e.g. Capapé *et al.*, 2008; Kousteni *et al.*, 2010). On the other hand, HSI values of maturing and adult females are higher than those of males,

which may be due to increased energy expenditure by females associated with the reproductive cycle. Indeed, the increase in HSI during the follicle maturation in the maturity stage is expected, since vitellogenin is produced in the females' liver. Hence, the variation in HSI values for females during different maturity stages is expected due to this vitellogenesis cycle (Craik, 1978), and have been previously documented in *S. canicula* (Kousteni *et al.*, 2010).

The newly estimated life history traits for *S. canicula* off northern Portugal fit with the described latitudinal variation pattern for the eastern North Atlantic Ocean and Mediterranean Sea. The results showed that small-spotted catsharks off the northern Portuguese coast reach a maximum TL of 644 mm, a value smaller than those recorded at greater latitudes along the eastern North Atlantic range but larger than those recorded within the Mediterranean Sea (see Table 1 for details).

The contrasting signal between eastern North Atlantic Ocean and Mediterranean Sea collections of *S. canicula* in life history may be due to differential responses to environmental factors related with latitude. This pattern conforms with Bergman's ecological generalization, that animals from higher latitudes or colder regions attain larger body sizes as a coping mechanism (Bergmann, 1848). This phenomena of size-latitude, or size-latitude driven factors, relationship has also been reported among marine species (Berke et al., 2013; Timofeev, 2001). Geographic variation in life history traits have been reported for other elasmobranchs, such as the bonnethead shark, *Sphyrna tiburo* (Carlson and Parsons, 1997; Lombardi-Carlson *et al.*, 2003; Parsons, 1993) and the starspotted dogfish, *Mustelus manazo* (Yamaguchi *et al.*, 2000). Carlson and Parsons (1997) observed that body size of the *S. tiburo* increased with increasing latitude. This variation could be due to individuals being locally adapted to distinct seasonal temperature and photoperiod conditions and thus have different life history strategies (Parsons, 1993). Yamaguchi *et al.* (2000) also noticed that the maturity of the *M. manazo* from Japan and Taiwan occurred at a larger size and older age in individuals from northern populations inhabiting lower water temperatures. The pattern observed in *S. canicula* is in accordance with the above findings, since eastern North Atlantic Ocean *S. canicula* inhabiting colder waters attain larger sizes and sexual maturity at larger body sizes compared to those in the Mediterranean inhabiting warmer waters.

On the other hand, the Mediterranean Sea is a more heterogeneous environment with varying water temperature, maximum depth and currents, as well as variation in

food availability (Lejeusne *et al.*, 2010a). Probably, the differences in maximum body size and L_{50} at maturity of *S. canicula* collections in the Mediterranean Sea are related to specific individual responses to different local environmental conditions. For instance, the observed spatial variability in life history parameters may be due to varying food availability whereby individuals may attain larger body sizes in more productive areas (Caddy *et al.*, 1995; García-Charton *et al.*, 2004). Thomason *et al.* (1996) shows how environmental factors, like temperature and photoperiod, influence growth in *S. canicula* embryos.

Management implications

Overfishing has been described as the primary cause for recent declines in elasmobranchs populations (Baum *et al.*, 2003; Baum and Myers, 2004; Carrier *et al.*, 2004). By-catch species of little economic interest can be driven to local depletion by fisheries targeting more productive species (Musick *et al.*, 2000). Moreover, slow-growing and late-maturing species tend to decrease in abundance when harvested at high rates (Adams, 1980; Jennings *et al.*, 1998). The above two conditions apply to the small-spotted catshark and this is of concern not only in Portuguese waters but along the entire species' range. In fact, declines in abundance in some catshark species (*Scyliorhinus* spp.) have already been described in the Adriatic Sea, where the current catch rates are only 2.4 - 10% of those reported in the 1940s (Barausse *et al.*, 2014).

Knowledge on the life history and stock structure of exploited species is of great importance for adequate fisheries management plans. Understanding the species biology, ecology and population structure is essential to prevent the collapse of stocks, as previously described in the northern cod *Gadus morhua* (Petitgas *et al.*, 2010; Walters and Maguire, 1996). The existence of multiple regional stocks can be hypothesized for the *S. canicula* based on the regional variability in life history traits, in addition to female philopatric behaviour and low dispersal rates observed in the species (Sims *et al.*, 2001). In fact, due to the particular life history traits of *S. canicula* in the Cantabrian Sea, Rodrıguez-Cabello *et al.* (2004) proposed that individuals from this region should be treated as a separate stock unit for fisheries management purposes. Further support for the existence of multiple regional stocks was provided by Barbieri *et al.* (2014) who found evidence of genetic differences between Mediterranean and eastern North Atlantic locations and a genetic population structuring of *S. canicula* within the Mediterranean Sea.

Fisheries management plans should take the regional variability in life history traits of *S. canicula* into account when estimating stock productivity and sustainable exploitation rates. Furthermore, the particular life history strategies of *S. canicula* such as low dispersal (Sims *et al*., 2001), female philopatric behaviour (Rodríguez-Cabello *et al.*, 2004) and presence of multiple isolated stocks, particularly in the Mediterranean (Barbieri *et al.* 2014; this study, Chapter 3), means that stock recovery based on immigration by neighbouring localities occurs over long time periods that are not compatible to the sustainability of fisheries operations. This is of particular relevance since regional declines in abundance of *S. canicula* have already been reported in the Adriatic Sea (Barausse *et al.*, 2014).

Limitations and Future work

Although the sampling method used here, i.e. collecting samples from commercial fisheries landings, was the most feasible given the time and resources available, it carried some limitations. The main limitation was the lack of small-sized, juvenile individuals in the monthly samples due to gear selectivity retaining individuals larger than the mesh size. By having a well-balanced sample size (i.e. including all sizes) every month throughout the year, one could better describe the reproductive cycle and egg-laying season of *S. canicula* from off the northern Portuguese shore. The ideal solution would be to participate in research cruises specifically targeting the species throughout the year, but in the absence of this option, one may increase sample size and coverage by visiting other closely located fishing ports. Nevertheless, knowing that no adult individuals below 483 mm were found, one could predict that every *S. canicula* specimen below that value is sexually immature, for the North Portuguese coast.

Another important sampling limitation that may be corrected in future work, is the precision and expertise of the observer. The initial lack of experience of the observer in assessing the correct development state of the internal reproductive organs resulted in some of the sampled individuals not being taken into account in the data analysis. Thus, it is suggested for future work that a training period should be conducted to ensure the consistency and accuracy of the data throughout the whole study.

Assessing age or size at sexual maturity is an important parameter used to identify separate fishing stocks (Begg *et al.*, 1999). Length-at-maturity indices for chondrichthyans should be used carefully, since the corresponding values vary significantly according to the criteria adopted for defining maturity (Braccini *et al.*, 2006). Because different criteria may be used among different authors to describe the sexual maturity of a species, any geographical pattern of life history variability may be incorrectly interpreted (Begg *et al.*, 1999). Hence, a common method to assign maturity should be accepted by all researchers and equally applied. Such methodology, when applied uniformly in different studies from geographically distinct areas, will help clarify if there indeed differences in life history strategies and identify the stock structure of any given species (Ivory *et al.*, 2005; Rodríguez-Cabello *et al.*, 2004). In a way, molecular genetics is a good example of a standardized methodology that is currently used across different locations in order to identify different stocks and could be explored in more depth in the future (Barbieri *et al.*, 2014; Carvalho and Hauser, 1995).

Analysing more locations within the Mediterranean Sea would also help to better understand the geographical pattern observed in life history traits variation in this region. Future work in the Mediterranean Sea should focus in studying not only the case of *S. canicula* but also other elasmobranchs. There is a lack of consistent and reliable data describing life history strategies and behaviour of sharks in the Mediterranean, a region of major importance since strong evidence indicates regional declines in elasmobranchs abundance have already occurred (Barausse *et al.*, 2014).

Another interesting research question is to isolate putative environmental variables that may influence the life history traits in *S. canicula.* Specifically, future studies could objectively test which environmental variables (e.g. water temperature, depth of occurrence, primary productivity) may be correlated with the observed regional variation in life history traits of the small-spotted catshark in the eastern North Atlantic and the Mediterranean. Here, latitude and longitude were used as proxies for changes in multiple environmental variables; a detailed approach as suggested above would be far more informative and allow the identification of key variables affecting the life history of *S. canicula* populations.

