UNIVERSIDADE DE LISBOA

FACULDADE DE CIÊNCIAS

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SKATES AND RAYS DIVERSITY, EXPLORATION AND CONSERVATION – CASE-STUDY OF THE THORNBACK RAY, *RAJA CLAVATA*

Bárbara Marques Serra Pereira

Doutoramento em Ciências do Mar

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Tese orientada por
Professor Auxiliar com Agregação Leonel Serrano Gordo e
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2010

The research reported in this thesis was carried out at the Instituto de Investigação das Pescas e do Mar (IPIMAR - INRB), Unidade de Recursos Marinhos e Sustentabilidade.

This research was funded by Fundação para a Ciência e a Tecnologia (FCT) through a PhD grant (SFRH/BD/23777/2005) and the research project EU Data Collection/DCR (PNAB).

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List of Abbreviations

Age	t
Akaike's Information Criterion	AIC
Average per cent error	APE
Barcode of Life Data System	BOLD
Basal plate	BP
Base pair	bp
Bayesian phylogenetic analysis	BA
Batch fecundity	Fbatch
Coefficient of determination	r^2
Coefficient of variation	CV
Cytochrome c oxidase subunit I	COI
Dipturus oxyrinchus	RJO
Direcção-Geral das Pescas e Aquicultura	DGPA
Disc width	DW
Disc length	DL
Ethylenediaminetetraacetic acid solution	EDTA
Fishing Strategies	FS
Flexible discriminant analysis	FDA
General Time-Reversible model	GTR
Gonadosomatic Index	GSI
Growth rate	k
Gutted weight	gW
Haplotype diversity	H_h
Hematoxylin and Eosin	H&E
Hepatosomatic index	HSI
Index of precision	D
Index of vacuity	%IV
Instantaneous Gompertz growth coefficient	g
International Council for the Exploration of the Seas	ICES
International Plan of Action for the conservation and management of sharks	IPOA-SHARKS
Length-at-first-maturity	L_{50}
Leucoraja circularis	RJI
Leucoraja naevus	RJN
Maximum number of follicles	Fmax
Minimum length of a mature female	Lmat
Maximum-likelihood	ML
Maximum-parsimony	MP
Minimum number of follicles	Fmin

Mitochondrial DNA	mtDNA
Neoraja iberica	RNI
Nucleotide diversity	π
Number of batches	Nbatch
Number of haplotypes	N_h
Number of segregating sites	S
Oviducal gland	OG
Partial fullness index	PFI
Percentage by number	%N
Percentage by weight	%W
Percentage frequency of occurrence	%O
Percent index of relative importance	%IRI
Periodic Acid-Schiff	PAS
Post-ovulatory follicles	POFs
Raja brachyura	RJH
Raja clavata	RJC
Raja maderensis	JFY
Raja microocellata	RJE
Raja miraletus	JAI
Raja montagui	RJM
Raja undulata	RJU
Residual mean square error	MSE
Rostroraja alba	RJA
Scientific, Technical and Economic Committee for Fisheries	STECF
Scyliorhinus canicula	SYC
Squalus acanthias	DGS
Tail length	CL
Tamura-Nei model	TrN
Theoretical age at 0 length	t_0
Theoretical asymptotic length	L_∞
Toluidine blue	TB
Total Allowable Catch	TAC
Total fecundity	Ftotal
Total length	TL
Total length-at-age t	TL_t
Total weight	TW
Tree-bisection-reconnection	TBR
Van Gieson stain	VG
von Bertalanffy growth function	VBGF
Working Group on Elasmobranch Fishes	WGEF

Agradecimentos

Esta tese é fruto de um excelente trabalho em equipa, e do apoio e carinho de todos os que me rodeiam. A todos eles agradeço pelo incentivo, colaboração, entusiasmo, conhecimento e amizade que me deram durante os últimos quatro anos e meio, que foram sem dúvida os anos mais importantes da minha vida. Quero no entanto realçar algumas dessas pessoas.

Em primeiro lugar quero agradecer aos meus orientadores Leonel Serrano Gordo e Ivone Figueiredo por aceitarem orientar-me neste doutoramento, pela amizade, por me apoiarem em todo o percurso e por tudo o que me ensinaram. Gostaria de agradecer à Ivone por me ajudar a crescer como cientista e por alimentar a minha vontade de saber e fazer sempre mais. Ela ensinou-me que devemos ser os primeiros a criticar o nosso próprio trabalho, a colocar as questões pertinentes que nos levam a pensar no ínfimo pormenor de cada trabalho, com o fim de aperfeiçoar tudo aquilo que produzimos como cientistas.

Gostaria de agradecer aos meus colegas de trabalho Teresa Moura, Inês Farias, Ana Rita Vieira, Carla Nunes, Catarina Maia, José Lago e Pedro Bordalo Machado por todo o apoio e companheirismo que faz de nós uma grande equipa, mesmo quando alguns dos elementos já seguiram outros caminhos. Quero agradecer em especial à Teresa e à Inês por me apoiarem e ajudarem ao longo de todo o processo, quer em cada publicação, como por assegurarem alguns dos trabalhos do serviço que não pude executar, por estar tão atarefada com a tese.

Quero agradecer à Doutora Graça Pestana e ao Doutor Yorgos Stratoudakis por me receberem na Unidade de Investigação de Recursos Marinhos e Sustentabilidade do IPIMAR, por disponibilizarem as condições necessárias para a realização deste projecto e por garantirem a obtenção de amostras e todo o material necessário nas diferentes fases do trabalho. Quero em especial agradecer ao Projecto EU Data Collection/, DCR (PNAB) por assegurar a maioria desse material.

Este doutoramento não poderia ser realizado sem uma bolsa de doutoramento, pelo que agradeço à Fundação para a Ciência e Tecnologia por ter aceite este meu projecto. Ao longo destes últimos quatro anos algumas instituições/projectos patrocinaram a minha ida a congressos internacionais, promovendo assim a divulgação do meu trabalho à comunidade científica e o aumento do meu conhecimento. Quero por esse motivo agradecer à Fundação

Luso-Americana (FLAD), International Council for the Exploration of the Sea (ICES) e ao Projecto COST Action FA0601. Não posso deixar de agradecer ao Doutor Miguel Neves dos Santos, Doutor Miguel Gaspar e Doutora Ivone Figueiredo do IPIMAR pela bolsa no âmbito do projecto PRESPO que me foi auferida no inicio deste ano. Sem esta bolsa não poderia ter prolongado o prazo de entrega desta tese de modo a conseguir alcançar todos os objectivos a que me propus no inicio do projecto e a terminar a tese com o rigor que pretendia.

Quero agradecer aos co-autores das publicações inseridas nesta tese, pois sem a sua ajuda, todo este trabalho não poderia ter sido possível: obrigada pela vossa ajuda e contribuição. Não posso deixar de agradecer também aos muitos amigos, colegas e revisores que me ajudaram na revisão desses artigos, nomeadamente na melhoria do inglês: Beverly Macewicz, Conor Nolan, Francisco Pina Martins, Jim Ellis, Joe Bizzarro, Karim Erzini, Megan Ellis (nee Storrie), Mónica Silva, Tom Blasdale, Nancy Brown-Peterson e Stacey Sakai. E ainda às minhas queridas amigas Andreia Búzio e Anabela Maia por me ajudar com o inglês na recta final da elaboração desta tese…obrigada pela vossa disponibilidade.

Não podia deixar de agradecer a todos os colegas do IPIMAR pelo excelente ambiente de trabalho e por me ajudaram em diversas etapas, quer na amostragem em laboratório, em lota, ou na obtenção de dados; em particular à Catarina Maia, Cristina Nunes, Dolores Antunes (Peniche), Emanuel Pombal (Matosinhos), Inês Farias, Hélder Antunes, João Oliveira, José Lago, Leonor Costa, Manuela Oliveira, Maria do Carmo Silva, Maysa Franco (estagiária), Paulo Castro (Matosinhos), Pedro Joyce (estagiário), Pedro Juliano, Rogélia Martins, Teresa Gama Pereira, Teresa Moura e Vera Sousa. Queria também agradecer ao Professor Fernando Afonso por proporcionar o estágio em histologia na Faculdade de Medicina Veterinária de Lisboa, e à técnica Rosário Jorge por tudo o que me ensinou no laboratório de patologia.

Um muito obrigada a todos os pescadores de Peniche que colaboraram comigo e que se mostraram sempre disponíveis para responder aos meus inquéritos. Quero ainda agradecer aos trabalhadores da DOCAPESCA de Peniche por toda a sua cooperação e simpatia.

Quero agradecer a todos os meus amigos por todo o apoio e por me afastarem do trabalho sempre que precisei de descansar. Aos meus queridos colegas/amigos do IPIMAR, pelos almoços, lanches, bolos e festas variadas, que aconteceram nos anos em que trabalhámos juntos. Às minhas queridas amigas Filipa e Margarida pelos nossos longos "lanches". À

Anabela, à Ana Sofia e à Andreia por estarem sempre presentes apesar de tão longe. À Carla por todas as horas de conversa sobre tudo e nada e pela companhia nas longas viagens a Peniche. Aos meus vizinhos, Ângela e Luís por todos os serões (mesmo que eu em muitos deles tenha adormecido), pelas patuscadas e pela vossa amizade.

Quero agradecer a toda a minha família. Mãe, obrigada por me apoiares sempre em tudo. Pai, obrigada por incentivares a ser quem sou hoje. Ao meu mano Bernardo por todo o carinho apesar de andarmos muitas vezes às turras. Milai e Luís, obrigada por todo o apoio, têm sido como uns pais para mim. Aos meus cunhadinhos, Nuno e Vanessa obrigada por todo o vosso companheirismo de irmãos. Aos meus tios Tinó e Carlos, obrigada por me acolherem tão bem; obrigada por me alojarem por tantas vezes no vosso "hotel", pois é a vocês e à vossa casa onde fui buscar alguma da minha inspiração para fazer todo este trabalho. Aos primos, Inês, Sara, Diana, Cafi, Nelson e João por todos os momentos de diversão.

Como não podia deixar de ser agradeço-te meu querido Ricardo… por me apoiares nas alturas de maior nervosismo (e não foram poucas), por me incentivares sempre em todos os momentos em que estive mais em baixo, por me ensinares a dar valor ao que faço e fazeres aumentar a minha auto-estima. Obrigada pelo carinho, pelo teu tempo, pelo respeito, pela tua dedicação… enfim por tudo.

Por último, quero dedicar esta tese ao meu querido avô João que infelizmente nos deixou a 26 de Setembro de 2009. Irei lembrar sempre com muito carinho todas as vezes que ele olhando para o imenso azul do mar me perguntava como decorria o meu "trabalho com as raias".

Abstract

Skates have been increasingly exploited in recent decades despite their recognized vulnerability to fishing (due to their life-history traits), and regardless of a lack of basic knowledge about their biology. The present thesis contributed to a great advance in the knowledge about skates in Portuguese waters, with special attention to the most abundant and commercially important species, the thornback ray *Raja clavata*. The main aims of the present thesis were to study: (i) skate fishery; (ii) skate biodiversity; and (iii) biological traits of the thornback ray. Skates are mainly caught by the artisanal fishery, and a fishing segment targeting skates was identified. They are landed in three mix species categories, and this incorrect identification has misleading a fluctuation in fish abundance of nine skate species. The blonde ray was the most abundant in landings, followed by the thornback ray and the undulate ray. Species were easily discriminated using the mitochondrial gene cytochrome c oxidase subunit I. The thornback ray was the species with the highest intraspecific genetic variability. Size conversion factors were obtained for six skate species, and proved to be helpful to discriminate between them. The thornback ray, blonde ray, spotted ray and cuckoo ray have generalized diets, feeding mainly on benthic prey, and changing their preferred prey items during their ontogenetic development. Dermal denticles were considered more accurate than vertebrae to assess age and growth of thornback ray. This species has a slow growth rate (k = 0.117 year^{-1}), large maximum size (L_∞ = 1280 mm), late maturation ($L_{50,\,F}$= 784 mm, around 8 years of age; $L_{50,\,M}$= 676 mm, around 6 years of age) and low fecundity (140 eggs per female per year). Great advances on the knowledge of the reproduction of this species were achieved, namely on the definition of reproductive phases and the development of the different reproductive structures. In conclusion, this thesis was pioneering in several fields of study, namely in the utilization of the COI gene to discriminate between NE Atlantic skate species, in the use of dermal denticles for ageing thornback ray and in describing the development of the oviducal gland of a skate species. In the future, it will be essential to extend the findings achieved in this thesis to the remaining species occurring in Portuguese waters, filling the gap of information about these fish for the southern region of the NE Atlantic, and to contribute to their accurate assessment.

Keywords: biodiversity, fishery, life cycle, Rajidae

Resumo

As raias são importantes elementos da comunidade bentónica. Tal como outras espécies de elasmobrânquios, as espécies de raia caracterizam-se por uma estratégia de vida de tipo selecção-K (grande longevidade, fecundidade reduzida e períodos de maturação longos). Algumas espécies caracterizam-se, ainda, pela realização de migrações de pequena escala e/ou permanência em habitats específicos para completar o seu ciclo de vida. Estas características tornam as espécies deste grupo vulneráveis à pressão exercida pela pesca.

Ao longo dos anos tem havido um crescente interesse pela exploração comercial de raias, também relacionado com o actual estado de quase sobreexploração de alguns stocks de peixes teleósteos tradicionalmente explorados. Esse interesse aumentou devido à ausência de legislação que as tornou numa pescaria alternativa e lucrativa. As raias representam uma importante fracção das capturas de elasmobrânquios em Portugal, e até recentemente, não existia discriminação específica dos desembarques. Actualmente, a União Europeia estabeleceu valores de captura total permitida (TACs) globais para raias para cada um dos estados membros, sendo de 1974 toneladas para Portugal. Foi ainda recomendada a discriminação de espécies de raia nos desembarques e definida a proibição de retenção a bordo de determinadas espécies (*Raja undulata*, *Rostroraja alba* e *Dipturus batis*). As raias são habitualmente desembarcadas como capturas acessórias da frota de pesca costeira. No NE Atlântico, incluindo Portugal, a raia-lenga *Raja clavata*, é uma das espécies mais abundantes e de maior importância comercial.

Embora vulneráveis à exploração, devido às suas características biológicas, são poucos os estudos realizados sobre estas espécies. Esta tese pretende contribuir para o aumento do conhecimento sobre raias, com especial ênfase na raia-lenga *R. clavata*, a espécie mais abundante na costa continental Portuguesa. Para cumprir esse objectivo, num quadro multidisciplinar, foram realizados estudos sobre: (i) caracterização das pescarias de raias em Portugal; (ii) caracterização da biodiversidade de raias da família Rajidae que ocorrem na costa Portuguesa, especificamente em termos de filogenia, morfometria e ecologia alimentar; (iii) caracterização biológica da espécie *R. clavata*, mais especificamente em relação à idade e crescimento e diferentes aspectos da reprodução.

A presente tese é composta por cinco capítulos, nos quais são debatidos três temas principais, relacionados com: (i) exploração pesqueira; (ii) biodiversidade; e (iii) características do ciclo de vida da espécie em estudo, *R. clavata*. A tese é apresentada sob a forma de uma colectânea de oito artigos, produzidos para responder directamente aos

objectivos propostos. No total, dois artigos encontram-se publicados, dois em publicação e os restantes encontram-se submetidos em fase de revisão, em revistas internacionais com arbitragem científica.

No Capitulo 1 (Introdução geral) é feito um enquadramento do grupo de espécies em estudo na Classe Chondrichtyes e sub-classe Elasmobrachii. Temas como a diversidade específica existente, as diferentes estratégias reprodutivas exibidas pelos peixes cartilagíneos e a distribuição global de raias, incluindo um enquadramento regional das espécies de raias existentes na costa continental Portuguesa e regiões insulares, são apresentados neste capítulo. Questões sobre a exploração pesqueira são abordadas, tal como os problemas e consequências da sua gestão. É realizado um levantamento dos principais estudos sobre a biologia da espécie *R. clavata* realizados até à actualidade, sendo os temas sobre distribuição, crescimento e reprodução os mais abordados.

No Capitulo 2 é feita uma caracterização preliminar sobre a pescaria de raias em Portugal. Verificou-se que as raias são maioritariamente desembarcadas pela pescaria artesanal, que opera com pequenas embarcações perto da costa. Foi identificado um segmento de pesca dirigido à pescaria de raias. Outros cinco segmentos de pesca foram identificados e caracterizados em termos de composição específica dos desembarques e artes de pesca utilizadas. Nos últimos anos, os desembarques anuais de raias mantiveram-se em cerca de 1600 toneladas, sendo realizados sob três categorias específicas, igualmente divididas em categorias de tamanho, e nas quais foram identificados problemas de identificação das espécies. Com base nos dados recolhidos, entre 2003 e 2008, pelo Programa Nacional de Amostragem Biológica do INRB-IPIMAR, a composição específica dos desembarques foi extrapolada, sendo verificado que a raia *R. brachyura* foi a mais abundante, seguida da *R. clavata* e *R. undulata*.

No Capítulo 3, o tema da biodiversidade de raias é abordado sob três aspectos: diferenciação genética, diferenciação morfotípica e ecologia alimentar. O primeiro estudo foi pioneiro em verificar a aplicabilidade do gene mitocondrial citocromo c oxidase I (COI) para a diferenciação especifica de algumas das espécies de raia do NE Atlântico, mais especificamente doze espécies de raia desembarcadas em Portugal continental. O COI foi insuficiente apenas em diferenciar entre as espécies *R. clavata* e *R. maderensis* e suspeita-se que a última seja mais um morfotipo de *R. clavata*, uma vez que esta apresentou elevados índices de diversidade intraespecifica. No segundo estudo, várias relações morfométricas, também denominadas factores de conversão, foram estimados para seis das principais espécies de raia que ocorrem nos desembarques. A sua aplicação para a discriminação de

espécies foi testada, sendo verificada a sua utilidade em casos em que persistam dúvidas na identificação de pares de espécies morfologicamente semelhantes. No terceiro estudo do Capítulo 3, baseado na análise de conteúdos estomacais, foi verificado que as quatro espécies mais abundantes na costa Portuguesa têm uma dieta generalista, baseada em espécies bentónicas. *R. brachyura* e *L. naevus* predam preferencialmente peixe, enquanto *R. clavata* e *R. montagui* preferem crustáceos, tais como camarões e caranguejos. Foi verificado que em todas as espécies ocorre uma mudança ontogénica na dieta, por volta dos 500 mm de comprimento.

O Capítulo 4 é dedicado ao estudo dos principais aspectos da biologia de *R. clavata*. O primeiro trabalho apresentado neste capítulo foi o primeiro a utilizar espinhos dérmicos para a determinação de idades nesta espécie, sendo demonstrado que estas estruturas produzem leituras de idade mais precisas do que o método tradicional que recorre às vértebras. Foram descritos vários tipos de espinhos dérmicos e com base em determinados critérios, foi seleccionado o tipo mais adequado para estudos de crescimento. Foram testadas várias técnicas de processamento e de leitura das diferentes estruturas. Foram ajustados dois modelos de crescimento (von Bertalanffy e Gompertz) aos dados de comprimento-idade, e os parâmetros do modelo de von Bertalanffy foram considerados mais representativos do padrão de crescimento da espécie. Em resumo, foi estimado que *R. clavata* é uma espécie de crescimento lento (k = 0.117 ano^{-1}), grande longevidade (L_∞ = 1280 mm) e que não existem diferenças significativas no crescimento entre sexos. A segunda parte deste capítulo é dedicada ao estudo da reprodução e encontra-se subdividida em três sub-capítulos. No primeiro estudo é proposta uma escala de maturação para elasmobrânquios ovíparos, cuja terminologia foi adaptada de uma escala usada para espécies de peixes teleósteos, num esforço de combater a multiplicidade de escalas existentes em trabalhos realizados com peixes. Paralelamente é apresentada uma descrição pormenorizada do processo de maturação de *R. clavata*, através de um estudo macroscópico e microscópico dos principais órgãos reprodutores de fêmeas e machos, bem como dos fenómenos relacionados com a gametogénese. Foi reconhecida a existência de um estado de regressão, baseado na presença de folículos pós-ovulatórios em fêmeas com ovários contendo apenas pequenos folículos e glândulas oviductais e útero de dimensões semelhantes a fêmeas em desova. O mesmo não foi verificado para os machos, pois estes parecem não regredir do estado maduro após atingida a maturação sexual. No segundo sub-capítulo são debatidas as principais questões relacionadas com a reprodução, tais como a definição das épocas de maturação, postura e cópula, estimativa da maturação e fecundidade. *R. clavata* tem uma maturação tardia ($L_{50, F}$=

784 mm; $L_{50, M}$= 676 mm). A desova, tal como a cópula ocorrem durante todo o ano, o que indica que esta espécie tem uma desova continua. A fecundidade é determinada, e foi estimada em cerca de 140 ovos por ano, por cada fêmea. A ocorrência de um estado de regressão e outro de regeneração em fêmeas foi corroborado neste trabalho, com base na análise dos valores do índice gonadossomático e das dimensões das glândulas oviductais e útero. O último estudo sobre reprodução aborda os processos subjacentes à encapsulação, mais especificamente o desenvolvimento e principais processos fisiológicos do órgão responsável por esse processo, i.e. as glândulas oviductais. Foi verificado que quando formadas, as glândulas oviductais começam a produzir as secreções que darão origem ao ovo encapsulado, sendo possível distinguir no seu interior, as zonas responsáveis pelos diferentes invólucros: geleias de origem mucopolissacárida, invólucro proteico e fibras proteicas evolvidas por muco. Foi ainda identificada a presença de esperma no interior das glândulas.

O Capítulo 5 apresenta uma integração dos principais resultados obtidos sob a forma de respostas às questões colocadas no inicio do trabalho. A discussão geral foca aspectos relacionados com a conservação e avaliação do estado dos stocks de raia.

Palavras-chave: biodiversidade, ciclo de vida, Portugal, pescaria, Rajidae.

List of Publications

This thesis comprises the scientific publications listed below. The author of this thesis is the first author in seven papers and co-author in one paper. All papers published or in press were included fulfilling the publishers' publication rights policies. The organization of the aforementioned scientific publications in this thesis is the following:

Chapter 2

Serra-Pereira, B., Figueiredo, I., Farias, I., Moura, T., Nunes C. and Gordo, L. S. Submitted. Fishing strategies of an artisanal Portuguese mixed-fishery landing skates (Rajidae). Journal of Applied Ichthyology.

Chapter 3

Serra-Pereira, B., Moura, Griffiths, A. M., Gordo, L. S. and Figueiredo, I. Submitted. Molecular barcoding of skates (Chondrichthyes: Rajidae) from the southern Northeast Atlantic Zoologica Scripta.

Serra-Pereira, B., Farias, I., Moura, T., Gordo, L.S., Santos, M. N. and Figueiredo, I. In Press. Morphometric ratios of six commercially landed skate species from the Portuguese continental shelf and their utility for identification. ICES Journal of Marine Science.

Farias, I., Figueiredo, I., Moura, T., Serrano Gordo, L., Neves. A. and Serra-Pereira, B. 2006. Diet comparison of four ray species *Leucoraja naevus, Raja brachyura, Raja clavata* and *Raja montagui* caught along the Portuguese continental coast. Aquatic Living Resources, 19, 105-114. doi: 10.1051/alr:2006010.

Chapter 4

Serra-Pereira, B., Figueiredo, I., Farias, I., Moura, T. and Gordo, L. S. 2008. Description of dermal denticles from the caudal region of *Raja clavata* and their use for the estimation of age and growth. ICES Journal of Marine Science, 65: 1701-1709. doi: 10.1093/icesjms/fsn167

Serra-Pereira, B., Figueiredo, I. and Serrano-Gordo, L. In press. Maturation of the gonads and reproductive tracts of the thornback ray (*Raja clavata*), with comments on the development of a standardized reproductive terminology for oviparous elasmobranchs. Marine and Coastal Fisheries: Dynamics, Management, and Ecosystem Science. (Special Section: Emerging issues and methodological advances in fisheries reproductive biology).

Serra-Pereira, B., Figueiredo, I. and Serrano-Gordo, L. Submitted. Maturation, fecundity and spawning strategy of the thornback ray, *Raja clavata*, from Portuguese waters. Marine Biology.

Serra-Pereira, B., Afonso, F., Farias, I., Joyce, P., Ellis, M., Figueiredo, I. and Serrano-Gordo, L. Submitted. Oviducal gland development in the thornback ray, *Raja clavata*. Helgoland Marine Research.

The candidate acknowledges that, although all the research was conducted in collaboration, she was fully involved in the planning, sampling, laboratory processes, data analysis and discussion of the results of all the works, as well as in their preparation and submission for publication.

Chapter 1

General Introduction

1. GENERAL INTRODUCTION

Cartilaginous fish are represented by the class Chondrichthyes. Within this class, there are two sub-classes: the elasmobranchs (sharks, skates and rays) and holocephalans (chimaeras, rat fish and elephant fish), represented by 60 living families, 185 genera and more than 1100 species (Compagno, 2005; Ebert and Compagno, 2007). The main features shared by this class comprise: (i) the simple endoskeleton of calcified cartilage, (ii) four to seven separate internal and external gill openings, (iii) no lungs or swim bladders, (iv) paired copulatory organs in males (claspers) as rearward extensions of the basal skeleton of the pelvic fins, and ensuing internal fertilization, and (v) a dermal skeleton of dermal denticles or placoid scales (toothlike structures with enameloid crowns and dentine bases) (Compagno, 1999). All Chondrichthyans are characterised by a highly K-selected life history, which consists on slow growth rates, late maturation, low fecundity and long generation times. Probably due to the long and independent evolutionary path within this group of species, their reproductive strategies have also evolved to become very diverse. Therefore, Chondrichthyans exhibit all major vertebrate reproductive modes, including: two types of oviparity, differing on the number of embryos and the time they spent inside the mother's body (single and multiple oviparity); and at least four types of viviparity, with different sources of nutrition for the developing embryo(s) (yolk-sac, limited histotrophy, lipid histotrophy, oophagy and placental viviparity) (Musick and Ellis, 2005).

Within the sub-class Elasmobranchii there are three orders that are commonly named together as rays and skates: Rajiformes (rays and skates), Torpediniformes (electric rays) and Myliobatiformes (stingrays). The order Rajiformes is the most diverse, consisting of 27 genera and possibly more than 245 species, assuming a large number of species is yet to be identified (Ebert and Compagno, 2007). It is important to note that, although without taxonomic connotation, in the literature Rajiformes are often termed as rays and skates, or just as skates or rays, based on the main morphological characteristics of the species. In general, skates refer to large species with long snouts, and rays refer to smaller species with short snouts.

Compared to other elasmobranchs, the high degree of species diversity exhibited by rays and skates, which contrasts with their restricted distribution and high number of endemic species, is remarkable given their relatively conservative dorso-ventrally flattened body

Figure 1.1. Skates from Portugal.
From the left to the right: (above) longnosed skate (*Dipturus oxyrinchus*), cuckoo ray (*Leucoraja naevus*), sandy ray (*Leucoraja circularis*), Iberian pigmy skate (*Neoraja iberica*), blonde ray (*Raja brachyura*) and thornback ray (*Raja clavata*); (below) small-eyed ray (*Raja microocellata*), brown ray (*Raja miraletus*), spotted ray (*Raja montagui*),undulate ray (*Raja undulata*), bottlenosed skate (*Rostroraja alba*) and Madeiran ray (*Raja maderensis*).

morphology and apparent restrictive habitat preference (Ebert and Compagno, 2007). Skates occur in all oceans from shallow coastal waters to abyssal regions (up to 3000 m), depending on the species. For those inhabiting the shelf and upper slope, most live on soft bottom, while others might be found in rocky bottoms (Stehmann and Bürkel, 1984; Ebert and Compagno, 2007). Skates are more diverse at higher latitudes and in deeper waters, and generally live in shallower waters towards the poles but prefer deeper depths in warm temperate and tropical regions (Ebert and Compagno, 2007). In mainland Portugal there are records of eleven skate species (Fig. 1.1): longnosed skate (*Dipturus oxyrinchus*), cuckoo ray (*Leucoraja naevus*), sandy ray (*Leucoraja circularis*), Iberian pigmy skate (*Neoraja iberica*), blonde ray (*Raja brachyura*), thornback ray (*Raja clavata*), small-eyed ray (*Raja microocellata*), brown ray (*Raja miraletus*), spotted ray (*Raja montagui*), undulate ray (*Raja undulata*) and bottlenosed skate (*Rostroraja alba*) (Stehmann and Bürkel, 1984; Machado *et al.*, 2004; Figueiredo *et al.*, 2007; Stehmann *et al.*, 2008). *N. iberica* is considered an endemic species from Iberian waters, occurring mainly in the southern Portuguese coast (Stehmann *et al.*, 2008). Other species of skates are known to occur on insular regions of the Portuguese Exclusive Economic Zone (EEZ; Madeira and Azores). These include the endemic species madeiran ray (*Raja maderensis*) (Fig. 1.1; Stehmann and Bürkel, 1984), and more occasionally, and only in the Azores waters, the pale ray (*Bathyraja pallida*), Richardson's ray (*Bathyraja richardsoni*), common skate (*Dipturus batis*), Shagreen ray (*Leucoraja fullonica*), deepwater ray (*Rajella bathyphila*) and Bigelow's ray (*Rajella bigelowi*) (ICES, 2009).

In marine ecosystems, the fishing impact on elasmobranch fish is currently a subject of increasing concern among international authorities (Stevens *et al.*, 2000) due to the consequences of overfishing reported for certain areas. The vulnerability of skate species to exploitation is highly dependent of their k-selected life history traits patterns (Dulvy *et al.*, 2000). The evaluation of skate vulnerability to fishing is commonly based on fishery catch rates. At intensified fishing regions, such as the west coast of the United Kingdom (Irish Sea, Bristol Channel and Celtic Sea) and North Sea, the remarkable stability shown by skate assemblage catch trends, masked population declines of some individual skate species. In that particular situation, larger species showed declined trends in their abundance and were replaced by smaller ones (Dulvy *et al.*, 2000). In those areas, local declines in population abundance caused by an intense use of trawling were not detected until several years after they took place (Walker and Hislop, 1998; Dulvy *et al.*, 2000). Between the most affected species was the common skate *Dipturus batis* (Brander, 1981; Walker and Heessen, 1996; Rogers and Ellis, 2000). In the North Sea, the severe decline of the longnosed skate and the

bottlenosed ray was also reported (Walker and Heessen, 1996). The nearest extinction of the barndoor skate *Dipturus laevis*, from British waters was well documented (Casey and Myers, 1998). Furthermore, the damaging effect of intense trawling exploitation was also reported for the strait of Sicily (Garofalo *et al.*, 2003).

Despite the consequences reported on the impact of intense fishing, the interest on skate fishery is still increasing and in 2006 reached 59% of the total reported landings (in weight) of elasmobranchs in the NE Atlantic, from bottom and pelagic fisheries (FAO, 2007). Worldwide, skates are caught in demersal fisheries (Walker, 1999; Agnew *et al.*, 2000; Ellis *et al.*, 2005). In Portugal, this fishery is mainly composed by the artisanal segment or mixed fishery and by the trawl segment (Machado *et al.*, 2004). Within the artisanal fishery, the main operated fishing gears are gill nets, trammel nets and longline (Machado *et al.*, 2004; Coelho *et al.*, 2005; Baeta *et al.*, 2010). In contrast to some other European countries, like the United Kingdom where localised fisheries target locally abundant mixed skate species assemblages (Walker *et al.*, 1997), in Portugal no skate target fisheries where yet identified. Skates and other elasmobranchs are a significant by-catch component of Portuguese artisanal fishery (Erzini *et al.*, 2002; Heessen, 2003; Coelho *et al.*, 2005; Baeta *et al.*, 2010). In the last decade, the average annual landings of skates in mainland Portugal was around 1500 tons (ICES, 2009), and their average first-sale value was about 3€ per kg (Machado *et al.*, 2004).

The skate status assessment in the Northeast Atlantic is dealt by the International Council for the Exploration of the Seas (ICES) Working Group on Elasmobranch Fishes (WGEF). Annually, this group collect data and information to describe the skate status by ICES area and prepare advice for further consideration by the advisory committees (e.g. ICES, 2007, 2008; ICES, 2009). Amongst the elasmobranchs, skates is the group for which there is less information available on their status , since for most of the species in the ICES areas there is no accurate delineation of stock structure (ICES, 2009). Generally, the WGEF collect updated information on skate landings, fisheries and fishery-independent survey data (e.g. ICES, 2009). In the EC, in addition to ICES, the Scientific, Technical and Economic Committee for Fisheries (STECF) also gives advice on skate status before any action is taken on fisheries management, as part of the EC plan to develop an International Plan of Action for the conservation and management of sharks, which comprises all Chondrichthyes (IPOA-SHARKS; FAO, 1999; Clarke, 2009). The overall objective of the IPOA-SHARKS is to ensure that Chondrichthyes catches from target and non-target fisheries are sustainable, whether they are industrial, artisanal or traditional fisheries. The current fields of action are: (i) the improvement of the identification and reporting, (ii) research-based conservation

measures, (iii) improved stakeholder awareness, (iv) adjustment of fishing effort and catches to available resources and (v) minimization of discards (EC, 2009).

In previous years, advice on skate fisheries was only provided for the North Sea, the only area where stock status assessment was conducted (Clarke, 2009). The overall advice was to reduce by-catch, and to continue to manage skates by a common total allowable catch (TAC) for all species (e.g. ICES, 2007). As advised, the actual EC management measures adopted under the Common Fisheries Policy for skates is a TAC for all skate species combined (e.g. 1974 tonnes for Portugal), even if the correct identification of skates landings at the species level being also demanded (EC No 43/2009, 2009). The on board retention and landing of undulate ray, longnosed skate, bottlenosed skate and common skate is prohibited for most ICES areas (EC No 43/2009, 2009), a measure considered inappropriate since it was adopted with no scientific background for some of the areas, e.g. the Iberian waters (ICES, 2009). Despite the obligation of species identification in landings, misidentification problems still persist (ICES, 2009). Another conservation measure impose regards the use of a minimum mesh size of 280 mm to target fisheries for skates using gillnets (Clarke, 2009).

The thornback ray, *Raja clavata* Linnaeus, 1758, is one of the most frequently landed skate species in the NE Atlantic, both in the north (e.g. Walker *et al.*, 1997; Dulvy *et al.*, 2000) and southern Europe (e.g. Machado *et al.*, 2004; Figueiredo *et al.*, 2007). This coastal species has a wide distribution from shallow waters to 700 m depth on a variety of substrata, occurring from Iceland, Norway and North Sea to South Africa and also in Mediterranean, Baltic and Black Sea (Fig. 1.2; Stehmann and Bürkel, 1984). Although reported for southern Africa, its occurrence in the area is uncertain. The thornback ray is an oviparous species, and despite its species-specific features, all its main life cycle traits are shared with those of other skates (Fig. 1.3). In the North Sea and eastern English Channel, thornback ray populations are relatively sedentary but undertake short migrations towards the coast during the reproductive season (Steven, 1936; Walker *et al.*, 1997; Hunter *et al.*, 2005; Hunter *et al.*, 2006). In the same region, adults move from deep waters to shallower areas where they mate and release the egg capsules. On the other hand, juveniles stay in the area during the first years of development, and then migrate to deeper waters. There are also some evidences that thornback ray could form single-sex aggregations on spawning grounds (Holden, 1975). In

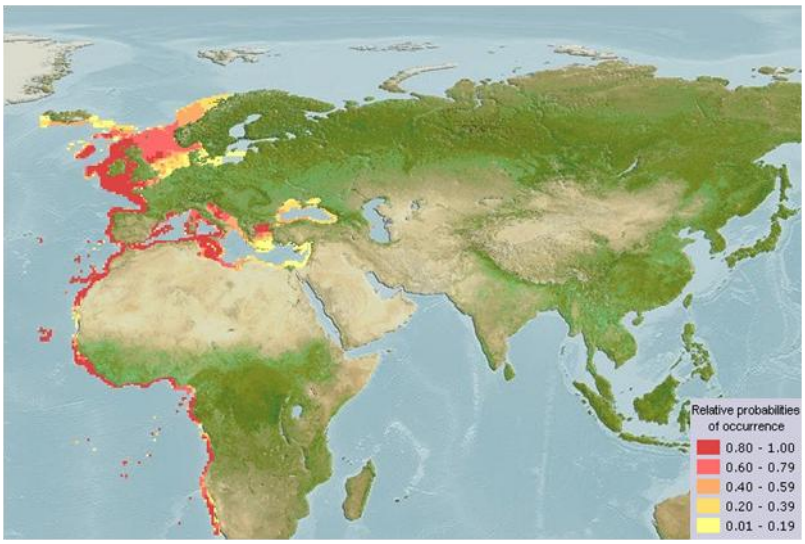

Figure 1.2. Overall distribution of the thornback ray, *Raja clavata*.
(from www.fishbase.org, 13/04/2010).

the British waters, spawning occurs from February to September, with maximum extrusion occurring in June (Holden *et al.*, 1971; Holden, 1975). In the SE Black Sea spawning lasts from May to December (Demirhan *et al.*, 2005). In the North Sea and in British waters, the thornback ray has a length-at-first-maturity of around 80% of its maximum size: females mature between 595 and 771 mm, whereas males mature between 540 and 679 mm (Nottage and Perkins, 1983; Ryland and Ajayi, 1984; Walker, 1999). Additionally, in British waters, estimates of fecundity range from 60 (Ryland and Ajayi, 1984) to 150 eggs per female per year (Holden *et al.*, 1971), and the egg-laying rate is constant during the peak of the spawning season with usually one pair of egg capsules laid in two consecutive days (Holden *et al.*, 1971; Ellis and Shackley, 1995). As in all skates, the embryo develops inside the egg capsule using yolk reserves for nourishment. For the thornback ray, the incubation time is estimated to be between 4.5 to 5.5 months, and the newborn hatch with a total length of 110-137 mm (Clark, 1922). In British waters, adult females and males can reach at least 1070 and 1016 mm, respectively (Holden, 1972; Nottage and Perkins, 1983). Regarding its diet, in northern Europe, the thornback ray has shrimps and brachyuran crabs as their main prey items (Holden and Tucker, 1974; Ajayi, 1982; Ellis *et al.*, 1996).

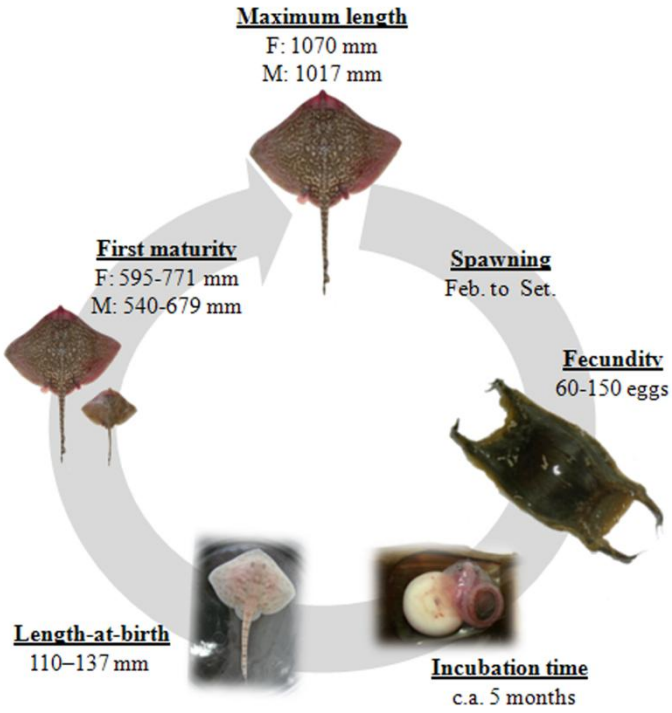

Figure 1.3. Thornback ray reproductive cycle.

1.1. Objectives

Skates have been increasingly exploited in recent decades despite their recognized vulnerability to fishing, and regardless of a lack of basic knowledge about their biology. This fact applies particularly to Southern Europe, where studies on the impact of fishing over skates' populations, and on the biology of some species, have only started to be developed in the last decade. In contrast, in the north of Europe (mainly in the UK and North Sea areas) these type of studies were carried out since the middle of the last century (e.g. Holden, 1972; Holden and Tucker, 1974; Holden, 1975; Walker, 1999; Hunter *et al.*, 2005). Besides taxonomic and systematic questions, high priority should also be given to address conservation issues of skate biodiversity. The present thesis intends to contribute to a great advance in some of these issues, focusing on the skates occurring in Portuguese waters. The main aims of this thesis focused on three issues: skate fisheries, skate biodiversity and skate life-history. In each of those issues, the general questions of interest were:

A. Skate fisheries

(i) How and how much are skates species landed in Portuguese landing ports?

(ii) Is it possible to discriminate fishing strategies within the fisheries that are catching skates?

(iii) Do identification problems still persist after the application of the EU legislation regarding skate landings discrimination by species?

B. Skate biodiversity

(iv) How diverse are the Portuguese waters in terms of skate species?

(v) Are molecular markers and body morphometry adequate to discriminate between skate species?

(vi) How diverse are the feeding habits of skates in Portuguese waters and what is their relationship with prey diversity?

C. Skate life-history – case study of the thornback ray

(vii) How different is the life strategy (growth and reproduction) of the species in Portuguese waters in relation to other areas around Europe?

(viii) How adequate are dermal denticles as ageing structures? Are the resultant age readings more accurate than those obtained with vertebrae?

(ix) Can we apply the reproductive terminology used for teleosts to describe the different reproductive phases of elasmobranch oviparous species, for example the thornback ray?

(x) Does length of first maturity differ between males and females?

(xi) Is the species a determined or indeterminate spawner? What is the mean fecundity?

(xii) What are the main physiological processes involved with maturation, egg encapsulation and extrusion?

1.2. Outline of the thesis

The thesis is presented as a compilation of eight scientific publications (two published articles, two in press and four submitted/under revision, in international journals with peer review), which directly address the objectives proposed, and is organized in five chapters.

In Chapter 2 the Portuguese fishery landing skates is characterized. The tendency of stability of the aggregated skate's landings was investigated using data from landing ports

along the Portuguese coast, from 1991 to 2005. Species identification problems in skate landings were identified. Furthermore, the mixed-nature of the fishery was described through the identification of possible Fishing Strategies, taken the example of the most important Portuguese landing port, in terms of skate's landings.

The biodiversity of skates in Portuguese waters is analyzed in Chapter 3, and includes three studies. The first study of this chapter was the first to present results on the ability of the mitochondrial gene cytochrome c oxidase subunit I to discriminate between the 12 skate species occurring in Portuguese waters (Portugal mainland, Madeira and Azores). The second study analysed the ability of body morphometry to discriminate between species. It also showed, for the first time, a compilation of size conversion factors (i.e. relationships of different body measurements) for the most important species occurring in Portuguese waters. The capability to solve discrimination problems between morphological similar *taxa*, like the two pairs of species, *R. montagui* and *R. brachyura*, and *R. clavata* and *R. maderensis* was highlighted in these two studies. The third study of this chapter focused on comparing the diet composition and feeding habits of four of the most important rajid species in Portuguese waters, including the thornback ray. An interesting dietary ontogenetic shift in all of the analysed species is described.

Chapter 4 is focused on study of the main biological traits of the thornback ray inhabiting the Portuguese waters. This chapter is subdivided into two sections: (i) age and growth; and (ii) reproduction (containing three sub-sections). In the first section a study is presented where, for the first time, dermal denticles are used for ageing thornback ray, and which proved that they are more accurate as ageing tools than vertebrae. Different processing techniques and reading methods were tested, and the different types of dermal denticles found in the thornback ray were described. The growth function that best describes thornback ray growth in Portuguese waters was selected. In the first sub-section about reproduction a maturity scale is proposed. This scale was adapted from the recent reproductive terminology for teleosts (Brown-Peterson *et al.*, in press) to oviparous elasmobranchs using the thornback ray as an example. A macroscopic and microscopic analysis describing development of the reproductive tract and the process of gametogenesis throughout the different reproductive phases was also performed and is included in this sub-section. In the second sub-section on reproduction, the main reproductive features of thornback ray in Portuguese waters were analyzed for the first time. The reproductive cycle was characterized, based on the alteration of the main reproductive organs with maturation. The duration of the maturation, mating and spawning seasons were determined, and the maturity ogives and fecundity were estimated.

Lastly, in the third subsection on reproduction, the processes underlying the oviducal gland development (organ responsible for the egg encapsulation), from the beginning of the differentiation to the extrusion of the egg capsules, were analysed utilizing histological and histochemical techniques. This was also a pioneering study once little was known about oviducal gland development.

To conclude, Chapter 5 presents a cohesive overview of the results obtained and a general discussion of the main issues associated with the conservation and assessment of skates.

Chapter 2

Skate fisheries

2. SKATE FISHERIES[1]

2.1. Abstract

In Portuguese continental ports, skates are caught by multispecies fisheries targeting other species. In recent years landing port authorities tried to separate skate landings by species, however identification problems persisted. Results from landing sampling program allows to conclude that 96% of landings assigned to cuckoo ray were correct, while for blonde ray and thornback ray these percentages were 54% and 0% respectively. Despite total annual landings of skates have been stable along years the sampling program showed difference on the landings between species. The blonde ray was the most abundant species in landings, followed by the thornback ray and undulate ray. Peniche was the major landing port for skates and it was selected to develop an approach for identifying fishing strategies. Based on composition of the landed species by fishing trip available from the sampling program six fishing strategies were identified. Each strategy was further characterized based on the vessel characteristics, type fishing gear, on main landed species, and species composition of skates. Among those strategies there was one targeting skates and in which large-mesh sized trammel net was the fishing gear. The direct application of this study would be the possibility to estimate the fishing effort (number of fishing trips) by skates, even when the information of skate landings at the species level is absent, since each FS would be characterize by a given skate species composition directly associated to the remaining species caught.

Keywords: artisanal fleet; cluster analysis; skate fishery; fishing strategies; Portugal; Rajidae.

2.2. Introduction

In recent years the interest on the commercial exploitation of rays and skates has increased in the NE Atlantic, probably as result on the current exploitation status of many teleosts

[1] Serra-Pereira, B., Figueiredo, I., Farias, I., Moura, T., Nunes C. and Gordo, L. S. Submitted. Fishing strategies of an artisanal Portuguese mixed-fishery landing skates (Rajidae). Journal of Applied Ichthyology.

traditionally exploited (Stehmann, 2002; Gallagher *et al.*, 2004). They represent over 40% of elasmobranch landings, reaching 59% in 2006 (FAO, 2007). Since several years, the ICES Working Group on Elasmobranch Fishes (WGEF) (ICES, 2008), highlights the difficulties in compiling assessment data on elasmobranchs, partially due to inadequate species-specific landing information, and lack of information on stock identity. The group acknowledged that a better sampling base must be established. Since 1994, the Portuguese Fisheries and Aquaculture General Directorate (Direcção-Geral das Pescas e Aquicultura, DGPA) has attempted to segregate skates by species in Portuguese landing ports, but segregation problems persist. Skates landings are also separated according to a commercial strategy which includes specimens' size (bigger species tend to have a higher value), and freshness.

Till recently, EU has not adopted any specific management measures for rays and skates. However in 2009 EU set Total Allowable Catch (TAC)'s for Rajidae together with obligation of Members States to report landings by species (Council regulation EC No 43/2009, 2009). The on board retention of undulate ray (*Raja undulata*), common skate (*Dipturus batis*) and bottlenosed skate (*Rostroraja alba*) is also prohibited, so fishers have the obligation to promptly release unharmed all specimens to the extent practicable, through the use of techniques and equipment that facilitate the rapid and safe release of this species.

In general, aggregated catch statistics for skates exhibit stable patterns (masking species specific declines) and tend to be disregarded in favor of other species displaying obvious sustainability problems (Dulvy *et al.*, 2000). But in reality, declines in larger species are accompanied by increase of smaller species in the community. Different skate species tend to occupy the same habitats, so that their geographical distribution is often overlapped (Walker *et al.*, 1997; Figueiredo *et al.*, 2007), as well as their feeding habits (Ellis et al., 1996; Farias et al., 2006). Skates are also known to be relatively sedentary, live in local concentrations with regular exchange of individuals, so that the majority of the species only undergo migrations for short distances (Walker *et al.*, 1997). For some species (e.g. thornback ray *Raja clavata*, spotted ray *Raja montagui*) juveniles tend to live in shallow coastal waters, whereas adults can move to more offshore areas (Walker *et al.*, 1997; Hunter *et al.*, 2005). Due to their relatively fixed distribution, their occurrence can be inferred based on associate species assemblages (Figueiredo *et al.*, 2007).

Skates are mainly landed as by-catch from various fisheries: demersal otter-trawl, gillnet and longline fisheries (Agnew *et al.*, 2000). Yet, there are some localised fisheries, e.g. off the British coast, targeting locally abundant species from the wider spatial rajid assemblage (Walker *et al.*, 1997). Since, rajid species are often so closely associated ecologically, it is not common to target one species in the fishery (Agnew *et al.*, 2000), so that they are often caught by mixed-fisheries. By definition, in mixed-fisheries it is sometimes difficult to identify if there are target species, since a wide range of species (varying with season and availability) are exploited by a given gear type (Jiménez *et al.*, 2004). Mixed fisheries may have different strategies according to vessel characteristics, local conditions, gear type, fishing ground and market demand.

The main objective of this study is to make a preliminary characterization of the Portuguese mixed-fishery landing skates. Using the information collected so far in landing ports at the fishing trip level, we aim to identify Fishing Strategies (FS, defined as a group of fishing trips operating similar fishing gears and landing a similar composition of species) based on the main species composition of landings (main target species) associated with the skates species further identified. The direct application of this study would be the possibility to estimate the fishing effort (number of fishing trips) by species, even when the information of skate landings at the species level is absent, since each FS would be characterize by a given skate species composition directly associated to the remaining species caught.

2.3. Material and Methods

2.3.1. Landing port selection

Data on annual landings (tonnes) of skates, from fishing ports along the Portuguese continental coast were provided by the DGPA for the period 1991–2009. Ports were divided into three regions (North, Central and South; see Figure 2.1). The port with the highest landings of skates was selected for this study. This was based on ports annual landed weight of "skates" against the total annual landed weight of "skates", plotted against year by region.

2.3.2. Skates landings

In order to understand and qualify identification problems currently occurring in Portuguese landings, skates landings were sampled in Peniche, between 2006 and 2008. Trips from the artisanal fleet were selected, and the landed species were identified. The commercial species categories (assigned by the DGPA based on the most abundant species) were also recorded: cuckoo ray, blonde ray, thornback ray and spotted ray. The annual landed weight by species was estimated for the period 2003 to 2008, based on the specific composition of skates landings, collected under the scope of the National Data Collection Program (PNAB, DCR), extrapolated to the combined annual landings, provided by the DGPA.

Sampling for the identification of FS was conducted at the selected landing port, on a monthly basis between January 2006 and July 2008, in the scope of PNAB, DCR. In each visit fishing vessels with landings of skates (designated as "fishing trips") were selected. From each fishing trip the name of the vessel, date of sampling, operated fishing gear(s) (provided by the fishermen), total landed weight and the landed weight by species were recorded. Each fish box containing skates was sampled, recording species, sex, total length, total weight and size category. Four size categories (T_1–T_4) were assigned by the fishing authorities, with T_1 and T_4 representing the largest and smallest size categories, respectively.

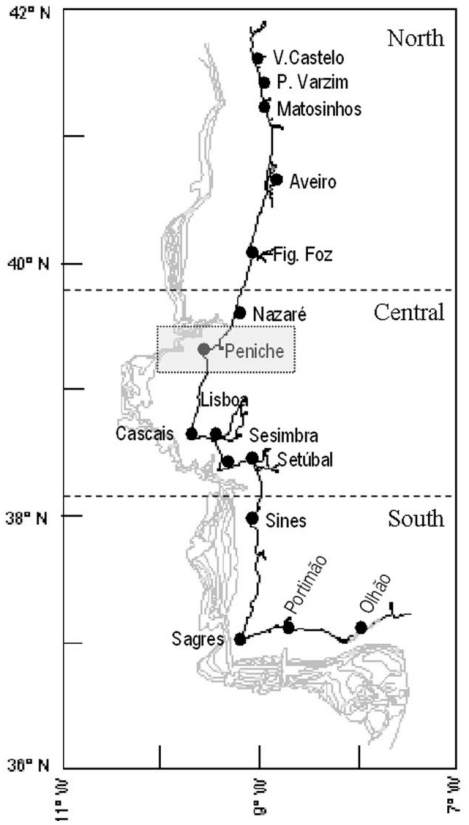

Figure 2.1. Location of the major ray and skate landing ports in mainland Portugal.
The sampling program was conducted in Peniche port, shaded.

2.3.3. Characterization of Fishing Strategies

The identification of possible FS using k-means clustering (Hartigan and Wong, 1979) was achieved through the allocation of each data record to one of the clusters to minimize the within cluster sum of squares. The first step was to identify, within our sampled trips, the top 15 landed commercial species (including groups of species) in terms of weight and/or percentage of occurrence. Next, our data was compiled to be further used in the cluster analysis, considering the following variables: (i) relative weight of the selected commercial species (species landed weight in relation to the total landed weight by trip); (ii) relative weight of each size category; and (iii) proportion of each species (species landed weight in relation to the total weight of skates, by trip). This data should provide the importance of each species or commercial species categories by FS, independently of the landed quantity. To select the most appropriate number of clusters Hartigan's test (Hartigan and Wong, 1979) was applied based on the comparison of explained variance between successive numbers of clusters. After identifying clusters, the FS were further characterized, taking into consideration: (i) vessel characteristics, (ii) fishing gears used, (iii) total landed weight, (iv) main landed species, and e) skate species identified in landings.

2.4. Results

Between 1991 and 2009 the total annual landings of "skates" remained quite stable around 1600 tonnes (Fig. 2.2). Value increased over the period until 2001, becoming relatively steady during the remaining years. In 2005 the maximum value was reached, almost 4.2 million Euros, representing an average of 2.5 Euro per kilo.

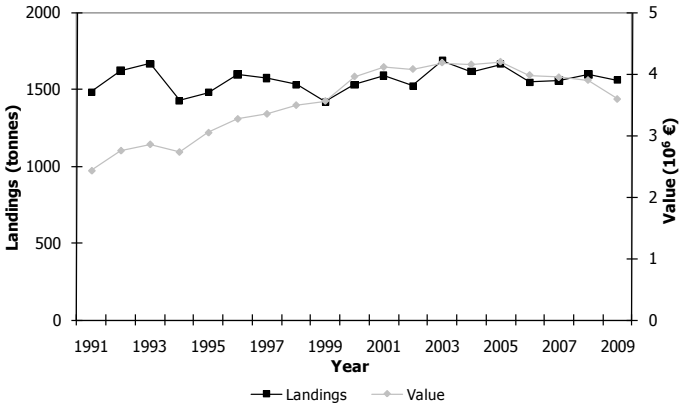

Figure 2.2. Portuguese annual landings (1991-2009) of the generic category "skates", total weight (tonnes) and value per kg (€).

2.4.1. Landing port selection

To select the target port for the sampling program, annual landings, from 1991 to 2005, were analysed for a total of 15 ports in mainland Portugal, with these ports accounting for approximately 84% of the Portuguese total annual "skates" landings for the period of our analysis. The annual landed weight of "skates" by port has been relatively stable in recent years (Fig. 2.3). Matosinhos was the most important port in the northern sector, with annual landings varying from 94–199 t, which represented <10% of the total "skates" landings. Skates were less important in the landings at ports in the southern sector (usually <5% of the total "skates" landings). Peniche was the most important in the central sector, with mean annual skate landings of 362 ± 32 t, and accounting for ca. 25% of the total "skates" landings. Hence, Peniche was selected for this study.

2.4.2. Skates landings

A total of nine species of skates were identified in landings (Table 2.1). The correspondence between the species categories assigned by the fishing authorities and the identified skate species landed is presented in Table 2.2a. In Peniche, during the sampling program, the landed category 'spotted ray' didn't occur. Within the remaining categories, species identification was not correctly made, apart from the cuckoo ray category, about 96% of which were identified correctly. Concerning the 'blonde ray category', only 54% were correctly identified, and five other species occurred in the mixture, of which thornback ray was the most frequent (24%). No specimens of thornback ray were observed in the 'thornback ray category', instead two large-bodied species were observed, longnosed skate (87%) and bottlenose skate (13%). Overall, nine skate species were observed in Peniche, the blonde ray was the most frequently landed species (50%), followed by thornback ray (20%) (Table 2.2b).

