UNIVERSITY OF MANCHESTER

THE APPLICATION OF GENETICS AND PROTEOMICS FOR THE CONSERVATION OF SHARKS AND THEIR RELATIVES

A thesis submitted to The University of Manchester for the degree of Doctor of Philosophy in the Faculty of Science and Engineering

Samantha Alison Hook

September 2019

Contents

List of Figures

List of Tables

List of Abbreviations

ACTB	Actin
AFLP	Amplified Fragment Length Polymorphisms
aDNA	Ancient DNA
BOLD	Barcode of Life Database
BLAST	Basic Local Alignment Search Tool
COL2	Collagen Type 2
COL5	Collagen Type 5
COL5α2	Collagen Type 5 (COLI) Alpha 2 (A2)
COLI	Collagen Type I
COLIα2	Collagen Type I (COLI) Alpha 2 (A2)
COLIαI	Collagen Type I (COLI) Alpha I (Ai)
COL2αI	Collagen Type II (COLI) Alpha I (Ai)
COL	Collagen
COI	Cytochrome C Oxidase I
DNA	Deoxyribonucleic Acid
dNTPs	Deoxyribonucleotide Triphosphate
ddH2O	Double-Distilled Water
eDNA	Environmental DNA
FISH-BOL	Fish Barcode of Life Campaign
h	Haplotype Diversity
IUCN	International Union for The Conservation Of Nature
IAM	Iodeacetamide
LC	Liquid Chromatography
LC-MS	Liquid Chromatography-Orbitrap Elite Tandem MS Analysis
MS	Mass Spectrometry
m/z	Mass to Charge Ratio
MALDI	Matrix Assisted Laser Desorption/Ionization
mRNA	Messenger
mtDNA	Mitochondrial DNA
MYS	Myosin
NCBI	National Centre for Biotechnology Information
NERC	Natural Environment Research Council
nDNA	Nuclear DNA
π	Nucleotide Diversity
PCR	Polymerase Chain Reaction
RFLP	Restriction Fragment Length Polymorphisms
STRs	Short Tandem Repeats
SSRs	Simple Sequence Repeats
SNPs	Single Nucleotide Polymorphisms
TPM	Tropomyosin
TUB	Tubulin
ZooMS	Zooarchaeology By Mass Spectrometry

Abstract

Sharks, rays and skates (elasmobranchs) are a group of cartilaginous fishes currently facing mass declines from overfishing. As of 2014, 25% of elasmobranch species were classified as threatened with extinction by The International Union of the Conservation of Nature (IUCN) Red List. It is estimated that 100 million are killed every year through illegal, unregulated and unreported (IUU) fishing, often for their fins which are in high demand for shark fin soup. As elasmobranchs have been on the planet for nearly 450 million years, the function that each species has within the environment is vital for the marine food web structure and function which ultimately regulates biomass density and therefore balancing ecosystem functions. However, more information is still required on the general biology and population structure of most elasmobranch species, which will assist in management at *in-situ* and *ex-situ* sites, to conserve populations.

To address some of these issues, this thesis uses a range of molecular techniques to investigate species identification and population dynamics by genetics and non-invasive/non-intrusive tagging methodologies. All samples collected and analysed were either a discard elasmobranch product from fish markets or from non-invasive/non-intrusive mucus swabs, which ensures each chapter has not contributed to the decline of wild populations. By focusing on non-invasive techniques, the thesis developed a new method of collecting non-intrusive DNA samples by mucus swabbing individuals underwater, collected by scuba divers. Non-intrusive and invasive discard product samples from the undulate ray *Raja undulata* revealed 6 new distinct genetic clusters throughout their distribution and the application of capture mark-recapture using photographs and recognition software provided consistent population sizes and evidence of female to female or female to male pairing between individuals. Photo recognition was also successfully applied to the small-spotted catshark *Scyliorhinus canicula* in early-stage development (within the first 60 weeks of life) as an alternative for invasive tagging, especially focusing on *ex-situ* conservation. Genetic analysis using microsatellite markers was applied to two species of captive sharks for *ex-situ* management and found genetically diverse populations with little signs of inbreeding. Wild samples of discard products of elasmobranchs and chimaeras (chondrichthyans) caught in Morocco in June 2015 and 2016 were utilized for two chapters. By employing DNA

barcoding methods and observational field methods, the first analysis of the diversity of chondrichthyans was conducted and provided evidence of targeted fishing for elasmobranchs and IUU fishing. These samples were also used to investigate the ability of identifying the proteins in elasmobranch fins, which could possibly be applied as an alternative method of identifying species. We found that 5 main protein groups define the composition of elasmobranch fins; collagen, actin, tubulin, tropomyosin and myosin. Finally, the use of genetics found the first evidence of twins and heteropaternal superfecundation, whereby twins were formed from two different fathers in the same egg.

Following the outcomes of the thesis, further research is advised to focus on revealing and understanding the full genome sequence of species to better understand individual characteristics and population differences, which may influence the their success or demise for future conservation. As elasmobranchs continue to decline, it is imperative that further studies are not only conducted but applied in order to conserve elasmobranchs for the future. The outcomes of this thesis have successfully generated a deeper understanding of the biology, connectivity and utilisation of elasmobranch populations in both *in-situ* and *ex-situ* sites. However, it is the responsibility of scientists and governments to continue the research into elasmobranch biology and population structure, to influence management, conservation and social change.

Declaration

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning. Joint authorship of published materials is listed at the beginning of each research chapter, however to the knowledge of the thesis author these have not been submitted by another student to this university or any other university or other institute of learning.

Copyright Statement

i. The author of this thesis (including any appendices and/or schedules to this thesis) owns certain copyright or related rights in it (the “Copyright”) and she has given The University of Manchester certain rights to use such Copyright, including for administrative purposes.

ii. Copies of this thesis, either in full or in extracts and whether in hard or electronic copy, may be made only in accordance with the Copyright, Designs and Patents Act 1988 (as amended) and regulations issued under it or, where appropriate, in accordance with licensing agreements which the University has from time to time. This page must form part of any such copies made.

iii. The ownership of certain Copyright, patents, designs, trademarks and other intellectual property (the “Intellectual Property”) and any reproductions of copyright works in the thesis, for example graphs and tables (“Reproductions”), which may be described in this thesis, may not be owned by the author and may be owned by third parties. Such Intellectual Property and Reproductions cannot and must not be made available for use without the prior written permission of the owner(s) of the relevant Intellectual Property and/or Reproductions. iv. Further information on the conditions under which disclosure, publication and commercialisation of this thesis, the Copyright and any Intellectual Property and/or Reproductions described in it may take place is available in the University IP Policy, in any relevant Thesis restriction declarations deposited in the University Library, The University Library’s regulations and in The University’s policy on Presentation of Theses.

Acknowledgements

Professional Acknowledgements

First and foremost, I would like to thank Dr Michael Buckley and Dr Holly Shiels for taking me on as a student in one of the most stressful periods of my PhD. From the start the doors to their offices were always open whenever I ran into a trouble or had a question about my research or writing. Jointly, they have consistently allowed this thesis to be my own work but have steered me in the right the direction when required. I would also like to thank Dr Cathy Walton for being a supervisor and mentor in genetics, and Dr John Fitzpatrick, Dr Jennifer Rowntree and Prof Richard Preziosi for starting my PhD journey before they left the University of Manchester early on in this process.

I owe a very important debt to Julie Samson, NERC DTP administrator and the cheerleader to all my academic work. Without her understanding ear, her ability to tackle issues head on and invaluable guidance on how overcome stressful situations, I would have not made it to the end of my PhD. Her kind and empathetic nature has saved me and many students within our cohort. On behalf of us all, we are eternally thankful. I would also like to thank Prof Geraint Vaughan for employing Julie and allowing her to throw some delicious lunches, as well as supporting and part managing the NERC DTP. And to my cohort, here is to the trips away, the lifelong friendships and the many games of werewolves keeping us all sane. A special thanks goes to the Natural Environment Research Council and the University of Manchester for funding my PhD including the lab experiments and my shark adventures. I would also like to thank Jean Denis-Hibbitt and the team at Sea Life, Merlin Entertainments. They have provided invaluable information throughout the duration of my PhD. As my CASE partner, I thank them for the funding which assisted in the expeditions to find undulate rays and the research in conservation genetics for captive management. I would like to thank everyone I met at the conference dinners, who have not only turned into collaborators, but lifelong friends. Without Martin and Sheilah, I would have never dreamed of seeing an undulate ray alive in the wild, and not only did they make this happen, but their enthusiasm and spirit for the species has shaped the way I think about future science. And

lastly Ana Verissimo, it was an honour to work with you in Portugal. I cannot thank you enough for your hard work and openness for collaboration.

I would like to give a special mention to Virginia Harvey, Noemi Procopio, Daniel Ripley, Karren Palmer and the Buckley Lab group for making it a cracking place to work. I am also especially grateful to The Sheils Lab group and their consistent lab meetings at 9 am every Friday. Not only did this ensure I was out of bed on a Friday morning and well-fed (with often worldwide breakfast choices), but it meant my week always ended on a high, reducing the troubles that every PhD student experiences on a day to day basis. Lastly, I would like to thank the huge support friendship and guidance given by Dr. David Orchard that has saved me on more than a few occasions.

Personal Acknowledgements

I am grateful to my mother Tracey, sister Emma, and grandparents Margaret and Keith, who have provided me with moral and emotional support. Their lack of ability to grasp what I am actually doing has been the inspiration of many of my science communication talks. I am also thankful to have such a supportive extended family, who have provided me with some great opportunities to unwind but who have also reminded me that I need to get a ‘real’ job. I am also thankful for my friends Tom, Emma, Chris, Sammy, Anna and Aoife for grounding me in an ever-challenging world. And lastly a thank you to Dale, for his attentive love and support. He has been my rock, shoulder to cry on, my travelling partner and my number one motivator; always believing in me even when I did not believe in myself.

Dedicated to my mother Tracey,

my sister Emma and my nana Margaret.

My female inspirations to be kind and dedicated on an unforgiving planet.

Samantha Alison Hook

samhook1205@gmail.com - samantha.hook@manchester.ac.uk - (+44) 7842055958

SUMMARY

I am a current PhD student (in submission) at the University of Manchester, specifically focused on the application of genetics for the conservation of elasmobranchs (sharks and rays) in wild and captive environments. My PhD has numerous interdisciplinary aspects consisting of collaborative three partnerships. As part of this collaborative work, I have produced numerous research chapters specifically for my project partners, meeting their requirements and deadlines to ensure the outcomes can be applied to the end-users. Aside from my NERC DTP grant, I have won a further £15,100 from five additional grants to either implement new research strategies, produce research for end-users or create and deliver a new course for sustainability with The Carbon Literacy Project.

RELEVANT EXPERIENCE

Employment	
Title: [1]Researcher, [2]Environmental Consultant	[1]Substance, Manchester 2019 to Present
Collaborations	
Research: The application of genetics to assess and manage populations of sharks and rays	Sea Life, UK 2015 - 2019
Funding	
Title: NERC DTP Funding **Earth pillar, awarded funding includes compulsory training, interdisciplinary research, and relevance to understanding, increasing or developing current research into earth sciences.**	Natural Environment Research Council DTP Funding, EAO, The University of Manchester, UK 2015-2019
Education	
Title: PhD Candidate **The application of genetics for the conservation and management of elasmobranchs (sharks and rays)**	The University of Manchester, UK School of Earth and Environmental Sciences (SEES) 2015 - 2019
Media	
Title: Invited speaker **Six-minute segment on the application of genetics for the conservation of the undulate ray, as part of the Undulate Ray Project**	“Beach Live: the Jurassic coast revealed”, BBC Four
Publications and Awards	
Award: Duke of Cambridge Commendation Award **Commendation for outstanding achievement in research in the field of scuba diving**	British Sub Aqua Club (BSAC), UK President of BSAC, HRH Duke of Cambridge, 2018

Employment

RESEARCHER

Substance; June 2019 - Present

Moving from an associate to a full-time position, as a researcher I work on marine research for clients including CEFAS (the Sea Angling Diary), Marine Management Organisation (recreational angler mapping) and Royal National Lifeboat Institution (station polling and analysis).

SUPPORT DEMONSTRATOR

The University of Manchester; January 2016 - Present

In this role I have demonstrated the use of practical equipment (including where relevant, software packages), experiments in molecular sciences including CRISPR, laboratory techniques (somewhat basic and more complex) and described scientific processes that form an element of an undergraduate course of study in line with specific learning objectives, set by main lecturers.

MANCHESTER ACCESS PROGRAM (MAP) TUTOR

The University of Manchester; January 2017 - Present

The Manchester Access Program is dedicated to assisting local college students (Year 12) who meet a specific academic and background criteria to achieve entry to the University of Manchester, or another research-intensive university, through the completion of a portfolio of work demonstrating specific knowledge and skills. I have tutored on the program for two consecutive years, which involves set regular tutor meetings, assistance with writing scientific reports and numerous reviews and marking students work.

ECOLOGICAL CONSULTANT

Arcadis, Warrington; March 2018 - Present

Working on a contractual basis I conduct ecological assessments on great crested newt *Triturus cristatus* and UK species of bat populations. This includes methods such as using non-intrusive mark-recapture data, habitat assessments to company's scope and use of Pettersson bat detectors.

RESEARCH ASSOCIATE

Substance; November 2017 – June 2019

This research has been part of the Sea Angling Diary: a project outsourced by CEFAS. Working as part of the team on a consultancy basis, I have been involved in surveying and networking with the sea angling community to encourage them in the participation of the Sea Angling Diary: a vital record of recreational sea angler's activity and catches.

SCIENTIFIC COORDINATOR (volunteer)

Fin Fighters; January 2016 - January 2018 (2 years 1 month)

I began as a scientific adviser for the charity. I set up numerous collaborations between the charity and the scientific community, endorsing the charities ability to provide in-field samples and knowledge. After a one-month research excursion to Morocco (2015) to investigate the illegal, unregulated and unreported (IUU) fishing I took a more senior role as coordinator where I continued to expand the scientific collaborations and received NERC DTP grants to continue the research expeditions through to 2016. Responsibilities of my role included networking, overseeing scientific research, conducting infield research, organizing large amounts of data, communicating knowledge to the general public and providing advisories on future projects. The project collaboration continues with the finalization of a documentary regarding the work.

ENVIRONMENTAL CONSULTANT

SK Environmental Solutions Ltd.; March 2017 - December 2017 (10 months)

PROJECT COORDINATOR – THE CITIZEN INSPECTOR NETWORK

The Black Fish Manchester, United Kingdom and Amsterdam, The Netherlands; November 2014 - December 2017 (3 years 2 months)

RESEARCH ASSISTANT

The University of Salford, UK; June 2014 - October 2016 (2 years 5 months)

PROJECT MANAGER AND LAB TECHNICIAN

REC; Resource and Environmental Consultancy Ltd.; March 2015 - September 2015 (7 months)

Collaborations

1. NERC CASE PARTNERSHIP SEA LIFE

A contracted collaboration between myself, the NERC Doctoral Training Program PhD (CASE partnership) and Merlin Entertainments to provide a first-rate, challenging research training experience, within the context of mutually beneficial research collaboration between academic and non-academic partner organisations.

Collaboration: September 2015 – September 2019; Placement duration: 3 months; Funding: £3,000 – Sea Life Trust

2. THE UNDULATE RAY PROJECT

A collaboration between myself and The Undulate Ray Project (ran by the NGO Stardis) as part of my PhD, to produce beneficial research for the conservation of the endangered undulate ray *Raja undulata* off the south coast of England. The collaboration has included sample collection, scuba diving and grant funding from the British Sub Aqua Club (BSAC). Duration: October 2016 – September 2019

3. ZSL

A collaboration of the Angel Shark Project to process non-intrusive samples collected in the UK to investigate the connectivity between the individuals in the UK and the relatedness between those to the Gran Canaries. Funded provided by the National Lottery and Welsh government. Duration: August 2018 – May 2019

4. THE CARBON LITERACY PROJECT

A collaboration to produce and deliver a new course titled “Carbon Literacy for Labs”, making the University of Manchester the University institute to provide such accreditation to their staff and students. Duration: September 2018 – January 2019

5. FIN FIGHTERS

A collaboration to conduct investigative fieldwork in Morocco on the illegal, unregulated and unreported fishing activity of sharks, rays and their relatives (chimaeras). The work commenced before the PhD and was concluded during the second year of the PhD to form Chapter 1. Duration: January 2015 – September 2017

Funding

Award Body	Funding Amount	Dates	Project Title
NERC Doctoral Training Partnership	PhD studentship: monthly stipend; £11,000 RTSG; tuition fees; training fees	Sep 2015 - June 2019	PhD Title: Application of genetics for the conservation of sharks and rays
Zoological Society of London	£3,400	Sep 2018 - May 2019	To investigate the genetic diversity of UK Angel Sharks
SEES Environmental Sustainability Grant	£2,700	Sep 2018 - Jan 2019	Carbon Literacy for Labs Project
Sea Life Trust	£3,000	2016- 2019	Application of genetics to understand captive populations of sharks and rays
HEIF- Eco-Innovation Voucher	£3,000	2016-2017	Development of marker use for external shark populations
HEIF- Eco-Innovation Voucher	£3,000	2015-2016	Development of markers to investigate current population of University of Manchester small-spotted catsharks
British Ecological Society	£500	2015	Research expedition to Madagascar as part of the Tropical Biology Association

Education

PHD CANDIDATE, BIOMOLECULAR SCIENCES, NERC DTP

The University of Manchester, UK
School of Earth and Environmental Sciences, 2015 – 2019

MASTERS OF SCIENCE BY RESEARCH

The University of Salford, UK
School of Environment & Life Sciences, 2014 – 2015

***Title: Genetic Variation and Parentage in the Ethiopian Wolf* Canis simensis**

BACHELOR OF SCIENCE (HONS)

The University of Salford, UK
Faculty of Life Sciences, 2009 – 2013

Title: Wildlife Conservation with Zoo Biology
Grade: First Class with Honours

GCSE'S and A LEVELS

Crompton House Sixth Form, UK
2002 – 2009
10 GCSE's A-C including Mathematics, Science and English
A level Subjects: Biology, Geography, ICT, Psychology

Research Contributions

INTERNATIONAL JOURNAL PUBLICATIONS

Sharks, rays and the application of conservation genetics
S. A. HOOK 1*
Biological Sciences Review, Hodder Education (2018)
1Faculty of Biological, Medical and Human Sciences, University of Manchester, Core Technology Facility, Manchester, M13 9NT, U.K.* Author for correspondence
ISBN: 978-1-4718-9040-6

Low mislabeling rates indicate marked improvements in European seafood market operations
S. MARIANI, *et al*.
Frontiers in Ecology and the Environment, (2015)
13.10: 536-540.

INTERNATIONAL MEETINGS- ORAL COMMUNICATIONS

FARO Conference 2017
"One fin too many; using DNA barcoding to identify illegal, unreported and unregulated chondrichthyan fishing in Morocco"
Samantha A Hook[1], Louise Ruddell[2], Michael Buckley[1], Andrew Griffiths[3]
Authors' affiliations: [1]University of Manchester, [2]Fin Fighters.org, Bristol, [3]University of Exeter

INTERNATIONAL MEETINGS- POSTER COMMUNICATIONS

Sharks International Conference, Joao Pessoa, Brazil 2018
"One fin too many; using DNA barcoding to identify illegal, unreported and unregulated chondrichthyan fishing in Morocco"
Samantha A Hook[1], Louise Ruddell[2], Michael Buckley[1], Andrew Griffiths[3]
Authors' affiliations: [1]University of Manchester, [2]Fin Fighters.org, Bristol, [3]University of Exeter

European Elasmobranch Association conference 2016
"The illegal unreported and unregulated fishing of sharks, rays and their relatives (Chondrichthyes) in Morocco"
Samantha A Hook[1], Louise Ruddell[2], Michael Buckley[1], Andrew Griffiths[3]
Authors' affiliations: [1]University of Manchester, [2]Fin Fighters.org, Bristol, [3]University of Exeter

NATIONAL MEETINGS- ORAL COMMUNICATIONS

DTP Conference 2018

"If you scratch my back, I'll scratch yours: non-intrusive DNA sampling of undulate rays *Raja undulata* off the coast of the UK"

Samantha Alison Hook[1], Martin Openshaw[2], Sheilah Openshaw[2], Michael Buckley[1]

Author Affiliations: [1]University of Manchester, [2]Stardis non-governmental organisation

The Future Directions in conservation science conference 2017

"One fin too many; using genetic barcoding to identify illegal shark and ray (elasmobranchii) fishing in Morocco"

Samantha A Hook[1], Louise Ruddell[2], Michael Buckley[1], Andrew Griffiths[3]

Authors' affiliations: [1]University of Manchester, [2]Fin Fighters.org, Bristol, [3]University of Exeter

DTP Conference 2017

"One Fin too Many"

Samantha Alison Hook[1]

Author Affiliations: [1]University of Manchester

National Aquarium Conference 2016

"Sharks, rays and genetic diversity"

Samantha Alison Hook[1]

Author Affiliations: [1]University of Manchester

Fisheries Society of the British Isles 2016

"Does genetic diversity measures predict IUCN status in elasmobranchs?"

Samantha A. Hook[1], John Fitzpatrick[2], Jennifer Rowntree[3]

Author affiliations: [1]University of Manchester, [2]Univerisity of Stockholm, [3]Manchester Metropolitan University

INVITED SPEAKER

Leviathan exhibition, Bluecoat Gallery, Liverpool 2019

"Empathy in and out of shark, ray and skate species"

Samantha Alison Hook[1]

Author Affiliations: [1]University of Manchester

Natural History of Marine Biology (Porcupine) Conference 2019

"Sharks, Rays and DNA: The use of DNA to uncover the secret lives of elasmobranchs"

Samantha Alison Hook[1], Martin Openshaw[2], Sheilah Openshaw[2]

Author Affiliations: [1]University of Manchester, [2]Stardis non-governmental organisation

Inshore Fisheries Conservation Authorities 2018

"The applications of genetics for shark and ray conservation"

Samantha A. Hook[1]

Authors' affiliations: [1]University of Manchester

Politecnico de Leiria, Portugal 2017

"Shark and Ray Genetics!"

Samantha A. Hook[1], Jean-Denis Hibbitt[2],

Author affiliations: [1]University of Manchester, [2]Merlin Animal Welfare and Development

Merlin Animal Welfare and Development 2016

"Shark and Ray Genetics!"

Samantha A. Hook[1], Jean-Denis Hibbitt[2], John Fitzpatrick[4]

Author affiliations: [1]University of Manchester, [2]Merlin Animal Welfare and Development, [3]Manchester Metropolitan University, [4]University of Stockholm

Amphibian and Reptile Group of South Lancashire Conference 2016

"How can genetic techniques help us understand great crested newt population dynamics?"

David Orchard[1], Samantha A. Hook[2], Robert Jehle[1]

Authors' affiliations: [1]University of Salford, [2]University of Manchester

Media and Public Engagement

PRESS RELEASES

BLUE PLANET UK, SEASON 1, EPISODE 2 BBC 1, 26TH OF MARCH 2019
"How a retired couple are researching undulate rays and contributing to science"

BSAC NEWSLETTER MARCH 2019
"Scuba diving with Undulate Rays"
Members newsletter

MIB NEWSLETTER NOVEMBER 2018
"Recognition of award announcement"
Internal newsletter

BSAC DUKE OF CAMBRIDGE AWARD PRESS RELEASE
"BSAC members receive Duke of Cambridge Scuba Award"

BBC FOUR, INVITED SPEAKER AUGUST 2018
"Beach Live: the Jurassic coast revealed"
Six-minute segment on the application of genetics for the conservation of the undulate ray, as part of the Undulate Ray Project on behalf of the University of Manchester.

NERC EAO DTP ARTICLE
"Reviewing Cohort 2 team building and management workshop"
Internal newsletter.

THE GUARDIAN UK EDITION ARTICLE 2015
"The Black Fish: undercover with the vigilantes fighting organised crime at sea"
Description: An article covering an undercover investigation into the illegal, unregulated and unreported fishing in Sicily Italy; investigation was ran by myself as part of The Black Fish CIN Coordinator role.

ORAL COMMUNICATIONS

PINT OF SCIENCE, MANCHESTER 2018
"Changing Perspectives: Why sharks are jawesome!"
Samantha A. Hook[1]
Author Affiliations: [1]University of Manchester

INTO THE BLUE FESTIVAL, MANCHESTER AIRPORT VISITORS CENTRE, 2016
"Sharks and Scientists! Into The Blue 2016"
Samantha A. Hook[1]
Author Affiliations: [1]University of Manchester

Certifications and Awards

CERTIFICATIONS

1. BRTISH SUB AQUA CLUB (BSAC) SPORTS DIVER (2011)
2. BRITISH SUB AQUA CLUB FOUNDATION INSTRUCTOR (BSAC IFC; 2011)
3. FIRST AID IN FIELD QUALIFIED (2016)
4. IUCN RED LIST ASSESSOR CERTIFIED (2017)
5. O2 ADMINISTRATION QUALIFIED (2017)
6. CARBON LITERACY FOR LABS CERTIFIED (2018)

DUKE OF CAMBRIDGE COMMENDATION AWARD Commendation for outstanding achievement in research in the field of scuba diving	November 2018
DTP CONFERENCE 2018 2ND PLACE COHORT PRESENTATION	June 2018
DTP CONFERENCE 2017 2ND PLACE 15 MINUTE PRESENTATION	June 2017

SECTION I: INTRODUCTION AND BACKGROUND

Chapter 1

Introduction and Literature Review

Biodiversity loss is one of the most critical environmental problems that threaten the natural world (Stachowicz *et al.*, 1999; Cardinale *et al.*, 2012; Hooper *et al.*, 2012). The claim that we are facing our sixth mass extinction is heavily supported by current extinction rates, which are thought to be at least 100 times faster than any pre-human background rate (Barnosky *et al.*, 2011; Pievani, 2014; Ceballos *et al.*, 2015; McCallum, 2015; Ceballos, Ehrlich and Dirzo, 2017; Sonne and Alstrup, 2019). Evidence suggests that humans are causing this sixth mass extinction through habitat fragmentation, climate change, introduction of non-native species, transmission of pathogens and overexploitation of resources (Barnosky *et al.*, 2011; Dulvy *et al.*, 2014; Pievani, 2014; Ceballos *et al.*, 2015; McCallum, 2015; Ceballos, Ehrlich and Dirzo, 2017; Sonne and Alstrup, 2019). Due to these high extinction rates and a lack of detailed knowledge about extant biodiversity, there is a high probability that a species will become extinct before it is even discovered (Mora *et al.*, 2011). To protect the environment, and ultimately human existence, a multitude of global conservation efforts are being applied. However, these conservation efforts to protect species have not slowed the rate of biodiversity loss (Butchart *et al.*, 2010; Hooper *et al.*, 2012; McCallum, 2015).

One of the habitat types most vulnerable to biodiversity loss is the oceans (Costello and Chaudhary, 2017). Due to climate change and overfishing, we are observing a rapid decline in healthy marine environments. It is estimated that we are losing on average 21% of the ocean's diversity, and that approximately 60% of all commercially fished stocks are overexploited, depleted or recovering from mass overfishing (Hooper *et al.*, 2012). The ocean is one of our main life supports, providing at least one third of the planet's oxygen and feeding roughly one billion people worldwide (Engelhaupt, 2007). Elasmobranchs, which include sharks, rays and skates are amongst the oldest ocean predators that are now most threatened by extinction (Stevens *et al.*, 2000; Dulvy *et al.*, 2014). As of 2014, roughly a

quarter of the world's elasmobranch species were classed as threatened with extinction (IUCN, 2014; Dulvy *et al.*, 2014). The largest threat to elasmobranchs is overfishing (Dulvy *et al.*, 2014). Typically, elasmobranchs are slow growing, with late sexual maturity and low reproductive fecundity (Dulvy *et al.,* 2014). The majority of elasmobranch life history traits fall under the *K*-selection theory, which characterises such species as having slower development/growth, lower resource thresholds, late reproduction, larger body sizes, later attainment of sexual maturity, longer life spans, low fecundity and longer gestation periods (Dulvy *et al.*, 2008). These traits, which were once an evolutionary success, render them particularly vulnerable to fishing pressures, slowing their ability to recover from mass depletion from overfishing (Stevens *et al.*, 2000; Carrier, Musick and Heithaus, 2010; Dulvy *et al.*, 2014; Almerón-Souza *et al.*, 2018). It is estimated that 100 million elasmobranchs are killed every year (Worm *et al.*, 2013) as a result of accidental by-catch and illegal, unreported and unregulated (IUU) fishing which contributes to 50% of global elasmobranch catch (Stevens *et al.*, 2000; Gilman, Brothers and Kobayashi, 2005; Worm *et al.*, 2013). Generally, elasmobranchs are defined as a key species, playing roles of predator and prey within each of their niche environments (Navia, Cortés and Mejía-Falla, 2010; Sekerci and Petrovskii, 2015; Navia *et al.*, 2017). The roles of the apex shark species have been found to be the most important within an environment, regulating biomass density and therefore balancing ecosystems (Stevens *et al.*, 2000; Navia, Cortés and Mejía-Falla, 2010; White and Sommerville, 2010; Sekerci and Petrovskii, 2015; Navia *et al.*, 2017). These roles are fundamental in the marine food web structure and function (Sekerci and Petrovskii, 2015; Navia *et al.*, 2017).

The following literature review provides an insight into the evolutionary biology of elasmobranchs, the potential illegal, unregulated and unreported fishing of elasmobranch species, and the current conservation legislation designed to reduce the risk of extinction. It explains the methods applied within conservation to uncover species identification, population dynamics and genetic health, and reviews new techniques which could be applied to species identification. Lastly, it summarises the aims and objectives of this thesis which investigates species identification and population dynamics providing new scientific information which it applies to conservation practise.