CHAPTER 3

POPULATION STRUCTURE OF *Scyliorhinus canicula* (Linnaeus, 1758) IN THE EASTERN NORTH ATLANTIC OCEAN AND MEDITERRANEAN SEA

Introduction

A population can be defined as "a group of individuals inhabiting close enough so that any member can reproduce with any other with virtually the same probability" (Waples and Gaggiotti, 2006). The study of life history trait variability may be useful in studying population structure, and a valuable asset to distinguish different population units (Rodríguez-Cabello *et al.*, 2004). However, life history traits can vary according to the time of the year and age of an individual (Carvalho and Hauser, 1995), limiting their usefulness in accurately identifying distinct populations. Molecular genetic markers are good complementary tools in studies of population structure, and can be used to unravel biological and ecological aspects of species that in other way would not be possible (Hedrick, 2001; Sunnucks, 2000).

Since the first published study of allozymes in Elasmobranchs by Smith (1986), several studies have used molecular markers to assess the genetic structure of Elasmobranchs and to better understand their population dynamics (Heist, 2004). Elasmobranchs´ population structure is usually influenced by their vagility as adults, since juvenile individuals tend to occupy their natal sites for a significant amount of time, before attaining older stages and move more actively (Frisk *et al.*, 2014). Hence, Elasmobranchs' population structure is often influenced by their philopatric behaviour during different life stages, and also by their vagility as adults (Musick *et al.*, 2004). In concordance, Schrey and Heist (2003) showed the lack of genetic structure on the shortfin mako, *Isurus oxyrinchus*, among the Atlantic, Pacific and Indic oceans, suggesting that the results were due to the great dispersal of both males and females and due to high levels of gene flow among regions. Moreover, Karl *et al.* (2011) documented for the bull shark, *Carcharhinus leucas*, which also has a high dispersal rate and long life span, some genetic differentiation among populations in the western

Atlantic ocean. The genetic differences detected at the mitochondrial level were not detected in the nuclear markers, suggesting that female philopatric behaviour may be causing this structuring. Another interesting case of population structure in a more fine scale is the one of the coastal blacktip shark, *Carcharhinus limbatus*, which show genetic differences between the Gulf of Mexico and the Atlantic coast of South Carolina. Where female philopatric behaviour and low dispersal rates seem to be driving the differentiation between the Gulf of Mexico and the coast of South Carolina (Keeney *et al.*, 2003).

The small-spotted catshark, *Scyliorhinus canicula* (Linnaeus, 1758) is a small coastal shark that is commercially exploited either as a target species or as by-catch of many fisheries along its range. Recently, Barausse *et al.* (2014) showed significant declines in catch rates of catsharks, *Scyliorhinus* spp., in the Adriatic Sea to 10.6% of the average 1940s levels. Despite this abrupt decline, there is limited knowledge of the species' population structure which compromises the the implementation of adequate stock management measures.

Differences in maximum size and L_{50} at maturity between eastern North Atlantic and Mediterranean *S. canicula* have been suggested as evidence of the existence of two subspecies (Compagno, 1984). Moreover, a morphometric study by (Muñoz-Chápuli *et al.*, 1984) stated that the differences between eastern North Atlantic and Mediterranean *S. canicula* individuals may underpin a genetic difference. Small-spotted catsharks exhibit limited dispersal (Rodríguez-Cabello *et al.*, 2004) and philopatric behaviour in female *S. canicula* has been suggested (Rodríguez-Cabello *et al.*, 1998), two factors that may contribute to population differentiation within the species range. Indeed, one previous study provided some evidence of strong genetic structuring of *S. canicula* within the Mediterranean Sea (Barbieri *et al.*, 2014).

The aim of this study is to assess the population structure of *S. canicula* in the eastern North Atlantic and Mediterranean Sea by means of mitochondrial DNA control region sequences and 12 nuclear microsatellite loci. The results may help define stocks and improve fisheries management plans throughout the species' range in the eastern North Atlantic and Mediterranean Sea.

Material and Methods

Tissue sampling

A total of 369 samples from *S. canicula* were collected from multiple locations along the eastern North Atlantic and Mediterranean Sea, from commercial fisheries landings and research surveys. Tissue samples were obtained from dorsal fins or muscle tissue and preserved in 96% ethanol at room temperature.

Table 6. Tissue sample collection of *S. canicula* from different areas of the eastern North Atlantic and Mediterranean Sea. TL Range, Total Length Range; F, Female(s); M, Males(s); N, Number of tissue samples; Sq, Sequences from (Gubili *et al.*, 2014).

Location	Abbreviation	Date	Capture Method	TL Range (mm)	Sex Ratio (F/F+M)	N	Sq
North eastern Atlantic							
North Sea	N. Sea	Fev 2011 - Dec 2013	Research vessel	261 – 695	0.67	*18*	*25*
Irish Sea	I. Sea	April 2014	Research vessel	378 – 691	0.5	*60*	-
British Islands	B. Isles	Aug 2007 – Jun 2008	-	250 – 692	0.53	-	*69*
Cantabrian Sea	Can. Sea	Apr 1997 - Oct 2010	Research vessel	480 – 670	0.5	*30*	-
North Portugal	N. PT	August 2013	Commercial fishery	436 – 644	0.31	*81*	*6*
South Portugal	S. PT	October 2013	Commercial fishery	398 – 583	0.53	*80*	*14*
Mediterranean							
Mallorca	Ma	December 2009	-	145 – 470	0.61	-	*40*
Gulf of Lion	G. Lion	-	Research vessel	399 – 499	0.46	*50*	-
Adriatic Sea	Adr. Sea	Nov 2011 - Jun 2013	Research vessel	325 – 505	0.50	*50*	*27*
Crete	Cr	May 2011	-	295 – 458	0.54	-	*29*

DNA extraction

Genomic DNA (gDNA) was extracted from each tissue sample using the EasySpin® Genomic DNA Tissue Kit (Citomed, Lisbon, Portugal), according to the manufacturer's instructions. Positive extractions were tested through gel electrophoresis of 2 µL of gDNA in an 0.8% agarose gel (w/v) stained with GelRed (0.175X) (Biotium, Inc., Hayward, CA, USA) and ran at ~300V for ~15 minutes in TBE 0.5X (Tris 89 mM, Boric Acid 89mM, EDTA 2 mM; pH 8.0). The gel was visualized under UV light on a Biorad Universal Hood II Quantity One 4.4.0. Extractions that showed strong bands were diluted in ultra-pure water to 1/2 or 1/5 in order to optimise

the DNA concentration and reduce the interaction of inhibitors during the subsequent polymerase chain reaction (PCR) amplification.

Mitochondrial DNA control region (CR) sequencing

Mitochondrial DNA control region (mtDNA CR) was amplified via PCR using the primers developed by (Gubili *et al.*, 2014). The sequence of the forward primer was ScyD1p was 5'- ATGACATGGCCCACATATCC - 3', and of the reverse primer was Scan2R was 5' – TTCTCTTCTCAAGACCGGGTA - 3'. The PCR for each sample was conducted in 10 µL total volume including: 5 µL of *Taq*™ PCR Master Mix (Qiagen), 3.6 µL of ultra-pure water (UPW), 0.4 µL of each primer at 10 µM and 0.6 µL of gDNA. The PCR temperature program followed an initial denaturation step at 94ºC for 3 min, then 35 cycles of denaturation at 93ºC for 30 sec, annealing at 53ºC for 30 sec and extension at 72ºC for 1 min, and a final step at 72ºC for 7 min. PCR products and a negative control were always checked by gel electrophoresis in 2% agarose gels (w/v) ran as described above. Prior to sequencing, mtDNA CR products were cleaned of excess primer and other inhibitors with ExoSap (USB Corporation, OH, USA) following the manufacturer's guidelines. The forward primer was utilized in the sequencing reaction using the BigDye cycle sequencing kit (Applied Biosystems, CA, USA), following the manufacturer's instructions. The clean PCR amplicons were sent out for Sanger sequencing at Macrogen Europe (Amsterdam, The Netherlands). The resulting electropherograms were manually edited and aligned using the Sequencher™ Version 4.1.4 software (Gene Codes Corporation; MI, USA).

In order to obtain a more comprehensive dataset in terms of sample size and geographical coverage, 210 additional mtDNA CR sequences from eastern North Atlantic and the Mediterranean Sea locations were obtained from Gubili *et al.* (2014) (Table 6).

Nuclear microsatellite genotyping

Twelve nuclear microsatellites developed specifically for *S. canicula* (Griffiths *et al.* 2011) were genotyped for all samples (Table 7). An initial PCR optimization procedure was conducted for each pair of primers prior to sample screening, using two individual samples, a gradient of annealing temperatures from 54 to 62ºC and different numbers of PCR cycles (from 30 to 35). Each reaction had a final volume of 5 µL and contained 2.5 µL of *Taq*™ PCR Master Mix (Qiagen; Hilden, Germany), 1.4 µL UPW,

0.25 µL of each primer (at a final concentration of 0.5 µM per reaction) and 0.6 µL of gDNA. The success and quality of the PCR results were run in a electrophoresis gel as described above. A ladder of known fragment length sizes (*Marker 5*, Eurogentec; Liège, Belgium) was run in each gel to confirm the amplicons' length.