The extrapolated annual landings of the six most abundant skate species (based on our own sampling) for the period 2003 to 2008 are presented in Figure 2.4. The blonde ray was previously the most abundant species however landings have declined, from ~800 tonnes in 2003, to ~500 in 2008. The thornback ray shows the opposite trend, becoming the most abundant species in 2008, increasing from ~300 tonnes to ~600 tonnes. Undulate ray landings have fluctuated around 100 tonnes, with an increase between 2003 and 2006, but declined since. The remaining species, the cuckoo ray, spotted ray and small-eyed ray, showed landings fluctuating below 150 tonnes in most recent years, with a decreasing trend.

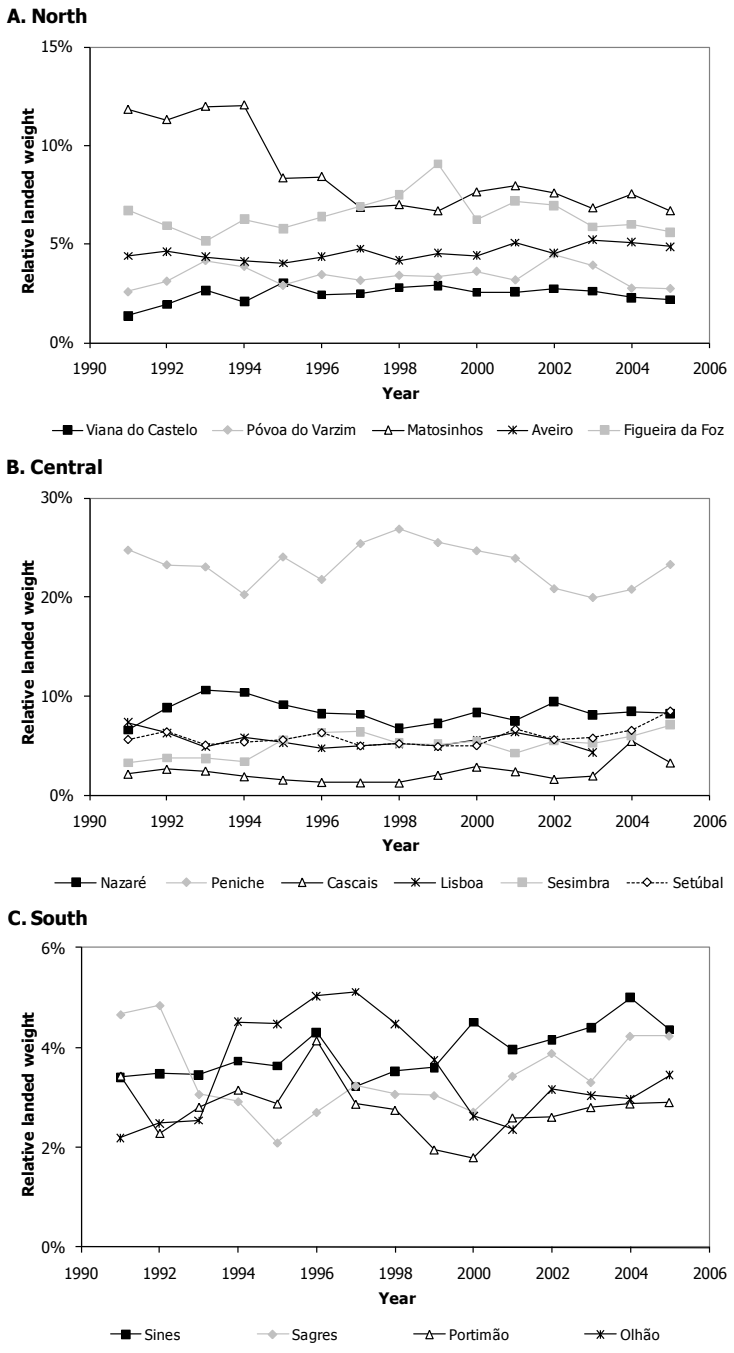

Figure 2.3. Trends in ray and skate landings (relative to total skate landings) by fishing ports in the northern, central and southern sectors.

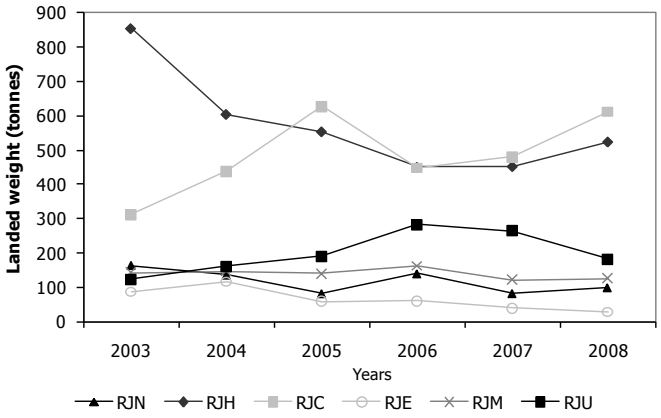

Figure 2.4. Estimated annual landed weight (tonnes) of the 6 most abundant skate species (see Table 2.1 for abbreviations used).

Table 2.1. List of skate species and of the most abundant species present in skate landings.
(FAO 3-alpha code, common name and scientific name).

FAO	Common name	Scientific name
RJO	Longnosed skate	*Dipturus oxyrinchus* (L.)
RJN	Cuckoo ray	*Leucoraja naevus* (Müller & Henle, 1841)
RJH	Blonde ray	*Raja brachyura* Lafont, 1873
RJC	Thornback ray	*Raja clavata* L.
RJE	Small-eyed ray	*Raja microocellata* Montagu, 1818
RJM	Spotted ray	*Raja montagui* Fowler, 1910
RJU	Undulate ray	*Raja undulata* Lacepède, 1802
RJA	Bottlenosed skate	*Rostroraja alba* (Lacepède, 1803)
SKA	Skates	Rajidae
MGR	Meagre	*Argyrosomus regius* (Asso, 1801)
COE	European conger	*Conger conger* L.
BSS	European seabass	*Dicentrarchus labrax* (L.)
CTB	Common two-banded seabream	*Diplodus vulgaris* Geoffroy Saint-Hilaire, 1817)
MNZ	Anglerfish	*Lophius* spp. L.
HKE	European hake	*Merluccius merluccius* (L.)
THS	Thickback soles	*Microchirus* spp.
MUR	Striped red mullet	*Mullus surmuletus* L.
OCC	Common octopus	*Octopus vulgaris* Cuvier, 1797
SBA	Axillary seabream	*Pagellus acarne* (Risso, 1827)
FOR	Forkbeard	*Phycis phycis* (Linnaeus, 1766)
TUR	Turbot	*Psetta maxima* (L.)
SYC	Lesser spotted dogfish	*Scyliorhinus canicula* (L.)
CTC	Common cuttlefish	*Sepia officinalis* L.
SOL	Common sole	*Solea solea* (L.)
HOM	Atlantic horse mackerel	*Trachurus trachurus* (L.)
BIB	Pouting	*Trisopterus luscus* (L.)
JOD	John dory	*Zeus faber* L.

Table 2.2. Sampled skate species landed into Peniche.
a) species composition within the species categories assigned by the fishing authorities (Blonde-ray, *Raja brachyura*; Thornback-ray, *Raja clavata*; and Cuckoo-ray, *Leucoraja naevus*); b) contribution of each species to total landings.

	A. Commercial species categories (%)			**B. Landed species (%)**
Landed Species	Blonde-ray	Thornback-ray	Cuckoo-ray	
Longnosed skate	0	87	0	1
Cuckoo ray	1	0	96	4
Blonde ray	54	0	0	52
Thornback ray	24	0	0	22
Small-eyed ray	7	0	0	6
Brown ray	0	0	1	0
Spotted ray	7	0	3	7
Undulate ray	7	0	0	7
Bottlenosed skate	0	13	0	0

2.4.3. Characterization of Fishing Strategies

A total of 252 fishing trips were sampled from the artisanal fleet of Peniche with landings of skates. Within the sampled trips, five types of fishing gears were operated: trammel net with meshes <200 mm, trammel net with meshes >200 mm, gillnets, longlines and pots. One or more active fishing gears may have been used during a fishing trip and, depending on vessel characteristics, fishing trips could be one or more days.

From a total of 76 commercial species categories, the following 19 were selected as the most abundant species in landings to be used in the cluster analysis (Table 2.1). These species are in addition to the nine skate species identified from port sampling and their four associated size categories. This resulted in a total of 32 input variables used in the clusters analysis.

Hartigan´s test applied to the results of K-means suggest that the use of six clusters [p-value: 9.8×10^{-7} (clusters 5 *vs.* 6); 1.00 (clusters 6 *vs. 7*)] was appropriate. The six clusters were then characterized as FS. Table 2.3 details their main features. The relative abundance in landings of the selected 19 commercial species categories and the nine species, in each fishing segment, are presented in Figures 2.5 and 2.6, accordingly.

2.4.4. Description of fishing strategies

FS1 was a longline fishery undertaken by small sized vessels (6–9 m). Large amounts of European conger were landed, representing ~70-80% of the total landed weight. Most of the skates landed were of large size, and the most frequent species were the blonde ray and the spotted ray.

Table 2.3. Characterization of the Fishing Strategies.
Based on: a) vessels characteristics (length, TAB and power); b) types of fishing gear used by FS (percentage of trips); c) total landed weight (25-75% quartiles); and d) skates landings in weight and percentage of each size category (25-75% quartiles).

	Fishing Strategy					
	1	**2**	**3**	**4**	**5**	**6**
n	14	29	60	36	41	66
Sampled vessels	9	16	29	22	16	27
Vessels characteristics						
Vessel length (m)	5.9 - 9.1	9.1 - 23	6.7 - 23	5.2 - 18	7.7 - 21.1	3.4 - 18
TAB	2.0 - 6.5	6.5 - 89.5	3.9 - 83.5	1.8 - 46.8	3.6 - 55.9	3.3 - 46.8
Power (hp)	27 - 73	36 - 440	36 - 435	20 - 250	36 - 440	360 - 250
Fishing Gear (%)						
Trammel net <200mm	0	69	62	75	83	77
Trammel net >200mm	0	28	33	25	46	18
Gillnet	0	28	8	3	2	9
Longline	100	10	18	6	2	5
Pots	0	24	35	36	10	32
Landed weight						
Total	106 - 372	96 - 547	96 - 273	45 - 136	51 - 161	73 - 199
Skates landings						
Skates (kg)	14.6 - 27.3	19.6 - 30.9	19.6 - 89.6	8.9 - 59.9	35.0 - 104.0	12.9 - 42.3
% T_1	42 - 75	22 - 41	8 - 38	16 - 60	25 - 52	28 - 72
% T_2	20 - 57	14 - 52	15- 43	27 - 42	19 - 44	22 - 48
% T_3	18 - 34	27 - 73	22 - 43	30 - 46	11 - 30	16 - 37
% T_4	19 - 26	40 - 88	26 - 59	21 - 51	13 - 27	20 - 45
Within cluster sum of squares	2.43	13.48	17.56	18.08	9.05	15.51

FS2 was a multi-gear group, with 69% of the trips included in this FS using trammel nets with small mesh alone or combined with other fishing gears. This FS contained the greatest landings (100-550 kg). The species landing composition was mixed because different gears were used. Common octopus (5-50%), John dory (5-35%) and anglerfish (5-40%) were the most abundant. Regarding skates, this FS was characterised by the presence of small size categories with important landings of cuckoo ray (25-75%) and spotted ray (30-70%).

FS3 was a multi-gear group, similar to FS2. It differed in the importance of skates, in this FS skates are the most abundant commercial species group (10-50%, opposing to the 5-15% from FS2). This FS also demonstrates a high abundance of thornback ray (60-90%). Common octopus was the second most abundant commercial category in landings (10-40%).

FS4 was mainly a trammel net fishery (both small and large mesh), with combined use of pots. The most abundant commercial species categories were skates (15-50%), European seabass (5-35%), common octopus (10-25%) and meagre (5-20%). In terms of skate species, longnosed skate, small-eyed ray and undulate ray attained the highest abundances, comparing with the remaining FSs.

FS5 used mainly trammel nets (85%) and particularly those of large-mesh sizes (46%), which are used to capture large skates (size categories T_1 and T_2). As a consequence this FS

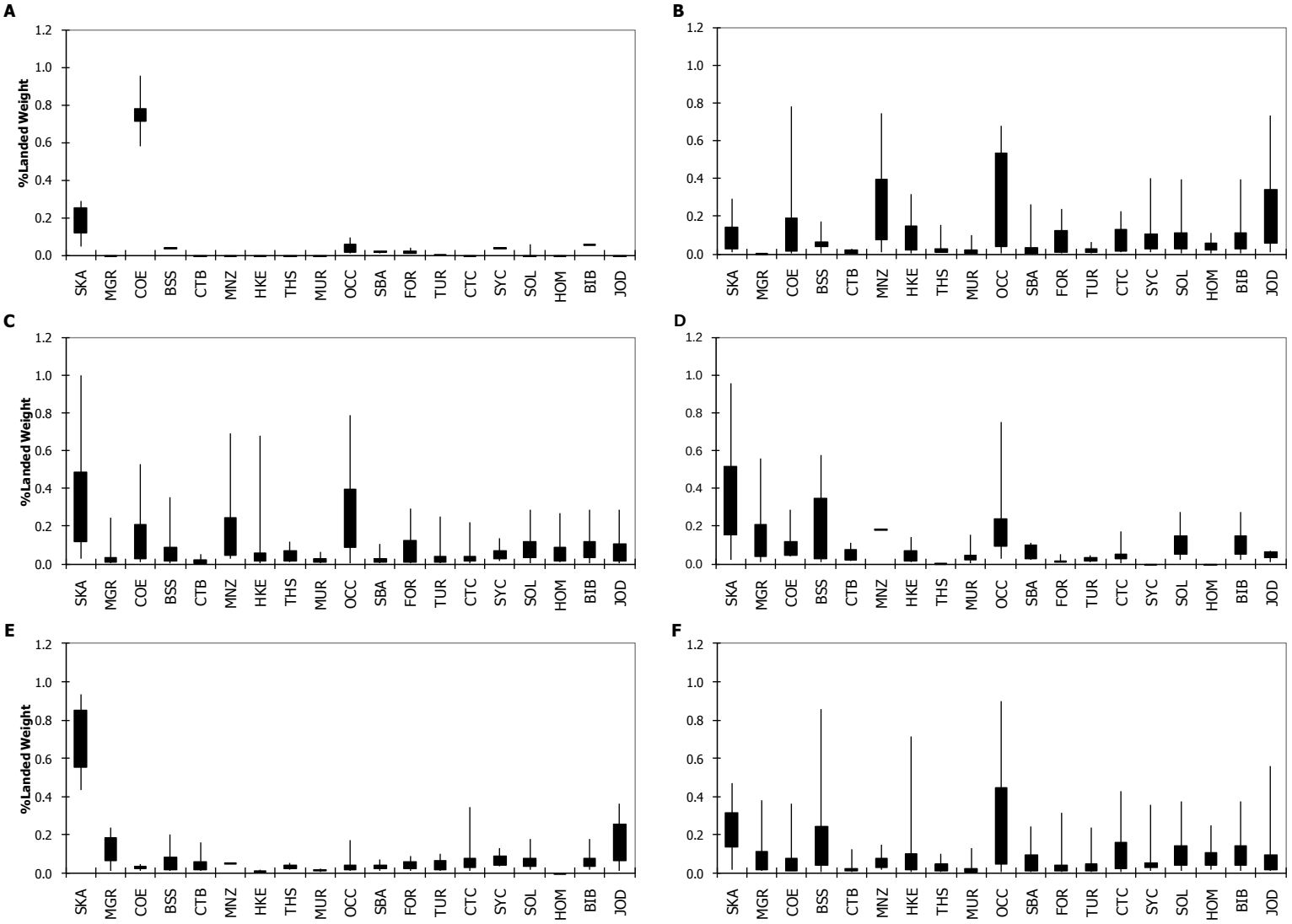

Figure 2.5. Percentage landed weight of the 19 selected commercial species (see Table 1 for abbreviations used) in each Fishing Segment (FS).
a) FS1; b) FS2; c) FS3; d) FS4; e) FS5; and f) FS6.

presented the highest abundances of skates (55-85%), of which the blonde ray is the most important (60-90%).

FS6 operated 77% with trammel nets with small mesh size, used alone or combined with pots. Those nets seem to be mainly used to catch common sole (5-20%), European seabass (5-25%) and skates (15-30%). Pots are responsible for the presence of common cuttlefish, common octopus (5-45%) and pouting (5-15%) in the landings. The most abundant skate species was blonde ray (70-100%), and consequently the large size category was also abundant (30-70%).

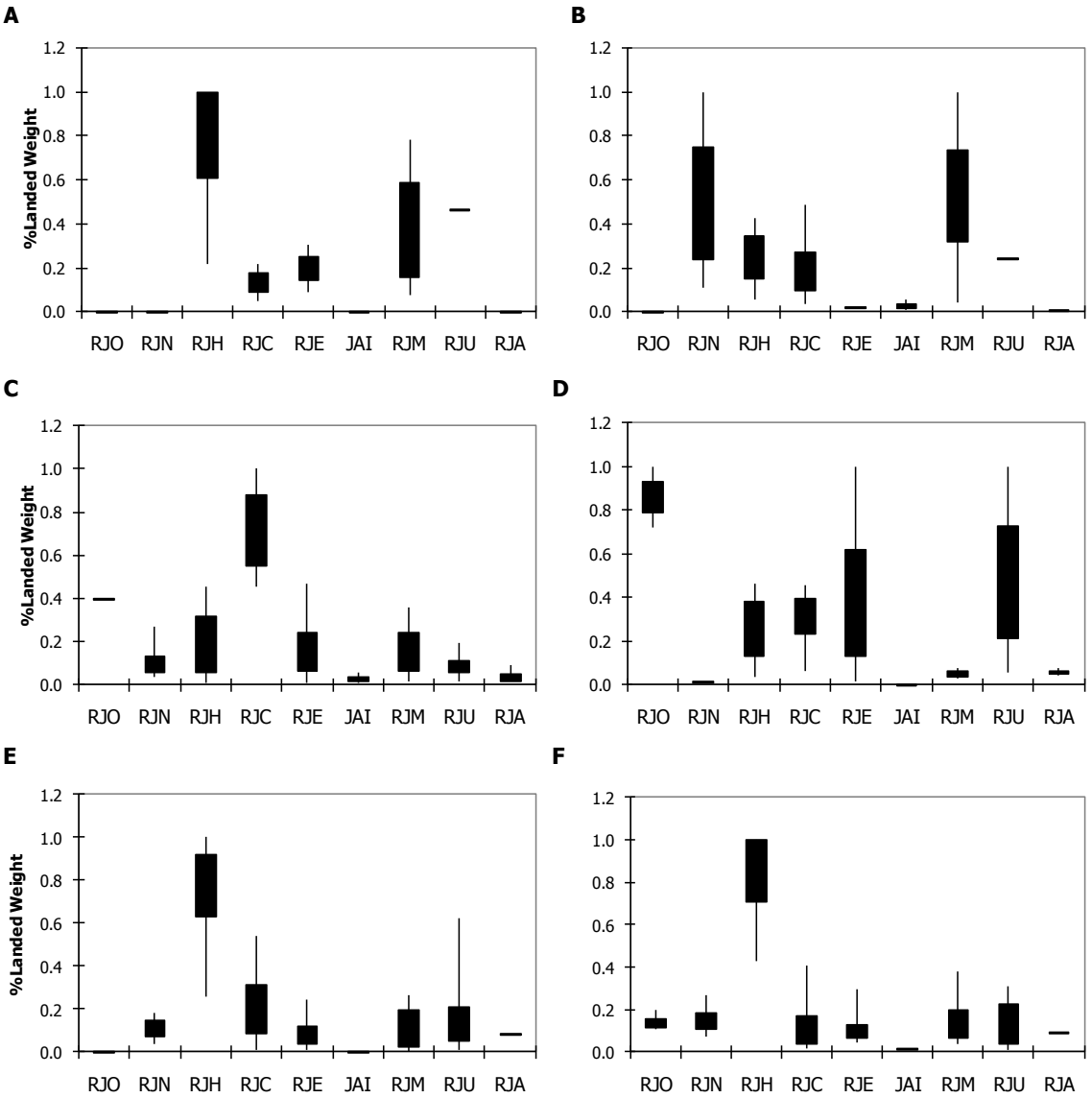

Figure 2.6. Percentage landed weight of the 9 ray and skate species (see Table 1 for abbreviations used) in each Fishing Segment (FS).
a) FS1; b) FS2; c) FS3; d) FS4; e) FS5; and f) FS6.

2.5. Discussion

Portuguese landings of the aggregated commercial species category "skates" have been stable (around 1600 tonnes) at least since the early '90s. The same trend has already been described for other areas, such as the North Sea (Walker and Heessen, 1996), UK (Dulvy *et al.*, 2000; Rogers and Ellis, 2000) and Mediterranean (Garofalo *et al.*, 2003). Following the same trend as seen in the Northern European countries (Dulvy *et al.*, 2000), the monetary value of catches showed a 1.5 fold increase from 1991 to 2005, when an average of 2.5 €.kg^{-1} was reached.

Despite the great effort of each EU member state to improve the data quality, some landings are still not discriminated by skate species (ICES, 2008). The segregation of "skates" landings in Portuguese ports, was made mainly by size categories (from small specimens, T_4, to larger specimens, T_1), regardless of the creation of commercial species categories (cuckoo ray, blonde ray and thornback ray). The cuckoo ray was the only species that was well identified and landed separately (96%). This fact could be related to its small size and the soft consistency of the flesh, in comparison to other species, which confers a reduced commercial value. The other landing species categories showed major identification problems, with the 'blonde ray category' seemingly used for a variety of coastal species, and the 'thornback ray category' containing more offshore species. The results obtained so far reinforce the need for proper training and user-friendly identification guides if reliable species-specific landings data are to be collected.

It is important to note that oscillations in species-specific landings observed in the present study were not analysed using a long term data-set, and large scale conclusions cannot be inferred. Nevertheless, the skate species occurring in Portuguese landings were identified. The blonde ray (*Raja brachyura*) was the most abundant species (500 and 800 tonnes), followed by the thornback ray (*Raja clavata*) (300 to 600 tonnes). The third most common species was the undulate ray (*Raja undulata*) (100 and 300 tonnes), and the remaining species, the cuckoo ray (*Leucoraja naevus*), spotted ray (*Raja montagui*) and small-eyed ray (*Raja microocellata*), were landed below 150 tonnes. The skate species composition in Portuguese landings seems to be similar to that observed in British waters, but quantitatively the thornback ray is largely the most important species in that area, c.a. 10% more abundant than the blonde ray (Dulvy *et al.*, 2000). The increasing importance of thornback ray in landings was also described for the strait of Sicily (Garofalo *et al.*, 2003). Undulate ray and

small-eyed ray seems to be more typically distributed along southern areas (Stehmann and Bürkel, 1984).

Skate landings in Portugal are mainly due to the artisanal fleet. Considering Peniche as the landing port model and representative for the whole country, the fleet is characterized by small size vessels with an average size of 12 m (5 to 23 m) operating near the coastline, using a variety of fishing gear types: trammel nets with different mesh sizes, gillnets, longline and pots. These fishing gears can be used alone or in combination in the same fishing trip, and could vary through the year for a given vessel. In contrast to other fleets, such as in the Irish Sea (Gallagher *et al.*, 2004), the proportion of skates landed by the otter trawl fleet (25%) is less than those from the artisanal fleet (75%) (Machado *et al.*, 2004).

The cluster analysis applied in the present paper proved to be adequate and consistent with other studies (ICES, 2003; Holley and Marchal, 2004), allowing the distinction of clusters that represent distinct fishing strategies (FSs), with minimal overlap between them. It is important to stress that this study had a simple objective of make a preliminary characterization of a local fishery landing skates. The 252 fishing trips used to characterize the fishery represents only a small portion of the annual landings, but due to the coverage throughout the year the months and the large number of vessels (63) analysed, they can be considered representative of the landings. In the future, this methodology may be extended to the remaining fishing ports, and consequently use those FSs as a sampling unit to estimate fishing effort for skates, instead of having a combined fishing effort (e.g. number of trips with skates landings). Since a given FS was characterized by an association of target species and skates species, a direct relationship with the main skate assemblage to each FS is possible to obtain, and therefore the universe of sampled trips could be extrapolated through a discriminant rule to the remaining fishing trips with the same characteristics (following Figueiredo *et al.*, 2007).

Trips with landings composed mainly by skates and rays should be carefully analysed because they represent the fleet segment that targets skates and rays, and therefore classified in the FS5. These trips, typically operating with large mesh trammel nets, are associated with a large quantity of blonde ray and also by the capture of other species, such as thornback ray, spotted ray and undulate ray. Trips operating with longline landing large quantities of European conger will be possibly classified as FS1. This FS will be used to estimate the fishing effort on large sized skates, mostly blonde ray. Fishing trips with large landings of European Seabass and meagre associate with the largest landings of undulate ray and small-

eyed ray are classified in FS4. The skate species landed by this FS indicates that most of the fishing trips were operated close to shore, with sandy substrate (Stehmann and Bürkel, 1984). Fishing trips operating with trammel nets and pots landing common octopus and European Seabass associated with large landings of blonde ray are classified in FS6. Finally, Multi-gear trips will be classified wither as FS2 or FS3. The first have the largest landings of anglerfish, common octopus and John Dory which are associated with large landings of cuckoo ray and spotted ray. This FS had also the greatest total landings (between 100 and 550 kg per trip), which could indicate the possible inclusion of multi-day trips (more than one day of fishing), possibly to deeper areas, suggested by the skate species composition. FS3 have a similar species composition to FS2, but it differs on the skate species composition, having the largest landings of thornback ray.

In conclusion, the cluster methodology used to define the FS of the fleets proved to be a good methodological approach. Moreover, this paper also contributes to increase the knowledge on Southern European skates fishery, mainly an artisanal mixed-fishery, which has very different characteristics from the better studied Irish Sea or North Sea fisheries. Since it was a preliminary study, it will be necessary to continue the sampling effort in ports to improve data quality and to see if future modification in FS occurs as a consequence of changes in the stocks abundance, and in local or international regulations. Inquiries to obtain more information on the fishing trips, mainly fishing grounds, depth, and time of setting and hauling of the gear, are already being applied. Another step should be to estimate/extrapolate landings to more detailed single species datasets, extend the knowledge to the remaining landing ports, obtain a standard characterization of the fleet by FS, identify which contributes to the main skate species landings and finally estimate the fishing effort by species. These procedures will be used for scientific advice and will facilitate the proper management strategies for skates on a species-specific base.

Chapter 3

Skate biodiversity

3. SKATE BIODIVERSITY

3.1. PHYLOGENY [1]

3.1.1. Abstract

Due to their vulnerability to fishing pressure, many species of skate (Rajidae) in the Northeast Atlantic are undergoing declines in abundance. The assessment of stock status and the subsequent proposal of management measures are quite often complicated by high levels of species diversity and endemism, coupled with morphological and ecological conservatism, which makes distinguishing between species difficult. In order to improve the identification of skates and investigate the phylogenetic position of endemic species the cytochrome c oxidase subunit I was sequenced in 12 species (*Dipturus oxyrinchus*, *Leucoraja naevus*, *Leucoraja circularis*, *Neoraja iberica*, *Raja brachyura*, *Raja clavata*, *Raja maderensis*, *Raja microocellata*, *Raja miraletus*, *Raja montagui*, *Raja undulata*, *Rostroraja alba*) inhabiting the Portuguese waters. Based on sequence divergence *R. maderensis* and *R. clavata* only differ by 1% of the 691 bp sequence, casting doubt on the recognition of *R. maderensis* (considered to be endemic to Madeira and the Azores), as a reproductively isolated species. Otherwise, there was clear phylogenetic support for the different genera and all the remaining species, although, in general, the genetic divergence was low compared to other chordates. In particular, COI analysis allowed clear identification of the morphologically similar species *R. brachyura* and *R. montagui*.

Keywords: barcoding, COI, *coxI*, cytochrome c oxidase, mitochondrial DNA, skate.

[1] Serra-Pereira, B., Moura, T., Griffiths, A. M., Gordo, L. S. and Figueiredo, I. Submitted. Molecular barcoding of skates (Chondrichthyes: Rajidae) from the southern Northeast Atlantic. Zoologica Scripta.

3.1.2. Introduction

Skates (order Rajiformes) are the most diverse group of Chondrichthyan fishes, including more than 250 species (Ebert and Compagno, 2007). Due to their high diversity, and global distribution (Stehmann and Bürkel, 1984; Ebert and Compagno, 2007), our knowledge of skate biology and ecology remains limited. This has been highlighted by the large numbers of newly described species that have been recorded (e.g. Stehmann *et al.*, 2008; Stevenson *et al.*, 2009), suggesting that the systematics of this group needs further investigation.

In common with other large elasmobranch fish, many skates also demonstrate a low resilience to fishing pressure (Walker and Hislop, 1998; Stevens *et al.*, 2000), which is due to their life history incorporating a long generation time, slow growth rate and low fecundity (Frisk *et al.*, 2001). Accordingly, overfishing has been blamed for the sharp declines in abundance that has occurred in the North Sea in many skate species, e.g. the common skate *Dipturus batis*, longnosed skate *Dipturus oxyrinchus* and bottlenosed ray *Rostroraja alba* (Walker and Hislop, 1998; Dulvy *et al.*, 2000). Still, skates remain a valuable commercial resource, representing more than 40% in weight of the reported landings of elasmobranchs in the Northeastern Atlantic, reaching 59% in 2006 (FAO, 2007), with skate species constituting an important by-catch of multi-gear shelf fishery across Europe (e.g. Walker *et al.*, 1997; Machado *et al.*, 2004).

Accurate species identification is critical for the design of sustainable fisheries and conservation management plans, since they are commonly implemented on a species-by-species basis, since not all species are equally sensitive to fishing pressure (Walker and Hislop, 1998). Until recently, this has not been done for landings of skates, where landings of different species were combined under a single category, such that serious declines in one species could effectively be masked by stable trends in more abundant groups (Dulvy *et al.*, 2000). In order to easily assess the trends in abundance of individual species, recent EU Regulation (EC No 43/2009, 2009) has been introduced to ensure that landings of some skates are recorded by species and not as aggregated group. In the specific case of Portuguese mainland fisheries, ten species are landed: *Dipturus oxyrinchus*, *Leucoraja naevus*, *Leucoraja circularis*, *Raja brachyura*, *Raja clavata*, *Raja microocellata*, *Raja miraletus*, *Raja montagui*, *Raja undulata* and *Rostroraja alba* (Machado *et al.*, 2004; Figueiredo *et al.*, 2007). Additionally, there are also reports of the occurrence of an endemic species found in Madeira and the Azores, *Raja maderensis* (Stehmann and Bürkel, 1984), and a new endemic species recently described for the continental waters to the south of Portugal, *Neoraja iberica*

(Stehmann *et al.*, 2008). Other species, also caught in Azores, include; *Bathyraja pallida, Bathyraja richardsoni*, *Dipturus batis*, *Leucoraja fullonica*, *Rajella bathyphila* and *Rajella bigelowi* (ICES, 2009).

Some of these species inhabiting the Portuguese waters, e.g. *L. naevus*, *R. miraletus* and *R. undulata*, show a unique dorsal colour pattern, which makes their identification straightforward, while distinguishing between other species with similar morphology and colouring can be difficult. In particular, *R. clavata* shows a remarkable variation in shape and colour patterns and a great similarity to *R. maderensis*. *Raja brachyura* and *R. montagui* also share a very similar dorsal coloration that can confuse their identification (Stehmann and Bürkel, 1984). Therefore, a complementary approach that may overcome occasional morphological ambiguities in the identification of species is to utilise molecular markers.

Mitochondrial DNA (mtDNA), has proven its utility in phylogenetic and phylogeographic studies of most animal groups, including marine fishes, due to a number of favourable characteristics, e.g. it is non-recombining and has a maternal mode of inheritance, (e.g. Valsecchi *et al.*, 2005b). More recently, mtDNA sequencing has been used for species identification and it has become widespread under the DNA Barcode initiative (e.g. Hebert *et al.*, 2003a; Hebert *et al.*, 2003b; Ward *et al.*, 2005; Moura *et al.*, 2008; Ward *et al.*, 2008a; Steinke *et al.*, 2009). The DNA barcode refers to the base pair (bp) sequence of a short (~650 bp), standard segment of the genome, that in animals, is part of the mitochondrial gene cytochrome c oxidase subunit I (COI) (Hebert *et al.*, 2003a). Because the COI gene mutates at evolutionarily rapid rates, comparison of COI sequences reveals differentiation, even at fine taxonomic levels, but it generally shows low levels of sequence divergence within species. By comparing the same short COI sequence across a wide diversity of taxonomic groups has produced a robust method for a global animal bioidentification system, providing a reliable and accessible solution to the problem of species identification for most animal groups (Hebert *et al.*, 2003a).

DNA barcoding has already applied to collections of skate from Alaska (Spies *et al.*, 2006), U.S. east coast (Alvarado Bremer *et al.*, 2005) and Australia (Ward *et al.*, 2005; Ward *et al.*, 2008b), but it has yet to be consistently utilised for the identification of skate in the northeast Atlantic. The present study examines the suitability of COI gene to discriminate among 12 skate species occurring in Portuguese waters, with particular attention to distinguish pairs of morphologically similar *taxa*: *R. montagui*/*R. brachyura*, and *R.*

clavata/*R. maderensis*. The resulting phylogeny will also be used to investigate taxonomic relationships between European skate species, specifically testing for consistency to previous morphological and molecular investigations. The relationship of the two endemic species relatively to the remaining *taxa* will also be highlighted.

3.1.3. Material and Methods

3.1.3.1. Sampling

Using the identification keys published by Stehmann and Bürkel (1984), a total of 41 individuals of 12 different skate species were identified; using: *D. oxyrinchus* , *L. circularis*, *L. naevus, N. iberica*, *R. brachyura*, *R. clavata*, *R. maderensis*, *R. microocellata*, *R. miraletus*, *R. montagui*, *R. undulata* and *R. alba.* In many juvenile specimens of *R. montagui* and *R. brachyura*, the identification based on morphological characters referenced in Stehmann and Bürkel (1984) was uncertain. The identification was also difficult when differentiating between *R. clavata* and *R. maderensis*. Therefore, the latter was validated using photographic records from specimens caught in the Azores, made available by the Department of Oceanography and Fisheries from the University of the Azores.

Muscle tissue samples were collected from all individuals (Table 3.1). The samples were obtained from commercial landings at the Peniche landing port, under the scope of the National Data Collection Program (PNAB, DCF) and from the Portuguese Institute for Marine Research (IPIMAR) annual research surveys. Most collection sites were located mainly along the Portuguese continental shelf. *Raja maderensis* specimen was collected by a commercial vessel operating in the semounts in the south-western Portuguese waters. All samples were stored at 4ºC in absolute ethanol.

3.1.3.2. DNA extraction, amplification ad sequencing

DNA was extracted from approximately 25 mg of tissue, using the *QIAGEN©, DNeasy Blood & Tissue Kit* (Qiagen, Crawley, United Kingdom), according to the manufacturer's protocol. PCR amplification was conducted in 25 µl reaction volumes containing the following reaction mix: ~50 ng of DNA sample, 10x reaction buffer, 1.5 mM MgCl, 0.2 mM dNTPs, 0.1 µM of each primers and 0.1 U of Taq Polymerase (Fermentas, Ontario, Canada).

To amplify the 5' end of the CO1 gene primers designed by Ward *et al.* (2005): FishF2 (5'TCGACTAATCATAAAGATATCGGCAC3') and FishR2 (5'ACTTCAGGGTGACCGAAGAATCAGAA3') were utilised in all species. The PCR thermal regime consisted of an initial denaturing step of 5 min at 95 ºC, followed by 35 cycles of denaturation (95 ºC, 30 secs), annealing (50 ºC, 60 secs) and extension (72 ºC, 60 secs) and a final extension step of 10 min at 72 ºC.

The PCR products were visualised on 1% agarose gels and then enzimatically purified using a modification of the Exo-SAP method (Werle *et al.*, 1994). The amplified products were sequenced both in forward and reverse directions with the dye labelled termination method (BigDye Terminator v3.1, Applied Biosystems, Inc.) on an ABI 3730XL sequencer.

Table 3.1. Summary of polymorphism statistics for the COI fragment.
n: number of samples; S: number of segregating sites; N_h: number of haplotypes; H_h: haplotype diversity (±sd); π: nucleotide diversity (±sd). * Statistical significant difference

Species	n	S	N_h	H_h	π (%)	Tajima's D	Fu and Li's D
D. oxyrinchus	4	0	1	0.00	-	-	-
L. circularis	2	0	1	0.00	-	-	-
L. naevus	4	3	3	0.83 (±0.22)	0.24	0.17 (p>0.10)	0.17 (p>0.10)
N. iberica	6	1	2	0.33 (±0.22)	0.05	-0.93 (p>0.10)	-0.95 (p>0.10)
R. brachyura	3	0	1	0.00	-	-	-
R. clavata	4	9	4	1.00 (±0.18)	0.87	2.21 (p>0.05)	2.21 (p<0.02)*
R. maderensis	1	-	-	-	-	-	-
R. microocellata	4	2	2	0.67 (±0.20)	0.19	1.89 (p>0.10)	1.89 (p>0.05)
R. miraletus	3	2	2	0.67 (±0.31)	0.19	-	-
R. montagui	5	2	3	0.80 (±0.16)	0.15	0.24 (p>0.10)	0.24 (p>0.10)
R. undulata	4	2	2	0.50 (±0.27)	0.15	-0.71 (p>0.10)	-0.71 (p>0.10)
R. alba	1	-	-	-	-	-	-

3.1.3.3. Data analysis

Sequences were aligned manually and edited using BioEdit 7.0.9.0 (Hall, 1999) and Sequencher 4.9 version (Gene Codes, 2009), before being deposited in GenBank (under accession numbers HM043182 to HM043222) and in Barcode of Life Data System [(BOLD, http://www.barcodinglife.org, see (Ratnasingham and Hebert, 2007)]. When doubts remain on species identification, the BOLD identification engine was used to compare the sequences obtained in the present study to those deposited by other authors. In the phylogenetic analyis,

Scyliorhinus canicula and *Squalus acanthias*, were included as outgroups (GenBank, accession numbers FM164483 and FM164433, respectively).

The nucleotide diversity indices (averaged among haplotypes and species), the number of segregating sites (S), the number of haplotypes (N_h), the haplotype diversity (H_h), the nucleotide diversity (π) and deviations from neutral expectations using the tests of Tajima (1989) and Fu and Li (1993) were calculated using MEGA 4.0 (Tamura *et al.*, 2007) and DnaSP 4.20.2 (Rozas *et al.*, 2003). The evolutionary divergence between pairs of species and genera were estimated using the maximum composite likelihood method in MEGA 4.0 software (Tamura *et al.*, 2007). Standard error estimates were obtained by a bootstrap procedure (500 replicates).

A matrix of genetic distance between haplotypes was calculated with PAUP using, with the most appropriate substitution model determined by MODELTEST 3.7 (Posada and Crandall, 1998). The Tamura-Nei model (TrN; Tamura and Nei, 1993) was selected based on Akaike Information Criterion (AIC) and presented a proportion of invariable sites of 0.651 and gamma correction of 2.895 (TrN+I+Γ). Phylogenetic relationships using maximum-parsimony (MP) and maximum-likelihood (ML) were inferred using PAUP 4.0b10 (Sinauer Associates, Inc. Publishers, Sunderland, MA, USA) (Swofford, 2001). For MP, a heuristic search was performed with starting trees obtained by random stepwise addition, with 100 replicates, and the tree-bisection-reconnection (TBR) branch swapping algorithm. TBR algorithm was also used in the ML analysis, with 100 random addition replicates at each optimisation step. In both analyses, 1000 bootstrap replicates were used to assess the robustness of the internal braches of the trees. Bayesian phylogenetic analysis (BA) was performed using MrBAYES 3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). The selected evolutionary model, based on MrMODELTEST (Nylander, 2004), was the General Time-Reversible model (GTR; Rodríguez *et al.*, 1990) with proportion of invariable sites of 0.648 and gamma correction of 2.683 (GTR+I+Γ). A Markov chain Monte Carlo analysis was applied, with four chains running for $1.5x10^5$ generations, saving the current tree every 100 generations. The Markov chains were initiated with a random starting tree using the default heating scheme. Subsequently, a consensus tree was produced (with a burn-in of 100 trees) that incorporated the Bayesian posterior probabilities of each node.

3.1.4. Results

3.1.4.1. Intra-specific variability

The 41 specimens were sequenced for the COI gene, producing a 691 bp read length for all species. Diversity indices were not estimated for *R. miraletus, D. oxyrinchus, L. circularis, R. brachyura, R. maderensis* and *R. alba* since each produced a single haplotype (Table 3.1). From the remaining species, *R. clavata* showed the highest diversity indices with 9 segregating sites present in four haplotypes (H_d =1.00±0.18). Both *L. naevus* and *R. montagui* presented three segregating sites in three and two haplotypes, respectively (H_d=0.83±0.22 and H_d=0.80±0.16), while *R. microocelata* and *R. miraletus* both presented two segregating sites in two haplotypes (H_d=0.67±0.20 and H_d=0.67±0.31, respectively). Nucleotide diversity (π) was low for most species (between 0.05% and 0.24%), with exception of *R. clavata* (π=0.87%). The values of Tajima's D and Fu and Li's D for this locus were not significantly different from zero, suggesting no statistical departure from neutral expectations. The only exception was the Fu and Li's D for *R. clavata* (Table 3.1).

3.1.4.2. Phylogenetic analysis

Considering the 691 bp sequence, 188 (27%) polymorphic sites were identified between the 12 skate species (Table 3.2); 144 were parsimony informative sites with two variants; 30 were sites with three variants; and 8 were sites with four variants. The majority of substitutions occurred in the third nucleotide position within codons (89%), and the rest were found in the first position. The nucleotide composition between genera was almost identical - 30.1% thymine; 28.5% cytosine; 24.2% of adenines; and 17.1% guanine.

Estimation of the levels of genetic divergence between pairs of species within genera showed that among *Raja* the genetic divergence ranged between 0.013 and 0.088, and among *Leucoraja* was 0.054 (Table 3.3). The lowest genetic divergence observed between any pair of species, occurred between *R. maderensis* and *R. clavata* (average of 0.013), and the highest was observed between *R. miraletus* and *N. Iberica* (0.061). When comparing individual specimens of *R. maderensis* and *R. clavata* the lowest pairwise divergence occurred between haplotypes JFY and RJC1 (0.007), which is similar to the range of values demonstrated between the other *R. clavata* haplotypes (range 0.006 to 0.007). According to

Table 3.2. Variable nucleotide sites in 691 bp consensus sequences of COI in 12 skate species.

Haplotype	**22 25 26 31 37 40 43 49 52 58 61 64 65 67 70 73 74 76 79 82 85 89 97 100 103 104 106 109 110 115 118 121 136 137 139 151 155 157 169 172 175 178 184 187 190 193 196 199 202 205 208 211 214**
D. oxyrinchus	T C T T T C A C G A T C C A T T T A C A A C A G **G** T C C C T C T T A T T T T T T G T A T A C C A T T T A C
L. circularis	. T . . . . G . . G G T T . . . C G T G . . . C **.** . A T T . T . . C . . . . T G T . C . . .
L. naevus	. T . . . . . . A G . T T G . . C G T G G . R C **.** . A . . . T . . C . . . . G T C C . G .
N. iberica	. . . C . T . T A . . . Y . . A . . T . . T . C **.** . . T . C T C A C . . . C C C . C . . G T G T C . . . T
R. brachyura	C T C . C . . A . . . . . . C C C . . . . . . C **.** A . . . . . . C . C C . . . . . C . . G . . . . C C . .
R. clavata	C T . . . . . A . . . . Y . Y . C . Y . . Y . C **.** A . . . . . . C . . C C . . Y . C . . G . . . . C C . .
R. maderensis	C T . . . . . A . . . . . . C . C . . . . . C **.** A . . . . . . C . . C C . . C . C . . G . . . . C C . .
R. microocellata	C T . . C . . A . . . . Y . C C C . . . . Y . C **.** A . . . . T . C . C C . . . . . . G . . . . . C C . .
R. miraletus	C T . . . . . A A . . . Y . Y . C . . . . . C **.** A . . . . T . C . . C . C . . . . . C G . A . . C . . T
R. montagui	C T . . C . . A . . . . Y . C . C . . . . . C **.** A . . . . . . C . . C C . . . A C . . G . T . . . C . .
R. undulata	C T . . . . . A . . . . . . C . C . . . . . C **.** A . . . . . . C . . C . . . . G T . . . C C . .
R. alba	. . . C . . . . T . . . . T . . . G C **A** . . . . C . . . C . C . . . C . . G . C C . . A

Haplotype	**220 226 229 232 235 238 241 244 250 253 256 265 271 274 277 280 281 283 286 292 295 298 301 304 307 310 311 316 319 322 328 331 334 337 340 343 346 349 352 355 358 361 364 367 370 373 374 376 385 388 391 401 404 411**
D. oxyrinchus	T A T C C G C A C G G C T T A T C G C T T C C C A C T G T C G C A A T A T C C C T T G **A** A C C G G G C C T
L. circularis	. . C A . A . . . . T . C . . C T . . A . T T . . . C A . . A G . C C . . . . . . C A . C . . C A A . . .
L. naevus	. G T G . A . . . A T . C . . . T A A A . T T . . . C A . . . A . C . . . . C A . C . . C A . . . C
N. iberica	. . . . T A . . T A C . C . . . T A . . A T A A . . . A . . A G G . . . . T . . . C A . . T . A T . . . .
R. brachyura	. . . . A A T G . C C T . C . . T A . . . . . G . . . G . A . G . . . A . . . . C . . . . . A C . . . .
R. clavata	. . C . A A C . . T C T . C . . T A T . . . . . G . . . . . A . G . . G A . . . . C . . . . T A C . . . .
R. maderensis	. . C . G A . . . T C T . C . . T A T . . . . . G . . . . . A . G . . G A . . . . C . . . . T A C . . . .
R. microocellata	. . . . A A . . . T C . . C . . T A . . . . . G . . A . . . . G . . . G . . . . C . . G . . A C A . . .
R. miraletus	A . C . A A . . . T C T . C . . T A T . . T T . . T . . . T . . . . A . . A C C C . . . . A C . T T .
R. montagui	. . . . A C . . . C C T . C G . T A T . . . . . G . . . . T . . . . A . . T . C . . . . . A C A . . .
R. undulata	. . C . A A . . . T C . . C . . T A T . . . . T A . G . . . A . T T . C A . . . . . T A . . .
R. alba	. . C . . A . . T A C T C . . . T A . . . T T A . T . A C . A T . . . . C A **G** . . T A A . . . .

Table 3.2. Continued.

Haplotype	403	404	406	409	415	418	421	422	424	427	430	431	433	436	439	442	443	451	457	460	463	472	475	478	481	487	490	493	496	499	502	508	511	514	515	520	523	526	529	532	535	538	541	544	545	547	548	550	553	559	565	568	571
D. oxyrinchus	A	A	**A**	T	T	T	C	T	**G**	A	T	A	T	A	T	T	C	C	C	C	C	T	T	C	G	A	A	A	C	T	G	G	A	C	T	C	G	G	A	T	C	T	A	T	**G**	C	T	A	T	A	C	A	C
L. circularis	.	.	.	C	C	C	T	.	.	.	C	.	.	C	C	C	T	.	.	.	.	C	C	.	A	.	.	.	.	A	A	A	G	T	.	.	.	.	T	C	T	C	.	.	.	.	.	.	G	.	.	.
L. naevus	G	.	.	C	C	.	T	C	.	.	.	.	.	C	.	C	T	.	.	.	.	C	C	.	A	.	.	.	.	A	A	A	.	T	.	.	.	A	T	C	T	C	.	.	.	.	.	.	G	.	.	T
N. iberica	.	G	.	.	.	.	.	C	.	.	.	.	.	.	.	T	.	T	T	.	.	.	.	A	T	.	.	.	.	A	A	.	.	.	T	A	A	T	C	T	.	G	.	.	T	C	T	A	G	.	.	A
R. brachyura	.	.	.	.	C	.	T	.	.	.	G	C	T	.	.	.	.	.	.	T	.	.	.	A	.	.	G	.	.	A	A	.	.	C	.	T	A	.	C	T	.	.	.	.	T	.	.	.	.	.	.	T
R. clavata	.	.	.	.	C	C	T	.	.	.	G	.	.	.	.	.	.	.	.	T	.	.	.	A	.	G	.	.	.	A	A	.	.	C	.	.	A	.	C	T	.	.	.	.	T	.	.	.	.	.	C	T
R. maderensis	.	.	.	.	C	A	T	.	.	.	G	.	.	.	.	.	.	.	.	T	.	.	.	A	.	G	.	.	.	A	A	.	.	C	.	.	A	.	C	T	.	.	.	.	T	.	.	.	G	.	C	T
R. microocellata	.	.	.	.	C	.	T	.	.	.	G	.	.	.	.	.	.	T	.	.	T	.	.	.	A	.	.	C	.	.	A	A	.	.	.	.	T	A	C	C	T	.	.	.	.	.	.	.	.	.	.	.	T
R. miraletus	.	.	.	.	C	.	T	.	.	G	.	G	C	.	.	.	.	.	.	T	.	C	.	A	C	.	.	T	.	A	A	.	A	C	T	.	.	C	C	T	.	.	C	.	.	.	.	.	.	A	.	.
R. montagui	.	.	.	.	C	.	.	.	.	.	.	G	.	.	.	C	.	.	T	.	T	.	.	.	A	.	.	.	.	.	A	A	.	.	C	.	.	.	C	C	T	.	.	.	.	T	.	.	.	.	.	.	T
R. undulata	.	.	.	.	C	.	T	.	.	.	C	.	C	.	.	.	.	.	.	T	.	.	.	T	A	.	.	.	.	.	A	A	.	.	C	.	C	A	.	C	T	.	.	.	.	.	.	.	R	T	.	.
R. alba	.	G	**T**	C	.	C	T	C	**A**	.	.	.	C	.	.	.	T	.	.	T	.	.	.	T	A	.	.	.	.	.	A	A	.	T	.	T	.	A	.	C	.	.	G	A	**A**	T	.	.	.	G	T	.	.

Haplotype	580	583	586	589	592	593	595	598	601	604	607	610	613	616	619	622	628	631	634	637	640	643	646	652	655	658	661	667	668
D. oxyrinchus	A	T	C	C	A	C	A	C	G	T	T	T	T	C	A	T	**T**	C	G	A	A	A	G	C	T	A	T	T	T
L. circularis	T	C	T	T	.	.	.	.	A	.	.	C	C	.	.	C	.	.	.	.	G	.	.	.	.	C	.	C	.
L. naevus	C	C	T	R	.	.	.	.	A	.	.	C	C	T	.	.	.	.	A	.	G	.	A	.	.	C	C	C	.
N. iberica	C	C	.	.	.	.	T	A	C	C	.	.	.	T	.	.	.	.	A	T	G	.	.	.	.	T	C	C	C
R. brachyura	T	C	.	T	.	.	T	.	.	C	C	.	.	.	G	.	.	.	C	T	.	.	A	.	.	C	.	.	.
R. clavata	C	C	.	T	R	.	.	.	.	.	.	.	C	.	.	.	.	.	M	T	.	G	.	.	C	.	.	Y	.
R. maderensis	C	C	.	T	.	.	.	T	.	.	.	.	C	.	.	.	.	.	C	T	.	G	.	.	C	.	.	.	.
R. microocellata	C	C	.	T	.	.	.	.	.	C	C	.	.	.	.	.	.	.	C	T	.	.	A	.	.	T	.	.	.
R. miraletus	T	.	.	T	.	T	.	.	A	.	C	.	.	.	.	.	.	.	T	T	G	.	A	A	.	.	.	.	.
R. montagui	C	C	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	A	.	T	.	.	C
R. undulata	T	C	.	T	.	.	T	T	A	.	.	.	.	.	.	.	.	T	A	T	.	.	A	.	.	.	.	Y	.
R. alba	T	C	.	.	.	.	T	A	A	C	.	.	C	.	.	A	**C**	.	.	.	.	.	A	.	.	T	C	C	C

Dots (.) denote nucleotide identity with *D. oxyrinchus* sequence (at the top). Singletones are highlighted in bold. Shaded sites indicate polymorphism within species: K=G or T; Y=C or T; R=A or G; M=A or C.

the BOLD database the sequence originating from *R. maderensis* had the highest degree of similarity to a specimen of *R. clavata* (99.69%). There was a high divergence between the endemic species *N. iberica* and all the species from the genus *Raja*, *Neoraja*, *Rostroraja* and *Dipturus* (from 0.102 to 0.161). When compared to other sequences from BOLD database, the *N. iberica* specimens showed a COI sequence 100% similar to *Neoraja caerulea* (and 99.85% similar to *Rajella fyllae*). The morphologically similar species *R. montagui* and *R. brachyura* showed a moderate divergence (0.057). Comparing the average levels of genetic divergence between genera (Table 3.4) the maximum divergence observed was between *Raja* and *Leucoraja* (0.143) and the minimum between *Raja* and *Dipturus* (0.930).

Table 3.3. Estimates of evolutionary divergence over sequence pairs between species.
Dipturus oxyrinchus (RJO), *Leucoraja circularis* (RJI), *Leucoraja naevus* (RJN), *Neoraja iberica* (RNI), *Raja brachyura* (RJH), *Raja clavata* (RJC), *Raja maderensis* (JFY), *Raja microocellata* (RJE), *Raja miraletus* (JAI), *Raja montagui* (RJM), *Raja undulata* (RJU) and *Rostroraja alba* (RJA). Standard error estimate(s) included in brackets.

	RJO	RJI	RJN	RNI	RJH	RJC	JFY	RJE	JAI	RJM	RJU
RJI	0.117 (0.026)										
RJN	0.124 (0.026)	0.054 (0.013)									
RNI	0.133 (0.028)	0.149 (0.031)	0.136 (0.029)								
RJH	0.098 (0.022)	0.158 (0.033)	0.153 (0.032)	0.145 (0.031)							
RJC	0.093 (0.020)	0.143 (0.030)	0.140 (0.029)	0.142 (0.030)	0.051 (0.011)						
JFY	0.093 (0.021)	0.147 (0.030)	0.143 (0.030)	0.141 (0.030)	0.052 (0.012)	0.013 (0.004)					
RJE	0.088 (0.020)	0.139 (0.029)	0.131 (0.028)	0.137 (0.029)	0.041 (0.011)	0.059 (0.014)	0.060 (0.014)				
JAI	0.110 (0.024)	0.148 (0.031)	0.146 (0.030)	0.161 (0.034)	0.088 (0.019)	0.086 (0.019)	0.088 (0.019)	0.085 (0.019)			
RJM	0.086 (0.020)	0.149 (0.032)	0.148 (0.031)	0.142 (0.030)	0.057 (0.013)	0.052 (0.013)	0.053 (0.013)	0.056 (0.013)	0.079 (0.018)		
RJU	0.090 (0.020)	0.129 (0.027)	0.136 (0.029)	0.132 (0.029)	0.063 (0.04)	0.063 (0.014)	0.059 (0.014)	0.068 (0.016)	0.079 (0.018)	0.071 (0.016)	
RJA	0.120 (0.025)	0.132 (0.028)	0.121 (0.026)	0.102 (0.023)	0.142 (0.028)	0.137 (0.028)	0.135 (0.028)	0.148 (0.031)	0.141 (0.029)	0.156 (0.032)	0.123 (0.026)

All the phylogenetic analyses produced trees with similar topologies that were also supported by high bootstap values (ML and MP), or high posterior probabilities (BA) (Fig. 3.1). The major difference between methods of tree construction was the early separation of *D. oxyrinchus*, in a separate clade from the remaining taxa (such that it occupied an ancestral position to all other skate species) in the Bayesian analysis. Otherwise, three main

evolutionary lineages were consistently recognized: the first with *Leucoraja* species, the second with *Neoraja* and *Rostroraja* and the third grouping all *Raja* species and *Dipturus*.