1.1 The evolutionary biology of elasmobranchs

Elasmobranchs are a diverse vertebrate subclass comprised of sharks and their relatives the batoids (guitarfishes, sawfishes, skates and rays) (Moy-Thomas, 1939; Fowler and Cavanagh, 2005). Elasmobranchs are one of two subclasses within the chondrichthyans, known commonly as the cartilaginous fishes; the second subclass is the Holocephali, also known as the modern chimaeroids. Today, there are as many as 1,250 species of chondrichthyans found in both fresh and marine environments across the globe. Elasmobranchii which is roughly translated to 'strap-gills' refers to five to seven gill slits which are a general trait throughout all elasmobranchs (Grigg, 1970; Maisey, 2012).

'Modern' elasmobranchs or Neoselachain elasmobranchs are the extant survivors of one of the earliest offshoots in the vertebrate evolutionary tree, dating back around 450 million years ago (Moy-Thomas, 1939; Maisey, Naylor and Ward, 2004; Naylor, Fedrigo and Andrés López, 2005). Neoselachain elasmobranchs are the result of concentrated bursts of adaptation throughout the Jurassic and Cretaceaous period (Mesozoic era), and a further split of sharks and batoids towards the end of the Devonain period (Sorenson, Santini and Alfaro, 2014). Sharks are characterised predominantly by their lack of a swim bladder (found in most bony fish) dermal denticle skin, an upper jaw detached from the cranium and continual production along with serial shedding of teeth (Grigg, 1970; Maisey, 2012). Batoids are further characterised by their 'dorsoventrally compressed bodies' (Schaefer and Summers, 2005), which range from rhomboidal to circular shapes (Compagno, 1977). Their pectoral fins are fused to the cranium and greatly enlarged, forming wing-like structures, which are used as the primary locomotor propulsors (Heine, 1992). Sharks and batoids now inhabit a vast array of aquatic environments, such as the Greenland sharks *Somniosus microcephalus* in the North Atlantic and Arctic Oceans (MacNeil *et al.*, 2012), bull sharks *Carcharhinus leucas* which can inhabit both fresh and salt water environments (Thorson, Cowan and Watson, 1973) and deepwater dwelling frilled sharks *Chlamydoselachus anguineus,* which exist between 500 and 1000 metres deep (Kubota, Shiobara and Kubodera, 1991). Today elasmobranchs exhibit some of the most unique morphologies in the world, with the greatest number of reproductive modes of all vertebrates.

All extant elasmobranchs employ internal fertilization and have adapted unique organ systems, claspers, siphon sacs and sperm storage, for reproduction (Pratt, Jr. and Carrier, 2001). Traditionally, the reproductive modes of elasmobranchs are defined as vivparity, ovovivparity and oviparity (Wourms, 1981; Wourms and Demski, 1993; Hamlett, 2001; Castro, 2013). Viviparity is a two stage nourishment system where a fertizilised egg is wrapped in a thin egg-like capsule and fed off a store of yolk (Wourms, 1981; Wourms and Demski, 1993; Hamlett, 2001; Castro, 2013). When the yolk supply is exhausted a placenta-like highly vascularised connection (psuedoplacenta) between the mother and offspring develops, providing nutrients and gas exchanged (Snelson Jr., Burgess and Roman, 2008). Once a foetus is fully developed the pseudoplacenta is broken and the offspring are born retaining a faint umbilical scar (Wourms, 1981; Wourms and Demski, 1993; Hamlett, 2001). Viviparity is estimated to have evolved at least 18 times among chondrichthyans (Wourms and Demski, 1993). Ovoviviparity can be divided to contain oophagy and/or intrauterine cannibalism or trophonemata (Snelson Jr., Burgess and Roman, 2008; Castro, 2013). Ovoviviparity is the development of offspring inside eggs within the mother, however there is no pseudoplacenta once a yolk store is exhausted (Wourms, 1981; Dodd, 1983; Musick and Ellis, 2005). Ovoviviparity with oophagy is when the offspring feed off unfertilized yoked ova once their yolk store is exhausted (Blackburn and Evans, 1985) and this can been seen in species such as porbeagle shark *Lamna nasus* (Campana, Gibson and Fowler, 2010), bigeye thresher shark *Alopias superciliosus* (Benjamin *et al.*, 2015) and the sand tiger shark, *Carcharias taurus* (Carlson *et al.*, 2009). Intrauterine cannibalism, also known as adelphophagy, “eating one’s brother”, or embryonic cannibalism is when the stronger (often more developed and larger) foetus will consume its smaller siblings during gestation (Chapman *et al.*, 2013), rather than unfertilized yoked ova as seen in oophagy (Musick and Ellis, 2005). The basking shark *Cetorhinus maximus,* the sand tiger shark *Carcharias taurus* (Grant *et al.*, 1993) and the porbeage shark *Lamna nasus* (Jensen et al. 2002) are examples of where both oophagy and intrauterine cannibalism can be observed (Compagno, 1984). Lastly oviparity is when fertilized eggs, encapsulated in an egg case with the entire nutrients the embryo will require, are released into the external environment (Hamlett, 2001; Snelson Jr., Burgess and Roman, 2008). These eggs are often referred to as mermaid purses and

are found in a vast array of shapes, sizes and external morphological differences which protect them while they develop in the ocean (Snelson Jr., Burgess and Roman, 2008).

Around 515 species (55%) of elasmobranchs are vivparious; 270 of batoids and 245-270 species of sharks (Wourms and Demski, 1993) and this is the most dominant mode of reproduction in elasmobranchs (Snelson Jr., Burgess and Roman, 2008). There has been a growing number in studies related to multiple paternities in elasmobranchs and this appears to be a common trait (Daly-Engel *et al.*, 2006; Marino *et al.*, 2015; Townsend *et al.*, 2015). Species include the leopard shark *Triakis semifasciata* (Nosal, Lewallen and Burton, 2013), gummy shark *Mustelus antarcticus* (Boomer *et al.*, 2013), bull shark *Carcharhinus leucas* (Pirog *et al.*, 2017) and the grey nurse sharks *Carcharias Taurus* (Townsend *et al.*, 2015). Multiple paternity and hybridization has also been found between two smooth-hound sharks *Mustelus mustelus and Mustelus punctulatus* (Marino *et al.*, 2015). Asexual reproductions, also known as facultative parthenogenesis or virgin births, have been documented in numerous captive and wild species such as the hammerhead shark *Sphyrna tiburo* (Chapman *et al.*, 2007), smalltooth sawfish *Pristis pectinata* (Fields *et al.*, 2015) and the white-spotted bamboo shark *Chiloscyllium plagiosum* (Feldheim *et al.*, 2010).

Elasmobranch young are born precocial, that is, often large and well developed (Wourms and Demski, 1993). The number of young is dependent on the species, for example the whale shark *Rhincondon typus* is ovoviviparous and has been found to carry up to 300 embryos at one time (Joung *et al.*, 1996) whereas the great white shark *Carcharodon carcharias* (also ovoviviparous) will produce between two and ten at one time (Blower *et al.*, 2012). Gestation for elasmobranchs is between six and two years (Snelson Jr., Burgess and Roman, 2008) and body size and sexual maturity is an interspecies variable. Some elasmobranchs such as the great white shark female can take up to 33 years before reaching sexual maturity (Robbins, 2007), but most species of elasmobranchs take around 3 and 8 years (Carrier, Musick and Heithaus, 2010).

The biological characteristics found in elasmobranch reproduction have previously been advantageous traits for their survival as they have enabled the group to grow and live at the carrying capacity without outcompeting for resources (Brown and Choe, 2019).

However, the combination of relatively slow growth rate, late maturity and long gestations (often producing very few young), has left elasmobranchs vulnerable to overfishing (Dulvy *et al.*, 2014). Overfishing has resulted in large declines of elasmobranch populations which in turn have had a negative effect on biodiversity within their environments. In order to recover elasmobranch populations, educational awareness and conservation efforts have begun to drive environmental policy across nations. The International Union for the Conservation of Nature (IUCN), The Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), The Convention of Migratory Species of Wild Animals (CMS) and the Working Group on Elasmobranch Fishes (WGEF) ran by International Council for the Exploration of the Sea (ICES) all play vital roles, which have seen new restrictions on conservation and better implementations of elasmobranch fisheries worldwide (WGEF, 2018; CITES, 2019b; CMS, 2019).

1.2 Overexploitation of elasmobranchs and policy implications

Elasmobranchs are utilized by humans in different ways across the globe; for example as a food source (Nalluri *et al.*, 2014), tourist attraction (Vianna *et al.*, 2012; Bentz *et al.*, 2014; Haas, Fedler and Brooks, 2017), biodiversity indicator (Dulvy *et al.*, 2014), killed for sport (Brunnschweiler and Ward-Paige, 2014) or culled in a method to reduce attacks (Trouwborst, Fleurke and Dubrulle, 2016). The greatest global economic benefactor from elasmobranch populations is the fishing industry, although due to levels of illegal, unregulated and unreported (IUU) fishing, the true revenue of the industry is unknown. It was estimated that around 97 million elasmobranchs were killed by the global fishing industry in 2010 alone however, due to IUU fishing this figure could be anywhere up to 273 million (Worm *et al.*, 2013). There has been an exponential growth in elasmobranch fishing since the 1950s, which has resulted in some species suffering up to a 90% decline in population size (Baum *et al.*, 2003; Baum and Myers, 2004). The exploitation of elasmobranchs for profit takes many forms, however, generally shark fin trade is the main target with an annual revenue of between $306 and $419 million USD since the year 2000 (Gallagher and Hammerschlag, 2011; Worm *et al.*, 2013).

As well as shark fins, the torso of a select proportion of captured individuals will end in markets, bringing between 20% and 60% of the price of tuna species (Bonfil, 1994; Ward-Paige *et al.*, 2012). Mislabelling of shark meats can also create substantial profits, such as in Brazil, where shark species were sold as grouper fish increase market revenue by 25% (Bornatowski, Braga and Barreto, 2018). In many cases mislabelling occurs to hide the true identity of protected species, which continue to be caught due to inadequate governance both regionally and internationally (Griffiths *et al.*, 2013). Unfortunately, elasmobranch populations are susceptible to poorly managed fisheries, which leave them exposed to overexploitation and IUU fishing. Of 173 commercially fished shark populations, comprising of 46 species, 87% were at high risk of ineffective fisheries management whilst the remaining 13% were classified as medium risk (Lack *et al.*, 2014). In order to combat elasmobranch population declines and IUU fishing, multiple commercially exploited species have been added to the CITES and CMS Appendices. These are two international treaties that have a legal mechanism for biodiversity conservation, supporting the management of elasmobranchs at a national and regional level (Zeng *et al.*, 2016). Currently, there are 183 party members, which consist of nearly every trading country, that have signed the CITES agreement. In doing so, they agree to participate in assessments and to implement the majority ruling of any listing made by CITES. Policy milestones for the protection of elasmobranchs vary between the two international bodies as seen in Table 1.1.

Table 1.1. An overview of the elasmobranch species held on either or both Appendices in The Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) and The Convention of Migratory Species of Wild Animals (CMS) international policy. Information derived from CITES, 2019 and CMS, 2019. App = Appendix, # C= number of countries

Species	CITES			CMS	
	App	# C	Year	App	Year
Alopias pelagicus	II	34	2017	II	2014
Alopias superciliosus	II	42	2017	II	2014
Alopias vulpinus	II	81	2017	II	2014
Anoxypristis cuspidata	I	22	2007	I, II	2014
Carcharhinus falciformis	II	111	2017	II	2014
Carcharhinus longimanus	II	40	2013	-	-
Carcharhinus obscurus	-	-	-	II	2017
Carcharodon carcharias	II	95	2005	I, II	2002
Cetorhinus maximus	II	61	2003	I, II	2005
Isurus oxyrinchus	-	-	-	II	2008
Isurus paucus	-	-	-	II	2008
Lamna nasus	II	50	2013	II	2008
Manta alfredi	II	42	2013	I, II	2014
Manta birostris	II	61	2013	I, II	2011
Manta birostris	-	-	-	I, II	2011
Mobula eregoodootenkee	II	30	2017	I, II	2014
Mobula hypostoma	II	20	2017	I, II	2014

Mobula japanica	II	54	2017	I, II	2014
Mobula kuhlii	II	15	2017	I, II	2014
Mobula mobular	II	21	2017	I, II	2014
Mobula munkiana	-	-	-	I, II	2014
Mobula rochebrunei	-	-	-	I, II	2014
Mobula tarapacana	-	-	-	I, II	2014
Mobula thurstoni	-	-	-	I, II	2014
Prionace glauca	-	-	-	II	2017
Pristis clavata	-	-	-	I, II	2014
Pristis pectinata	-	-	-	I, II	2014
Pristis pristis	-	-	-	I, II	2014
Pristis zijsron	-	-	-	I, II	2014
Rhincodon typus	-	-	-	I, II	2017, 1999
Rhinobatos rhinobatos	-	-	-	I, II	2017
Rhynchobatus australiae	-	-	-	II	2017
Sphyrna lewini	-	-	-	II	2014
Sphyrna mokarran	-	-	-	II	2014
Squalus acanthias	-	-	-	II	2008
Squatina squatina	-	-	-	I, II	2017

The policy and law of elasmobranch fishing depends on species and regional stock assessments. Decisions are based on scientific data and fisheries assessments, often presented by organisations such as WGEF, CITES and CMS (WGEF, 2018; CITES, 2019b; CMS, 2019). These organisations hold international treaty meetings whereby representatives from those countries and institutions involved will present evidence to inform assessments on specific species. The most recent is the new CITES agreement, published in August 2019, for increased protection from trade of a further 18 shark and ray species, comprising of mako sharks, wedgefishes and guitarfishes (CITES, 2019a). This agreement uses a system of three appendices to categorise those species subject to international trade or controls. Appendix I includes species defined as threatened with extinction by The IUCN Red List, appendix II species are not necessarily threatened with extinction, but policies are required to avoid overexploitation, and appendix III controls trade, as opposed to being a licensing system (CITES, 2019b). Appendices I and II prohibits all international trade including export and re-export of specimens without an authorised license (CITES, 2019b). There are currently 16 species published within protected by CITES, with a further 18 species to be implemented in the forthcoming months (Table 1.1) (CITES, 2019a; CMS, 2019). Like CITES, the CMS contains two Appendices; Appendix I, refers to endangered migratory species, and Appendix II refers to migratory species conserved through agreements between countries (CMS, 2018, 2019). The most recent meetings for the CMS and WGEF were held in 2017, providing information and determining new species assessments for either Appendix I or II (Table 1.1) (CITES, 2019a; CMS, 2019). At the last

WGEF meeting in 2017, 24 stocks across 14 defined areas were assessed for one or more species, resulting in either updated information, updated assessment or both (CITES, 2019a). These key international agreements are implemented by national legislation in individual countries, effecting import and export trade. Most famously, the “Fins Naturally Attached” policy was passed in the EU this provides some of the world’s most effective protection for sharks. This policy was adopted 2003 and amended in 2013, prohibiting the practise shark finning by ensuring all fins are naturally attached upon landing thus increasing the ability to manage and regulate shark fin trade (EUR-Lex, 2016). Prior to 2013, the EU had only required that both the fins and carcasses had to be landed at the same time creating room for IUU fishing. The new amendment to the policy had the potential to have a marked impact on legal shark fishing in Europe, as Spain has been the largest exporter of shark fin to Hong Kong (Clarke, 2004). The UK is also contributing to shark product economy, importing roughly 1,016 tonnes which equates to $3.2 million USD between 2002 and 2012 (Dent and Clarke, 2015). Despite the legal finning regulations applied world-wide, the volume of shark fins found and traded within regional or global markets have not reduced (Worm *et al.*, 2013).

In some jurisdictions, sharks can be more profitable alive than dead. In the Bahamas, shark tourism produces approximately $113.8 million USD annually (Bornatowski, Braga and Barreto, 2018) and a single reef shark can generate $1.9 million USD in its lifetime (Vianna *et al.*, 2012). The establishment of a non-consumptive markets for elasmobranchs has grown exponentially across the globe and are now found in at least 29 countries (Bornatowski, Braga and Barreto, 2018), generating a revenue of roughly $314 million USD annually (Bornatowski, Braga and Barreto, 2018). In 2009, the island of Palau in the Pacific ocean became the first country to create a shark sanctuary, banning shark fishing and the trade of shark items (including fins) (Vianna *et al.*, 2012). Since then, 10 countries have followed suit, realising the economic and ecological importance of protecting and maintaining local elasmobranch populations (The PEW Charitable Trust, 2017). As well as having an ecological importance, these countries have researched the economic values of each shark, discovering the ecotourism industry massively outweighs that of the shark fishing and finning trade (The PEW Charitable Trust, 2017). Despite the economic and

ecological importance, elasmobranchs continue to face a widespread decline, and this is being monitored by The International Union for the Conservation of Nature (IUCN) Red List of Endangered Species™, hereafter referred to as The IUCN Red List.

1.3 Extinction risk assessment

The IUCN was founded in 1948 and has developed to be the largest global conservation network and is the leading authority on the environment and sustainable development (Baillie *et al.*, 2004). One of the most successful projects founded by the IUCN is that of the World Wildlife Fund (WWF). Along with other organisations, the IUCN established the WWF in 1961 as an international fundraising organisation, working to support the conservation movement on a worldwide scale (The IUCN, 2018). The IUCN's approach has created a basis for conservation which is used by many zoos, aquariums and scientific researchers.

The IUCN Red List is the world's most comprehensive information source for the conservation status for all evaluated species worldwide (Baillie *et al.*, 2004). The IUCN Red List is a powerful tool which can aid the direction of conservation and management, including in-situ and ex-situ conservation, and international and national policy (The IUCN, 2010). It adopts the traditional role of identifying species that are at risk of extinction, whilst providing information and analysis on status trends and threats to a species (The IUCN, 2018). It aims to establish the basis from which species status should be monitored, providing a global context to establish conservation management and priorities at a local level, and to continue monitoring the status to identify status change (The IUCN, 2018). The aim is to increase the number of species assessed to 160,000 by 2020 (The IUCN, 2018). As of August 2019, it has evaluated more than 105,700 species, of which 28,000 are threatened with extinction. These include 14% of birds, 25% of mammals, 33% of corals, 34% of conifers and 40% of amphibians (The IUCN, 2018). The total number of species which exist on the planet is not known, however the number of animal species is estimated to be approximately 8.7 million and current taxonomic classification represents only a fraction of this total (Mora *et al.*, 2011).

In order to implement effective conservation methods and achieve the aims of The IUCN Red List, the process of evaluation and the definitions of criteria must be widely understood. Prior to 1994, The IUCN Red List contained a subjective category of threatened species list (The IUCN, 2001; Baillie *et al.*, 2004; Vié *et al.*, 2009). The new system adopted in 1994 made its categories more relevant to conservation. This led to increased recognition by governmental and non-governmental organisations (NGO's) (The IUCN, 2001; Baillie *et al.*, 2004; Vié *et al.*, 2009). The IUCN categories consist of Not Evaluated (NE), Data Deficient (DD), Least Concern (LC), Near Threatened (NT), Vulnerable (VU), Endangered (EN), Critically Endangered (CE), Extinct in the Wild (EW) and Extinct (EX) as seen in Figure 1.1 (The IUCN, 2010). Species threatened with extinction fall into the categories CR, EN and VU, while least concerned species fall under either NT or LC (Figure 1.1) (The IUCN, 2001, 2010). Species listed in DD or NE indicate that there has currently been no assessment of the risk of extinction, however should not be treated as if they were not threatened (Baillie *et al.*, 2004; Vié *et al.*, 2009; The IUCN, 2010). These two categories are either a result of an absence of resources or data, and it could be argued that it is more appropriate to give these categories the same degree and attention as those threatened with extinction (The IUCN, 2010). Liberal use of DD is discouraged and a clear amount of evidence must be provided before assigning DD to a species evaluated (Baillie *et al.*, 2004; Vié *et al.*, 2009; The IUCN, 2010).

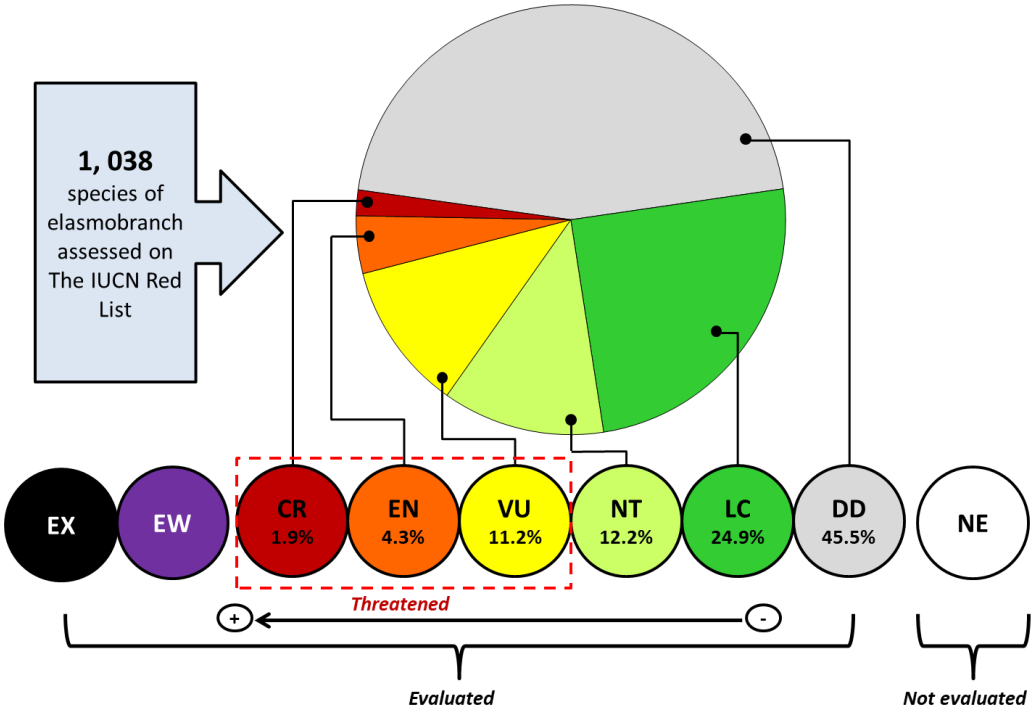

Figure 1.1. An adaptation of The IUCN Red List structure, with the current information available for elasmobranchs (IUCN, 2019)Not Evaluated (NE), Data Deficient (DD), Least Concern (LC), Near Threatened (NT), Vulnerable (VU), Endangered (EN), Critically Endangered (CE), Extinct in the Wild (EW) and Extinct (EX). No elasmobranch species have yet been classified as Extinct (Ex) or Extinct in the Wild (EW) by the IUCN Red List. The current number of Not Evaluated (NE) is not reported.

The evaluation for each species is often estimated with a considerable level of uncertainty due to the natural variation, terminology, research and measurement error that can be found in data available (The IUCN, 2001, 2018). A single global category, as seen in Figure 1.1, must be selected for each species; however there are regional assessments if valid data is available that assess populations (The IUCN, 2001; Dulvy *et al.*, 2014). The factors which categorises a species as threatened with extinction fall under three criteria: 1. reduction in species population size; 2. loss of geographical range and habitat; 3. number of mature individuals. For example, a CR species would have more than or equal to 80% over 10 years (or three generations) reduction, EN will have a more than or equal to 70% reduction and VU will have more than or equal to 50% reduction in population size (The IUCN, 2001, 2018; Dulvy *et al.*, 2014). The IUCN Red List criteria are only applied when evidence concerning numbers trends and distributions can be provided (The IUCN, 2001, 2018; Dulvy *et al.*, 2014). In the case of elasmobranchs, the most recent overall assessment in 2014 shows that a quarter of all sharks and batoids were threatened with extinction (Figure 1.1) (Dulvy *et al.*, 2014).

Currently the IUCN Red List offers the best basis for prioritizing species for conservation action. To further the evaluation efforts and provide a leadership for the conservation of threatened chondrichthyan species, the Shark Specialist Group (SSG) was established in 1991 by the IUCN Species Survival Commission, a dedicated science-based network of volunteer experts with the primary goal of reducing loss of diversity on earth (The IUCN SSG, 2019). The SSG is now one of the largest specialist groups in the IUCN (comprising of 128 active members of volunteer experts) and its aim is to secure management, conservation and, where necessary, the recovery of chondrichthyan species by assembling scientific knowledge and technology to deliver "*knowledge that enables action*" (The IUCN SSG, 2019). The biggest contribution made by the SSG outside of The IUCN Red List has been the recent "*Global Strategy for the Conservation of Sharks and Rays (2015-2025)*" (Bräutigam *et al.*, 2015). As co-chairs, providing knowledge for the planning process, the process also involved a team of experts and NGO's across the globe which aims to save elasmobranch species from extinction by managing fisheries sustainably and ensuring responsible trade and consumption of elasmobranch products (Bräutigam *et al.*, 2015). The fundamental elements within the strategy revolve around improvements in data collection and scientific investigation and an increase in political and financial investment to improve governance frameworks and methods in elasmobranch conservation (Bräutigam *et al.*, 2015).

In order to meet the aims of the Global Strategy for the Conservation of Sharks and Rays by 2025, there is a need for a development of research on those species classified as special interest, including those most threatened with extinction, data deficient or not assessed (Bräutigam *et al.*, 2015). Research areas for improving knowledge to better inform conservation status within The IUCN Red List include; taxonomy, current population trends including size and distribution, life history traits and ecology, human use, threats and actions (IUCN, 2012). Overall, if more research is conducted into these areas, higher numbers of species will be properly assessed, giving correct information to assist government policies and to end overexploitation of elasmobranchs.

1.4 Applied methods of conservation genetics

Conservation genetics is the application of molecular and evolutionary genetics to study biodiversity conservation (Frankham, 2010a). This field of study can be applied to answer multiple questions such as species identification, hybridization, population structures and genetic diversity (Mendonça *et al.*, 2009; Frankham, 2010a; Cruz *et al.*, 2014). Genetic diversity is most commonly defined as a representation of the essential raw material necessary for a species to evolve and adapt to changing environments (Frankham, Ballou and Briscoe, 2004). In order for a species to succeed through time, individuals must conserve enough genetic material in order to adapt under natural environmental pressures, as part of natural selection (Conner and Hartl, 2004; Frankham, 2010a). When a population or entire species begins to lose genetic diversity, often caused by population fragmentation and inbreeding, it becomes vulnerable to extinction risks (Frankham, 2010b). In order to determine how elasmobranchs will overcome large population declines and therefore loss of genetic diversity, regional and species population analyse at a genetic level must be conducted. The techniques applied can be used to investigate DNA at an environmental or population/individual level.

1.4.1 DNA based techniques

DNA (Deoxyribonucleic acid) is defined by the Oxford dictionary (2010) as a "*self-replicating material which is present in nearly all living organisms as the main constituent of chromosomes*". In essence, DNA is the genetic information which equates to the traits, qualities and features that characterise an animal. DNA in animals is found in either the nucleus or mitochondrion organelles and can be used to investigate a number of biological traits about individuals or populations. Nuclear DNA (nDNA) is inherited equally from both parents, while mitochondrial DNA (mtDNA) is generally thought to be maternally inherited (Martin, Naylor and Palumbi, 1992; Frankham, Ballou and Briscoe, 2004). Both nDNA and mtDNA have been used in elasmobranch species for the study of population structure and behaviour (Carrier, Musick and Heithaus, 2004; Stéphan *et al.*, 2014; Le Port *et al.*, 2016; Domingues, Hilsdorf and Gadig, 2017; Larson, Daly-Engel and Phillips, 2017). In elasmobranchs, mtDNA has been shown to evolve slower than any other taxon (Martin,

Naylor and Palumbi, 1992), often leading research towards more nDNA based questions and techniques for taxonomic and population assessment.

To answer any question requiring extensive genetic information the most appropriate markers must first be selected. There are a variety of markers that have been used in elasmobranch research, including Restriction Fragment Length Polymorphisms (RFLPs) (Mendonça *et al.*, 2009), Amplified Fragment Length Polymorphisms (AFLPs) (Zenger *et al.*, 2006; Suárez-Moo *et al.*, 2013), Microsatellites (Griffiths *et al.*, 2010; Gerotto, 2013; Maduna *et al.*, 2014), Single Nucleotide Polymorphisms (SNPs) (Carrier, Heithaus and Simpfendorfer, 2017; Pazmiño *et al.*, 2017; O'Connell *et al.*, 2019) . Due to the advances in technology, methodologies are constantly updated, advanced or replaced to investigate new or existing studied species. With the exception of DNA barcoding, RFLPs, AFLPs, microsatellites and SNPs fall under DNA (or genetic) fingerprinting (Coulson *et al.*, 2011; Domingues *et al.*, 2019). Fingerprinting provides an individual's specific DNA pattern to profile individuals collected from body tissues or fluid in order to identify and distinguish relationships between individuals within one or more populations (Hoelzel, 1998). The techniques within fingerprinting can produce complex results that ultimately help answer questions relating to population dynamics and genetic health.