The 12 successfully amplified microsatellite loci were combined in a single multiplex PCR and the forward primers were labeled with different fluorescent dyes (FAM, VIC, NED or PET; Applied Biosystems; CA, USA; Table 7). The multiplex was further optimized for primer concentration (primer forward per reaction: 0.008 – 0.031 µM; primer reverse per reaction: 0.08 – 0.31 µM; see Table 7 for details) using two to four samples and a negative control. The multiplex of microsatellite loci was amplified via PCR in a reaction volume of 10 µL, containing 5 µL of *MyTaq*™ PCR Master Mix (Qiagen), 3 µL of UPW, 1 µL of Primer Mix (see Table 7 for details) and 1 µL of gDNA. The final PCR temperature program followed an initial denaturation step at 95ºC for 15 min followed by 35 cycles of denaturation at 94ºC for 30 sec, annealing at 60ºC for 90 sec and extension at 72ºC for 45 sec, and a final extension step at 72ºC for 30 min. Depending on the strength of the PCR reaction, the amplicons were diluted from 0.28X to 0.6X to avoid an excess of fluorescence. Subsequently, 10 µL of deionized formamide and 0.2 µL of internal size standard (Genescan-500 LIZ, ABI) were added to 1 µL of diluted PCR product and run on an ABI 3130xl Genetic Analyzer (AB Applied Biossystems; CA, USA). The resulting electropherograms were analyzed with the Genemapper software 4.1. (Applied Biosystems) to score the microsatellite alleles.

Table 7. Summary of analysed microsatellite markers for *S. canicula*; from Griffiths *et al.* (2011) Tail, tail fluorescence; SR, product sequence length range; T, annealing temperature in PCR; F Final, final concentration of forward primer per reaction; R Final, final concentration of reverse primer per reaction.

Locus	RM	Tail	SR (bp)	T (ºC)	F Final (µM)	R Final (µM)
Scan02	(TG)9	NED	104-128	60	0.008	0.08
Scan03	(AT)9	FAM	170-180	60	0.012	0.12
Scan04	(AC)9	VIC	237-247	60	0.0097	0.097
Scan05	(GA)9	NED	176-186	60	0.008	0.08
Scan06	(TG)9	PET	204-224	60	0.0307	0.307
Scan09	(AT)9	VIC	114-116	60	0.008	0.08
Scan10	(TC)9	NED	249-260	60	0.008	0.08
Scan12	(AG)9	FAM	99-107	60	0.008	0.08
Scan13	(TG)11	VIC	174-188	60	0.008	0.08
Scan14	(CA)9	NED	277-284	60	0.015	0.15
Scan15	(CA)15	FAM	228-246	60	0.008	0.08
Scan16	(TG)9	PET	256-268	60	0.017	0.17

Data analysis - Mitochondrial DNA CR

MtDNA CR diversity indices such as the number of polymorphic sites, number of haplotypes (H), haplotype diversity (*h*), nucleotide diversity (π) and mean number of pairwise differences between haplotypes (*k*) were calculated in DnaSP version 5.10.01 (Librado and Rozas, 2009). The mtDNA CR haplotype network was estimated using the median joining algorithm (Bandelt *et al.*, 1999) implemented in the NETWORK version 4.6.1.2 (Fluxus Technology Ltd.; from http://www.fluxus-engineering.com). The mtDNA CR haplotype network shows how haplotypes are shared among sample collections, the frequency of each haplotype in the total sample, and the haplotype relationships.

Levels of among-sample genetic differentiation were calculated by means of pairwise F_{ST} based on haplotype frequencies of the mtDNA CR using Arlequin version 3.5.1.2 software (Excoffier and Lischer, 2011). *P* values were estimated with 10 000 permutations of the data. The probability of rejecting the null hypothesis (H_0=no genetic differentiation) for each pairwise test was corrected via a standard Bonferroni correction of the critical α value in order to reduce false positives (Type I error):

$$\frac{\alpha \text{ value}}{\text{N}i}$$

being α value the standard 0.05 significance value and $\text{N}i$ the number of comparisons in the analysis.

An analysis of molecular variance (AMOVA) was also carried out in Arlequin as a way to infer the population structure of the *S. canicula*. A first scenario tested for the global panmixia (Table 8 Scenario A). Two other scenarios of more detailed population structure were tested: the first tested how genetic variation is divided between the eastern North Atlantic Ocean and the Mediterranean Sea sample groups (Table 8 Scenario B); the second scenario tested the same hierarchical division but between the eastern North Atlantic sample collections plus Mallorca sample collection (eastern North Atlantic plus Mallorca) Vs remaining Mediterranean sample collections (Mediterranean, i.e. Gulf of Lion plus Adriatic Sea plus Crete) (Table 8 Scenario C). A detailed summary of these scenarios is illustrated in Table 8. Afterwards, a final scenario tested for panmixia among collections from the eastern North Atlantic Ocean alone.

Table 8. Summary of scenarios tested for analysis of molecular variance (AMOVA) in order to infer population structure and panmixia for *S. canicula*. Each colour represents the assemblage of sampling collections that form a group to be tested. Location abbreviations follow Table 6.

Location	Secnario A	Scenario B	Scenario C	Scenario D
N. Sea				
I. Sea				
B. Isles				
Can. Sea				
N. PT				
S. PT				
Ma				
G. Lion				
Adr. Sea				
Cr				

Data analysis - Nuclear microsatellites

GeneAlEx version 6.5 (Peakall and Smouse, 2012) was used to check if the microsatellite allele distribution was in accordance with Hardy-Weinberg equilibrium (HWE) expectation, and to test for Linkage Disequilibrium (LD) between loci across samples as well as within sample collections. Additionally, the inbreeding coefficient (F_{IS}), observed and expected heterozygosity and the average number of alleles per sample collection were also estimated in GeneAlEx. Micro-Checker version 2.2.3 (Van Oosterhout *et al.*, 2004) was utilised to check for genotyping errors, stuttering, allele dropout and null alleles. Estimation of allelic diversity standardized by sample size, i.e. allelic richness was calculated using FSTAT version 2.9.3.2 (Goudet, 2001).

Genetic differentiation at the nuclear microsatellite loci was visualized by means of a factorial correspondence analysis (FCA) implemented in Genetix version 4.05.2 software (Belkhir *et al.*, 1996) using the multilocus microsatellite genotypes of all individuals. In a FCA, the multilocus genotypes were positioned in a n-dimensional space according to their allelic composition in a way that genotypes with similar composition are closely located to one another.

Levels of among-sample genetic differentiation were calculated by means of pairwise F_{ST} based the nuclear microsatellite allelic frequencies using Arlequin. *P* values were estimated using 10 000 permutations of the data. The probability of rejecting the null hypothesis (H_0=no genetic differentiation) for each pairwise test was

corrected via a standard Bonferroni correction of the critical α value and was calculated through the same means as above.

An AMOVA was carried out in Arlequin to infer the population structure at the nuclear microsatellites loci according to the scenarios described in Table 8, except for scenario C for which no data was available for Mallorca.

Structure version 2.3.4 software (Falush *et al.*, 2003; Pritchard *et al.*, 2000) was used to infer the number *K* of populations and assign individuals to each *K* population based on their allelic composition. The *K* values ranged from 1 to 7 using an admixture model with default parameters. Three replicates were run for each *K* value using 500 000 burnin steps and 1 000 000 Markov chain Monte Carlo (MCMC) steps. The optimal *K* was estimated using Structure Harvester web version 0.6.94 (Earl & vonHoldt 2012; http://taylor0.biology.ucla.edu/structureHarvester/#) based on Evanno *et al.* (2005) Detla-K method.

Geographical analysis

Isolation-by-distance was tested with the "MASS" package (Venables and Ripley, 2002) in R correlating geographical distance between sample collections and pairwise F_{ST} values based on mtDNA CR haplotype frequencies and nuclear microsatellite allelic frequencies. Geographical distance was calculated by the nearest distance uniting two collections' regions that was over 400m in depth, resorting to the European Marine Observation and Data Network bathymetry analysis tool (EMODnet © Bathymetry, 2014; at http://portal.emodnet-bathymetry.eu/).