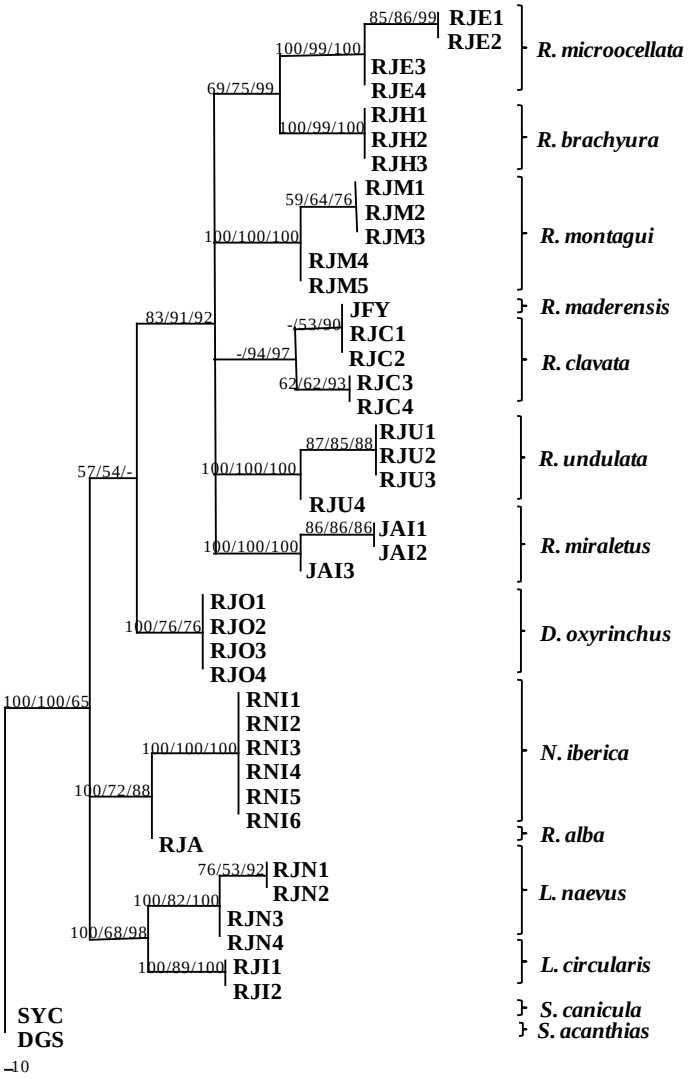

Figure 3.1. Maximum likelihood tree based on COI sequences from the 12 skate species.
Dipturus oxyrinchus (RJO), *Leucoraja circularis* (RJI), *Leucoraja naevus* (RJN), *Neoraja iberica* (RNI), *Raja brachyura* (RJH), *Raja clavata* (RJC), *Raja maderensis* (JFY), *Raja microocellata* (RJE), *Raja miraletus* (JAI), *Raja montagui* (RJM), *Raja undulata* (RJU) and *Rostroraja alba* (RJA). Nodal support is indicated: the first number refers to maximum parsimony bootstrap values, the second to maximum likelihood bootstrap values and third to Bayesian posterior probabilities. The same tree topology was obtained with the three different inference methods, except for the relationship between *Raja* and *Dipturus*, and *R. clavata* and *R. maderensis* (-). All trees were rooted using the homologous COI sequence of *Scyliorhinus canicula* (SYC) and *Squalus acanthias* (DGS) as outgroup (GenBank Acc. Num. FM164483 and FM164433).

The isolation of the newly described endemic species, *N. iberica* from the remaining species occurring in Portuguese waters (Fig.3.1) was well supported with high relative sequence divergences (Table 3.3). Whilst the phylogenetic analysis produced robust support for each species of skate, *R. maderensis* and *R. clavata* were not separated into different

clades, although it should be noted that each of the four specimens of *R. clavata* produced a distinct haplotype (Table 3.2). Similarly, *R. maderensis* presented a distinct haplotype, with five variable sites from RJC1 and nine variable sites from RJC2 (Table 3.2).

Interestingly, the COI sequencing also identified the misclassification of sample RJM5, resulting in its reclassification from *R. brachyura* to *R. montagui.*

Table 3.4. Estimates of evolutionary divergence over sequence pairs between genus. Standard error estimate(s) are between brackets.

	Raja	*Dipturus*	*Leucoraja*	*Neoraja*
Dipturus	0.093 (0.019)			
Leucoraja	0.143 (0.028)	0.121 (0.025)		
Neoraja	0.142 (0.028)	0.133 (0.027)	0.140 (0.027)	
Rostroraja	0.142 (0.028)	0.120 (0.025)	0.124 (0.024)	0.102 (0.021)

3.1.5. Discussion

The present study provides further evidence of the suitability of the COI gene for species identification, being the first to present results for north east Atlantic skate species. Its application to those morphological conservative and difficult to distinguish species of skate has allowed most species to be successfully differentiated. The estimates of genetic divergence between pairs of species were generally low (Hebert *et al.*, 2003b), but within the same magnitude to those observed for other elasmobranchs (e.g. Moura *et al.*, 2008; Smith *et al.*, 2008; Ward *et al.*, 2008b). Yet, they were above than the 2% value of intra-specific divergences considered optimal for species discrimination (Hebert *et al.*, 2003b). Therefore, this study provides a framework of simple PCR-based assays that can be easily applied when problems concerning species identification arise. This may become increasingly relevant if the European Union continues to distinguish between species of skate within catch records, especially as the EU Regulation now prohibits landing of many threatened species within the Community (EC No 43/2009, 2009).

The only case where COI sequencing failed to differentiate between previously recognised species involved *R. clavata* and *R. maderensis*. *Raja maderensis* possesses a very similar COI sequence to *R. clavata* specimens (99.69% similarity in BOLD database) and the phylogenetic analysis failed to separate these *taxa* into different clades. Based on these

results, two hypotheses appear likely: (i) *R. maderensis* is a distinct species, although genetically closely related to *R. clavata*; or (ii) *R. maderensis* is another morphotype of *R. clavata*. For a number of reasons it seems that the second hypothesis is the most probable, i.e. the results casts doubt on the recognition of *R. maderensis* as a distinct species. First, *R. maderensis* its only mentioned in a two publications on systematic (Stehmann and Bürkel, 1984; McEachran and Dunn, 1998), and no further work has been completed into the biology of the species, that could prove distinct life history traits from *R. clavata*. Second, *R. clavata* has been shown to demonstrate both high levels of intra-specific genetic diversity (this study; Valsecchi *et al.*, 2005b; Chevolot *et al.*, 2006) and morphological variation (Stehmann and Bürkel, 1984; Serra-Pereira *et al.*, in press-a), so that the divergence observed between *R. clavata* specimens was sometimes higher than the divergence observed between *R. clavata* and *R maderensis*. Therefore, *R. maderensis* could represent one of *R. clavata* morphotypes. The two species are suggested to coexist in the north east Atlantic, and although *R. maderensis* is endemic of Madeira and Azores (Stehmann and Bürkel, 1984; Fock *et al.*, 2002), the level of sequence divergence is not enough to consider two distinctive species (less that the 2% value previously suggested as optimal for describing distinct species). Yet, it cannot be excluded the hypothesis of existence of isolated populations/stocks (*R. clavata* and *R. maderensis* specimens were collected probably more than 1000 km distance apart). Additionally, skates are known to have long generation times, which means that sister *taxa* need more time to appear monophyletic in the analysis of mtDNA sequences (Tinti *et al.*, 2003), and previous studies utilising mtDNA to study divergence closely related skate species have similarly failed to distinguish between them (e.g. *Raja asterias* and *Raja polystigma*, Tinti *et al.*, 2003; Pasolini *et al.*, 2006).

Neoraja iberica showed a distinct separation from the remaining species of genus *Raja*, *Neoraja*, *Rostroraja* and *Dipturus* occurring in Portuguese waters. According to the BOLD database, specimens identified as *Neoraja iberica* (Stehmann *et al.*, 2008) had COI sequence that was identical to the only specimen of *Neoraja caerulea* included in the database (and for which the details of capture are not publically available). Although the latter is considered endemic off Ireland and Iceland and never described in Portuguese waters (Stehmann and Bürkel, 1984), the results obtained and possibility of synonymy between both *taxa* are inconclusive, and further molecular studies should be developed with all the pigmy ray species (genus *Neoraja*) in order to validate the position of *N. iberica* as distinctive species.

COI sequencing clearly separate *R. brachyura* and *R. montagui* (evolutionary divergence of 6%). These species are of particular interest as misidentifications often occurs between them (especially amongst juveniles), due to their similar colouration pattern (Stehmann and Bürkel, 1984). In this study the analysis of the COI fragment suggests that misidentification from morphology alone occurred in one case; a specimen of *R. montagui* was incorrectly identified as *R. brachyura*.

Phylogenetic analyses showed that all *taxa* formed strongly supported reciprocally monophyletic clades (except for *R.* maderensis), with the identification of 11 distinct species. Levels of genetic divergence among species within genera were on average 0.06 for *Raja* and 0.05 for *Leucoraja*. These values are higher than those observed for the *Bathyraja* genus (Spies *et al.*, 2006; Smith *et al.*, 2008) which may be explained by the higher morphological variability demonstrated by species of *Raja* and *Leucoraja* (Stehmann and Bürkel, 1984). However, the analysis of the COI region did not give sufficient phylogenetic resolution to infer evolutionary relationships between closely related species, particularly between *taxa* within the same genus.

More broadly within Rajidae, the COI nucleotide sequences separated species belonging to well separated genus in three clades: (i) *Leucoraja*; (ii) *Rostroraja* and *Neoraja*; and (iii) *Dipturus* and *Raja*. This accords well with previous, morphology based taxonomy (McEachran and Miyake, 1990; McEachran and Dunn, 1998), the only difference being the position of *Rostroraja* in the tree, in which is positioned in the same clade with *Raja* and *Dipturus*, rather than with *Neoraja*. Within the tree, *Rostoraja* is represented by a single species, *R. alba* (Compagno, 2005), thus the difference in its position could be due to poor taxonomic coverage in the current study. Since the two genera (*Rostroraja* and *Neoraja*) are morphologically very distinct, future studies should focus on better taxonomic coverage that may resolve their phylogenetic relationship.

The results of our analysis generally supports previous studies, which have utilised a range of mtDNA sequences for phylogenetic inference between overlapping collections of European skate species (Tinti *et al.*, 2003; Valsecchi *et al.*, 2005a; Pasolini *et al.*, 2006; Turan, 2008; Iglésias *et al.*, 2009; Griffiths *et al.*, 2010). The phylogenetic relationships between species and genera show a very similar arrangement to those of obtained by most studies (Valsecchi *et al.*, 2005a; Pasolini *et al.*, 2006; Iglésias *et al.*, 2009; Griffiths *et al.*, 2010). Generically, the same three clades described above, are present. Among *Raja* spp. the pairs of species *R. brachyura*/*R. microocellata* seem to present the lower divergence level. The most significant differences occur in comparison to: (i) Tinti *et al*.'s (2003) 16S

phylogeny, where *R. miraletus* was positioned further from the remaining *Raja* species than *D. oxyrinchus*, which was included within the same clade; and (ii) to Turan (16S rDNA; , 2008) and Iglésias *et al*. (tRNA-Phe, 12S rDNA, tRNA-Val and 16S rRNA; , 2009) studies's, in which *Rostroraja* was placed in a clade isolated from the remaining genus, and in an ancestral position to all other skate species and not aside with *Neoraja.* Compared to those phylogenies published for skate *taxa* originating in the Mediterranean Sea with 16S ribosomal DNA (rDNA) (Tinti *et al.*, 2003; Turan, 2008), the results obtained in the present study were more robust (higher bootstrap values) and accord much more closely with recent analyses incorporating longer sequence reads and broader collections of *taxa* (Valsecchi *et al.*, 2005a; Iglésias *et al.*, 2009; Griffiths *et al.*, 2010).

This study concludes that COI is a reliable tool for skate species identification. Uncertainty concerning the taxonomic distinctiveness of *R. maderensis* remains; the sample did yield a unique haplotype, but this is perhaps not surprising given the high levels of genetic diversity demonstrated by *R. clavata* and the fact the level of sequence divergence remained below the critical 2% suggested as designating distinct groups. Further sampling of these two closely related groups is therefore required, particularly from examples of *R. clavata* that exist in sympatry with *R. maderensis*, around the Azores. COI sequencing proved to be useful when problems surrounding species identification occurred, as in the case of morphologically similar species like *R. montagui* and *R. brachyura*.

3.2. MORPHOMETRY [1]

3.2.1. Abstract

European skate landings have traditionally been reported under a generic landing category, because of problems with species identification. To address this data deficiency, the ICES Working Group on Elasmobranch Fishes compiled conversion factors, including the relationships between different body measurements, for the main elasmobranch species. Size conversion factors for six common NE Atlantic skate species, *Leucoraja naevus*, *Raja*

[1] Serra-Pereira, B., Farias, I., Moura, T., Gordo, L.S., Santos, M. N. and Figueiredo, I. In Press. Morphometric ratios of six commercially landed skate species from the Portuguese continental shelf and their utility for identification. ICES Journal of Marine Science.

brachyura, *R. clavata*, *R. miraletus*, *R. montagui*, and *R. undulata* are compiled, and the capability of morphometric data to assist species discrimination is evaluated, highlighting the case of similar species such as *R. brachyura* and *R. montagui*. The estimated size conversion factors displayed some variability between areas and sexes for most species, the allometric relationship between weight and total length did not differ significantly between sexes, and some morphometric ratios proved adequate in discriminating between rajid species (misclassification error 0.12). *Leucoraja naevus* was fully discriminated from the remaining species. Species with a similar dorsal colour, e.g. *R. brachyura* and *R. montagui*, showed good discrimination based on their morphometry, with just 6–11% misclassification between the two.

Keywords: biometry, fisheries, Portugal, Rajidae, size conversion factors, skates

3.2.2. Introduction

Skates (order Rajiformes) are one of the most speciose elasmobranch orders, and include at least 27 genera and more than 245 species (Ebert and Compagno, 2007). They are found in all oceans, from shallow coastal waters to abyssal regions. Of those inhabiting the shelf and upper slope, some live on soft bottom, others on coarser substrata (Stehmann and Bürkel, 1984; Ebert and Compagno, 2007). Skates are important elements of the marine biodiversity, but they are highly vulnerable to commercial exploitation. In 2006, skate catches amounted for ~59% of the total reported landings (by weight) of elasmobranchs in the NE Atlantic (FAO, 2007).

The external morphology and colour of skates is, in many cases, sufficient to discriminate between species inhabiting the NE Atlantic. Nevertheless, for commercial reasons, the different species are often landed under a generic landing category (Dulvy *et al.*, 2000; ICES, 2007). During a pilot sampling programme conducted at ports in mainland Portugal, eight species of skate were identified: cuckoo ray *Leucoraja naevus*, blonde ray *Raja brachyura*, thornback ray *Raja clavata*, small-eyed ray *Raja microocellata*, brown ray *Raja miraletus*, spotted ray *Raja montagui*, undulate ray *Raja undulata*, and bottlenosed skate *Rostroraja alba* (Machado *et al.*, 2004). In addition, three other species, which are either rare or absent from landings, have been described for this geographic area: longnosed skate *Dipturus oxyrinchus*, sandy ray *Leucoraja circularis* (Figueiredo *et al*., 2007), and Iberian pigmy skate

Neoraja iberica (Stehmann *et al.*, 2008). The present work focuses on six of the most common and widely studied species of this assemblage (*L. naevus*, *R. brachyura*, *R. clavata*, *R. montagui*, *R. miraletus*, and *R. undulata*). Identification keys are available for all six (e.g. Stehmann and Bürkel, 1984) to allow for their correct identification. Although the typical specimens of *L. naevus*, *R. miraletus*, and *R. undulata* are difficult to misidentify, because of their unique dorsal colouration, there may be problems distinguishing between the other three, mainly because of the remarkable variability within *R. clavata*, and the similarity between *R. montagui* and *R. brachyura*. *Leucoraja naevus*, *R. miraletus*, and *R. montagui* are small species that reach a maximum length of about 70 cm (Holden, 1972; Du Buit, 1975), maturing at lengths of ~60 cm (Walker, 1999). All three are potentially more resilient to fishing pressure than larger species, such as *R. brachyura*, *R. clavata*, and *R. undulata*, which can reach lengths of >100 cm (Holden, 1972; Moura *et al.*, 2007; Serra-Pereira *et al.*, 2008).

One of the terms of reference for the 2007 ICES Working Group on Elasmobranch Fishes (WGEF) referred to the need to compile conversion factors for elasmobranch species, but little information was made available (ICES, 2007). Conversion factors are commonly used to estimate values from one or more known body measurements. Therefore, morphometric conversions are particularly helpful when, for example, a specimen is damaged, or when dealing with commercially pre-processed specimens (e.g. wings only, tail off, head off, or a combination of these), in which not all morphometric traits can be measured. As conversion factors differ between species, they may also serve as a tool for species identification, especially for persons with limited taxonomic expertise, or for accurate identification of problematic specimens. This is particularly important at landing ports where fish need to be identified on site.

The present study aimed to (i) estimate relationships between different body measurements (i.e. size conversion factors) in order to increase the information available for six common NE Atlantic skate species in Portuguese waters, and (ii) investigate the ability of these measurements to discriminate between species, in order to provide additional tools for assisting in the identification of skates on the Portuguese shelf and in other parts of the NE Atlantic.

3.2.3. Material and Methods

Specimens were sampled on a monthly basis between February 2001 and June 2008, from commercial landings in three ports along the Portuguese continental coast: Matosinhos in the north, Peniche in the centre, and Portimão in the south (Fig. 3.2). From these ports, skates are caught in a variety of gears, including trammel- and gillnets, longlines, trawls, and traps.

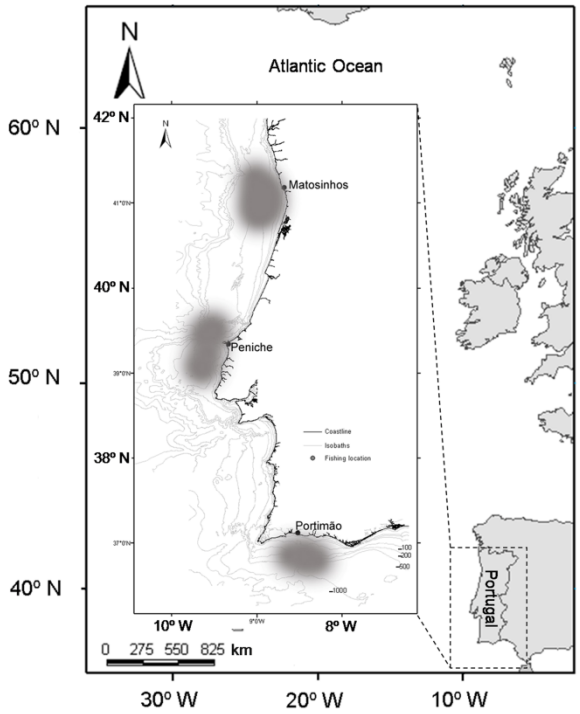

Figure 3.2. Map of the NE Atlantic with detail of the study location off Portugal.
The landing ports (Matosinhos, Peniche, and Portimão) and the main fishing areas (shaded grey) are identified, and bathymetric contours (isobaths) are provided in m.

For each boat sampled, a subsample of the total skate landings was selected randomly. For each specimen, the species was identified and the following size measurements were recorded to the nearest millimetre (mm; see Figure 3.3): total length (TL), disc width (DW), disc length (DL), and tail length (CL). Although all the measurements are shown in Figure 2 on the ventral view of a skate, TL, DW, and DL were measured on linear axes under the individual, as viewed from the dorsal side. DL and CL were only available for specimens landed in Peniche. Specimens were also sexed and weighed (total weight, TW, and gutted weight, gW) to an accuracy of 1–10 g.

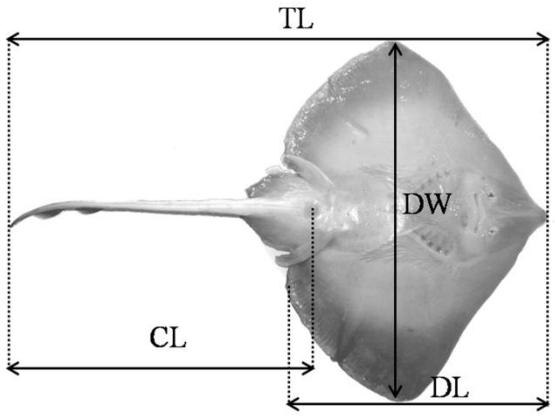

Figure 3.3. Measurements recorded to the nearest 1 mm on linear axes for each fish.
Total length (distance from the tip of the snout to the end of the tail, TL), disc width (maximum distance between the wing tips, DW), disc length (distance from the tip of the snout to the posterior edge of the disc, DL), and tail length (distance from the cloaca to the end of the tail, CL).

3.2.3.1. Data analysis

For each species an exploratory analysis of morphometric ratios (DW:TL, DL:TL, CL:TL, and DL:DW) was performed by sex and area (landing port), whenever there were sufficient data available for two variables. *Raja miraletus* from the north and centre, and *L. naevus* and *R. undulata* from the south were not considered for analysis owing to the small sample sizes by sex ($n < 10$). No *R. brachyura* were available in samples from the south.

To investigate the influence of area and sex on the morphometric ratio DW:TL, TL was grouped into 10 cm size bins (TL classes), and a linear model was adjusted to each species' dataset. The factor TL class is expected to be a major contributor to the variability of this morphometric ratio, so a model that considers the factors area and sex nested on the factor TL class was constructed:

$$\text{Ratio} = \mu + \beta_{\text{Area}} + \alpha_{Sex} + \gamma_{\text{Area*Sex}} + \varphi_{\text{Area*Sex*Lengthclass}} + \varepsilon, \quad (1)$$

assuming that $\varepsilon \sim N(0, \sigma^2)$. The other morphometric ratios were only available for central Portugal, so the nested model initially proposed was:

$$\text{Ratio} = \mu + \beta_{\text{Sex}} + \alpha_{\text{Sex*Lengthclass}} + \varepsilon, \quad (2)$$

under the assumption that $\varepsilon \sim N(0, \sigma^2)$. The assumptions of the models, particularly variance homogeneity and normality, were investigated through an analysis of the residuals.

A stepwise algorithm was used to select the factors to be included in the model. Model selection was based on the Akaike Information Criterion (AIC), according to which, competing models may be ranked for a given dataset, and the one with the lowest AIC value is considered to be the most appropriate (Venables and Ripley, 2002).

Based on the statistical significance of the adjustments above, two procedures were used to estimate the conversion factors: (i) if the model selected explained >50% of the total variance, the size conversion factor was estimated from the combination of the different levels of the factors with a significant effect, using Equations (1) and (2); (ii) if the model captured only a small part of the total variance (<50%), the conversion factors were estimated by adjusting a linear model to the pairs of measurements under analysis, without considering the effect of the previous factors (Sex, Area, and TL class). The least squares method was used to estimate the parameters of the expressions $TL \sim aDW + b$, $TL \sim aCL + b$, $TL \sim aDW + b$, and $DW \sim aDL + b$, where *a* is the intercept and *b* the slope.

The allometric relationships between TW and TL, and between gW and TL were adjusted, and the parameters estimated by a nonlinear least squares method (Venables and Ripley, 2002): $TW \sim aTL^b$ and $gW \sim aTL^b$, where *a* is the initial growth coefficient or condition factor and *b* represents the growth rate. Likelihood ratio tests (Draper and Smith, 1981) were applied to compare the parameter estimates between sexes for the two length–weight relationships.

A flexible discriminant analysis (FDA; Hastie *et al.*, 1981) was applied to investigate whether a simple combination of morphometric ratios was adequate to discriminate between species. All available species were used in these analyses. The FDA is a method for multigroup classification, and rules are built to predict the class membership of an item (species) based on several predictors, in this case morphometric ratios. The morphometric ratios used as body shape descriptors were: DW:TL (whole body shape), DL:DW (disc shape), and CL:DL (relative tail size).

3.2.4. Results

In all, 2009 fish were sampled, and Table 3.5 summarizes the number sampled by species, sex and geographical area, and their size ranges.

Table 3.5. Summary of the data available by species, sex and area, provided as ranges for each morphometric measurement.

Species	Sex	Area	*n*	TL	TW	gW (g)	DW (mm)	DL (mm)	CL (mm)
JAI	F	South	16	329-488	-	-	228-336	-	-
	M	South	22	313-492	-	-	215-310	-	-
RJC	F	North	150	420-905	200-7750	-	305-608	-	-
		Centre	155	489-934	623-6710	585-5810	345-669	248-489	269-464
		South	22	362-793	900-3800	-	263-588	-	-
	M	North	133	410-875	450-4140	-	295-562	-	-
		Centre	80	458-870	540-3870	480-3435	321-565	226-428	260-443
		South	30	315-850	1000-3200	-	212-547	-	-
RJH	F	North	68	380-975	400-8520	-	297-715	-	-
		Centre	148	376-1061	304-10680	283-9160	268-771	205-588	230-514
	M	North	67	389-1005	400-8700	-	284-723	-	-
		Centre	114	412-1005	421-7290	387-6404	292-692	240-554	260-495
RJM	F	North	33	368-702	270-2340	-	257-459	-	-
		Centre	185	390-702	388-2583	360-2306	285-483	212-373	240-346
		South	18	331-575	-	-	235-412	-	-
	M	North	23	412-651	450-1980	-	281-430	-	-
		Centre	97	408-612	455-1575	420-1450	292-412	209-319	226-336
		South	20	366-522	-	-	253-354	-	-
RJN	F	North	23	497-718	700-2460	-	270-409	-	-
		Centre	224	448-691	460-2135	435-1975	249-399	215-340	250-353
	M	North	23	501-682	720-2410	-	275-401	-	-
		Centre	130	439-672	317-2250	298-2120	259-384	205-329	250-346
RJU	F	North	19	538-860	1300-6500	-	407-595	-	-
		Centre	91	517-933	814-7291	748-6197	347-655	282-512	286-447
	M	North	24	480-860	650-5000	-	355-550	-	-
		Centre	94	522-959	640-6230	570-5630	348-602	279-507	266-452

TL, total length; TW, total weight; gW, gutted weight; DW, disc width; DL, disc length; CL, tail length; JAI, *R. miraletus*; RJC, *R. clavata*; RJH, *R. brachyura*; RJM, *R. montagui*; RJN, *L. naevus*; RJU, *R. undulata*.

3.2.4.1. Morphometric variation within species

Apart from the variability observed between species, differences in morphometric ratios (DW:TL, DL:TL, CL:TL and DL:DW) were also observed within species (Fig. 3.4). *Leucoraja naevus* was clearly the species showing the most distinctive shape, relative to the other species. It has a narrower disc in relation to body length (lowest DW:TL) and the disc is longer than wide (higher DL:DW). *Raja montagui* showed the following morphometric similarities with the other three species: DL:TL and CL:TL with *R. clavata*; DL:DW with *R. brachyura*; and DW:TL with *R. miraletus.*

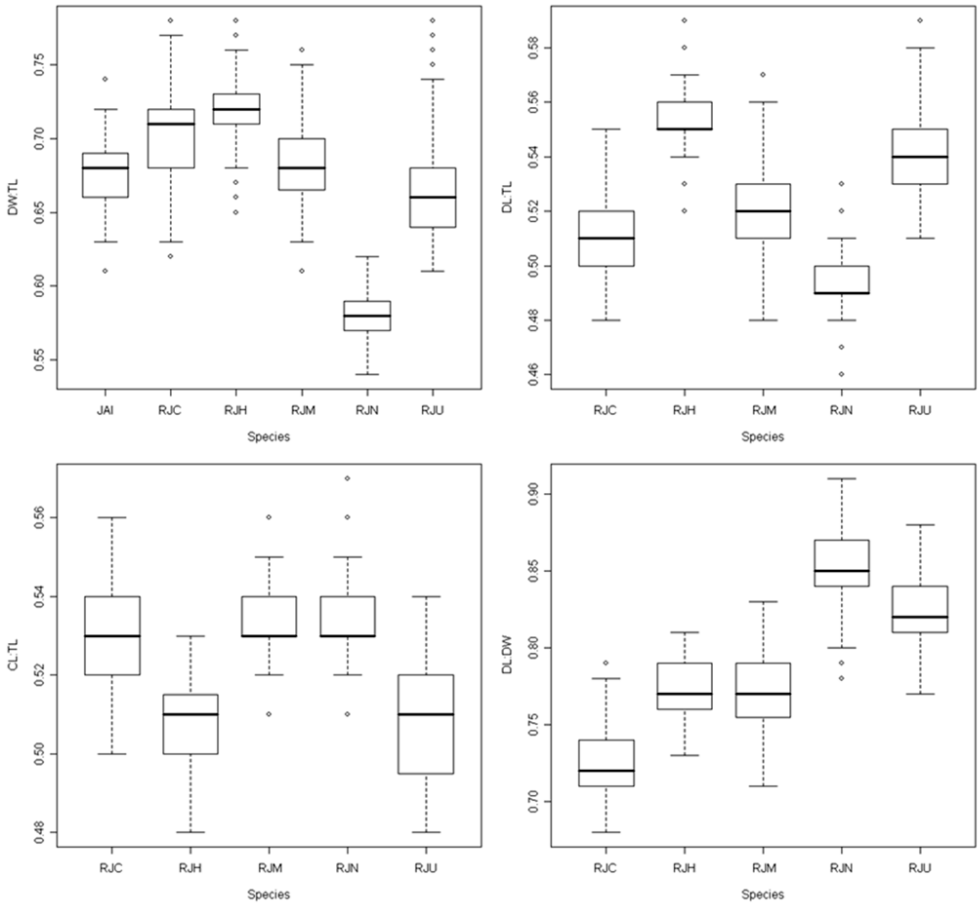

Figure 3.4. Boxplots showing species-specific variation in the morphometric ratios DW:TL, DL:TL, CL:TL, DL:DW.
JAI, *R. miraletus*; RJC, *R. clavata*; RJH, *R. brachyura*; RJM, *R. montagui*; RJN, *L. naevus*; RJU, *R. undulata*. Boxplot statistical entries: maximum, 3rd (75%) quartile, median, 1st (25%) quartile, and minimum, and the open circles represent outliers.

Nested models for the DW:TL ratio, considering the interactions of area, sex and TL classes, were statistically significant for *R. clavata*, *R. montagui*, and *R. undulata* (p-value<0.001). Nested models for the CL:TL ratio, considering the interaction between sex and TL classes, were statistically significant for all species except *R. undulata* (p-value<0.01). The nested model for DL:DW, considering the interaction between sex and TL classes, was only significant for *R. brachyura* (p-value<0.001). The size conversion factors estimated for the aforementioned nested models are presented in Table 3.6. Variations between area, sex, and TL class were observed, but there was no general trend.

For the remaining ratios (i.e. TL~DW for *R. miraletus*, *R. brachyura*, and *L. naevus*, TL~CL for *R. undulata*, TL~DL for all species but *R. miraletus*, and DW~DL for all species except *R. miraletus* and *R. brachyura*) there were linear relationships (Table 3.7). The estimates of the parameters for these relationships can be used as conversion factors between

Table 3.6. Estimates of the nested models for the morphometric ratios (DW:TL, CL:TL and DL:DW), by sex, area (N, north; C, centre; S, south), and TLclass.

Species	Ratio	R^2	TLclass (mm)	Females			Males		
				N	C	S	N	C	S
RJC	**DW:TL**	0.56	300	0.715	0.716	0.717	0.642	0.666	0.703
			400	0.722	0.710	0.707	0.717	0.710	0.723
			500	0.711	0.717	0.725	0.704	0.703	0.692
			600	0.721	0.719	0.745	0.682	0.699	0.680
			700	0.715	0.727	0.728	0.663	0.673	0.674
			800	0.709	0.717	-	-	-	0.640
	CL:TL	0.56	400		0.540			0.557	
			500		0.536			0.457	
			600		0.527			0.537	
			700		0.515			0.540	
			800		0.522			0.540	
RJH	**CL:TL**	0.54	400		0.515			0.510	
			500		0.513			0.519	
			600		0.523			0.510	
			700		0.500			0.513	
			800		0.495			0.500	
			900		-			-	
			1000		0.480			-	
	DL:DW	0.51	300		0.765			0.81	
			400		0.753			0.765	
			500		0.752			0.757	
			600		0.76			0.766	
			700		0.771			0.776	
			800		0.782			0.787	
			900		0.78			0.788	
			1000		0.772			-	
RJM	**DW:TL**	0.51	300	0.7	0.74	0.714	0.644	0.662	0.701
			400	0.6933	0.702	0.707	0.682	0.676	0.704
			500	0.702	0.687	0.705	0.66	0.655	0.69
			600	0.684	0.688	-	-	-	-
			700	0.65	0.69	-	-	-	-
	CL:TL	0.52	400		0.539			0.534	
			500		0.533			0.546	
			600		0.523			0.551	
RJN	**CL:TL**	0.51	400		0.55			0.55	
			500		0.532			0.524	
			600		0.524			0.521	
RJU	**DW:TL**	0.72	400	0.664	0.664		0.726	0.726	
			500	0.74	0.673		0.706	0.769	
			600	0.707	0.678		0.69	0.769	
			700	0.706	0.676		0.653	0.759	
			800		0.662		0.606	0.738	

TL, total length; DW, disc width; DL, disc length; CL, tail length; RJC, *R. clavata*; RJH, *R. brachyura*; RJM, *R. montagui*; RJN, *L. naevus*; RJU, *R. undulata*.

the various morphometric measurements. The coefficient of determination was high for all adjusted linear models ($R^2 > 0.85$).

According to the results of the likelihood ratio test, the differences between the estimated parameters of the length~weight models (TW~TL and gW~TL) adjusted by sex were not statistically different at a 95% confidence level, for all species. The parameter estimates of the nonlinear models TW~TL and gW~TL are also presented in Table 3.7, respectively. The estimated allometric coefficients were between 2.86 (*R. undulata*) and 3.58 (*L. naevus*) for TW~TL, and between 3.02 (*R. clavata*) and 3.49 (*L. naevus*) for gW~TL.

Table 3.7. Parameter estimates of the linear models for the pairs of linear distances (DW~TL, CL~TL, DL~TL, and DL~DW), and the nonlinear models, $W \sim aTL^b$ and $gW \sim aTL^b$, with standard errors around the estimates presented in parenthesis.

Relationship	Parameter	JAI	RJC	RJH	RJM	RJN	RJU
TL~DW	*a*	-33.02 (27.18)		-17.78 (5.66)		51.89 (7.46)	
	b	1.60 (0.10)		1.43 (0.01)		1.57 (0.02)	
	R^2	0.88		0.98		0.93	
TL~CL	*a*						-34.66 (17.36)
	b						2.07 (0.05)
	R^2						0.98
TL~DL	*a*		45.69 (8.83)	32.62 (5.30)	54.50(7.86)	66.37 (8.88)	2.23 (12.77)
	b		1.83 (0.02)	1.73 (0.01)	1.72(0.03)	1.78 (0.03)	1.84 (0.03)
	R^2		0.97	0.99	0.94	0.91	0.96
DW~DL	*a*		10.12 (6.95)		11.75 (5.96)	17.98 (5.04)	37.72 (7.32)
	b		1.36 (0.02)		1.26 (0.02)	1.11 (0.02)	1.13 (0.02)
	R^2		0.96		0.93	0.92	0.96
$W \sim aTL^b$	*a*		5.20×10^{-6} (1.77×10^{-6})	1.98×10^{-6} (5.07×10^{-7})	3.44×10^{-7} (1.53×10^{-7})	1.55×10^{-7} (7.45×10^{-8})	1.92×10^{-5} (8.61×10^{-6})
	b		3.05 (0.05)	3.20 (0.04)	3.47 (0.07)	3.58 (0.08)	2.86 (0.07)
$gW \sim aTL^b$	*a*		5.71×10^{-6} (2.23×10^{-6})	3.22×10^{-6} (8.12×10^{-7})	8.03×10^{-7} (3.79×10^{-7})	2.51×10^{-7} (1.27×10^{-7})	4.91×10^{-6} (2.90×10^{-6})
	b		3.02 (0.06)	3.11 (0.04)	3.32 (0.07)	3.49 (0.08)	3.04 (0.09)

TL, total length; DW, disc width; DL, disc length; CL, tail length; W, total weight; gW, gutted weight; JAI, *R. miraletus*; RJC, *R. clavata*; RJH, *R. brachyura*; RJM, *R. montagui*; RJN, *L. naevus*: RJU, *R. undulata*.

3.2.4.2. Species discrimination using morphometric analysis

In a first FDA procedure, the species and sexes were entered as discriminant classes. The FDA results revealed no sexual dimorphism in the morphometric ratios for most of the species studied (Table 3.8).The misclassification error was 0.33. The species with the lowest misclassification between sexes was *R. montagui* (10% for females and 0% for males), followed by *R. clavata* (16% for males and 21% for females). Yet, *R. montagui* females were misclassified as *R. clavata* males in 20% of cases and as *R. brachyura* females in 14%. The

species with the greatest misclassification between sexes were *R. brachyura* and *L. naevus*, for both of which most males were identified as females, 92% and 77% accordingly.

Table 3.8. Results of the flexible discriminant analysis (FDA) between five skate species and sexes (F, female; M, male).

	RJC (F)	**RJC (M)**	**RJH (F)**	**RJH (M)**	**RJM (F)**	**RJM (M)**	**RJN (F)**	**RJN (M)**	**RJU (F)**	**RJU (M)**
RJC (F)	78	21	2	0	0	5	0	0	0	0
RJC (M)	16	54	0	0	20	0	0	0	0	0
RJH (F)	6	4	91	92	14	10	0	0	0	0
RJH (M)	0	0	0	0	0	0	0	0	0	0
RJM (F)	0	21	7	4	66	10	0	0	6	0
RJM (M)	0	0	0	0	0	75	0	0	6	0
RJN (F)	0	0	0	0	0	0	97	77	0	10
RJN (M)	0	0	0	0	0	0	2	23	0	5
RJU (F)	0	0	0	4	0	0	0	0	61	43
RJU (M)	0	0	0	0	0	0	2	0	28	43

Each column corresponds to the identified species and sexes and the rows correspond to the classification made by the FDA model. RJC, *R. clavata*; RJH, *R. brachyura*; RJM, *R. montagui*; RJN, *L. naevus*; RJU, *R. undulata*. The cells of the matrix represent the percentage of the classification. Total misclassification error, 0.33.

In the second procedure, only species were entered as discriminant classes. In this case, the morphometric ratios (DW:TL, DL:DW and CL:DL) proved to be adequate for discriminating rajid species (Table 3.9). The misclassification error was 0.12. *Leucoraja naevus* was always discriminated from the other case study species. The greatest proportion of misclassifications was between *R. clavata* (15%) and *R. montagui* (29%). The former was misclassified as *R. montagui*, and the latter as either *R. clavata* or *R. brachyura.*

Table 3.9. Results of the flexible discriminant analysis (FDA) between five species of skate.

	RJC	**RJH**	**RJM**	**RJN**	**RJU**
RJC	85	1	18	0	0
RJH	3	91	11	0	5
RJM	12	6	71	0	5
RJN	0	0	0	100	8
RJU	0	1	0	0	82

Each column corresponding to the species and the rows to the classification made by the FDA model.RJC, *R. clavata*; RJH: *R. brachyura*; RJM, *R. montagui*; RJN, *L. naevus*; RJU, *R. undulata*. The cells of the matrix represent the percentage of the classification. Total misclassification error, 0.12.

3.2.5. Discussion

Following the call made by the ICES WGEF (ICES, 2007), size conversion factors were estimated for the six skate species studied here. A clear definition of the measurements, including anterior and posterior reference points and how the distance between these two points was measured, allow future application of the results without additional bias (Francis, 2006). As proposed, the longest longitudinal axis (TL) was used in most size conversion factors, because it is considered to be the best index of size (Francis, 2006). In some species (e.g. *R. clavata, R. montagui*, and *R. undulata*) differences were recorded between sexes, areas, and size classes. These differences should be taken into consideration when applying the conversion factors to other subsets, and for that reason, the sex, area, sample size, and length range of dependent and independent variables was reported for each species. Only limited morphometric information has been presented by other authors, typically in association with reproduction and growth studies, with the TL~W and TL~DW relationships the most frequently reported (Du Buit, 1975; Nottage and Perkins, 1983; Ryland and Ajayi, 1984; Coelho and Erzini, 2002). Jardas (1975) was the only author to focus on the morphometry of *R. clavata*, but he did not provide conversion factors. Pallaoro *et al.* (2005) presented the W~TL relationship for *R. clavata* and *R. miraletus*. Comparing the results from different studies, it is evident that, even for the same species, the conversion factors can vary between areas and sexes. The TL~W relationship is merely indicative for a species, because weight varies with size class, and many external factors may also influence the total weight of an individual, and hence influence the TL~W relationship, including maturation, spawning period, and food intake (Bagenal and Tesch, 1978). Moreover, the size range used in an analysis may also account for any differences observed between various studies. For that reason, the TL~W relationships are only really applicable for the size range examined (Petrakis and Stergiou, 1995). Data on gutted weight are limited, and such data could be usefully collected in future studies elsewhere in European waters. The gutted weight could be more informative when comparing fish from different sampling areas exposed to different environmental conditions.

The results indicate that morphometric ratios may be considered a useful, simple, and reliable additional tool for helping with species discrimination. They can be applied successfully in cases where recurrent doubts remain on the separation of species, as is often the case between some specimens of *R. montagui* and *R. brachyura*, which can share a similar colour pattern, but do show distinct body morphometry, with a low percentage of

misclassification between them. Also, if less specialized workers encounter problematic animals in the field, the collection of more information on the morphometry of such fish could serve as a useful tool for subsequently validating identification, without the need for examining more complex diagnostic characters (e.g. the number of rows of teeth, or clasper structure). Three morphometric ratios (DW:TL, DL:DW, and CL:DL) proved to be adequate for discriminating the six rajid species under study, according to FDA. The measurements proved to be 100% successful in identifying *L. naevus*, a species belonging to a different genus from the remaining five species, and therefore characterized by a defined morphotype, with narrow, long disc, and a long tail. *Raja montagui* and *R. clavata* provided the most cases of misclassification; both these species have a diamond-shaped disc that could be a reason for the observed misclassification given by FDA.

3.3. FEEDING ECOLOGY [1]

3.3.1. Abstract

Data on the diet of species are important for understanding ecosystem dynamics and are fundamental for the implementation of recent approaches in stock assessment and consequently for the establishment of more ecological management measures. In mainland Portugal, as in most European countries, skates and rays represent an important proportion of commercial landings. The four main species landed are *Raja clavata* and *Raja brachyura*, followed by *Leucoraja naevus* and *Raja montagui*. This paper analyses their diets based on the examination of stomach contents. Food items were identified to the lowest identifiable taxon and were further assembled into major taxonomic groups designated as prey. Intra- and interspecific comparisons were made according to size and sex. All four species had generalized diets with differences in prey preference among them. Decapods and bony fish were the most frequent prey. Furthermore, an ontogenetic dietary shift was evident in all species at around 45−55 cm total length. Both intra- and interspecific differences observed seem to be related to size and morphological characteristics of the species, as well as type of

[1] Farias, I., Figueiredo, I., Moura, T., Serrano Gordo, L., Neves. A. and Serra-Pereira, B. 2006. Diet comparison of four ray species *Leucoraja naevus, Raja brachyura, Raja clavata* and *Raja montagui* caught along the Portuguese continental coast. Aquatic Living Resources, 19, 105-114. doi: 10.1051/alr:2006010.

dentition. These variations allow different species, as well as small and large specimens from the same species, to exploit a larger diversity of habitats.

Keywords: Feeding ecology, Dietary composition, Ontogenetic dietary shift, Ray, Skate, Elasmobranch fish, Atlantic Ocean.

3.3.2. Introduction

Diet studies are important for the understanding of species biology and ecology, since the quality and quantity of food are exogenous factors that directly affect species growth and, indirectly, their maturation and mortality (Wootton, 1990; Stergiou and Karpouzi, 2001). Feeding ecology studies are commonly based on the analysis of stomach contents of collected specimens, since the use of direct methods is usually difficult or even impossible for fish species (Assis, 1992; Cortés, 1997). These studies also represent an essential tool for the comprehension of some population phenomena, such as migrations, competition and physiological variations, and consequently for the understanding of fluctuations in stock abundance (Assis, 1992). The quantification of diets and trophic relationships are also fundamental inputs for the implementation of ecosystem models (Herrán, 1988; Stergiou and Karpouzi, 2001).

The commercial interest in cartilaginous fishes has increased worldwide in recent decades, mainly due to the depletion of many commercial bony fish stocks together with an increasing interest on elasmobranch muscle, cartilage, liver oil and fins (Stehmann, 2002). In the North-eastern Atlantic, skates and rays represent more than 40% of elasmobranch landings and within these the order Rajiformes is particularly important due not only to their diversity but also to their economical value (Walker, 1999).

In Portugal, as in most European countries, rays and skates landings are recorded as aggregate landings, and are not usually differentiated into species. A sampling program on skates and rays landings carried on along the Portuguese continental shelf showed that *Raja clavata* and *Raja brachyura* were the most commonly landed species, whereas *Raja montagui* and *Leucoraja naevus* represented nearly 40% of the remaining sampled specimens (Machado *et al.*, 2004). In previous diet studies carried out at North European waters, the main prey for *R. clavata* were shrimps and brachyuran crabs, for *R. brachyura* were bony fish and shrimps, for *R. montagui* were mysids and other small crustaceans, and for *L. naevus*

were small crustaceans and bony fish (Holden and Tucker, 1974; Du Buit, 1978; Ajayi, 1982; Ellis *et al.*, 1996). In African Atlantic waters, *R. clavata* was found to feed mostly on lobsters and fish (Ebert *et al.*, 1991). Former studies along the Portuguese coast pointed out shrimps and brachyuran crabs as the most common prey for the four species, being fish also important in *L. naevus* (Marques and Ré, 1978; Cunha *et al.*, 1987). In the Azorean waters, fish, brachyuran crabs and mysids were identified in *R. clavata* stomachs (Gomes *et al.*, 1998).

The present paper studies the diet composition and feeding habits of four of the most important rajid species landed in Portuguese ports: *Raja clavata* Linnaeus 1758; *R. brachyura* Lafont 1873; *R. montagui* Fowler 1910; and *Leucoraja naevus* (Müller & Henle 1841). This study is based on stomach contents analysis and includes intra- and interspecific comparisons according to size and sex.

3.3.3. Material and Methods

3.3.3.1. Sampling

For this study, stomachs of *Raja clavata* (n = 159), *R. brachyura* (n = 97), *R. montagui* (n = 127) and *Leucoraja naevus* (n = 135) were analysed. Samples were collected from commercial landings and from scientific trawl surveys carried out by IPIMAR, between 2001 and 2005. For each specimen, the following informationwas recorded: total length (*TL*, mm), total weight (g), and sex.

The food categories that composed the stomach contents were identified to the lowest possible taxonomic level, counted and weighted. The main prey categories were grouped into major taxonomic groups and assembled to the following prey: polychaetes (Polychaeta); unidentified crustaceans (Crustacea); amphipods (Crustacea: Amphipoda); mysids (Crustacea: Mysidacea); isopods (Crustacea: Isopoda); decapods (Crustacea: Decapoda); shrimps (Crustacea: Decapoda: Dendrobranchiata and Caridea); anomurans (Crustacea: Decapoda: Anomura); lobsters (Crustacea: Decapoda: Macrura); brachyuran crabs (Crustacea: Decapoda: Brachyura); cephalopods (Cephalopoda); and bony fish (Osteichthyes). Minor prey-taxa: Algae, Cnidaria, Sipuncula, Bivalvia, Gastropoda, and Echinodermata were categorized as “Others”, and weighted and counted as a unique food category. Each food category was further designated as prey. Unidentified material was excluded from the analysis since its occurrence was negligible.

Specimens from each sex were grouped into two major length groups designated as "small" (TL < 50 cm) and "large" (TL ≥ 50 cm). This value is close to the length at first maturity of *R. clavata*, *R. montagui* and *L. naevus*. Although the length at first maturity of *R. brachyura* is estimated at about 90 cm (Walker, 1999), the size limit used was also 50 cm due to the insufficient number of large individuals available in the samples.

3.3.3.2. Data analysis

3.3.3.2.1. Overall diet

For each species, the index of vacuity (%IV) was determined, by sex, as the percentage of empty stomachs in the whole sample of stomachs. A stomach was considered to be empty when it only contained either nothing or only a small amount of digested and unidentified material, sediment or endoparasites.

To evaluate the importance of each prey, the following indices were determined: (i) percentage by number (%N); (ii) percentage by weight (%W); (iii) percentage frequency of occurrence (%O); and (iv) percent index of relative importance (%IRI). By expressing the IRI as a percentage, it constitutes a robust estimator of the relative importance of each prey and facilitates comparisons between different prey (Cortés, 1997).

A χ^2 test was used to test the null hypothesis of no differences between sexes on the number of occurrence of each prey for each predator species. A univariate t-test was used to test the null hypothesis of no differences on the weight of each prey between sexes. In both tests, a 5% significance level was adopted.

3.3.3.2.2. Prey importance and feeding strategy

Three-dimensional diagrams were constructed for each predator species and major length group by displaying the stomach contents in terms of %N, %W and %O (Cortés, 1997). This approach illustrates the diet in terms of prey importance, by distinguishing between dominant and rare prey, and also according to predator feeding strategy, by differentiating between generalist and specialist diets. In this graphical approach any point located close to 100% O, 100% N and 100% W corresponds to a dominant food item, whereas a point located near the origin of axes corresponds to a rare food item. Furthermore the existence of a cluster of points located close to 100% O and the origin of at least one of the other two axes

corresponds to a generalized diet. Alternatively, a cluster near 100% O and 100% for at least one of the other indices corresponds to a specialized diet.

To evaluate ontogenetic dietary shifts, plots of mean partial fullness index (PFI) vs. *TL* class (10 cm length classes) were done by sex (Lilly and Rice, 1983). PFI was determined according to:

$$PFI_i = \frac{1}{n}\sum_{j=1}^{n}\frac{W_{ij}}{(TL_j)^3}\times 10^4 \text{ ,}$$

where *Wi j* is the weight of the *i*th prey in the *j*th stomach, *TLj* is the total length of the *j*th predator (in cm) and *n* is the total number of sampled stomachs. Length was used instead of weight since the former is not influenced by changes in muscle, liver, gonads and stomach contents. This index has the advantage of not being strongly influenced by either the frequent occurrence of small prey or by the rare presence of large prey (Lilly and Rice, 1983).

In order to get some insight about trophic differences between the four predator species further divided by length group, a cluster analysis was applied using Schoener's (1970) dissimilarity index and Ward's clustering method. This index was determined using percentage by weight (%W) as a diet measure (Wallace, 1981):

$$S = \sum_{i=1}^{n}\inf(W_{ip}, W_{iq}) \text{ ,}$$

where *Wip* is the weight of prey item *i* found in stomach *p* relative to the total weight of prey items, *Wiq* is the same for predator *q* and inf(*Wip*,*Wiq*) is the infimum between the two values. When resource availability data are absent, this index appears to be the most accurate to estimate diet overlap (Wallace, 1981). It has been demonstrated that cluster analyses provide an efficient and relatively simple way of comparing data from feeding studies (Ross, 1978). In this graphical representation, the smallest linkages indicate the greatest similarities between the diets.

3.3.4. Results

3.3.4.1. Overall diet

Stomach composition of the four ray species is presented in Table 3.10, which also includes, if available, information on the main prey's habitat. Some prey were highly digested and consequently difficult to identify. Some of the brachyuran crabs, cephalopods and fish

presented marks of teeth on the carapaces and bodies. *L. naevus* presented indices of vacuity both by sex higher than in the other three species (Table 3.11). In *R. clavata* and *R. brachyura*, the index was higher in males, whereas was it greater in females of the other two species. In *L. naevus*, the value of the index for females was almost twice that for males. Few specimens had everted stomachs (two of each of *R. clavata* and *R. montagui* and one of *L. naevus*).

Table 3.10. Overall diets of the four ray species.
(*Raja clavata*, *R. brachyura*, *R. montagui* and *Leucoraja naevus*) identified to species level, with available information on the main habitat of prey.

Prey-taxon	Habitat	*Raja clavata*	*Raja brachyura*	*Raja montagui*	*Leucoraja naevus*
ANNELIDA					
POLYCHAETA					
Glycera spp.	shallow sublittoral	x		x	
Nephthys spp.	shallow to deep water	x	x	x	x
Sigalion spp.	low water		x	x	
Leanira spp.		x		x	
Eupanthalis kinbergi				x	
ARTHROPODA					
CRUSTACEA					
OSTRACODA	most benthic		x		
COPEPODA			x		x
MALACOSTRACA		x	x	x	x
CUMACEA					x
EUPHAUSIACEA					x
AMPHIPODA			x		
Ampelisca brevicornis	intertidal and sublittoral	x		x	
Ampelisca spinipes		x		x	
Ampelisca unidentata		x		x	
Ampelisca armoricana				x	
Ampelisca spooneri				x	
Ampelisca sarsi				x	
Hippomedon denticulatus	sublittoral; shallow water			x	x
Hippomedon oculatus					x
MYSIDACEA					
Lophogaster typicus		x		x	x
Gastrosaccus normani			x		
Paramysis arenosa				x	
ISOPODA					
Conilera cylindracea	sublittoral	x		x	
Cirolana cranchi	offshore			x	
Eurydice pulchra	intertidal	x		x	x
Eurydice spinigera	sublittoral		x	x	x
Eurydice affinis	intertidal			x	
DECAPODA					
DENDROBRANCHIATA					
Solenocera membranacea	20−700 m deep	x		x	x

Table 3.10. Continued.

Prey-taxon	Habitat	*Raja clavata*	*Raja brachyura*	*Raja montagui*	*Leucoraja naevus*
ARTHROPODA					
CRUSTACEA					
DECAPODA					
CARIDEA					
Pasiphaea sivado	10−600 m deep	x			
Alpheus glaber	30−40 m deep	x		x	
Alpheus macrocheles	littoral or sublittoral		x		
Athanas nitescens	phanerogamic prairies	x			
Processa canaliculata	70−600 m deep	x	x		x
Processa edulis	phanerogamic prairies	x			
Processa intermedia	rare	x			
Processa macrophthalma	shallow water	x		x	
Processa mediterranea	about 200 m deep	x	x		
Processa elegantula	rare; 30−40 m deep		x		
Processa nouveli holthuisi	rare; 20−230 m		x		
Processa modica				x	
Chlorotocus crassicornis	50−600 m deep	x		x	x
Pandalina brevirostris	20−30 m to 100 m deep		x		
Aegaeon lacazei	200−400 m deep	x			x
Pontophilus spinosus	10−200 m deep	x			
Pontophilus norvegicus	200−500 m deep			x	
Crangon crangon	benthic; shallow water		x	x	
MACRURA					
Callianassa tyrrhena	shallow water to 1 m deep	x			
Scyllarus arctus				x	
ANOMURA					
Pagurus bernhardus	coastal to 500 m deep	x			
Anapagurus spp.		x			
Galathea intermedia	30−40 m deep	x	x		
Munida rutlanti	80−500 m deep	x			x
Munida intermedia	300−400 m deep			x	
BRACHYURA					
Corystes cassiveluanus	10−20 m deep	x			
Atelecyclus rotundatus	20−90 m deep	x		x	
Atelecyclus undecimdentatus	shallow water to 30 m deep			x	
Thia scutellata	4−20 m deep	x	x	x	
Pirimela denticulata	near coast to up to 200 m deep			x	
Polybius henslowi	shallow water to 200 m deep	x	x	x	
Liocarcinus depurator	shallow water to 300 m deep	x		x	
Liocarcinus marmoreus	shallow water to 200 m deep	x			
Liocarcinus pusillus	shallow water to 200 m deep		x		
Pinnotheres pinnotheres	commensal with bivalves and ascids	x		x	
Goneplax rhomboides	shallow water to 400 m deep	x		x	
Eurynome aspera	10−550 m deep	x			

Table 3.10. Continued.