1.4.2 DNA fingerprinting methodologies

Traditionally, RFLPs were used in mitochondrial DNA (mtDNA) for the genetic analysis on both an individual and population level. They can be used to identify species (Martin, Naylor and Palumbi, 1992; Mendonça *et al.*, 2009), estimate gene diversity (Heist, Graves and Musick, 1995), population structure, and investigate hybridization, gene flow, introgression allopolypoidy and autopolupoidy (Carrier, Musick and Heithaus, 2010). RFLPs use bacterial restriction enzymes to cut specific sequence motifs (usually 4 to 6 bases) from a segment of DNA (Bermingham, Seutin and Ricklefs, 1996). Mutations or changes in the number of nucleotides between individuals can be seen by identifying different fragment patterns in the DNA. The detection of these changes is viewed through agarose or polyacrylamide gels, which separate the fragments by size. There are two methods to complete RFLPs; either to amplify the DNA using a polymerase chain reaction (PCR) and

use restriction enzymes to cut the DNA at specific sequence motifs, or to cut sections of the DNA with restriction enzymes and use a labelled homologous sequence to probe the DNA (Bermingham, Seutin and Ricklefs, 1996; Mendonça *et al.*, 2009). The drawback of RFLPs is that they require a large quantity of DNA and are shown to be time consuming and expensive, with no amplification through polymerase chain reaction (PCR) methods. Although there have been further developments to reduce these disadvantages, RFLPs have generally been replaced by newer techniques which include different marker types (Wolfe and Liston, 1998). A similar technique to RFLPs is Amplified, Fragment Length Polymorphism (AFLPs), which involve selective amplification of restriction fragment sets from genomic DNA by generating double digestion and cutting the DNA with restriction enzymes (Vos *et al.*, 1995). A two phased PCR protocol is conducted within the AFLP approach, and separated either on gels or an automated sequencer (Bonin, Pompanon and Taberlet, 2005). Similar to the RFLPs, AFLPs need a high quantity of DNA and are not as informative as newer fingerprinting techniques. Previously, both RFLPs and AFLPs have been used to analyse populations, including breeding studies and gene mapping (Suárez-Moo *et al.*, 2013) as well as species identification (Zenger *et al.*, 2006).

More recently the RFLP and AFLP techniques have been replaced by microsatellite markers, which build genetic maps of a species with improved success and at a reduced cost. Microsatellites, also known as simple sequence repeats (SSRs) or short tandem repeats (STRs), are tandem repetitive elements of short sequences (usually di-, tri-, or tetra-nucleotide) (Hamada *et al.*, 1984). These short sequences hold variations in the repeat copy number, resulting in a profusion of distinguishable alleles (Avise, 1994; Byrne and Avise, 2012) and have been utilized in both prokaryotic and eukaryotic genomes (Bhargava and Fuentes, 2010). Generally, microsatellites consist of between two and six nucleotides, which display a specific level of polymorphism that is based on the differences in number of repeat motifs between individuals (Bhargava and Fuentes, 2010; Byrne and Avise, 2012). Microsatellite markers rely on a PCR for amplification and types of electrophoresis gel's and/or ABI series genotyping to detect success of the PCR (Ewen *et al.*, 2000). To score the repetitive elements of short sequences (fragments), the microsatellite repeat motifs are observed on electropherograms obtained from multichannel electrophoresis equipment. The

microsatellite repeat motifs are usually fluorescently labelled within the primers for detection with the capillary electrophoresis equipment. The main reason for the popularity change from RFLPs and AFLPs to use of microsatellites was due to the high polymorphism and number of alleles present at a single microsatellite locus, and secondly due to the ability to analyse genotypes by simple PCRs without the use of restriction enzymes (Vignal *et al.*, 2002).

Whole genome mapping and the use of single nucleotide polymorphisms (SNPs) as genetic markers are the most recent development in identifying population structure and variation (Edwards *et al.*, 2007; Vieira *et al.*, 2016). Specifically, SNPs produce high frequency, mutation rates on coding regions of sequenced DNA, making them the overall method of choice for genetic studies (Edwards *et al.*, 2007). The method to aquire SNP markers is often more costly than other traditional methods of reviewing populations as it required large genome sequences of 2 or more individuals to identify difference between regions for primer development (Edwards *et al.*, 2007). Generally, SNPs can provide a more in-depth analysis, often increasing information regarding sub-structures of metapopulations which can be missed in other techniques by providing more variance between samples (Manuzzi *et al.*, 2019). Despite this, microsatellites have been shown to evade DNA corrections that occur during the repair system of coding DNA, meaning a variety of alleles can exist on a single targeted region (Vieira *et al.*, 2016). This is argued to provide more information than SNPs, while this is dependent on the number of markers tested (Vieira *et al.*, 2016).

1.4.3 DNA barcoding

In animals DNA barcoding targets a region within the mtDNA to provide information regarding the species including identification and haplotype numbers. DNA barcoding in all fish genetic research (including elasmobranchs) involves using PCR and sequencing techniques with universal primers which amplify most fish species. The mitochondrial cytochrome c oxidase I (COI) gene for fish identification was first used by Bartlett and Davidson (1991). Since then the technique has increased dramatically with more than 11063 species of fish sequenced at the COI gene (Ward *et al.*, 2005; Steinke and Hanner, 2011; Ward, 2012). FISH-BOL (the Fish Barcode of Life Campaign) is a collaboration around the

globe to sequence around 648 base pair region of the mitochondrial COI gene for all fish and for these to be uploaded into BOLD (the Barcode of Life Data System) (Ward *et al.*, 2005; Steinke and Hanner, 2011). DNA barcoding research shows that the method can discriminate between 98-99% of fish species that have already been examined so far (Holmes et al. 2008). DNA barcoding has been used in a number of forensic methods, including identification of batoid products (Coulson *et al.*, 2011; Cerutti-Pereyra *et al.*, 2012; Hunter, 2016; Bineesh *et al.*, 2017) and identification of shark fins (Holmes, Steinke and Ward, 2009; Fields, Abercrombie, *et al.*, 2015; Steinke *et al.*, 2017; Almerón-Souza *et al.*, 2018).

1.4.4 Sampling collection and storage

The three methods commonly used to collect DNA are referred to as destructive, invasive and non-intrusive sampling. A destructive sampling method ultimately results in an animal being destroyed for the collection of tissue (Taberlet and Luikart, 1999), and researchers are now less likely to choose this method either because of animal welfare and rights, or that the species is too threatened. Invasive sampling often requires the animal to be captured in order to remove blood or tissue (Taberlet and Luikart, 1999). Non-intrusive sampling involves collecting DNA from a source that is left in the environment, or elsewhere, and does not cause disturbance to the animal (Waits and Paetkau, 2005). Table 1.2 has a descriptive list of the sample and sampling methods which can be used for collecting DNA in elasmobranchs. It is advised that as soon as samples have been collected they should be stored in a buffer or 100% ethanol and maintained at temperatures of between -20˚C and -80˚C (Smith *et al.*, 2014). At these temperatures DNA is less likely to decompose and therefore can be analysed over a greater length of time, however, there are many more methods of DNA preservation. This will enable historic samples to be compared with those of the future, demonstrating potential changes over time (Annas, 1993).

Table 1.2. A description of the types of successful sample collection and storage for extraction of DNA from elasmobranchs

Sampling	Method	Condition	Use	F	B	EtOH	Publication
Fin Clip	Invasive	Alive	All	Y	Y	Y	Lewallen, Anderson and Bohonak, 2007
	Destructive	Dead					Clarke *et al.*, 2006
Tail Clip	Invasive	Alive	All	Y	Y	Y	Cerutti-Pereyra *et al.*, 2012
	Destructive	Dead					

Barbs	Invasive	Alive	Batoids	Y	Y	Y	Janse, Kappe and Van Kuijk, 2013
	Destructive	Dead					
Scrub	Non-invasive	Alive	All	N	N	Y	Kashiwagi *et al.*, 2015
	Invasive						
Faecal	Non-invasive	Alive	All	Y	Y	Y	Wetherbee and Gruber, 1993
	Invasive						
Blood	Invasive	Alive	All	Y	Y	N	Lee *et al.*, 2000
	Destructive	Dead					
Internal Organs	Destructive	Dead	All	Y	Y	Y	Wang and Yang, 2004
Muscle Biopsy	Invasive	Alive	All	Y	Y	Y	Chan *et al.*, 2003
	Destructive	Dead					
Oviducal Gland	Destructive	Dead	All	Y	Y	Y	Griffiths *et al.*, 2012

Use, All = all elasmobranchs, F = Frozen, B = Buffer, EtOH = 100% ethanol, Y = Yes, N = No

It is important when studying population genetics that a proportionate number of individuals are sampled (Smith *et al.*, 2014). Population genetics and the theories behind their practical application to conservation can ultimately help reduce the risk of a species becoming endangered (Frankham, 2010a). For example, in *ex-situ* conservation, such as aquariums, it is possible analyse the population dynamics within a captive population and apply the findings to studbook management and breeding programmes.

It is becoming common practise to use genetics to analyse captive populations due to social and governing organisations, such as the European Association of Zoos and Aquariums (EAZA), encouraging better captive management (EAZA, 2017). When keeping species threatened with extinction in captivity, genetic data can investigate and resolve inbreeding or outbreeding depressions, and maintain a genetically healthy captive stock. There are very few studies of the application of genetics for elasmobranch conservation; nevertheless investigations have identified new evidence of multiple paternities in single litters and even parthenogenesis. An example of captive paternity testing is seen in the white-spotted eagle rays *Aetobatus narinari* conducted by Janse et al. (2013). This work was also classified as a non-invasive, whereby the barbs, which can regrow, were removed from individuals. Controversially, this research described the method as an animal friendly, ideal non-invasive sampling technique. However, the implications of removing stings is not fully understood (Janse, Kappe and Van Kuijk, 2013). Kashiwagi *et al.*, (2015) successfully sampled and amplified DNA from manta rays in the wild using a tooth brush attached to an extendable pole, displaying less invasive alternative for sampling batoids.

With multiple sampling methods now available, there is a growing shift towards the use of non-invasive techniques to identify and review new or existing populations. However, to identify an individual, invasive source material is often required. In cases where DNA is

too costly to extract or not available due to natural or human-made decay, alternative methods for species identification prevail. These methods often fall under forensic scenarios, whereby the identification and evidence of the samples collected need to be investigated. An example for elasmobranchs such as sharks could be to investigate ingredients in cooked materials, such as shark fin soup.

1.4.5 Species identification using proteins

In cases of forensic identification, analysing proteins is a more recent developing approach than DNA, as proteins are able to withstand natural and biochemical processes which generally degrade DNA (Virkler and Lednev, 2009), which can also help studies investigate biological age-related information. More specifically, for identification purposes, the protein collagen has been the most successful marker in techniques in much older archaeological and palaeontological specimens that suffer much worse DNA preservation issues (Buckley, 2018). This has been best demonstrated by the creation of a technique called Zooarchaeology by Mass Spectrometry (ZooMS), a method of extracting and visualising archaeological collagen peptide mass fingerprints using mass spectrometry (MS) to achieve faunal identification (Buckley *et al.*, 2009, 2010, 2014; Collins *et al.*, 2010; Buckley, 2018). The use of MS is a technique to analyse biological and chemical molecules based on their mass to charge ratio (*m/z*). The *m/z* is calculated in MS by charging sample molecules through ionization and detecting them in a gaseous phase by the mass analyser once accelerated in an electric field within a vacuum, also known as the flight tube (Harvey, 2016). When analysing proteins, the most common method of mass analyser is the time-of-flight, whereby the time it takes for ions to fly through the flight tube calculates the *m/z* (Harvey, 2016). When analysing proteins such as collagen, Matrix Assisted Laser Desorption/Ionization (MALDI) time-of-flight MS is used to produce peptide mass fingerprints (Harvey, 2016). The study of proteins using these methods is often referred to as proteomics, and is present throughout in most studies that identify species from proteins (Buckley, 2018).

Collagen is defined as the fibrous protein found between various connective tissues that creates the physiological structure and function in skin, bone and cartilage (Gay and

Miller, 1983). Collagen, in its present extracellular matrix, accounts for nearly 30% of the total proteins found in animal bodies, making it good source for biomaterial (Kim, 2012). It has been shown that collagen formation was established over 500 million years ago and is now found across the animal kingdom in various physiological forms (Kadler *et al.*, 1996). Overall, it has been found to survive longer in archaeological samples, and through processes such as cooking, when compared with other bone protein (Buckley *et al.*, 2009, 2010, 2014). More specifically the Collagen Type I (COLI) alpha I (αI) chains has been proven to show enough variation in the amino acid sequence for species identification and phylogenetic inferences (Buckley *et al.*, 2009, 2010, 2014; Collins *et al.*, 2010; Buckley, 2018). The COLI is a molecule made up of three polypeptide alpha (α) chains; of a highly repetitive amino acid sequence, which fold into a triple-helix (Kadler *et al.*, 1996). There are two α1 chains, of identical amino acid sequence, and one slightly shorter α2 chain that has a different sequence (Kadler *et al.*, 1996). Previously, the protein sequences for collagen have been isolated and characterised in shark, batoid and holocephali skin, muscle and cartilage (Nomura, 2004; Hwang *et al.*, 2007; Bae *et al.*, 2008). In studies of protein extraction from waste material provided from the fishing industry, such as the skin of elasmobranchs, the majority of collagens identified were Type I, therefore displaying its availability in decaying products (Kawaguchi, 1985; Yoshimura *et al.*, 2000; Nomura, 2004; Hwang *et al.*, 2007).

Along with the COLI molecule, other collagen and proteins are highly present within the composition of individual samples, such tropomyosin, a skeletal muscle protein (Hayley *et al.*, 2008) and COL Type II (COLII), a mineralized cartilage protein (Coates *et al.*, 1998). Similar to COLI, COLII is composed of three identical peptide chains, that fold to create a triple-helix molecule (Kadler *et al.*, 1996). In chondrichthyans, the evolution of COLII has resulted in the strong and rigid endoskeleton made entirely from calcified cartilage is thought to be a primitive vertebrate characteristic (Coates *et al.*, 1998; Ehrlick, 2015). The calcification matrix of the COLII in chondrichthyes is found throughout the body, as cortical mineralised areas covering cartilage surfaces, such as the vertebra body, remodel a normal cartilage matrix and mineral deposition (Ørvig, 1951). The collagen composition depends on the kind of calcification: (a) early stage mineralisation, also known as globular calcification, (b) granular calcification, (c) areolar calcification, which only found in the vertebral centre of

elasmobranchs (Ørvig, 1951; Kemp and Westrin, 1979). Despite the high presence of collagen in elasmobranchs, there are very few peptide sequences available in order to reference for studies into species identification when searched through the Basic Local Alignment Search Tool (BLAST) on Genbank (www.ncbi.nlm.nih.gov/nucloetide).

Species identification through collagen fingerprinting uses the same principle as DNA barcoding; that there is enough variation among species for successful species identification. Both techniques are generally more reliable than morphological identification, especially in cases where samples are presented in partial forms such as archaeological bone (Harvey, Daugnora and Buckley, 2018), and processed foods (Kim, 2012). Collagen extraction methodologies generally vary depending on the sample type, whether it is skin, bone or cartilage (Gay and Miller, 1983; Nagai and Suzuki, 2000; Buckley *et al.*, 2009). ZooMS has yet to be been widely applied to elasmobranchs and the technique is fairly new. Due to the elasmobranchii cartilaginous form, it is unclear whether the current methods under development will be as successful. For example, previous studies into the extraction of collagen found that the denaturation of bullhead shark *Heterodontus japonicus* collagen in skin took place at approximately 25°C (Nagai and Suzuki, 2000). If the fibres found in shark fin soup denature at the same temperature, then any protein identification post processing would not be possible. However, as this has yet to be fully explored, the success of collagen as a fingerprint/barcoding method in elasmobranchs is unknown. Furthermore, it is unclear whether collagen fingerprinting will prevail over other techniques when reviewing modern samples, as DNA technology is advancing rapidly and successfully identifying processed species using a range of barcoding methods (Fields, Abercrombie, *et al.*, 2015; Cardeñosa *et al.*, 2017; Hellberg, Isaacs and Hernandez, 2019).

1.4.6 Population dynamics and genetic health

Population genetics is the application of molecular and evolutionary genetics to a single species with one or more distinct populations. In genetics a population is defined as a group of existing interbreeding individuals, where population genetics can be applied to study relationships, population dynamics and the effects of environmental pressures (Hedrick, 2011). Conservation genetics is often a key part of population genetics, and both

are fields which have had an exponential growth in use over the past 25 years, turning theory based concepts into an empirical discipline (Ouborg *et al.*, 2010; Hedrick, 2011). Genetic markers have opened the fields of conservation and population genetics. These markers can be used to test population size, dynamics, gene flow, individual relatedness and the extent of fine scale genetic structuring such as genetic differentiation and genealogical relationships (Nei and Kumar, 2000; Schmidt *et al.*, 2009; Carrier, Musick and Heithaus, 2010; Verissimo *et al.*, 2011; Fox *et al.*, 2018; Manuzzi *et al.*, 2019).

Genetic variation is present in natural populations of species in one form or another. Genetic variations are found in the genotype of the individuals and often are reflected in the individual's phenotype, including everything from traits such as hair colour to genetically derived disabilities (Frankham, Ballou and Briscoe, 2004). The fundamental concept of genetic variation is measured by the diversity of gene frequencies, also known as genetic diversity. As well as this, genetic variation occurs via mechanisms such as sexual reproduction and forms of genetic drift in a population. Genetic diversity represents an amount of change in DNA sequences and is altered by natural selection factors such as mutation, selection and genetic drift (Frankham, Ballou and Briscoe, 2004). The presence of heritable genetic diversity determines the ability a population has to respond and overcome selective pressures from environmental changes among other selective forces. If genetic diversity is present some individuals will reproduce.

When measuring genetic diversity it is important to consider the amount of intraspecific diversity. Intraspecific diversity is typically divided into two types: intrapopulation diversity where genetic variation is measured within a single population of one species and interpopulation diversity where genetic variation is measured among multiple populations of one species (Frankham, Ballou and Briscoe, 2004). Interpopulation diversity can occur on significant levels if a population becomes separated and there is no migration between the two, and therefore there is no exchange of gametes or gene flow (Frankham, Ballou and Briscoe, 2004; Frankham, 2010b; Hedrick, 2011). This is known as population fragmentation. The reduction of available habitat and habitat fragmentation which leads to population fragmentation can have negative effects on the interpopulation diversity as gene

flow and gamete exchange as important for maintaining genetic variation (Frankham, 2010b).

Loss of genetic variation can have serious effects on a population; in wild populations with a random mating system it can lead to a level of inbreeding which gives a higher number of closely related individuals than expected (Frankham, Ballou and Briscoe, 2004). This can decrease a species 'viability, vigour or growth in progeny' (Klug et al. 2007) and is also known as inbreeding depression. Inbreeding depression creates individuals which can be vulnerable to disease, have a level of reduction in biological fitness and lower offspring survival rates. Inbreeding depression is a result of increased homozygosity for deleterious alleles. The number of deleterious alleles present in a gene pool is called genetic load. Inbreeding levels can be measured by the Hardy-Weinberg equilibrium and the inbreeding coefficient *F*.

In a balanced or symmetrical population there is random mating, no mutation, migration or selection and therefore the alleles and genotype frequencies remain at equilibrium (Frankham, Ballou and Briscoe, 2004). This is called the Hardy-Weinberg equilibrium and this provides a measure of which populations can be tested against to detect deviation from random mating, selection, levels of inbreeding or outbreeding, and estimating the dominant alleles (Frankham, Ballou and Briscoe, 2004). For example in the case of inbreeding, the expected number of heterozygotes decreases and the number of homozygotes increase relative to the Hardy-Weinberg equilibrium (Baker, 2008; Carrier, Heithaus and Simpfendorfer, 2017). A deficiency in the expected number of heterozygotes relative to Hard-Weinberg equilibrium is the most common tool used to measure the level of non-random mating (Raymond and Rousset, 1995; Rousset, 2008). Hard-Weinberg assumes that there is a large population size, with no migration, equal fertility of parent genotypes and equal fertilizing capacity of gametes, random union of gametes and equal survival of all offspring, no mutation and normal Mendelian segregation of alleles (Raymond and Rousset, 1995; Rousset, 2008). Mendel's law of segregation and recombination explains how genetic characteristics are transmitted from one generation to the next, based

on the principle that genetic material is divided in the reproductive cells, and offspring receives one gene from each of the parent (Baker, 2008; Frankham, 2010b).

Divergence rates (*d*) (also known as mutation rates) estimate the evolutionary rate in which mutations occur within DNA and, refers to the divergence of the mitochondrial DNA (mtDNA) (Frankham, Ballou and Briscoe, 2004). Differences in divergence rates among species and habitats is likely to depend on many factors such as competition (Meyer and Kassen, 2007). Keeney and Heist (2006) found blacktip sharks *Carcharhinus limbatus* have a *d* equalling to 0.43% per million years (MY) of the control region in mtDNA. This was nearly half the *d* found for the hammerhead sharks *Sphyrna lewini*, which was calculated at 0.8% per MY by Duncan *et al.* (2006). These figures have been used as the basis for further studies and reviews into evolutionary divergence, including genetics, speciation and biogeography of chondrichthyans (Boomer *et al.*, 2012, 2013; Castillo-Páez *et al.*, 2014; Bester-van der Merwe and Gledhill, 2015). This is an extremely slow evolutionary divergence, especially when compared to that of mammals. For instance, in Eutherian mammals the calculated divergence rate for mtDNA control region was 82% per MY (Tamura, 1992). Haplotype (*h)* and nucleotide (*π)* diversity is calculated most commonly from sequences found in the mitochondrial DNA (mtDNA). Haplotype and nucleotide diversity is the most commonly published estimate for calculating diversity with a population (Goodall-Copestake, Tarling and Murphy, 2012). There are multiple mtDNA target regions or entire genome analysis to measure these two diversities, however, the most common for elasmobranchs are the control region (CR) and cytochrome c oxidase subunit I (COI) regions.

1.4.7 Reviewing the drivers in elasmobranch genetic diversity

A common application of conservation and population genetics is to assess the genetic diversity within a species to determine the health and therefore ability to overcome environmental pressures. Without sufficient genetic diversity, it is thought that a population or species may not have enough variability to adapt (Spielman *et al.*, 2004). In large populations, individuals typically have extensive genetic diversity and are more resilient to extinction pressures. In contrast species that exist in small populations often have reduced

genetic diversity and can be more prone extinction, likely due to inbreeding effects (Frankham, 2003; Spielman *et al.*, 2004; O'Grady *et al.*, 2006).

The relationships between elasmobranch life history traits and extinction risk, categorised by the IUCN, have previously been analysed by Dulvy et al. (2014). Body size, minimum depth at which individuals spent their time and depth range were found to display positive correlations with extinction risk (IUCN category) (Dulvy *et al.*, 2014). In other marine fishes, empirical evidence suggests that maturation and body size are the best predictors of extinction risk (Reynolds *et al.*, 2005). It is assumed that maximum body size is a predictor of IUCN status and therefore extinction risk because of the relationship between body size and natural rate of shark and ray population increase (Dulvy *et al.*, 2014). Body size is also an indicator of extinction risk in mammals, as impacts from intrinsic and environmental factors sharply increase above a body mass of 3 kilograms (kg) (Cardillo *et al.*, 2005). Just under half of all sharks have a fork body length over 100 cm and weight over 3 kg (Shiffman and Hammerschlag, 2016). For example the blue shark *Prionace glauca* which is the most heavily fished species of shark that reaches average sexual maturity at 220 cm or a body mass of roughly 98.72 kg, has suffered an estimated 60% population decline over 15 years (Baum, Kehler and Myers, 2005; Stevens, 2009). Despite this sharp decline the blue shark is only classified as Near Threatened by The IUCN Red List (Stevens, 2009).

Very little research has been conducted into the genetic diversity and the risk of extinction of elasmobranchs. In a previous study, Spielman *et al.* (2004) found that genetic diversity was lower in 77% of 170 threatened taxa when compared to related non-threatened taxa. However, only 35 species of poikilotherms including fish, amphibians and reptiles were analysed in this data set with no reference to elasmobranch genetic diversity (Spielman *et al.*, 2004). Previously it has been hypothesized that species were driven to extinction before effects on genetic diversity was observed (Lande, 1988). If elasmobranchs follow a similar pattern to the taxa analysed in Spielman *et al.* (2004), there should be significant differences between genetic diversity of threatened and non-threatened species. In addition, if elasmobranch life history traits, such as body size and late maturity are influencing IUCN

category, future management could include such parameters as either a predictor value for extinction risk category and/or genetic diversity in elasmobranchs.

By conducting a literature review of available genetic diversity levels measured microsatellite and mtDNA analysis, it was found that 63 species representing 21 families and seven orders of elasmobranchs from 129 publications contain data for mitochondrial DNA genetic diversity levels (representing 16,424 individual elasmobranchs), microsatellite genetic diversity levels (representing 13,714 individual elasmobranchs), or both (Supplementary Table 1). All species within this literature have an IUCN Red List assessment threat category, and basic scientific information on the life history traits. Genetic heterozygosity values from publications with microsatellite analysis were available in the literature for a total of 46 species and estimates of mitochondrial genetic diversity for a total of 37 species. There are a further ten species that have been studied for genetic diversity levels (either mitochondrial, nuclear using microsatellites or both) classified by the IUCN Red List to be data deficient. Furthermore, within the literature, a total of 20 species have both heterozygosity and mitochondrial genetic diversity levels as seen in Supplementary Table 1.

1.5 Aims and objectives of this thesis

As emphasized throughout this thesis, there are many aspects of elasmobranch biology that are yet to be explored. In particular, there is a lack of data within specific geographical regions, or for a certain species, causing low levels of protection which grants unrestricted IUU fishing and thus population declines. Even when an elasmobranch species has a specific level of protection, a lack of resources prevents independent research into true costs and benefits of protection. As a species threatened with extinction continues to decline, or becomes regionally extinct, *ex-situ* conservation bodies such as zoos and aquariums define their role in conservation as increasing the number of individuals to save a species. With the little information available for the general genetic health of elasmobranchs and the low success of reproducing some elasmobranchs in aquariums, it can be questioned whether aquariums can maintain the same conservation stances as they hold for other taxa such as reintroductions in mammals, birds and amphibians (Toone and Wallace, 1994; Daly *et al.*, 2008; Xia *et al.*, 2014). The key issues that are defined as having insufficient information and/or resources are:

- General biology of some species of elasmobranchs
- Population status including health, connectivity, number of individuals and regional threats
- Regional management implemented by governments; inadequate funds, technical ability and politics inadvertently assisting IUU fishing
- *Ex-situ* conservation management

In order to address some of these key issues, my research focuses on the application of molecular biology techniques in genetics and proteomics to initially explore various methods of species and individual identification (Section II) as well as population genetics (Section III) in elasmobranchs. Genetic and proteomic methods of species identification were applied to elasmobranchs from Morocco because this is an unstudied region which continues to maintain strong trade with the EU despite any prior knowledge of possible IUU fishing. Alternative methods of identifying a species and individuals without the reliance on DNA are also investigated. This includes the use of proteins for species

identification and the use of spot pattern recognition, validating the methods using already developed DNA techniques. To understand population dynamics, the management of a species with different levels of protection across its range are investigated. To address this, novel methods of individual identification and population analysis were used to determine how levels of protection affect health. The use of genetics to manage captive species is also investigated in two large aquariums, and the application of genetics for ex situ conservation of elasmobranchs is reviewed. This thesis presents new findings on basic elasmobranch biology which strengthen the case for targeted conservation action. To meet these aims this thesis is structured as follows:

Section II: Species Identification

Chapter 2. Identifying chondrichthyans in Morocco using DNA

In this chapter, the first investigation into species diversity of chondrichthyans along the Atlantic coast of Morocco is investigated with the use of field and COI barcoding techniques. Field research was conducted over June and July in 2015 and 2016 in collaboration with a non-government organisation (NGO) Fin Fighters. The results of this chapter aim to indicate the current level of chondrichthyan fishing and IUU fishing within the region.

Chapter 3. Extracting proteins for elasmobranch identification

A new protocol is investigated for the identification of elasmobranchs using proteins. This could revolutionise the enforcement of IUCN regulations by confidently identifying processed elasmobranch products, such as those within shark fin soup. The protocol provides the first stages of simulating natural decay and possible identification of elasmobranch proteins in processed foods. It specifically focuses on the collagen regions as a species identifier.

Chapter 4. Identifying individual sharks by non-invasive techniques

This chapter explores the possibility of applying spot pattern recognition to identify elasmobranchs in their early developmental stages. Individual identification is then validated

using microsatellite analysis. This protocol provides a method that can be applied to other populations, or possibly other species, to determine individual identification post hatching.

Section III: Species Genetics

Chapter 5. The investigation of undulate ray population dynamics

In this chapter microsatellite analyse are used to investigate population dynamics of the undulate ray *Raja undulata* across its entire distribution. To complete the research, a new successful method of sample collection was developed which included non-intrusive swabbing of resting rays by scuba divers. Spot pattern recognition of data collected from 2012 to 2018 was used to investigate the relationship between individuals and population size at a single site using capture-mark-recapture methods. Collaboration with The Undulate Ray Project and Sea Life (Merlin Entertainments) is included, as both organisations have applied this research to educate and inform on populations, both at a single-site and across their distribution.

Chapter 6. Using microsatellites to conserve captive sharks

This chapter again uses microsatellite technology to investigate the potential application of genetics in the captive breeding of sharks. The possibility of applying this type of genetic conservation to other species in order to manage captive populations is discussed. These recommendations have been acted upon by Sea Life (Merlin Entertainments) UK and Meeresmuseum, Germany.

Chapter 7. The discovery of twins in three elasmobranch species

In this chapter new information on the reproductive biology of elasmobranchs is provided by discovering twins in three oviparous elasmobranch species. Here we define twins as the case of two individuals within one egg case. This occurrence has only previously been reported in a few other elasmobranch species, and the occurrence has never been genetically investigated. Here, the relationship between these individuals using genetic markers (microsatellites) was analysed and multiple conclusions on why such twins occur are provided.

Chapter 8. Discussion and conclusions

A critical evaluation of the results of this thesis is conducted, assessing its contribution to elasmobranch biology and conservation. This work is concluded with recommendations for further research which is essential to provide a credible scientific basis for future elasmobranch conservation.

1.6 Journal Format

The thesis is presented in the University of Manchester journal format, in accordance with the set rules and regulations. Chapter 4 has been published in the Journal of Fish Biology on the 15th of October 2019. Chapter 7 has been published in PLoS ONE on the 2nd of December 2019. Chapters 5 and 6 have been submitted and are currently under review within the chosen journals. All chapters are presented in the thesis in accordance with the University of Manchester rules and regulations and have been adapted from the thesis style to suit the journal's formatting rules and regulations.