Results

Genetic diversity - Mitochondrial DNA CR

A total of 556 mtDNA CR sequences were analyzed including 346 sequences obtained *de novo* and 210 from Gubili *et al.* (2014). Forty different haplotypes were found differing in 23 polymorphic positions of which 19 were parsimony informative and 4 were singletons. The average number of nucleotide differences between haplotypes was 1.669±0.965, and the overall nucleotide and haplotype diversities were 0.0040 and 0.826, respectively. The Cantabrian Sea sample collection showed the highest haplotype diversity (*h*=0.85; Table 9) and mean number of differences between haplotypes (*k*=1.83; Table 9), while on the other hand, Crete showed the lowest values of both indices (*h*=0.59 , *k*= 1.01; Table 9).

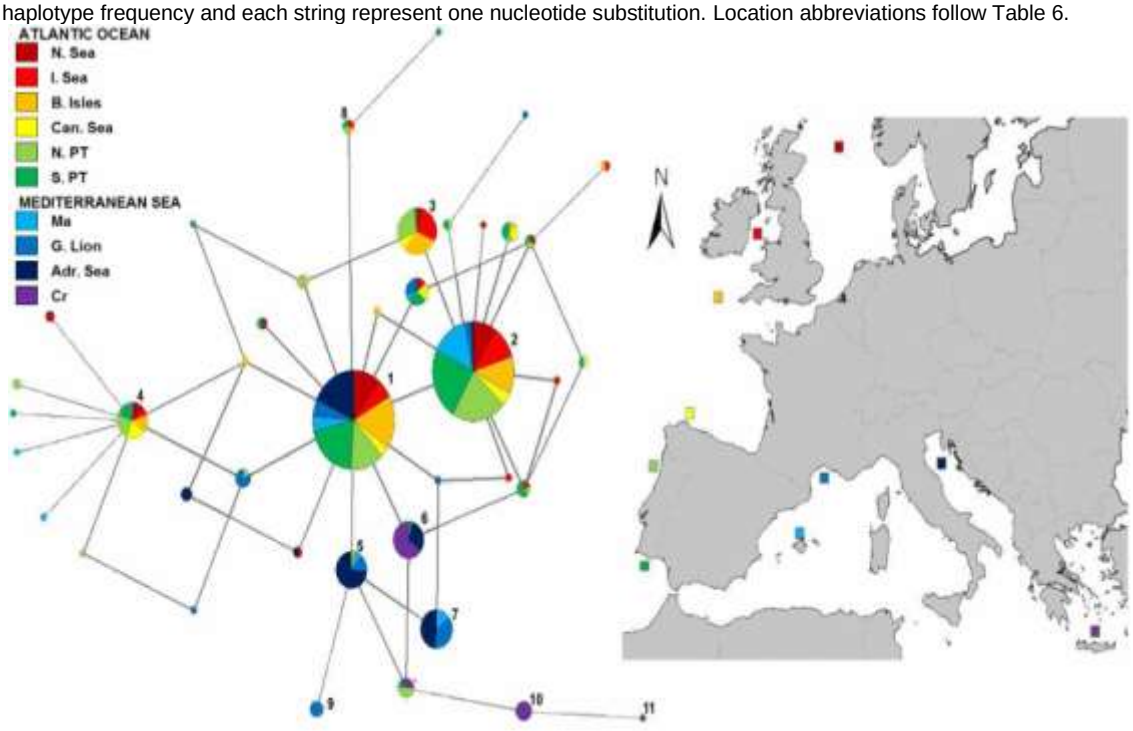
Table 9. Genetic diversity indices for *S. canicula*, from all mtDNA CR haplotypes. *N,* number of samples [sequences obtained *de novo*]; *H*, number of haplotypes (unique haplotypes); *h*, haplotype diversity; *π*, nucleotide diversity; *k*, mean number of pairwise differences between haplotypes. Location abbreviations follow Table 6.

	N	***H***	***h***	***π***	***k***
Eastern North Atlantic					
N. Sea	*43* [*18*]	9 (1)	0.72	0.0032	1.28
I. Sea	[*46*]	11 (3)	0.79	0.0034	1.36
B. Isles	*69*	11 (2)	0.74	0.0031	1.25
Can. Sea	[*27*]	7 (0)	0.85	0.0046	1.83
N. PT	*87* [*81*]	14 (2)	0.78	0.0039	1.55
S. PT	*93* [*79*]	17 (3)	0.73	0.0032	1.27
Mediterranean					
Ma	*40*	8 (2)	0.66	0.0033	1.33
G. Lion	[*45*]	10 (4)	0.87	0.0046	1.84
Adr. Sea	*77* [*50*]	7 (1)	0.74	0.0030	1.19
Cr	*29*	4 (2)	0.59	0.0025	1.01

The mtDNA CR haplotype network demonstrated that the two most frequent haplotypes (haplotypes 1 and 2 in Figure 29) are shared between all eastern North Atlantic and Mediterranean populations, excluding Crete. These haplotypes also occupy a central position in the network and differ from the remaining haplotypes by up to four mutational steps. Some haplotypes are more common or exclusive to eastern North Atlantic sample collections (haplotypes 3 and 4 in Figure 29, e.g.) while others are more frequent in the Mediterranean collections (haplotypes 5 to 7 in Figure 29, e.g.). Moreover, 10 haplotypes are shared among eastern North Atlantic sample

collections while only one haplotype is exclusive to the Mediterranean collections (haplotype 7 in Figure 29).

Figure 29. Haplotype network of the mitochondrial DNA CR region for *S. canicula*. Circle diameter is proportional with haplotype frequency and each string represent one nucleotide substitution. Location abbreviations follow Table 6.



Pairwise F_{ST} values for the mtDNA CR suggest differentiation between the Mediterranean and the eastern North Atlantic sample collections (F_{ST} range: 0.068-0.338; p<1.1E-3; Table 10) except for most comparisons involving Mallorca. Interestingly, Mallorca shows significant genetic differentiation from the remaining Mediterranean sample collections (F_{ST} range: 0.130-0.374; p<1.1E-3; Table 10).

F_{ST} values also show no genetic differentiation among eastern North Atlantic sample collections (F_{ST} range: -0.007-0.034; p>1.1E-3; Table 10). In contrast, significant genetic differentiation was found among all Mediterranean collections (F_{ST} range: 0.068-0.374; p<1.1E-3; Table 10), except between the Adriatic Sea and the Gulf of Lion (F_{ST} =0.056; p>1.1E-3; Table 10).

Table 10. F_{ST} values among sample collections of *S. canicula* based on mitochondrial DNA CR haplotype frequencies **Bold** values indicate statistical significance after Bonferroni correction for multiple tests (α=0.001). Location abbreviations follow Table 6.

	N. Sea	I. Sea	B. Isles	C. Sea	N. PT	S. PT	Mal	G. Li	Adr Sea	Cr
N. Sea	-									
I. Sea	0.033	-								
B. Isles	-0.001	0.015	-							
C. Sea	0.020	0.009	0.021	-						
N. PT	0.012	-0.005	0.013	0.006	-					
S. PT	-0.007	0.034	0.020	0.022	0.011	-				
Mal	0.059	0.053	**0.092**	0.067	0.031	0.032	-			
G. Li	**0.099**	**0.104**	**0.100**	**0.068**	**0.100**	**0.105**	**0.130**	-		
Adr Sea	**0.117**	**0.161**	**0.109**	**0.129**	**0.149**	**0.137**	**0.202**	0.056	-	
Cr	**0.338**	**0.305**	**0.323**	**0.283**	**0.295**	**0.320**	**0.374**	**0.261**	**0.278**	-

The AMOVA analysis on “scenario A” (i.e. global panmixia) rejected the null hypothesis (Table 11), with 19.59% of the variation at the mtDNA CR being due to differences among populations. AMOVA on “Scenario B” (Table 8) analysis revealed significant genetic differences between eastern North Atlantic and Mediterranean collections, where 15.12% of the variance for the mtDNA CR data is due to differences between the groups; and only 10.42% of the variance is due to differences among sample collections within groups. The AMOVA on “Scenario C” (Table 8) showed greater genetic differentiation between eastern North Atlantic collections, including Mallorca, and the remaining Mediterranean collections: 22.65% of the variance in mtDNA CR data is due to differences between the two groups and only 7% is due to differences among sample collections within groups. Finally, the hypothesis of panmixia within the eastern North Atlantic sample collections was also rejected, with only 1.76% of the variance being due to differences among collections within the eastern North Atlantic Ocean.

Table 11. Analysis of molecular variance (AMOVA). Fixation indices for mitochondrial DNA CR sequence by haplotype frequencies. All *P*-values <0.05. Scenarios following Table 8.