Prey-taxon	Habitat	*Raja clavata*	*Raja brachyura*	*Raja montagui*	*Leucoraja naevus*
MOLLUSCA					
BIVALVIA		x		x	x
GASTROPODA		x	x	x	x
CEPHALOPODA					
Sepia spp.	demersal	x			
Alloteuthis subulata	neritic and demersal; sandy bottom	x	x	x	
Loligo vulgaris	nectobenthic; surface to 550 m deep	x	x		x
Loligo forbesii	nectobenthic; surface to 400 m deep		x		
Histioteuthis spp.	oceanic x	x			
Illex coindetii	neritic; surface to 1100 m deep	x	x		
Todarodes sagitattus					x
Eledone cirrhosa	benthic; 45−580 m deep	x			
ECHINODERMATA		x			x
CHORDATA					
OSTEICHTHYES					
Sardina pilchardus	coastal pelagic; 25−55 m deep		x	x	x
Argentina sphyraena	continental shelf to 450 m or deeper	x		x	
Belone belone	epipelagic; neritic	x			
Micromesistius poutassou	mesopelagic; 160−3000 m deep	x	x	x	x
Trisopterus luscus	adults offshore; 30−100 m deep	x	x		
Merluccius merluccius	semi-benthic; 100−300 m deep	x			
Trachurus trachurus	sandy bottom in 100−200 m deep	x	x	x	
Pomatoschistus minutus	inshore; sand; to about 20 m deep	x			
Scomber scombrus	pelagic; up to 200−250 m deep	x			
Lepidotrigla cavillone	muddy sands; 30−450 m deep	x			
Echiichthys vipera	littoral and benthic		x		
Trachinus draco	littoral and benthic			x	
Gymnammodytes semisquamatus	offshore over shell-gravel		x	x	x
Callionymus maculatus	benthic; sandy bottoms; 45−650 m deep		x		
Citharus linguatula	benthic or continental shelf		x		
Lepidorhombus boscii	depths down to 700−800 m		x		
Arnoglossus spp.	benthic			x	

Table 3.11. Number of sampled stomachs (n) by species, sex (F: females; M: males) and major length group (S: small, *TL* < 50 cm; L: large, *TL* ≥ 50 cm) and index of vacuity estimates (%IV).

			Raja clavata	*Raja brachyura*	*Raja montagui*	*Leucoraja naevus*
F	n	S	11	5	32	27
		L	62	56	33	55
		%IV	0	1.6	17.1	4.6
M	n	S	11	5	34	20
		L	75	31	28	33
		%IV	3.5	2.8	9.4	1.6

3.3.4.2. Prey importance and feeding strategy

For the four ray species, no significant differences in occurrence and weight were found between sexes (Table 3.12) and therefore feeding strategy plots were constructed by combining data from females and males. Feeding strategy three-dimensional plots for each

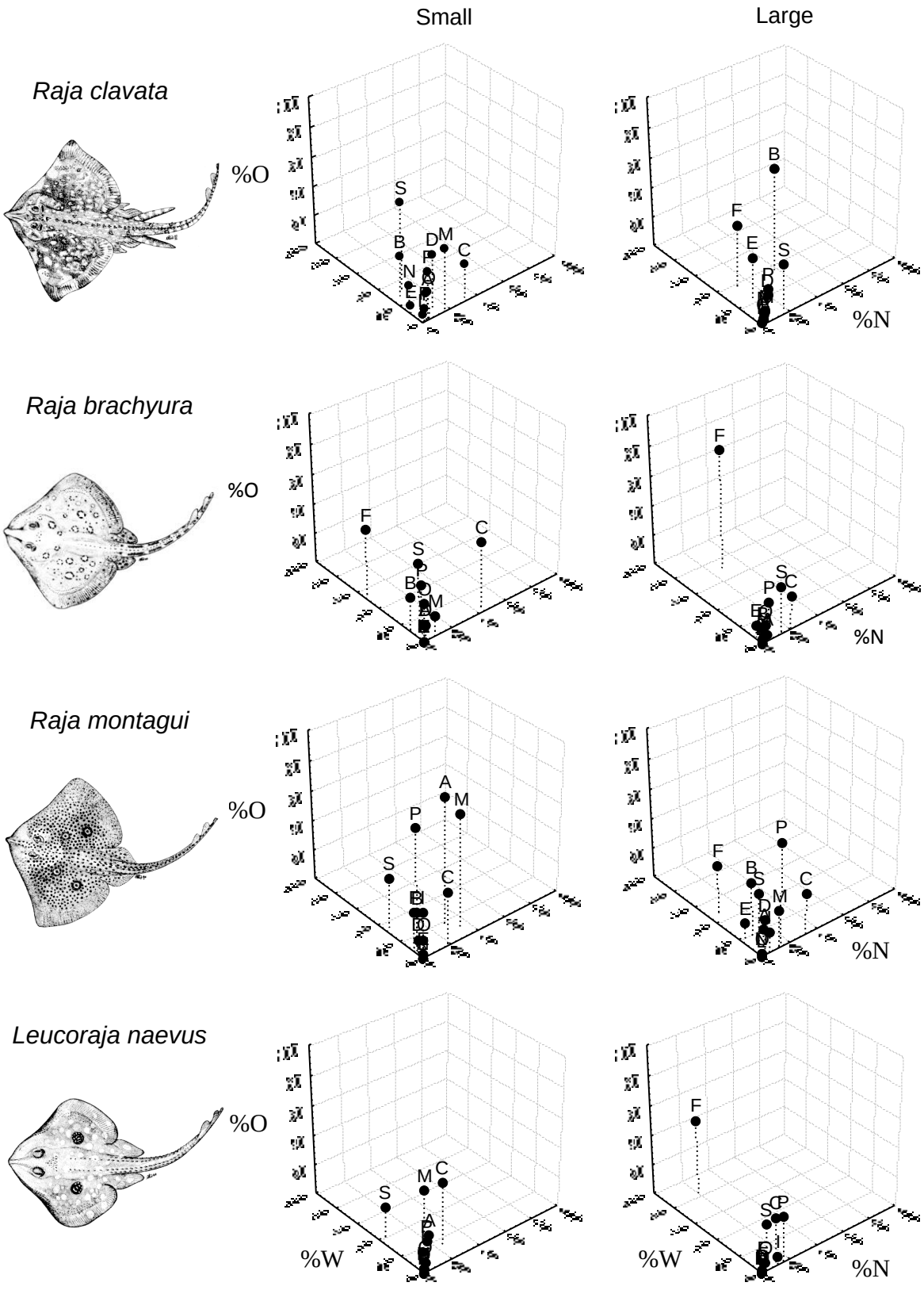

Figure 3.5. Three-dimensional representations of the feeding habits of small (TL < 50 cm) and large (TL ≥ 50 cm) *Raja clavata*, *R. brachyura*, *R. montagui* and *Leucoraja naevus*.
Prey codes – P: Polychaetes; C: Unidentified crustaceans; A: Amphipods; M: Mysids; I: Isopods; D: Decapods; S: Shrimps; L: Lobsters; N: Anomurans; B: Brachyuran crabs; E: Cephalopods; F: Bony fish; O: Others. Fish drawing adapted from Bauchot (1987). Percentage by number (%N), by weight (%W) and frequency of occurrence (%O).

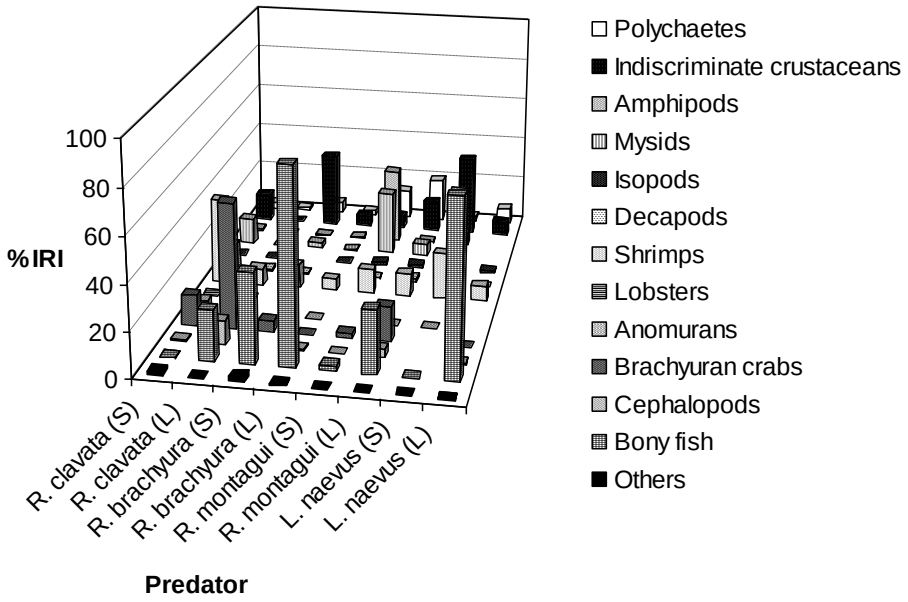

Figure 3.6. Index of relative importance (%IRI) by species and major length group.
(S: small, *TL* < 50 cm, L: large, *TL* ≥ 50 cm) of *Raja clavata*, *R. brachyura*, *R. montagui* and *Leucoraja naevus*.

rajid species are presented and summarized with information on frequently identified species and their habitats (Figure 3.5, Table 3.13). These results are further supported by %IRI values (Fig. 3.6). For small *R. clavata*, the most important prey were indiscriminate crustaceans and shrimps, namely the benthic shrimp *Solenocera membranacea*. Besides these prey, large specimens fed also on bony fish and brachyuran crabs, within which *Polybius henslowi* (pelagic) was the most common item. For *R. brachyura*, bony fish followed by indiscriminate crustaceans and shrimps were the main prey both for small and large individuals (Fig. 3.6). For the former, *Crangon crangon* (benthic shrimp that lives in shallow waters) was the most common item, while large specimens fed predominantly on benthic offshore prey like the smooth sandeel *Gymnammodytes semisquamatus* and the small shrimp *Processa canaliculata* (Figure 3.5; Table 3.13). Polychaetes and small intertidal crustaceans (e.g. *Ampelisca* spp. and *Lophogaster typicus*) were the most important prey for *R. montagui*, and showed the highest values of %IRI (Fig. 3.6). Large specimens also fed on bony fish like *Micromesistius poutassou* (mesopelagic). For *L. naevus*, indiscriminate and small crustaceans, like *L. typicus* and *S. membranacea*, were the most important prey for small specimens. For large individuals, polychaetes and the bony fish *G. semisquamatus* were also important food items (Figs. 3.5 and 3.6; Table 3.13).

For all ray species, the plots of mean PFI versus predator's total length class (Fig. 3.7) suggested ontogenetic shifts in their diets at lengths of about 45−55 cm in both sexes. Smaller

Table 3.12. Estimated statistics for testing differences between sexes in number of occurrence and weight for each major length group.
(S - small, TL < 50 cm; L - large, TL ≥ 50 cm). χ^2=20.03 with α=0.05 for occurrence. p>0.05 for weight. r - H_0 is rejected; nr - H_0 is not rejected.

Index	*Raja clavata*				*Raja brachyura*				*Raja montagui*				*Leucoraja naevus*			
	S		L		S		L		S		L		S		L	
Occurrence	7.78	nr	12.23	nr	10.24	nr	14.43	nr	11.11	nr	10.33	nr	12.52	nr	7.69	nr
Weight	0.39	nr	0.24	nr	0.37	nr	0.80	nr	0.24	nr	0.43	nr	0.16	nr	0.16	nr

males and females of *R. clavata* fed mainly on polychaetes, mysids, and various other small crustaceans, but with low values of mean PFI. For specimens larger than 45 cm, the values of mean PFI increased and cephalopods, bony fish and brachyuran crabs were the main prey. For females, two modal peaks were evident at lengths of about 50 and 75 cm. For males, there are also two marked peaks but at around lengths of 60 and 70 cm.

Fish were a major prey item for all sizes of *R. brachyura* (Fig. 3.7). Excluding bony fish, polychaetes were the most common prey for females with lengths from 45 to 65 cm, and shrimps and brachyuran crabs prevailed in males from 35 to 45 cm. Cephalopods were the most important prey for both sexes for specimens larger than 50 cm.

For small females of *R. montagui* (Fig. 3.7), the most important prey were various crustaceans and polychaetes, while large females predated primarily on fish. For males, although shrimps were equally important for large and small specimens, for the former, cephalopods, brachyuran crabs and fish also showed high values of mean PFI.

Table 3.13. Feeding habits by species and major length group.

Length group		***Raja clavata***	***Raja brachyura***	***Raja montagui***	***Leucoraja naevus***
Small	Most important prey	Indisc. crustaceans Shrimps	Shrimps Bony fish Indisc. crustaceans	Polychaetes Amphipods Mysids	Shrimps Mysids Indisc. crustaceans
	Examples of identified species	*Solenocera membranacea* (1)	*Crangon crangon* (2)	*Ampelisca* spp. (3); *L. typicus* (3)	*Lophogaster typicus* (3) *S. membranacea* (1)
Large	Most important prey	Brachyuran crabs (dominant) Bony fish Indisc. crustaceans Shrimps	Bony fish (dominant) Shrimps Indisc. crustaceans	Polychaetes; Indiscriminate crustaceans; Bony fish.	Bony fish (dominant) Polychaetes Indisc. crustaceans Shrimps
	Examples of identified species	*Polybius henslowi* (4)	*Processa canaliculata* (5) *G. semisquamatus* (5)	*Micromesistius poutassou* (6)	*G. semisquamatus* (5)

Habitat: (1) benthic, (2) benthic, shallow waters, (3) suprabenthic, intertidal to sublittoral, (4) pelagic, (5) benthic offshore, (6) mesopelagic.

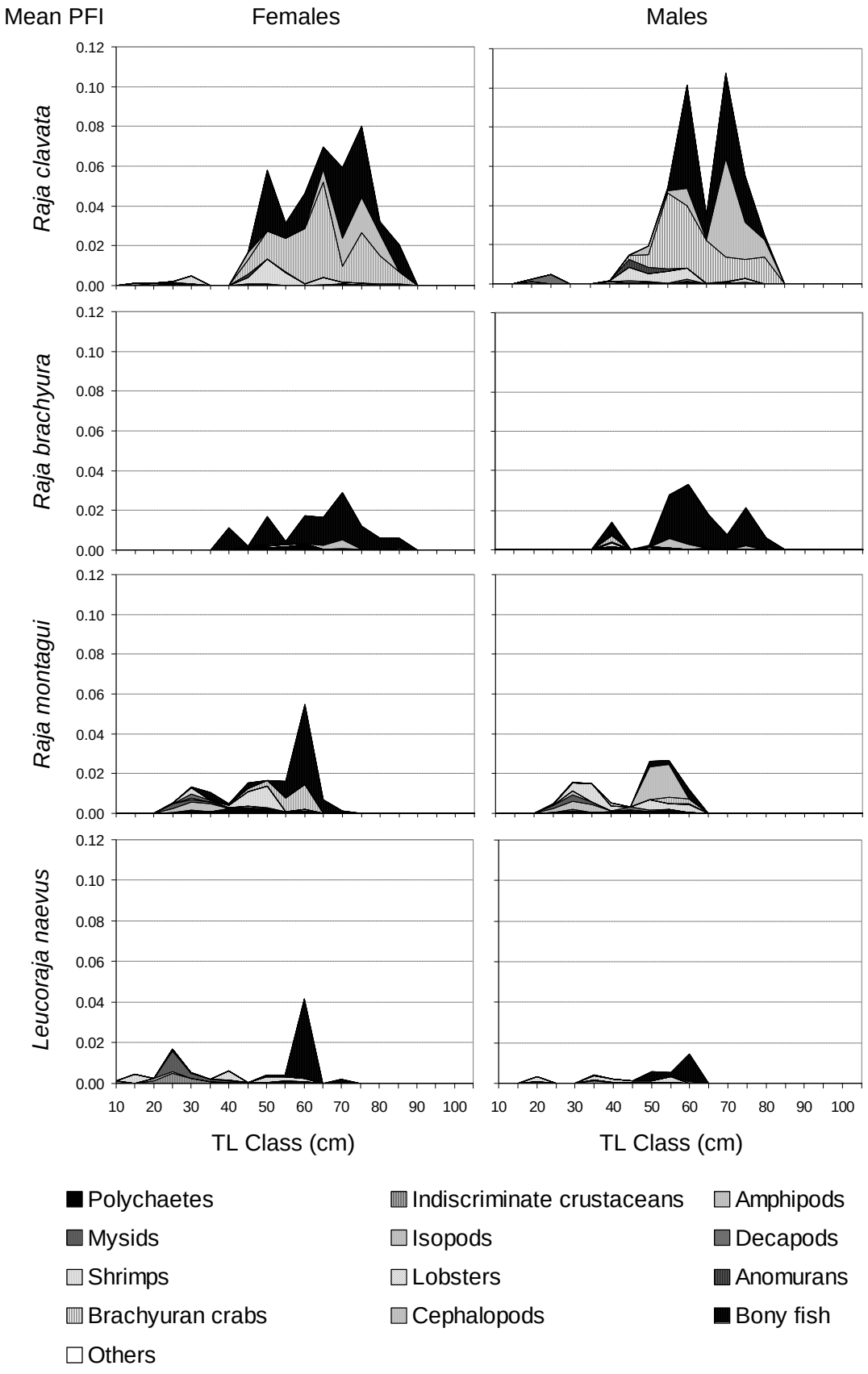

Figure 3.7. Mean partial fullness index (PFI) vs. total length (TL, cm) class of females (left column) and males (right column) of *Raja clavata*, *R. brachyura*, *R. montagui* and *Leucoraja naevus*.

For *L. naevus* specimens larger than 45 cm (Fig. 3.7), fish were the dominant prey, with high values of mean PFI. Mysids were important for females with lengths ranging from 25 to 35 cm. Shrimps also showed relatively high values for females with 10−20 and 35−60 cm length and for males with about 15−25 and 30−60 cm length.

The dendrogram (Fig. 3.8) illustrates the similarity in the diets of small and large rays of the four species analysed. "Large" and "small" length groups were separated into two major clusters. Small *L. naevus*, small *R. montagui* and small *R. clavata* were grouped into one cluster. Large *R. brachyura* and large *L. naevus* were clustered together, showing a high level of similarity. Small *R. brachyura* was further linked to this cluster. Large *R. clavata* and *R. montagui* had similar diets and were relatively similar to the diets of the preceding group.

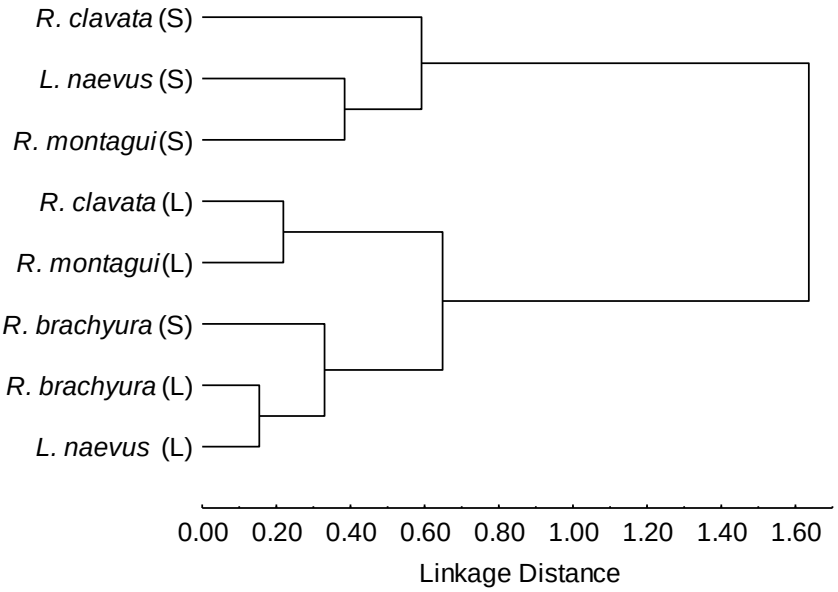

Figure 3.8. Cluster analysis of prey similarity between species (*Raja clavata*, *R. brachyura*, *R. montagui* and *Leucoraja naevus*) divided by major length group (S: small, TL < 50 cm, L: large, TL ≥ 50 cm).
Ward's method, dissimilarity matrix based on Schoener's (1970) index. Similarity was determined from percentage by eight of each prey.

3.3.5. Discussion

The occurrence of large prey, like brachyuran crabs, cephalopods and bony fish, with marks of teeth on their carapaces and bodies may suggest that they had been chewed prior to ingestion. This feeding strategy is known to occur in *R. clavata*, *R. montagui* and *L. naevus* (Daan *et al.*, 1993). Furthermore it was observed that small and soft prey, like polychaetes and some small crustaceans were more easily digested. The most obvious consequences of

the occurrence of highly digested prey were the difficulty in identifying some specimens, and the underestimation in weight of some prey.

The high index of vacuity recorded for *L. naevus* has also been reported in other diet studies of this species (Holden and Tucker, 1974; Ellis *et al.*, 1996). Piscivorous species are generally found to possess a relatively high index of vacuity, and this may be because fish have a higher nutritional value than crustaceans and so it is not necessary to feed so frequently; are digested more rapidly than invertebrates; or because feeding is restricted by success in prey capture (Ellis *et al.*, 1996). Despite the small proportion of everted stomachs observed, the full stomach eversion, followed by its swallowing, could also be a factor contributing for the occurrence of empty stomachs. This mechanism has been described for rays and other elasmobranchs and allows removing parasites, indigestible material, toxic food and remains of gastric mucosa and mucus (Sims *et al.*, 2000; Brunnschweiler *et al.*, 2005).

The analysis of feeding strategy plots highlighted the generalized diets exhibited by all four ray species with a higher incidence of benthic preys. In general, results were similar to those presented for the species in other Atlantic areas (Holden and Tucker, 1974; Du Buit, 1978; Marques and Ré, 1978; Ajayi, 1982; Cunha *et al.*, 1987; Ebert *et al.*, 1991; Ellis *et al.*, 1996; Gomes *et al.*, 1998).Cephalopods and fish, especially Gadiformes and Clupeiformes, were more frequent in the diets of larger specimens and in species that attain larger maximum lengths, like *R. clavata* and *R. brachyura*. This may result from the fact that larger individuals are more active predators and their swimming capacities allow to catch faster prey and also to forage along the water column (Ebeling, 1988).

Results indicated the existence of an ontogenetic dietary shift at around classes 45−55 cm for the four analysed species, despite all attaining different maximum lengths and lengths at first maturity. This fact suggests that this shift is more dependent on size than on other life history characteristics. The relationship between predator's size and mouth dimensions in rays has been frequently stated to be correlated with their diets and their degree of prey specialization (Du Buit, 1978; Walker, 1999; Scharf *et al.*, 2000). Small *L. naevus*, *R. montagui* and *R. clavata* had relatively narrow diets, possibly limited by mouth size and swimming capacity, being grouped together in a distinct cluster. Small and large *R. brachyura* showed a very similar diet, both feeding mainly on bony fish. The type of dentition is also accepted to affect the type of diet. Cusped teeth are prevalent in piscivorous rays, whereas molariform teeth are better suited to feeding on crustaceans and other hard invertebrates (Du Buit, 1978). *R. brachyura* and *L. naevus*, which mainly feed on fish and are clustered together, have both cusped teeth (Du Buit, 1978). Large *R. clavata* and *R. montagui*,

which were included in the same cluster, feed mostly on brachyuran crabs and fish and both have molariform teeth (Du Buit, 1978).

In all four species, ontogenetic shifts were in general characterized by changes from small to larger and faster prey, from benthic to semi-pelagic feeding habits, from shallow to offshore waters and from crustacean-dominated diets to a more piscivorous diet. The main changes were from benthic shrimps to pelagic crabs for *R. clavata*; from benthic teleosts to offshore teleosts and occasionally pelagic teleosts for *R. brachyura*; from sublittoral supra-benthic prey to mesopelagic teleosts for *R. montagui*; and from mysids and benthic shrimps to mesopelagic and offshore benthic teleosts for *L. naevus*. Similar shifts have been also recorded for these species in other geographic areas (e.g. Steven, 1930; Holden and Tucker, 1974; Ajayi, 1982; Daan *et al.*, 1993; Walker, 1999).

Chapter 4

Skate life-history – case-study of the thornback ray, *Raja clavata*

4. SKATE LIFE-HISTORY - case-study of the thornback ray, *Raja clavata*

4.1. AGE AND GROWTH [1]

4.1.1. Abstract

This work is a response to a lack of knowledge of the biology of *Raja clavata* in southern European waters, particularly in terms of age and growth. Two structures were analysed: dermal denticles and vertebral centra. Six types of dermal denticle were identified in the tail. Among those, small thorns were the most suitable for age determination owing to their fixed position, persistence throughout their lifespan, and defined growth-band pattern. Caudal thorns were more accurate than vertebral centra for age determination and were therefore selected as the most appropriate structure for ageing *R. clavata*. Based on edge analysis, annual band deposition was verified. The birthdate was established as 1 June based on the prevalence of hyaline edges in age-0 class specimens: prevalence peaked in May and June. Both von Bertalanffy and Gompertz growth models were fitted to age-at-length data, but the former was considered more appropriate based on similarity between the estimated L_∞ and the observed maximum size. No significant differences in growth parameters were observed between sexes. The estimated growth parameters were L_∞ = 1280 mm, k = 0.117 year^{-1}, and t_0 = 20.617 years. The maximum age estimated for *R. clavata* was 10 years, for a female of length 835 mm.

Keywords: caudal thorns, edge analysis, precision analysis, Rajidae, thornback ray, vertebrae.

[1] Serra-Pereira, B., Figueiredo, I., Farias, I., Moura, T. and Gordo, L. S. 2008. Description of dermal denticles from the caudal region of *Raja clavata* and their use for the estimation of age and growth. ICES Journal of Marine Science, 65: 1701-1709. doi: 10.1093/icesjms/fsn167

4.1.2. Introduction

Skates and rays represent an important proportion of Portuguese mixed fishery landings. They are caught mainly by the artisanal fleet operating with trammel-nets, gillnets, and longlines, but also as bycatch in demersal trawls. As K-strategists, they are particularly vulnerable to depletion as a result of fishing activity. As several studies have shown, the most vulnerable species to exploitation are those with larger maximum sizes, later maturation, and lower rates of potential population increase (Dulvy *et al.*, 2000). *Raja clavata*, commonly known as the thornback ray, is one of these examples, and one of the most abundant ray species in the Northeast Atlantic (Walker and Hislop, 1998). In the past 50 years, its abundance has declined, mainly as a consequence of fishing pressure (Walker and Hislop, 1998).

In recent decades, many authors have recognized the ecological importance of *R. clavata* and have carried out studies on its biology. Reproduction peaks from April to July (Holden, 1975), and populations are relatively sedentary but undertake short migrations towards the coast during the reproductive season (Steven, 1936). The estimated length-at-first maturity for females is 595–771 mm and for males 540–679 mm (Nottage and Perkins, 1983; Ryland and Ajayi, 1984; Walker, 1999). Estimates of fecundity range from 60 (Ryland and Ajayi, 1984) to 150 eggs per female per year (Holden *et al.*, 1971). Laboratory experiments have shown that juveniles hatch after 4.5–5.5 months at total lengths (TLs) of 110–137 mm (Clark, 1922). Adult females and males can reach at least 1070 and 1016 mm, respectively (Holden, 1972; Nottage and Perkins, 1983). *Raja clavata* differs in its growth characteristics from other Rajidae in having marked differences in the maximum size and growth rate between sexes, which may be related to the time of maturation (Holden, 1972; Walker, 1999; Whittamore and McCarthy, 2005).

Age determination is basic to understanding the population dynamics of exploited species and essential for many stock assessments, providing estimates of growth rates and longevity by species and area. Concentric growth bands have been documented in the vertebral centra of many species of rays and skates (Daiber, 1960; Davis *et al.*, 2007). For *R. clavata*, ages are assessed using this method (Taylor and Holden, 1964; Ryland and Ajayi, 1984; Fahy, 1989; Walker, 1999; Whittamore and McCarthy, 2005), and growth rates are estimated from tagging data (Holden, 1972) and by length frequency analysis (Brander and Palmer, 1985). Nonetheless, problems have been found with the use of vertebrae to age elasmobranchs, because vertebrae in different parts of the vertebral column may have different numbers of

growth increments (Natanson and Cailliet, 1990). This phenomenon may result from biological differences in the development of vertebrae or from the differing resolution of the methods used to examine increments (Officer *et al.*, 1996). To ensure the accuracy of age determination, validation must be included in age and growth studies. For *R. clavata*, the annual deposition of a pair of bands (hyaline and opaque) has been validated with tagging experiments using tetracycline (Holden and Vince, 1973; Ryland and Ajayi, 1984).

More recently, caudal thorns have been used to age some species of skates and rays from different parts of the world (Gallagher and Nolan, 1999; Henderson *et al.*, 2005; Gallagher *et al.*, 2006; Davis *et al.*, 2007; Matta and Gunderson, 2007; Moura *et al.*, 2007). Previously, caudal thorns and other dermal denticles had been used in taxonomic and phylogenetic studies to discriminate fresh and fossilized rajids (Stehmann and Bürkel, 1984; Gravendeel *et al.*, 2002). Dermal denticles are placoid scales, formed from minerals deposited by epidermal and dermal cells: dentine inside and vitrodentine outside. Typically they consist of a basal plate (BP) embedded in the dermis, a neck connecting the BP with the crown, and an exposed spiny crown (Kemp, 1999). During growth, the band most recently formed is deposited under the BP, and a new band added at the distal margin of the thorn, such that in longitudinal sections an overlay of inverted cones inside the proto-thorn is apparent (Gallagher *et al.*, 2005). In *R. clavata*, the entire dorsal surface is covered by dermal denticles of different shape and distributed according to a variable pattern. The smaller denticles are referred to as prickles, the medium-sized ones as thorns, and the large ones with a heavy BP as bucklers (Stehmann and Bürkel, 1984). These structures were first used in age determination by Gallagher and Nolan (1999), for four species of *Bathyraja* from the Falkland Islands. Those authors concluded that caudal thorns could be removed and cleaned more easily than vertebrae and that their removal had little or no effect on the commercial value because there was no need to cut the ray open. Moreover, they stated that the location of the first growth band was easy to identify, in contrast to other structures, increasing the accuracy of the age estimate. Other authors have also applied this technique: Henderson *et al.* (2005) for *Bathyraja albomaculata* from the Falkland Islands, Gallagher *et al.* (2006) for *Amblyraja radiata* off Greenland, Matta and Gunderson (2007) for *Bathyraja parmifera* from the eastern Bearing Sea, Davis *et al.* (2007) for *Bathyraja trachura* from the Pacific coast of USA, and Moura *et al.* (2007) for *Raja undulata* from the Portuguese continental coast.

The aims of the present study were to test the suitability of the different types of dermal denticle found in the tail of *R. clavata* for age determination, using different processing techniques and reading methods; to compare age estimates from vertebral centra and dermal

denticles and identify the most accurate ageing tool; and to study the growth of *R. clavata* from the waters off mainland Portugal.

4.1.3. Material and Methods

4.1.3.1. Sampling

Specimens of *R. clavata* were collected between June 2003 and October 2007 from: the Portuguese Fisheries Institute (IPIMAR) bottom-trawl research surveys carried out along the Portuguese continental coast; and from landings of two commercial artisanal fleets operating with trammel-nets, gillnets and longlines, one in northern Portugal (Matosinhos) and the other in the centre (Peniche).

For each fish, TL and disc width (mm) were measured, total weight (g) recorded, and the sex and maturity stage assigned according to the maturity scale proposed by Stehmann (2002) for oviparous elasmobranchs. Vertebrae were obtained either from the post-scapular vertebral region or from the tail. Dermal denticles were also obtained from the tail. Both structures were stored frozen before processing.

4.1.3.2. Processing techniques

Portions containing vertebrae were prepared according to two techniques. In the first, vertebrae were cleaned in bleach for 30 min, passed through running water, and then dried at room temperature for 24 h. The remaining tissue was removed with a scalpel. The second technique was adapted from Gallagher and Nolan (1999) and consisted of cleaning the vertebrae in 5% buffered trypsin solution (pH 7.5) for 16 h, followed by immersion in water at 30ºC. Vertebrae were finally rinsed with distilled water and dried. To clarify the growth-band pattern, two methods were used, submitting the vertebral centra to (i) burning, using an oven at 200ºC for 10 min, or (ii) immersion in 5% ethylenediaminetetraacetic acid solution (EDTA) for 10 min (Gallagher and Nolan, 1999).

Gallagher and Nolan's (1999) technique was adapted for dermal denticles. Different times and temperatures of immersion in 5% buffered trypsin solution were tested: (i) 30ºC for 16 h; (ii) 50ºC for 1 h; (iii) 50ºC for 20 min; and (iv) 80ºC for 20 min. Protocol steps were similar to those for vertebral centra: cleaning with distilled water and band-pattern enhancement with EDTA solution for 10 min.

4.1.3.3. Age and growth

Vertebral centra and dermal denticles were observed with an Olympus SZX9 stereomicroscope at x28 magnification. When the structure did not fit into the image at x28, a magnification of x18 was used for measurement purposes. The images were captured and digitized using a SONY DFW-SX910 digital camera and TNPC 4.1 image analysis software (Noesis, 2002). Different techniques for reading vertebral centra and dermal denticles of *R. clavata* were used: (i) applying liquid clarifiers, such as EDTA and glycerine, to the structures and (ii) using transmitted or reflected light.

Vertebral centra and different types of dermal denticle were compared for age determination, using subsamples of 21 and 107 fish, respectively. For vertebral centra, bands were counted on the axis showing the most distinctive band pattern. For dermal denticles, the posterior region behind the crown insertion was selected as the main reading axis. When doubts remained, the secondary axis was considered to corroborate the first band count. For assignment of age, the birthdate was assumed to be 1 June (according to edge-analysis results), and one opaque and one hyaline band were assumed to be laid down annually in the centra (Holden and Vince, 1973; Ryland and Ajayi, 1984). This assumption was also adopted when ageing dermal denticles. Using a subsample (of 107 fish from a total of 272 fish analysed), ages were assigned by two independent and experienced readers. The first reader replicated the age reading using dermal denticles for the estimation of intra-reader variability. Ageing precision was analysed by applying four statistical measures: average per cent error (APE; Beamish and Fournier, 1981), coefficient of variation (*CV*), index of precision (*D*; Chang, 1982), and percentage of agreement between readers. The consistency between the two readings was analysed, and when the differences were evident, the structures were re-examined by both readers until consensus was reached. If a consensus on age could not be reached, that fish was removed from the study.

To determine the relationship between dermal denticle growth and somatic growth, measurements were recorded using the digital images acquired on TNPC 4.1 (Noesis, 2002), as shown in Figure 4.1: (i) BP length, along the anterior–posterior axis of the crown; (ii) maximum BP width, transversely to the length; (iii) height, vertically from the tip of the crown to the BP; (iv) crown length, from the base to the tip; and (v) crown projection angle, the opening from the anterior edge of the BP to the tip of the crown, with the origin on the posterior edge of the BP.

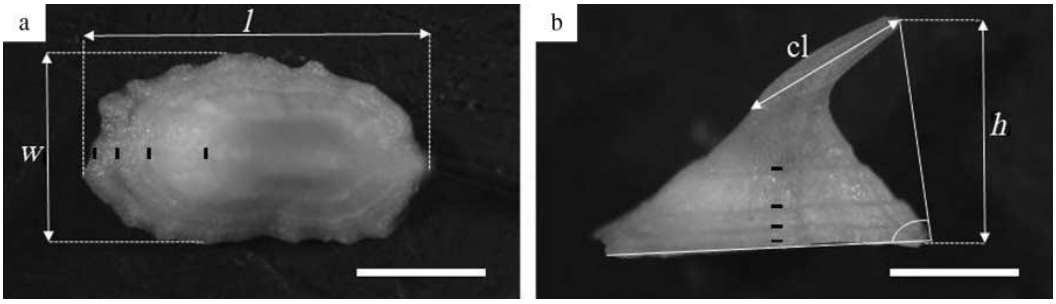

Figure 4.1. Caudal thorn of a 2-year-old, 297-mm TL male in **(a)** superior and **(b)** lateral view.
Measurements are BP length (*l*), width (*w*), and height (*h*), and crown length (*cl*) and angle. The black marks correspond to hyaline bands. Band-counting criteria, from the crown to the edge, were applied as follows: the first band corresponds to the proto-thorn margin, the second and third are birthmarks from the first and second years, and the fourth mark is the beginning of the third year, but because the sampling date were before the birthdate, this band was not considered (scale bar = 1 mm).

Edge analysis was applied to validate the periodicity of band formation. This method is used to characterize the margin of the age structure over time, opaque or translucent, to discern seasonal changes in growth (Cailliet *et al.*, 2006). The selected fish for this task were collected over a 3-year period covering all age groups, and edge analysis was made without prior knowledge of the sampling date. The percentage of each edge type (opaque or hyaline) was plotted against month.

The von Bertalanffy growth function (VBGF; Equation 1) and the Gompertz growth model (Equation 2) were fitted to length-at-age data based on the best ageing structure:

$$\mathrm{TL}_t = L_\infty(1-\mathrm{e}^{-k(t-t_0)}), \quad (1)$$

$$\mathrm{TL}_t = L_\infty \mathrm{e}^{-\mathrm{e}^{(-g(t-t_0)}}, \quad (2)$$

where TL_t is the total length-at-age t (mm), L_∞ the theoretical asymptotic length (mm), k the growth rate (year^{-1}), g the instantaneous Gompertz growth coefficient (year^{-1}), t the age (years), and t_0 the theoretical age at 0 length (years). The parameters were estimated for males and females separately using the statistical software R 2.5.1. for Windows (R Project for Statistical Computing, 2007). The growth parameters were compared using Hotelling's T^2 test (Bernard, 1981), and if no statistical differences were found, data were pooled and a new growth model was fitted. To select the most appropriate growth model, the biological meaning of the estimated parameters and the goodness-of-fit were taken into account. Goodness-of-fit was evaluated by residual mean square error (*MSE*), Akaike's Information Criterion (*AIC*; Shono, 2000), and the coefficient of determination (r^2).

4.1.4. Results

In all, 272 *R. clavata* were sampled (Fig. 4.2), from which 143 were females with 145–913 mm TL, and 129 were males with 148–870 mm TL.

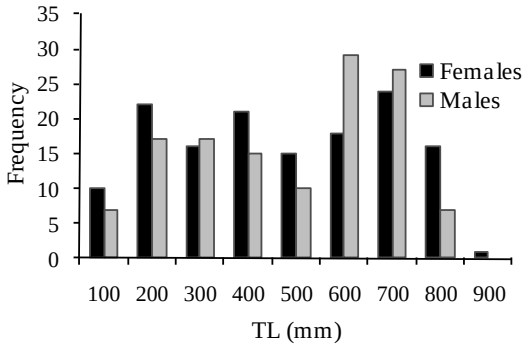

Figure 4.2. Length frequency distribution by sex of *R. clavata* sampled for age assessment.

4.1.4.1. Processing techniques

For vertebral centra, the removal of the surrounding tissues was more effective with the trypsin solution than with bleach; the centra were ready for enhancement without any additional care. The band pattern became more visible after burning. However, care in drying the centra and removing all trypsin in running water was needed, because the heat of the oven activates the enzyme, which corrodes the edges.

For dermal denticles, immersion in trypsin solution in a water bath at 50ºC for 20 min was the best technique. At a lower temperature (30ºC), the results were similar, but the process was more time-consuming. Dermal denticles became yellowish and dull with the water bath at 80ºC, and the BP edge corroded, making identification of the band pattern more difficult or even impossible.

The best procedure for reading the growth bands in vertebral centra was by observation under transmitted light and clarification with EDTA. For dermal denticles, age was easily assigned only with transmitted light, but clarifiers needed to be used with caution, because the opaque bands near the edge easily became translucent.

4.1.4.2. Types of thorn and their suitability for age determination

The three main types of caudal denticles defined by Stehmann and Bürkel (1984) were found in the caudal region: prickles, thorns, and bucklers (Fig. 4.3).

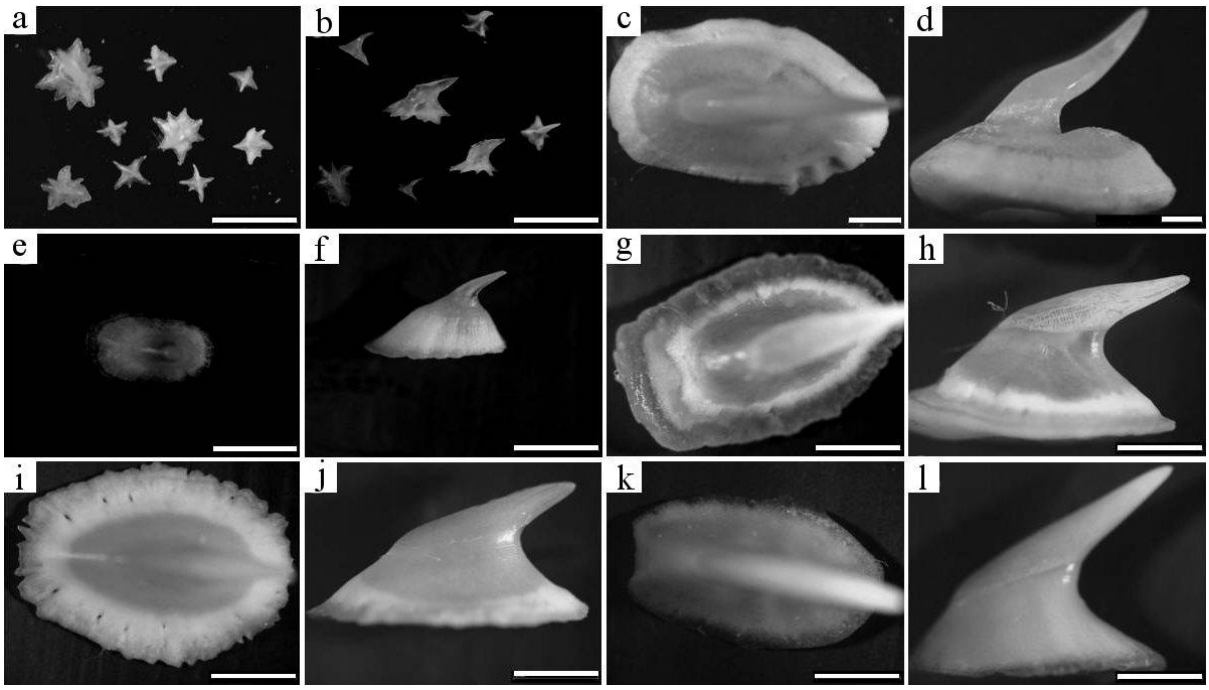

Figure 4.3. Types of dermal denticles observed in the caudal region of *R. clavata.*
(a and b) prickles, leaf shaped, (c and d) bucklers, and (e–l) thorns of different type: (e and f) small; (g and h) large with rectangular BP; (i and j) large with oval BP; (k and l) large with large crown and narrow BP. For each type pair of panels, images are presented first from the above then from the side (scale bar = 2 mm; note that bucklers were photographed at lower magnification).

Prickles (Figs. 4.3a and 4.3b) were found in all age classes. Like other dermal denticles, they seemed to grow with the individual. However, because of their small size (2.32±1.01 mm) and irregular BP, observation of growth bands was difficult. Moreover, there is no certainty about their persistence throughout a fish's lifetime.

Bucklers (Figs. 4.3c and 4.3d) were found along the lateral surface of the tail, starting in early adults with a TL > 400 mm. These are large dermal denticles (8.58±2.14 mm), with oval and heavy BP, which is initially narrow and thin, but that thickens with time. As they are not found throughout the life of the fish, they could not be used for age determination.

Thorns are distributed along the midline of the caudal region of juveniles and adults, but in the lateral line only of adults (TL > 400 mm). Thorns have an intermediate size between the other two types (5.01±1.76 mm length). Four types of caudal thorns were observed in *R. clavata* and their description was made according to size and characteristics of the BP and the length and angle of the crown. "Small thorns" (Figs. 4.3e and 4.3f) have a yellowish to grey crown in younger fish and an orange one in older specimens. In the first years of growth, the BP is oval, small, and thin. As fish size increases, the BP grows like an overlay of cones, and became proportionally larger than the crown. The small thorns are found throughout the life of the fish (from 148 to 845 mm TL), and their dimensions are listed in Table 4.1. The second

type is the "large thorn with rectangular BP" (Figs. 4.3g and 4.3h), which is characterized by a large orange crown. The BP is generally square to rectangular, with a long posterior region behind the crown where growth bands are prolonged and better observed. They were found in fish with 270–891 mm TL, and their dimensions are also listed in Table 4.1. The third type, "large thorns with oval BP" (Figs. 4.3i and 4.3j), also has an orange or sometimes greyish crown. The crown and the BP connection are longer than in the previous type. The BP is oval, with the region behind the crown generally compressed and shortened, compared with the rectangular thorn type. As a consequence of the shape of the thorn, visualization of growth bands was more difficult in that region and better observed laterally. This thorn type was observed in fish with 195–892 mm TL, and their dimensions are given in Table 4.1. The fourth type, "large thorns with a large crown and narrow BP" (Figs. 4.3k and 4.3l), generally has an orange crown. The BP is small and soft, and the first band broad and opaque, followed by a narrow hyaline band. As with the other thorn types, this kind was also sampled in young and adult fish (215–744 mm TL); again their dimensions are given in Table 4.1.

Table 4.1. Dimensions of the four types of thorn: (A) small thorns; (B) large thorns with a rectangular BP; (C) large thorns with an oval BP; (D) large thorns with a large crown and narrow BP.

Thorn type	BP height (mm)	BP length (mm)	BP width (mm)	Crown length (mm)	Crown projection angle (º)
A	1.62±0.27	2.66±0.56	1.41±0.38	1.30±0.20	82.72±11.02
B	4.24±0.73	6.62±1.33	4.66±1.09	4.63±1.07	85.61±6.97
C	4.29±0.54	7.38±1.40	4.66±1.09	4.63±1.07	85.61±6.97
D	4.24±1.15	1.16±1.15	3.32±0.91	5.15±0.81	113.79±18.42

To facilitate understanding the position and frequency of the different types of dermal denticle, the tail of one fish is shown in Figure 4.4. Small thorns alternate with large thorns with rectangular BP. Large thorns with an oval BP and large thorns with a large crown and a narrow BP are placed laterally to the small thorns. It is also easy to identify the bucklers in the lateral region, and the prickles covering the entire surface of the tail.

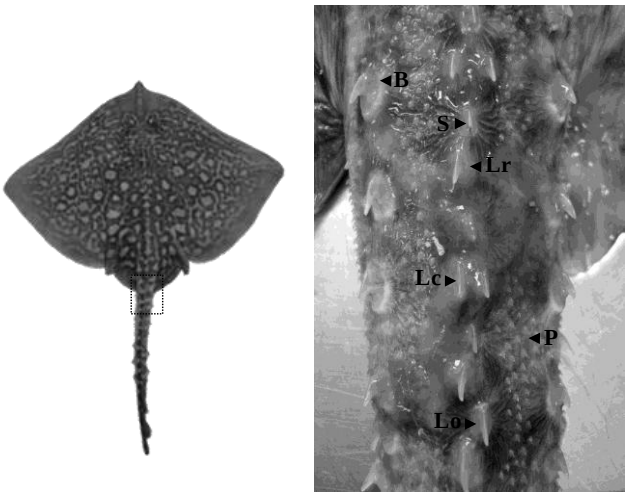

Figure 4.4. Location of the six types of dermal denticle present in the caudal region.
B, buckler; *S*, small thorn; *Lr*, large thorn with rectangular BP; *Lc*, large thorn with large crown and narrow BP; *P*, prickle; *Lo*, large thorn with oval BP.

Considering all the types of dermal denticle, we concluded that prickles, bucklers, and thorns with a narrow BP were not suitable for age determination, given their BP shape (prickles) and the fact that they are not present during the whole life of the fish (bucklers and thorns with narrow BP). Although the remaining types seemed to be suitable for age determination, the small thorns were considered the best because of (i) their uniform position in the tail, (ii) their persistence, and (iii) the defined pattern of growth bands.

Additionally, there was a good relationship between fish TL and thorn length for the three types of thorn considered to be suitable for age determination (Fig. 4.5).

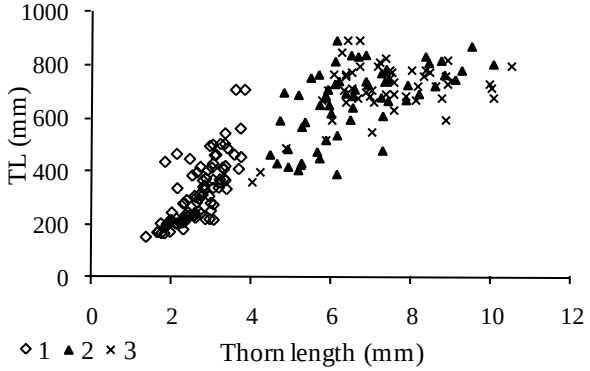

Figure 4.5. Relationship between TL (mm) and thorn length (mm) for three types of caudal thorn.
(1) small; (2) large with rectangular BP; and (3) large with oval BP.

4.1.4.3. Age and growth

After selecting the type of caudal thorn, a subsample of 21 fish was used to compare age estimates derived from vertebral centra and caudal thorns from the same fish. The band

patterns of the two types of structure were similar, and age readings consistent, to an agreement of 80.95% (r^2 = 0.97). In fact, only in ages 0, 1, 6, and 9, a deviance on the age assigned with the two structures was recorded, in one specimen each.

The application of ageing statistical measures (*APE*, *V*, and *D*) on the observations made by different readers (Table 4.2) showed that caudal thorns tended to yield lower values, indicating greater reproducibility, than vertebral centra. Additionally, intra and inter-reader variability was analysed for caudal thorns (Fig. 4.6), revealing great consistency between readings (both 98%, considering an error of ±1 year). Consequently, caudal thorns were chosen for age determination.

Table 4.2. Ageing precision statistical measures applied to age readings made by two independent readers, using caudal thorns and vertebral centra: *APE*, *CV*, *D*, and percentage agreement between readers.

Structure	*n*	*APE*	*CV*	*D*	% Agreement between readers
Vertebral centra	21	8.6	12.2	8.6	57
Caudal thorns	107	2.3	3.2	2.3	79

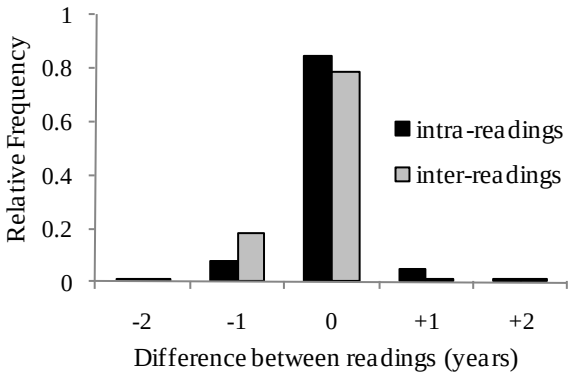

Figure 4.6. Intra- and inter-reader variability in age readings based on *R. clavata* caudal thorns.

For age assessment, two criteria were considered based on embryonic development and edge analysis: (i) the first, postembryonic hyaline band is always formed after an opaque band and a translucent band and is visible after the hyaline edge of the proto-thorn (Fig. 4.1); and (ii) ages were corrected according to the birthdate, 1 June, based on the prevalence of hyaline edges in age-0 fish and the maximum occurrence of hyaline edges in May and June (Fig. 4.7). There were no hyaline edges in July because of sample composition (only older adults were sampled that month).

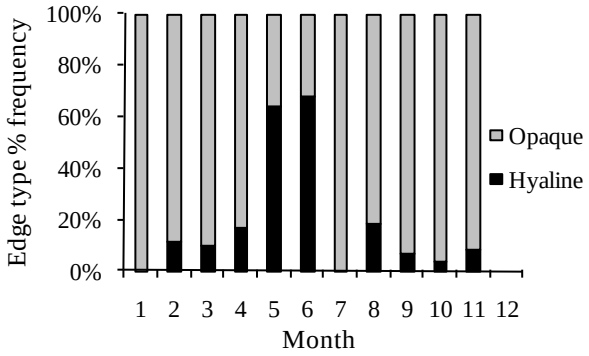

Figure 4.7. Monthly variation in caudal thorn edge types (n = 264).

The von Bertalanffy and Gompertz growth parameters estimated for length-at-age data, for the whole sample and by sex, are listed in Table 4.3. From 272 fish, 251 were used in the estimates (129 females and 122 males): ten fish were removed as a result of deficient processing, five were removed because there was no consensus on age between readers, and six age-0 fish were removed because of the scarcity of smaller fish.

Table 4.3. Estimated growth parameters from age-at-length data for male and female *R. clavata* separately and combined, caught off mainland Portugal, using the VBGF and the Gompertz model.

Growth model	**von Bertalanffy**			**Gompertz**		
	All	**Females**	**Males**	**All**	**Females**	**Males**
Length range (mm)	[195, 913]	[199, 913]	[195, 870]	[195, 913]	[199, 913]	[195, 870]
n	251	129	122	251	129	122
L_∞ (mm)	1 280	1 407	1 171	966.5	1 014	906.4
k (year^{-1})	0.117	0.097	0.142	–	–	–
g (year^{-1})	–	–	–	0.298	0.266	0.347
t_0 (year)	–0.617	–0.880	–0.358	0.581	0.500	0.715
MSE	60.50	63.76	56.87	59.25	62.51	55.49
AIC	2 776.79	1 443.08	1 337.12	2 766.32	1 437.98	1 331.12
R^2	0.953	0.951	0.957	0.955	0.953	0.959

L_∞, theoretical asymptotic length; *k*, growth rate; *g*, Gompertz growth coefficient, t_0, theoretical age at zero length; MSE, residual mean square error; AIC, Akaike's Information Criterion; R^2, coefficient of determination.

No statistically significant differences in growth parameters were found between sexes for either growth model (von Bertalanffy: $T^2 = 5.20$, $T^2_0 = 7.99$; $p > 0.05$; Gompertz: $T^2 = 6.11$, $T^2_0 = 7.99$; $p > 0.05$), and so data were pooled and new von Bertalanffy and Gompertz growth models were fitted (Fig. 4.8). According to *MSE*, *AIC*, and r^2 criteria, the fits of the two growth models were similar. However, we preferred the VBGF model because one of its parameters, L_∞, seemed to be closer to the maximum species length known.

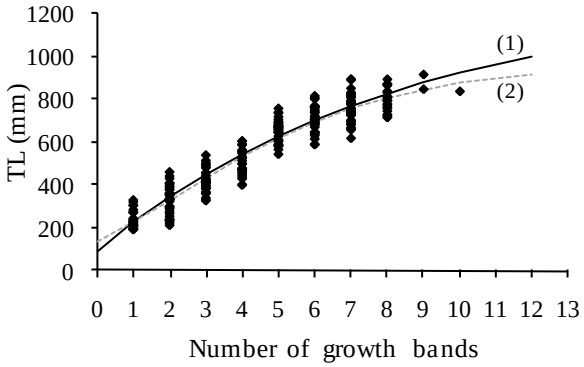

Figure 4.8. Age-at-length data derived from *R. clavata* thorn observations and the fitted (1) von Bertalanffy growth model (dark line) and (2) the modified version of the Gompertz model (dashed line).

4.1.5. Discussion

With the decline in catches of fish traditionally targeted, rays and skates have become increasingly valuable for commercial fishers. Comparative studies of historical data from the 20th century have shown how the abundances of larger elasmobranch species have declined in European waters (Rogers and Ellis, 2000). Up-to-date studies on the biology of rays and skates, especially large species, are therefore necessary to evaluate the current state of elasmobranch stocks in European waters. The present study is a contribution to the knowledge of the biology of *R. clavata*, one of the species most affected by overfishing over the past few decades (Walker and Hislop, 1998). The absence of studies from southern Europe is also a key shortcoming, because all previous age and growth studies of *R. clavata* were on fish caught in northern European waters (Taylor and Holden, 1964; Holden, 1972; Ryland and Ajayi, 1984; Brander and Palmer, 1985; Fahy, 1989; Walker, 1999; Whittamore and McCarthy, 2005), and most of them were based on counting growth bands in the vertebral centra.

According to Campana (2001), the difficulty in band identification in age and growth studies is related to: (i) the processing technique; (ii) the nature of the structure being analysed (e.g. the extent of calcification); and (iii) the species being studied. The influence of the processing technique, combined with reading procedures, was the first issue to be analysed. The effectiveness of the dermal denticle processing technique using trypsin solution at 50ºC can be explained by the fact that the maximum functioning rate of trypsin at atmospheric pressure is attained at this temperature, which coincides with the denaturation process of the enzyme (Fraser and Johnson, 1951). Staining, for example, with silver nitrate

(Gallagher and Nolan, 1999), was not applied in this work. The fact that caudal thorns have to be read just after staining, because of loss of definition of the band pattern, made it impractical because: (i) the same thorn had to be read more than once; (ii) the reading was not always made on the same day as processing; (iii) the use of staining was more time-consuming and expensive. The reading protocol with reflected light can be applied to such other species as *Leucoraja naevus* (Gravendeel *et al.*, 2002), with its flattened denticles and broad and thin BP.