1.7 References

Ahonen, H., Harcourt, R. G. and STOW, A. J. (2009) 'Nuclear and mitochondrial DNA reveals isolation of imperilled grey nurse shark populations (*Carcharias taurus*)', *Molecular Ecology*. Blackwell Publishing Ltd, 18(21), pp. 4409–4421. doi: 10.1111/j.1365-294X.2009.04377.x.

Almerón-Souza, F. *et al.* (2018) 'Molecular Identification of Shark Meat From Local Markets in Southern Brazil Based on DNA Barcoding: Evidence for Mislabeling and Trade of Endangered Species', *Frontiers in Genetics*. Frontiers, 9, p. 138. doi: 10.3389/fgene.2018.00138.

Andreotti, S. *et al.* (2016) 'An integrated mark-recapture and genetic approach to estimate the population size of white sharks in South Africa', *Marine Ecology Progress Series*, 552, pp. 241–253. doi: 10.3354/meps11744.

Annas, G. J. (1993) 'Privacy Rules for DNA Databanks', *JAMA*. American Medical Association, 270(19), p. 2346. doi: 10.1001/jama.1993.03510190102034.

Avise, J. C. (1994) *Molecular Markers, Natural History and Evolution*. Boston, MA: Springer US. doi: 10.1007/978-1-4615-2381-9.

Bae, I. *et al.* (2008) 'Biochemical properties of acid-soluble collagens extracted from the skins of underutilised fishes', *Food Chemistry*. Elsevier, 108(1), pp. 49–54. doi: 10.1016/J.FOODCHEM.2007.10.039.

Baillie, J. E. *et al.* (2004) *A Global Species Assessment*. Edited by J. E. M. Baillie, C. Hilton-Taylor, and S. N. Stuart. The IUCN Species Survival Commission.

Baker, C. S. (2008) 'A truer measure of the market: the molecular ecology of fisheries and wildlife trade.', *Molecular ecology*, 17(18), pp. 3985–98. doi: 10.1111/j.1365-294X.2008.03867.x.

Barnosky, A. D. *et al.* (2011) 'Has the Earth's sixth mass extinction already arrived?', *Nature*. Nature Publishing Group, 471(7336), pp. 51–57. doi: 10.1038/nature09678.

Bartlett, S. E. and Davidson, W. S. (1991) 'Identification of *Thunnus* Tuna Species by the Polymerase Chain Reaction and Direct Sequence Analysis of their Mitochondrial Cytochrome *b* Genes', *Canadian Journal of Fisheries and Aquatic Sciences*. NRC Research Press Ottawa, Canada , 48(2), pp. 309–317. doi: 10.1139/f91-043.

Baum, J. *et al.* (2009) *Sphyrna lewini. The IUCN Red List of Threatened Species*, *e.T39385A10190088.* doi: http://dx.doi.org/10.2305/IUCN.UK.2007.RLTS.T39385A10190088.en.

Baum, J. *et al.* (2015) *Carcharhinus longimanus. The IUCN Red List of Threatened Species*, *e.T39374A85699641.* doi: http://dx.doi.org/10.2305/IUCN.UK.2015.RLTS.T39374A85699641.en.

Baum, J. K. *et al.* (2003) 'Collapse and Conservation of Shark Populations in the Northwest Atlantic', *Science*. American Association for the Advancement of Science, 299(5605), pp. 389–392. doi: 10.1126/science.1079777.

Baum, J. K., Kehler, D. and Myers, R. A. (2005) 'Robust estimates of decline for pelagic shark populations in the northwest Atlantic and Gulf of Mexico', *Fisheries*, 30(10)..

Baum, J. K. and Myers, R. A. (2004) 'Shifting baselines and the decline of pelagic sharks in the Gulf of Mexico', *Ecology Letters*. John Wiley & Sons, Ltd (10.1111), 7(2), pp. 135–145. doi: 10.1111/j.1461-0248.2003.00564.x.

Benjamin, D. *et al.* (2015) 'First Report on Recruits of Bigeye Thresher Shark *Alopias Superciliosus* (Laminiformes: Alopidae) with Largest Birth Size from Indian Waters',

International Journal for Research in Applied Science and Engineering Technology, 1(4).

Bentz, J. *et al.* (2014) 'Shark Diving in the Azores: Challenge and Opportunity', *Tourism in Marine Environments*, 10(1), pp. 71–83. doi: 10.3727/154427314X14056884441789.

Bermingham, E., Seutin, G. and Ricklefs, R. (1996) 'Regional approaches to conservation biology: RFLPs', *DNA sequence, and Caribbean birds*, pp. 104–124.

Bester-van der Merwe, A. E. and Gledhill, K. S. (2015) 'Molecular species identification and population genetics of chondrichthyans in South Africa: current challenges, priorities and progress', *African Zoology*. Taylor & Francis, 50(3), pp. 205–217. doi: 10.1080/15627020.2015.1063408.

Bhargava, A. and Fuentes, F. F. (2010) 'Mutational Dynamics of Microsatellites', *Molecular Biotechnology*. Humana Press Inc, 44(3), pp. 250–266. doi: 10.1007/s12033-009-9230-4.

Bineesh, K. K. *et al.* (2017) 'DNA barcoding reveals species composition of sharks and rays in the Indian commercial fishery', *Mitochondrial DNA Part A*. Taylor & Francis, 28(4), pp. 458–472. doi: 10.3109/19401736.2015.1137900.

Bitalo, D. N. *et al.* (2015) 'Differential gene flow patterns for two commercially exploited shark species, tope (*Galeorhinus galeus*) and common smoothhound (*Mustelus mustelus*) along the south–west coast of South Africa', *Fisheries Research*. Elsevier, 172, pp. 190–196. doi: 10.1016/J.FISHRES.2015.07.003.

Blackburn, D. and Evans, H. (1985) 'The evolution of fetal nutritional adaptations', *Journal of Zoology*.

Blower, D. *et al.* (2012) 'Population genetics of Australian white sharks reveals fine-scale spatial structure, transoceanic dispersal events and low effective population sizes', *Marine Ecology Progress Series*, 455, pp. 229–244. doi: 10.3354/meps09659.

Bonfil, R. (1994) *Overview of world elasmobranch fisheries*. Food and Agriculture Organization of the United Nations.

Bonin, A., Pompanon, F. and Taberlet, P. (2005) 'Use of Amplified Fragment Length Polymorphism (AFLP) Markers in Surveys of Vertebrate Diversity', *Methods in Enzymology*. Academic Press, 395, pp. 145–161. doi: 10.1016/S0076-6879(05)95010-6.

Boomer, J. J. *et al.* (2012) 'Genetic divergence, speciation and biogeography of Mustelus (sharks) in the central Indo-Pacific and Australasia', *Molecular Phylogenetics and Evolution*. Academic Press, 64(3), pp. 697–703. doi: 10.1016/J.YMPEV.2012.05.024.

Boomer, J. J. *et al.* (2013) 'Frequency of Multiple Paternity in Gummy Shark, *Mustelus antarcticus*, and Rig, *Mustelus lenticulatus*, and the Implications of Mate Encounter Rate, Postcopulatory Influences, and Reproductive Mode', *Journal of Heredity*. Narnia, 104(3), pp. 371–379. doi: 10.1093/jhered/est010.

Boomer, J. J. and Stow, A. J. (2010) 'Rapid isolation of the first set of polymorphic microsatellite loci from the Australian gummy shark, *Mustelus antarcticus* and their utility across divergent shark taxa', *Conservation Genetics Resources*. Springer Netherlands, 2(S1), pp. 393–395. doi: 10.1007/s12686-010-9274-6.

Bornatowski, H., Braga, R. R. and Barreto, R. P. (2018) 'Elasmobranchs Consumption in Brazil: Impacts and Consequences', in. Springer, Cham, pp. 251–262. doi: 10.1007/978-3-319-56985-7_10.

Bräutigam, A. *et al.* (2015) *Global Sharks and Rays Initiative Global Priorities for Conserving Sharks and Rays: A 2015- 2025 Strategy*.

Brown, J. L. and Choe, J. C. (2019) 'Behavioral Ecology and Sociobiology', *Encyclopedia of Animal Behavior*. Academic Press, pp. 103–108. doi: 10.1016/B978-0-12-809633-8.20838-8.

Brunnschweiler, J. M. and Ward-Paige, C. A. (2014) 'Shark fishing and tourism', *Oryx*. Cambridge University Press, 48(4), pp. 486–487. doi: 10.1017/S0030605313001312.

Buckley, M. *et al.* (2009) 'Species identification by analysis of bone collagen using matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry', *Rapid Communications in Mass Spectrometry*. John Wiley & Sons, Ltd, 23(23), pp. 3843–3854. doi: 10.1002/rcm.4316.

Buckley, M. *et al.* (2010) 'Distinguishing between archaeological sheep and goat bones using a single collagen peptide', *Journal of Archaeological Science*. Academic Press, 37(1), pp. 13–20. doi: 10.1016/J.JAS.2009.08.020.

Buckley, M. *et al.* (2014) 'Species identification of archaeological marine mammals using collagen fingerprinting', *Journal of Archaeological Science*. Academic Press, 41, pp. 631–641. doi: 10.1016/J.JAS.2013.08.021.

Buckley, M. (2018) 'Zooarchaeology by Mass Spectrometry (ZooMS) Collagen Fingerprinting for the Species Identification of Archaeological Bone Fragments', in *Zooarchaeology in Practice*. Cham: Springer International Publishing, pp. 227–247. doi: 10.1007/978-3-319-64763-0_12.

Burgess, G. H. (2009) *Carcharhinus brevipinna. The IUCN Red List of Threatened Species*, *e.T39368A10182758.* doi: http://dx.doi.org/10.2305/IUCN.UK.2009-2.RLTS.T39368A10182758.en.

Burgess, H. G. and Branstetter, S. (2009) *Carcharhinus limbatus. The IUCN Red List of Threatened Species*, *e.T3851A10124862*. doi: http://dx.doi.org/10.2305/IUCN.UK.2009-2.RLTS.T3851A10124862.en.

Butchart, S. H. M. *et al.* (2010) 'Global biodiversity: indicators of recent declines.', *Science (New York, N.Y.)*. American Association for the Advancement of Science, 328(5982), pp. 1164–8. doi: 10.1126/science.1187512.

Byrne, R. J. and Avise, J. C. (2012) 'Genetic mating system of the brown smoothhound shark (*Mustelus henlei*), including a literature review of multiple paternity in other elasmobranch species', *Marine Biology*. Springer-Verlag, 159(4), pp. 749–756. doi: 10.1007/s00227-011-1851-z.

Cailliet, G. M. *et al.* (2016) *Squatina californica. The IUCN Red List of Threatened Species*, *e.T39328A80671059.* doi: http://dx.doi.org/10.2305/IUCN.UK.2016-2.RLTS.T39328A80671059.en.

Camargo, S. M. *et al.* (2016) 'Structure and Genetic Variability of the Oceanic Whitetip Shark, *Carcharhinus longimanus*, Determined Using Mitochondrial DNA', *PLOS ONE*. Edited by R. Cimmaruta. Public Library of Science, 11(5), p. e0155623. doi: 10.1371/journal.pone.0155623.

Campana, S., Gibson, A. and Fowler, M. (2010) 'Population dynamics of porbeagle in the northwest Atlantic, with an assessment of status to 2009 and projections for recovery', *ICCAT Collect Vol Sci Pap*, 65(6), pp. 2109–2182.

Cardeñosa, D. *et al.* (2017) 'A multiplex PCR mini-barcode assay to identify processed shark products in the global trade', *PLOS ONE*. Edited by M. Hajibabaei. Public Library of Science, 12(10), p. e0185368. doi: 10.1371/journal.pone.0185368.

Cardeñosa, D., Hyde, J. and Caballero, S. (2014) 'Genetic Diversity and Population Structure of the Pelagic Thresher Shark (*Alopias pelagicus*) in the Pacific Ocean: Evidence for Two Evolutionarily Significant Units', *PLoS ONE*. Edited by M. Hart. Public Library of Science, 9(10), p. e110193. doi: 10.1371/journal.pone.0110193.

Cardillo, M. *et al.* (2005) 'Multiple Causes of High Extinction Risk in Large Mammal Species', *Science*, 309.

Cardinale, B. J. *et al.* (2012) 'Biodiversity loss and its impact on humanity', *Nature*. Nature Publishing Group, 486(7401), pp. 59–67. doi: 10.1038/nature11148.

Carlisle, A. B. *et al.* (2015) *Triakis semifasciata. The IUCN Red List of Threatened Species*, *e.T39363A80672743*. doi: http://dx.doi.org/10.2305/IUCN.UK.2015-4.RLTS.T39363A80672743.en.

Carlson, J. *et al.* (2009) 'An update on the status of the sand tiger shark, *Carcharias taurus* in the northwest Atlantic Ocean'.

Carlson, J., Wiley, T. and Smith, K. (2013) *Pristis pectinata (errata version published in 2019). The IUCN Red List of Threatened Species*, *e.T18175A141791261*. doi: http://dx.doi.org/10.2305/IUCN.UK.2013-1.RLTS.T18175A141791261.en.

Carrier, J. C., Heithaus, M. R. and Simpfendorfer, C. A. (2017) *Shark research : emerging technologies and applications for the field and laboratory*.

Carrier, J. C., Musick, J. A. and Heithaus, M. R. (2010) *Sharks and their relatives II : biodiversity, adaptive physiology, and conservation*. CRC Press/Taylor & Francis.

Carrier, J., Musick, J. and Heithaus, M. (2004) *Biology of Sharks and Biology of Marine Birds*.

Castillo-Páez, A. *et al.* (2014) 'Strong Population Structure and Shallow Mitochondrial Phylogeny in the Banded Guitarfish, *Zapteryx exasperata* (Jordan y Gilbert, 1880), from the Northern Mexican Pacific', *Journal of Heredity*. Narnia, 105(1), pp. 91–100. doi: 10.1093/jhered/est067.

Castro, J. (2013) 'A primer on shark reproduction for aquarists', *Reproduction of Marine Life, Birth of New Life! Investigating the Mysteries of Reproduction*.

Ceballos, G. *et al.* (2015) 'Accelerated modern human–induced species losses: Entering the sixth mass extinction', *Science Advances*. American Association for the Advancement of Science, 1(5), p. e1400253. doi: 10.1126/sciadv.1400253.

Ceballos, G., Ehrlich, P. R. and Dirzo, R. (2017) 'Biological annihilation via the ongoing sixth mass extinction signaled by vertebrate population losses and declines.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 114(30), pp. E6089–E6096. doi: 10.1073/pnas.1704949114.

Cerutti-Pereyra, F. *et al.* (2012) 'Identification of Rays through DNA Barcoding: An Application for Ecologists', *PLoS ONE*. Edited by S.-O. Kolokotronis. Public Library of Science, 7(6), p. e36479. doi: 10.1371/journal.pone.0036479.

Chan, R. *et al.* (2003) 'Application of DNA-based techniques for the identification of whaler sharks (Carcharhinus spp.) caught in protective beach meshing and by recreational', *Aquatic Communications*.

Chapman, D. D. *et al.* (2004) 'Predominance of genetic monogamy by females in a hammerhead shark, *Sphyrna tiburo*: implications for shark conservation', *Molecular Ecology*, 13(7), pp. 1965–1974. doi: 10.1111/j.1365-294X.2004.02178.x.

Chapman, D. D. *et al.* (2007) 'Virgin birth in a hammerhead shark', *Biology Letters*. The Royal SocietyLondon, 3(4), pp. 425–427. doi: 10.1098/rsbl.2007.0189.

Chapman, D. D. *et al.* (2011) 'Genetic Diversity Despite Population Collapse in a Critically Endangered Marine Fish: The Smalltooth Sawfish (*Pristis pectinata*)', *Journal of Heredity*, 102(6), pp. 643–652. doi: 10.1093/jhered/esr098.

Chapman, D. D. *et al.* (2013) 'The behavioural and genetic mating system of the sand tiger shark, *Carcharias taurus*, an intrauterine cannibal.', *Biology letters*. The Royal Society, 9(3), p. 20130003. doi: 10.1098/rsbl.2013.0003.

Chevolot, M. *et al.* (2005) 'Characterization and isolation of DNA microsatellite primers in *Raja clavata* L. (thornback ray, Rajidae)', *Molecular Ecology Notes*. John Wiley & Sons, Ltd (10.1111), 5(2), pp. 427–429. doi: 10.1111/j.1471-8286.2005.00951.x.

Chevolot, M. *et al.* (2006) 'Population structure of the thornback ray (*Raja clavata* L.) in British waters', *Journal of Sea Research*. Elsevier, 56(4), pp. 305–316. doi: 10.1016/J.SEARES.2006.05.005.

Chevolot, M. *et al.* (2008) 'Temporal changes in allele frequencies but stable genetic diversity over the past 40 years in the Irish Sea population of thornback ray, *Raja clavata*', *Heredity*. Nature Publishing Group, 101(2), pp. 120–126. doi: 10.1038/hdy.2008.36.

CITES (2019a) 'Consideration of Proposals for Amendment of Appendices I and II', in *CoP18 Prop. 44*. Colombo, Sri Lanka. Available at: https://cites.org/sites/default/files/eng/cop/18/prop/19032019/E-CoP18-Prop-44.pdf.

CITES (2019b) *How CITES works | CITES*, *Convention on International Trade in Endangered Species of Wild Fauna and Flora*. Available at: https://www.cites.org/eng/disc/how.php

Clarke, S. (2004) *Shark Product Trade in Hong Kong and Mainland China and Implementation of the CITES Shark Listings*, *TRAFFIC*. Hong Kong, China.

Clarke, S. C. *et al.* (2006) 'Identification of Shark Species Composition and Proportion in the Hong Kong Shark Fin Market Based on Molecular Genetics and Trade Records', *Conservation Biology*. John Wiley & Sons, Ltd (10.1111), 20(1), pp. 201–211. doi: 10.1111/j.1523-1739.2005.00247.x.

CMS (2018) *Memorandum of understanding on the conservation of migratory sharks*, *3rd Meeting, Monaco*.

CMS (2019) *Appendix I & II of CMS*, *Convention on the Conservation of Migratory Species of Wild Animals*. Available at: https://www.cms.int/en/page/appendix-i-ii-cms.

Coates, M. I. *et al.* (1998) 'Spines and tissues of ancient sharks', *Nature*. Nature Publishing Group, 396(6713), pp. 729–730. doi: 10.1038/25467.

Coelho, R. *et al.* (2009) *Raja undulata. The IUCN Red List of Threatened Species*, *e.T161425A5420694.* doi: http://dx.doi.org/10.2305/IUCN.UK.2009-2.RLTS.T161425A5420694.en.

Collins, M. *et al.* (2010) 'ZooMS: the collagen barcode and fingerprints', *Spectroscopy Europe*, 22(2).

Compagno, L. J. (1984) *Sharks of the world / 2. Carcharhiniformes.* Food and Agriculture Organization of the United Nations.

Compagno, L. J. V. (1977) 'Phyletic Relationships of Living Sharks and Rays'. Narnia, 17(2), pp. 303–322. doi: 10.1093/icb/17.2.303.

Compagno, L. J. V., Pogonoski, J. and Pollard, D. (2009) *Glyphis glyphis. The IUCN Red List of Threatened Species*, *e.T39379A10221801.* doi: http://dx.doi.org/10.2305/IUCN.UK.2009-2.RLTS.T39379A10221801.en.

Conner, J. K. and Hartl, D. L. (2004) 'A primer of ecological genetics.', *A primer of ecological genetics.* Sinauer Associates Incorporated.

Conrath, C. (2009) *Mustelus canis. The IUCN Red List of Threatened Species*, *e.T39359A10215463.* doi: http://dx.doi.org/10.2305/IUCN.UK.2005.RLTS.T39359A10215463.en.

Cook, S. F. and Compagno, L. J. V. (2009) *Hexanchus griseus. The IUCN Red List of Threatened Species*, *e.T10030A3155348*. doi: http://dx.doi.org/10.2305/IUCN.UK.2005.RLTS.T10030A3155348.en.

Corrigan, S., Kacev, D. and Werry, J. (2015) 'A case of genetic polyandry in the shortfin mako *Isurus oxyrinchus*', *Journal of Fish Biology*. John Wiley & Sons, Ltd (10.1111), 87(3), pp. 794–798. doi: 10.1111/jfb.12743.

Cortés, E. *et al.* (2006) *Sphyrna tiburo. The IUCN Red List of Threatened Species*, *e.T39387A2921446.* doi: http://dx.doi.org/10.2305/IUCN.UK.2016-2.RLTS.T39387A2921446.en.

Costello, M.J. and Chaudhary, C., 2017. Marine biodiversity, biogeography, deep-sea gradients, and conservation. *Current Biology*, *27*(11), pp.R511-R527.

Coulson, M W *et al.* (2011) 'DNA barcoding of Canada's skates.', *Molecular ecology resources*, 11(6), pp. 968–78. doi: 10.1111/j.1755-0998.2011.03034.x.

Coulson, M. W. *et al.* (2011) 'DNA barcoding of Canada's skates', *Molecular Ecology Resources*. John Wiley & Sons, Ltd (10.1111), 11(6), pp. 968–978. doi: 10.1111/j.1755-0998.2011.03034.x.

Cruz, V. P. *et al.* (2014) 'First identification of interspecies hybridization in the freshwater stingrays', *Conservation Genetics*. doi: 10.1007/s10592-014-0642-8.

Daly-Engel, T. S. *et al.* (2006) 'Assessment of multiple paternity in single litters from three species of carcharhinid sharks in Hawaii', *Environmental Biology of Fishes*, 76(2–4), pp. 419–424. doi: 10.1007/s10641-006-9008-5.

Daly-Engel, T. S. *et al.* (2007) 'Frequency of multiple paternity in an unexploited tropical population of sandbar sharks (*Carcharhinus plumbeus*)', *Canadian Journal of Fisheries and Aquatic Sciences*, 64(2), pp. 198–204. doi: 10.1139/f07-005.

Daly, G. *et al.* (2008) 'Reintroduction of the Green and Golden Bell Frog *Litoria aurea* to Pambula on the south coast of New South Wales', *Australian Zoologist*. Royal Zoological Society of NSW , 34(3), pp. 261–270. doi: 10.7882/AZ.2008.003.

Dent, F. and Clarke, S. (2015) *State of the global market for shark products Technical Coordinator-Sharks and Bycatch ABNJ (Common Oceans) Tuna Project Western and Central Pacific Fisheries Commission Pohnpei, The Federated States of Micronesia*. Rome.

Dibattista, J. D. *et al.* (2008) 'Are indirect genetic benefits associated with polyandry? Testing predictions in a natural population of lemon sharks', *Molecular Ecology*. John Wiley & Sons, Ltd (10.1111), 17(3), pp. 783–795. doi: 10.1111/j.1365-294X.2007.03623.x.

DING, S.-X. *et al.* (2009) 'Characterization of 12 polymorphic microsatellite loci for the whitespotted bamboo shark (*Chiloscyllium plagiosum* Bennett)', *Molecular Ecology Resources*. Blackwell Publishing Ltd, 9(5), pp. 1398–1400. doi: 10.1111/j.1755-0998.2009.02676.x.

Dodd, J. M. (1983) *Reproduction in Cartilaginous Fishes (Chondrichthyes)*, *Fish Physiology*. Academic Press.

Domingues, R. R. *et al.* (2019) 'Use of mucus as a non-invasive sampling method for DNA barcoding of stingrays and skates (batoid elasmobranchs)', *Journal of Fish Biology*, 94(3), pp. 512–516. doi: 10.1111/jfb.13919.

Domingues, R. R., Hilsdorf, A. W. S. and Gadig, O. B. F. (2017) 'The importance of considering genetic diversity in shark and ray conservation policies', *Conservation Genetics*. Springer Netherlands, pp. 1–25. doi: 10.1007/s10592-017-1038-3.

Dostalova, A. *et al.* (2009) *Rhizoprionodon terraenovae. The IUCN Red List of Threatened Species*, *e.T39382A10225086*. doi: http://dx.doi.org/10.2305/IUCN.UK.2009-2.RLTS.T39382A10225086.en.

Dudgeon, C. L. *et al.* (2006) 'Polymorphic microsatellite loci for the zebra shark *Stegostoma fasciatum*', *Molecular Ecology Notes*. Blackwell Publishing Ltd, 6(4), pp. 1086–1088. doi: 10.1111/j.1471-8286.2006.01442.x.

Dudgeon, C. L., Simpfendorfer, C. and Pillans, R. D. (2016) *Stegostoma fasciatum. The IUCN Red List of Threatened Species*, *e.T41878A68645890*. doi: http://dx.doi.org/10.2305/IUCN.UK.2016-3.RLTS.T41878A68645890.en.

Dulvy, N. K. *et al.* (2006) *Dipturus batis. The IUCN Red List of Threatened Species*, *e.T39397A10198950*. doi: http://dx.doi.org/10.2305/IUCN.UK.2006.RLTS.T39397A10198950.en.

Dulvy, N. K. *et al.* (2008) 'You can swim but you can't hide: the global status and conservation of oceanic pelagic sharks and rays', *Aquatic Conservation: Marine and Freshwater Ecosystems*, 18(5), pp. 459–482. doi: 10.1002/aqc.975.

Dulvy, N. K. *et al.* (2014) 'Extinction risk and conservation of the world's sharks and rays.', *eLife*. eLife Sciences Publications, Ltd, 3(e00590), p. e00590. doi: 10.7554/eLife.00590.

Dulvy, N. K. *et al.* (2015) *Raja maderensis. The IUCN Red List of Threatened Species*, *e.T161459A48939762*. doi: http://dx.doi.org/10.2305/IUCN.UK.2015-1.RLTS.T161459A48939762.en.

Duncan, K. M. *et al.* (2006) 'Global phylogeography of the scalloped hammerhead shark (Sphyrna lewini)', *Molecular Ecology*. John Wiley & Sons, Ltd (10.1111), 15(8), pp. 2239–2251. doi: 10.1111/j.1365-294X.2006.02933.x.

EAZA (2017) *EAZA Population Management Manual*.

Edwards, D. *et al.* (2007) 'What Are SNPs?', in *Association Mapping in Plants*. New York, NY: Springer New York, pp. 41–52. doi: 10.1007/978-0-387-36011-9_3.

Ehrlick, H. (2015) *Biological Materials of Marine Origin*. Volume 4. Edited by N. G. Gorb and C. Albrecht. Germany: Springer. doi: 10.1007/978-94-007-5730-1.

Ellis, J. *et al.* (2007) *Raja montagui. The IUCN Red List of Threatened Species*, *e.T63146A12623141*. doi: http://dx.doi.org/10.2305/IUCN.UK.2007.RLTS.T63146A12623141.en.

Ellis, J. *et al.* (2009) *Scyliorhinus canicula. The IUCN Red List of Threatened Species*, *e.T161399A5415204.* doi: http://dx.doi.org/10.2305/IUCN.UK.2009-2.RLTS.T161399A5415204.en.

Ellis, J. (2016) *Raja clavata. The IUCN Red List of Threatened Species*, *e.T39399A103110667.* doi: http://dx.doi.org/10.2305/IUCN.UK.2016-3.RLTS.T39399A103110667.en.

Ellis, J., Dulvy, N. and Walls, R. (2015) *Leucoraja naevus. The IUCN Red List of Threatened Species*, *e.T161626A48949434.* doi: http://dx.doi.org/10.2305/IUCN.UK.2015-1.RLTS.T161626A48949434.en.

Engelhaupt, E. (2007) 'Farming the Deep Blue Sea', *Environmental Science and Technology*, pp. 4188–4191.

EUR-Lex (2016) *Report From The Commission To The European Parliament and The Council on the operation of Council Regulation (EC) No 1185/2003 on the removal of fins of sharks on board vessels, as amended by Regulation (EU) No 605/2013, and on the international developments in this field*. European Commission.

Ewen, K. R. *et al.* (2000) 'Identification and Analysis of Error Types in High-Throughput Genotyping', *The American Journal of Human Genetics*, 67(3), pp. 727–736. doi: 10.1086/303048.

Farrell, E. D. *et al.* (2014) 'Multiple paternity in the starry smooth-hound shark *Mustelus asterias* (Carcharhiniformes: Triakidae)', *Biological Journal of the Linnean Society*, 111(1), pp. 119–125. doi: 10.1111/bij.12179.

Farrugia, T. J. *et al.* (2016) *Pseudobatos productus. The IUCN Red List of Threatened Species*, *e.T60171A104004394.* doi: http://dx.doi.org/10.2305/IUCN.UK.2016-3.RLTS.T60171A104004394.en.

Feldheim, K. A. *et al.* (2007) 'Polymorphic microsatellite markers for studies of the conservation and reproductive genetics of imperilled sand tiger sharks (*Carcharias taurus*)', *Molecular Ecology Notes*. Blackwell Publishing Ltd, 7(6), pp. 1366–1368. doi: 10.1111/j.1471-8286.2007.01888.x.

Feldheim, Kevin A. *et al.* (2010) 'Genetic tools to support the conservation of the endangered smalltooth sawfish, *Pristis pectinata*', *Conservation Genetics Resources*. Springer Netherlands, 2(1), pp. 105–113. doi: 10.1007/s12686-010-9175-8.

Feldheim, K. A. *et al.* (2010) 'Shark Virgin Birth Produces Multiple, Viable Offspring', *Journal of Heredity*. Narnia, 101(3), pp. 374–377. doi: 10.1093/jhered/esp129.

Feldheim, K. A., Gruber, S. H. and Ashley, M. V. (2002) 'The breeding biology of lemon sharks at a tropical nursery lagoon', *Proceedings of the Royal Society of London. Series B: Biological Sciences*. The Royal Society, 269(1501), pp. 1655–1661. doi: 10.1098/rspb.2002.2051.

Fergusson, I., Compagno, L. J. V. and Marks, M. (2009) *Carcharodon carcharias. The IUCN Red List of Threatened Species*, *e.T3855A10133872.* doi: http://dx.doi.org/10.2305/IUCN.UK.2009-2.RLTS.T3855A10133872.en.