Scenario	Fixation indices		
	F_{CT}	F_{SC}	F_{ST}
A	-	-	0.196
B	0.151	0.123	0.255
C	0.226	0.090	0.296
D	-	-	0.013

Genetic diversity - Nuclear microsatellites

From the twelve microsatellite markers, *Scan 14* was removed from further analysis. The decision was made as the locus was associated with high levels of stutter and secondary products in several samples. The remaining, eleven nuclear microsatellite loci were genotyped for 307 *S. canicula*, of which 15 individuals had missing data at one to three loci. MicroChecker analyses indicated the presence of null alleles at the *Scan03* locus for the Adriatic, Irish Sea, South Portugal and Gulf of Lion sample collections, and at the *Scan13* locus for the South Portugal collection. For all the populations and loci, after Bonferroni correction (corrected α=0.0045) Hardy-Weinberg disequilibrium was observed for: *Scan03* in the Irish Sea and North Portugal and *Scan13* in South Portugal. The loci were not in linkage disequilibrium. The mean number of alleles per locus was 9.45, which varied from a minimum of three (*Scan09*) to a maximum of 13 alleles (*Scan06*). The mean allelic richness (R_S) was similar across collections and ranged between 4.89 and 5.70 (Table 12). The South Portugal collection showed the highest values of observed (H_O=0.63) and expected heterozygosity (H_E=0.64), and the highest number of private alleles (*Private A*=7). On the other hand, the Adriatic Sea collection had the lowest observed and expected heterozygosity values (Table12). North Sea, Cantabrian Sea and Gulf of Lion had no exclusive alleles.

Table 12. Genetic diversity indices for *S. canicula*, across all nuclear microsatellite loci. *N,* number of samples; H_O, observed mean heterozygosity; H_E, expected mean heterozygosity; F_{IS}, fixation index; *Mean A*, mean number of alleles; *Private A*, private number of alleles; *Mean* R_S, mean allelic richness (corrected to *N*=18). Location abbreviations follow Table 6.

	N	***H_O***	***H_E***	***F_{IS}***	***Mean A***	***Private A***	***Mean R_s***
Eastern North Atlantic							
N. Sea	*18*	0.60	0.59	0.010	5.55	0	4.89
I. Sea	*56*	0.62	0.64	0.021	7.18	3	5.43
Can. Sea	*26*	0.62	0.63	0.013	5.82	0	5.70
N. PT	*61*	0.62	0.63	0.010	7.00	3	5.55
S. PT	*61*	0.63	0.64	0.029	7.55	7	5.53
Mediterranean							
G. Lion	*44*	0.62	0.63	0.010	6.27	0	5.65
Adr Sea	*41*	0.59	0.59	0.008	5.82	2	5.27

The FCA showed that the multilocus microsatellite genotype diversity could be explained by four factors (Figure 30a), of which the first two explained 61% of the variability. According to the FCA, microsatellite genotypes data show two different clusters between eastern North Atlantic and Mediterranean populations, as well as between the two Mediterranean collections (i.e. Gulf of Lion and Adriatic Sea) (Figure 30a). Moreover, considering only eastern North Atlantic populations (Figure 30b), the FCA showed different clusters comprising North Sea/Irish Sea, Cantabrian Sea/North Portugal and South Portugal. Nevertheless, there was still considerable overlap among all eastern North Atlantic collections.

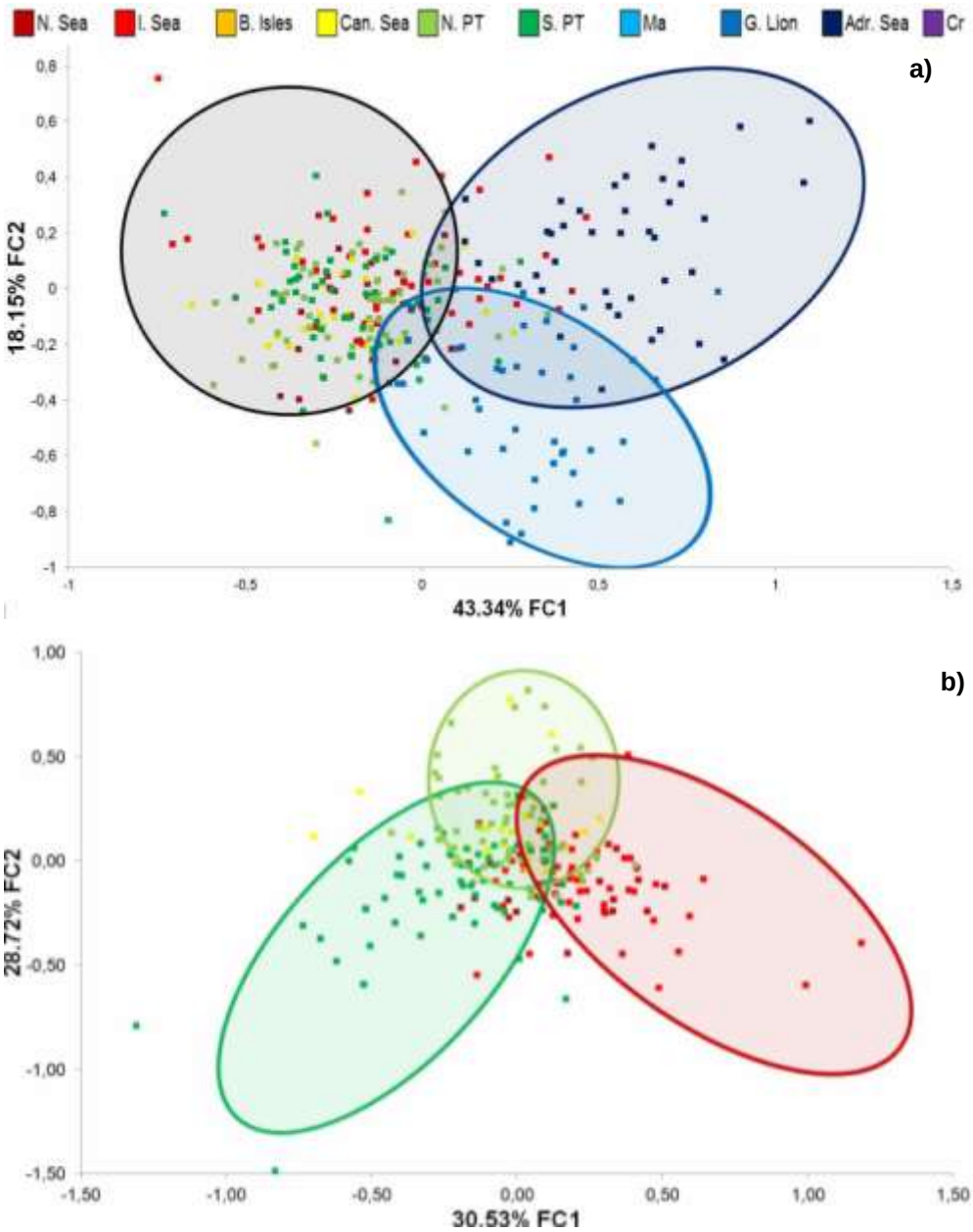


Figure 30. Factorial correspondence analysis of multilocus microsatellite genotypes from eastern North Atlantic and Mediterranean collections a), and from eastern North Atlantic collections only b). Location abbreviations follow Table 6.

Pairwise F_{ST} values at the nuclear microsatellite loci revealed significant genetic differentiation between the eastern North Atlantic Ocean and the Mediterranean Sea collections, with the exception of the Gulf of Lion and the North Sea (F_{ST} =0.014, p>0.002; Table 13). No genetic differentiation was detected among eastern North Atlantic Ocean collections (F_{ST} range: -0.001-0.007; p>0.002; Table 13). Conversely, significant genetic differentiation was detected between the two Mediterranean Sea collections.

Table 13. F_{ST} values among sample collections of *S. canicula* based on nuclear microsatellite allelic frequencies across 11 loci. **Bold** values indicate statistical significance after Bonferroni correction for multiple tests (α=0.002). Location abbreviations follow Table 6.

	N. Sea	I. Sea	C. Sea	N. PT	S. PT	G. Lion	Adr. Sea
N. Sea	-						
I. Sea	-0.001	-					
C. Sea	0.003	0.003	-				
N. PT	0.008	0.002	-0.001	-			
S. PT	0.007	0.002	0.005	0.003	-		
G. Lion	0.014	**0.025**	**0.034**	**0.027**	**0.029**	-	
Adr. Sea	**0.060**	**0.048**	**0.074**	**0.062**	**0.058**	**0.036**	-

The AMOVA analysis on "scenario A" (i.e. global panmixia) rejected the null hypothesis (Table 14), with 2.35% of the nuclear microsatellites loci being due to differences among populations. AMOVA on "Scenario B" (Table 8) analysis revealed significant genetic differences between eastern North Atlantic and Mediterranean collections, where . 2.88% of the variance for the nuclear microsatellites loci is due to differences between the groups; and only 0.94% of the variance is due to differences among sample collections within groups. The hypothesis of panmixia within the eastern North Atlantic sample collections was also rejected, with only 0.29% of the variance being due to differences among collections within the eastern North Atlantic Ocean.