Other authors who assessed age using dermal denticles did not mention the presence of different types of caudal thorn in *R. clavata*. The difficulty in distinguishing the different types of thorn was related to the fact that in the median and parallel tail rows, the thorns between neighbouring zones change their shape gradually, which increases the variability of their shape (Gravendeel *et al.*, 2002). In terms of dimension, the rectangular BP thorns are smaller than the oval BP thorns. The shape of these two types seems to be influenced by the space available for them to grow between thorns. As a consequence, the shape was sometimes hard to distinguish, so the thorn type was difficult to assign. Nonetheless, the long connection between the crown and BP in the second type was always easy to detect, and it was used as a diagnostic character. Although caudal thorns are securely embedded in the caudal tissue overlying the spinal column (Gallagher *et al.*, 2005), all thorn types seem to drop out during the life of a fish, and according to Meunier and Panfili (2002) may even be replaced over time. Our study, however, does not corroborate the results of those authors, because gaps and marks were found along the tails of both young and old adult *R. clavata*. Small and large thorns (excluding the thorns with a narrow BP) were found on fish of all length classes, and can be used for age determination. Nevertheless, in terms of growth-band readability, the small thorns seem to be the most suitable.

Results from the analysis of thorn dimension revealed a positive correlation between *R. clavata* somatic growth and thorn growth, as observed by Gallagher *et al.* (2005) for *Bathyraja brachyurops*. In both these species, thorn length correlates well with TL. These observations indicate that the surface ridges on caudal thorns represent a near stasis of somatic growth, and the broader bands periods of more rapid growth (Gallagher *et al.*, 2005). The precision measures (*APE*, *CV*, and *D*) demonstrated that age estimates obtained with caudal thorns were more reproducible than those based on vertebral centra. In fact, the estimated values for caudal thorns are below the precision limit established by Campana (2001), with *APE* <5.5% and *CV* <7.6%. Based on all these results, the use of caudal thorns is proposed for future age and growth studies of *R. clavata*. In addition to precision, caudal

thorns can be more easily obtained at fish markets or in the field with minimal damage to the fish. In the latter situation, it is also possible to apply known-age and marked-fish validation methods, removing caudal thorns and marking each fish, before releasing them alive. The poor results for vertebral centra were related to the difficulty of obtaining good contrast between hyaline and opaque bands, and consequently in assigning a consistent birthdate. The same difficulty was identified by other authors (Daiber, 1960; Brander and Palmer, 1985).

The process of estimating fish age has two major sources of error: that associated with the structure being examined and that attributable to the subjectivity inherent in age estimation. To avoid some of this subjectivity, validation is necessary. As absolute age validation was not possible in the present study, two procedures (Campana, 2001) were implemented: (i) determination of the age of first increment formation; and (ii) verification of increment periodicity across the entire age range. The sampling of recently hatched fish of 145–210 mm TL from March to June allowed validation of the first-formed band, and the edge analysis allowed validation of increment periodicity. As reported by other authors for vertebral centra (Holden and Vince, 1973; Ryland and Ajayi, 1984), the laying down of a pair of bands in caudal thorns is annual. The translucent band appeared mainly in May and June, during the peak of the reproductive season (Holden, 1975), and the opaque band was observed during the other months of the year.

For growth estimation, age-0 fish were not used in the analysis because only the largest individuals of this age class (Lt > 145 mm) were available. If these data had been used, then an overestimate of L_∞ and a consequent underestimate of the growth rate (k) would be likely to occur. The maximum age observed in this study was 10 years, and the maximum fish length sampled was 913 mm TL. The same dimensions have been reported for British waters by Ryland and Ajayi (1984), but the maximum age attained by these animals differs according to author, with 16 and 9 years reported by Ryland and Ajayi (1984) and Whittamore and McCarthy (2005), respectively. In terms of growth rate by sex, there was no statistical difference between the growth parameters, although females in our sample were generally bigger than males. Other authors have made the same observation.

Following Cailliet *et al.* (2006), two distinct growth models were applied to the dataset: the VBGF and the Gompertz growth model. The VBGF produced more reliable growth parameters, considering the life history of the studied species. Variations in life-history traits between geographically separated populations of skates and rays are not unusual. There is a slight difference between the estimated growth parameters for thornback ray and those presented in the past by other authors from the North and Irish Seas and the English Channel

(Table 4.4). Off mainland Portugal, however, the thornback ray seems to attain a larger L_∞ and follow a slower *k*. The differences between growth parameters estimated here and those obtained by other authors could be related to compensatory mechanisms attributable to the sharp decrease in the abundance of thornback ray in northern European waters (Dulvy *et al.*, 2000; Rogers and Ellis, 2000). Off Portugal, however, there is no clear evidence yet that the population of *R. clavata* is declining (Machado *et al.*, 2004).

Table 4.4. Parameters of the VBGF estimated by other authors for *R. clavata* in European waters.

Source	Area	Sex	*n*	L_{max} (mm)	L_∞ (mm)	*k* (year^{-1})	t_0 (years)
Taylor and Holden (1964)	British waters	F	85	920	1 273	0.10	–2.50
		M	61	770	883	0.22	–1.30
Holden (1972)	Irish Sea and Bristol Channel	F	234	1070	1 281	0.09	–1.32
		M	206	870	856	0.21	–0.60
Ryland and Ajayi (1984)	Bristol Channel	Both	2143	990	1 392	0.09	–2.63
Brander and Palmer (1985)	Irish Sea	Both	1125	1060	1 050	0.22	0.45
Fahy (1989)	Irish Sea	F	1504		1 078 - 1 200	0.15 - 0.26	–1.01 to 0.05
		M	783		968 - 1 043	0.19 - 0.24	–1.36 to 0.32
Walker (1999)	North Sea	F	51	940	1 180	0.14	–0.88
		M	41	860	980	0.17	–0.43
Whittamore and McCarthy (2005)	North Wales	F	135	945	1 176	0.16	–0.71
		M	54	778	1 009	0.18	–0.99

4.2. REPRODUCTION

4.2.1. REPRODUCTIVE TERMINOLOGY [1]

4.2.1.1. Abstract

There is the need for unified terminology for reproductive phase assignment across fish *taxa*, regardless of the reproductive strategy involved. Reproductive terminology already adopted for teleosts has been applied to oviparous elasmobranchs of both sexes. A historical

[1] Serra-Pereira, B., Figueiredo, I. and Serrano-Gordo, L. In press. Maturation of the gonads and reproductive tracts of the thornback ray (*Raja clavata*), with comments on the development of a standardized reproductive terminology for oviparous elasmobranchs. Marine and Coastal Fisheries: Dynamics, Management, and Ecosystem Science. (Special Section: Emerging issues and methodological advances in fisheries reproductive biology)

review of the terminologies used by previous authors and how these correspond to the new terminology is presented. Five reproductive phases were considered: immature, developing, spawning capable (which includes an actively spawning sub-phase), regressing and regenerating. Using the example of the oviparous elasmobranch thornback ray, the different phases were described based on both macro- and microscopic features of the reproductive tract, including ovaries, oviducal glands and uterus in females, and testes, claspers and sperm ducts in males. For the last two phases, only regressing females were observed. Records from other species suggest that all of the phases can be found in oviparous elasmobranchs, depending on the reproductive strategy of the species.

4.2.1.2. Introduction

Knowledge of elasmobranch reproductive cycles is still scarce. Therefore details on reproductive cycles for most elasmobranch species and standardized reproductive terminology are not yet available. New standardized terminology for teleost reproduction has only recently been proposed by Brown-Peterson *et al.* (in press), despite increased knowledge of teleost reproduction for a significant number of species. For oviparous teleost species, the reproductive cycle is divided into five reproductive phases: immature, developing, spawning capable (which includes an actively spawning sub-phase), regressing (ending of spawning season), and regenerating (preparation for the next season). Given the high number of current classifications, the standardization of reproductive terminology across different fish taxa is of great importance in order to allow comparisons among different studies (Brown-Peterson *et al.*, in press).

For elasmobranchs there are several reproductive terminologies. One proposed by Stehmann (2002) for oviparous species has been adopted by some authors (e.g. Costa *et al.*, 2005; Moura *et al.*, 2007). It divides the reproductive cycle into three ovarian phases (immature, maturing and mature), and three uterine phases (active, advanced and extruding). Stehmann's terminology is one of the most complete terminologies, since it differentiates spawning from mature females, whereas the remaining terminologies generally considered only three phases: immature, adolescent and mature (Table 4.5; e.g. Richards *et al.*, 1963; Walmsley-Hart *et al.*, 1999; Ebert, 2005; Coelho and Erzini, 2006; Ruocco *et al.*, 2006; Kyne *et al.*, 2008). Most studies on reproductive development of oviparous elasmobranchs relied only on macroscopic features (e.g. Richards *et al.*, 1963; du Buit, 1976; Ebert, 2005; Oddone

and Vooren, 2005; Ruocco *et al.*, 2006; Ebert *et al.*, 2008a; Ebert *et al.*, 2008b), yet some good descriptive work on gonadal histology are also available (e.g. Stanley, 1966; Hamlett *et al.*, 1998; Andreuccetti *et al.*, 1999; Hamlett *et al.*, 2005; Lutton *et al.*, 2005). However, these works generally only applied to adult specimens in the spawning capable phase. Barone *et al.* (2007) were the only to apply histology to improve the description of the reproductive phases. However, the authors noted that their work was not complete since the phases described were based only on gonadal development.

All elasmobranchs have internal fertilization, so all species require specialized behavioural, morphological and physiological mechanisms to ensure the success of fertilization (Hamlett and Koob, 1999). Skates are oviparous, producing eggs enclosed in hard egg capsules, which are released into the water, 0.5-2 days after the beginning of egg encapsulation (Holden *et al.*, 1971; Ellis and Shackley, 1995). After extrusion, parental care is absent. The skate reproductive tract has adapted to oviparity by allowing egg encapsulation and sperm storage in the oviducal gland (Hamlett *et al.*, 1998), as well as egg capsule sclerotization via quinone tanning in the uterus (Koob and Cox, 1990). In general, the complex life cycle of oviparous elasmobranchs is translated into an extended reproductive cycle. In addition to the batoid family Rajidae, this type of reproductive strategy is also shared by most charchariniform catsharks of Scyliorhinidae, the bullhead sharks of Heterodontiformes and the orectolobiform carpetsharks of Parascycilliidae, Hemiscyllidae and Stegostomatidae (Compagno *et al.*, 2005; Musick and Ellis, 2005). It is important to note that the scyliorhinids *Halaelurus* spp. and some Orectolobiformes share a different type of oviparity, the multiple or retained oviparity, which differ on the retention of multiple eggs in the oviduct for most part of development and consequent extrusion of the eggs containing well-advanced embryos (Compagno, 1990; Dulvy and Reynolds, 1997). The long reproductive cycle of oviparous species is associated with high energy requirements, which is related to greater ovarian follicle size prior to ovulation in females. For most species, mature size is reached at least one year after hatching, and may take up to seven years, as in the case in the thornback ray, *Raja clavata* (Serra-Pereira *et al.*, 2008).

In skates, the embryo develops inside the egg capsule using yolk reserves for nourishment. The gestation period is species-specific. The thornback ray is the most abundant species in NE Atlantic landings (Dulvy *et al.*, 2000). Spawning occurs between February and September, in British waters (Holden *et al.*, 1971; Holden, 1975). Incubation time is estimated to be five months (Ellis and Shackley, 1995). Compared to the majority of teleosts, this species has a number of K-strategist characteristics, including: late maturation, around

80% of the maximum size (Walker, 1999; Whittamore and McCarthy, 2005); slow growth (k=0.117 year^{-1}; Serra-Pereira *et al.*, 2008); large maximum sizes (adult females and males can reach at least 1070 and 1016 mm total length, respectively; Holden, 1972; Nottage and Perkins, 1983) ; and low fecundity (the maximum estimate for thornback ray is an average of 140-150 eggs per female per year; Holden *et al.*, 1971; Holden, 1975).

The main objectives of this paper are: (i) to describe the reproductive tract development and gametogenesis in rajid species, in particularly focusing on the thornback ray; (ii) to adapt the recent reproductive terminology for teleosts (Brown-Peterson *et al.*, in press) to oviparous elasmobranchs (excluding retained oviparity), using the thornback ray as an example, in an effort to unifying the reproductive terms used among all fish studies. This will be accomplished through macroscopic and microscopic analysis of female and male reproductive structures, following the work developed by Barone *et al.* (2007). The standardize terminology will not be extended to retained oviparity, since little is known about their development, and therefore different reproductive adaptations may occur.

4.2.1.3. Material and Methods

4.2.1.3.1. Sampling

Thornback ray samples were collected between 2004 and 2008 from: (i) landings of Portuguese commercial artisanal fleets (Matosinhos and Peniche), under the scope of the National Data Collection Program (PNAB, DCR), and (ii) IPIMAR bottom-trawl research surveys carried out along the Portuguese continental shelf.

The reproductive organs of both sexes, ovaries, oviducal glands, uteri, testes and sperm ducts (both epididymis and vas deferens), were extracted and preserved in 10% buffered formaldehyde.

4.2.1.3.2. Histological procedures

Sections of reproductive organs were extracted and processed using an automated tissue processor (Leica TP1020, Germany) according to the standard protocol (Bancroft and Gamble, 2002). Samples were embedded in paraffin wax blocks using a standard heated paraffin embedding system (Leica EG 1140H, Germany). The paraffin blocks were then sliced at 3-5 μm of thickness, using a sliding microtome (Leica SM 2000 R, Germany) or a

rotary microtome (Leica RM2125RT, Germany). The following staining techniques were used to analyze the histological structure of the oviducal gland: (i) Hematoxylin and Eosin (H&E); (ii) Toluidine blue (TB); (iii) Periodic Acid-Schiff (PAS); and (iv) combined Alcian blue and PAS (PAS/AB). Histological protocols followed Bancroft and Gamble (2002).

Histological slides were observed with a stereo microscope (Olympus SZX9, USA) and an optic microscope (Carl Zeiss Axioplan 2 imaging, Germany). Images were obtained using the imaging software TNPC 4.1 and AxioVision 4.1, respectively.

Descriptions of the different reproductive phases for females and males based on macro- and microscopic features of the oviparous thornback ray reproductive system during maturation were performed. A total of 183 samples of thornback rays were observed. The current reproductive phases described by Stehmann (2002) were adapted in order to accommodate the terminology recently proposed by Brown-Peterson *et al.* (in press).

4.2.1.4. Results and Discussion

4.2.1.4.1. Comparison of terminologies used for oviparous elasmobranchs

The terminology commonly used to describe different reproductive phases in oviparous elasmobranchs is variable among authors (Table 4.5).

The term *immature* is used by most authors to designate specimens with small gonads and undeveloped reproductive tract (e.g. Richards *et al.*, 1963; Walmsley-Hart *et al.*, 1999; Stehmann, 2002; Coelho and Erzini, 2006; Ruocco *et al.*, 2006; Barone *et al.*, 2007; Kyne *et al.*, 2008; Frisk and Miller, 2009). In females ovaries do not have visible follicles, the uterus is undeveloped and oviducal glands are absent; in males claspers are shorter than the pelvic fins, the testes are small and do not have visible lobes and sperm ducts are undeveloped. The term "juvenile" is also used to designate immature specimens (Ivory *et al.*, 2004; Ebert, 2005; Ebert *et al.*, 2006). Other authors (e.g. Stehmann, 1987; Templeman, 1987; Sulikowski *et al.*, 2005) even used the term "immature" to classify all the specimens prior to maturation, both immature and developing.

The *developing* phase (Brown-Peterson *et al.*, in press) is used to designate specimens in pre-spawning conditions. In females ovaries have small follicles and the uterus and oviducal glands are developing; in males claspers are larger than pelvic fins, testes have visible lobes and sperm ducts are developing. The developing phase is also termed "adolescent"

Table 4.5. Comparison between the reproductive phases terminology adopted for oviparous elasmobranchs studies.
The one from Brown-Peterson *et al*. (in press) is highlighted as being the one proposed to standardize the terminology used across all oviparous elasmobranchs.

Authors	**Number of phases**	**Maturity scale terminology**					
Brown-Peterson et al. (in press)	*5*	*Immature*	*Developing*	*Spawning capable*	*(Actively spawning sub-phase)*	*Regressing*	*Regenerating*
Frisk and Miller (2009)	4	Immature	Adolescent	Onset mature	Functional mature		
Barone *et al.* (2007)	6	Immature	Virgin/ Maturing	Mature	Extruding	Resting	
Coelho and Erzini (2006), Ruocco *et al.* (2006)	3	Immature	Maturing		Mature		
Ebert (2005) [1], Ebert *et al.* (2006)	3	Juvenile	Adolescent		Mature		
Ivory *et al.* (2004)	4	Juvenile	Maturing (adolescent)	Mature (adult)	Running/Laying Resting (adult)		
Stehmann (2002)[2], Templeman (1987), Sulikowski *et al.* (2005)	6	Immature (juvenile)	Maturing (adolescent)	Mature (adult)	Active/ Advanced/ Extruding		
Walmsley-Hart *et al.* (1999)[3]	3	Immature (juvenile)	Immature (subadult)		Mature (adult)		
Stehmann (1987)[4]	2	Immature			Mature		
Richards *et al.* (1963), Kyne *et al.* (2008)	3	Immature	Adolescent		Mature		

[1] Followed by Ebert *et al.* (2008a, 2008b)
[2] Followed by Costa *et al.* (2005) and Moura *et al.* (2007)
[3] Followed by Colonello *et al.* (2007) and Quiroz *et al.* (2009)
[4] Followed by Whittamore and McCarthy (2005) and Demirhan *et al.*(2005)

(Richards *et al.*, 1963; Ebert, 2005; Ebert *et al.*, 2006; Kyne *et al.*, 2008; Frisk and Miller, 2009), "maturing" (Stehmann, 2002; Ivory *et al.*, 2004; Coelho and Erzini, 2006; Ruocco *et al.*, 2006; Barone *et al.*, 2007), "immature-subadult" (Walmsley-Hart *et al.*, 1999) and "virgin" for males (Barone *et al.*, 2007). Barone *et al.* (2007) subdivided developing males into virgin and maturing, the former describing males with soft claspers and developing testes and sperm ducts, and the latter only characterized by hardened claspers.

The *spawning capable* phase (Brown-Peterson *et al.*, in press) is used to designate adult specimens in reproductive conditions. This phase is most often termed as "mature" (e.g. Richards *et al.*, 1963; Stehmann, 1987; Templeman, 1987; Walmsley-Hart *et al.*, 1999; Ebert, 2005; Sulikowski *et al.*, 2005; Coelho and Erzini, 2006; Ebert *et al.*, 2006; Ruocco *et al.*, 2006; Kyne *et al.*, 2008). In oviparous elasmobranchs, the spawning capable phase refers to specimens capable of reproducing, to designate females with ovaries full of large vitellogenic follicles, and well developed uterus and oviducal glands, and males with hard and enlarged claspers, enlarged testis full of developed lobes, and developed sperm ducts). Terms used instead of spawning capable are "onset mature" (Frisk and Miller, 2009) and "mature" (Stehmann, 2002; Ivory *et al.*, 2004; Barone *et al.*, 2007). In oviparous elasmobranchs, *actively spawning* sub-phase is use to describe females with egg capsules inside the uterus, and males with reddish and swollen clasper glans and sperm flowing in the sperm ducts and seminal vesicle. This sub-phase has previously been called "functional mature" for both sexes (Frisk and Miller, 2009), "active" (Stehmann, 2002) or "running" for males (Ivory *et al.*, 2004), and "extruding" (Barone *et al.*, 2007), "laying/resting" (Ivory *et al.*, 2004) or "active/advanced/extruding" for females, depending on the stage of development of the egg capsule (Stehmann, 2002).

The *regressing* phase, also termed "resting" (Barone *et al.*, 2007), is used to identify mature adults that cease spawning. In oviparous elasmobranch females in this phase have ovaries containing follicles with different sizes, post-ovulatory follicles, and small oviducal glands, whereas males have large and hard claspers and undeveloped testis. This term was only applied in the rajid *Raja asterias* (Barone *et al.*, 2007). Ivory *et al.* (2004) used the term "resting" to identify adult females in spawning condition without further description of an actual "resting" phase in *Scyliorhinus canicula*. The *regenerating* phase was never applied to oviparous elasmobranchs.

4.2.1.4.2. Females

The thornback ray possesses two ovaries, each located on the distal surface of the epigonal organ, and containing developing follicles distributed on its surface (Fig. 4.9).

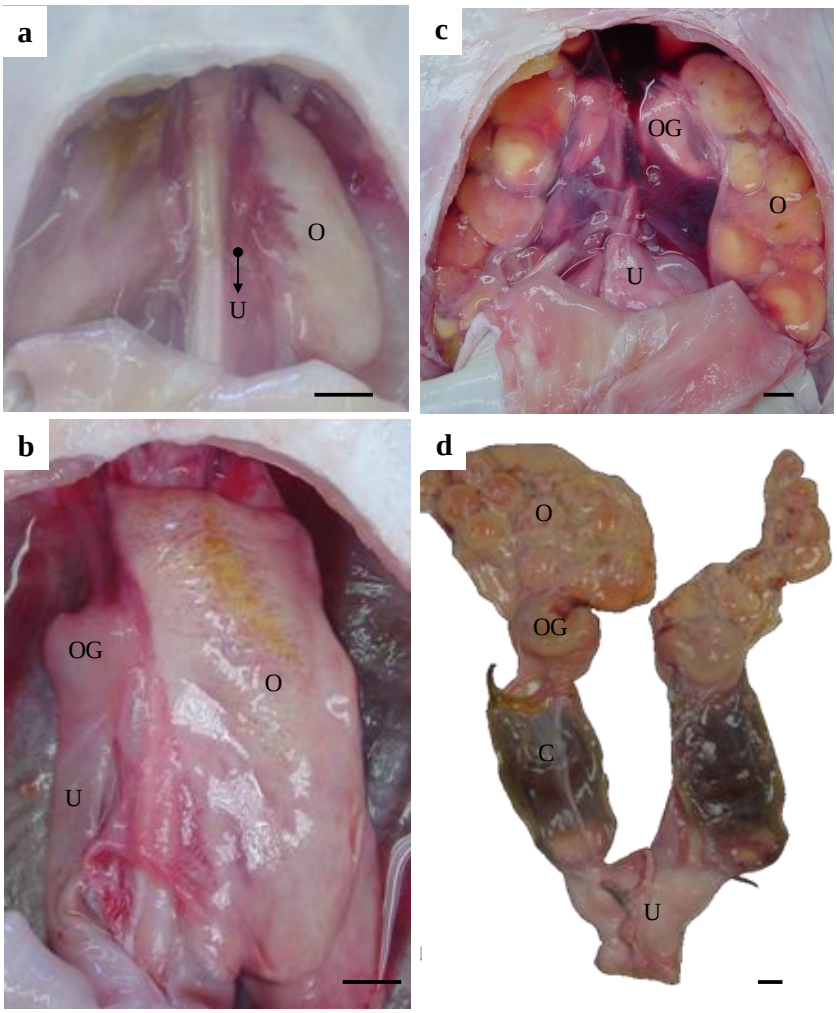

Figure 4.9. Macroscopic reproductive phases in females.
a) immature; b) developing; c) spawning capable; and d) actively spawning sub-phase. (O: ovary, U: uterus, OG: oviducal gland C: egg capsule)

Macroscopically, the ovary is not distinguishable from the epigonal organ, since the latter is a thin layer surrounding the gonadal tissue. This ovary is classified as an external ovary according to Pratt (1988). Ovaries possess follicles in all stages of development, with no dominant stage (Fig. 4.9), a sign of asynchronous follicle development, with the result that thornback ray is most likely a batch spawner (Murua and Saborido-Rey, 2003). As in all other skates, each oviduct opens into an oviducal gland (Fig. 4.9). The uterus is composed of a pair of anterior ducts connected anteriorly to the oviducal gland and converging in a unique posterior duct, which opens to the exterior through the cloaca (Fig. 4.9). The main structure

of the reproductive tract, including the position of the epigonal organ is shared by all rajid species, as well as by oviparous sharks, such as *Scyliorhinus canicula* (Stehmann, 2002).

The *immature* phase is clearly identified in the thornback ray. Macroscopically, it is not possible to identify follicles in the ovary, nor can the oviducal glands be distinguished from the uterus which is very thin (Fig. 4.9a). However, microscopic analysis shows that the epigonal organ dominates most of the gonad. The epigonal organ, an autonomous lymphomyeloid tissue, is highly vascularized, with the presence of different types of blood cells; leukocytes are the most abundant, mainly consisting of granulocytes and lymphocytes (Fig. 4.10a). Ovarian follicles (i.e. oocyte and associated membranes, surrounded by the epigonal organ) are located under the germinal epithelium and under the tunica albuginea, a thin layer of connective tissue, to which they seem to be connected, especially during early development (Fig. 4.10b). No oogonia are present. In skates, as well as in all other elasmobranchs, oogenesis occurs early in life, and oogonia are only observed during embryonic development (Prisco *et al.*, 2002; McMillan, 2007; Prisco *et al.*, 2007). In immature females the first two stages of ovarian follicles observed are primordial and primary follicles. Ovarian follicle structure changes during development. The primordial follicles (less than 0.3 mm) consist of a primary oocyte surrounded by a single layer of flattened follicle cells (squamosus cells) (Fig. 4.10c). Primordial follicles are transformed into primary follicles (diameter between 0.3 and 1 mm) in which the oocyte increases in size and the follicular epithelium thickens into a columnar epithelium, containing two types of cells: small cells and large or intermediate cells (Fig. 4.10d). The primary follicle stage is intermediate between primordial and pre-vitellogenic follicles. No analogy could be made with teleosts since their follicles transform from primary to cortical alveolar oocytes, which differentiate just prior to vitellogenesis, and therefore are not present in immature females. In summary, in oviparous elasmobranchs, immature females seem to have, in fact, some gamete development occurring in the ovaries. This is not expected to occur in this phase, based on what is known for teleosts (McMillan, 2007). But regarding the long reproductive cycle of thornback ray and all other oviparous elasmobranchs, in immature females, follicles seem to undergo a premature somatic growth of the oocyte and proliferation of follicular epithelium cells, without transformation of the oocyte internal structure. Since this could only be detected with histology, the macroscopic criterion was maintained as a main character to classify immature females.

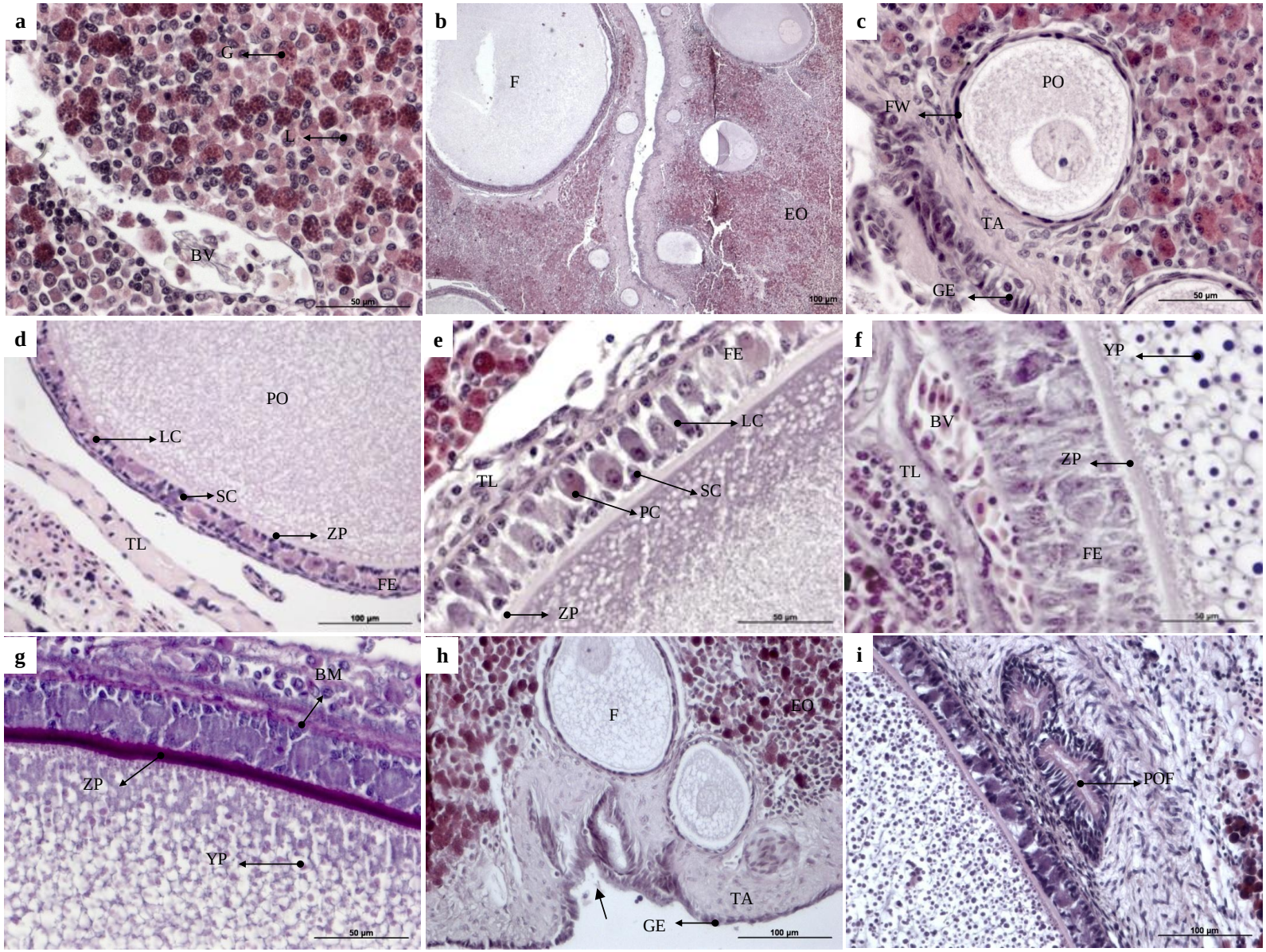
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Figure 4.10. The ovary. (previous page)
a) highly vascularized epigonal organ (BV: blood vessel), containing granulocytes (G) and lymphocytes (L). H&E. Scale bar = 50 µm; b) ovary in a developing phase, showing follicles (F) in different stages of development, surrounded by the epigonal organ (EO). H&E. Scale bar = 100 µm; c) primordial follicle (105 µm in diameter) composed by a primary oocyte (PO) surrounded by squamosus cells; follicular wall (FW) attached to the germinal epithelium (GE) and tunica albuginea (TA). H&E. Scale bar = 50 µm; d) primary follicle (790 µm in diameter) composed by a primary oocyte (PO) surrounded by the zona pellucida (ZP), a pseudostratified columnar follicular epithelium (FE), containing small cells (SC) and large cells (LC), and more externally by the thecal layers (TL). H&E. Scale bar = 50 µm; e) pre-vitellogenic follicle (1031 µm in diameter) composed by an oocyte surrounded by the zona pellucida (ZP), follicular epithelium (FE), containing small cells (SC), pyriform cells (PC) and large cells (LC), and more externally by the thecal layers (TL). H&E. Scale bar = 50 µm; f) vitellogenic follicle (3480 µm diameter) with visible yolk platelets (YP) inside the cytoplasm, thicker zona pellucida (ZP) and follicular epithelium (FE), and vascularized (BV: blood vessel) thecal layer (TL). H&E. Scale bar = 50 µm; g) vitellogenic follicle (4100 µm diameter) (BM: basement membrane, ZP: zona pellucida, YP: yolk platelets). PAS+. Scale bar = 50 µm; h) deformations on the germinal epithelium (GE). H&E. Scale bar = 100 µm; i) post-ovulatory follicles (POF) in a developing female. H&E. Scale bar = 100 µm.

The *developing* phase occurs in females with total length between 300 to 700 mm. In contrast to what is known for teleosts (e.g. Tyler and Sumpter, 1996; Jalabert, 2005), in elasmobranchs the developing phase is not a fast process; it seems to last at least one year in sharks (e.g. Costa *et al.*, 2005; Ebert *et al.*, 2006), skates and rays (e.g. Ebert, 2005; Coelho and Erzini, 2006; Moura *et al.*, 2007; Ebert *et al.*, 2008a; Ebert *et al.*, 2008b; Frisk and Miller, 2009), and lasts up to 6 years in the case of the thornback ray (Serra-Pereira *et al.*, 2008). This long period of maturation is a major feature of all elasmobranchs (Frisk *et al.*, 2001). A number of significant changes occur during the maturation process. Macroscopically, ovaries initially contain only pre-vitellogenic follicles (< 4 mm), and the oviducal gland starts to differentiate from the uterus as a white-coloured bean-shaped structure (Fig. 4.9b). At the end of this phase, the ovaries of females develop large vitellogenic follicles (< 15 mm) and oviducal glands near full development, very similar to the subsequent reproductive phase. The subdivision of the developing phase, although facultative, allows for a better idea of the reproductive phase by differentiating between a female that is just starting to develop or is almost reaching the spawning capable phase. The term “Early Developing” should be used for females with ovaries containing only white follicles less than 2 mm in diameter and oviducal glands that are absent or beginning to form (whitish). “Mid Developing” should refer to females with ovaries containing yellow follicles less than 8 mm in diameter (commonly less than 30 follicles) and developing oviducal glands. Lastly, “Late Developing” should be used for females containing ovaries with a great quantity of yellow follicles (commonly more than 30 follicles) with diameter less than 15 mm and oviducal glands completely formed.

In the developing phase, primordial and primary follicles persist in the ovary. Pre-vitellogenic follicles are observed (diameter > 1 mm), which are larger in size and have

thicker follicular epithelia. Pre-vitellogenic follicles also contain pyriform and round cells with lipid-like substances in the epithelium, in addition to small and large cells (Fig. 4.10e). Small cells are known to grow into large cells, which subsequently transform into pyriform cells (Andreuccetti *et al.*, 1999). The pyriform cell apex connects with the oocyte, forming an intercellular bridge through which cytoplasmic constituents are transferred into the oocyte (Andreuccetti *et al.*, 1999). This type of follicle development from primordial to vitellogenic follicles, is similar to that described for oviparous (e.g. Andreuccetti *et al.*, 1999; Barone *et al.*, 2007) and viviparous elasmobranchs (e.g. Prisco *et al.*, 2002; Prisco *et al.*, 2007). In teleosts, the early-developing pre-vitellogenic phase corresponds to that in which cortical alveolar oocytes are formed, which is a main difference from elasmobranchs, since this type of follicle never occurs in elasmobranch ovaries (e.g. Lutton *et al.*, 2005; Barone *et al.*, 2007; McMillan, 2007).

Ovarian follicles approximately 2.5 mm in diameter begin the vitellogenesis process (Fig. 4.10f), which consists of: formation of yolk platelets, pseudostratification of follicular epithelium, and increase in peripheral vascularization between the thecal layers and the follicular epithelium. The peripheral vascularization is related to the transport of yolk precursors into the oocyte (Andreuccetti *et al.*, 1999). In the thornback ray, the basement membrane, the zona pellucida and the yolk platelets were markedly stained with PAS+ (Fig. 4.10g). Vitellogenesis seems to start in follicles around a similar size among rajids (e.g. Barone *et al.*, 2007) and other elasmobranchs (Prisco *et al.*, 2002; Prisco *et al.*, 2007).

The pair of oviducal glands starts to differentiate in the developing phase. The gland tubules form from the lumen and expand to the entire gland. In the late developing phase, the oviducal gland is fully developed and all the secretory zones are distinguished (Serra-Pereira *et al.*, 2008). The uterus is composed of a very broad lamina propria (connective tissue) and is slightly vascularized. Internally, the uterus structure is arranged into invaginations and covered by simple columnar epithelium (Figs. 4.11a-b).

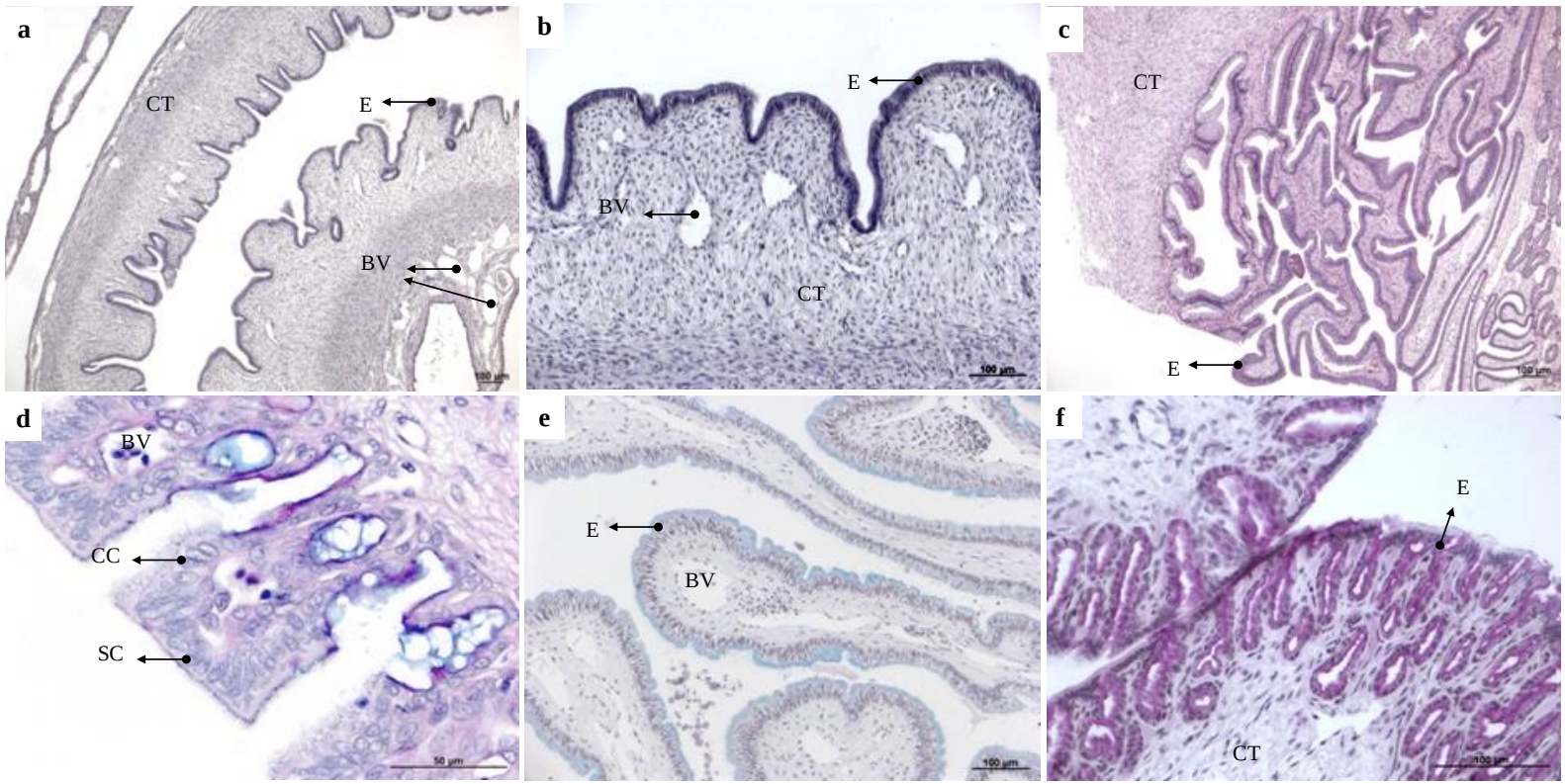

Figure 4.11. The uterus.
a) immature uterus composed by vascularized (BV: blood vessel) connective tissue (CT) covered by simple columnar epithelium (E). H&E. Scale bar = 100 µm; b) surface detail of an immature uterus. H&E. Scale bar = 100 µm; c) spawning capable uterus logitudinal folds. H&E. Scale bar = 100 µm; d) longitudinal fold detail, showing the two types of cells: ciliated cells (CC) and secretory cells (SC). PAS/AB. Scale bar = 50 µm; e) spawning capable uterus with AB+ secretions inside the epithelial cells. PAS/AB. Scale bar = 100 µm; f) spawning capable uterus with PAS+ secretions inside the epithelial cells. PAS/AB. Scale bar = 100 µm.

Spawning capable thornback ray females are observed year-round in Portuguese waters. These females possess ovaries filled with follicles in different development stages, including large vitellogenic follicles greater than 15 mm in diameter. Also, their oviducal glands are completely formed and their uteri are enlarged (Fig. 4.9c). The maximum follicle diameter in the thornback ray is 40 mm. The maximum follicle diameter observed in the ovaries seems to be related to the maximum size of the species. Among rajids, smaller species, such as *Leucoraja naevus* (du Buit, 1976) and *Dipturus polyommata* (Kyne *et al.*, 2008), attain a maximum follicle size less than 30 mm in diameter. Species with similar maximum sizes as the thornback ray also attain similar follicle diameters (e.g. *Atlantoraja cyclophora*; Oddone and Vooren, 2005), whereas larger species tend to have larger follicles, around 50 to 60 mm in diameter (e.g. *Raja rhina*; Ebert *et al.*, 2008b). Based on follicle composition following ovulation, the thornback ray may be a batch spawner. Further studies should be developed in this field to clarify the type of fecundity of this species, as well as other rajid species.

In this study, histological slides with follicles larger than 6 mm were not analyzed. However, Andreuccetti *et al.* (1999) observed large follicles, close to ovulation size in *Raja asterias*. In those follicles, the follicular epithelium was reduced in thickness, compared to previous stages, and only a few number of small cells and scattered large cells persisted. Pyriform cells disappeared by apoptosis and round cells were reduced in size and disappeared prior to ovulation.

Deformations of the germinal epithelium were observed in various cross sections along the ovary, in all reproductive phases (Fig. 4.10h). These deformations seem to relate to detachment of larger follicles from the periphery and subsequent movement inside the ovary and cannot be related to ovulation since they occurred prior to the spawning phase. The oviducal gland possesses tubules filled with secretions including within the lumen (Serra-Pereira *et al.*, Submitted). Sperm bundles were observed at the interior of the female's oviducal gland (Serra-Pereira *et al.*, Submitted). The uterus showed an increase in invaginations (Fig. 4.11c). The simple epithelium changed into undulating surface epithelium, composed of ciliated cells, with basal elongated nuclei, and secretory cells with apical globulous nuclei (Fig. 4.11d). Vascularization increased both near the folds and near the external surface of the uterus. The blood vessels reached the tip of each fold, as shown in Figures 4.11d and 4.11e. In this reproductive phase, the uterus produce secretions through the epithelial secretory cells, which were sulphated acid mucins (AB+) (Fig. 4.11e) and neutral mucins (PAS+) (Fig. 4.11f).

The *actively spawning* sub-phase corresponds to all the uterine phases (active/advanced/extruding) described by Stehmann (2002), which are collectively associated with capsule formation (Fig. 4.9d). Thornback ray in the actively spawning sub-phase are observed year-round, in Portuguese waters. In other areas the spawning season is also extended; between February and September in UK coastal waters (Holden *et al.*, 1971; Holden, 1975), and between May and December in the SE Black Sea (Demirhan *et al.*, 2005). A continuous spawning reproductive strategy seems to be the most common among rajids (e.g. Richards *et al.*, 1963; du Buit, 1976; Walker, 1999; Oddone and Vooren, 2005), as well as some catsharks (Compagno *et al.*, 2005). Yet, it is important to note that not all adult females were observed in that condition at the same time, so an asynchrony within the population must occur.

The actively spawning sub-phase is identified by the presence of egg capsules in the uterus. The ovulation of follicles occurred at diameters around 30 mm. After being fertilized, the egg was surrounded by the following series of envelopes produced by the oviducal gland: (i) sulphated acid and neutral mucin secreted by the club zone (hydrodynamic support); (ii) second layer of jelly secreted by the papillary zone, composed by sulphated acid and neutral mucins; (iii) third layer of jelly, a sulphated acid mucin secreted by the papillary zone (lubricant and bounding layer); (iv) hard egg envelope, proteic, secreted by the baffle zone; and (v) surface hairs (chemically similar to the capsule), coated with mucous secretions that cover the exterior of the capsule (sulphated acid mucins), produced by the terminal zone (Serra-Pereira *et al.*, Submitted). The chemical nature of the different egg surroundings is similar among oviparous species (Hamlett *et al.*, 2005). In this reproductive phase, the uterus possess highly vascularised folds with ciliated microvilli and branched tubular glands, producing sulphated acid and neutral mucins (AB+ and PAS+) (Figs. 4.11d-f). Due to its structure and secretions, the uterus has a great contribution to the capsule surface structure and chemistry (Hamlett *et al.*, 2005), facilitating biochemical processes for capsule sclerotization, including provision of oxygen and absorption of water (Koob and Hamlett, 1998; Hamlett *et al.*, 2005). The whole process of capsule formation to oviposition is a very fast process. In the thornback ray it may last one to two days (Holden *et al.*, 1971; Ellis and Shackley, 1995).

Post-ovulatory follicles (POFs) (Fig. 4.10i) are only observed in females initially assigned to the developing phase, characterized by ovaries with follicles smaller than 1 mm, and enlarged oviducal gland and uterus. This combination of characteristics represented 30 to 40% of the females after attaining the first maturity size. Since POFs should not be found in

the developing phase and should instead be observed after spawning (Brown-Peterson *et al.*, in press), it is suggested that these females could be, in fact, in a *regressing* phase. A regressing phase has already been described in the following rajids: in *Raja asterias*, based on the presence of POFs in females with small oviducal glands (Barone *et al.*, 2007); in *Raja clavata* based on the cessation of egg laying from October to January (Holden, 1975); adult females of the following species, *Bathyraja aleutica*, *B. lindbergi* and *B. minispinosa*, with inactive and atrophied ovaries (Ebert, 2005).In other oviparous elasmobranchs a regressing phase must also occur, especially in those species with short spawning seasons (Compagno *et al.*, 2005). In species with continuous spawning, the regressing phase seems to occur at an individual level (Oddone and Vooren, 2005). In the thornback ray, as well as in other oviparous elasmobranchs (e.g. Barone *et al.*, 2007), small, white pre-vitellogenic follicles persist in the ovaries across all reproductive phases, which seems to be a follicle reserve that may contribute to future spawning episodes.

Although not identified in the present study, the *regenerating* phase should be considered as a reproductive phase. Since in oviparous elasmobranchs the reproductive tract seems not to regress to a phase where only primary growth follicles are found in the ovary (Brown-Peterson *et al.*, in press), the regenerating phase could be used to classify adult females prior to follicle growth. In this phase, females have ovaries full of small follicles, as well as enlarged oviducal glands and uteri. A regenerating period was already described in *Atlantoraja cyclophora*, based on GSI values and the presence of females with white follicles in length classes where vitellogenesis and egg deposition occurred (Oddone and Vooren, 2005). In that study the regenerating period was termed "resting period". Further investigation will be needed to better characterize this phase.

In summary, the reproductive terminology used in teleosts seems to be adaptable to oviparous elasmobranch females. Yet, some differences in the characterization of the different phases should be taken into account, including the following: (i) Immature phase: follicles in a more advanced stage than primary growth oocytes can be found in immature females, i.e. "primary follicles" in which some proliferation of the thecal and follicular epithelium cells is observed. Gamete development seems to be more extended in time, which could be related to the longer reproductive cycles and higher longevity of this species; (ii) Developing phase: longer duration of the developing phase, characterized by the occurrence of pre-vitellogenic follicles with major differences from those identified in teleosts, i.e. cortical alveolar (Brown-Peterson *et al.*, in press), which are absent from elasmobranchs; (iii)

Spawning capable phase: no hydrated oocytes are observed in elasmobranchs; (iv) Actively spawning sub-phase: after being fertilized the ovulated egg is surrounded by a series of mucins and by a proteic capsule secreted by the oviducal gland, whose activity must be triggered by a complex hormonal regulation (Hamlett *et al.*, 2005); (v) Further histological analysis should be made to better characterize the regressing and regenerating phases.

4.2.1.4.3. Males

Testes have a lobular surface and are surrounded by the epigonal organ (Fig. 4.12). Similar to the condition in females, the epigonal organ is a thin layer surrounding the male gonad. The development of sperm in each lobe is radial, developing from the germinal zone in the center to the periphery of the lobe and diametric, across the testis, from the dorsal to the ventral surface. This type of development leads to a compound testis, according to Pratt's nomenclature (1988). The main reproductive structure is similar among all rajid species and oviparous sharks (Stehmann, 2002).

In *immature* males, the claspers are flexible and shorter than the pelvic fins (Fig. 4.13a). Testes are homogeneous or have small lobules in the dorsal surface (Fig. 4.12a). The sperm ducts, the epididymis and vas deferens, are very thin and hardly differentiated with a naked eye. Microscopically, spermatogenesis starts both from the germinal zone or germinal papilla located in the center of the lobe and dorsally in the testis (Figs. 4.14a-b). Following Parsons and Grier (1992) spermatocyst classification, at this reproductive phase only stage I (primordial germ cell or gonocytes), stage II (spermatogonia) and stage III (primary spermatocytes) spermatocysts were observed (Figs. 4.14c-e). Spermatocysts are spherical units composed by germ cells and Sertoli cells surrounded by an acellular basal lamina (Fig. 4.14d). In all elasmobranchs, the germ cells of only a single developmental stage are associated with a Sertoli cell at any given time, which then degenerate after the development is complete (Stanley, 1966). Stage I spermatocysts, located beneath the coelomic epithelium in the germinal zone, consist of loosely organized gonocytes or primary spermatagonia, some of which are already bound to a basement membrane (Fig. 4.14c). In Stage II spermatocysts,

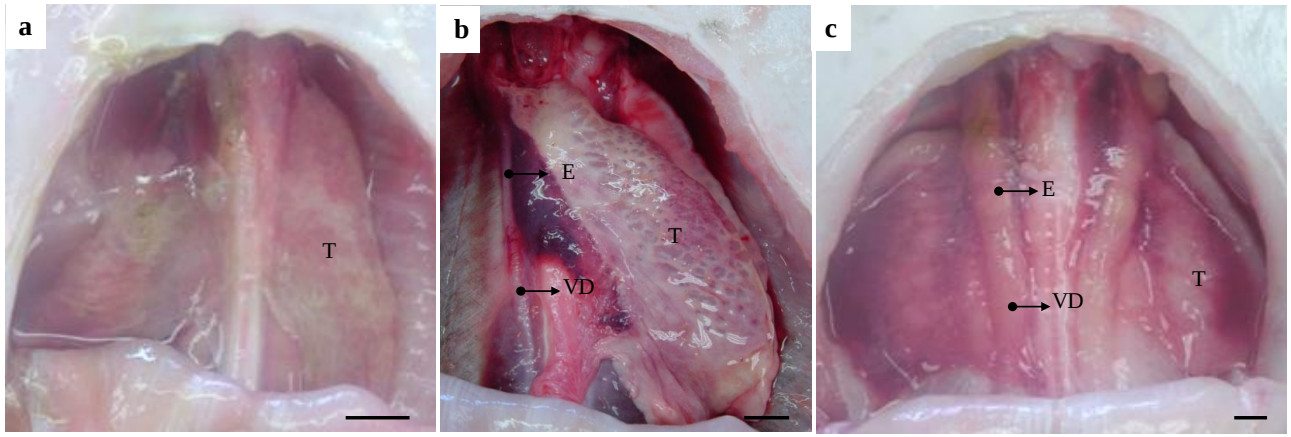

Figure 4.12. Macroscopic reproductive phases in males.
a) immature; b) developing; c) spawning capable. (T: testis; E: epididymis, VD: vas deferens).

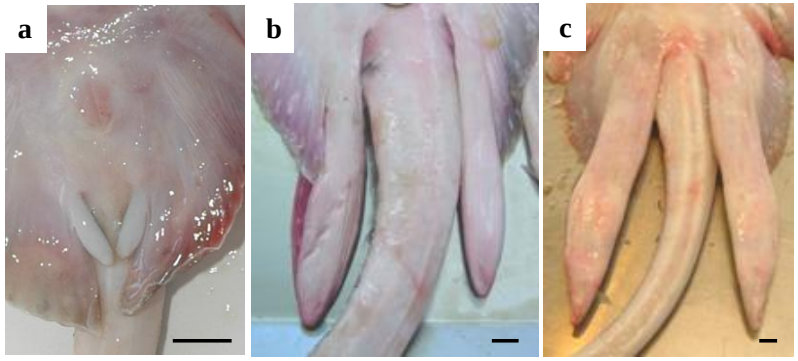

Figure 4.13. External reproductive phases in males, based on clasper growth.
a) immature; b) developing; c) spawning capable.

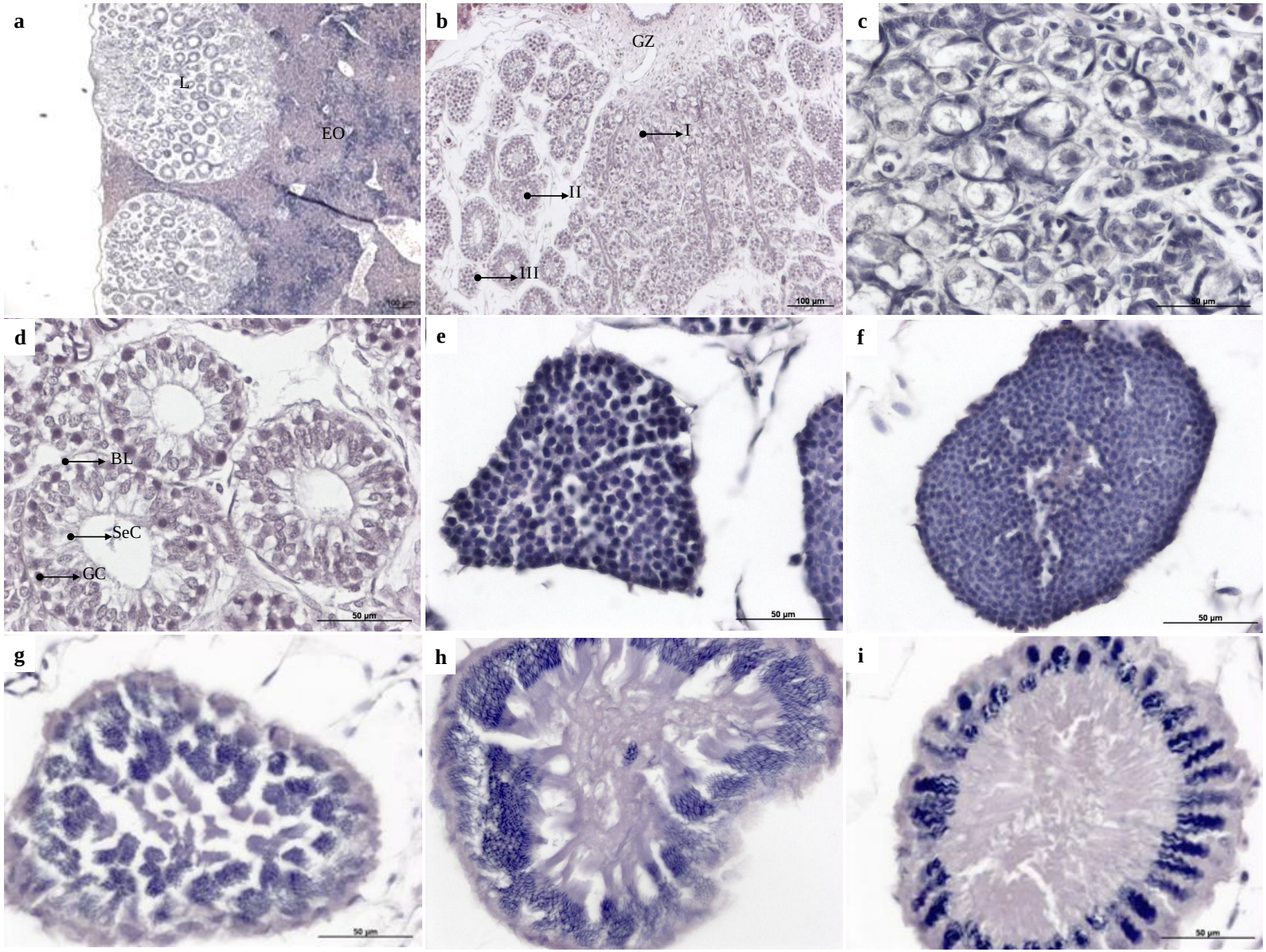
a
L
EO
b
GZ
I
II
III
c
d
BL
SeC
GC
e
f
g
h
i

Figure 4.14. The testis. (previous page)
a) immature testis with small lobules (L) starting to differentiate, surrounded by the epigonal organ (EO). H&E. Scale bar = 100 μm; b) first stages of the spermatogenensis in a lobe from an immature testis, starting from the germinal zone (GZ): I: gonocytes, II: spermatogonia, III: primary spermatocyte. H&E. Scale bar = 100 μm; c) stage I: gonocyte. H&E. Scale bar = 50 μm. d) stage II: spermatogonia, composed by germ cells (GC) and Sertoli cells (SeC), surrounded by a acellular basal lamina (BL). Scale bar = 50 μm; e) stage III: primary spermatocyte. H&E. Scale bar = 50 μm; f) stage IV: secondary spermatocyte. H&E. Scale bar = 50 μm; g) stage V: spermatid. H&E. Scale bar = 50 μm; h) stage VI: immature sperm. H&E. Scale bar = 50 μm; i) stage VII: mature sperm. H&E. Scale bar = 50 μm.

the spermatogonia and Sertoli cells divide and the spermatocyst enlarge; the spermatogonia are aligned beneath the basement membrane and the Sertoli cell nuclei also start to migrate to the periphery of the spermatocyst, surrounding a central lumen (Fig. 4.14d). Stage III spermatocysts consist of primary spermatocytes resulting from the first meiotic division of spermatogonia; primary spermatocytes have large nuclei and fill the entire spermatocyst; Sertoli cells remain in the periphery (Fig. 4.14e).