Fields, A. T., Abercrombie, D. L., *et al.* (2015) 'A Novel Mini-DNA Barcoding Assay to Identify Processed Fins from Internationally Protected Shark Species', *PLOS ONE*. Edited by A. Stow. Public Library of Science, 10(2), p. e0114844. doi: 10.1371/journal.pone.0114844.

Fields, A. T., Feldheim, K. A., *et al.* (2015) 'Facultative parthenogenesis in a critically endangered wild vertebrate', *Current Biology*. Cell Press, 25(11), pp. R446–R447. doi: 10.1016/J.CUB.2015.04.018.

Finucci, B. and Kyne, P. M. (2018) *Mustelus lenticulatus. The IUCN Red List of Threatened Species*, *e.T39356A116736541.* doi: http://dx.doi.org/10.2305/IUCN.UK.2018-2.RLTS.T39356A116736541.en.

Fitzpatrick, S. *et al.* (2011) 'Development and characterization of 10 polymorphic microsatellite loci for the blue shark, *Prionace glauca*, and their cross shark-species

amplification', *Conservation Genetics Resources*. Springer Netherlands, 3(3), pp. 523–527. doi: 10.1007/s12686-011-9395-6.

Fordham, S. *et al.* (2016) *Squalus acanthias. The IUCN Red List of Threatened Species*, *e.T91209505A2898271*. doi: http://dx.doi.org/10.2305/IUCN.UK.2016-1.RLTS.T91209505A2898271.en.

Fowler, S. L. (2009) *Cetorhinus maximus. The IUCN Red List of Threatened Species*, *e.T4292A10763893*. doi: http://dx.doi.org/10.2305/IUCN.UK.2005.RLTS.T4292A10763893.en.

Fowler, S. L. and Cavanagh, R. (2005) *Sharks, rays and chimaeras : the status of the Chondrichthyan fishes: status survey*. IUCN.

Fox, G. *et al.* (2018) 'Bespoke markers for ex-situ conservation : application , analysis and challenges in the assessment of a population of endangered undulate rays', *Jzar*, 6(2), pp. 50–56.

Frankham, R. (2003) 'Genetics and conservation biology', *Comptes Rendus Biologies*. Elsevier Masson, 326, pp. 22–29. doi: 10.1016/S1631-0691(03)00023-4.

Frankham, R. (2010a) 'Challenges and opportunities of genetic approaches to biological conservation', *Biological Conservation*. Elsevier, 143(9), pp. 1919–1927. doi: 10.1016/J.BIOCON.2010.05.011.

Frankham, R. (2010b) 'Inbreeding in the wild really does matter', *Heredity*, 104(2), pp. 124–124. doi: 10.1038/hdy.2009.155.

Frankham, R., Ballou, J. D. (2004) *A primer of conservation genetics*. Cambridge University Press.

Gallagher, A. J. and Hammerschlag, N. (2011) 'Global shark currency: the distribution, frequency, and economic value of shark ecotourism', *Current Issues in Tourism*. Routledge , 14(8), pp. 797–812. doi: 10.1080/13683500.2011.585227.

Gay, S. and Miller, E. J. (1983) 'Overview: What Is Collagen, What Is Not', *Ultrastructural Pathology*. Taylor & Francis, 4(4), pp. 365–377. doi: 10.3109/01913128309140589.

Geraghty, P. T. *et al.* (2013) 'Population Expansion and Genetic Structure in *Carcharhinus brevipinna* in the Southern Indo-Pacific', *PLoS ONE*. Edited by N. Salamin. Public Library of Science, 8(9), p. e75169. doi: 10.1371/journal.pone.0075169.

Gerotto, V. (2013) *Genetic variability and differentiation in the Mediterranean endemic starry ray Raja asterias (Delaroche, 1809)*. Biologia marina [LM-DM270] - Ravenna.

Gilman, E., Brothers, N. and Kobayashi, D. R. (2005) 'Principles and approaches to abate seabird by-catch in longline fisheries', *Fish and Fisheries*. Blackwell Science Ltd, 6(1), pp. 35–49. doi: 10.1111/j.1467-2679.2005.00175.x.

Giresi, M. *et al.* (2012) 'Isolation and characterization of microsatellite markers for the blacknose shark, *Carcharhinus acronotus*', *Conservation Genetics Resources*. Springer Netherlands, 4(1), pp. 141–145. doi: 10.1007/s12686-011-9494-4.

Goodall-Copestake, W. P., Tarling, G. A. and Murphy, E. J. (2012) 'On the comparison of population-level estimates of haplotype and nucleotide diversity: a case study using the gene cox1 in animals', *Heredity*. Nature Publishing Group, 109(1), pp. 50–56. doi: 10.1038/hdy.2012.12.

Grant, R. *et al.* (1993) 'Reproduction and embryonic development of the sand tiger shark, *Odontaspis taurus* (Rafinesque)', *Fishery Bulletin*, 81(2), pp. 201–225.

Griffiths, A. M. *et al.* (2010) 'Molecular markers reveal spatially segregated cryptic species in a critically endangered fish, the common skate (*Dipturus batis*).', *Proceedings. Biological sciences / The Royal Society*, 277(1687), pp. 1497–503. doi: 10.1098/rspb.2009.2111.

Griffiths, A. M. *et al.* (2012) 'First analysis of multiple paternity in an oviparous shark, the small-spotted catshark (*Scyliorhinus canicula* L.)', *Journal of Heredity*. Oxford University Press, 103(2), pp. 166–173. doi: 10.1093/jhered/esr112.

Griffiths, A. M. *et al.* (2013) 'DNA barcoding unveils skate (Chondrichthyes: Rajidae) species diversity in "ray" products sold across Ireland and the UK', *PeerJ*. PeerJ Inc., 1, p. e129. doi: 10.7717/peerj.129.

Grigg, G. C. (1970) 'Use of the first gill slits for water intake in a shark', *Journal of Experimental Biology*, 59(3), pp. 569–574.

Gubili, C. *et al.* (2009) 'Concordance of genetic and fin photo identification in the great white shark, *Carcharodon carcharias*, off Mossel Bay, South Africa', *Marine Biology*. Springer-Verlag, 156(10), pp. 2199–2207. doi: 10.1007/s00227-009-1233-y.

Gubili, C. *et al.* (2012) *Global perspectives on the biology and life history of the white shark*. CRC Press.

Haas, A. R., Fedler, T. and Brooks, E. J. (2017) 'The contemporary economic value of elasmobranchs in The Bahamas: Reaping the rewards of 25 years of stewardship and conservation', *Biological Conservation*. Elsevier, 207, pp. 55–63. doi: 10.1016/J.BIOCON.2017.01.007.

Hamada, H. *et al.* (1984) 'Characterization of genomic poly(dT-dG).poly(dC-dA) sequences: structure, organization, and conformation.', *Molecular and cellular biology*. American Society for Microbiology Journals, 4(12), pp. 2610–21. doi: 10.1128/mcb.4.12.2610.

Hamlett, W. (2001) 'Reproduction in Fish. Encyclopaedia of Life Sciences'.

Harvey, V. L. (2016) *Proteomic Analyses of Sub-Fossil Vertebrate Remains from the Cayman Islands | Research Explorer | The University of Manchester*. University of Manchester.

Harvey, V. L., Daugnora, L. and Buckley, M. (2018) 'Species identification of ancient Lithuanian fish remains using collagen fingerprinting', *Journal of Archaeological Science*. Academic Press, 98, pp. 102–111. doi: 10.1016/J.JAS.2018.07.006.

Harvey, V. L., Keating, J. N. and Buckley, M. (2019) *Collagen Type 1 sequence diversity and evolution in fishes*. University of Manchester.

Hayley, M. *et al.* (2008) 'Shark skeletal muscle tropomyosin is a phosphoprotein', *Journal of Muscle Research and Cell Motility*. Springer Netherlands, 29(2–5), pp. 101–107. doi: 10.1007/s10974-008-9143-z.

Hedrick, P. W. (2011) *Genetics of populations*. Jones and Bartlett Publishers.

Heine, C. (1992) *Mechanics of flapping fin locomotion in the cownose ray, Rhinoptera bonasus (Elasmobranchii: Myliobatidae).* Duke University .

Heist, E. J., Graves, J. E. and Musick, J. A. (1995) 'Population Genetics of the Sandbar Shark (*Carcharhinus plumbeus*) in the Gulf of Mexico and Mid-Atlantic Bight', *Copeia*, 1995(3), p. 555. doi: 10.2307/1446752.

Heist, E., Musick, J. and Graves, J. E. (1996) 'Mitochondrial DNA Diversity And Divergence Among Sharpnose Sharks, *Rhizoprionodon Terraenovae*, From The Gulf Of Mexico And Mid-Atlantic Bight', *Fishery Bulletin*, 94(4).

Hellberg, R. S., Isaacs, R. B. and Hernandez, E. L. (2019) 'Identification of shark species in commercial products using DNA barcoding', *Fisheries Research*. Elsevier, 210, pp. 81–88. doi: 10.1016/J.FISHRES.2018.10.010.

Helyar, S. *et al.* (2011) 'New microsatellite loci for the longnose velvet dogfish *Centroselachus crepidater* (Squaliformes: Somniosidae) and other deep sea sharks', *Conservation Genetics Resources*. Springer Netherlands, 3(1), pp. 173–176. doi: 10.1007/s12686-010-9316-0.

Hernández, S. *et al.* (2014) 'Evidence for multiple paternity in the school shark *Galeorhinus galeus* found in New Zealand waters', *Journal of Fish Biology*, 85(5), pp. 1739–1745. doi: 10.1111/jfb.12490.

Heupel, M. (2009) *Carcharhinus melanopterus. The IUCN Red List of Threatened Species*, *e.T39375A10219032.* doi: http://dx.doi.org/10.2305/IUCN.UK.2009-2.RLTS.T39375A10219032.en.

Hoelzel, A. R. (1998) 'Genetic structure of cetacean populations in sympatry, parapatry, and mixed assemblages: implications for conservation policy', *Journal of Heredity*. Narnia, 89(5), pp. 451–458. doi: 10.1093/jhered/89.5.451.

Holmes, B. H., Steinke, D. and Ward, R. D. (2009) 'Identification of shark and ray fins using DNA barcoding', *Fisheries Research*. Elsevier, 95(2–3), pp. 280–288. doi: 10.1016/J.FISHRES.2008.09.036.

Hooper, D. U. *et al.* (2012) 'A global synthesis reveals biodiversity loss as a major driver of ecosystem change', *Nature*. Nature Publishing Group, 486(7401), pp. 105–108. doi: 10.1038/nature11118.

Hunter, K. (2016) 'The Development of Molecular Techniques for the Conservation of Captive Elasmobranchs', pp. 0–59.

Huveneers, C. and Ishihara, H. (2016) *Hemitrygon akajei. The IUCN Red List of Threatened Species*, *e.T60148A104113240*. doi: http://dx.doi.org/10.2305/IUCN.UK.2016-3.RLTS.T60148A104113240.en.

Hwang, J.-H. *et al.* (2007) 'Purification and characterization of molecular species of collagen in the skin of skate (*Raja kenojei*)', *Food Chemistry*. Elsevier, 100(3), pp. 921–925. doi: 10.1016/J.FOODCHEM.2005.10.046.

IUCN (2012) 'Research Needed Classification Scheme (Version 2.0)', pp. 3–4.

IUCN (2014) *A quarter of sharks and rays threatened with extinction | IUCN*, *International Union of the Conservation of Nature*. Available at: https://www.iucn.org/content/quarter-sharks-and-rays-threatened-extinction

Janse, M., Kappe, A. L. and Van Kuijk, B. L. M. (2013) 'Paternity testing using the poisonous sting in captive white-spotted eagle rays *Aetobatus narinari*: a non-invasive tool for captive sustainability programmes', *Journal of Fish Biology*, 82(3), pp. 1082–1085. doi: 10.1111/jfb.12038.

Johnson, G. J., Pillans, R. D. and Stevens, J. D. (2019) *Carcharhinus tilstoni. The IUCN Red List of Threatened Species*, *e.T41739A68613771*. doi: http://dx.doi.org/10.2305/IUCN.UK.2019-1.RLTS.T41739A68613771.en.

Joung, S.-J. *et al.* (1996) 'The whale shark, *Rhincodon typus*, is a livebearer: 300 embryos found in one "megamamma" supreme*', *Environmental Biology of Fishes*, 46, pp. 219–223.

Kadler, K. E. *et al.* (1996) 'Collagen fibril formation.', *The Biochemical journal*. Portland Press Limited, 316 (Pt 1)(1), pp. 1–11. doi: 10.1042/bj3160001.

Karl, S. A. *et al.* (2011) 'Phylogeography and conservation of the bull shark (*Carcharhinus leucas*) inferred from mitochondrial and microsatellite DNA', *Conservation Genetics*. Springer Netherlands, 12(2), pp. 371–382. doi: 10.1007/s10592-010-0145-1.

Kashiwagi, T. *et al.* (2012) 'Development and characterization of ten microsatellite loci for the reef manta ray *Manta alfredi*', *Conservation Genetics Resources*. Springer Netherlands, 4(4), pp. 1055–1058. doi: 10.1007/s12686-012-9705-7.

Kashiwagi, T. *et al.* (2015) 'Evaluating manta ray mucus as an alternative DNA source for population genetics study: underwater-sampling, dry-storage and PCR success', *PeerJ*. PeerJ Inc., 3, p. e1188. doi: 10.7717/peerj.1188.

Kawaguchi, T. (1985) 'Chemical nature of collagen in the placoid-scale dentine of the blue shark, *Prionace glauca* L.', *Archives of Oral Biology*. Pergamon, 30(5), pp. 385–390. doi: 10.1016/0003-9969(85)90064-0.

Keeney, D. B. *et al.* (2005) 'Microsatellite and mitochondrial DNA analyses of the genetic structure of blacktip shark (*Carcharhinus limbatus*) nurseries in the northwestern Atlantic, Gulf of Mexico, and Caribbean Sea', *Molecular Ecology*. John Wiley & Sons, Ltd (10.1111), 14(7), pp. 1911–1923. doi: 10.1111/j.1365-294X.2005.02549.x.

Keeney, D. B. and Heist, E. J. (2006) 'Worldwide phylogeography of the blacktip shark (*Carcharhinus limbatus*) inferred from mitochondrial DNA reveals isolation of western Atlantic populations coupled with recent Pacific dispersal', *Molecular Ecology*. John Wiley & Sons, Ltd (10.1111), 15(12), pp. 3669–3679. doi: 10.1111/j.1365-294X.2006.03036.x.

Kemp, N. E. and Westrin, S. K. (1979) 'Ultrastructure of calcified cartilage in the endoskeletal tesserae of sharks', *Journal of Morphology*. John Wiley & Sons, Ltd, 160(1), pp. 75–101. doi: 10.1002/jmor.1051600106.

Kim, S.-K. (2012) *Marine cosmeceuticals : trends and prospects*. CRC Press.

King, J. R. *et al.* (2015) 'Genetic analysis of stock structure of blue shark (*Prionace glauca*) in the north Pacific ocean', *Fisheries Research*. Elsevier, 172, pp. 181–189. doi: 10.1016/J.FISHRES.2015.06.029.

Kubota, T., Shiobara, Y. and Kubodera, T. (1991) 'Food habits of the frilled shark *Chlamydoselachus anguineus* collected from Suruga Bay, central Japan.', *Nippon Suisan Gakkaishi*. 57(1), pp. 15–20. doi: 10.2331/suisan.57.15.

Kulka, D. W. *et al.* (2009) *Amblyraja radiata. The IUCN Red List of Threatened Species*, *e.T161542A5447511*. doi: http://dx.doi.org/10.2305/IUCN.UK.2009-2.RLTS.T161542A5447511.en.

Kyne, P. M. *et al.* (2006) *Aetobatus narinari. The IUCN Red List of Threatened Species*, *e.T39415A10231645.* doi: http://dx.doi.org/10.2305/IUCN.UK.2006.RLTS.T39415A10231645.en.

Kyne, P. M. and Burgess, G. H. (2006) *Chiloscyllium plagiosum. The IUCN Red List of Threatened Species*, *e.T60222A12325334.* doi: http://dx.doi.org/10.2305/IUCN.UK.2006.RLTS.T60222A12325334.en.

Kyne, P. M., Carlson, J. and Smith, K. (2013) *Pristis pristis (errata version published in 2019). The IUCN Red List of Threatened Species*, *e.T18584848A141788242*. doi: http://dx.doi.org/10.2305/IUCN.UK.2013-1.RLTS.T18584848A141788242.en.

Kyne, P. M., Rigby, C. and Simpfendorfer, C. (2013) *Pristis clavata (errata version published in 2019). The IUCN Red List of Threatened Species*, *e.T39390A141790455*. doi: http://dx.doi.org/10.2305/IUCN.UK.2013-1.RLTS.T39390A141790455.en.

Kyne, P. M., Sherrill-Mix, S. A. and Burgess, G. H. (2006) *Somniosus microcephalus. The IUCN Red List of Threatened Species*, *e.T60213A12321694.*

Lack, M. *et al.* (2014) 'Development of a Rapid Management-Risk Assessment Method for Fish Species through its Application to Sharks', *Report to the Department of Environment, Food and Rural Affairs Defra contract No. MB0123*.

Lande, R. (1988) 'Background and mass extinctions: the alternation of macroevolutionary regimes.', *Science (New York, N.Y.)*. American Association for the Advancement of Science, 231(4734), pp. 129–33. doi: 10.1126/science.231.4734.129.

Larson, S. *et al.* (2009) 'Microsatellite loci within sixgill sharks, *Hexanchus griseus*', *Molecular Ecology Resources*. John Wiley & Sons, Ltd (10.1111), 9(3), pp. 978–981. doi: 10.1111/j.1755-0998.2009.02531.x.

Larson, S. *et al.* (2011) 'Relatedness and polyandry of sixgill sharks, *Hexanchus griseus*, in an urban estuary', *Conservation Genetics*. Springer Netherlands, 12(3), pp. 679–690. doi: 10.1007/s10592-010-0174-9.

Larson, S. E., Daly-Engel, T. S. and Phillips, N. M. (2017) 'Review of Current Conservation Genetic Analyses of Northeast Pacific Sharks', *Advances in Marine Biology*. Academic Press, 77, pp. 79–110. doi: 10.1016/BS.AMB.2017.06.005.

Lee, S. S. *et al.* (2000) 'Rearrangement of immunoglobulin genes in shark germ cells.', *The Journal of experimental medicine*. Rockefeller University Press, 191(10), pp. 1637–48. doi: 10.1084/jem.191.10.1637.

Lessa, R. *et al.* (2006) *Rhizoprionodon porosus. The IUCN Red List of Threatened Species*, *e.T61407A12473033.* doi: http://dx.doi.org/10.2305/IUCN.UK.2006.RLTS.T61407A12473033.en.

Lewallen, E. A., Anderson, T. W. and Bohonak, A. J. (2007) 'Genetic structure of leopard shark (*Triakis semifasciata*) populations in California waters', *Marine Biology*. Springer Berlin Heidelberg, 152(3), pp. 599–609. doi: 10.1007/s00227-007-0714-0.

MacNeil, M. A. *et al.* (2012) 'Biology of the Greenland shark *Somniosus microcephalus*', *Journal of Fish Biology*. John Wiley & Sons, Ltd (10.1111), 80(5), pp. 991–1018. doi: 10.1111/j.1095-8649.2012.03257.x.

Maduna, S. N. *et al.* (2014) 'Microsatellite cross-species amplification and utility in southern African elasmobranchs: A valuable resource for fisheries management and conservation', *BMC Research Notes*. BioMed Central, 7(1), p. 352. doi: 10.1186/1756-0500-7-352.

Maisey, J. G. (2012) 'What is an "elasmobranch"? The impact of palaeontology in understanding elasmobranch phylogeny and evolution', *Journal of Fish Biology*. John Wiley & Sons, Ltd (10.1111), 80(5), pp. 918–951. doi: 10.1111/j.1095-8649.2012.03245.x.

Maisey, J. G., Naylor, G. J. and Ward, D. J. (2004) 'Mesozoic elasmobranchs, neoselachian phylogeny and the rise of modern elasmobranch diversity', *Mesozoic Fishes*, 3, pp. 17–56.

Manuzzi, A. *et al.* (2019) 'Population genomics and phylogeography of a benthic coastal shark (*Scyliorhinus canicula*) using 2b-RAD single nucleotide polymorphisms', *Biological Journal of the Linnean Society*. Narnia, 126(2), pp. 289–303. doi: 10.1093/biolinnean/bly185.

Marino, I. *et al.* (2015) 'Multiple paternity and hybridization in two smooth-hound sharks', *Scientific Reports*. Nature Publishing Group, 5(1), p. 12919. doi: 10.1038/srep12919.

Marino, I. A. M. *et al.* (2015) 'New Molecular Tools for the Identification of 2 Endangered Smooth-Hound Sharks, *Mustelus mustelus* and *Mustelus punctulatus*', *Journal of Heredity*. Narnia, 106(1), pp. 123–130. doi: 10.1093/jhered/esu064.

Marshall, A. *et al.* (2018) *Mobula alfredi (amended version of 2011 assessment). The IUCN Red List of Threatened Species*, *e.T195459A126665723*. doi: http://dx.doi.org/10.2305/IUCN.UK.2011-2.RLTS.T195459A126665723.en.

Martin, A. P., Naylor, G. J. P. and Palumbi, S. R. (1992) 'Rates of mitochondrial DNA evolution in sharks are slow compared with mammals', *Nature*. Nature Publishing Group, 357(6374), pp. 153–155. doi: 10.1038/357153a0.

Massa, A. *et al.* (2006) *Mustelus schmitti. The IUCN Red List of Threatened Species*, *e.T60203A12318268*. doi: http://dx.doi.org/10.2305/IUCN.UK.2006.RLTS.T60203A12318268.en.

McCallum, M. L. (2015) 'Vertebrate biodiversity losses point to a sixth mass extinction', *Biodiversity and Conservation*. Springer Netherlands, 24(10), pp. 2497–2519. doi: 10.1007/s10531-015-0940-6.

McCauley, L. *et al.* (2004) 'Characterization and isolation of DNA microsatellite primers in the spiny dogfish (*Squalus acanthias*)', *Molecular Ecology Notes*, 4(3), pp. 494–496. doi: 10.1111/j.1471-8286.2004.00724.x.

Mendes, N. J. *et al.* (2015) 'Microsatellite loci in the oceanic whitetip shark and cross-species amplification using pyrosequencing technology', *Conservation Genetics Resources*. Springer Netherlands, 7(2), pp. 585–589. doi: 10.1007/s12686-015-0435-5.

Mendonça, F. F. *et al.* (2009) 'Identification of the shark species *Rhizoprionodon lalandii* and *R. porosus* (Elasmobranchii, Carcharhinidae) by multiplex PCR and PCR-RFLP techniques', *Molecular Ecology Resources*. John Wiley & Sons, Ltd (10.1111), 9(3), pp. 771–773. doi: 10.1111/j.1755-0998.2009.02524.x.

Mendonça, F. F. *et al.* (2013) 'Diversity and genetic population structure of the Brazilian sharpnose shark *Rhizoprionodon lalandii*', *Aquatic Conservation: Marine and Freshwater Ecosystems*. John Wiley & Sons, Ltd, 23(6), pp. 850–857. doi: 10.1002/aqc.2342.

Meyer, J. R. and Kassen, R. (2007) 'The effects of competition and predation on diversification in a model adaptive radiation', *Nature*. Nature Publishing Group, 446(7134), pp. 432–435. doi: 10.1038/nature05599.

Mora, C. *et al.* (2011) 'How Many Species Are There on Earth and in the Ocean?', *PLoS Biology*. Edited by G. M. Mace. Public Library of Science, 9(8), p. e1001127. doi: 10.1371/journal.pbio.1001127.

Morgan, M. *et al.* (2009) *Carcharhinus acronotus. The IUCN Red List of Threatened Species*, *e.T161378A5410167*. doi: http://dx.doi.org/10.2305/IUCN.UK.2009-2.RLTS.T161378A5410167.en.

Mourier, J. *et al.* (2013) 'Genetic Network and Breeding Patterns of a Sicklefin Lemon Shark (*Negaprion acutidens*) Population in the Society Islands, French Polynesia', *PLoS ONE*. Edited by G. Sorci. Public Library of Science, 8(8), p. e73899. doi: 10.1371/journal.pone.0073899.

Mourier, J. and Planes, S. (2013) 'Direct genetic evidence for reproductive philopatry and associated fine-scale migrations in female blacktip reef sharks (*Carcharhinus melanopterus*) in French Polynesia', *Molecular Ecology*. John Wiley & Sons, Ltd (10.1111), 22(1), pp. 201–214. doi: 10.1111/mec.12103.

Moy-Thomas, J. A. (1939) 'The early evolution and relationships of the elasmobranchs', *Biological Reviews*, 14(1), pp. 1–26. doi: 10.1111/j.1469-185X.1939.tb00921.x.

Murray, B. W. *et al.* (2008) 'Mitochondrial cytochrome b variation in sleeper sharks (Squaliformes: Somniosidae)', *Marine Biology*. Springer-Verlag, 153(6), pp. 1015–1022. doi: 10.1007/s00227-007-0871-1.

Musick, J. A., Grubbs, R. D., *et al.* (2009) *Carcharhinus obscurus. The IUCN Red List of Threatened Species*, *e.T3852A10127245.* doi: http://dx.doi.org/10.2305/IUCN.UK.2009-2.RLTS.T3852A10127245.en.

Musick, J. A., Stevens, J. D., *et al.* (2009) *Carcharhinus plumbeus. The IUCN Red List of Threatened Species*, *e.T3853A10130397*. doi: http://dx.doi.org/10.2305/IUCN.UK.2009-2.RLTS.T3853A10130397.en.

Musick, J. and Ellis, J. (2005) 'Reproductive evolution of chondrichthyans', in Hamlett, W. and Jamieson, B. (eds) *Reproductive Biology and Phylogeny of Chondrichthyes: Sharks, Batoids and Chimaeras*, pp. 45–79.

Mycock, S. G., Lessa, R. and Almeida, Z. (2006) *Sphyrna tudes. The IUCN Red List of Threatened Species*, *e.T60202A12318061*. doi: http://dx.doi.org/10.2305/IUCN.UK.2006.RLTS.T60202A12318061.en.

Nagai, T. and Suzuki, N. (2000) 'Isolation of collagen from fish waste material — skin, bone and fins', *Food Chemistry*. Elsevier, 68(3), pp. 277–281. doi: 10.1016/S0308-8146(99)00188-0.

El Nagar, A. *et al.* (2010) 'Characterisation of polymorphic microsatellite markers for skates (Elasmobranchii: Rajidae) from expressed sequence tags', *Conservation Genetics*. Springer Netherlands, 11(3), pp. 1203–1206. doi: 10.1007/s10592-009-9919-8.

Nalluri, D. *et al.* (2014) 'Methylmercury in dried shark fins and shark fin soup from American restaurants', *Science of The Total Environment*. Elsevier, 496, pp. 644–648. doi: 10.1016/J.SCITOTENV.2014.04.107.

Nance, H. A., Daly-Engel, T. S. and Marko, P. B. (2009) 'New microsatellite loci for the endangered scalloped hammerhead shark, *Sphyrna lewini*', *Molecular Ecology Resources*. Blackwell Publishing Ltd, 9(3), pp. 955–957. doi: 10.1111/j.1755-0998.2008.02510.x.

Navia, A. F. *et al.* (2017) 'How many trophic roles can elasmobranchs play in a marine tropical network?', *Marine and Freshwater Research*. CSIRO PUBLISHING, 68(7), p. 1342. doi: 10.1071/MF16161.

Navia, A. F., Cortés, E. and Mejía-Falla, P. A. (2010) 'Topological analysis of the ecological importance of elasmobranch fishes: A food web study on the Gulf of Tortugas, Colombia', *Ecological Modelling*. Elsevier, 221(24), pp. 2918–2926. doi: 10.1016/J.ECOLMODEL.2010.09.006.

Naylor, G., Fedrigo, O. and Andrés López, J. (2005) *Phylogenetic Relationships among the Major Lineages of Modern Elasmobranchs*.

Nei, M. and Kumar, S. (2000) *Molecular evolution and phylogenetics*. Oxford University Press.

Nomura, Y. (2004) 'Properties and utiliztion of shark collagen', *Developments in Food Science*. Elsevier, 42, pp. 147–158. doi: 10.1016/S0167-4501(04)80017-7.

Nosal, A. P., Lewallen, E. A. and Burton, R. S. (2013) 'Multiple paternity in leopard shark (*Triakis semifasciata*) litters sampled from a predominantly female aggregation in La Jolla, California, USA', *Journal of Experimental Marine Biology and Ecology*. Elsevier, 446, pp. 110–114. doi: 10.1016/J.JEMBE.2013.05.002.

O'Bryhim, J. R. *et al.* (2015) 'Development of microsatellite markers for globally distributed populations of the threatened silky shark, *Carcharhinus falciformis*', *Conservation Genetics Resources*. Springer Netherlands, 7(2), pp. 463–465. doi: 10.1007/s12686-014-0396-0.

O'Connell, K. A. *et al.* (2019) 'A Tale of Two Skates: Comparative Phylogeography of North American Skate Species with Implications for Conservation', *Copeia*. The American Society of Ichthyologists and Herpetologists , 107(2), p. 297. doi: 10.1643/CG-18-114.

O'Grady, J. J. *et al.* (2006) 'Realistic levels of inbreeding depression strongly affect extinction risk in wild populations', *Biological Conservation*. Elsevier, 133(1), pp. 42–51. doi: 10.1016/J.BIOCON.2006.05.016.