Table 14. Analysis of molecular variance (AMOVA). Fixation indices for 11 microsatellite loci by distance matrix. All *P*-values <0.05. Scenarios following Table 8.

Scenario	Fixation indices		
	F_{CT}	F_{SC}	F_{ST}
A	-	-	0.024
B	0.029	0.038	0.010
D	-	-	0.003

Results from Structure indicates that the most likely *K* value is 2 (Figure 31) corresponding to two different clusters: an eastern North Atlantic cluster composed by North Sea, Irish Sea, Cantabrian Sea, North and South Portugal collections; and a Mediterranean Sea cluster composed by Gulf of Lion and Adriatic Sea collections (Figure 32). Additionally, some individuals sampled from the eastern North Atlantic

Ocean show an inferred ancestry that is dominantly Mediterranean, and vice versa (Table 15).

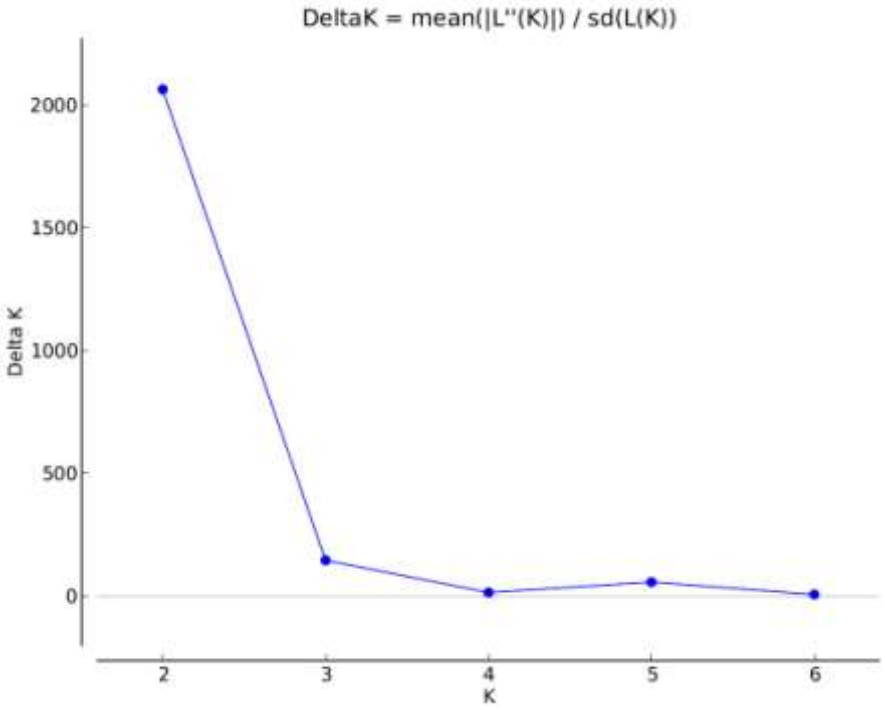


Figure 31. Delta-K calculated from Evanno *et al.* (2005) method.

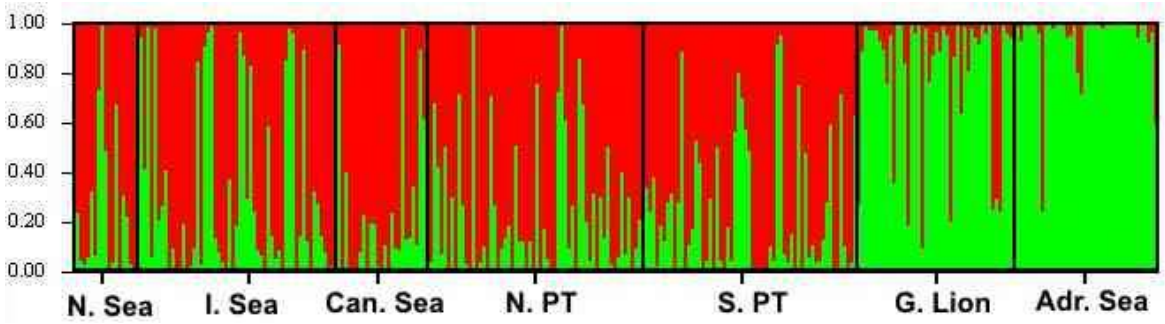


Figure 32. Bar plot from Structure clustering method using the genotypes from 11 microsatellite loci, *K*=2.

Table 15. Proportion of inferred ancestry between eastern North Atlantic Ocean and Mediterranean Sea samples. *N*=number of genotyped individuals used. Locations abbreviations follow Table 6.

Sample Collection	N	% Individuals assigned to a clusters	
		Eastern North Atlantic	Mediterranean
Eastern North Atlantic			
N. Sea	*18*	71.9	28.1
I. Sea	*56*	66.0	34.0
Can. Sea	*26*	77.4	22.6
N. PT	*61*	76.1	23.9
S. PT	*61*	75.4	24.6
Mediterranean			
G. Lion	*44*	19.3	80.7
Adr. Sea	*41*	5.1	94.9

Geographic analysis

An isolation-by-distance pattern was detected by both the mtDNA CR and nuclear microsatellites loci, based on pairwise F_{ST} values among all *S. canicula* collections and their respective geographical distances (mtDNA: R^2=0.403; p=1.7E-6, Figure 33a; Microsatellites: R^2=0.577; p=3.9E-5, Figure 33b). On the other hand, no significant correlation was observed between the same pairwise F_{ST} values among *S. canicula* collections from the eastern North Atlantic Ocean alone and latitudinal values (mtDNA: R^2=-0.0329; p=0.47, Figure 34a; Microsatellites: R^2=0.294; p=0.061, Figure 34b). Nevertheless, there was a tendency for pairwise F_{ST} values of nuclear microsatellite allelic frequencies to increase with increasing geographical distance (Figure 34b).