Like in females, it seems that the relatively long maturation process of oviparous elasmobranchs is translated in early male gamete development in the immature phase, since more advanced stages than primary spermatogonia are observed (Brown-Peterson *et al.*, in press). This fact has also been described in other elasmobranchs like the two deep water sharks *Centroscymnus coelolepis* and *Centroscymnus squamosus* (Girard *et al.*, 2000). In these deep-water species, males macroscopically classified as immature showed more advanced stages (secondary spermatocysts and spermatids) than primary spermatocysts, when analyzed microscopically.

In the *developing* phase, claspers are enlarged, but still flexible. Claspers are longer than pelvic fins (Fig. 4.13b) and the number of lobules in the testes increases (Fig. 4.12b). Microscopically, all the spermatocyst stages coexist, including: stage I (primordial germ cell or gonocytes), stage II (spermatogonia), stage III (primary spermatocytes), stage IV (secondary spermatocytes) in which primary spermatocytes divide and form secondary spermatocytes with small, round nuclei and condensed chromosomes (Fig. 4.14f); stage V (spermatids) consisting of spermatids, produced after the second meiotic division of secondary spermatocytes, showing elliptical nuclei and emerging flagella, and separated and unorganized inside the spermatocyst (Fig. 4.14g); stage VI (early sperm) spermatids that have undergone spermiogenesis, are transformed into more elongated immature sperm, forming loose bundles, with heads facing the basement membrane and tails projecting toward the lumen. (Fig. 4.14h); and stage VII (mature sperm) which consists of mature sperm organized in tight bundles associated with Sertoli cells arranged in the periphery (Fig. 4.14i). Both the

epididymis and vas deferens are already visible and start to coil as maturation advances (Fig. 4.12b).

In the *spawning capable* phase, males have claspers that remain rigid upon reaching maturity, due to their hard cartilages, and attain their maximum length (Fig. 4.13c). The testes are completely formed (Fig. 4.12c), and are filled with lobules containing all the spermatocyst stages, with a greater proportion of those stages V to VII (spermatids to mature sperm) than observed in the previous phase. If no differences exist among elasmobranchs, the distribution of spermatocysts in this species should be homogeneous across the gonad (Maruska *et al.*, 1996). The mature sperm or spermatozoa exit the testis via efferent ducts. The sperm is then transported to the claspers through the epididymis and vas deferens to the seminal vesicles. The epididymis (Figs. 4.15a-b) shows a simple columnar epithelium, folded into villosities, each containing a blood vessel inside. In the lumen, sperm arranged in bundles are observed, surrounded by sparse liquid, probably secreted by the Sertolli cells. The vas deferens (Figs. 4.15c-d) is composed of a simple columnar ciliated epithelium, also folded into villosities. Not all villosities contained a blood vessel. The lumen was filled with seminal liquid containing abundant sperm bundles and round structures similar to primary spermatocytes (Fig. 4.15d).

Males are assigned to the *actively spawning* sub-phase based on the appearance of the claspers. The internal appearance of the claspers is reddish and swollen after copulation; and completely filled sperm ducts, so that when sliced, the sperm spills out of the ducts. Like in teleosts, the actively spawning sub-phase cannot be distinguished through histology (e.g. Brown-Peterson *et al.*, in press).

There was no evidence of *regressing* and *regenerating* phases in males. In other words, males with hard, enlarged claspers with regressing gonads or those that appeared reproductively inactive were not observed in thornback ray. In fact, males seem to not have a reproductive cycle, but rather progress from immature through spawning one time, and then remain in the spawning or spawning capable phases for the rest of their lives. This suggests that active spermatogenesis is always occurring in males once they have reached sexual maturity, which is completely different from what is known in the teleosts. In other elasmobranchs, such as *Raja asterias* (Barone *et al.*, 2007) males with large claspers and

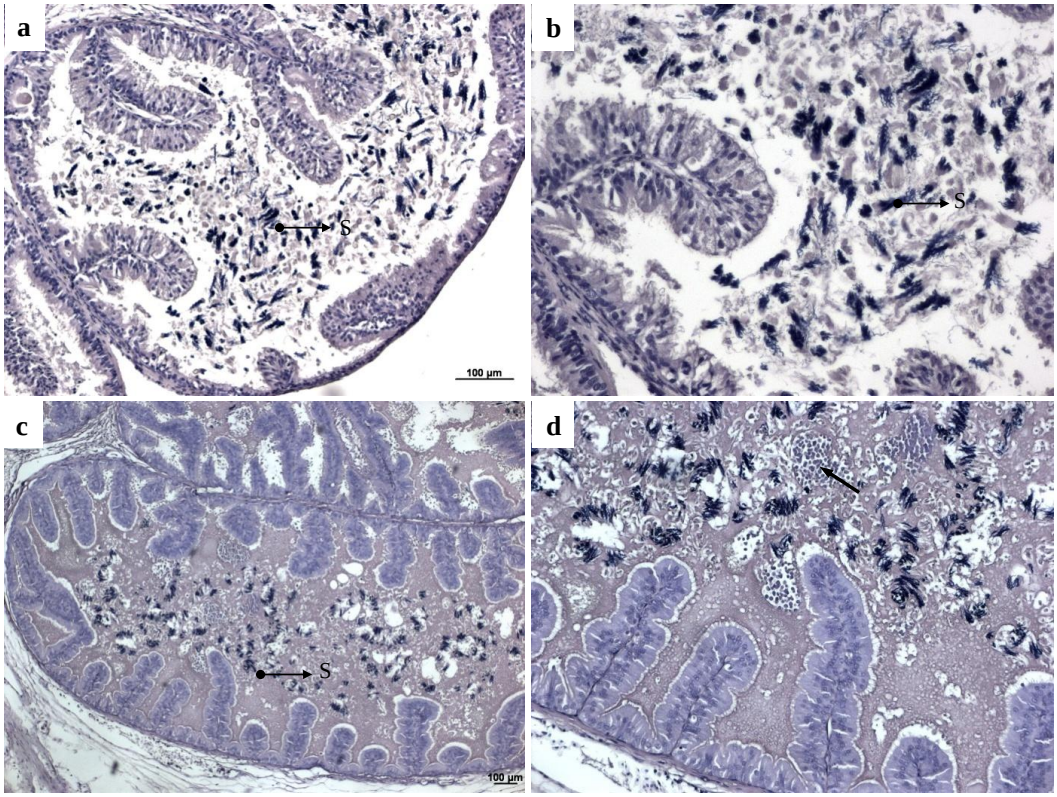

Figure 4.15. Sperm ducts.
a) epididymis: in the spawning capable phase; sperm bundles (S) surrounded by Sertoli cell material. H&E, scale bar = 100 µm; b) detail of a villosity in the epididymis. H&E, scale bar = 100 µm; c) vas deferens: in the spawning capable phase; sperm bundles (S) surrounded by a dense seminal liquid. H&E Scale bar = 100 µm; d) detail of the vas deferens showing the ciliated villosities and the sperm and structures similar to primary spermatocytes (arrow) disperse in the lumen. H&E, scale bar = 100 µm.

small testes were described to occur, and correspond to a regressing phase. As a result, although the regressing phase was not observed in the thornback ray, it should be considered as a reproductive phase for males in order to apply to species in which it occurs.

In summary, the reproductive terminology used in teleosts seems to be adaptable to oviparous elasmobranch males. Yet, the following differences should be taken into account. (i) In skates, spermatogenesis occurs in seminiferous follicles arranged inside lobules, each containing a germinal epithelium. This is a distinct organization from that found in most teleosts, where testis are arranged in elongated branching seminiferous tubules instead of follicles, and lacking a permanent germinal epithelium (Matty, 1985). (ii) Immature phase: spermatogenesis seems to be triggered earlier in the development, compared to teleosts, since primary spermatocysts are observed in immature testis and more advanced stages are observed in other elasmobranch species. (iii) Spawning capable phase: occurrence of internal fertilization, in which the sperm is released inside the female through claspers, instead of

Table 4.6. New proposal for a reproductive terminology for oviparous elasmobranchs, applied to skates, based on the new terminology from Brown-Peterson *et al.* (in press) and the reproductive phases proposed by Stehmann (2002).
The macroscale and microscale features, for both females and males, are presented for each phase. Microscale features were based on thornback ray reproductive development. The measurements presented are specific for thornback ray.

Phase	Macroscale	Microscale
FEMALES		
Immature	Ovaries small, whitish and homogeneous; undistinguishable ovarian follicles. Absent oviducal gland and thread-like narrow uterus.	Ovary with primordial (smaller than 0.3 mm) and primary follicles (0.3 to 1 mm) connected to the germinal epithelium and tunica albuginea. Uterus composed mainly by connective tissue, covered by simple columnar epithelium with some invaginations; some blood vessels present.
Developing*	Ovaries enlarged with small follicles in different stages of development, sometimes restricted to the anterior part of the ovary. Developing oviducal gland. Enlarged uterus. *A subdivion could be considered **Early developing**: ovary with only white follicles with less than 2 mm, oviducal absent or beginning to form (whitish); **Mid developing**, ovary with yellow follicles below 8 mm (commonly below 30 follicles), developing oviducal gland; **Late developing**: ovary with a great quantity of yellow follicles (commonly above 30) with diameter less than 15 mm, oviducal gland completely formed.	Ovary with primordial, primary, pre-vitellogenic and vitellogenic follicles (smaller than 15 mm). Oviducal gland can show only the beginning of gland tubules formation or be completely formed, with differentiation of the four secreting zones, depending on the advance in maturation. Beginning of secretions production in the oviducal gland and uterus. Uterus more invaginated and vascularized.
Spawning capable	Large ovaries with large yolked follicles that can reach around 40 mm in diameter. Oviducal gland and uterus fully developed.	Follicles in all stages can be observed in the ovary. Secretions present in the gland tubules of the oviducal gland. Uterus highly invaginated, showing longitudinal folds producing secretions to the lumen.
Actively Spawning sub-phase	Large yolked-egg may be present in the oviducal gland. Egg capsule present in the uterus and attached or not to the oviducal gland. Capsules may be starting to be produced or be fully formed, hardened and dark; present in one or both uterus.	POFs can be present in the ovary. Oviducal gland tubules full of secretion materials. Secretions also present in the gland lumen.
Regressing	Large ovaries with follicles not occupying the entire surface. Large vitelogenic follicles may be present. Oviducal gland completely formed and expanded uterus. Can be mistaken with the developing phase.	Follicles in all stages can be observed in the ovary. POFs present in the ovary. Oviducal gland completely formed but without secretions in the lumen. Uterus completely formed and with production of secretions.
Regenerating	Ovaries full of small follicles, enlarged oviducal glands and uterus.	(not analysed)

Table 4.6. Continued.

Phase	Macroscale	Microscale
MALES		
Immature	Claspers flexible and small, shorter than or as long as the pelvic fins. Testes small, sometimes with lobules already visible. Sperm ducts straight and thread-like.	Testis containing spematocysts only in stages I, II and III.
Developing	Claspers extended, longer than the tip of the pelvic fin, with calcifying skeleton but still soft and flexible. Testes enlarged with developing lobules. Sperm ducts beginning to coil. Males with only one of previous features should also be classified under this phase.	Testis containing spematocysts in all stages. Sperm ducts start to differentiate vilosities. No sperm is observed inside the ducts.
Spawning capable	Claspers enlarged, longer than the tip of the pelvic fin; fully formed and rigid. Testes enlarged, filled with developed lobules and often redish in colour. Sperm ducts tightly coiled and filled with sperm.	Testis containing spematocysts in all stages. Stages V-VII are more abundant than in the developing stage. Sperm ducts composed by villosities full with seminal liquid, more dense in the vas deferens. Sperm bundles observed in the lumen.
Actively Spawning sub-phase	Glans claspers reddish, dilated and swollen. Sperm flowing in the sperm ducts.	Same as Spawning Capable.
Regressing	Claspers enlarged, longer than the tip of the pelvic fin; fully formed and rigid. Small testes with few visible lobules.	(not analysed)
Regenerating	This phase is not known to occur in oviparous elasmobranchs	

being released to the sea through short gonoducts. (iv) Once a male attains maturity it seems not to regress to a previous phase in some species such as the thornback ray, whereas in other species they have a regressing phase.

In conclusion, the reproductive terminology adopted from Brown-Peterson *et al.* (in press) proved to be adequate when applied to oviparous elasmobranchs for both females and males, using the thornback ray as an example. A direct application of previous terminologies was accomplished. A summary of the main macroscale and microscale features by reproductive phases, described along the present study, is presented in Table 4.6. A more detailed description considering particular features of other oviparous elasmobranch species should be achieved by future studies, as well as an adaptation of the same terminology to viviparous species. The need for a more detailed terminology that includes regressing and regenerating phases had already been mentioned by other authors (e.g. Ebert *et al.*, 2008b), since the common misclassification of adult elasmobranch specimens as developing, i.e. as "immature" for the purposes of fitting maturity ogives, rather than "mature", as they had already spawn at

least once, could lead to overestimation of the length of 50% maturity and to biased estimates of the population's reproductive potential and growth (Ebert *et al.*, 2008b).

4.2.2. MATURATION, FECUNDITY AND SPAWNING STRATEGY [1]

4.2.2.1. Abstract

In Portuguese waters, spawners of thornback ray were found throughout the year. The maturation process was divided into three main phases and these were described, using the information on gonad weight, oviducal and uterus width in females and on gonad weight, clasper length and sperm ducts width in males. The duration of developing stage was estimated to last up to 6 year and females attain length-at-first-maturity at 784 mm while males at 676 mm, which corresponds to 8 and 6 years. A resting stage was identified, for females larger than length-at-first-maturity and characterized by low Gonadossomatic Index and well developed oviducal glands and uterus. In the ovaries, follicle development was asynchronous. Fecundity was determinate with batch episodes, with about 35 eggs per batch. During spawning season a total of four batch episodes occur meaning that the total fecundity was around 140 eggs per female.

4.2.2.2. Introduction

Skates (Rajidae) have an oviparous reproductive mode, with internal fertilization, which involves pre-copulatory courting behavior (Luer and Gilbert, 1985). Before egg extrusion, here referred as spawning, a series of complex processes occur in reproductive structures other than the ovary, which are not observed in the egg production of gonochoristic, oviparous teleost fishes. The oviducal gland, an organ derived from the oviduct, is the site for the egg encapsulation, which consists on the production of egg investments and egg envelop, the transport of the fertilized eggs and sperm storage (Hamlett *et al.*, 1998). The resultant egg

[1] Serra-Pereira, B., Figueiredo, I. and Serrano-Gordo, L. Submitted. Maturation, fecundity and spawning strategy of the thornback ray, *Raja clavata*, from Portuguese waters. Marine Biology.

capsule is species specific and, once produced, the eggs are directly released to the sea. Further embryo development does not involve maternal care.

Studies on the reproduction of skates are essential to assess the status of the populations and consequently for the proposal of management measures, using approaches that enter into consideration the species life cycle. These studies include the temporal delimitation of the reproductive processes such as maturation, mating and spawning, the estimation of length-at-first-maturity and fecundity. To succeed on some of these goals it is crucial that maturity scales clearly distinguish the different phases and that the criteria can be objectively used by different users and be comparable across fish *taxa*. For the Northeastern Atlantic species the maturity scale proposed by Stehmann (2002) for cartilaginous fishes has been commonly used. Its major criticism is related to the failure to differentiate between non reproductive adult females and males from those that had never reached maturity. Recently Brown-Peterson *et al.* (2007) proposed a unified reproductive terminology for fishes that should be applied across fish taxa. According to this all the maturity stages from immature to resting were contemplated and could be applied to different fish regardless from the reproductive mode. A recent study (Serra-Pereira *et al.*, in press-b) proposes an analogy between these two scales, adapted to oviparous elasmobranchs.

The thornback ray, *Raja clavata*, is the most abundant rajid in the NE Atlantic (Dulvy *et al.*, 2000) and one of the most important in European landings. In Portugal, it represents between 23% and 37% of the total landed weight of the combination of species that are landed under the generic category of "skates" (Machado *et al.*, 2004). Its maximum total length was estimated at 1 m, which corresponds to a longevity of 10 years (Serra-Pereira *et al.*, 2008). The actual EU management measure adopted under Common Fisheries Policy to skates is a combined TAC for all skates species, additionally for some ICES areas the on board retention for some of the species is prohibited (EC No 43/2009, 2009). However this additional measure has been considered inappropriate since it was adopted with no scientific background (ICES, 2009).

Studies on reproduction of the thornback ray gave different estimates of the reproductive parameters between geographical areas. The duration of the spawning season is an example, being the estimates also variable within the same region (Table 4.7). In UK coastal waters, Holden *et al.* (1971) and Holden (1975) estimated a spawning period between February and September, with a peak in June, Ryland and Ajayi (1984) and Brander and Palmer (1985) referred that it starts later, May and March, respectively. In the SE Black Sea spawning lasts from May to December (Demirhan *et al.*, 2005). The reproductive behavior of thornback ray

can involve seasonal migrations, as reported for the Southern North Sea; adults move from deep waters to shallower areas where they mate and release the egg capsules; juveniles stay in the area during the first years of development, and then migrate to deeper waters (Hunter *et al.*, 2006). The egg-laying rate is constant during the peak of the spawning season, usually one pair of egg capsules laid in two consecutive days (Holden *et al.*, 1971; Ellis and Shackley, 1995). Female's length-at-first-maturity ranges from 67 to 77 cm total length, while for males it ranges from 59 to 68 cm total length (Nottage and Perkins, 1983; Ryland and Ajayi, 1984; Walker, 1999). Like the other rajids, the thornback ray presents low fecundity, with estimates between 60 and 150 eggs per female per year, in UK waters (Holden *et al.*, 1971; Holden, 1975; Ryland and Ajayi, 1984). The gestation period is estimated to last about 5 months (Clark, 1922; Ellis and Shackley, 1995).

Table 4.7. Thornback ray estimates of length of 50% maturity (L_{50}), for males (M) and females (F), fecundity and duration of the spawning season, in different areas of the NE Atlantic.
Estimates obtained in the present study from both direct and indirect methods are presented.

Author (year)	Area	L_{50} (F)	L_{50} (M)	Fecundity	Spawning Season
Holden *et al.* (1971), Holden (1975)	Eastern England, UK			142-150	February-September (peak: June)
Jardas (1973)	Adriatic Sea	730*	540*		
Nottage and Perkins (1983)	Solway Firth, UK	624*	618*	-	-
Ryland and Ajayi (1984)	Carmarthen Bay, UK	595*	605*	62-74	May-September
Brander and Palmer (1985)	Irish Sea				March-September (peak: June)
Walker (1999)	North Sea	771	679		-
Whittamore and McCarthy (2005)	Caernarfon Bay, UK	705	588	-	-
Demirhan *et al.* (2005)	SE Black Sea	667	640		May-December
Present study	Portugal	(699)* 784	(590)* 676	136/115 (35)**	All year

*Minimum length of a mature thornback ray (Lmat)
** Batch fecundity estimate

As summarized previously, most of the information on the reproduction of thornback ray has been published in the 70's and 80's, mainly to UK waters and with few recent updates despite the increasing fishing effort that the species has been suffering. This study intends to resume all the available information on the reproductive biology of the thornback ray and update it with recent knowledge on the Portuguese coast. Regarding the latter, it intends to verify if this species has a different life strategy in Portuguese waters, comparing to the data available for the UK region and Black Sea. To achieve this goal the main lines are: (i) the delimitation of the maturation, mating and spawning seasons; (ii) the characterization of the

reproductive cycle, based on the alteration of the main reproductive organs with maturation, including the identification of regressing/regenerating females and males; (iii) the estimation of maturity ogives for both sexes; and (iv) the estimation of fecundity.

4.2.2.3. Material and Methods

4.2.2.3.1. Sampling

Thornback ray samples of all size classes were collected between 2003 and 2008, from three IPIMAR yearly bottom-trawl research surveys carried out along the Portuguese continental shelf (March, June and October). Large individuals that have potentially reached maturity were sampled monthly, each year, from January to November, and also in December in 2003, from landings of commercial artisanal fleets at the north (Matosinhos) and at the centre (Peniche) of Portugal, under the scope of the National Data Collection Program (PNAB, DCR).

For each specimen the total length (TL) and disc width (DW) were measured (mm). The total weight (TW), the liver weight, the gonad weight and the gutted weight (gW) were recorded (g). The sex and maturity stage was assigned according to the maturity scale proposed by Stehmann (2002) adapting the terminology from Brown-Peterson et al. (2007): (i) immature, (ii) developing, (iii) spawning capable and (iv) spawning (Table 4.8; according to Serra-Pereira *et al.*, in press-b). A regressing or regenerating stage was adopted.

The diameter of yolked follicles, identified by their yellow colour, was measured (mm), in both ovaries. Oviducal glands height, width and length were measured, as well as, the uterus width at the anterior and posterior regions (Fig. 4.16). Egg capsules were measured (mm) in length, width, thick, anterior horn length, anterior inter-horn width, posterior horn length, posterior inter-horn width and weighted (g) full and empty (Fig. 4.16). Regarding males, the width of both the epididimus and vas deferens was measured (Fig. 4.17).

Table 4.8. Maturity stages description applied to skates, based on the new terminology from Brown-Peterson *et al.* (2007) and the maturity scale proposed by Stehmann (2002), whose terminology is indicated between brackets.

Stages	Description
FEMALES	
Immature (Immature, juvenile)	Ovaries small, whitish and homogeneous, without follicle differentiation. Absent oviducal gland and narrow uterus.
Developing (Maturing, adolescent)	Ovaries enlarged with small follicles in different stages of development, sometimes restricted to the anterior part of the ovary. Yellow follicles present. Oviducal gland in differentiation. Enlarged uterus.
Spawning capable (Mature, adult)	Large ovaries with large follicles that can reach 4 cm in diameter. Oviducal gland and uterus fully developed.
Spawning (Active/ Advanced/ Extruding)	Ovaries and oviducal gland similar to the spawning capable stage. Large yolked egg may be present in the oviducal gland. Egg capsule present in the uterus and attached or not to the oviducal gland. Capsules may be starting to be produced or be fully formed, hardened and dark.
Regressing or Regenerating	Large ovaries with follicles not occupying the entire surface. Oviducal gland completely formed and expanded uterus. Can be mistaken with the developing stage.
MALES	
Immature (Immature, juvenile)	Claspers flexible and small, shorter than the tip of the pelvic fin. Testes small, sometimes with lobules already visible. Sperm ducts straight and thread-like.
Developing (Maturing, adolescent)	Claspers extended, longer than the tip of the pelvic fin, with soft and flexible skeleton. Testes enlarged with developing lobules. Sperm ducts beginning to coil.
Spawning capable (Mature, adult)	Claspers fully formed and rigid. Testes enlarged, filled with developed lobules and often redish in colour. Sperm ducts tightly coiled and filled with sperm.
Spawning (Active)	Glands claspers reddish, dilated and swollen. Testis similar to the mature stage. Sperm flowing in the sperm ducts.

4.2.2.3.2. Data analysis

Gonadosomatic Index (GSI) and hepatosomatic index (HSI) were estimated in relation to the total gW. Both indexes were analysed by month and combining spawning capable and spawning individuals from both sexes, since both stages include adult skates reproductively capable.

The measurements taken from the gonads, oviducal glands and uterus in females and from gonads, claspers and sperm ducts in males were analysed by TL and by maturity stage in order to characterize the maturation process. The null hypothesis of no differences on the width between the two oviducal glands in females and between the epididimus and vas deferens in males were tested with Student's t-test (Zar, 1996).

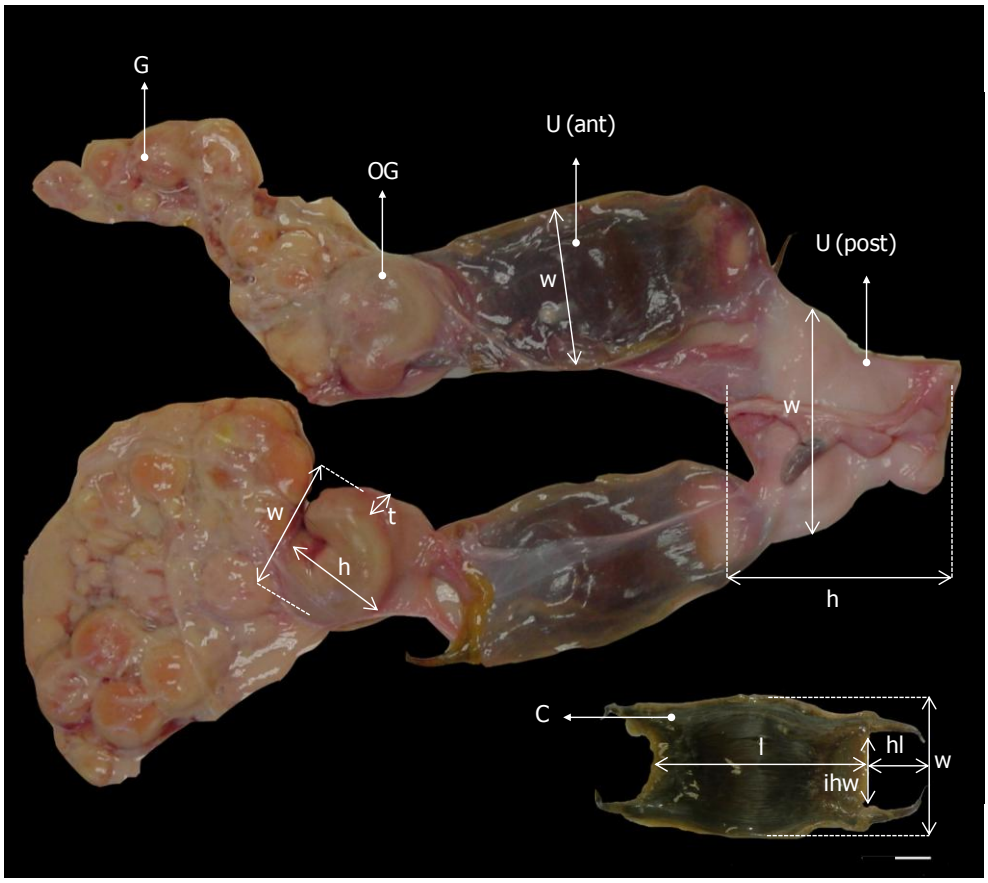

Figure 4.16. Reproductive system of a female in the advanced stage.
O: ovary; OG: oviducal gland; U (ant): uterus (anterior portion); U (post): uterus (posterior portion); C: egg capsule. Egg capsule with the dorsal surface up, covered with hairs (lower right). The measurements made in the oviducal glands, uterus and capsules are presented: w: width; h: height; t: thickness; l: length; hl: horns length; ihw: inter-horn width. Scale bar = 1 cm.

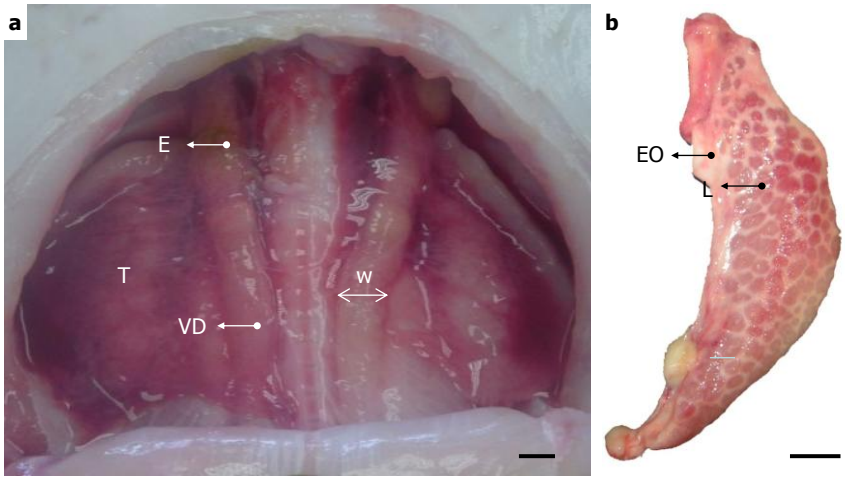

Figure 4.17. Male reproductive system.
a) male in the spawning stage: T: testis, E: epididimus, VD: vas deferens. The width (w) measured to both the epididimus and vas deferens is presented. Scale bar = 1 cm; b) testis of a male in the maturing stage, with the lobes (L) not completely enlarged, surrounded by epigonal organ (EO). Scale bar = 1 cm.

Based on GSI, oviducal gland and posterior uterus widths estimates, females in the developing stage were assigned to the resting or regenerating stages.

Maturity ogives were adjusted to females and males through Generalized Linear Models (GLMs; McCullagh and Nelder, 1989) with a binomial error distribution and a logit link:

$$\log(\frac{p}{1-p}) = -a + bl + \varepsilon, \quad (1)$$

where p is the proportion of fish in a mature condition by length class (l). Estimates of the length-at-first-maturity (L_{50}) and of the slope at L_{50} were derived as:

$$L_{50} = -a/b, \quad (2)$$

$$Slope = b/4. \quad (3)$$

Goodness-of-fit was evaluated by the coefficient of determination (r^2) and ANOVA test (Zar, 1996).

Fecundity was defined as the total number of eggs released per female during the spawning season and it was estimated as the total number of yolked follicles counted in both ovaries. The null hypothesis of no differences in the number of ovarian follicles between ovaries was tested with χ^2 test (Zar, 1996). Fecundity was determined based on females in the spawning capable stage prior spawning, i.e. with high GSI (GSI>2). To avoid underestimates of fecundity females that had already extruded eggs were excluded. Batch fecundity (*Fbatch*), i.e. the number of follicles spawned in each batch (Murua and Saborido-Rey, 2003) was estimated as the median number of yolked follicles with diameter larger than 10 mm, further named as *batch follicles*. This threshold was adopted based on the analysis of follicle size frequency distribution considering that only the yolked follicles with diameter larger than that threshold will be released during the subsequent batch. Assuming that all yolked follicles present in females in spawning capable or spawning stages will be released and no more yolked follicles will be produced till the end of its spawning process, the total fecundity (*Ftotal*) was estimated as:

$$Ftotal = F\max - F\min, \quad (4)$$

where *Fmax* is the maximum number of follicles observed in spawning females and *Fmin* is the minimum number of follicles observed in spawning females. The number of batches (*Nbatch*) per female was then estimated:

$$Nbatch = \frac{Ftotal}{Fbatch}, \quad (5)$$

In this estimate, it was assumed that *Fbatch* is maintained relatively constant throughout batching episodes and that all the follicles remaining in the ovaries near *Fmin* will not be spawned in the ongoing season. Probably they will be retained in the ovary and enter into atresia afterwards.

An indirect method to estimate *Ftotal*, already applied to skate species, like the thornback ray (Holden, 1975), the spotted ray, *Raja montagui*, and the little skate, *Raja erinacea* (Walker, 1999), based on the relative frequency of spawning females per month was applied to data. Only females with TL higher than the minimum length of a mature female (*Lmat*) were considered.

$$Fecundity = \sum_{m} \left[\left(P_m / P\max \right) * \left(N_m * E \right) \right], \quad (6)$$

where P_m is the proportion of spawning females per month, *Pmax* is the maximum egg laying rate during the month with highest proportion of spawning females, N_m is the number of days per month and E is the average egg laying rate, considering 0.5 eggs per female, based on the observations made by Holden (1975). Due to sampling problems, P_m=0 in some months. In this case, partial fecundity was estimated from equation (6) and then extrapolated to 12 months to estimate *Ftotal*.

χ^2 test was used (Zar, 1996) to compare the relative frequencies of *batch follicles* at 2 mm diameter classes by quarter. For this analysis only females in the spawning and spawning capable stages were considered. The assumption of independence on the number of follicles between size classes per quarter was considered.

4.2.2.4. Results

A total of 1767 specimens of thornback ray were sampled (Table 4.9), being 49% females and 51% males. 26% were collected from landings (TL ranged from 320 to 934 mm), and 74% were collected during research cruises (TL ranged from 125 to 1050 mm).

4.2.2.4.1. Reproductive seasonality

Table 4.9 resumes the main characteristics by maturity stage for both sexes. Relative frequencies by maturity stage are presented in Figures 4.18a-b, for females (Fig. 4.18a) and males (Fig. 4.18b). Spawning capable and spawning females (with TL ranging from 699 to 934 mm) and males (with TL ranging from 590 to 1050 mm) occurred throughout the year

(Figs. 4.18c-d). The only exceptions were in April, when spawning females were absent, and in December, when only five immature females (with TL ranging from 557 to 657 mm) and seven immature males (with TL ranging from 566 to 753 mm) were sampled. Monthly fluctuations were observed on female and male GSI and HSI (Fig. 4.19 and Table 4.9). Developing females (Table 4.10), with TL smaller than 800 mm, showed high GSI levels and its variance was also high, especially after September. High GSI levels, i.e. above 0.70, were registered for length classes greater than 800 mm during all months of the year but the highest variability on GSI was registered between March and May. Restricting to spawning capable and spawning females, the higher GSI levels were evident around August and September (Fig. 4.19a). Female HSI did not greatly differ along the year (HSI~7), but the lowest level was registered in October (HSI=5.7) (Fig. 4.19b). In males GSI was around 0.9 during all the months of the year (Fig. 4.19c). Male HSI levels greater than 5 occurred from January to May while values less than 5 occurred in the remaining months (Fig. 4.19d).

The relationship of gonad weight versus TL by maturity stage is presented in Figure 4.20. In females (Fig. 4.20a), the maximum gonad weight was 284 g, observed in a spawning capable female with 805 mm TL. From Figure 4.20a analysis, three phases in the increase rate of gonad weight with TL were observed: (i) slow increase rate for females with TL smaller than 600 mm (immature and early developing); (ii) a moderate increase rate for females with TL between 600 and 750 mm (immature, developing and spawning capable); and (iii) a fast increase rate in females with TL larger than 750 mm (spawning capable and spawning). Oviducal glands were evident in females with TL larger than 700 mm. Since no significant differences on oviducal gland width between the right and left gland were observed ($t=0.15$, $p=0.88$, $d.f.=320$), the analysis proceeded using only the right gland. The relationship between the oviducal gland width and TL is presented in Figure 4.21. In the developing stage most of females had an oviducal gland width smaller than 30 mm. In the spawning capable stage the average oviducal gland width was 35 mm and in the spawning stage it was about 40 mm. The maximum oviducal gland width was 50 mm. The uterus also showed a positive relationship with TL as maturity increases (Fig. 4.22). As observed for gonads, its growth seemed to have three phases for the four measurements (Fig. 4.22a-d): (i) a slow increase rate in females with TL smaller than 700 mm; (ii) a moderate increase rate in females with TL between 700 and 800 mm; and (iii) a fast increase rate in females with TL larger than 800 mm TL.

Table 4.9. Ranges of total length (TL, in mm), indices values (Gonadossomatic Index, GSI, and Hepatossomatic Index, HSI) and gonad weight (GW, in g) by maturity stage and by sex.
For females is also presented: oviducal gland width in mm, posterior uterus width in mm. For males is also presented: clasper length in mm, sperm ducts width in mm.

		Immature	Developing	Spawning capable	Spawning
Females	n	522	228	56	55
	TL (mm)	138-840	367-965	735-934	699-924
	GSI	<1.4	0.1-2-1	0.7-5.9	0.9-5.6
	HSI	0.4-10.1	1.5-13.6	2.3-10.7	2.5-8.8
	GW (g)	<50	1-95	19-284	24-243
	Ov. gland (mm)	-	14-38	24-45	32-49
	Uterus (mm)	2-45	3-90	31-77	37-67
Males	n	605	99	120	82
	TL (mm)	125-756	316-912	590-1050	610-875
	GSI	0.6-0.8	0.6-1.2	0.6-1.3	0.7-1.5
	HSI	0.8-9.3	1.2-10.8	1.1-10.2	1.8-8.6
	GW (g)	<17	1-33	6-66	13-38
	Clasper (mm)	9-100	16-215	130-268	175-241
	Ducts (mm)	>7	2-12	8-14	8-14

Table 4.10. GSI variation, by month and length class, in developing females above 500 mm TL.
Average estimates and the coefficient of variation (CV) between brackets are presented.

	Lenght class (mm)							
Months	**500-549**	**550-599**	**600-649**	**650-699**	**700-749**	**750-799**	**800-849**	**>850**
1			0.86	0.68	0.64	0.69	2.11	
2	0.24				0.68 (0.37)	0.81 (0.36)	1 (0.05)	0.7
3	0.37 (0.38)	0.33 (0.01)	0.43	0.45 (0.17)	0.54 (0.36)	0.73 (0.21)	0.99 (0.49)	1.28
4	0.34	0.75	0.4	0.63 (0.18)	0.6 (0.45)	0.6 (0.26)	0.79 (0.5)	
5		0.54 (0.13)	0.42	0.49 (0.27)	0.94 (0.63)	0.65 (0.12)	0.8 (0.44)	
6		0.37		0.37	0.54 (0.35)	0.61 (0.02)	0.65 (0.3)	0.41 (0.16)
7			0.55 (0.13)	0.51 (0.07)	0.56 (0.2)	0.6 (0.21)	0.72 (0.31)	0.56
8	0.38			0.6	0.62 (0.14)	0.5 (0.46)	0.28	
9					0.59 (0.32)	0.55 (0.05)	0.78 (0.27)	0.84
10	0.5	0.31	0.49	0.66 (0.6)	0.77 (0.34)	0.53 (0.38)	0.76 (0.08)	0.68 (0.42)
11	0.43			0.9 (0.59)	0.84 (0.6)	0.92 (0.35)	0.46 (0.2)	0.99

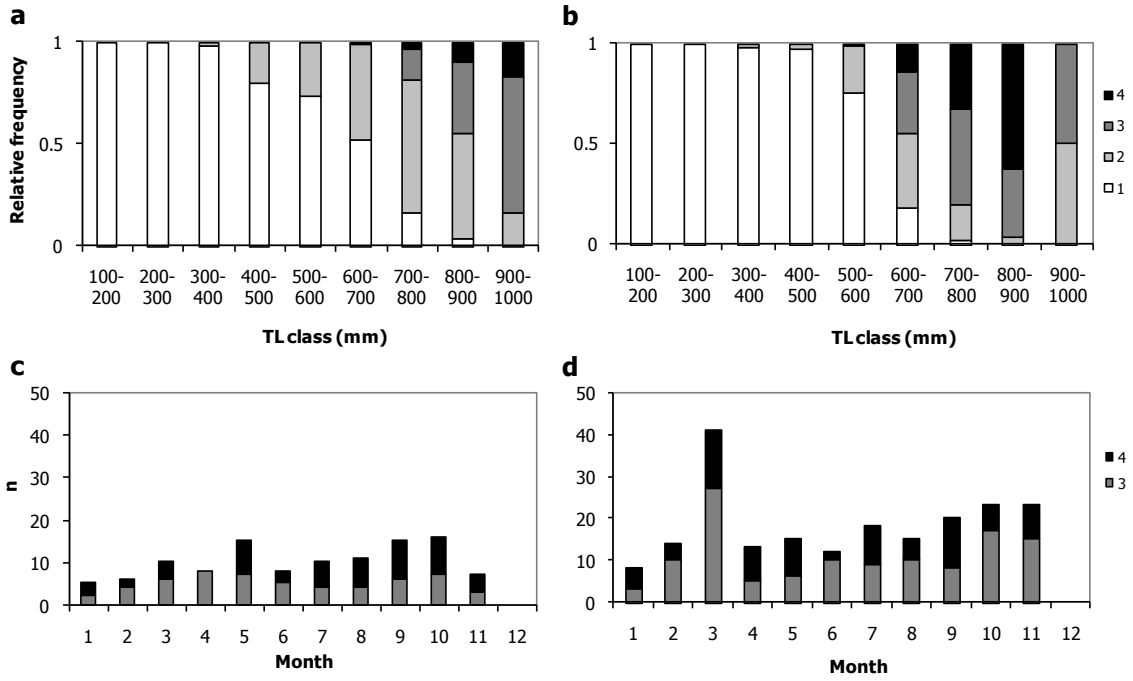

Figure 4.18. Sample composition.
a-b) Relative size frequency distribution of females (a) and males (b) by maturity stage; c-d) Monthly frequency of females (c) and males (d) in the spawning capable and spawning stages. Maturity stages: 1: immature, 2: developing, 3: spawning capable, and 4: spawning.

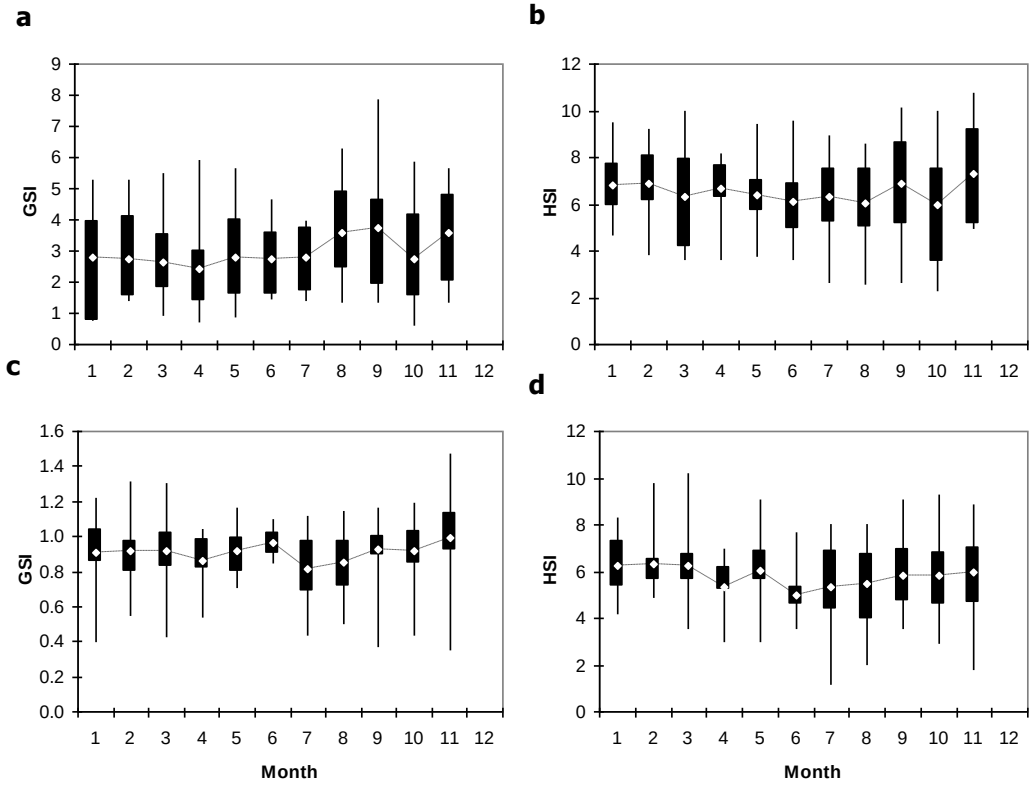

Figure 4.19. Thornback ray indices by month, considering the spawning and spawning capable stages combined.
a) Female GSI; b) female HIS; c) male GSI; and d) male HSI.

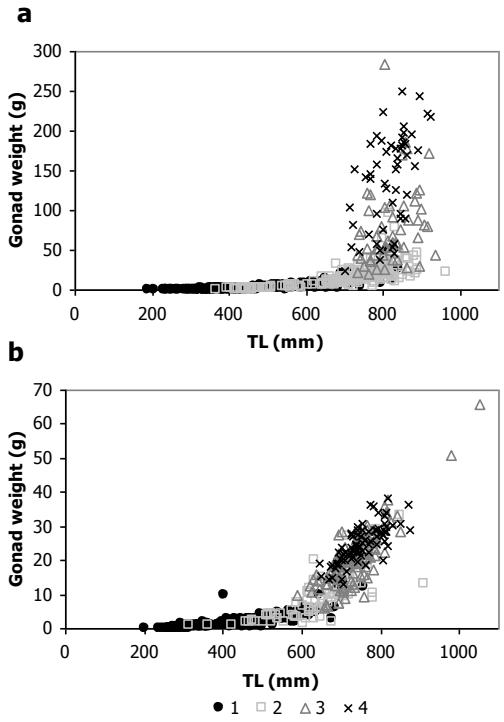

Figure 4.20. Relationship between gonad weight (g) and total length (TL, mm).
a) females, and b) males, by maturity stage (1: immature, 2: developing, 3: spawning capable, 4: spawning)

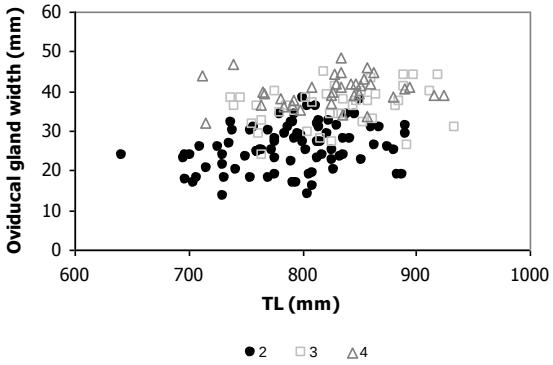

Figure 4.21. Relationship between the oviducal gland width (mm) and total length (TL, mm), by maturity stage.
(2: developing, 3: spawning capable, 4: spawning)

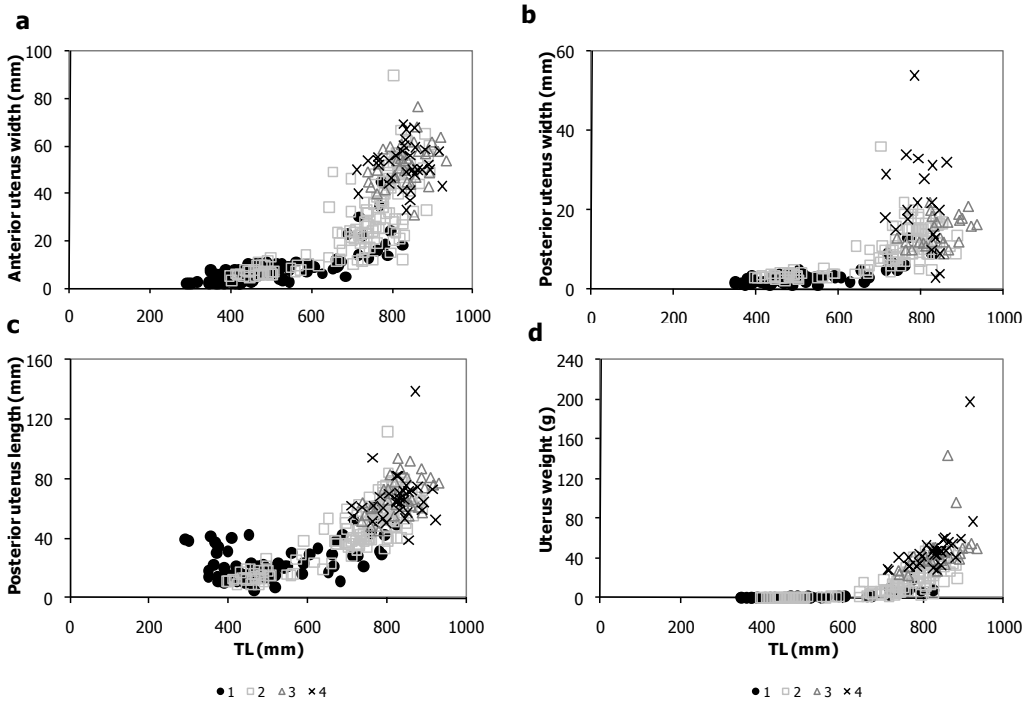

Figure 4.22. Relationship between uteri measurements and total length (TL, mm), by maturity stage. (1: immature, 2: developing, 3: spawning capable and 4: spawning): a) anterior uterus width (mm); b) posterior uterus width (mm); c) posterior uterus length (mm); and d) uterus weight (g).

For large females (TL>800 mm) in the developing stage, with low GSI (GSI<0.78), the widths of the oviducal gland and of uterus were similar to those of spawning females, i.e, above 24 mm and 40 mm respectively. Although previously assigned to the developing stage it was obvious that these females already had spawned once, and were consequently assigned to a new stage, the resting stage. In other hand, females with high GSI (GSI>0.78) that were previously assigned to the developing stage, and showed oviducal gland and uterus widths similar to those of spawning females, were assigned to the regenerating stage, since they already show signs of gonad growth, due to an increase of yolked follicles. A total of 39 females were assigned to these stages and since they had already spawned once in their lives were considered as mature.

In males the rate of gonad development presented a similar pattern to that observed in females (Fig. 4.20b): (i) a slow increase rate in males with TL smaller than 600 mm TL (immature and developing); (ii) a moderate increase rate in males with TL between 600 and 700 TL (mostly developing); and (iii) a fast increase rate in males with TL larger than 700 mm (late developing to spawning). In the case of claspers their length showed a positive relationship with TL (Fig. 4.23a): (i) slow increase rate in males with TL smaller than 400 mm TL (immature); (ii) a fast increase rate in males with TL between 400 and 600 mm

(developing); and (iii) a moderate increase rate in males with TL larger than 600 mm TL (spawning capable and spawning), when claspers become calcified. No significant differences on width between epididimus and vas deferens were observed (t=-0.96, p=0.34, d.f.=191), so the analysis proceeded using only the epididimus. Sperm ducts showed only two-phased growth, both in width (Fig. 4.23b) and weight (Fig. 4.23c): slow growth below 600 mm and fast growth above 600 mm.

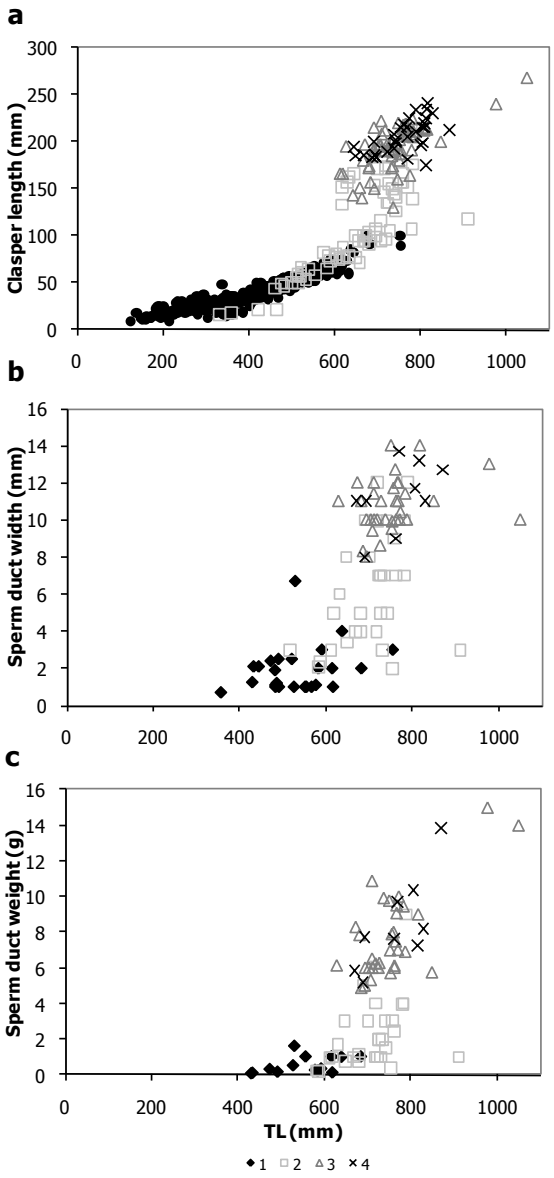

Figure 4.23. Males reproductive structures growth with total length (TL, mm), by maturity stage.
(1: immature, 2: developing, 3: spawning capable and 4: spawning): a) clasper length (mm); b) sperm ducts width (mm); and c) sperm ducts weight (g).

4.2.2.4.2. Maturity

The smallest mature female measured 699 mm TL, while the smallest mature male measured 590 mm TL. The adjusted maturity ogives for each sex are represented in Figure

4.24. The estimated L_{50} was 784 mm for females (Fig. 4.24a) (r^2=0.79; F=500.73, p<0.01; df=859) and 676 mm for males (Fig. 4.24b) (r^2=0.82; F=892.18, p<0.01; df=904). The adoption of resting and regenerating stages for females, morphologically distinguishable from developing stage, allowed a better adjustment of the maturity ogive model, since more large sized females were considered as mature because according to this criterion they had already spawn once.

Based on previous age results (Serra-Pereira *et al.*, 2008) those lengths of first maturity correspond to ages of about 7 years for females and 6 years for males.

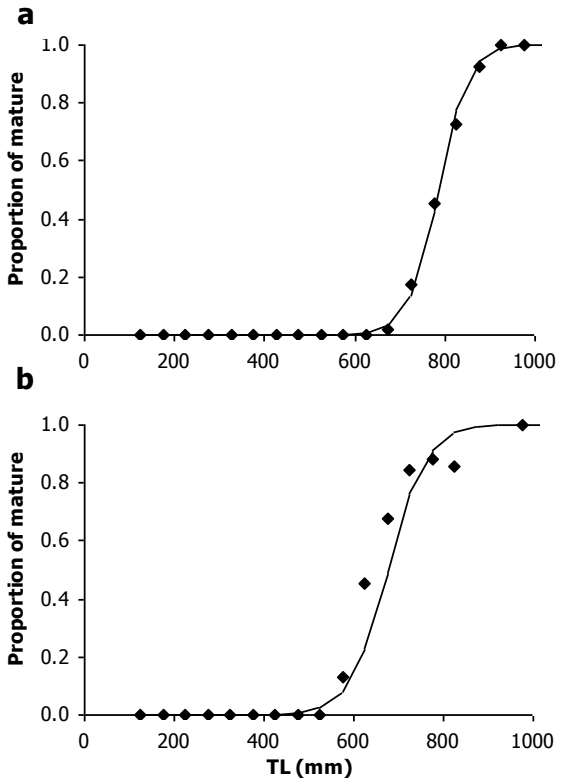

Figure 4.24. Maturity ogives.
a) females, b) males. The solid curve represents the estimated logistic curve and the dots represent the observed proportion of mature.

4.2.2.4.3. Fecundity

There were no significant differences between the number of yolked follicles in the right and left ovaries (χ^2=59.67, p=0.34, d.f.=56). Relative frequency distributions by ovarian follicle diameter classes for developing, spawning capable and spawning stages are presented in Figure 4.25a. In all different stages there was a large number of small follicles (<10 mm). The largest ovarian follicle class of developing females was 14-16 mm while spawning capable and spawning females contained follicles in every diameter classes (from 2-4 to >30). The largest follicle diameter sampled was around 35 mm, which corresponds to the same

diameter of the fertilized egg inside early egg capsules. In spawning capable and spawning females the follicle size frequency distribution was unimodal and skewed to left, with mode around 4-6 mm; the spawning female follicles distribution presented a heavier right tail.