O'Leary, S. J. *et al.* (2015) 'Genetic Diversity of White Sharks, *Carcharodon carcharias*, in the Northwest Atlantic and Southern Africa', *Journal of Heredity*. Narnia, 106(3), pp. 258–265. doi: 10.1093/jhered/esv001.

O'Leary, S. J., Feldheim, K. A. and Chapman, D. D. (2013) 'Novel microsatellite loci for white, *Carcharodon carcharias* and sandtiger sharks, *Carcharias taurus* (order Lamniformes)', *Conservation Genetics Resources*. Springer Netherlands, 5(3), pp. 627–629. doi: 10.1007/s12686-013-9866-z.

Ørvig, T. (1951) 'Histologic studies of placotlerms and fossil elasmobranchs. I. The endoskeleton, with remarks on the hard tissues of lower vertebrates in general.', *Ark Zoology*, 2, pp. 321–454.

Ouborg, N. J. *et al.* (2010) 'Conservation genetics in transition to conservation genomics', *Trends in Genetics*. Elsevier Current Trends, 26(4), pp. 177–187. doi: 10.1016/J.TIG.2010.01.001.

Ovenden, J. R. *et al.* (2009) 'The extent of population genetic subdivision differs among four co-distributed shark species in the Indo-Australian archipelago', *BMC Evolutionary Biology*. BioMed Central, 9(1), p. 40. doi: 10.1186/1471-2148-9-40.

Ovenden, J. R., Street, R. and Broderick, D. (2006) 'New microsatellite loci for Carcharhinid sharks (*Carcharhinus tilstoni* and *C. sorrah*) and their cross-amplification in other shark species', *Molecular Ecology Notes*. John Wiley & Sons, Ltd (10.1111), 6(2), pp. 415–418. doi: 10.1111/j.1471-8286.2005.01254.x.

Pardini, A. T. *et al.* (2000) 'Isolation and characterization of dinucleotide microsatellite loci in the Great White Shark, *Carcharodon carcharias*', *Molecular Ecology*. John Wiley & Sons, Ltd (10.1111), 9(8), pp. 1176–1178. doi: 10.1046/j.1365-294x.2000.00954-4.x.

Pazmiño, D. A. *et al.* (2017) 'Genome-wide SNPs reveal low effective population size within confined management units of the highly vagile Galapagos shark (*Carcharhinus galapagensis*)', *Conservation Genetics*. Springer Netherlands, 18(5), pp. 1151–1163. doi: 10.1007/s10592-017-0967-1.

Pereyra, S. *et al.* (2010) 'Low genetic diversity and population structure of the narrownose shark (*Mustelus schmitti*)', *Fisheries Research*. Elsevier, 106(3), pp. 468–473. doi: 10.1016/J.FISHRES.2010.09.022.

Pérez-Jiménez, J. *et al.* (2016) *Mustelus henlei. The IUCN Red List of Threatened Species*, *e.T161648A80672263*. doi: http://dx.doi.org/10.2305/IUCN.UK.2016-2.RLTS.T161648A80672263.en.

Phillips, N. M. *et al.* (2009) *Microsatellite and mitochondrial DNA assessment of the genetic diversity and population structure of the Freshwater Sawfish, Pristis microdon, and the Green Sawfish, Pristis zijsron, respectively, in Australian waters: preliminary results.*

Phillips, N. M. *et al.* (2011) 'Population genetic structure and genetic diversity of three critically endangered Pristis sawfishes in Australian waters', *Marine Biology*. Springer-Verlag, 158(4), pp. 903–915. doi: 10.1007/s00227-010-1617-z.

Pierce, S. J. and Norman, B. (2016) *Rhincodon typus. The IUCN Red List of Threatened Species*, *e.T19488A2365291.* doi: http://dx.doi.org/10.2305/IUCN.UK.2016-1.RLTS.T19488A2365291.en.

Pievani, T. (2014) 'The sixth mass extinction: Anthropocene and the human impact on biodiversity', *Rendiconti Lincei*. Springer Milan, 25(1), pp. 85–93. doi: 10.1007/s12210-013-0258-9.

Pillans, R. (2003) '*Negaprion acutidens*. The IUCN Red List of Threatened Species', in *SSG Australia & Oceania Regional Workshop*. e.T41836A10576957. doi: http://dx.doi.org/10.2305/IUCN.UK.2003.RLTS.T41836A10576957.en.

Pillans, R., Stevens, J. D. and White, W. T. (2009) *Carcharhinus sorrah. The IUCN Red List of Threatened Species*, *e.T161376A5409506.* doi: http://dx.doi.org/10.2305/IUCN.UK.2009-2.RLTS.T161376A5409506.en.

Pirog, A. *et al.* (2017) 'First evidence of multiple paternity in the bull shark (*Carcharhinus leucas*)', *Marine and Freshwater Research*. CSIRO PUBLISHING, 68(1), p. 195. doi: 10.1071/MF15255.

Pollard, D. and Smith, A. (2009) *Carcharias taurus. The IUCN Red List of Threatened Species*, *e.T3854A10132481*. doi: http://dx.doi.org/10.2305/IUCN.UK.2009-2.RLTS.T3854A10132481.en.

Le Port, A. *et al.* (2016) 'Characterisation of eleven new polymorphic microsatellite markers for the coastal stingray *Dasyatis brevicaudata* (Dasyatidae Hutton 1875), and cross-amplification in seven dasyatid species', *Biochemical Systematics and Ecology*. Pergamon, 65, pp. 234–237. doi: 10.1016/J.BSE.2016.02.009.

Pratt, Jr., H. L. and Carrier, J. C. (2001) 'A Review of Elasmobranch Reproductive Behavior with a Case Study on the Nurse Shark, *Ginglymostoma Cirratum*', *Environmental Biology of Fishes*. Kluwer Academic Publishers, 60(1/3), pp. 157–188. doi: 10.1023/A:1007656126281.

Ramírez-Macías, D. *et al.* (2009) 'Isolation and characterization of microsatellite loci in the whale shark (*Rhincodon typus*)', *Molecular Ecology Resources*. John Wiley & Sons, Ltd (10.1111), 9(3), pp. 798–800. doi: 10.1111/j.1755-0998.2008.02197.x.

Raymond, M. and Rousset, F. (1995) 'GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism', *Journal of Heredity*, 86, pp. 248–249.

Reardon, M. *et al.* (2009) *Alopias pelagicus. The IUCN Red List of Threatened Species*, *e.T161597A5460720*. doi: http://dx.doi.org/10.2305/IUCN.UK.2009-2.RLTS.T161597A5460720.en.

Reynolds, J. D. *et al.* (2005) 'Biology of extinction risk in marine fishes', *Proceedings of the Royal Society B: Biological Sciences*, 272(1579), pp. 2337–2344. doi: 10.1098/rspb.2005.3281.

Rigby, C. L. *et al.* (2017) *Carcharhinus falciformis. The IUCN Red List of Threatened Species*, *e.T39370A117721799*. doi: http://dx.doi.org/10.2305/IUCN.UK.2017-3.RLTS.T39370A117721799.en.

Rigby, C. L. *et al.* (2019) *Isurus oxyrinchus. The IUCN Red List of Threatened Species*, *e.T39341A2903170*. doi: http://dx.doi.org/10.2305/IUCN.UK.2019-1.RLTS.T39341A2903170.en.

Robbins, R. L. (2007) 'Environmental variables affecting the sexual segregation of great white sharks *Carcharodon carcharias* at the Neptune Islands South Australia', *Journal of Fish Biology*. John Wiley & Sons, Ltd (10.1111), 70(5), pp. 1350–1364. doi: 10.1111/j.1095-8649.2007.01414.x.

Rousset, F. (2008) 'Genepop'007: a complete reimplementation of the Genepop software for Windows and Linux', *Molecular Ecol Resources*, 8, pp. 103–106.

Rus Hoelzel, A. *et al.* (2006) 'Low worldwide genetic diversity in the basking shark (*Cetorhinus maximus*)', *Biology Letters*. The Royal SocietyLondon, 2(4), pp. 639–642. doi: 10.1098/rsbl.2006.0513.

Sandoval-Castillo, J. *et al.* (2004) 'Cryptic isolation of Gulf of California shovelnose guitarfish evidenced by mitochondrial DNA', *Marine Biology*. Springer-Verlag, 145(5), pp. 983–988. doi: 10.1007/s00227-004-1378-7.

Schaefer, J. T. and Summers, A. P. (2005) 'Batoid wing skeletal structure: Novel morphologies, mechanical implications, and phylogenetic patterns', *Journal of Morphology*. John Wiley & Sons, Ltd, 264(3), pp. 298–313. doi: 10.1002/jmor.10331.

Schmidt, J. V. *et al.* (2009) 'Low Genetic Differentiation across Three Major Ocean Populations of the Whale Shark, *Rhincodon typus*', *PLoS ONE*. Edited by R. DeSalle. Public Library of Science, 4(4), p. e4988. doi: 10.1371/journal.pone.0004988.

Schrey, A. W. and Heist, E. J. (2003) 'Microsatellite analysis of population structure in the shortfin mako (*Isurus oxyrinchus*)', *Canadian Journal of Fisheries and Aquatic Sciences*. NRC Research Press Ottawa, Canada , 60(6), pp. 670–675. doi: 10.1139/f03-064.

Schultz, J. K. *et al.* (2008) 'Global phylogeography and seascape genetics of the lemon sharks (genus Negaprion)', *Molecular Ecology*. John Wiley & Sons, Ltd (10.1111), 17(24), pp. 5336–5348. doi: 10.1111/j.1365-294X.2008.04000.x.

Sekerci, Y. and Petrovskii, S. (2015) 'Mathematical Modelling of Plankton–Oxygen Dynamics Under the Climate Change', *Bulletin of Mathematical Biology*. Springer US, 77(12), pp. 2325–2353. doi: 10.1007/s11538-015-0126-0.

Sellas, A. B. *et al.* (2015) 'Population Structure and Seasonal Migration of the Spotted Eagle Ray, *Aetobatus narinari*', *Journal of Heredity*. Narnia, 106(3), pp. 266–275. doi: 10.1093/jhered/esv011.

Serena, F. *et al.* (2009) *Mustelus mustelus. The IUCN Red List of Threatened Species*, *e.T39358A10214694*. doi: http://dx.doi.org/10.2305/IUCN.UK.2009-2.RLTS.T39358A10214694.en.

Serena, F. *et al.* (2015) *Raja asterias. The IUCN Red List of Threatened Species*, *e.T63120A48913317*. doi: http://dx.doi.org/10.2305/IUCN.UK.2015-1.RLTS.T63120A48913317.en.

Serena, F., Mancusi, C. and Ellis, J. (2009) *Mustelus asterias. The IUCN Red List of Threatened Species*, *e.T39357A10214084.* doi: http://dx.doi.org/10.2305/IUCN.UK.2009-2.RLTS.T39357A10214084.en.

Shiffman, D. S. and Hammerschlag, N. (2016) 'Preferred conservation policies of shark researchers', *Conservation Biology*. John Wiley & Sons, Ltd (10.1111), 30(4), pp. 805–815. doi: 10.1111/cobi.12668.

Simpfendorfer, C. (2013) *Pristis zijsron (errata version published in 2019). The IUCN Red List of Threatened Species*, *e.T39393A141792003.* doi: http://dx.doi.org/10.2305/IUCN.UK.2013-1.RLTS.T39393A141792003.en.

Simpfendorfer, C. and Burgess, G. H. (2009) *Carcharhinus leucas. The IUCN Red List of Threatened Species*, *e.T39372A10187195.* doi: http://dx.doi.org/10.2305/IUCN.UK.2009-2.RLTS.T39372A10187195.en.

Smale, M. J. *et al.* (2009) *Raja miraletus. The IUCN Red List of Threatened Species*, *e.T161599A5461484.* doi: http://dx.doi.org/10.2305/IUCN.UK.2009-2.RLTS.T161599A5461484.en.

Smale, M. J. (2009) *Triaenodon obesus. The IUCN Red List of Threatened Species*, *e.T39384A10188990.* doi: http://dx.doi.org/10.2305/IUCN.UK.2005.RLTS.T39384A10188990.en.

Smith, M. *et al.* (2014) *The Elasmobranch Husbandry Manual: Captive Care of Sharks, Rays and their Relatives*. Columbus, Ohio.

Snelson Jr., F. F., Burgess, G. H. and Roman, B. L. (2008) 'The Reproductive Biology of Pelagic Elasmobranchs', in Camhi, M. D., Pikitch, E. K., and Babcock, E. A. (eds) *Sharks of the Open Ocean. Biology* . Fisheries and Conservation, pp. 24–53.

Sonne, C. and Alstrup, A. K. O. (2019) 'Discussion: Early life and lessons learned from mass extinctions', *Environmental Research*. Academic Press, 172, pp. 444–445. doi: 10.1016/J.ENVRES.2019.02.040.

Sorenson, L., Santini, F. and Alfaro, M. E. (2014) 'The effect of habitat on modern shark diversification', *Journal of Evolutionary Biology*. John Wiley & Sons, Ltd (10.1111), 27(8), pp. 1536–1548. doi: 10.1111/jeb.12405.

Spielman, D. *et al.* (2004) 'Does Inbreeding and Loss of Genetic Diversity Decrease Disease Resistance?', *Conservation Genetics*. Kluwer Academic Publishers, 5(4), pp. 439–448. doi: 10.1023/B:COGE.0000041030.76598.cd.

Stachowicz, J. J. *et al.* (1999) 'Species Diversity and Invasion Resistance in a Marine Ecosystem', *Science*. American Association for the Advancement of Science, 286(5444), pp. 1577–1579. doi: 10.1126/science.286.5444.1577.

Steinke, D. *et al.* (2017) 'DNA analysis of traded shark fins and mobulid gill plates reveals a high proportion of species of conservation concern', *Scientific Reports*. Nature Publishing Group, 7(1), p. 9505. doi: 10.1038/s41598-017-10123-5.

Steinke, D. and Hanner, R. (2011) 'The FISH-BOL collaborators' protocol', *Mitochondrial DNA*. Taylor & Francis, 22(sup1), pp. 10–14. doi: 10.3109/19401736.2010.536538.

Stéphan, E. *et al.* (2014) 'Length at maturity , conversion factors , movement patterns and population genetic structure of undulate ray (*Raja undulata*) along the French Atlantic and English Channel coasts : preliminary results', pp. 1–16.

Stevens, J. *et al.* (2000) 'The effects of fishing on sharks, rays, and chimaeras (chondrichthyans), and the implications for marine ecosystems', *ICES Journal of Marine Science*. Oxford University Press, 57(3), pp. 476–494. doi: 10.1006/jmsc.2000.0724.

Stevens, J. (2003) 'entroselachus crepidater. The IUCN Red List of Threatened Species', in *SSG Australia & Oceania Regional Workshop*. e.T46864A11087086. doi: http://dx.doi.org/10.2305/IUCN.UK.2003.RLTS.T46864A11087086.en.

Stevens, J. (2009) *Prionace glauca. The IUCN Red List of Threatened Species*, *e.T39381A10222811.* doi: http://dx.doi.org/10.2305/IUCN.UK.2009-2.RLTS.T39381A10222811.en.

Stevens, J. and Correia, J. P. S. (2003) 'Centroscymnus coelolepis. The IUCN Red List of Threatened Species', in *SSG Australia & Oceania Regional Workshop*.

e.T41747A10552910. doi: http://dx.doi.org/10.2305/IUCN.UK.2003.RLTS.T41747A10552910.en.

Suárez-Moo, P. de J. *et al.* (2013) 'High genetic connectivity in the Atlantic sharpnose shark, *Rhizoprionodon terraenovae*, from the southeast Gulf of Mexico inferred from AFLP fingerprinting', *Fisheries Research*. Elsevier, 147, pp. 338–343. doi: 10.1016/J.FISHRES.2013.07.003.

Sundström, L. F. (2015) *Negaprion brevirostris. The IUCN Red List of Threatened Species*, *e.T39380A81769233.* doi: http://dx.doi.org/10.2305/IUCN.UK.2015.RLTS.T39380A81769233.en.

Taberlet, P. and Luikart, G. (1999) 'Non-invasive genetic sampling and individual identification', *Biological Journal of the Linnean Society*. Narnia, 68(1–2), pp. 41–55. doi: 10.1111/j.1095-8312.1999.tb01157.x.

Taguchi, M. *et al.* (2013) 'Development of 15 polymorphic microsatellite markers for the shortfin mako, *Isurus oxyrinchus*, and cross-species amplification in lamniforme sharks', *Conservation Genetics Resources*. Springer Netherlands, 5(3), pp. 675–678. doi: 10.1007/s12686-013-9880-1.

Taguchi, M. *et al.* (2015) 'Population genetic structure and demographic history of Pacific blue sharks (*Prionace glauca*) inferred from mitochondrial DNA analysis', *Marine and Freshwater Research*. CSIRO PUBLISHING, 66(3), p. 267. doi: 10.1071/MF14075.

Tamura, K. (1992) 'Estimation of the number of nucleotide substitutions when there are strong transition-transversion and G+C-content biases.', *Molecular Biology and Evolution*. Narnia, 9(4), pp. 678–687. doi: 10.1093/oxfordjournals.molbev.a040752.

The IUCN (2001) *IUCN red list categories and criteria*. IUCN--The World Conservation Union.

The IUCN (2010) *IUCN Red List of Threatened Species Summary*. Available at: https://pdfs.semanticscholar.org/6205/dd7286002d8e83718e962db481416a4e9296.pdf

The IUCN (2018) *IUCN 70 years: The International Union for the Conservation of Nature*. France. Available at: https://portals.iucn.org/library/sites/library/files/documents/2019-007-En.pdf

The IUCN SSG (2019) *Position Statements and Advice Documents*, *The International Union for the Conservation of Nature*. Available at: https://www.iucnssg.org/statements-and-documents.html

The PEW Charitable Trust (2017) *Protections for Threatened Migratory Sharks*. Available at: https://www.pewtrusts.org/-/media/assets/2017/10/gsc_protections_for_threatened_migratory_sharks.pdf

Thorson, T. B., Cowan, C. M. and Watson, D. E. (1973) 'Body Fluid Solutes of Juveniles and Adults of the Euryhaline Bull Shark *Carcharhinus leucas* from Freshwater and Saline Environments', *Physiological Zoology*. University of Chicago Press , 46(1), pp. 29–42. doi: 10.1086/physzool.46.1.30152514.

Toone, W. D. and Wallace, M. P. (1994) 'The extinction in the wild and reintroduction of the California condor (*Gymnogyps californianus*)', in *Creative Conservation*. Dordrecht: Springer Netherlands, pp. 411–419. doi: 10.1007/978-94-011-0721-1_23.

Townsend, R. *et al.* (2015) 'Multiple paternity in captive grey nurse sharks (*Carcharias taurus*): implications for the captive breeding of this critically endangered species', *Pacific Conservation Biology*. CSIRO PUBLISHING, 21(2), p. 122. doi: 10.1071/PC14909.

Trouwborst, A., Fleurke, F. and Dubrulle, J. (2016) 'Border Fences and their Impacts on Large Carnivores, Large Herbivores and Biodiversity: An International Wildlife Law Perspective', *Review of European, Comparative & International Environmental Law*. John Wiley & Sons, Ltd, 25(3), pp. 291–306. doi: 10.1111/reel.12169.

Valsecchi, E. *et al.* (2004) 'Rapid Miocene-Pliocene dispersal and evolution of Mediterranean rajid fauna as inferred by mitochondrial gene variation', *Journal of Evolutionary Biology*. John Wiley & Sons, Ltd (10.1111), 18(2), pp. 436–446. doi: 10.1111/j.1420-9101.2004.00829.x.

Verissimo, A. *et al.* (2011) 'Frequency of Multiple Paternity in the Spiny Dogfish *Squalus acanthias* in the Western North Atlantic', *Journal of Heredity*. Oxford University Press, 102(1), pp. 88–93. doi: 10.1093/jhered/esq084.

Veríssimo, A., Mcdowell, J. R. and Graves, J. E. (2010) 'Global population structure of the spiny dogfish *Squalus acanthias*, a temperate shark with an antitropical distribution', *Molecular Ecology*. John Wiley & Sons, Ltd (10.1111), 19(8), pp. 1651–1662. doi: 10.1111/j.1365-294X.2010.04598.x.

Vianna, G. M. S. *et al.* (2012) 'Socio-economic value and community benefits from shark-diving tourism in Palau: A sustainable use of reef shark populations', *Biological Conservation*. Elsevier, 145(1), pp. 267–277. doi: 10.1016/J.BIOCON.2011.11.022.

Vié, J.-C. *et al.* (2009) *Wildlife in a changing world : an analysis of the 2008 IUCN red list of threatened species*. IUCN.

Vieira, M. L. C. *et al.* (2016) 'Microsatellite markers: what they mean and why they are so useful', *Genetics and Molecular Biology*. Sociedade Brasileira de Genética, 39(3), pp. 312–328. doi: 10.1590/1678-4685-GMB-2016-0027.

Vignal, A. *et al.* (2002) 'A review on SNP and other types of molecular markers and their use in animal genetics', *Genetics Selection Evolution*. BioMed Central, 34(3), p. 275. doi: 10.1186/1297-9686-34-3-275.

Vignaud, T. M. *et al.* (2014) 'Genetic structure of populations of whale sharks among ocean basins and evidence for their historic rise and recent decline', *Molecular Ecology*. John Wiley & Sons, Ltd (10.1111), 23(10), pp. 2590–2601. doi: 10.1111/mec.12754.

Virkler, K. and Lednev, I. K. (2009) 'Analysis of body fluids for forensic purposes: From laboratory testing to non-destructive rapid confirmatory identification at a crime scene', *Forensic Science International*. Elsevier, 188(1–3), pp. 1–17. doi: 10.1016/J.FORSCIINT.2009.02.013.

Vos, P. *et al.* (1995) 'AFLP: a new technique for DNA fingerprinting', *Nucleic Acids Research*. Narnia, 23(21), pp. 4407–4414. doi: 10.1093/nar/23.21.4407.

Waits, L. P. and Paetkau, D. (2005) 'Noninvasive genetic sampling tools for wildlife biologists: a review of applications and recommendations for accurate data collection', *The Journal of Wildlife Management*. John Wiley & Sons, Ltd, 69(4), pp. 1419–1433. doi: 10.2193/0022-541X(2005)69[1419:NGSTFW]2.0.CO;2.

Walker, T. I. *et al.* (2006) *Galeorhinus galeus. The IUCN Red List of Threatened Species*, *e.T39352A10212764.* doi: http://dx.doi.org/10.2305/IUCN.UK.2006.RLTS.T39352A10212764.en.

Walker, T. I. (2016) *Mustelus antarcticus. The IUCN Red List of Threatened Species*, *e.T39355A68634159.* doi: http://dx.doi.org/10.2305/IUCN.UK.2016-1.RLTS.T39355A68634159.en.

Wang, J. Y. and Yang, S.-C. (2004) 'First records of Pacific sleeper sharks (*Somniosus pacificus* in the subtropical waters of eastern Taiwan', *Bulletin of Marine Science*.

University of Miami - Rosenstiel School of Marine and Atmospheric Science, 74(1), pp. 229–236.

Ward-Paige, C. A. *et al.* (2012) 'Recovery potential and conservation options for elasmobranchs', *Journal of Fish Biology*. John Wiley & Sons, Ltd (10.1111), 80(5), pp. 1844–1869. doi: 10.1111/j.1095-8649.2012.03246.x.

Ward, R. D. *et al.* (2005) 'DNA barcoding Australia's fish species.', *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, 360(1462), pp. 1847–57. doi: 10.1098/rstb.2005.1716.

Ward, R. D. (2012) 'FISH-BOL, A Case Study for DNA Barcodes', in. Humana Press, Totowa, NJ, pp. 423–439. doi: 10.1007/978-1-61779-591-6_21.

Wetherbee, B. M. and Gruber, S. H. (1993) 'Absorption Efficiency of the Lemon Shark *Negaprion brevirostris* at Varying Rates of Energy Intake', *Copeia*, 1993(2), p. 416. doi: 10.2307/1447140.

WGEF (2018) *Report of the Working Group on Elasmobranch Fishes (WGEF)*. Lisbon, Portugal. Available at: http://ices.dk/sites/pub/Publication Reports/Expert Group Report/acom/2018/WGEF/02 WGEF Report 2018_Executive_Summary.pdf

White, W. T. (2006) *Aetobatus flagellum. The IUCN Red List of Threatened Species*, *e.T60119A12306888.* doi: http://dx.doi.org/10.2305/IUCN.UK.2006.RLTS.T60119A12306888.en.

White, W. T. and Sommerville, E. (2010) 'Elasmobranchs of Tropical Marine Ecosystems', in Carrier, J. C., Musick, J. A., and Heithaus, M. R. (eds) *Sharks and Their Relatives II: Biodiversity, Adaptive Physiology, and Conservation*. 2nd edn. Boca Raton, FL: CRC Press/Taylor & Francis Group, pp. 159–240.

Whitney, N. M. *et al.* (2012) 'Oceanic dispersal in a sedentary reef shark (*Triaenodon obesus*): genetic evidence for extensive connectivity without a pelagic larval stage', *Journal of Biogeography*. John Wiley & Sons, Ltd (10.1111), 39(6), pp. 1144–1156. doi: 10.1111/j.1365-2699.2011.02660.x.

Wolfe, A. D. and Liston, A. (1998) 'Contributions of PCR-Based Methods to Plant Systematics and Evolutionary Biology', in *Molecular Systematics of Plants II*. Boston, MA: Springer US, pp. 43–86. doi: 10.1007/978-1-4615-5419-6_2.

Worm, B. *et al.* (2013) 'Global catches, exploitation rates, and rebuilding options for sharks', *Marine Policy*. Pergamon, 40, pp. 194–204. doi: 10.1016/J.MARPOL.2012.12.034.

Wourms, J. P. (1981) 'Viviparity: The Maternal-Fetal Relationship in Fishes', *American Zoologist*. Narnia, 21(2), pp. 473–515. doi: 10.1093/icb/21.2.473.

Wourms, J. P. and Demski, L. S. (1993) 'The reproduction and development of sharks, skates, rays and ratfishes: introduction, history, overview, and future prospects', in. Springer, Dordrecht, pp. 7–21. doi: 10.1007/978-94-017-3450-9_1.

Wynen, L. *et al.* (2009) 'Mitochondrial DNA supports the identification of two endangered river sharks (*Glyphis glyphis* and *Glyphis garricki*) across northern Australia', *Marine and Freshwater Research*. CSIRO PUBLISHING, 60(6), p. 554. doi: 10.1071/MF08201.

Xia, C. *et al.* (2014) 'Reintroduction of Przewalski's horse (*Equus ferus przewalskii*) in Xinjiang, China: The status and experience', *Biological Conservation*. Elsevier, 177, pp. 142–147. doi: 10.1016/J.BIOCON.2014.06.021.

Yagishita, N. and Yamaguchi, A. (2009) 'Isolation and characterization of eight microsatellite loci from the longheaded eagle ray, *Aetobatus flagellum* (Elasmobranchii,

Myliobatidae)', *Molecular Ecology Resources*. John Wiley & Sons, Ltd (10.1111), 9(3), pp. 1034–1036. doi: 10.1111/j.1755-0998.2009.02568.x.

Yoshimura, K. *et al.* (2000) 'Preparation and Dynamic Viscoelasticity Characterization of Alkali-Solubilized Collagen from Shark Skin'. American Chemical Society. doi: 10.1021/JF990389D.

Zeng, Y. *et al.* (2016) 'DNA barcoding of Mobulid Ray Gill Rakers for Implementing CITES on Elasmobranch in China', *Scientific Reports*. Nature Publishing Group, 6(1), p. 37567. doi: 10.1038/srep37567.

Zenger, K. R. *et al.* (2006) 'Widespread Utility of Highly Informative AFLP Molecular Markers across Divergent Shark Species', *Journal of Heredity*. Narnia, 97(6), pp. 607–611. doi: 10.1093/jhered/esl044.