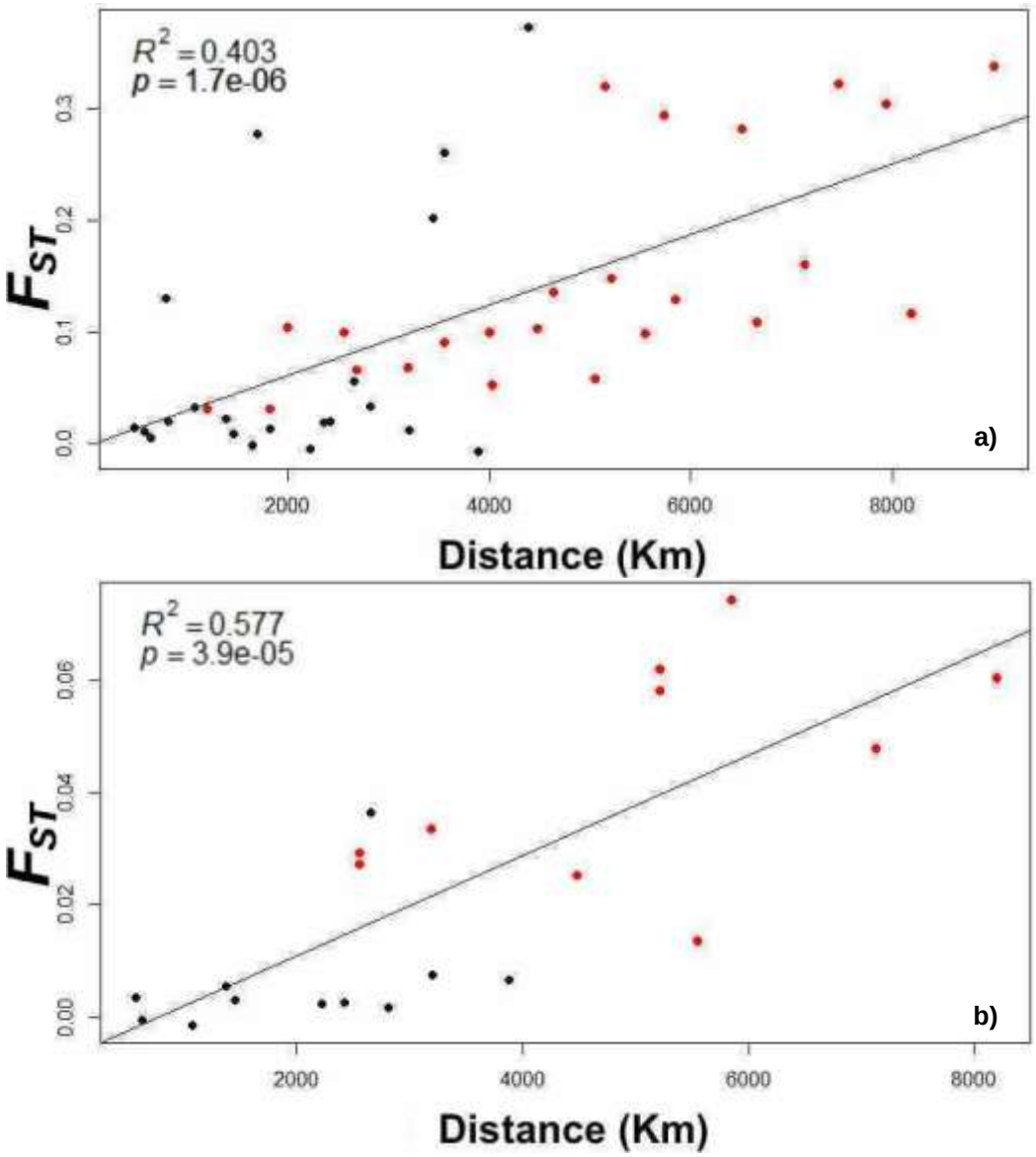


Figure 33. Relationship between genetic distance values and respective geographical distance among *S. canicula*, sample collections. F_{ST} values for the mitochondrial DNA CR sequence a) and F_{ST} values for the nuclear microsatellites loci b). Red dots represent geographical differences between eastern North Atlantic Ocean and Mediterranean Sea collections.

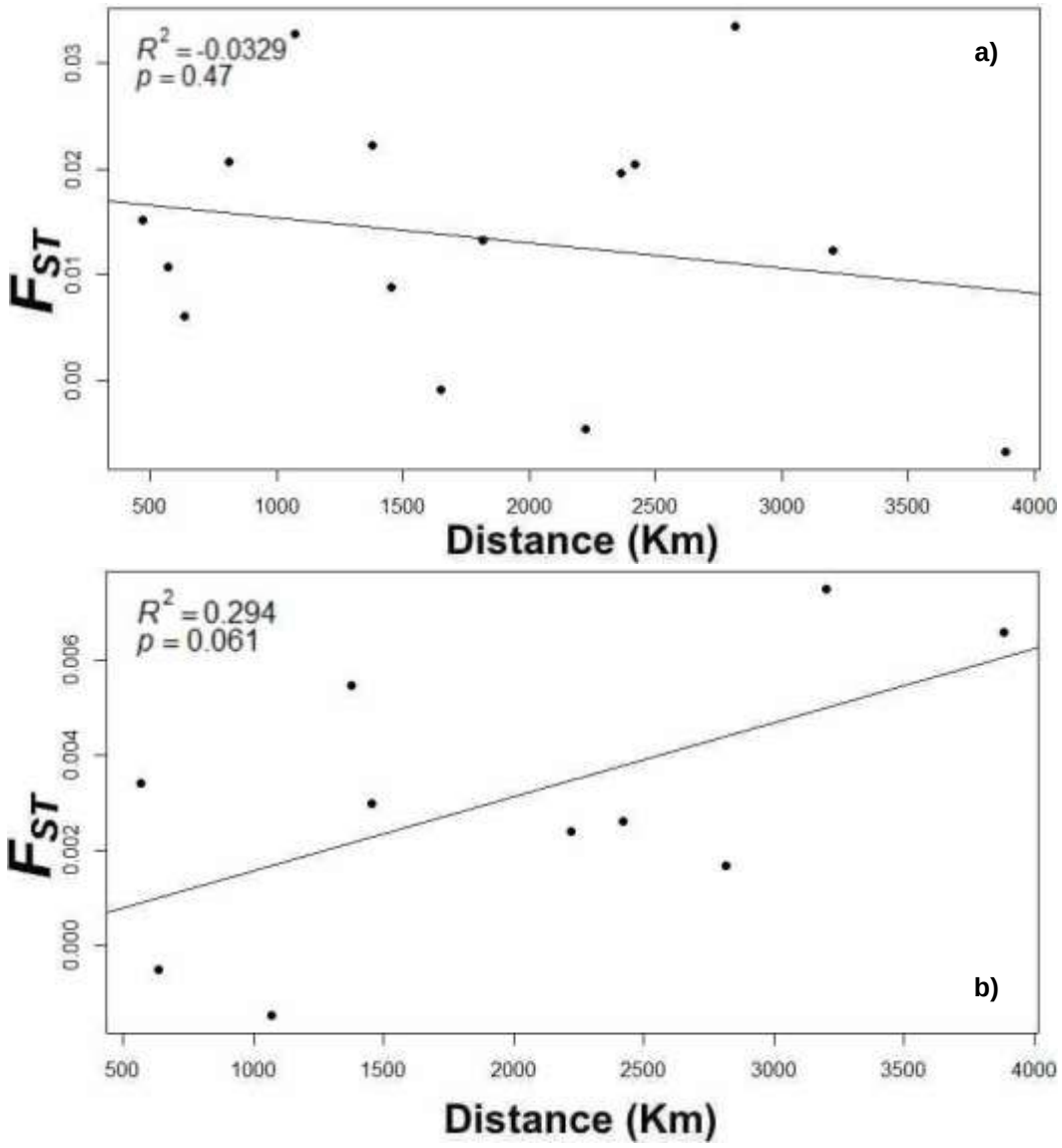


Figure 34. Relationship between genetic distance values and respective geographical distance among *S. canicula*, sample collections within the eastern North Atlantic Ocean. F_{ST} values for the mitochondrial DNA CR sequence a) and F_{ST} values for the nuclear microsatellites loci b).

Discussion

Molecular markers revealed a complex population structure for *S. canicula* throughout its distribution in the eastern North Atlantic and Mediterranean. Significant genetic differentiation exists between the eastern North Atlantic and Mediterranean collections. Barbieri *et al.* (2014) also showed genetic differentiation between eastern North Atlantic and Mediterranean populations of *S. canicula*. Other studies on elasmobranchs also reported genetic differentiation between the eastern North Atlantic and the Mediterranean, and suggested that the strait of Gibraltar works as a barrier to dispersal or has had that function in the past (Chevolot, 2006; Griffiths *et al.*, 2011b). Nevertheless, for *S. canicula*, the Strait of Gibraltar is not a barrier to gene flow and the Balearic Sea may serve as a contact zone between the eastern North Atlantic Ocean and the Mediterranean sea. This hypothesis is supported by the results on the Mallorca collection: no genetic differentiation among eastern North Atlantic collections and Mallorca was found except between Mallorca and British Islands, in addition including Mallorca into the eastern North Atlantic group increases the difference between the eastern North Atlantic and the Mediterranean groups.

MtDNA and nuclear microsatellite pairwise F_{ST} results also showed no significant genetic difference among the eastern North Atlantic sample collections. Nevertheless, the FCA on the microsatellite data suggests some population structure along the eastern North Atlantic: three clusters were recovered namely a northern cluster (North Sea and Irish Sea), a central cluster (Cantabrian Sea and northern Portugal) and a southern cluster (southern Portugal). The AMOVA analyses also rejected the hypothesis of panmixia within the eastern North Atlantic, suggesting population structure may be present. Since the pairwise F_{ST} is a more conservative fixation index, it may not be detecting this gradual latitudinal gradient in genetic differences for the *S. canicula* in the eastern North Atlantic. Even though, no signal of isolation by distance was detected in the eastern North Atlantic for either type of molecular markers.

Both mtDNA CR and the nuclear microsatellites loci showed significant genetic differences among Mediterranean sample collections. Pairwise F_{ST} tests show significant genetic differences among all collections within the Mediterranean except between Gulf of Lion and the Adriatic sample collections where genetic differences were detected at the nuclear microsatellite loci but not at the mtDNA CR. These results

are not in concordance with the female philopatric behavior described for the species (Sims *et al.*, 2001), since it would result in structuring at the mitochondrial level.

The heterogeneity of habitats in the Mediterranean Sea due to different water currents (Lejeusne *et al.*, 2010b) and areas of greater food availability (García-Charton *et al.*, 2004) may be isolating individuals in discrete suitable areas. Additionally, the Mediterranean has varying depths that may limit the connectivity among *S. canicula* that are distributed above 400 m depth. These extrinsic factors, allied to the species low dispersal (Rodríguez-Cabello *et al.*, 2004) may be isolating *S. canicula* populations in the Mediterranean.