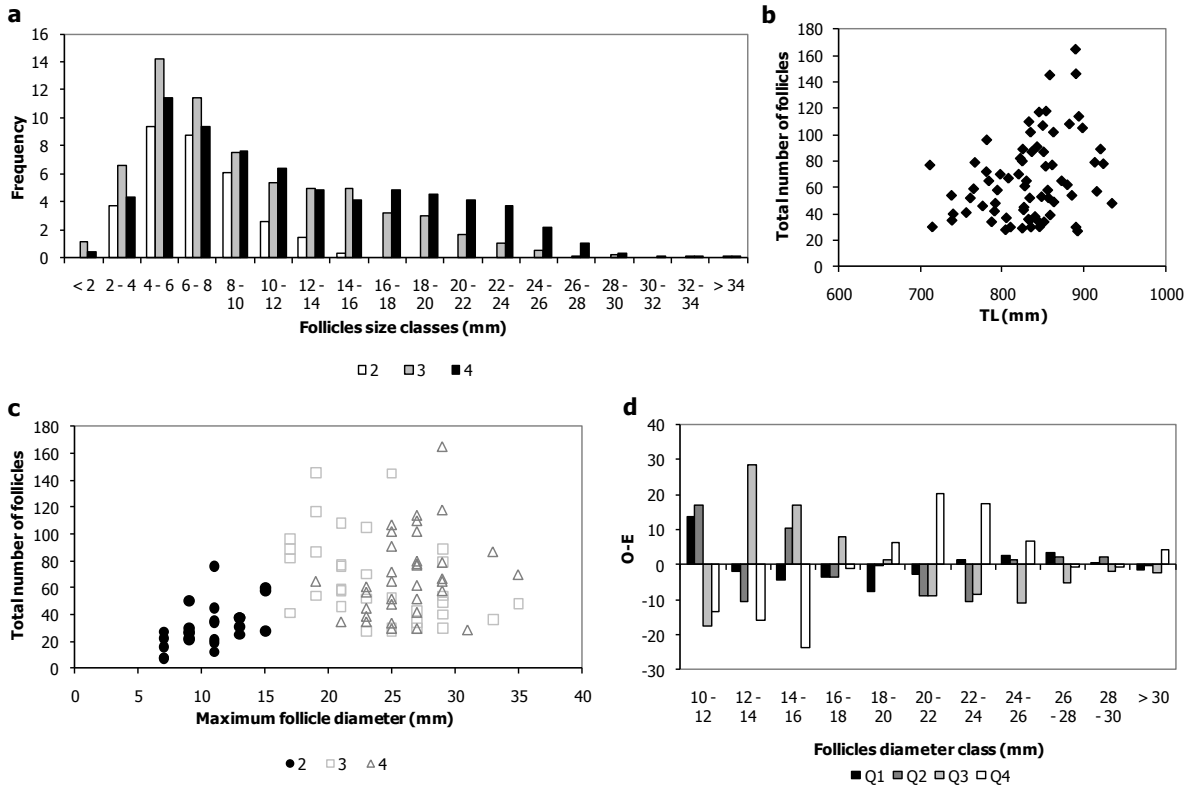

Figure 4.25. Ovarian fecundity.
a) size frequency histogram of the average number of follicles per diameter class (2 mm), by maturity stage; b) relationship between the total number of ovarian follicles in the ovaries and total length (TL), in spawning and spawning capable females; c) relationship between the total number of follicles and the diameter of the largest follicle in the ovary, by maturity stage; d) difference between observed and expected number of follicles (O-E) by 2 mm diameter class, by quarter (Q1 to Q4) (2: developing, 3: spawning capable and 4: spawning).

No significant size dependency on the total number of *batch follicles* was observed (r^2=0.07, n=33, in spawning capable females; r^2=0.18, n=35, in spawning females) (Fig. 4.25b). As a consequence, the proposition of a fecundity model depending on body size seemed inappropriate. Once the L_{50} was attained, the number of *batch follicles* in the ovaries varied between females (4 to 81 follicles); the maximum total number of follicles was observed in a spawning capable female measuring 890 mm TL (165 follicles). In the developing stage, the number of follicles in the ovary was positively related with the maximum follicle diameter (Fig. 4.25c). When the maturity is attained, a relation between those two parameters seemed not to exist. The median *Fbatch* was estimated in 35 eggs per female ($P_{0.25}$=26; $P_{0.75}$=48). The maximum *Ftotal* of thornback ray was estimated in 136 eggs

per female, being *Fmax*=165 eggs and *Fmin*=29 eggs. The total number of four batches was estimated to occur in thornback ray.

The fecundity estimated using the indirect method was 96.1 eggs per females, considering Lmat= 699 mm TL (Table 4.11) and the reproductive period between January and November. Since no spawning females were sampled in April, *Ftotal* was extrapolated for 12 months (~115 eggs per female).

Table 4.11. Fecundity estimates for thornback ray, according to the indirect method. $Fecundity = \sum_{m}[(P_m / P\max)*(N_m * E)]$, where *n* is the total number of adult females (TL > Lmat), P_{cs} is the proportion of spawning capable females, P_m is the proportion of spawning females and F_m is the fecundity per month. Total fecundity corresponds to fecundity (Jan-Nov) extrapolated for 12 months.

Month	n	P_{sc}	P_m	F_m
1	8	0.625	0.375	14.9
2	19	0.316	0.105	3.8
3	44	0.227	0.091	3.6
4	21	0.381	0	0.0
5	26	0.577	0.308	12.2
6	30	0.267	0.1	3.8
7	20	0.5	0.3	11.9
8	19	0.579	0.368	14.6
9	23	0.652	0.391	15.0
10	38	0.421	0.237	9.4
11	22	0.318	0.182	7.0
12	0	0	0	0.0
			Fecundity (Jan-Nov)	96.1
			Total fecundity	115.3

The analysis of the relative frequencies of *batch follicles* by diameter class showed great fluctuations along the different quarters (Fig. 4.25d). Under the null hypothesis, in the first and second quarters the observed frequencies of small follicles (diameter class = [10-12] mm) were larger than the expected ones, but not significant (χ^2=2.17, p≈0.05). At the third quarter the frequency observed of medium-sized follicles (diameter class = [12-18] mm) was significantly larger than the expected (χ^2=7.29, p<0.01), while in the fourth quarter the observed large sized-follicles (diameter class [20-22] mm, χ^2=8.26, p<0.01; [20-22] mm, χ^2=7.52, p<0.01) and largest follicles (diameter > 30 mm) were significantly greater than the expected ones (χ^2=10.42, p<0.01).

One to two egg capsules were commonly observed inside spawning thornback ray females (one in each portion of the anterior uterus). Thornback ray egg capsules were rectangular-shaped, dark brown, covered with a large amount of fibres in both sides and with two thorns

in each edge. In average they measured 72 mm in length and 52 mm in width (Fig. 4.26a). The posterior horns are larger (around 30 mm), than the anterior horns (around 23 mm) (Fig. 4.26a). The egg capsule contents, i.e. the egg jelly plus the egg varied in weight between 11 and 19 g (Fig. 4.26b). Within the 55 spawning females sampled in the present study, three females contained four egg capsules, two in each portion of the anterior uterus. It was assumed to be a malformation, since the two most anterior egg capsules were reduced in size (length around 48 mm), and didn't contained a fertilized egg inside, only egg jelly.

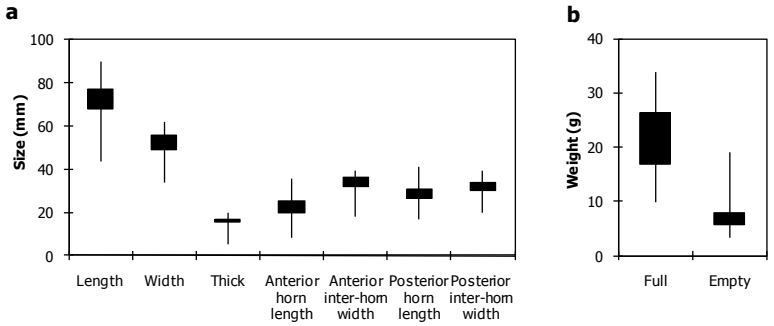

Figure 4.26. Egg capsules measurements.
a) size (mm) and b) weight (g).

4.2.2.5. Discussion

This study provides new information on the reproductive cycle of thornback ray in Portuguese continental waters where it reproduces during the whole year. The delimitation of the different reproductive seasons was based both on the occurrence of the different maturity stages by sex along the months of the year, and on evidences brought by the analysis of the growth of the different reproductive organs of both females and males.

4.2.2.5.1. Reproductive seasonality

In females, the developing stage, i.e. the pre-maturation period is a very long stage since females are mature at about 6 years old (Serra-Pereira *et al.*, 2008). The developing stage comprised females, with 300 to 700 mm total length, and with ovaries containing only pre-vitellogenic follicles with diameter smaller than 4 mm, to females almost reaching maturity, with large vitellogenic follicles, at about 15 mm. Females in the spawning capable and spawning stages have a similar TL (around 700 mm). The overlap in length of females at developing stage and spawning capable or spawning stage leads to admit that the maturation

of the largest eggs, and the transition of females from final developing stage to spawning conditions, is a relatively fast process. This requires a high energy allocation that can be explained by the fast increase in the gonad weight. The high GSI variance observed in developing females with TL below 800 mm, after September, suggests that in this time of the year, females assigned to this stage could be either maturing for the first time or be almost attaining maturity. These results also re-enforces the idea of a long developing stage. The growth increase rates across maturation observed in all the reproductive organs supports the three-stage maturity phases observed in all rajids, and the existence of an extended period to achieve maturation (e.g. Ebert, 2005; Oddone and Vooren, 2005; Frisk and Miller, 2009).

Females and males in the spawning capable and spawning stages were observed throughout the year. Thus in Portugal the spawning is continuous with no distinct spawning season and with mating episodes occurring all year round. In other areas spawning season are also extended; between February and September in UK coastal waters (Holden *et al.*, 1971; Holden, 1975), and between May and December in the SE Black Sea (Demirhan *et al.*, 2005) (Table 4.7). A continuous spawning reproductive strategy seems to be most common among rajid species like the cuckoo ray, *Leucoraja naevus*, in the Celtic Sea (du Buit, 1976), little skate (Richards *et al.*, 1963), thorny skate, *Amblyraja radiata* (Walker, 1999), *Atlantoraja cyclophora* in south Brazil (Oddone and Vooren, 2005). Other rajid species, however, have a distinct reproductive strategy with a well defined and shorter spawning season, like the undulate ray, *Raja undulata* (Coelho and Erzini, 2006; Moura *et al.*, 2007) and clearnose skate, *Raja eglanteria* (Richards *et al.*, 1963).

Although no resting period was detected for the population, the recovery after spawning seems to be triggered at the individual level. Females in a resting and regenerating stage were identified. The high variance observed in the GSI of females with TL>800 mm, is due to the mixture with females that are preparing to spawn and those that will not contribute for the ensuing cycle and that are possibly in the resting or regenerating stage. The resting stage was assigned to those large females originally assigned to developing stage but with low GSI and presenting an oviducal gland and posterior uterus widths similar to spawning females. Whereas the regenerating stage was assigned to those female with the same characteristics but with high GSI, since they have adult characteristics and an increasing gonad weight. A resting period was already described to occur in other skate species: thornback ray cease spawning for 4 months, from October to January in British waters (Holden, 1975); *Atlantoraja cyclophora* females from large TL classes, where vitellogenesis and egg deposition occurred, presented white follicles and presented low GSI values (Oddone and

Vooren, 2005); and starry ray, *Raja asterias* females with small oviducal glands presented POFs (Barone *et al.*, 2007).

In males no regressing stage was observed. Yet, it was detected a male with TL clearly above the L_{50}, 912 mm, presenting characteristics of a developing male. Due to the small size of the claspers (118 mm) and its flexible state, it is possible that this specimen never attained maturity or it has reached a terminal regressing stage and entered on a senescent stage. However in other species, e.g. starry ray (Barone *et al.*, 2007) a resting stage was identified.

4.2.2.5.2. Maturity

In Portuguese waters mature specimens were found with a minimum TL of 699 mm for females and 590 mm for males, and the L_{50} was achieved at 784 mm in females and 676 mm in males. It is common in skates, including the thornback ray, that the male mature with TLs smaller than females (Table 4.7). Prior to Walker (1999) all the maturity estimates were based on the Lmat, i.e., minimum length of a mature female or male. At present study Lmat estimates obtained for females were smaller than the one from Jardas (1973) but larger than the other two (Nottage and Perkins, 1983; Ryland and Ajayi, 1984), although the length ranges were similar. For males the Lmat estimates were smaller than the latter and larger than Jardas' (1973). In both sexes of thornback ray the L_{50} estimates were larger than those available for the species from other studies, but closer to Walker (1999). The fact that in southern European waters, taken the example of the Portuguese coast (ICES area IXa) both Lmat and L_{50} estimates were high, could be a sign of a healthier stock of thornback ray.

Females achieving maturity at >81% of the maximum TL observed is characteristic of k-strategist species (Pianka, 1970). This level is similar to the ones observed for species from the genus *Raja* (e.g. Walker, 1999; Coelho and Erzini, 2006; Barone *et al.*, 2007), *Leucoraja* (e.g. Walker, 1999), *Amblyraja* (e.g. Walker, 1999), *Bathyraja* (e.g. Ebert, 2005; Ruocco *et al.*, 2006) and *Atlantoraja* (e.g. Oddone and Vooren, 2005). Males achieve maturity at >64% of the maximum TL, which is lower than the one presented for other rajid species (e.g. Ebert, 2005). This difference could be due to the larger maximum length observed for males (1050 mm TL) in the present study compared to other areas, like the North Sea (e.g. 820 mm TL; Walker, 1999), Adriatic Sea (e.g. 765 mm TL; Jardas, 1975) or Black Sea (e.g. 950 mm TL; Demirhan *et al.*, 2005). Apart from the regional differences in size composition of the populations, the differences in the maximum length sampled in each area could also be due to fishing pressure or gear selectivity.

4.2.2.5.3. Fecundity

Follicles at different developmental stages were commonly observed in the same ovary, with no dominance of a particular follicles stage, indicating that the follicle development in the thornback ray is asynchronous, as defined by Murua and Saborido-Rey (2003). Furthermore, as in other rajid species, like the white-dotted skate *Bathyraja albomaculata* (Ruocco *et al.*, 2006) and the yellownose skate, *Dipturus chilesis* (Quiroz *et al.*, 2009), there were no statistical differences on the number of ovarian follicles between the two ovaries. This species did not show significant size-dependency relation in the total number of follicles in the ovaries, which could indicate that once a female reach maturity the maximum fecundity could be achieved. Despite the population has a continuous spawning along the year the results obtained, in particular the existence of resting stage females, suggest that individually the species behave as a determinate spawner. In fact the coexistence, in time, of females with the same TL but different fecundities corresponding to the beginning, middle, or end of spawning, indicates different spawning rhythms within the population. This occurrence, different from what is theoretical expected (Shine, 1988), i.e. larger the species higher the fecundity, was already observed in rajids, like the little skate, the winter skate, *Leucoraja ocellata* (Frisk and Miller, 2009), big skate, *Raja binoculata*, longnose skate, *Raja rhina* (Ebert *et al.*, 2008b).

The first method used to estimate fecundity was a direct method based on the number of follicles present in the ovaries of females in pre-spawning stage. The small difference between *Fmin* and *Fbatch* supports the selection of the threshold of follicle larger than 10 mm diameter applied in our estimates and consequently the robustness of the applied method. The estimated number of batches was corroborated by the analysis of the individual follicle frequency distribution in spawning females. When analysing female by female, a consistent number of no more than four batches was observed to be individualized in the ovaries, and females in the spawning stage with large yolked follicles did not contained less than 29 eggs in the ovaries, leading to assume that the remaining follicles could enter in atresia and be reabsorbed by the ovaries. The knowledge on the atretic process in skate species should be more explored in future researches, in order to validate its occurrence. The indirect method was used to compare the estimates between methods and with other studies (e.g. Holden *et al.*, 1971; Holden, 1975). The many assumptions associated with this method, like a single and individualized peak of spawning (represented by *Pmax*), not very adequate to a continuous spawner, a constant egg laying rate of 0.5 eggs per female per day in the peak of spawning and a proportional egg laying rate in the remaining months, make it a less realistic

estimate when applied to the thornback ray, due to its life cycle features. Yet it could serve as an approximate fecundity estimate when do data of the follicle size frequency distribution is available, since it gives an approximate average fecundity per female within the population. Fecundity estimates are a difficult parameter to be found in reproduction studies on skates. This fact could be due to logistic involved in eggs counts, on the difficulty to obtain undamaged ovaries during collection or after extended periods of time following specimens capture and processing, limiting the number of specimens from which accurate counts could be obtained (Ebert *et al.*, 2008b). Comparing to other authors (Table 4.7) our estimate, using the direct method, is close to that from Holden (1975) and Holden *et al.* (1971). However, while in this study a continuous spawning season thought the year was observed, Holden (1975) and Holden *et al.* (1971) limited the spawning season to 8 months, which suggests that although the values obtained were approximate, the egg laying rate seems to be lower than the presented by their study, leading to lower fecundity by month. As advised by Ryland and Ajayi (1984) fecundity was underestimated in their study, due to the inclusion of females that had already spawned.

The smallest frequency of large follicles (diameter larger than 30 mm) and the high frequency of medium-sized follicles (from 12 to 18 mm) observed in the third quarter, along with the GSI observed in those months, point towards a larger spawning effort by the population in the fourth quarter, when the largest follicles were observed. The diameter of larger follicles (around 35 mm) observed before spawning was similar to the one of fertilized eggs inside egg capsules at early stage of embryonic development. This indicates that that maximum yolk size is achieved just prior to ovulation, like was observed in other rajid species, like the little skate and the winter skate, in which the largest 5% of the follicles in the ovaries was larger than the average size inside the eggs (Frisk and Miller, 2009). In some specimens a gap was observed between small-sized follicles and large follicles. In specimens reaching the end of the spawning season, the stock of yolked follicles was much shorter comparing to others with full ovaries. Thus, the number of yolked follicles seems to be predestined to be released during a spawning season, being those above 10 mm the ones that seems to be released per batch. Those facts, together with the large amount of energy that is necessary to produce the offspring on each season, lead us to infer that the fecundity of the thornback ray could be a determinate fecundity (Murua and Saborido-Rey, 2003). By definition, in this type of fish, the number of yolked follicles remaining in the ovary decrease with each spawning event or batch, since the standing stock of yolked follicles is not replaced during the spawning season (Murua and Saborido-Rey, 2003).

The high fecundity estimates obtained for the thornback ray, could lead to suggest a higher resilience to fishing than other skate species with similar maximum length (e.g. Holden *et al.*, 1971; Ryland and Ajayi, 1984). Even so, due to their lower growth rates, longer generation times (Serra-Pereira *et al.*, 2008) and late maturation, and consequent high sensitivity, their sustainable exploration could not be disregarded. Special attention should be given to females, since they mature very late in their lives. The high sensitivity and low resilience of the thornback ray to fishing pressure was already described by Walker and Hislop (1998). A more refined investigation should be made in the future, in order to investigate the occurrence of possible differences within populations. Due to the doubts that persist on the fecundity of this species, further investigations should be performed.

4.2.3. OVIDUCAL GLAND DEVELOPMENT [1]

4.2.3.1. Abstract

The reproductive processes of chondrichthyans are complex. The study of oviducal gland development throughout maturation is vital for the understanding of their reproductive cycle. This study makes the first contribution of this subject regarding skates. In the oviparous thornback ray, *Raja clavata*, the oviducal gland starts to develop early in the developing stage with the formation of the glandular region and folding of the epithelium into four zones: club, papillary, baffle and terminal. The definitive form is achieved at the latest developing stage, when the secretions start to be produced and are stored inside the gland tubules. Histological staining techniques alternative to Haematoxylin and Eosin (e.g. Periodic Acid-Schiff and Alcian blue), revealed that the jellies produced by the club and papillary zones were composed of neutral and sulfated acid mucins. The last row of gland tubules of the papillary zone presented a high concentration of sulfated acid mucins. The baffle zone responsible for the production of the proteic egg envelope layers represented 60 to 80% of the glandular area of the oviducal gland. Sperm were observed in bundles at the deeper recesses of the baffle zone in the maturation process, and were also detected as isolated cells near the lumen during

[1] Serra-Pereira, B., Afonso, F., Farias, I., Joyce, P., Ellis, M., Figueiredo, I. and Serrano-Gordo, L. Submitted. Oviducal gland development in the thornback ray, *Raja clavata*. Helgoland Marine Research.

capsule formation. The terminal zone was composed of mixed gland tubules, serous (producing protein fibres) and mucous glands (producing sulfated acid mucins).

Keywords: maturation; oviparous; Portugal; Rajidae; reproduction.

4.2.3.2. Introduction

The class of chondrichthyan fish include two subclasses, the elasmobranchs (sharks, skates and rays) and the holocephalans (chimaeras). In this class, there is a great diversity of reproductive strategies, oviparity and several types of viviparity, but all share internal fertilization (Musick and Ellis, 2005). Skates are oviparous and release to the sea fertilized eggs enclosed in a hard egg covering, a capsule (Musick and Ellis, 2005). The embryonic development takes place inside the capsule, without using yolk reserves. Depending on the species, the estimated incubation time lasts from 4.5 to 14 months, and for the skate the thornback ray, *Raja clavata*, it is approximately 5 months (Clark, 1922).

Capsule formation is a process that involves an important organ from the reproductive tract of the females, the oviducal gland (OG), which skates share with most chondrichthyans. The OG is an organ derived from the oviduct that develops between the oviduct and the uterus. The important physiological functions that the OG is responsible for are: (i) the production of egg investments; (ii) the formation of the tertiary egg coverings, including the hard egg capsule of oviparous; (iii) the transport of fertilized eggs; and (iv) the possible sperm storage in some species (Hamlett *et al.*, 1998). Each of these functions is, in general, the result of the activity of different zones of the OG.

The OG of most chondrichthyans shares the same internal organization. The oviparous small-spotted catshark, *Scyliorhinus canicula*, was the first to be studied (e.g. Threadgold, 1957; Rusaouën, 1976; Knight *et al.*, 1993) and most of the knowledge on the morphology and functionality of the OG was recorded for this species. In general the OG is made from numerous simple tubular secretory glands. In the tubules, secretions are produced, differently according to the zone, transported through secretory ducts and extruded to the main lumen between lamellae into transverse grooves. The terminology for the zonation of the OG adopted in this work follows the one proposed by Hamlett *et al.* (1998). According to these authors, from the anterior to the posterior area, there are four distinct zones, named in agreement with shape of surface lamellae, as seen in longitudinal section when viewed with

light microscope, or its position in the OG: club, papillary, baffle and terminal zone. Club and papillary zones are referred to the profile of the surface layer when viewed via light microscopy. Club is characterized by club-shaped lamellae, whereas papillary has elongate digit-shaped lamellae. Baffle refers to the baffle plates that form the lips of extrusion of tertiary egg envelopes. The secretory materials are produced in simple tubular gland secretory cells that merge into secretory ducts and that end by a pair of baffle plates, the spinneret region. The extruded secretions end up within the transverse grooves where the fibres are assembled. Plateau projections are situated between adjacent rows of tubules, surrounding the transverse grooves. Terminal refers to its end position and it is characterized by not being organized into lamellae, but consisting of isolated, scattered tubules. The tubular glands are short and simple and are composed of mucous cells, serous cells or a mixture of the two types of cells (Hamlett *et al.*, 1998; Hamlett *et al.*, 2005).

The first step in the formation of the egg covering is the enclosure of the fertilized egg inside an egg jelly layer secreted by the club and papillary zones (Rusaouën, 1976; Hamlett *et al.*, 1998). In skates, the egg jelly leaves the capsule after approximately one third of its development through the slits laterally located at each horn or tendril of the egg capsule, opening the capsule to embryo-assisted flow of sea water (Long and Koob, 1997). The egg jelly serves as a structural device to hydrodynamically support the egg and the developing embryo, and is not the substantial source of carbohydrate nutrition for early development (Koob and Straus, 1998). In addition, it is believed that the egg jelly produced by the papillary zone functions as a lubricant during encapsulation, reducing the friction between the fluid and the forthcoming capsule (Hamlett *et al.*, 1998; Hamlett *et al.*, 2005).

The baffle zone produces and secretes a complex material, a network of fibres that forms the tertiary egg covering. The secreted material consists of a sulphur-containing protein called prokeratine, which is chemically close to keratin (Rusaouën, 1976). In some species of sharks the egg covering also contains collagen (Krishnan, 1959). The egg capsule undergoes a sclerotization by quinone tanning, due to the presence of a phenolic protein (catechol) in the uterus, and an oxidation of the cathecol to quinone by the enzymes, tyrosine hydroxylase and cathecol oxidase (Threadgold, 1957; Koob and Cox, 1990). Egg capsule morphology of oviparous chondrichthyans are species specific and offer an effective protection to the fertilized egg/embryo due to an extreme toughness and strength, flexibility, moderate extensibility and high permeability to low molecular weight substances and ions (Knight and Feng, 1994a, b; Knight *et al.*, 1996).

The process of secretion of the egg envelope components is also very complex. First, the proteins and enzymes involved in the capsule formation are continuously produced and stored in a prepolymerized condition as storage granules which are surrounded by a membrane. These granules are located within the secretory cells of the gland tubules. At the final stage, the membranes of the storage granules coalesce with the membrane of the secretory cell (at the apical surface). The granule contents are then released to the lumen of gland tubules, through a merocrine secretion process. Inside the lumen, the polymerization process, triggered by an ionic change of the environment, transforms the secreted material into coalescent strands (fibrillogenesis). The material is transported to the spinneret, by ciliary action and secreted around the egg jelly surface as parallel oriented fibrils closely packed together. During the stabilization process fibrils suffer a progressive decrease in thickness with water loss, and consequent increase in resistance, improving embryo's protection (Knight *et al.*, 1993; Hamlett *et al.*, 1998).

The terminal zone, the last zone of the OG, is responsible for the formation of surface hairs coated with mucous secretions that cover the exterior of the capsule in some oviparous species [e.g. the holocephalan *Callorhynchus milli* (Smith *et al.*, 2004) and the rajid *R. erinacea* (Hamlett *et al.*, 1998)]. When oviparous egg capsule is extruded to the environment, it becomes covered with e.g. sand and other sea debris, due to the presence of the sticky hairs that serve as anchors and assist to camouflage the egg capsule (Hamlett *et al.*, 2005). The terminal zone is also the site of sperm storage in some chondrichthyans (e.g. Pratt, 1993; Fishelson and Baranes, 1998; Hamlett *et al.*, 1998; Smith *et al.*, 2004; Hamlett *et al.*, 2005; Storrie *et al.*, 2008). The terminal zone also produces and secretes a lubricating mucous to assist passage of the egg (Hamlett *et al.*, 2005).

No previous studies have described the structural development of a skate's OG. Storrie (2004) was the only to describe the structural development of the OG throughout different maturity stages, when studying the OG of the aplacental viviparous gummy shark, *Mustelus antarticus* and Nalini (1940) when studying the oviparous grey bambooshark, *Chiloscyllium griseum*. The main aim of the current study was to describe the processes underlying OG development in an oviparous Rajid, from the beginning of differentiation to the extrusion of the egg capsules. The nature of the secretions produced by the different gland zones at the different maturity stages was identified through special histological staining techniques alternative to Haematoxylin and Eosin. To better understand this physiological process in the reproductive strategy of rajids, the thornback ray, *Raja clavata*, was selected as a model. This

skate is the most abundant species in the NE Atlantic (Walker and Hislop, 1998), and only a few reproductive studies are available.

4.2.3.3. Material and Methods

4.2.3.3.1. Sampling

The thornback ray, *Raja clavata*, has been routinely sampled since 2004 by the Portuguese Fisheries Institute (IPIMAR), under the scope of the National Data Collection Program (PNAB, DCR). From February 2004 to June 2008 female *R. clavata* were collected from: (i) IPIMAR bottom-trawl research surveys carried out along the Portuguese continental shelf, and (ii) landings of commercial artisanal fleets operating with trammel nets, gillnets or longline, along northern (Matosinhos) and central (Peniche) Portugal.

For each specimen the total length (TL) and disc width (DW) were measured (mm). The total weight (TW) (g) was recorded. The specimen was sexed and a maturity stage was assigned by applying the maturity scale proposed by Stehmann (2002) for oviparous elasmobranchs, and adapting the universal reproductive terminology proposed in Brown-Peterson *et al.* (2007) (Table 4.12). The oviducal glands (OG) of each specimen were removed, measured in mm (width, height and thickness; Fig. 4.27a), weighed (0.01 g) and preserved in 10% buffered formaldehyde. The relationship of maturity and morphological characteristics of the OG was analyzed using ANOVA and Tukey's HSD (Honestly Significant Difference) tests.

4.2.3.3.2. Histological procedures

The modifications in the histological structure of the OG and the presence and nature of the secretions produced during the development process were analysed using a selection of one OG per specimen, covering all the maturity stages. Sagittal sections of about 3 mm thick were removed from the middle of the OGs (Fig. 4.27b). The samples were processed using an automated tissue processing machine (Leica TP1020, Germany). The protocol consisted of: (i) dehydration through a series of alcohols from 70% to absolute ethanol; (ii) clearing with xylene; and (iii) impregnation and embedding in paraffin wax. Embedding of the samples in paraffin wax blocks was made using the heated paraffin embedding system (Leica EG 1140H, Germany). The paraffin blocks were then sliced, in sagittal sections, at 3-5 μm of

Table 4.12. Maturity scale for oviparous elasmobranch females adapted from Stehmann (2002) and using the reproductive terminology from Brown-Peterson *et al.* (2007).
The terminology used by Stehmann (2002) is indicated between brackets. Both macroscopic and histological descriptions are presented.

Stages	Macroscopic	Histological
Immature (immature, juvenile)	Ovaries small, whitish and homogeneous; undistinguishable ovarian follicles. Absent oviducal gland and thread-like narrow uterus.	Ovary with primordial (smaller than 0.3 mm) and primary follicles (0.3 to 1 mm) connected to the germinal epithelium and tunica albuginea. Uterus composed mainly by connective tissue, covered by simple columnar epithelium with some invaginations; some blood vessels present.
Developing (maturing, adolescent)	Ovaries enlarged with small follicles in different stages of development, sometimes restricted to the anterior part of the ovary. Yellow follicles present. Oviducal gland in differentiation. Enlarged uterus.	Ovary with primordial, primary, pre-vitellogenic and vitellogenic follicles (smaller than 15 mm). Oviducal gland can show only the beginning of gland tubules formation or be completely formed, with differentiation of the four secreting zones, depending on the advance in maturation. Beginning of secretions production in the oviducal gland and uterus. Uterus more invaginated and vascularized.
Spawning capable (mature, adult)	Large ovaries with large yolked follicles that can reach around 40 mm in diameter. Oviducal gland and uterus fully developed.	Follicles in all stages can be observed in the ovary. Secretions present in the gland tubules of the oviducal gland. Uterus highly invaginated, showing longitudinal folds producing secretions to the lumen.
Spawning (Active/ Advanced/ Extruding)	Ovaries and oviducal gland similar to the spawning capable phase. Large yolked-egg may be present in the oviducal gland. Egg capsule present in the uterus and attached or not to the oviducal gland. Capsules may be starting to be produced or be fully formed, hardened and dark; present in one or both uterus.	Follicles in all stages can be observed in the ovary. Post-ovulatory follicles can be present in the ovary. Oviducal gland tubules full of secretion materials. Secretions also present in the gland lumen. Uterus producing secretions to the lumen.

thickness, using a sliding microtome (Leica SM 2000 R, Germany) and a rotary microtome (Leica RM2125RT, Germany). Different staining techniques were tested to analyse the histological structure of the OG: (i) Hematoxylin and Eosin (H&E), stains the nucleus black and the cytoplasm pink; and (ii) Toluidine blue (TB), a metachromatic dye with a blue nuclear counterstain. Additional staining procedures were used to investigate the chemical nature of the secretions produced by the different glandular zones: (i) Periodic Acid-Schiff (PAS), with and without diastase, to detect neutral mucins (PAS+ structures stained red); (ii) combined Alcian blue and PAS to detect sulfated acid and neutral mucins (PAS/AB) (PAS+ structures stained red, AB+ stained blue and PAS+AB+ stained in different intensities of purple); (iii) Van Gieson stain (VG) to detect collagen, stained in red. Histological staining

protocols used by Bancroft and Gamble (2002) were followed, with some adaptations to improve the results: (i) in PAS and PAS/AB staining techniques, sections were covered with Schiff's solution, for about 8-10 minutes, instead of 15 minutes, since longer times caused background staining; (ii) in PAS/AB staining, after staining with AB, sections were covered with 1% Periodic acid, for 3 minutes, instead of 5 minutes; and (iii) in VG staining, sections were covered in Van Gieson solution for 5 minutes instead of 3 minutes.

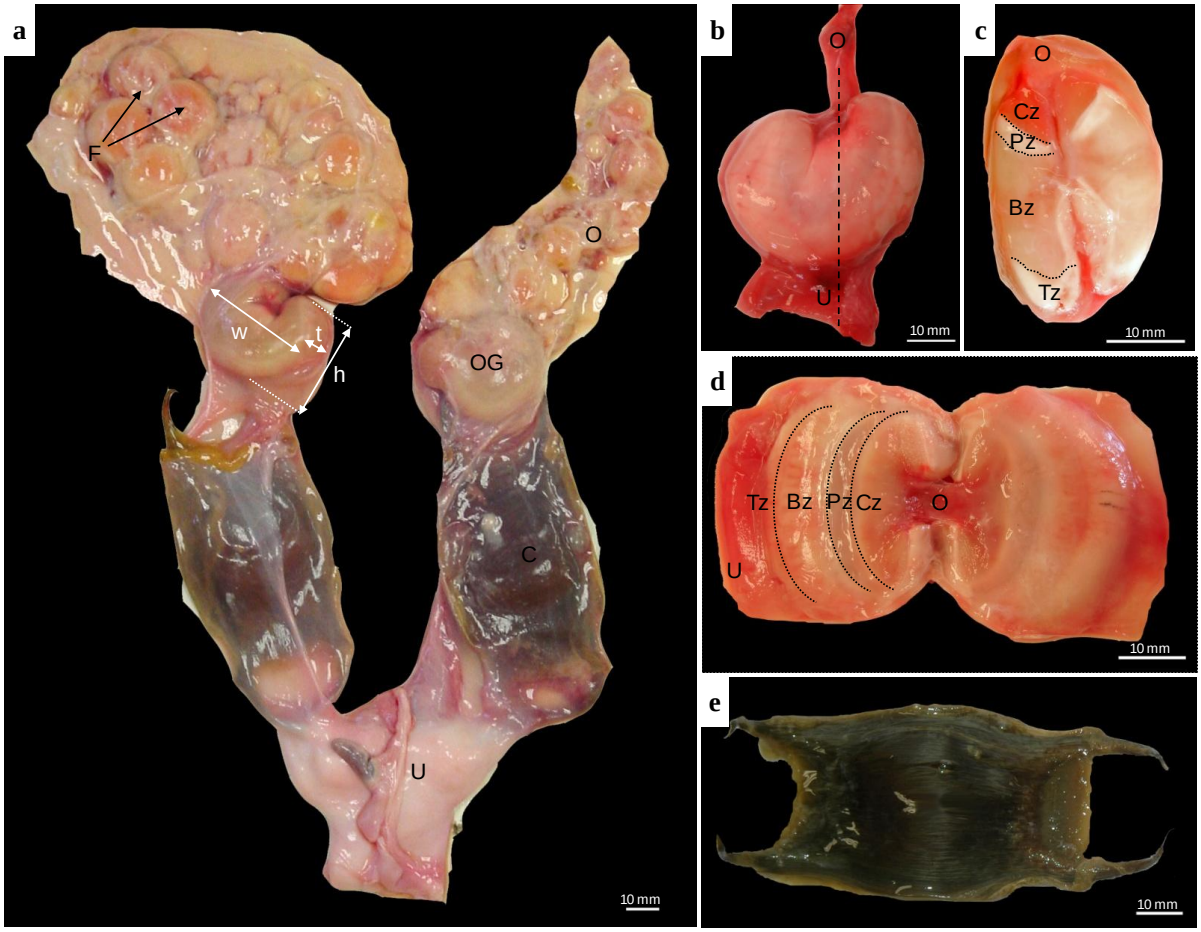

Figure 4.27. Reproductive system of a female *Raja clavata*, with details of the oviducal gland and egg capsule. a) Reproductive tract of a female *Raja clavata* at the spawning stage of maturity. O: ovary; F: follicles; OG: oviducal gland; C: capsule; U: uterus. The measurements made on the OGs are represented: w: width; h: height; t: thickness. b) External anatomy of the oviducal gland (OG) at the spawning stage. In the upper end it communicates with the oviduct (O) and in the low end it communicates with the uterus (U). The dotted line in the center indicates the position of sagittal sectioning. c) Sagittal section of the OG at the spawning stage, showing the different zones from anterior (O: oviduct) to posterior: club zone (Cz); papillary zone (Pz); baffle zone (Bz); terminal zone (Tz). d) Internal view of the two halves of the OG at the spawning stage, displaying the different gland zones, from the oviduct (O) to the uterus (U): club zone (Cz); papillary zone (Pz); baffle zone (Bz); terminal zone (Tz). e) Egg capsule with the dorsal surface up, which is covered with hairs.

The histological slides were observed using a stereo microscope (Olympus SZX9, USA) and an optic microscope (Carl Zeiss Axioplan 2 imaging, Germany). The former was used to

observe the whole sagittal section of the gland and the latter was used to analyse the gland structure in more detail. Images were obtained using a Sony DFW-SX910 camera and the imaging software TNPC 4.1 used with the stereo microscope and a Zeiss AxioCam MRc camera and the imagining software AxioVision 4.1 used with the optic microscope. AxioVision 4.1 was also used to measure several aspects of the OG, such as the diameter and wall thickness (from the lumen to the periphery) of the tubular glands. Other measurements were taken from histological sections of the OGs, using TNPC 4.1, in order to evaluate the growth and the final area occupied by each zone of the OG. The surface length, or distance along the surface of the OG lumen lined with lamellae, for each type of lamellae, and the area occupied by the tubular glands was measured by zone.

4.2.3.4. Results

A total of 142 female *Raja clavata* were used for studying the development of the OG. The reduced number of females with developed oviducal gland (OG) specimens was due to an overrepresentation of immature females in landings. The female reproductive tract in the spawning stage of maturity is represented in Figure 4.27a. At this stage, it was possible to observe the two ovaries filled with large yolked follicles. The OGs, located between the oviduct and the uterus, were completely formed (40 mm width) at this stage (Fig.4.27b). They were composed of two identical bean-shaped halves surrounding a flattened lumen. The succession of the different zones of an OG from the oviduct to the anterior uterus was observed both in sagittal section (Fig. 4.27c) and in the frontal view (internal view), the two halves of the OG separated (Fig. 4.27d). Inside the uterus, two egg capsules were in development, still flexible and without surface fibres. A fully formed capsule of *R. clavata* is shown in Figure 4.27e. The capsule was rectangular in shape, dark brown in coloration with two short horns on each distal extremity and the dorsal and ventral surfaces were covered by a high density of fibres. In most examples these features made the egg capsule opaque.

4.2.3.4.1. Macroscopic development

As female mature, the OG increased in size (Figs. 4.28a-c). In the immature stage or stage 1 the OG was not differentiated, so no measurements were collected. All the morphological characteristics (width, height and thickness) were demonstrated to be statistically different between maturity stages (Table 4.13). No statistical differences were observed within the

spawning stage, if the three stages from Stehmann's (2002) maturity scale were considered (Table 4.13). During the developing stage (stage 2) the gland was visible and the values of the three measurements were the lowest of all maturing stages. The full development, in terms of macroscopic development, was achieved in the spawning stage, when the median was highest for the three dimensions. Some overlap of OG measurement across stages occurred because females of the same TL showed great variation in OG size (Fig. 4.28d).

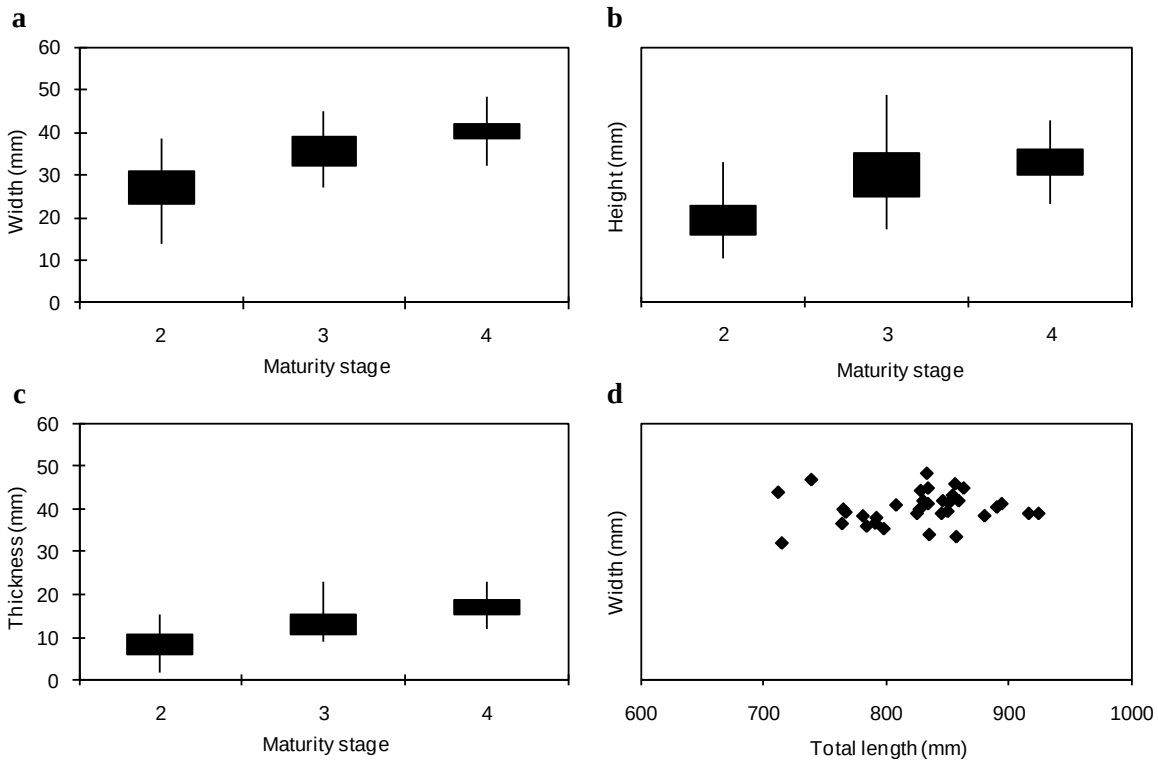

Figure 4.28. Oviducal gland (OG) measurements by maturity stage.
(2: developing; 3: spawning capable; 4: spawning): a) width; b) height; c) thickness. d) Relation between the OG width and the specimen total length.

4.2.3.4.2. Microscopic structure

A total of 70 OGs were selected for histological examination. Glands were removed from females at different stages of development. Figure 4.29 presents the general appearance of OG during development: (i) immature (Fig. 4.29a); (ii) early developing (Fig. 4.29b); (iii) mid developing (Fig. 4.29c); and (iv) spawning capable (Fig. 4.29d).

In an *immature stage*, there was no visible differentiation of the OG from the rest of the reproductive tract (Fig. 4.29a). The duct contained some lamellae and the lumen was lined by simple columnar epithelium cells. In cross section, the reproductive duct measured 24.52±4.73 μm in thickness.

Table 4.13. Statistical results on the effect of maturity on the morphological characteristics of the oviducal gland (width, height and thickness).
a) Maturity stages considering Brown-Peterson *et al.* (2007): 2: developing; 3: spawning capable; 4: spawning; b) Spawning stage subdivided by three stages according to Stehmann (2002): A: active; B: advanced; C: extruding.

	ANOVA		Tukey's HSD test (a)						Tukey's HSD test (b)					
	All		2 vs. 3		2 vs. 4		3 vs. 4		A vs. B		A vs. C		B vs. C	
	n	p-value	n	p-value	n	p-value	p-value	n	n	p-value	n	p-value	n	p-value
Width	138	$<2.2x10^{-16}$	104	0.00	97	0.00	75	0.01	20	0.22	25	0.97	23	0.50
Height	138	$<2.2x10^{-16}$	104	0.00	97	0.00	75	0.00	20	0.60	25	1.00	23	0.64
Thickness	102	$<2.2x10^{-16}$	104	0.00	97	0.00	75	0.00	20	0.87	25	1.00	23	0.92

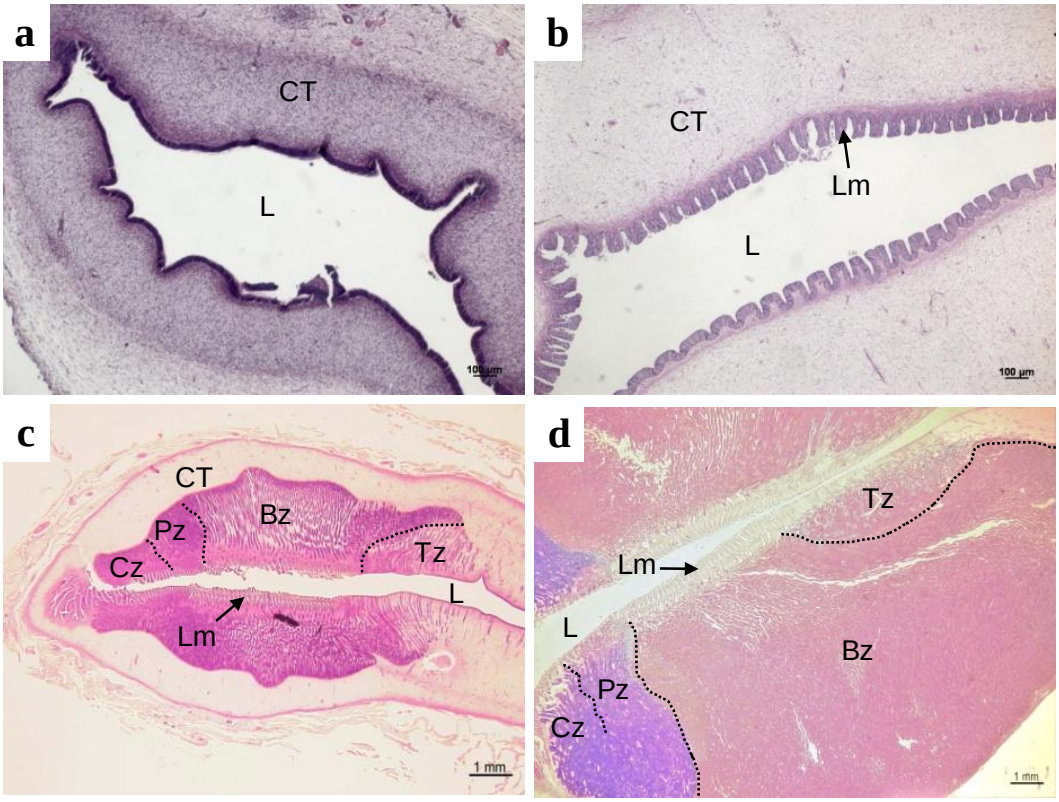

Figure 4.29. Sagittal sections of the oviducal gland (OG) of *Raja clavata*, at the different stages of development.
a) Undifferentiated OG in an immature female. H&E. b) Beginning of the development of the OG in an early developing female. H&E. c) Lamellae, tubular glands formation and differentiation of the four distinct zones in a developing female. H&E. d) Totally developed OG with full difertiation of the four secretory zones in a spawning capable female. PAS/AB. CT: connective tissue; L: lumen; Lm: lamellae; Cz: club zone; Pz: papillary zone; Bz: baffle zone; Tz: terminal zone.

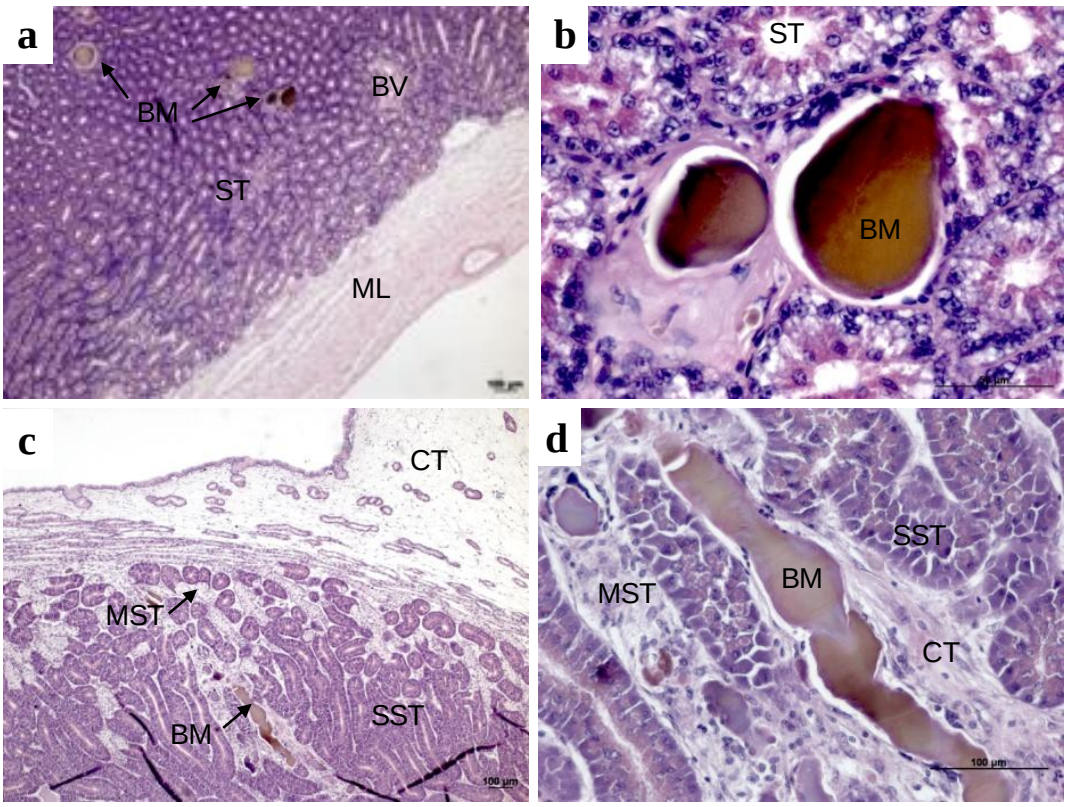

Figure 4.30. Brown material accumulations.
a) Deep recesses of the baffle zone in a developing female. H&E. b) Detail of the brown material observed in the baffle zone in a developing female. H&E. c) Terminal zone in a spawning female. H&E. d) Detail of the brown material observed in the terminal zone in a spawning female. BM: brown material; ST: secretory tubules; BV: blood vessel; ML: muscle layer; CT: connective tissue; MST: mucous secretory tubule; SST: serous secretory tubule.

During the *early developing stage*, OG growth was visible as an expansion of the duct between the oviduct and uterus. Uniform lamellae were observed along the entire section of the OG (Fig. 4.29b). At this stage, the simple and unbranched tubular glands have started to differentiate. The differentiation of the tubular glands in the whole OG began at the lumen, and evolved to eventually occupy the majority of the OG (as seen in Figure 4.29c). The tubular glands were supported by loose connective tissue which was reduced as the tubular glands proliferated throughout the OG. In the developing stage, it was possible to distinguish serous and mucous glands prior to the differentiation of the distinct lamellae of each zone. In cross section, the tubular glands had a diameter of 57.27±11.55 μm and a wall thickness of 20.54±4.89 μm. Near the serous glands, where the baffle zone will be originated, some homogeneous brown material accumulations were sometimes visible (Figs. 4.30a and 4.30b). None of the staining techniques used in this study produced a positive result to identify the brown material. At this stage, the smooth muscular tissue layer surrounding the OG was thin

and with a small number of muscular fibres (496.42±39.61μm). Staining with VG produced negative results, indicating that the OG did not contain collagen.

In the *late developing stage*, the OG was fully differentiated into four zones (Fig. 4.29d): club, papillary, baffle, and terminal. The zones were identified according to the shape of the lamellae lining the lumen (first column in Figure 4.31) and by the distinct secretory tubules (general appearance in the second column in Figure 4.31 with cellular detail in the third column in Figure 4.31). The tubular glands have become large (74.89±7.11 μm) and their walls thick (23.39±4.58 μm). The club zone had club shaped lamellae (Fig. 4.31a). The surface epithelium of the lamellae, similar in the four zones, was composed of ciliated cells and secretory cells. Similarly, the tubular glands (Fig. 4.31b) also contained two types of cells: (i) sustentacular ciliated cells with elongated apical nuclei; and (ii) secretory cells with, large, globular, basal nuclei (Fig. 4.31c). The papillary zone had digit shaped lamellae (Fig. 4.31d). Most of the tubular glands were very similar to the ones in the club zone, with the exception of the caudal-most papillary tubules, adjacent to the baffle zone, that were more vacuolated (Fig. 4.31e). As in the club zone, the epithelium of the tubules contained ciliated cells and secretory cells (Fig. 4.31f). The baffle zone had two types of epithelial projections: a pair of small folds in the spinneret region, the baffle plates, surrounded by a pair of large folds, the plateau projections (Fig. 4.31g). A blood vessel was observed within each plateau projection. The number of transverse grooves ranged from 25 to 30, in each half of the gland. The epithelium of the serous tubular glands was composed of secretory cells and ciliated cells (Figs. 4.31h and 4.31i). The cytoplasm of the secretory cells was packed with numerous secretory granules that measured 0.94±0.28 μm in diameter (Fig. 4.31i). The terminal zone (Fig. 4.31j) consisted of elongated tubular glands and a regular surface epithelium with unequal spacing of the secretory duct openings. Two types of tubular glands were identified, those composed of serous secretory cells (similar to those in the baffle zone) containing secretory granules and those composed of mucous secretory cells (frothy, vacuolated cells) (Fig. 4.31k). Both types of tubular glands had an epithelium lining the lumen, composed of ciliated cells and secretory cells (Fig. 4.31l). The first secretions produced in the four zones of the OG stained lightly, and then increased in intensity when maturity approached the spawning capable stage. The glandular activity of the papillary zone was PAS+ and AB+ (Figs. 4.32a and 4.32b). The terminal zone began its activity also in the late developing stage, staining AB+ (Figs. 4.32c and 4.32d).

In the *spawning capable stage***,** the secretory tubules had thicker walls as a consequent of more secretions stored inside their cells. The granules of the secretory cells of the club zone

stained PAS+ and AB+ (Figs. 4.33a and 4.33b). The secretory tubules of the papillary zone produced three distinct types of mucins as identified by their differential staining (Fig. 4.33c): a) the majority of the tubules were PAS+ and AB+ (Fig. 4.33d); b) the region near the lumen was intensely PAS+ (Fig. 4.33e), or PAS+ and AB+ in some specimens; and c) the most-caudal row of secretory tubules near the baffle zone were AB+ (Fig. 4.33f). The baffle zone did not react to any of the special staining techniques tested in the present work (Fig. 4.33g). In some spawning capable females, the secretory tubules deep in the baffle zone (near the muscle tissue) contained almost no secretory materials stored inside. In the terminal zone, mucous glands were AB+ and serous glands were PAS- and AB- (Figs. 4.33h and 4.33i). Secretions were also detected in the lumen of the gland tubules in: (i) the papillary zone (Fig. 4.34a); (ii) the baffle zone, both in the tubular glands (Fig. 4.34b) and secretory ducts (Fig. 4.34c); and (iii) the terminal zone (Fig. 4.34d).

At *spawning stage*, the tubular glands attained the largest sizes in diameter 94.03±14.02 μm and wall thickness 43.43±8.85 μm. The secretory granules inside the serous gland tubules of the baffle and terminal zones measured 1.22±0.25 μm. The secretory tubule lumens, closest to the lumen of the whole OG, were filled with secretions in all four zones. In spawning females, brown material accumulations were also observed, in the baffle and terminal zones (Figs. 4.30c and 4.30d).

4.2.3.4.3. Presence of sperm

Sperm were observed inside the OG of 13 females, from the developing to the extruding stage. Since only two to four sections per specimen were observed for most females, this does not imply the absence of sperm in the remaining females. From the developing to the extruding stage, sperm were observed as laterally aligned bundles in the deep recesses of the baffle zone tubules, adjacent to the muscle tissue, mainly located in the anterior portion of the OG (Figs. 4.35a and 4.35b). These tubules containing sperm were composed of both secretory and sustentacular ciliated cells. In the extruding stage, non-aggregated individual sperm were also observed inside the gland tubules near the spinneret in the baffle zone (Figs. 4.35c and 4.35d). Non-aggregated individual sperm was observed in the terminal zone tubules, in one specimen.