Supplementary Table 1: An overview of the data extracted from the literature review into the current measures of genetic diversity on mitochondrial DNA and nuclear DNA (with the use of microsatellites only). The IUCN Red List category including trends and extinction risk is also referenced. All data is up to date as of the 26th of August 2019. **mt#, number of mitochondrial studies, n#, number of nuclear DNA studies, Pp, Total number of populations studied, N, number of individuals analyse, NH, number of haplotypes, H, average number of haplotypes found, h, haplotype diversity, π, nucleotide diversity, Ho, observed heterozygosity, He, expected heterozygosity. IUCN, The IUCN Red List category (LC, Least Concern; NT, Near Threatened; VU, Vulnerable; E, Endangered; CR, Critically Endangered), EX, Extinction risk (Threatened with Extinction, TWE or Non-threatened, NNT), T, species population trend (U, unknown; D, decreasing; S, stable, I, increasing)**

Species	**mt#**	**n#**	***Pp***	***N***	***N* H**	**H**	***h***	***π***	H_o	H_e	**GD Ref**	**IUCN**	**EX**	**T**	**IUCN Ref**
Aetobatus flagellum	0	1	1	24	-	-	-	-	0.465	0.481	Yagishita and Yamaguchi, 2009	EN	TWE	D	White, 2006
Aetobatus narinari	2	1	8	763	50	10	0.683	0.005	0.726	0.739	Sellas *et al.*, 2015	NT	NNT	D	Kyne *et al.*, 2006
Alopias pelagicus	1	1	9	618	19	19	0.419	0.116	0.675	0.729	Cardeñosa, Hyde and Caballero, 2014	VU	TWE	D	Reardon *et al.*, 2009
Carcharhinus acronotus	0	1	1	32	-	-	-	-	0.491	0.507	Giresi *et al.*, 2012	NT	NNT	D	Morgan *et al.*, 2009
Carcharhinus brevipinna	1	0	2	430	37	37	0.677	0.001	-	-	Geraghty *et al.*, 2013	NT	NNT	U	Burgess, 2009
Carcharhinus falciformis	0	1	1	53	-	-	-	-	0.552	0.709	O'Bryhim *et al.*, 2015	VU	NNT	D	Rigby *et al.*, 2017
Carcharhinus leucas	1	2	3	249	14	14	0.760	0.003	0.612	0.6189	Wynen *et al.*, 2009; Karl *et al.*, 2011	NT	NNT	U	Simpfendorfer and Burgess, 2009
Carcharhinus limbatus	3	1	14	1058	69	17	0.636	0.002	0.500	0.500	Keeney *et al.*, 2005	NT	NNT	U	Burgess and Branstetter, 2009
Carcharhinus longimanus	0	1	1	28	-	-	-	-	0.542	0.700	Mendes *et al.*, 2015; Camargo *et al.*, 2016	VU	TWE	D	Baum *et al.*, 2015
Carcharhinus melanopterus	0	1	1	264	-	-	-	-	0.572	0.581	Mourier and Planes, 2013	NT	NNT	D	Heupel, 2009
Carcharhinus obscurus	2	1	10	308	32	16	0.720	0.005	0.623	0.732	Ovenden *et al.*, 2009	VU	TWE	D	Musick, Grubbs, *et al.*, 2009
Carcharhinus plumbeus	1	1	10	398	67	67	0.959	0.005	0.538	0.573	Daly-Engel *et al.*, 2006, 2007	VU	TWE	D	Musick, Stevens, *et al.*, 2009
Carcharhinus sorrah	1	2	3	177	12	12	0.600	0.003	0.5188	0.544	Ovenden, Street and Broderick, 2006; Ovenden *et al.*, 2009	NT	NNT	U	Pillans, Stevens and White, 2009
Carcharhinus tilstoni	0	1	1	79	-	-	-	-	0.649	0.408	Ovenden, Street and Broderick, 2006	LC	NNT	S	Johnson, Pillans and Stevens, 2019
Carcharias Taurus	2	3	9	609	27	3	0.479	0.002	0.726	0.786	Feldheim *et al.*, 2007; Ahonen, Harcourt and Stow, 2009; O'Leary, Feldheim and Chapman, 2013	VU	TWE	U	Pollard and Smith, 2009
Carcharodon carcharias	6	6	12	1319	118	17	0.699	0.007	0.649	0.635	Pardini *et al.*, 2000; Gubili *et al.*, 2009, 2012; Blower *et al.*,	VU	TWE	U	Fergusson, Compagno and

										2012; O'Leary, Feldheim and Chapman, 2013; O'Leary *et al.*, 2015; Andreotti *et al.*, 2016				Marks, 2009
Centroscymnus coelolepis	0	1	1	211	-	-	-	-	0.770	0.770	Veríssimo, Mcdowell and Graves, 2010	NT	NNT	U	Stevens and Correia, 2003
Centroselachus crepidater	0	1	2	20	-	-	-	-	0.511	0.515	Helyar *et al.*, 2011	LC	NNT	U	Stevens, 2003
Cetorhinus maximus	2	0	6	674	133	7	0.577	0.001	-	-	Rus Hoelzel *et al.*, 2006	VU	TWE	D	Fowler, 2009
Chiloscyllium plagiosum	0	1	1	34	-	-	-	-	0.620	0.693	Ding *et al.*, 2009	NT	NNT	U	Kyne and Burgess, 2006
Hemitrygon akajei	1	0	3	107	28	28	0.939	0.007	-	-	Ding *et al.*, 2009	NT	NNT	U	Huveneers and Ishihara, 2016
Dipturus batis	1	1	2	144	15	8	0.455	0.001	0.316	0.388	El Nagar *et al.*, 2010a	CE	TWE	D	Dulvy *et al.*, 2006
Galeorhinus galeus	1	2	7	285	38	38	0.920	0.007	0.636	0.664	Hernández *et al.*, 2014; Bitalo *et al.*, 2015	VU	TWE	D	Walker *et al.*, 2006
Glyphis glyphis	1	0	3	93	12	12	0.760	0.000	-	-	Wynen *et al.*, 2009	EN	TWE	D	Compagno, Pogonoski and Pollard, 2009
Hexanchus griseus	0	2	2	467	-	-	-	-	0.477	0.749	Larson, Tinnemore and Amemiya, 2009a; Wynen *et al.*, 2009; Larson *et al.*, 2011	NT	NNT	U	Cook and Compagno, 2009
Isurus oxyrinchus	0	3	5	647	-	-	-	-	0.738	0.782	Schrey and Heist, 2003; Gubili *et al.*, 2012; Taguchi *et al.*, 2013; Corrigan, Kacev and Werry, 2015	EN	TWE	D	Rigby *et al.*, 2019
Leucoraja naevus	0	1	1	17	-	-	-	-	0.542	0.516	El Nagar *et al.*, 2010a	LC	NNT	U	Ellis, Dulvy and Walls, 2015
Mobula alfredi	0	1	1	60	-	-	-	-	0.477	0.480	Kashiwagi *et al.*, 2012	VU	TWE	D	Marshall *et al.*, 2018
Mustelus antarcticus	0	2	2	357	-	-	-	-	0.684	0.681	Boomer and Stow, 2010; Boomer *et al.*, 2013	LC	NNT	S	Walker, 2016
Mustelus asterias	0	1	1	127	-	-	-	-	0.745	0.735	Farrell *et al.*, 2014	LC	NNT	U	Serena, Mancusi and Ellis, 2009
Mustelus canis	0	1	1	91	-	-	-	-	0.501	0.525	Giresi *et al.*, 2012	NT	NNT	U	Conrath, 2009
Mustelus henlei	0	1	1	213	-	-	-	-	0.783	0.695	Byrne and Avise, 2012	LC	NNT	U	Pérez-Jiménez *et al.*, 2016
Mustelus lenticulatus	0	1	1	75	-	-	-	-	0.614	0.621	Boomer *et al.*, 2013	LC	NNT	S	Finucci and Kyne, 2018
Mustelus mustelus	0	2	6	125	-	-	-	-	0.636	0.515	Bitalo *et al.*, 2015; Marino *et al.*, 2015	VU	TWE	D	Serena *et al.*, 2009

Mustelus schmitti	1	0	2	198	22	11	0.226	0.002	-	-	Pereyra *et al.*, 2010	EN	TWE	D	Massa *et al.*, 2006
Negaprion acutidens	1	2	7	156	4	4	0.280	0.001	0.601	0.653	Schultz *et al.*, 2008; Mourier and Planes, 2013; Mourier *et al.*, 2013	VU	TWE	D	Pillans, 2003
Negaprion brevirostris	1	3	2	1876	11	11	0.780	0.006	0.757	0.793	Feldheim, Gruber and Ashley, 2002; Dibattista *et al.*, 2008; Schultz *et al.*, 2008	NT	NNT	U	Sundström, 2015
Prionace glauca	1	4	10	1022	16	16	0.920	0.005	0.604	0.613	Ovenden *et al.*, 2009; Fitzpatrick *et al.*, 2011; King *et al.*, 2015; Taguchi *et al.*, 2015	NT	NNT	U	Stevens, 2009
Pristis clavata	1	0	1	73	15	15	0.489	0.006	-	-	Phillips *et al.*, 2011	EN	TWE	D	Kyne, Rigby and Simpfendorfer, 2013
Pristis pristis	1	0	1	149	18	18	0.650	0.004	-	-	Phillips *et al.*, 2009	CE	TWE	D	Kyne, Carlson and Smith, 2013
Pristis pectinata	0	2	2	167	-	-	-	-	0.849	0.838	Feldheim *et al.*, 2010; Chapman *et al.*, 2011	CE	TWE	D	Carlson, Wiley and Smith, 2013
Pristis zijsron	1	1	3	109	9	9	0.555	0.003	0.810	0.838	Phillips *et al.*, 2009	CE	TWE	D	Simpfendorfer, 2013
Raja asterias	1	0	3	18	2	2	0.290	0.009	-	-	Valsecchi *et al.*, 2004	NT	NNT	D	Serena *et al.*, 2015
Raja clavata	3	4	24	1934	61	20	0.616	0.005	0.659	0.680	Chevolot *et al.*, 2005, 2006, 2008; El Nagar *et al.*, 2010b	NT	NNT	D	Ellis, 2016
Raja maderensis	1	0	2	37	4	4	0.482	0.001	-	-	Valsecchi *et al.*, 2004	VU	TWE	D	Dulvy *et al.*, 2015
Raja miraletus	1	0	3	18	2	2	0.170	0.003	-	-	Valsecchi *et al.*, 2004	LC	NNT	S	Smale *et al.*, 2009
Raja montagui	0	1	1	23	-	-	-	-	0.588	0.661	El Nagar *et al.*, 2010b	LC	NNT	S	Ellis *et al.*, 2007
Amblyraja radiata	1	0	2	3	3	3	1.000	0.009	-	-	Chevolot *et al.*, 2006; Coulson *et al.*, 2011	EN	TWE	D	Kulka *et al.*, 2009
Raja undulata	0	1	9	108	-	-	-	-	0.683	0.714	Fox *et al.*, 2018	EN	TWE	D	Coelho *et al.*, 2009
Rhincodon typus	3	2	35	1254	273	25	0.993	0.011	0.601	0.623	Ramírez-Macías *et al.*, 2009; Schmidt *et al.*, 2009; Vignaud *et al.*, 2014	EN	TWE	D	Pierce and Norman, 2016
Pseudobatos productus	1	0	4	64	17	17	0.767	0.119	-	-	Sandoval-Castillo *et al.*, 2004	NT	NNT	D	Farrugia *et al.*, 2016
Rhizoprionodon porosus	2	0	3	385	75	19	0.660	0.003	-	-	Mendonça *et al.*, 2013	LC	NNT	S	Lessa *et al.*, 2006
Rhizoprionodon terraenovae	1	0	2	80	24	12	0.762	0.003	-	-	Heist, Musick and Graves, 1996	LC	NNT	U	Dostalova *et al.*, 2009
Scyliorhinus	0	1	1	150	-	-	-	-	0.648	0.648	Griffiths *et al.*, 2010	LC	NNT	S	Ellis *et al.*, 2009

canicula														
Somniosus microcephalus	1	0	1	16	7	7	0.775	0.002	-	-	Murray *et al.*, 2008	NT	NNT	U	Kyne, Sherrill-Mix and Burgess, 2006
Sphyrna lewini	2	2	18	451	33	17	0.708	0.011	0.667	0.724	Nance, Daly-Engel and Marko, 2009; Ovenden *et al.*, 2009	EN	TWE	U	Baum *et al.*, 2009
Sphyrna tiburo	0	1	1	119	-	-	-	-	0.654	0.686	Chapman *et al.*, 2004	LC	NNT	S	Cortés *et al.*, 2006
Sphyrna tudes	1	0	1	55	6	3	0.139	0.000	-	-	Duncan *et al.*, 2006	VU	TWE	D	Mycock, Lessa and Almeida, 2006
Squalus acanthias	1	3	5	909	103	103	0.839	0.009	0.515	0.656	McCauley *et al.*, 2004; Larson, Tinnemore and Amemiya, 2009b; Veríssimo, Mcdowell and Graves, 2010	VU	TWE	D	Fordham *et al.*, 2016
Squatina californica	0	1	1	3	-	-	-	-	0.631	0.59	Larson, Tinnemore and Amemiya, 2009b	NT	NNT	D	Cailliet *et al.*, 2016
Stegostoma fasciatum	1	1	2	75	8	8	0.720	0.140	0.768	0.752	Dudgeon *et al.*, 2006	VU	TWE	D	Dudgeon, Simpfendorfer and Pillans, 2016
Triaenodon obesus	1	0	2	310	15	15	0.550	0.002	-	-	Whitney *et al.*, 2012	NT	NNT	U	Smale, 2009
Triakis semifasciata	0	1	1	471	-	-	-	-	0.858	0.851	Nosal, Lewallen and Burton, 2013	LC	NNT	U	Carlisle *et al.*, 2015

SECTION II: SPECIES IDENTIFICATION

Chapter 2

In deep water: investigating the shark, skate, ray and chimaera (chondrichthyan) fishing in Morocco using genetic barcoding and observational research

S.A. Hook*[1], A. Griffith[2], M. Brierley[3], M. Buckley[1]

[1]School of Earth and Environmental Sciences, Manchester Institute of Biotechnology, University of Manchester, Manchester, M1 7DN, UK

[2]The University of Exeter, Exeter, EX4 4PY

[3]BBC Natural History Unit, Bristol, BS8 2LR

*Corresponding Author: Samantha A. Hook samhook1205@gmail.com

Author Contributions

SAH designed the project, conducted the sampling, genetic analysis and wrote the paper, AG provided sampling equipment, MBrierley assisted in sampling and photography, MBuckley supervised SAH and provided the lab space. All authors contributed equally to the review of the paper.

2.1 Abstract

Chondrichthyans, comprising of sharks, rays, skates (elasmobranchs) and chimaeras are a diverse class of fishes found across the globe that have been evolving for approximately 450 million years. The main threat to chondrichthyans is overfishing, either through regulated or illegal, unreported or unregulated (IUU) methods. Morocco is a marine rich area, with over 70 species of elasmobranchs and an unknown number of chimaeras. Throughout history, Morocco has been reliant on the marine environment to both fish from and for trade, however very little is published on the fisheries on chondrichthyans along its coastline. Here, we use visual observation and DNA sampling to investigate the species diversity of chondrichthyans along the Atlantic coast of Morocco. Using FISH-BOL COI barcoding found in the mitochondrial DNA (mtDNA), we identified 1 chimaera, 13 shark and 11 batoid species from 113 chondrichthyan samples based, on a ≥ 95% sequence similarity criterion against Genbank and the Barcode of Life Database. The average evolutionary divergence between the species barcoded was 0.29% and phylogenetic tree analysis displayed close similarities with others published. We determined that despite a good coverage of the mtDNA, more gene regions are required to complete a more accurate phylogenetic tree. Lastly, we found 29% of the total number of species was classified as threatened with extinction by The IUCN Red List of Endangered Species and a further 20% were data deficient and 3% were not evaluated. Through observations and literature review we found no evidence of effective monitoring, a trade of elasmobranch products, in particular deep-sea species, and evidence of shark fin trade.

Keywords: elasmobranchs, IUCN, CITES, longlining, phylogenetics

2.2 Introduction

The chondrichthyes are a diverse class consisting of elasmobranchs (sharks, skates and rays) and holocephalans (chimaeras), equating to around 1,200 species (Compagno, 2005; Naylor *et al.*, 2012). Of the current 1,000 species of elasmobranchs, one quarter are classified as threatened with extinction by the International Union for the Conservation of Nature (IUCN) Red List of Threatened Species™ (IUCN, 2014). As one of the oldest classes on the planet, existing for nearly 450 million years (Blomme *et al.*, 2006), chondrichthyans have proceeded to become some of the largest and most wide-ranging apex predators of the oceans (Vélez-Zuazo and Agnarsson, 2011). Their decline is also one of the most challenging to document due to their habitat and variety in life-history traits. Despite our lack of knowledge we are currently fishing elasmobranchs at alarming rates, with roughly 100 million sharks killed every year (Worm *et al.*, 2013).

The demand for food is increasing and it is estimated that 1 billion people rely on fish as their primary food source (Engelhaupt, 2007). There has been a rise in the number of fisheries despite globally 60% of commercially important fish stocks are categorised as either ‘depleted’, ‘overexploited’ or ‘recovering’ (FAO, 2012). New technology and better equipment means fishing vessels are delving into unknown territory, such as deep-sea fishing, and are able to fish for longer periods of time in harsher weather conditions (Roberts, 2002). The impacts of fishing pressures on ocean predators such as elasmobranchs are largely unknown (Dulvy *et al.*, 2008). Targeted and untargeted elasmobranch fishing is a great concern worldwide due to the groups low productivity in relation to teleost fish (Stevens *et al.*, 2000); accidental by-catch and illegal, unreported and unregulated (IUU) fishing contributes to 50% of global elasmobranch catch (Gilman, Brothers and Kobayashi, 2005; FAO, 2012; Worm *et al.*, 2013). Of the chondrichthyans, sharks are the most overexploited species due to the high demand in shark fin which is considered a prestigious delicacy in some Asian cultures (Man, Wu and Wong, 2014), shortly followed by the batoids (skates and rays). Due to the recent development in deep-sea fishing holocephalans (chimaeras) are being caught more regularly and the future direction of fishing this group is not yet known (Techera and Klein, 2014).

Coastal tropical regions of the world's oceans hold the highest shark and ray species diversity (Carrier, Musick and Heithaus, 2010; White and Sommerville, 2010), which is often reflected in the fishery captures. The North African coastal country of Morocco is described as a hotspot for batoids, housing more than 58 species off its coastline (Guisande *et al.*, 2013). Similarly, there are roughly 70 known species of shark (Guisande *et al.*, 2013) and an unknown diversity of chimaera which either reside or migrate through the waters in this region. Blue shark for example have prolonged residency in areas on the west coast of Morocco from El- Jadida to Essouria (Queiroz *et al.*, 2012). Morocco borders the North Atlantic Sea and Mediterranean Sea (geographical subarea of the Southern Alboran Sea) which creates a mass of fishing opportunity, industry and therefore wealth. Today, the European Union (EU) pays over €30 million per year for their fisheries partnership agreement with Morocco which has been in place under different protocols since 1995 (Cullberg and Swedish Society for Nature Conservation, 2009; The European Commission, 2013). The agreement allows the EU to fish in both Moroccan and Western Sahara waters, despite Morocco have no legal rights to the Western Sahara's fishing grounds (Zunes and Mundy, 2010). A similar agreement is in place with the Chinese government however, there remains a lack of transparency on the amount of money paid by China for this right. The cost is thought to be a lower than that paid by the EU, driving down the cost of fish from this region (Belhabib *et al.*, 2015). Historically, Japan and South Korean began commercially fishing and finning elasmobranchs in Morocco in 1960s along with cephalopods and pelagic tuna (Baddyr and Guenette, 2001). The increasing fishing effort on the same resources by Asia and the EU suggests increasing competition to secure the fishing grounds in Morocco (Plague, 2001; Milano, 2006). A recent study into the IUU fish caught by the Chinese in Moroccan waters between the years 2000 and 2010 found that 43% of the landed value of fish originating from Morocco was illegal (Belhabib *et al.*, 2015). Despite agreeing to decrease their quota by 40%, European fleets have also continued to illegally increase their catches by 5% in Morocco and the Western Sahara (Belhabib *et al.*, 2015; Pauly and Le Manach, 2015). Morocco has begun to improve their fishing sector with a view of tripling their income by 2020 through a number of means including: raising the volume of fish

production (including aquaculture); increasing the number of fish landing ports; and raising the value of seafood exports (Moroccan Investment Development Agency, no date).

With the knowledge of the value and importance of their fisheries, the Moroccan government has signed numerous declarations and agreements to protect certain species and restrict trade of specimens that may threaten their survival. For example, Morocco is currently an active member of the International Commission for the Conservation of Atlantic Tunas (ICCAT), the Conservation for Migratory Species (CMS) and the Convention on International Trade in Endangered Species (CITES). All three affiliations have a level of protection for one or more elasmobranchii species, which prohibits either landing, trade or both. Numerous reports claim the country is still rife with mass amounts of IUU fishing, including driftnets, despite international prohibitions (CoC, 2015).

In this study, we investigate the potential exploitation of chondrichthyans in ports along the Atlantic coast of Morocco by confirming the identification of chondrichthyan samples taken as part of studies into IUU fishing conducted by the non-government organisation (NGO) (www.finfighters.org). The NGO is part of a large collaboration of non-profit organisations investigating IUU fishing across the EU and trading partners, and specialise in elasmobranch species to increase the knowledge of landing regions and species distributions. Alongside this, we analyse the DNA polymorphisms on the Cytochrome c oxidase I (COI) region on the mitochondria of samples taken from chondrichthyan species identified in the field with two or more individual samples, and compare the single sequence species with the closest matching sequence using DNA reference databases. Lastly we evaluate the genetic barcodes and in-field evidence for indications of IUU fishing under any acts of the ICCAT, CMS and CITES.

2.3 Methods

2.3.1 Study location and Sampling

Samples were collected over the period of two years; between the months of June and July in 2015 and 2016, in accordance with the NGO Fin Fighters annual investigative period. All sites (with the exception of Agadir and Larache) were sampled for duration of four

to six days. Due to the new prohibitions implemented in 2016, both Agadir and Larache were only sampled once (Agadir in 2015 and Larache in 2016) (Figure 2.1).

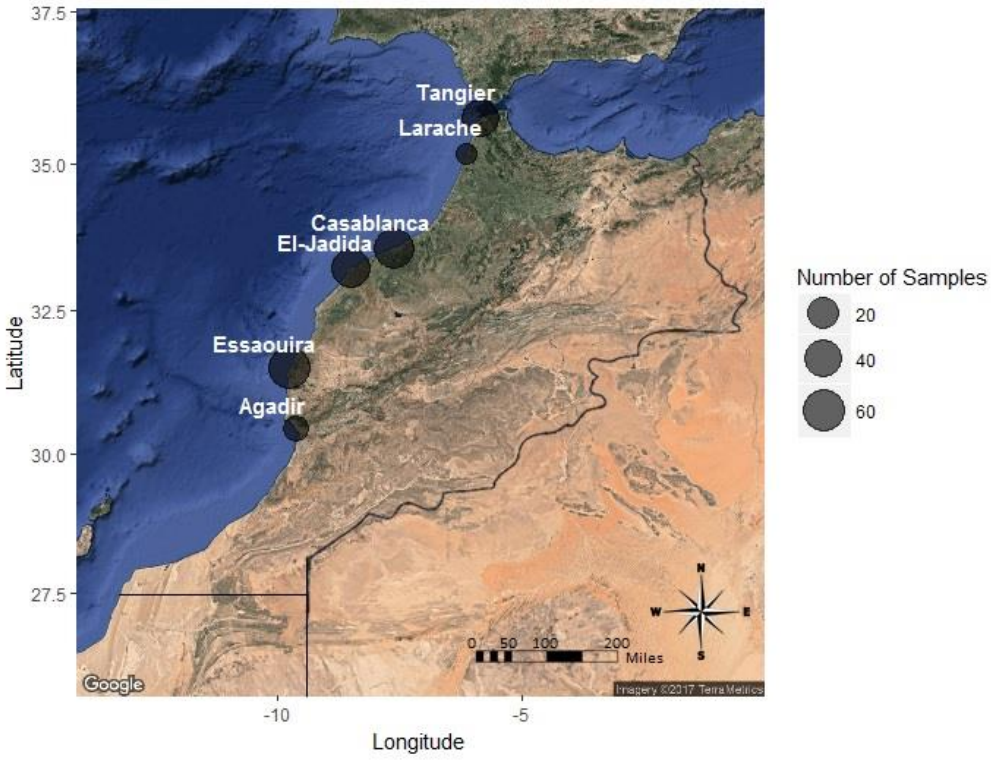

Figure 2.1. Google adapted map of sample sites and number of samples (created using ggmap and ggplot in R version 3.2.1). Locality follows the recommendations by Steinke and Hanner (2010; latitude and longitude with the use of GPS).

2.3.2 Specimen documentation, imaging and sample collection

Fin clip samples were acquired from 230 individual shark, batoid or chimaera species. Samples were stored in RNALater and held at 4 ˚C until transported to the lab where samples were held at -80 ˚C. Date and time of collection, location and individual identification at either species or genus level were recorded for all samples. Identification levels (as defined by Steinke and Hanner (2011)) ranged between Level 1 at which specimen identification is highly reliable to Level 5 at which the identification was superficial, depending on the condition of the sample. Where possible, the measurements of individuals were recorded; body mass (kilograms), total length (from nose to tip of tail; centimetres), fork length (from nose to base of tail; centimetres), width (between the point of which pectoral fins meet the torso; centimetres), wing diameter (widest part of the ray; centimetres), sex and level of maturity (stiffening of the claspers or signs of offspring). Because samples were collected from working fishing ports and markets, it was not possible to take photographs

and record all parameters in some instances. Information follows that of Steinke and Hanner (2010) ensuring sequences and information, including photographs, can be added to the FISH-BOL project.

2.3.3 DNA extraction, amplification and sequencing

DNA was extracted using Qiagen DNA blood and tissue extraction kit protocol. Approximately 655 base pairs (bp) of the Cytochrome c oxidase I (COI) gene were amplified using a same combinations as in Ward *et al.*, (2005) primer designs:

FishF1 (5'-TCAACCAACCACAAAGACATTGGCAC-3')

FishR1 (5'-TAGACTTCTGGGTGGCCAAAGAATCA-3')

FishF2 (5′-TCGACTAATCATAAAGATATCGGCAC-3′)

FishR2 (5′-ACTTCAGGGTGACCGAAGAATCAGAA-3′)

DNA amplification conditions follow Steinke and Hanner (2011) protocol to ensure sequences can be added to the FISH-BOL project; 12.5 µl Polymerase Chain Reaction (PCR) mix contained 1.25 µl of 10x PCR buffer (PCR grade $MgCl^2$), 0.125 µl of each primers (either combination F1 and R1 or F2 and R2, 10mM), 0.25 µl of a dNTP mix (10mM), 0.125 µl of DNA Taq Polymerase, 0.5-2 µl of DNA template (50ng) and 10.125-8.625 PCR grade ultrapure double-distilled H_2O (ddH_2O). Thermal cycler conditions were adapted from that of Steinke and Hanner (2010); one cycle for 5 minutes at 95˚C (denaturation), followed by 35 cycles of 0.5 minutes at 95˚C (denaturation), 0.5 minutes at 54˚C (annealing) and 1 minute at 72˚C (amplification), completed by 10 minutes at 72˚C (final amplification stage) and then held at 4˚C. PCR products were loaded on a 1.5% agarose gel and products which were visible were sent for sequencing.

2.3.4 DNA polymorphisms and evolutionary divergence

The PCR products were prepared to 10 ng/µl and sent to be sequenced by the in-house DNA facility at the University of Manchester which uses Sanger sequencing. Once the sequences were obtained, the results were first reviewed using BioEdit version 7.2.5 (Hall, 1999). Sequences were uploaded onto the Nucleotide Basic Local Alignment Search Tool

(BLAST) on Genbank (www.ncbi.nlm.nih.gov/nucloetide) and Barcode of Life Database (BOLD) (www.boldsystems.org) systems as part of the international barcode of life. A criterion of 95% similarity threshold was held upon each sequence in either nucleotide search tools as there are no present uploaded samples from the area of Morocco. Sequence information was uploaded under the recommendations of Steinke and Hanner (2011) to contribute to the current global taxonomic authority file of the campaign FISH-BOL (Froese and Pauly, 2017). In BioEdit, ClustalW full multiple alignment bootstrap NJ Tree of 1000 runs was conducted to align sequences of the same species (Higgins, 1997; Hall, 1999). The DNA polymorphisms were analysed in DNAsp (Rozas *et al.*, 2003) between the aligned regions. DNA polymorphisms included the number of segregating sites, the number of haplotypes, haplotype (gene) diversity, and standard deviation (the square root of sampling variance (Nei, 1987)). In samples with only one barcode region, BOLD and GenBank were used to determine the closest matching sequence (measured as a percentage (%)) and the region of which the sample was fished (country and ocean). Finally, we reviewed the estimates of evolutionary divergence between all species, using the longest COI sequences found in each species in Mega X (Kumar *et al.*, 2018). Analyses were conducted using the Maximum Composite Likelihood model (Tamura, Nei and Kumar, 2004).

2.3.5 Phylogenetic analysis

All phylogenies were conducted on sequence data aligned through ClustalW (Higgins, 1997) in Bioedit (Hall, 1999). We conducted two phylogenetic analysis to determine the maximum likelihood evolutionary history between the 113 COI barcodes collected. To determine which maximum likelihood model for each tree would fit our each set of data we used the Mega X (Kumar *et al.*, 2018). Our first analysis was then completed using the Maximum Likelihood method and Hasegawa-Kishino-Yano model (Hasegawa, Kishino and Yano, 1985) with gamma distribution (5 categories (+G, parameter = 0.3548)). We then randomly selected one sequence from each species within our CO1 barcoded list, totalling in 25 nucleotide sequences and ran a further analysis under a General Time Reversible model (Nei and Kumar, 2000) also with a gamma (G) distribution (5 categories (+G, parameter = 0.4868)). Both trees were calculated under a bootstrap consensus tree inferred from 500 replications (Felsenstein, 1985). Both trees were rooted to *Chimaera*

opalescens, as this was the furthest distantly related individual within the chondrichthyan phylogeny.

2.4 Results

From the two sampling trips (June 2015 and June 2016) we collected 230 samples and visually detected 31 species from six different locations including fish markets and dock markets along the North-Atlantic coast of Morocco (Table 2.1, Figure 2.1). DNA barcoding determined 113 chondrichthyan samples based on a ≥ 95% sequence similarity criterion in Genbank and BOLD, representing one chimaera, 13 sharks and 11 batoids.

Figure 2.2. Photographs of sharks and shark-fin sample collection on the dockside markets and indoor fish markets. Top left, large mature shortfin mako shark Isurus oxyrinchus (El- Jadida, June 2016) Top right, the jaw of a butchered shortfin mako Isurus oxyrinchus (Tangier, June 2016), Bottom left, gutted deep-sea sharks Centrophorus squamosus, Centroscymnus coelolepis and Centrophorus granulosus (Agadir, June 2015), Bottom right, collection of fins for shark-fin trade (Essouira, June 2015).

From the observed and sampled species list, we found 29% of the total number of species was classified as threatened with extinction by The IUCN Red List of Endangered Species, hereafter referred to as The IUCN Red List (Dulvy *et al.*, 2014). A further 20% were

data deficient and 3% were not evaluated (Table 2.1). We also found 39% of the species were decreasing globally in the wild and only 20% were classified as stable. Of the nine species classified as threatened with extinction a third were found at three or more locations, and over 55% were observed in a juvenile life stage.