Conservation implications

The life history patterns of elasmobranchs, i.e. slow growth rates, large adult sizes, late reproduction and low fecundity, makes this group highly susceptible to overfishing (Hoenig and Gruber, 1990). Evident cases of different life history strategies in the *S. canicula,* like varying growth rates (Henderson and Casey, 2001), maximum size and size at maturity (see Table 1 in Chapter 2), along with strong genetic structure within the Mediterranean Sea and the hypothesis of possible structuring within the eastern North Atlantic show that *S. canicula* forms separate distinct stocks. These data are extremely helpful for planning a long-term effective management and sustainability of stocks at the local scale (Barbieri *et al.*, 2014; Ward, 2000). Fisheries management should take into account that different stocks are present in the Balearic Sea, Gulf of Lion and Crete and conservation strategies should be made for these locations separately. Also, this study shows that the eastern North Atlantic may harbour more than one stock unit but more data are needed to clarify the population structure in this region.

Future work

In order to better understand the eastern North Atlantic - Mediterranean connectivity, increasing the sample size and number of regions studied for the microsatellite markers analysis in the Balearic Sea would help to ascertain the population dynamics for *S. canicula* in the contact zone. Additionally, it would be relevant to add *S. canicula* samples from northern Africa (e.g. off Marroco, Tunisia, Algeria, or Egypt) to further assess the eastern North Atlantic - Mediterranean

connectivity. Moreover, adding more samples from a broader area along the latitudinal range of the species, i.e. south to Senegal, would shed some light on the putative latitudinal population structuring suggested by the FCA analysis.

General Discussion

The life history traits of small-spotted catsharks from off the northern Portuguese coast (eastern North Atlantic) fit with the previous pattern described for the species where individuals from the Mediterranean Sea attain smaller maximum sizes and reach sexual maturity at smaller sizes than those in the eastern North Atlantic coast (Bendiab *et al.*, 2012; Capapé *et al.*, 2014, 2008a, 1991; Capapé and Zaouali, 1977; Ellis and Shackley, 1995; Henderson and Casey, 2001; Ivory *et al.*, 2005; Jennings *et al.*, 1999; Kousteni *et al.*, 2010; Leloup and Olivereau, 1951; Mendes *et al.*, 2004; Rodríguez-Cabello *et al.*, 1998; Zupanovic, 1961). Moreover, within the eastern North Atlantic, the above two life history traits estimated from off the Portuguese coast fit with the overall pattern of decreasing maximum size and size at maturity with decreasing latitude also described for *S. canicula* (Ellis and Shackley, 1995; Henderson and Casey, 2001; Ivory *et al.*, 2005; Jennings *et al.*, 1999; Leloup and Olivereau, 1951; Rodríguez-Cabello *et al.*, 1998). Concordantly, significant genetic differentiation was detected at the mtDNA CR and 11 nuclear microsatellite loci between *S. canicula* from the eastern North Atlantic and the Mediterranean Sea.

The latitudinal variability in life history traits observed along the eastern North Atlantic Ocean has no correspondence to a regional substructuring of *S. canicula* since no discrete population units were detected between the North Sea and the coast of Portugal. Nonetheless, the AMOVAs for the mtDNA CR and the 11 microsatellites reject the hypothesis of panmixia within the eastern North Atlantic. Additionally, although no signal of significant isolation by distance is shown in the results, there is a tendency for genetic difference at the nuclear microsatellites level to increase with increasing distance. Moreover, the FCA results on the multilocus microsatellite genotypes suggest that some structure may be present at the nuclear microsatellite level: the allelic composition are positioned forming three clusters within the eastern North Atlantic ocean, showing a latitudinal gradient of genetic differentiation among North Sea/Irish Sea, Cantabrian Sea/North Portugal and South Portugal. This suggested pattern of genetic differentiation, seems to fit the described pattern for maximum size and size at 50% maturity. Where *S. canicula* individuals from the North Sea/Irish Sea have similar maximum body sizes and L_{50} values (Henderson and Casey, 2001; Jennings *et al.*, 1999) that are different from the Cantabrian Sea and coast off Portugal values (Rodríguez-Cabello *et al.*, 1998; and results from this study).

Interestingly, no significant genetic differentiation was detected between Mallorca (western Mediterranean) and the eastern North Atlantic collections, except for the comparison including the British Islands. Thus, gene flow between the Atlantic and the Mediterranean collections appears to occur or to have occurred until recently, implying that the Balearic Sea may work as a contact zone for the species. Despite this fact, individuals from a near region of Mallorca, the Algerian Sea, show lower maximum body size and L_{50} values than the *S. canicula* from the coast off Portugal (Bendiab *et al.*, 2012). Hence, the genetic pattern seems to not match the differences in life history traits. Although, having a better sample that could describe the reproductive biology and life history characters from Mallorca would help to ascertain this issue.

Moreover, these findings suggest that the Strait of Gibraltar does not work as a barrier for the dispersal of *S. canicula* individuals. As it is in the case of the long nose skate, *Dipturus oxyrinchus*, for which (Griffiths *et al.*, 2011b) described for having a population structure consisting of two populations, the eastern North Atlantic and the Mediterranean (Mallorca). Suggesting that the genetic differentiation could be due to *D. oxyrinchus* dispersal being affected by the shallow waters in the Strait of Gibraltar.

In the Mediterranean, the maximum size and size at 50% maturity seem to be correlated with longitude – *S. canicula* from eastern regions of the Mediterranean Sea (Adriatic and Aegean Sea) seem to have smaller maximum body sizes and attain sexual maturity at shorter sizes (Kousteni *et al.*, 2010; Finotto *et al.*, in prep.) than individuals from westerner regions (Capapé *et al.*, 2014, 2008a, 1991; Capapé and Zaouali, 1977). The results from the F_{ST} based on the mtDNA CR and the microsatellites allelic frequencies also suggest that a genetic differentiation is present among Gulf of Lion, Adriatic Sea and Crete. Therefore, a correlation between genetic differences and life history variation within the Mediterranean may be present. Based on the molecular markers' results and the pattern of variation in life history traits, the Mediterranean Sea gathers more differentiated stocks of *S. canicula* than the eastern North Atlantic; as previously suggested by Barbieri *et al.* (2014).

Conservation and management implications

Based on the results from this study, no genetic differentiation was detected along the eastern North Atlantic coast. Nevertheless, such finding should not preclude the application of local management efforts for *S. canicula*, since individuals from distinct areas show different life history strategies and should be considered as separate management units (Rodríguez-Cabello *et al.*, 2004). Growth rates for *S. canicula* seem to be the same for the eastern North Atlantic, i.e. from the west coast of Ireland and Irish Sea to the Cantabrian Sea (Henderson and Casey, 2001; Ivory *et al.*, 2005; Rodríguez-Cabello *et al.*, 2005b). In the Mediterranean growth rates seem to follow the same pattern and do not have significant differences among different regions (Bendiab *et al.*, 2012; Capapé and Zaouali, 1977; Rodríguez-Cabello *et al.*, 1998). Thus suggesting that, regions showing smaller sizes at maturity, like the eastern Mediterranean regions, may have *S. canicula* individuals maturing at younger ages. Such hypothesis should be addressed in future studies by incorporating growth estimates in age at maturity studies.

Moreover, significant genetic differences were detected for *S.canicula* within the Mediterranean. These results suggest that distinct fisheries management stocks should be implemented in the Gulf of Lion and Crete. The F_{ST} values based on mtDNA show that the Adriatic Sea and Gulf of Lion are not genetically different, opposing to the results from the microsatellites. This is not in concordance with Barbieri *et al.* (2014) results, which show a significant genetic difference among the Adriatic population and the remaining populations in the Mediterranean and suggests that it should be treated as a unique stock. Although, Barbieri *et al.* (2014) grouped sample collections form regions far apart within the Mediterranean, and that may be biasing the results. Nevertheless, more effort by fisheries management should be implemented in order to implement conservation strategies for the Adriatic Sea, since abundance in catsharks (*Scyliorhinus* spp.) have been declining (Barausse *et al.*, 2014).

Future work

Studying the reproductive biology of *S. canicula* in the Mallorca coast and describe its maximum size and size at maturity could give valuable information whether the life history traits in the region fit the pattern described for the Atlantic or the Mediterranean. Additionally, this would shed some light on the dynamic of populations

in this contact zone (the Balearic Sea) for *S. canicula* between the Atlantic and the Mediterranean.

Studying genes directly linked with growth and sexual maturity along with assessing environmental factors, may help in the future to understand the pattern of regional phenotypic variability and the factors underlying its evolution for the species.

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