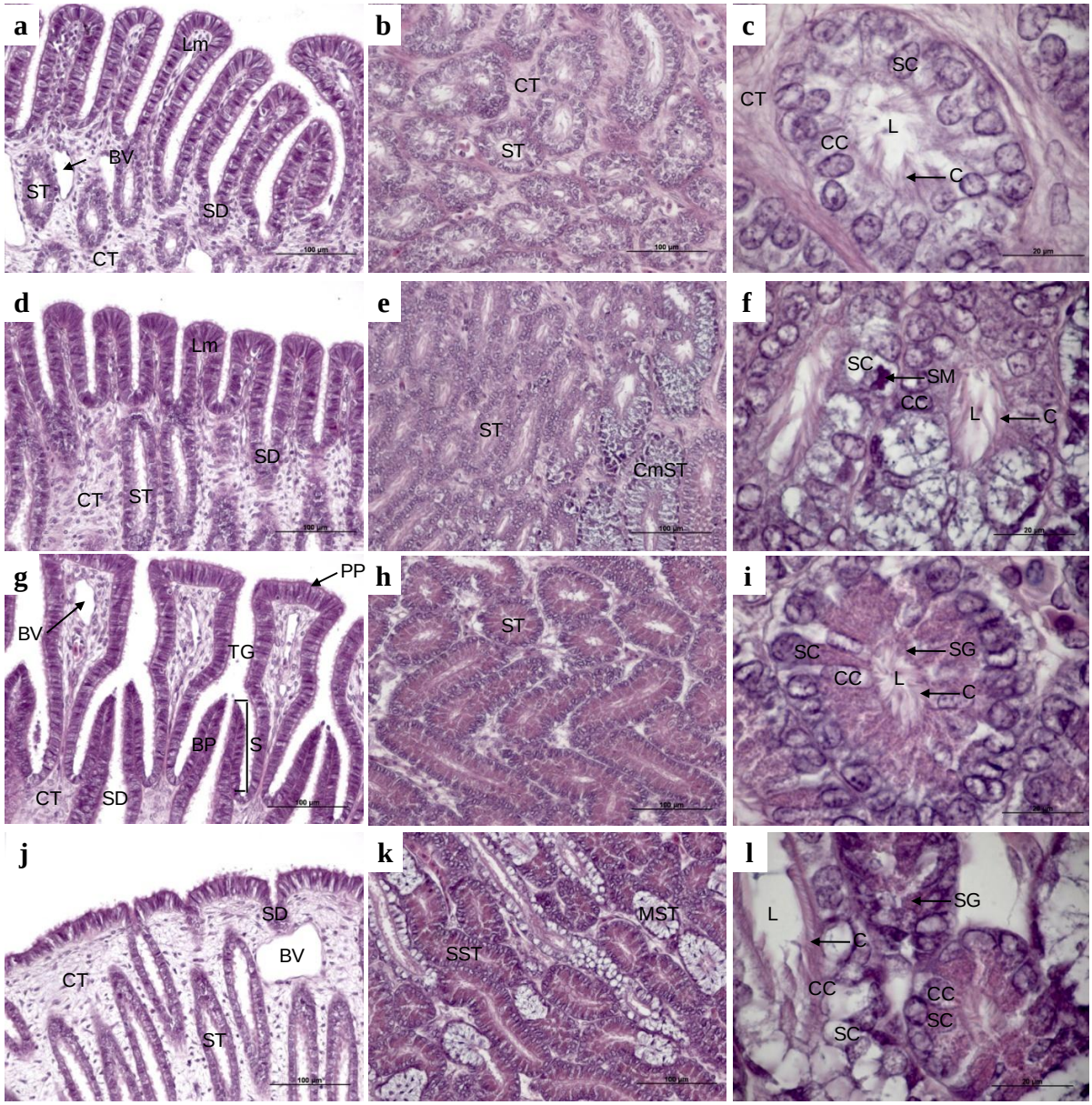

Figure 4.31. Differentiated zones of the oviducal gland in the late developing stage.
H&E. a) Club zone lamellae. b) Club zone secretory tubules. c) Detail of a secretory tubule, in the club zone, with cilated and secretory cells. d) Papillary zone lamellae. e) Papillary zone secretory tubules. The caudal-most papillary tubules adjacent to the baffle zone are distinct from the remaining tubules. f) Detail of a secretory tubule, in the papillary zone, with ciliated cells, and secretory cells. Secretory material stored inside secretory cells. g) Baffle zone lamellae. The secretory ducts open into the lumen through the spinneret region, composed by two baffle plates. The baffle plates are surrounded by another pair of large folds, the plateau projections, lining the transverse groove. h) Baffle serous secretory tubules. i) Detail of a secretory tubule, in the baffle zone, with ciliated cells and secretory cells. Secretory cells contain secretory granules. j) Structural organization of the terminal zone displaying the regular surface epithelium and the elongated tubular glands opening into the lumen. k) Terminal zone secretory tubules, with both mucous and serous secretory cells. l) Detail of the two types of secretory tubules, in the terminal zone, mucous and serous. Both tubules present ciliated cells and secretory cells. Secretory cells of serous tubules present secretory granules. Lm: lamellae; BV: blood vessel; ST: secretory tubule; SD: secretory duct; CT: connective tissue; SC: secretory cell; CC: ciliated cell; L: Lumen; C: cilia; CmST: caudal-most secretory tubules on papillary zone; SM: secretory material; PP: plateau projection; TG: transverse groove; BP: basal plate; S: spinneret; SG: secretory granules; SST: serous secretory tubules; MST: mucous secretory tubule.

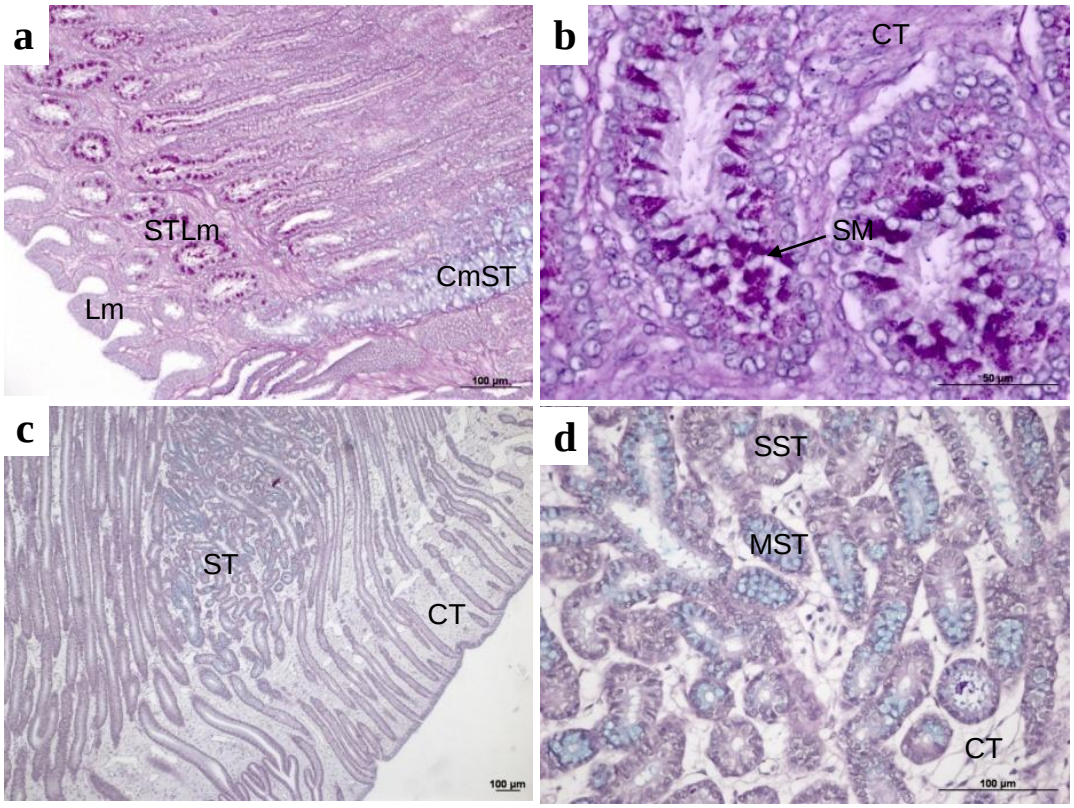

Figure 4.32. Secretory material first produced in the oviducal gland of a developing female.
PAS/AB. a) Papillary zone with AB+ secretory material produced by the most-caudal row of tubules and PAS+ secretory material produced by the tubules near the lamellae. b) Detail of secretory tubule near the lamellae of the papillary zone with PAS+ secretory material stored inside secretory cells. c) Terminal zone with AB+ secretory products inside mucous glands. d) Detail of the mucous secretory tubules producing AB+ secretory material. Lm: lamellae; STLm: secretory tubules near the lamellae in papillary zone; CmST: caudal-most secretory tubules in papillary zone; ST: secretory tubule; CT: connective tissue; SM: secretory material; SST: serous secretory tubules; MST: mucous secretory tubule.

4.2.3.4.4. Histological measurements

The surface length and glandular area of each zone of the OG increased in size with maturity (Fig. 4.36). The maximum surface length occurred earlier than the associated glandular area. The surface length of the club zone and papillary zones each represented about 12% of the total extension of all lamellae (Fig. 4.36a). The maximum surface length of the baffle zone was found very early in maturation, and represented about 35% of the total surface length. Similarly, the terminal zone average 37% of the total surface length. When analysing the glandular growth of the different maturity stages (Fig. 4.36b), the baffle zone was most often the largest, occupying 60 to 80% of the total glandular area. The club and papillary zones together represented 10 to 20%. They were analysed together, because of the difficulty in distinguishing the limits between them. The terminal zone was the only glandular zone that decreased in relative size with maturation, from 12-17% in the developing stage to 5-7% in the extruding stage.

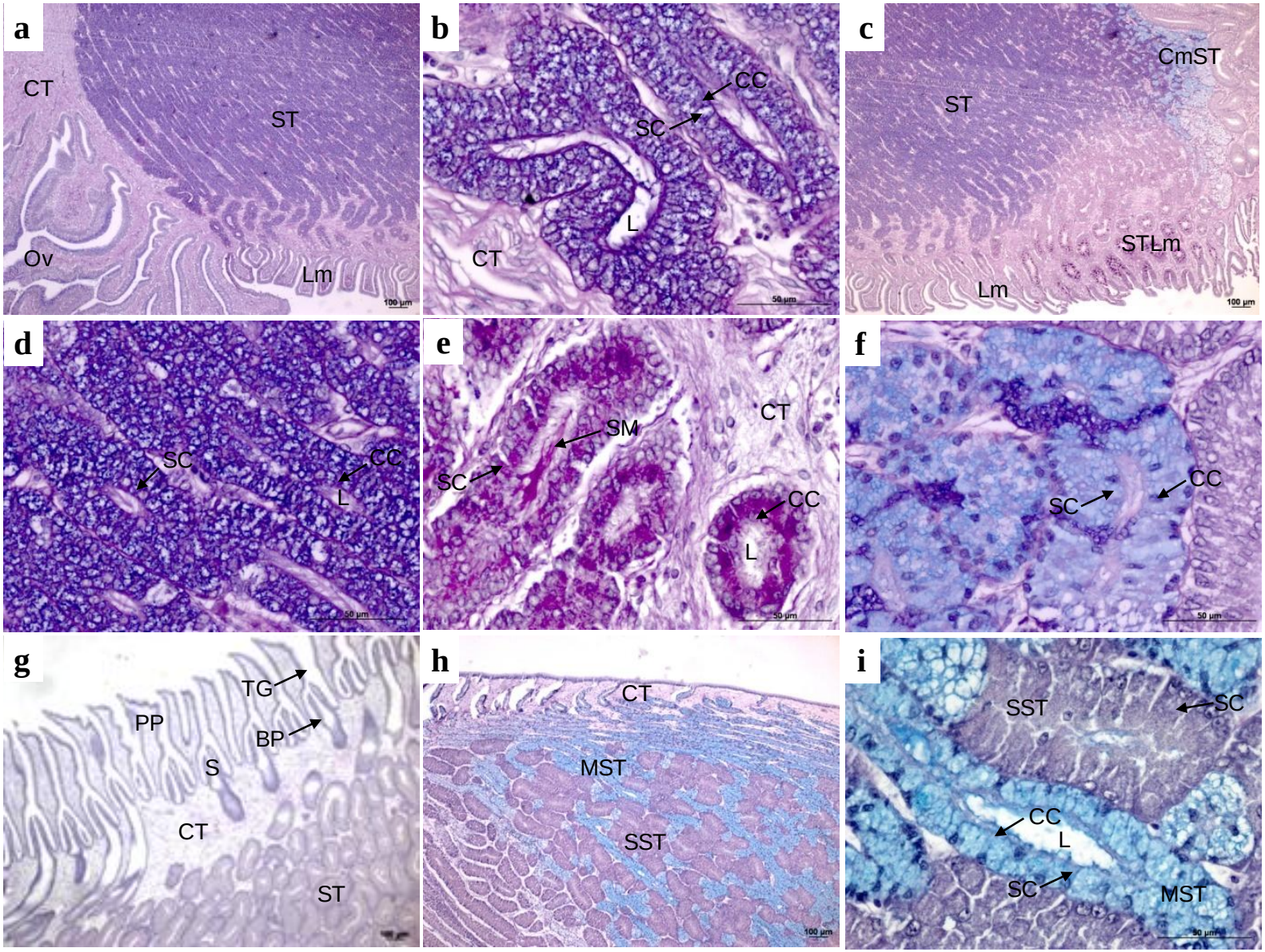
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Figure 4.33. Secretory material produced by the secretory tubules from the four zones of the oviducal gland of a spawning capable/spawning female. (previous page)
PAS/AB. a) Club zone with PAS+ and AB+ secretory material stored inside the tubules. b) Secretory tubules of the club zone with PAS+ and AB+ secretory material stored inside the secretory cells. c) Structural organization of the different regions of the papillary zone. d) Main secretory tubules of the papillary zone, containing PAS+ and AB+ secretory material. e) Papillary secretory tubules near the lamellae containing PAS+ secretory material. f) Caudal-most papillary tubules containing AB+ secretory material. g) Baffle zone secretory tubules PAS- and AB-. h) Terminal zone mucous secretory tubules containing AB+ secretory material and serous secretory tubules PAS- and AB-. i) Serous secretory tubules containing AB+ secretory material stored inside secretory cells. CT: connective tissue; ST: secretory tubules; Lm: lamellae; Ov: oviduct; SC: secretory cell; CC: ciliated cell; L: Lumen; STLm: secretory tubules near the lamellae on papillary zone; CmST: caudal-most secretory tubules on papillary zone; SM: secretory material; PP: plateau projection; TG: transverse groove; BP: basal plate; S: spinneret; SST: serous secretory tubules; MST: mucous secretory tubule.

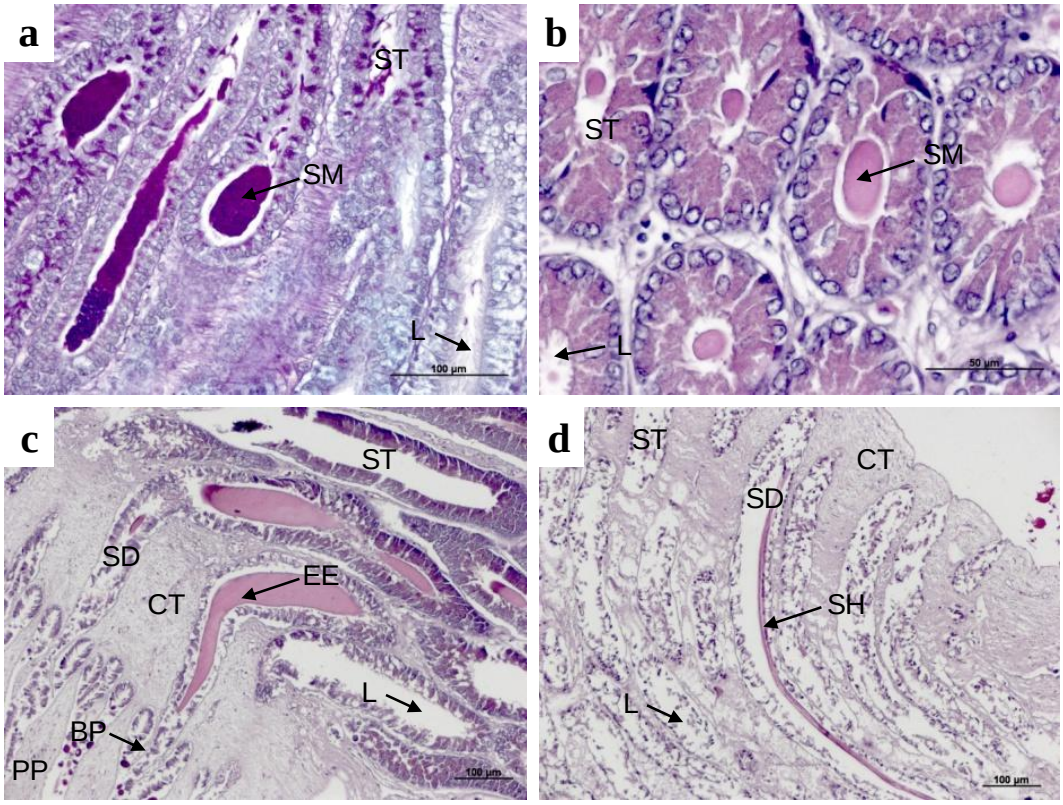

Figure 4.34. Secretory material accumulated in the tubules lumen of spawning capable females.
a) Papillary zone containing PAS+ secretory material. PAS/AB. b) Baffle zone tubular glands containing egg envelope material. H&E. c) Baffle zone secretory ducts secreting the egg envelop material to the gland lumen. H&E. d) Terminal zone secretory ducts secreting the surface hairs to the gland lumen. H&E. ST: secretory tubule; SM: secretory material; L: Lumen; SD: secretory duct; CT: connective tissue; EE: egg envelope; PP: plateau projection; BP: basal plate. SH: surface hair.

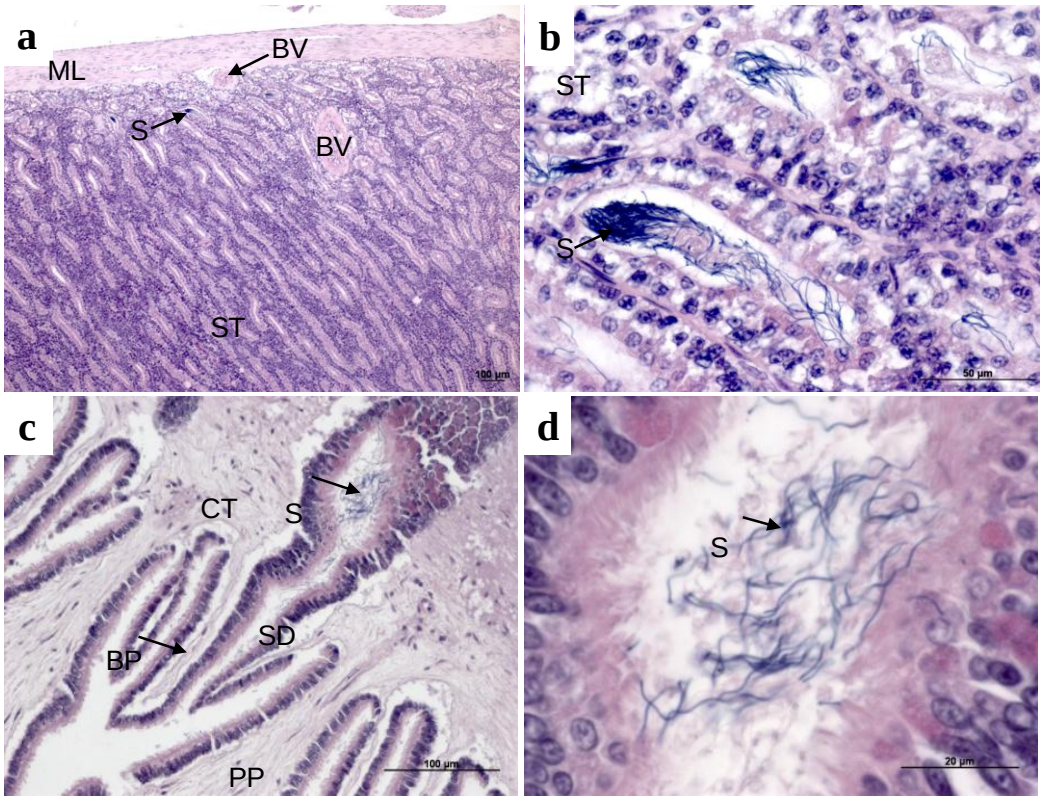

Figure 4.35. Sperm observed inside the oviducal gland.
H&E. a) Sperm bundles inside the tubules of the baffle zone deep in the oviducal gland, in the late developing stage. b) Detail of the tubules containing sperm in the deep recesses of the baffle zone in the late developing stage. c) Non-aggregated individual sperm inside the secretory ducts of the baffle zone in spawning capable females. D) Detail of the secretory duct containing sperm, in the spawning capable stage. ML: muscle layer; BV: blood vessel; S: sperm; ST: secretory tubules; L: lumen; CT: connective tissue; SD: secretory duct; BP: basal plate; PP: plateau projection.

4.2.3.5. Discussion

The main morphology and functionality features of the oviducal gland (OG) are virtually identical between all chondrichthyans. Only numbfishes, a family (Narcinidae) of electric rays from the order Torpediniformes, lack this organ (Prasad, 1945). Oviparous species have the largest OGs in chondrichthyans (Hamlett *et al.*, 2005). The OG of *Raja clavata* is one of the biggest and is similar in size to the OG from the grey bambooshark, *Chiloscyllium griseum* (50 mm height and 38 mm width; Nalini, 1940), and the catshark, *Scyliorhinus canicula* (35 mm height and 20 mm width; Knight *et al.*, 1996). Within the same family, the OG is virtually similar, although small histological differences could be observed. Oviparous species have species specific egg capsules (i.e. different shape and size). In the species that have egg capsules with surface hairs (e.g. *R. clavata*, *R. erinacea*, and *Raja eglanteria*), the terminal zone possesses both mucous and serous glands, while the latter are absent from

those species with smooth egg capsules [e.g. *S. canicula* (Knight *et al.*, 1996; Hamlett *et al.*, 2005)].

This study is the first to describe the OG development throughout the reproductive cycle of skates. In developing *R. clavata* females, mucins were first visualized in the club, papillary and terminal zones. Therefore, it could be concluded that this stage is when the secretory cells of the gland tubules start to produce secretion materials. In the spawning capable stage, the secretory tubules enlarge as a result of the increase in size and number of the secretory granules stored inside the secretory cells. Later, the secretory material is released into the tubular glands lumen, and is transported through the secretory ducts by ciliary action of the ciliated cells in the epithelium of the tubules. The secretory material is transformed from secretory granules into a coalescent strand within the tubular lumen, and is visible in every zone of the *R. clavata* OG. When the coalescent strands pass through the spinneret regions of the baffle zone, they become ribbon like in form, and are integrated in the network of packed fibrils that will compose the future egg envelope (Knight *et al.*, 1993).

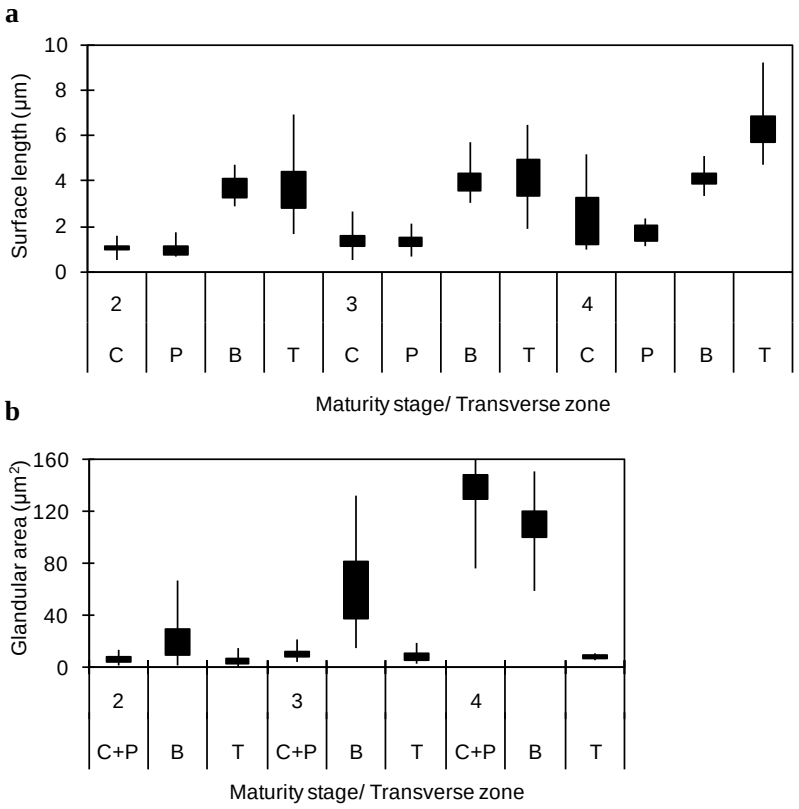

Figure 4.36. Measurements of the different zones.
(C: club; P: papillary; C+P: club and papillary; B: baffle; T: terminal) of the oviducal gland by maturity stage (2: developing; 3: spawning capable; 4: spawning). a) Surface fold length (µm). b) Glandular area (µm^2). Boxes limit the percentiles 25 and 75. Bars stands for the upper and lower value observed.

The chemical nature of the secretions produced by the different gland zones was investigated using special histological staining protocols/techniques. The secretions of the club, papillary and terminal zones all contained mucins. In spawning capable females, the tubular glands from the club and papillary zones were filled with neutral and sulfated acid mucins, which stained positively with PAS and AB. In the papillary zone, three distinct areas were identified: (i) one area showed similar staining as the club zone, PAS+ and AB+, and included the majority of the papillary gland tubules; (ii) another area close to the lamellae contained many neutral mucins that stained intensely with PAS+, and (iii) the third area was located close to the junction with the baffle zone and produced sulfated acid mucins that markedly stained with AB+. Most of the jelly produced by the papillary zone seemed to have a final major input of neutral mucins by the second type of gland tubules, which could influence the final viscosity of the secreted product. In fact, according to Koob and Straus (1998) the jellies produced by the club and papillary zones showed different viscosities, the latter being more viscous than the layer of jelly closest to the egg. Its differential viscosity result from a different chemical composition, with higher concentrations of galactosamine, glucosamine, galactose and fucose, and consequent higher viscosity inside the horns (egg jelly produced by the papillary zone), and lower concentration and lower viscosity near the egg (egg jelly produced by the club zone). Future studies need to focus on where and in what quantities each of these four carbohydrates are produced in the club and papillary zones, to fully understand the dynamic of the oviducal gland. In the papillary zone, the secretory material produced in the last row of tubules showed a different chemical composition (only sulphated acid mucins) from the material produced in the remaining tubules. This feature was already described to occur in other chondrichthyan species, in both oviparous, like *C. griseum* (Nalini, 1940) and the elephant fish, *Callorhynchus milli* (Smith *et al.*, 2004), and viviparous, like the gummy shark, *Mustelus antarticus* (Storrie, 2004). It is believed that the thin layer of egg jelly, produced by the caudal-most papillary tubules, function as a bonding layer and lubricant between the egg investments secreted by the club and papillary zones, and the egg envelop secreted by the baffle zone (Nalini, 1940).

The larger number of transverse grooves in the baffle zone provides for a thicker egg covering (Knight *et al.*, 1993; Hamlett *et al.*, 1998). In general, oviparous species, like *R. clavata* (25 to 30 transverse grooves), *C. griseum* (74 transverse grooves; Nalini, 1940) and *S. canicula* (20 transverse grooves; Hamlett *et al.*, 2005) have more transverse grooves than viviparous species. In the aplacental viviparous spiny dogfish, *Squalus acanthias*, the baffle zone has considerably fewer transverse grooves, and consequently the egg covering is

reduced to a flexible egg candle that surrounds the embryos and disappears prior to parturition. In the extreme case of aplacental viviparous, like the yellow spotted stingray, *Urolophus jamaicensis*, the baffle zone is completely absent and no egg covering is produced (Hamlett *et al.*, 1998). Besides the differences in structure chondrichthyan OGs may also produce secretory material with different chemical compositions. The secretory material produced by the baffle zone in *R. clavata* was not identified as one of the mucins tested in the present study. The observation of secretory cells with the cytoplasm packed with numerous spherical granules about 1.22 μm in diameter, which is characteristic of cells specializing in protein secretion (Knight *et al.*, 1993; Junqueira and Carneiro, 1995), suggests that the secretions produced in the baffle zone were proteins, as described for other species (Knight *et al.*, 1993; Koob and Cox, 1993). Studies on the OG of *R. erinacea* showed that the six major proteins, present in the granules, all contain high levels of glycine, serine, proline and tyrosine (Koob and Cox, 1993). The application of Van Gieson stain showed further that the granules were not composed of collagen. This constituent was also absent in *R. erinacea* but present in the egg covering of oviparous sharks, such as *S. canicula* (Knight *et al.*, 1993) and *Chiloscyllium griseum* (Krishnan, 1959).

The chemical nature of the homogeneous brown material observed in the baffle and terminal zones could not be identified. No positive results were obtained for each of tested staining techniques. Despite being observed in a very early stage of the gland development, the texture and colour of these accumulations are similar to the tanned egg covering (Threadgold, 1957; Koob and Cox, 1990, 1993). Storrie (2004) observed in the gummy shark, *Mustelus antarticus*, egg envelop material being secreted from the baffle zone. Although pink in color, the appearance resembled the brown material secretions in this study, and may lead one to infer that both could have the same origin.

Sperm storage in female chondrichthyans, particularly sharks and holocephalans, has been largely confirmed by histological analysis [e.g. in the Oman shark *Iago omanensis* (Fishelson and Baranes, 1998); in the elephant fish *Callorhinchus milli* (Smith *et al.*, 2004); in the gummy shark *Mustelus antarticus* (Storrie *et al*., 2008)]. Sperm storage occurs inside specialized tubules (sperm storage tubules, SST) located in the terminal zone. The secretory cells present in the SST produced secretions that contributed to the support, nourishment, and maintenance of the sperm during the storage period (Hamlett *et al.*, 2002a; Hamlett *et al.*, 2002b; Storrie, 2004; Storrie *et al.*, 2008). The tubules also contained ciliated cells, which are responsible for the transport of sperm into and out of the tubules.

In the case of Rajids, sperm has been histologically detected as nonnagregated, individual sperm in the baffle zone near the gland lumen, and rarely in the deeper regions of the gland of *R. erinacea* (Hamlett *et al.*, 1998). Sperm storage has been experimentally inferred through female specimens of the blonde ray, *Raja brachyura* (Clark, 1922) and of *R. clavata* (Ellis and Shackley, 1995) kept in captivity. In the two experiments, females were isolated from males and were able to lay fertilized eggs after 5 (*R. brachyura*) and 13 (*R. clavata*) weeks of isolation.

In the present study on the OG of *R. clavata*, non-aggregated, individual sperm were occasionally found in the tubules of the baffle and terminal zones near the lamellae, and more frequently, sperm bundles were observed in the tubules at the deep recesses of the baffle zone. The presence of sperm in the baffle zone of developing females indicates that mating occurs before maturity is reached, which was also reported in other chondrichthyans [e.g. *M. antarticus* (Storrie, 2004; Storrie *et al.*, 2008)]. The baffle tubules containing sperm bundles, which are characteristic of SSTs (Hamlett *et al.*, 1998; Hamlett *et al.*, 2002a; Hamlett *et al.*, 2002b; Storrie, 2004; Hamlett *et al.*, 2005; Storrie *et al.*, 2008), have both secretory and ciliated cells. In *R. clavata*, the secretory function may only be related to egg envelop secretion rather than to sperm maintenance because the tubules resemble the remaining secretory baffle tubules, and were not surrounded by highly vascularised connective tissue, a main SST characteristics presented in previous studies (e.g. Hamlett *et al.*, 2005; Storrie *et al.*, 2008). Thus, the sperm observed could be the result of a recent mating episode and not from sperm that was stored for an extended period of time. Hence, further studies should be conducted in order to widen the observations made in the present study on *R. clavata* and to other Rajid species that occur in Portuguese waters, in order to investigate histologically the sperm storage facts reported in previous studies that were based on pregnant captive females (Clark, 1922; Ellis and Shackley, 1995).

In *R. clavata*, the terminal zone seems only to be responsible for the formation of hair filaments on the surface of the capsule, and is not involved with sperm storage. As observed, this zone is composed of mixed gland tubules (two different types of gland tubules) that contain both mucous and serous glands. First the mucous section of the tubules produce secretions, then as the emerging hair filaments, produced by the serous glands, pass through the mucoid region they gain a coat of sticky material that will serve the purpose of attaching debris for camouflage (Hamlett *et al.*, 2005). In aplacental species, the terminal zone is only composed of mucous glands and SSTs (Hamlett *et al.*, 1998). As described for other chondrichthyans (e.g. Smith *et al.*, 2004; Hamlett *et al.*, 2005; Storrie *et al.*, 2008), the

terminal zone is not organized into lamellae. Instead it consists of isolated, scattered tubules, which, in the case of species that have egg capsules with surface hairs it determines the positioning of those hairs. In the case of the *R. clavata* that produces an egg capsule full of surface hairs, the terminal zone is much extended in terms of its surface.

In conclusion, the formation of the egg capsule in *R. clavata* is a continuous process involving the oviducal gland. Capsule formation starts at the developing stage, when the OG becomes completely formed and all four zones begin to produce and secrete the relevant materials. There are no great variations in the size of the tubular glands within the spawning stage, if the three stages included in the maturity stage from Stehmann (2002) are considered. In fact, all three stages are related to the production of the capsule which is a very fast process (not more than one day for *R. clavata*) (Ellis and Shackley, 1995). The sperm, observed inside the OG from developing to extruding females, did not seem to be stored. Some questions remain to be answered about the chemical nature of the secretions produced by the OG, and need future investigation. Given the complexity and variability of the reproductive strategies among chondrichthyans, it would be important to expand this histological investigation to other species in order to better understand their life cycles.

Chapter 5

General discussion and conclusions

5. GENERAL DISCUSSION AND CONCLUSIONS

Global trends of overfishing and biodiversity loss coupled with an increasing interest in skate harvesting and low resilience of these taxa have raised concerns around this group. The present thesis contributed to the increase in knowledge about skates in Portuguese waters, especially on the biology of thornback ray *Raja clavata*, filling the gap of information about these fish for the southern region of the NE Atlantic. This research has produced important new findings on three major issues, successfully answering the questions posed in the beginning of this thesis (see Chapter 1).

The first issue addressed concerned the characterization of skate's fishery (Chapter 2).

(i) How and how much are skates species landed in Portuguese landing ports?

In Portugal, the proportion of skates landed by the artisanal fleet (75%) is higher than that by the otter trawl fleet (25%), contrary to what is known for northern Europe (e.g. Dulvy *et al.*, 2000). Since the launch of the sampling program in 2001 to identify species composition of Portuguese landings (Machado *et al.*, 2004), the landing ports of Matosinhos (in the north) and Peniche (in the centre) continue to be the most important in terms of skate landings, with average catches around 133 tonnes and 362 tonnes, respectively. Great progress was made on the identification of the main characteristics of the fleet catching skates with this thesis. In the landing port of Peniche, the artisanal fleet was characterized by small size vessels with an average size of 12 m (5 to 23 m) operating near the coastline. Five types of fishing gears were identified to catch skates: trammel net with meshes <200 mm, trammel net with meshes >200 mm, gillnets, longlines and pots. Usually, fishing trips last one day, and depending on vessel characteristics one or more actively fishing gears are operated simultaneously.

Portuguese total annual landings for all skates combined has been stable, around 1600 tonnes for the last two decades. While the total income from skate landings has increased from 2.4 to 4.2 million Euros (average of 2.3 Euro per kilo), which represents a 1.5 fold increase. These trends are similar to what has been described for other areas, such as the North Sea (Walker and Heessen, 1996), United Kingdom (Dulvy *et al.*, 2000; Rogers and Ellis, 2000) and Mediterranean (Garofalo *et al.*, 2003). Species specific landings were extrapolated from the data collected under the National Sampling Program. Between 2003 and 2008, the blonde ray (*Raja brachyura*) was the most abundant species, with landings between 500 and 800 tonnes. The thornback ray (*Raja clavata*) was the second most abundant species, with landings from 300 to 600 tonnes. The third most common species was

the undulate ray (*Raja undulata*), with landings between 100 and 300 tonnes. The remaining species, the cuckoo ray (*Leucoraja naevus*), spotted ray (*Raja montagui*) and small-eyed ray (*Raja microocellata*), were landed below 150 tonnes.

(ii) Is it possible to discriminate fishing strategies within the fisheries that are catching skates?

The difficulty to estimate fishing effort by skate species led to the development of a procedure that identifies fishing strategies (FS) in the artisanal fisheries responsible for skate landing. This information could be used in future studies as a sampling unit, instead of an estimated combined fishing effort for all skates (e.g. number of trips with skate landings). Cluster analysis was successful in identifying those FSs, and six groups were defined based on the information collected in landing ports, including fisherman feedback, and further characterized according to an association of target species and skates species. One FS was identified to target skates, using frequently large mesh trammel nets to catch large skates, mainly blonde ray. Another FS operated solely with longline, and their largest landings were of European conger and large sized skates, mostly blonde ray. Two multi-gear fisheries were identified with trips using all gear types: one FS, possibly including multi-day trips, showed large landings of cuckoo ray and spotted ray and also anglerfish, common octopus and John Dory; whereas, the other FS showed the largest landings of thornback ray. The remaining identified FSs operated with trammel nets and pots, differing on the skate landings composition: one FS operated near the coast, containing the largest landings of undulate ray and small-eyed ray; and the other landed mainly large blonde ray.

(iii) Do identification problems still persist after the application of the EU legislation regarding skate landings discrimination by species?

After the implementation of the legislation that ensured that skates should not to be landed as aggregated catches (EC No 43/2009, 2009), the species categories that were created (cuckoo ray, blonde ray and thornback ray), continue to show a mixture of species, representing a misclassification of: 4% in cuckoo ray, 46% in blonde ray and 100% in thornback ray. In the species category named “blonde ray” a mixture of coastal species was identified, whereas in the “thornback ray” category was used for offshore species. Nine species were observed to be commonly landed in the Peniche landing port under those three species categorie: (i) cuckoo ray *Leucoraja naevus*, brown ray *Raja miraletus* and spotted ray *Raja montagui* in the cuckoo ray category; (ii) blonde ray *Raja brachyura*, thornback ray

Raja clavata, undulate ray *Raja undulata*, small-eyed ray *Raja microocellata*, spotted ray and cuckoo ray in the blonde raycategory; and (iii) Longnosed skate *Dipturus oxyrinchus* and bottlenosed skate *Rostroraja alba* in the thornback ray category.

There is more uncertainty in the assessment of skates than for most other commercial fish species. The fact that severe problems are still occurring in discriminating skate species from landings even after the imposition of the new legislation (EC No 43/2009, 2009), is one of the main impasses for an accurate assessment. Due to this uncertainty, the assessment of species specific landings continues to be made through extrapolation of the data collected under the National Sampling Program. Based on the morphological differences between the main species landed in Portugal, it is thought that even the fishermen are aware of their biodiversity and know to some extent how to differentiate them. However, disparity of commercial value between some of the species, perhaps due to the flesh consistency or even their external appearance, could be one major social barrier that could be blocking the implementation of this new rule (species are landed with their ventral side up, which prevent their prior identification by the buyers). This thesis results underline the importance of promoting awareness initiatives in landing ports that stress the importance of this new legislation (EC No 43/2009, 2009) and enforces collection of species-specific information. Only this, will allow for a more accurate landings database, and consequently give a more accurate insight on stock levels for the different major species landed. This type of initiatives already proved to be successful in the United Kingdom where a collaborative approach to assess and manage these species was implemented through an association established between fishermen, processors, scientists and conservationists, named Skate and Ray Producers' Association (SRPA) (SEAFISH, 2010).

In addition to a general characterization of skate biodiversity from Portuguese waters, this thesis provides new evidences for the use of complementary tools (through molecular markers and body morphometry) for species discrimination, in order to answer the identification problems verified in landings (Chapter 3).

(iv) How diverse are the Portuguese waters in terms of skate species?

This thesis contributed to consolidate and update the information available on the biodiversity of skates occurring in Portuguese waters (Machado *et al.*, 2004; Coelho *et al.*, 2005; Figueiredo *et al.*, 2007; Baeta *et al.*, 2010). As mentioned earlier, nine species were

identified in landings, and three additional species are also found to occur in Portugal: sandy skate *Leucoraja circularis*, Iberian pigmy skate *Neoraja iberica* and Madeiran ray *Raja maderensis*. The last two species are endemic, the former is described to occur in the southern mainland region, and the latter is only found in the insular regions of Madeira and Azores. Due to its geographical location, other species, are also caught exclusively in the Azores, and they were consequently not analysed in the present thesis: pale ray *Bathyraja pallida,* Richardson's ray *Bathyraja richardsoni*, common skate *Dipturus batis*, Shagreen skate *Leucoraja fullonica*, Deepwater ray *Rajella bathyphila* and Bigelow's ray *Rajella bigelowi* (ICES, 2009).

In this thesis the mitochondrial gene cytochrome c oxidase subunit I (COI) was used for the first time to identify those 12 Northeast Atlantic skates. *Raja clavata* was the species showing the highest diversity indices for this gene in agreement with studies using other molecular markers, such as nuclear microsatellite loci and mitochondrial cytochrome b sequences (Chevolot *et al.*, 2006) and mitochondrial DNA control region (Valsecchi *et al.*, 2005).

The diversity of skates was also analysed in terms of size conversion factors. DW:TL, DL:TL, CL:TL and DL:DW were obtained for *L. naevus*, *R. brachyura*, *R. clavata*, *R. montagui*, *R. miraletus*, and *R. undulata,* most of them published here for the first time. The majority of these size conversion factors were variable between areas and sexes, yet no sexual dimorphism was observed on the allometric relationship between weight and total length.

(v) Are molecular markers and body morphometry adequate to discriminate between skate species?

Molecular markers and body morphometry proved to be successful in species discrimination and their use is recommended when problems persist in distinguishing between very similar species, especially juveniles.

The genetic divergence between pairs of species was generally low (Hebert *et al.*, 2003) and within the same magnitude of those observed for other elasmobranchs (e.g. Moura *et al.*, 2008; Smith *et al.*, 2008; Ward *et al.*, 2008), and above than the 2% value of intra-specific divergences considered optimal for species discrimination (Hebert *et al.*, 2003). The only exception was verified between *R. clavata* and *R. maderensis* (1.3% genetic divergence), i.e. the results casts doubt on the recognition of *R. maderensis* as a distinct species. The insufficient data available about *R. maderensis* (Stehmann and Bürkel, 1984; McEachran and

Dunn, 1998) and the high levels of intra-specific genetic diversity (Chapter 3.1; Valsecchi *et al.*, 2005; Chevolot *et al.*, 2006) and morphologic variation (Chapter 3.2; Stehmann and Bürkel, 1984) of *R. clavata* lead to conclude that the most probable hypothesis is that *R. maderensis* is another morphotype of *R. clavata*.

In terms of body morphometry the misclassification between species was low (13%). *Leucoraja naevus* showed the most distinctive shape (narrower disc in relation to body length, and disc longer than wide) and was fully discriminated from the remaining species. In species where recurrent misidentification cases occur (*R. brachyura* and *R. montagui*) this tool proved to be very successful.

(vi) How diverse are the feeding habits of skates in Portuguese waters and what is their relationship with prey diversity?

R. clavata, *R. brachyura*, *L. naevus* and *R. montagui* have generalized diets, feeding on benthic prey that live from shallow waters to depths of 500 to 700 m, which could be an advantage to their adaptation to changes in the environment. Their most frequent prey-*taxa* were decapods (crabs and shrimps) and fish. No significant differences were found in the diet between sexes. Inter and intraspecific differences seem to be related to size and morphological characteristics of the species, as well as type of dentition (Du Buit, 1978). *R. brachyura* and *L. naevus* have cusped teeth and feed mostly on fish, while *R. clavata* and *R. montagui* have molariform teeth and feed on crustaceans. These differences allow them to exploit a larger diversity of habitats, avoiding intraspecific competition, namely between small and large specimens, as well as interspecific competition.

An ontogenetic dietary shift was evident in all species at around 45-50 cm total length class, associated to a close direct relationship between predator's size and mouth dimensions (e.g. size, shape and mechanisms of mouth and teeth), swimming capacity and visual accuracy, already described for other geographical areas (Steven, 1930; Holden and Tucker, 1974; Ajayi, 1982; Daan *et al.*, 1993). The observed ontogenetic shifts were characteristically from small (e.g. mysids, isopods, amphipods and polychaetes) to larger and faster prey (e.g. cephalopods, fish and crabs), from benthic to semi-pelagic feeding habits, from shallow to offshore waters and from crustaceans to fish.

As shown in the previous two chapters the thornback *R. clavata* is an important species in Portuguese waters both in terms of commercial importance, since it is an important component of the catch in demersal mixed-fisheries (Chapter 2), and in terms of biodiversity

(Chapter 3), being the most diverse species both in terms of genetic pool, morphometry or feeding habits. As hypothesized, it was crucial to increase the knowledge on the biology of this species, in the southern area of the Northeast Atlantic where information was completely unavailable, since there were found large differences to the northern stocks data. This thesis contributes greatly in this area (Chapter 4), especially on issues regarding growth and reproduction.

(vii) How different is the life strategy (growth and reproduction) of the species in Portuguese waters in relation to other areas around Europe?

Based on edge analysis, an annual deposition of growth bands was verified to occur in thornback ray, which is corroborated by previous findings using tetracycline in mark recapture methods to validate the growth of thornback ray in British waters (Holden and Vince, 1973). Von Bertalanffy growth model was considered more appropriate than Gompertz, on the basis of similarity between the estimated L_∞ and the observed maximum size. No significant differences in growth parameters were observed between sexes. Off mainland Portugal, the thornback ray seems to attain a larger L_∞ (L_∞ = 1280 mm) and follow a slower k (k = 0.117 year^{-1}) compared to the parameters estimated for northern European populations, such as the North Sea (Walker, 1999) and around the British Isles (Taylor and Holden, 1964; Holden, 1972; Brander, 1981; Ryland and Ajayi, 1984; Fahy, 1989; Whittamore and McCarthy, 2005). The maximum age observed for *R. clavata* was 10 years, for a female of length 835 mm.

In terms of reproduction, in Portugal thornback ray is a continuous spawner, with no clear spawning or mating seasons, with both spawning females (699-934 mm TL) and males (590-1050 mm TL) found throughout the year. Although with an apparent resting period, an extended spawning season for this species was also observed in British waters (between February and September; Holden *et al.*, 1971; Holden, 1975) and in the SE Black Sea (between May and December; Demirhan *et al.*, 2005). In both sexes of thornback ray length-at-first-maturity estimates were larger than those available for this species from other areas, like the Adriatic Sea (Jardas, 1973) and British waters (Nottage and Perkins, 1983; Ryland and Ajayi, 1984), but closer to that estimated for the North Sea (Walker, 1999).

(viii) How adequate are dermal denticles as ageing structures? Are the resultant age readings more accurate than those obtained with vertebrae?

Results on the use of dermal denticles to age thornback ray are presented for the first time in ths thesis. Six types of dermal denticles were identified in the tail of the thornback ray, with small thorns the most suitable for age determination owing to their fixed position, persistence throughout their lifespan, and defined growth-band pattern. When compared, caudal thorns were more accurate than vertebral centra for age determination, i.e. age estimates were more reproducible, and therefore the use of caudal thorns is proposed for future age and growth studies focusing on thornback ray. The most effective processing technique for dermal denticles was the immersion in trypsin solution in a water bath at 50ºC for 20 min. The best procedure for reading the growth bands was by observation under transmitted light. Other advantages exist on the use of such structures for ageing skates, since they can be more easily obtained at fish markets or in the field with minimal damage to the fish, so it is also possible to apply known-age and marked-fish validation methods, through removal of caudal thorns and marking of each fish, before releasing them alive.

(ix) Can we apply the reproductive terminology used for teleosts to describe the different reproductive phases of elasmobranch oviparous species, for example the thornback ray?

Due to the need to unify the assignment of maturation phases in data collection programs (until the present day, at least ten terminologies were applied to elasmobranch species; e.g. Richards *et al.*, 1963; Walmsley-Hart *et al.*, 1999; Stehmann, 2002; Ebert, 2005; Frisk and Miller, 2009) a standardize terminology already adopted for teleosts was applied successfully to skates: immature, developing, spawning capable, spawning, regressing and regenerating. Although this terminology was tested only with thornback ray, it is believed that no problems will occur when extended to other rajid species or even to other oviparous elasmobranchs. This terminology is particular useful since accurate reproductive information is the basis for the assessment of the population status of exploited species, especially when little information is available about population structure, as is the case of skates.

Until the present thesis, the regressing and regenerating phases were only assigned to skates in a recent study regarding the starry ray *Raja asterias* from the Mediterranean (Barone *et al.*, 2007). Although no resting period was detected for the stock of thornback ray from Portuguese waters, the recovery after spawning seems to be triggered at the individual level. The regressing phase was therefore assigned to those large females originally assigned

to developing stage presenting a low GSI (ovaries containing follicles smaller than 1 mm and post-ovulatory follicles) and an oviducal gland (width > 24 mm) and posterior uterus (width > 40 mm) similar to that from spawning females, which indicates that they had already spawned once in their lives. The regenerating phase was assigned to those female with the same characteristics but with high GSI, since they have adult characteristics and an increasing gonad weight. Until now, no evidence of regressing and regenerating phases were observed in thornback ray males. They seem not to have a reproductive cycle, but rather progress from immature through spawning one time, and then remain in the spawning or spawning capable phases for the rest of their lives. Active spermatogenesis is always occurring in males once they have reached sexual maturity.

(x) Does length-at-first-maturity differ between males and females?

In Portugal, thornback ray females mature latter than males, i.e. females attain length-at-first-maturity at 784 mm (smallest mature female measured 699 mm TL) while males at 676 mm (smallest mature male measured 590 mm TL), which corresponds to 8 and 6 years.

The growth increase rates across maturation observed in all the reproductive organs supports that the three-stage maturity phases observed in all rajids also occurs in the thornback ray (e.g. Ebert, 2005; Oddone and Vooren, 2005; Frisk and Miller, 2009). In females, the maturation process was divided into three main phases, defined according to the growth of the different reproductive organs (ovaries, oviducal glands and uteri): (i) first phase with slow growth increase rate (immature and early developing, TL<700 mm); (ii) second phase with moderate growth increase rate (immature, developing and spawning capable, 700<TL<800 mm); (iii) third phase with fast growth increase rate (spawning capable and spawning, TL>800 mm). In males, the maturation process was also divided into three main phases, but the time when they were achieved differed between the internal and external reproductive organs (testes and sperm ducts *vs.* claspers): (i) first phase with slow growth increase rate (testes and sperm ducts: immature and early developing, TL<600 mm; claspers: immature, TL<400 mm); (ii) second phase with moderate growth increase rate (testes and sperm ducts: developing males, 600<TL<700 mm; claspers: developing, 400<TL<600 mm); (iii) third phase with fast growth increase rate (testes: late developing to spawning males, TL>700 mm; claspers: spawning capable and spawning, TL>600 mm).

(xi) Is the species a determined or indeterminate spawner? What is it's the mean fecundity?

Thornback ray is a determinate spawner and the asynchronous development of follicle in the ovary lead to conclude that the eggs are released in batch episodes, with about 35 eggs per batch. During spawning season a total of four batch episodes occur meaning that the mean total fecundity was around 140 eggs per female. The maximum total number of follicles was observed in a spawning capable female measuring 890 mm TL (165 follicles). The smallest frequency of large follicles (diameter larger than 30 mm) and the high frequency of medium-sized follicles (from 12 to 18 mm) observed in the third quarter, along with the high GSI observed in those months, point towards a larger spawning effort by the population in the fourth quarter, when the largest follicles were observed.

(xii) What are the main physiological processes involved with maturation, egg encapsulation and extrusion?

During maturation the reproductive system of females undergoes a series of transformations that will allow the formation of encapsulated eggs when attaining maturity. The oviducal gland is the organ responsible for its formation, and in this thesis a study was conducted for the first time to understand the function of this organ in the thornback ray during all the maturation process. Oviducal glands start to develop early in the developing stage, becoming visible in females larger than 700 mm TL, the definitive form is achieved at the latest developing stage, when the secretions start to be produced and are stored inside the gland tubules. Different zones are identified according to the shape of the lamellae lining the lumen and by the distinct secretory tubules: club, papillary, baffle and terminal (Hamlett *et al.*, 1998). Ovulation occurs at diameters around 30 mm; after being fertilized, the egg is surrounded by a series of envelopes produced by the oviducal gland: (i) sulphated acid and neutral mucin secreted by the club zone (hydrodynamic support); (ii) second layer of jelly secreted by the papillary zone, composed by sulphated acid and neutral mucins; (iii) third layer of jelly, a sulphated acid mucin secreted by the papillary zone (lubricant and bounding layer); (iv) hard egg envelope, proteic, secreted by the baffle zone; and (v) surface hairs (chemically similar to the capsule), coated with mucous secretions that cover the exterior of the capsule (sulphated acid mucins), produced by the terminal zone. Just before the extrusion, the uterus contributes to the egg capsule structure and chemistry (Threadgold, 1957; Koob and Cox, 1990, 1993), producing sulphated acid and neutral mucins. The thornback ray produces one to two egg capsules at a time. The egg capsules are rectangular-shaped, dark

brown, covered with a large amount of fibres in both sides and with two thorns in each edge. In average they measured 72 mm in length and 52 mm in width.

Sperm was observed in the baffle zone of developing females indicating that mating occurs before maturity is reached. Sperm were observed in bundles at the deeper recesses of the baffle zone in the maturation process, and were also detected as isolated cells near the lumen during egg capsule formation. The sperm observed could be the result of a recent mating episode or short duration storage (Hamlett *et al.*, 2005; Storrie *et al.*, 2008).

Skates in general, and thornback ray in particular, have demonstrated to be one of the most resilient groups among all Chondrichthyans. Their compensatory mechanisms to fishing pressure are related to a combination of different factors. As shown in this thesis (Chapter 4) for the thornback ray and in previous studies for this and other skate species (e.g. Walker, 1999; Frisk *et al.*, 2001; Frisk and Miller, 2009), despite a late maturation (around 80% of the maximum TL), skates present the highest fecundity values and the more extended spawning periods within Chondrichthyans. Yet, the higher fecundity seems not to be sufficient when the fishing pressure is very intense (Brander, 1981; Walker and Hislop, 1998). Instead, it seems that net recruitment rate and juvenile survival are the most important compensatory mechanisms, increasing the resilience to fishing pressure (Brander, 1981; Walker and Hislop, 1998). Those main features give them the ability to be more efficient when responding to more intense harvesting. Yet, the sedentary nature of skate populations and high endemism (possibly up to 55% of the species) (McEachran, 1990), opposed to some sharks that are highly migratory tend to form local populations with limited movement (Hunter *et al.*, 2005; Chevolot *et al.*, 2006), which poses a major problem to their survival, when under intense local harvesting. There are no national or EU measures to protect juvenile skates (e.g. minimum landing size). One problem of the implementation of such measure, is that, due to the high biodiversity of species occurring in the same area (Chapter 3), and to their distinct life history traits (small species *vs.* large species), the adoption of a minimum landing size for all species could also have negative effects. If the conservation measure was towards the protection of large bodied species, which are, as known, more threatened to fishing pressure (Dulvy *et al.*, 2000), it would indirectly increase the discards of smaller ones, and therefore possible unbalance the ecosystem dynamics. To find the most appropriate solution to this problem it will be essential to have a good understanding of the life history dynamics of each individual species. After the great goals achieved in this thesis, the first step will be to extend the data on main biological traits gathered for thornback ray to the remaining skate species

occurring in Portuguese waters. In the future, some major guidelines should also be to help define population structure of all skate species, throughout the use of more refined tools, such as population genetics and mark-recapture studies. Only with this type of information it will be possible towards progress to a more accurate assessment of this group of species.

Chapter 6

References

6. References

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