Table 2.1. The observed and barcoded species from the North Atlantic coastline of Morocco. Years: from which year the species was observed and a sample was collected. Location, AD, Agadir, CB, Casablanca, EJ, El-Jadida, ES, Essouira, LC, Larache, TG, Tangier, Life Stages, MT, Mature, JV, Juvenile, IUCN, LC, Least Concern, NT, Near Threatened, VU, Vulnerable, EN, Endangered, CR, Critically Endangered, DD, Data Deficient, NA, Not Assessed. ↑, increasing, —, stable, ↓, decreasing, UK, Unknown. *CITES Appendix listing, and/or CMS agreement.

Common name	Scientific name	Years	Location	Life Stages	IUCN	Trend
Big-eyed Thresher Shark	*Alopias superciliosus**	2015, 2016	AD, ES, TG	MT, JV	VU	↓
Gulper Shark	*Centrophorus granulosus*	2015, 2016	CB	MT	DD	UK
Leafscale Gulper Shark	*Centrophorus squamosus*	2015, 2016	LC, TG	MT, UK	VU	↓
Portuguese Dogfish	*Centroscymnus coelolepis*	2016	CB	MT	NT	UK
Rabbit Fish	*Chimaera monstrosa*	2016	EJ, LC	MT	NT	—
Opal Chimaera	*Chimaera opalescens*	2016	EJ	MT	NE	UK
Kitefin Shark	*Dalatias licha*	2015	TG	JV	NT	UK
Tortonese's Stingray	*Dasyatis tortonesei*	2015, 2016	ES	MT	DD	UK
Birdbeak Dogfish	*Deania calcea*	2015, 2016	CB, LC	MT	LC	↑
Common Skate	*Dipturus batis*	2015	AD	MT	CR	↓
School Shark	*Galeorhinus galeus*	2015, 2016	ES, EJ	MT, JV, UK	VU	↓
Atlantic Sawtail Shark	*Galeus atlanticus*	2015, 2016	TG	MT, JV	NT	UK
Blackmouth Catshark	*Galeus melastomus*	2015, 2016	CB, ES, EJ	MT, JV	LC	—
Bluntnose Sixgill Shark	*Hexanchus griseus*	2016	CB	MT, JV	NT	UK

Shortfin Mako	*Isurus oxyrinchus**	2015, 2016	ES, EJ, TG	MT, JV, UK	VU	↓
Cuckoo Ray	*Leucoraja naevus*	2015, 2016	AG	MT	LC	UK
Sandy Ray	*Leucoraja Circularis*	2016	AD, ES	MT	EN	↓
Smooth Hound	*Mustelus mustelus*	2015, 2016	EJ	MT	VU	↓
Blue Shark	*Prionace glauca*	2015, 2016	CB, ES, EJ, TG	MT, JV, UK	NT	UK
Blonde Skate	*Raja brachyura*	2015, 2016	ES, EJ, TG	MT, JV	NT	↓
Thornback Ray	*Raja clavata*	2015, 2016	CB, EJ	MT, JV	NT	↓
Small-eyed Ray	*Raja microocellata*	2015, 2016	CB, ES	MT	NT	↓
Brown Ray	*Raja miraletus*	2015	AG, CB, TG	MT	LC	—
Spotted Ray	*Raja montagui*	2015, 2016	ES	MT, JV	LC	—
Undulate Ray	*Raja undulata*	2015, 2016	AG, CB, EL, ES, LC, TG	MT, JV	EN	↓
Common Guitar Fish	*Rhinobatos rhinobatos**	2015, 2016	CB, EJ	JV, UK	EN	↓
Small-spotted Catshark	*Scyliorhinus canicula*	2015, 2016	AG, CB, ES, EJ, TG	MT, JV, UK	LC	—
Knifetooth Shark	*Scymnodon ringens*	2016	CB	MT	DD	UK
Common Torpedo Ray	*Torpedo torpedo*	2016	AG, EJ	MT	DD	—
Marbled Electric Torpedo Ray	*Torpedo marmorata*	2015, 2016	ES	MT, UK	DD	UK
Velvet Dogfish	*Zameus squamulosus*	2016	CB	MT	DD	UK

By sampling in 2015 and 2016, the number of successfully barcoded samples increased from 59 to 113. We were also able to visit a new location (Larache) in 2016. We barcoded one tissue sample from a fish steak collected from Tangier fish market on the 7th

of June 2016 that was confirmed to be swordfish by both GenBank and BOLD. Due to a language barrier and no viable labelling on any products, this sample is most likely a product of misidentification within the field.

Table 2.2. Summary of polymorphism statistics for the COI fragment. n, number of samples; S, number of segregating sites; Nh, number of haplotypes; Hh, haplotype diversity (±SD); π, nucleotide diversity, Sim (%), closest sequence similarity percentage (%), R – O, Region of closes similar sequence (R- O, Med-Mediterranean, NA, North-Atlantic, BB, Bay of Bengal, SA, South-Atlantic, TS, Tasmanian Sea, AS, Arabian Sea), Ref, BOLD Reference BD:, Genbank Reference (BLAST) BL

Species	*n*	*S*	N_h	H_h	π (%)	*Sim (%)*	*R - O*	Ref
Alopias superciliosus	1	–	–	–	–	100	India - BB	BD: GBGC11452-13
Centrophorus granulosus	1	–	–	–	–	96	Malta - Med	BL: KY909356.1
Centrophorus squamosus	2	11	2	1.00 (±0.50)	0.05	–	–	–
Centroscymnus coelolepis	1	–	–	–	–	100	Tasmania - TS	BD: FOA136-04
Chimaera opalescens	2	3	2	1.00 (±0.50)	0.01	–	–	–
Dalatias licha	1	–	–	–	–	100	France - NA	BD: ANGBF419-12
Dasyatis tortonesei	1	–	–	–	–	100	Italy - Med	BD: ELAME119-09
Deania calcea	2	2	2	1.00 (±0.50)	0.00	–	–	–
Galeorhinus galeus	5	49	3	0.70 (±0.22)	0.05	–	–	–
Galeus atlanticus	1	–	–	–	–	100	Portugal - NA	BD: MLFPI004-09
Isurus oxyrinchus	1	–	–	–	–	100	Oman - AS	BD: GEP092-12
Leucoraja naevus	1	–	–	–	–	100	Portugal - NA	BD:FCFP004-05
Mustelus mustelus	3	13	3	1.00 (±0.27)	0.06	–	–	–
Prionace glauca	2	7	2	1.00 (±0.50)	0.05	–	–	–
Raja brachyura	2	19	2	1.00 (±0.50)	0.05	–	–	–
Raja clavata	5	5	3	0.70 (±0.22)	0.00	–	–	–
Raja microocellata	4	0	1	0.00 (±0.00)	0.00	–	–	–
Raja miraletus	1	–	–	–	–	97	Angola - SA	BD: ELAME729-09
Raja montagui	1	–	–	–	–	100	Portugal - NA	BD: FCFP006-05
Raja undulata	33	18	5	0.23 (±0.10)	0.01	–	–	–
Rhinobatos rhinobatos	1	–	–	–	–	94	Israel - Med	BL: KF564313.1
Scyliorhinus canicula	34	45	10	0.51 (±0.11)	0.04	–	–	–
Torpedo marmorata	5	27	4	0.90 (±0.16)	0.02	–	–	–
Torpedo torpedo	1	–	–	–	–	100	Israel - Med	BD: BIM093-13
Zameus squamulosus	2	7	2	1.00 (±0.50)	0.01	–	–	–

Whilst the majority of the samples produced nearly all of the bases in the 655 bp COI region, there were 16 sequences that generated between 50% and 70% less bp's than expected, with the shortest sequence equalling to only 100 bp. Despite short reads, the sequences produced sufficient to find a GenBank or BOLD matches or both. In our results, GenBank was able to successfully identify 111 sequences, while BOLD identified 101

sequences. When GenBank and BOLD could identify the sequences, the match was the same species.

Table 2.3. The estimates of evolutionary divergence between sequences as calculated in Mega X (Kumar et al., 2018) using the maximum likelihood model (Tamura, Nei and Kumar, 2004). This analysis involved 25 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option) and there were a total of 682 positions in the final dataset.

		01	02	03	04	05	06	07	08	09	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
02	*Mustelus mustelus*	0.15																							
03	*Galeus atlanticus*	0.21	0.26																						
04	*Prionace glauca*	0.19	0.29	0.21																					
05	*Alopias superciliosus*	0.28	0.24	0.28	0.31																				
06	*Isurus oxyrinchus*	0.30	0.20	0.29	0.28	0.18																			
07	*Scyliorhinus canicula*	0.28	0.23	0.25	0.29	0.25	0.30																		
08	*Torpedo marmorata*	0.24	0.32	0.29	0.30	0.25	0.30	0.17																	
09	*Torpedo torpedo*	0.34	0.31	0.35	0.34	0.38	0.40	0.26	0.15																
10	*Centroscymnus coelolepis*	0.36	0.31	0.31	0.26	0.40	0.39	0.30	0.28	0.37															
11	*Zameus squamulosus*	0.40	0.35	0.34	0.32	0.42	0.41	0.33	0.29	0.41	0.11														
12	*Dalatias licha*	0.35	0.29	0.33	0.28	0.30	0.35	0.33	0.31	0.34	0.17	0.18													
13	*Deania calcea*	0.36	0.28	0.31	0.27	0.33	0.38	0.28	0.30	0.33	0.15	0.18	0.16												
14	*Centrophorus granulosus*	0.45	0.44	0.35	0.35	0.40	0.46	0.38	0.35	0.45	0.23	0.27	0.23	0.15											
15	*Centrophorus squamosus*	0.34	0.33	0.30	0.28	0.27	0.30	0.28	0.34	0.34	0.15	0.18	0.18	0.12	0.13										
16	*Rhinobatos rhinobatos*	0.39	0.28	0.37	0.26	0.44	0.41	0.35	0.27	0.35	0.28	0.28	0.29	0.28	0.38	0.28									
17	*Leucoraja naevus*	0.25	0.27	0.31	0.31	0.27	0.24	0.28	0.29	0.29	0.29	0.28	0.32	0.28	0.35	0.32	0.23								
18	*Raja miraletus*	0.41	0.27	0.30	0.30	0.40	0.38	0.34	0.27	0.41	0.27	0.26	0.30	0.31	0.40	0.30	0.24	0.15							
19	*Raja undulata*	0.40	0.30	0.31	0.30	0.37	0.36	0.33	0.27	0.38	0.28	0.29	0.28	0.29	0.39	0.32	0.23	0.15	0.08						
20	*Raja brachyura*	0.31	0.33	0.33	0.33	0.31	0.29	0.33	0.31	0.31	0.33	0.36	0.33	0.35	0.41	0.36	0.27	0.23	0.15	0.12					
21	*Raja microocellata*	0.35	0.27	0.27	0.28	0.30	0.33	0.34	0.27	0.34	0.33	0.32	0.27	0.29	0.41	0.31	0.29	0.14	0.13	0.12	0.10				
22	*Raja clavata*	0.39	0.34	0.29	0.32	0.33	0.34	0.33	0.28	0.33	0.32	0.31	0.30	0.32	0.39	0.34	0.26	0.18	0.08	0.08	0.12	0.06			
23	*Raja montagui*	0.37	0.29	0.29	0.29	0.34	0.32	0.33	0.29	0.35	0.30	0.29	0.31	0.32	0.39	0.31	0.26	0.16	0.09	0.07	0.12	0.06	0.05		
24	*Dasyatis tortonesei*	0.43	0.28	0.33	0.33	0.41	0.41	0.35	0.29	0.38	0.27	0.26	0.31	0.32	0.41	0.31	0.24	0.27	0.26	0.24	0.32	0.30	0.26	0.26	
25	*Chimaera opalescens*	0.40	0.29	0.32	0.34	0.31	0.35	0.34	0.30	0.35	0.31	0.32	0.28	0.29	0.37	0.33	0.29	0.26	0.27	0.26	0.29	0.26	0.27	0.27	0.23

The overall average evolutionary distance between each COI barcoded sequences was 0.29. This analysis involved 25 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option) and there were a total of 682 positions in the final dataset.

2.4.1 DNA trees analysis results

From the two evolutionary analyses conducted in Mega X (Kumar *et al.*, 2018), we found that the tree with one randomly selected COI barcode for each species had the highest percentage of replicate trees in which the associated taxa were clustered together in the bootstrap test (500 replicates) (Felsenstein, 1985) (Figure 2.3).

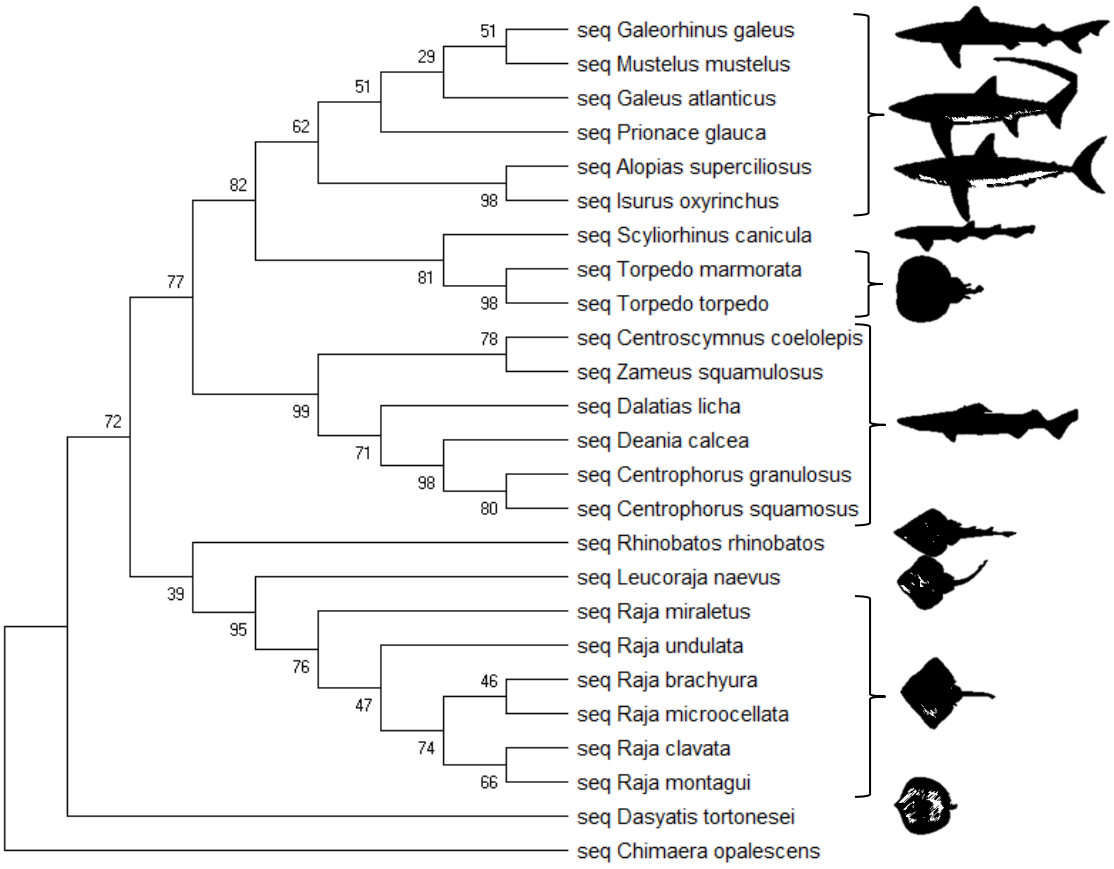

Figure 2.3. The evolutionary analysis by Maximum Likelihood method and General Time Reversible model (Nei and Kumar, 2000). A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.4868)). There were a total of 682 positions in the final dataset. Silhouettes were drawn through GIMP v 2.10.10 and collated onto the phylogenetic tree.

Initial tree(s) for the heuristic search were obtained automatically by applying Neighbour-Joining (NJ) and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The first evolutionary analysis was conducted under the Hasegawa-Kishino-Yano model (Hasegawa, Kishino and Yano, 1985) in Mega X (Kumar *et al.*, 2018), and defined the clades within the species with multiple barcodes (Supplementary Figure 2.1). This analysis involved 113 nucleotide sequences and there were a total of 705 positions in the final dataset.

2.5 Discussion

Here we have conducted the first investigation into elasmobranch fisheries along the Atlantic coast of Morocco, creating a detailed analysis of species observed and conducting a COI barcoding analysis on the species samples sampled. By barcoding the c.650 bp region of the mitochondrial DNA (mtDNA) COI barcode we discriminated the differences between the samples and therefore identified all111 samples used. Of the species observed and samples, we show that 29% are threatened with extinction, with a further 39% decreasing in the wild, indicating that conservation management is required in order to save these rare and declining species.

DNA barcoding as a method of species ID is a common technique with a high number of applications across the marine environment. The various barcoding techniques are playing a more important role to define new species or investigate catch. Reference material in the forms of sequence data from as many species as possible is a necessity in order to determine species identification, on both a global and regional level. Within the chondrichthyans there were only four records of elasmobranchii species on the BOLD database to have been barcoded within Morocco (Ratnasingham and Hebert, 2007; Steinke and Hanner, 2011); two unknown Squalus species, one *Etmopterus pusillus* and one *Pteroplatytrygon violacea*. In our study we increased this number by sequencing 113 individuals from 25 species landed in six ports along the Morocco's North Atlantic coast. The lack of reference material poses an issue if barcode regions were used to define populations for management.

Although the FISH-BOL Project and BOLD have been keen players in the identification of fish species (Ward, Hanner and Hebert, 2009), the size in base pairs of the COI region has proven to be difficult in certain sample types, demanding different barcoding methods for species identification. These alternative barcoding regions often still investigate the COI gene and it is used for most animal life (Dawnay *et al.*, 2007). For degraded elasmobranch template DNA, such as found in processed shark fin products that have undergone either desiccation, boiling or both for shark fin soup, shorter assay regions are required to yield to short sequences, often between 110 and 130 bp (Fields *et al.*, 2015). Mini-barcoding as an adaptation to the FISH-BOL protocol, is providing novel opportunities to identify shark fin soup samples (Fields *et al.*, 2015) , elasmobranch liver oil pills and even skincare products (Cardeñosa *et al.*, 2017). Mini-barcodes have also revolutionised the ability to detect elasmobranch species through environmental DNA (Bakker *et al.*, 2017). By using mini-barcoding however, there is a loss of variable regions along the 650 bp region of the COI gene that can often provide extra information, such as regional haplotype from which individuals were fished. Ultimately, it has been found to be more effective to use the original FISH-BOL protocol, and use the resulting sequence, which ultimately can vary in size, but still provide species identification (Dawnay *et al.*, 2007; Ward, Hanner and Hebert, 2009; Griffiths *et al.*, 2013; Steinke *et al.*, 2017; Hellberg, Isaacs and Hernandez, 2019). Within our study, despite samples being collected as either fresh or discarded with slight desiccation, certain samples provided base pair lengths of between 50% and 70% less than expected. If further investigation were to be conducted on specimens that had been processed further, it could be determined whether mini-barcodes are required (Hellberg, Isaacs and Hernandez, 2019).

The average degree of evolutionary divergence between these 25 species was 0.29%. Previously, evolutionary divergence were found to be 0.35% for 111 chondrichthyan species (Bineesh *et al.*, 2017) and 0.37% for 210 species of chondrichthyans (Ward *et al.*, 2008). Therefore, it can be assumed that by increasing the number of chondrichthyan species, the level of evolutionary divergence will also increase. By sequencing a *Chimaera opalescens* we were able to successfully root a COI an evolutionary analysis tree from the maximum likelihood method. Overall our tree displayed interesting traits of evolutionary

divergence, placing the Torpedo genus within the shark branches at an 82% certainty. We know however this is not the case, and Torpedo genus falls within the batoids under elasmobranchii classification (Naylor, Fedrigo and Andrés López, 2005). From the likelihood analysis combining mitochondrial and nucleotide data published for all chondrichthyans conducted by Naylor *et al.,* (2005), we see that the placement of specific families using the COI barcode only is less likely to be the true phylogenetic tree. Mitochondrial DNA can become saturated between species at a nucleotide and codon level, causing evolutionary differentiation for phylogenetic analysis (Naylor, Fedrigo and Andrés López, 2005). Differences between mtDNA are better observed when the whole genome is present, rather than reliance on one gene, such as our COI barcoding region. Despite using one mitochondrial gene region, our tree was able to collate orders between species and generally grouped sharks and batoids separately once rooted.

Generally, there is a lower level scientific research within developing countries such as Morocco, especially in areas involving the marine environments. We found chondrichthyan fishing is prevalent along the Atlantic coast of Morocco representing both elasmobranchs and holocephalans, and although larger markets had fisheries enforcement staff, we found no evidence of documenting fishing activity. We also noted that there were a high amount of juveniles present and fins were separated from a range of sized elasmobranchs for the fin trade. By sampling the areas in June 2015 and 2016, we increased the number of species visually observed, our number of samples and therefore the diversity of species identified. As the months were consistently the same over the two years, it would be recommended to further investigate the species landed at other months of the year. We observed that every known town and city we visited along the Moroccan Atlantic coast had a certain level of primary (developing commercial) fishing activity; however, the majority were artisanal fisheries which work often with longlines. By engaging with the local workers of the town of Essaouira we learnt that the target fishing has adapted as species diversity has changed during an average person's lifetime. They speculated that it was due to the Moroccan government sale of its fishing rights, which has allowed large commercial fishing vessels to catch fish continuously along the entire coast for months at a time. This was said to be causing the local fishing economy to diversify into deep-sea

fishing, as populations at the surface depleted and we found this was evident in the number of deep-sea chondrichthyans collected, including *Centrophorus squamosus, Centroscymnus coelolepis*, *Centrophorus granulosus* and *Chimaera opalescens,* of which are present between 200 m and 4,000 m in depth (Compagno, 1984; Compagno and Niem, 1998; Daley, Stevens and Graham, 2002).

These shark species, along with other apex and mesopredators, are prime capture examples of longliners in artisanal fisheries (Ward and Myers, 2005; Gilman *et al.*, 2016). Longline fisheries can have specific gear, tailored for higher trophic species to be captured, for example circle hooks increase elasmobranch catch as the method has up to 1.2 times relative risk of capture (Gilman *et al.*, 2016). Further evidence of demersal longlining was found with the number of batoids on sale on the docks, indicating target fisheries within Essaouira, however, hook type was not identified. Historically the majority of elasmobranch catch is a secondary industry to other more desirable species, such as sardines in Essaouira. The upwelling environment and plentiful prey, attracts apex predators which increases the probability of their capture (Roy, Cury and Kifani, 1992; Galego Fernandes *et al.*, 2005). As commercial fisheries continue to increase, it is highly probable that the sardine numbers will decline. This could result in a reduce amount of prey within the marine environment and increase the number of localised target fisheries for predators (Ward and Myers, 2005; Gilman *et al.*, 2016).

To ensure the future of elasmobranch populations, management is a necessity, accounting for both localised and foreign commercial fisheries utilising the Moroccan coast. Morocco has large financial gains from the availability of populations, from both the EU and Asia (Zunes and Mundy, 2010; The European Commission, 2013; Belhabib *et al.*, 2015). From our analysis, it is clear species threatened with extinction are being caught. As IUU fishing is a major cause in the decline of chondrichthyans, and as Morocco is a key environment for roughly 70 species of elasmobranchs and unknown amount of holocephalans, it is important that the country abides by the regulations set in place under the agreements which ensure trading.

2.6 Acknowledgements

The author would like to thank Fin Fighters.org for allowing them to join on their expeditions to Morocco. Without this, we would not have been able to conduct this research as efficiently.

2.7 References

Baddyr, M. and Guenette, S. (2001) 'The Fisheries off the Atlantic Coast of Morocco 1950 - 1997', *Fisheries Centre Research Reports*, 9(3).

Bakker, J. *et al.* (2017) 'Environmental DNA reveals tropical shark diversity in contrasting levels of anthropogenic impact', *Scientific Reports*. Nature Publishing Group, 7(1), p. 16886. doi: 10.1038/s41598-017-17150-2.

Belhabib, D. *et al.* (2015) 'Euros vs. Yuan: Comparing European and Chinese Fishing Access in West Africa', *PLOS ONE*. Edited by A. C. Tsikliras. Public Library of Science, 10(3), p. e0118351. doi: 10.1371/journal.pone.0118351.

Bineesh, K. K. *et al.* (2017) 'DNA barcoding reveals species composition of sharks and rays in the Indian commercial fishery', *Mitochondrial DNA Part A*. Taylor & Francis, 28(4), pp. 458–472. doi: 10.3109/19401736.2015.1137900.

Blomme, T. *et al.* (2006) 'The gain and loss of genes during 600 million years of vertebrate evolution', *Genome Biology*. BioMed Central, 7(5), p. R43. doi: 10.1186/gb-2006-7-5-r43.

Cardeñosa, D. *et al.* (2017) 'A multiplex PCR mini-barcode assay to identify processed shark products in the global trade', *PLOS ONE*. Edited by M. Hajibabaei. Public Library of Science, 12(10), p. e0185368. doi: 10.1371/journal.pone.0185368.

Carrier, J. C., Musick, J. A. and Heithaus, M. R. (2010) *Sharks and their relatives II : biodiversity, adaptive physiology, and conservation*. CRC Press/Taylor & Francis.

CoC (2015) *Compliance Committee: Working group on illegal, unreported and unregulated (IUU) fishing in the GFCM area*. Marrakech, Morocco.

Compagno, L. J. (1984) *Sharks of the world / 2. Carcharhiniformes.* Food and Agriculture Organization of the United Nations.

Compagno, L. J. V. (2005) 'Checklist of living Chondrichthyes', *Reproductive biology and phylogeny of Chondrichthyes : sharks, batoids and chimaeras*. Science Publishers, pp. 503–548.

Compagno, L. J. V. and Niem, V. H. (1998) 'Squalidae', in Niem, K. E. C. and V. H. (ed.) *FAO species identification guide for fishery purposes. The living marine resources of the Western Central Pacific. Volume 2. Cephalopods, crustaceans, holothurians and sharks.* Rome: FAO, pp. 1213–1232.

Cullberg, M. 168356 and Swedish Society for Nature Conservation, S. (Sweden) eng (2009) 'To draw the line. A report about EU fisheries agreements in West Africa'. Stockholm (Sweden) SSNC.

Daley, R. K., Stevens, J. D. and Graham, K. (2002) 'Catch analysis and productivity of the deepwater dogfish resource in southern Australia'. Hobart, Tas., CSIRO Div. of Marine Research.

Dawnay, N. *et al.* (2007) 'Validation of the barcoding gene COI for use in forensic genetic

species identification', *Forensic Science International*. Elsevier, 173(1), pp. 1–6. doi: 10.1016/J.FORSCIINT.2006.09.013.

Dulvy, N. K. *et al.* (2008) 'You can swim but you can't hide: the global status and conservation of oceanic pelagic sharks and rays', *Aquatic Conservation: Marine and Freshwater Ecosystems*. John Wiley & Sons, Ltd., 18(5), pp. 459–482. doi: 10.1002/aqc.975.

Dulvy, N. K. *et al.* (2014) 'Extinction risk and conservation of the world's sharks and rays.', *eLife*. eLife Sciences Publications, Ltd, 3, p. e00590. doi: 10.7554/eLife.00590.

Engelhaupt, E. (2007) 'Farming the Deep Blue Sea', *Environmental Science and Technology*, pp. 4188–4191.

FAO (2012) *The State of World Fisheries and Aquaculture 1999*. Rome.

Felsenstein, J. (1985) 'Confidence limites on phylogenies: an approach using the bootstrap', *Evolution*. John Wiley & Sons, Ltd (10.1111), 39(4), pp. 783–791. doi: 10.1111/j.1558-5646.1985.tb00420.x.

Fields, A. T. *et al.* (2015) 'A Novel Mini-DNA Barcoding Assay to Identify Processed Fins from Internationally Protected Shark Species', *PLOS ONE*. Edited by A. Stow. Public Library of Science, 10(2), p. e0114844. doi: 10.1371/journal.pone.0114844.

Froese, R. and Pauly, D. (2017) *FishBase*, *World Wide Web electronic publication*. Available at: http://www.fishbase.org/.

Galego Fernandes, P. *et al.* (2005) 'Anthropogenic features in the Sines (Portugal) and Essaouira (Morocco) coastal aquifers: a comparative study of their hydrochemical evolution by a Principal Component Analysis.' Consejo Superior de Investigaciones Científicas - Consejo Superior de Investigaciones Científicas.

Gilman, E. *et al.* (2016) 'A cross-taxa assessment of pelagic longline by-catch mitigation measures: conflicts and mutual benefits to elasmobranchs', *Fish and Fisheries*. John Wiley & Sons, Ltd (10.1111), 17(3), pp. 748–784. doi: 10.1111/faf.12143.

Gilman, E., Brothers, N. and Kobayashi, D. R. (2005) 'Principles and approaches to abate seabird by-catch in longline fisheries', *Fish and Fisheries*. Blackwell Science Ltd, 6(1), pp. 35–49. doi: 10.1111/j.1467-2679.2005.00175.x.

Griffiths, A. M. *et al.* (2013) 'DNA barcoding unveils skate (Chondrichthyes: Rajidae) species diversity in "ray" products sold across Ireland and the UK', *PeerJ*. PeerJ Inc., 1, p. e129. doi: 10.7717/peerj.129.

Guisande, C. *et al.* (2013) 'Factors affecting species richness of marine elasmobranchs', *Biodiversity and Conservation*. Springer Netherlands, 22(8), pp. 1703–1714. doi: 10.1007/s10531-013-0507-3.

Hall, T. . (1999) 'BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT'. Nucl.Acids. Symp, (41), pp. 95–98.

Hasegawa, M., Kishino, H. and Yano, T. (1985) 'Dating of the human-ape splitting by a molecular clock of mitochondrial DNA', *Journal of Molecular Evolution*. Springer-Verlag, 22(2), pp. 160–174. doi: 10.1007/BF02101694.

Hellberg, R. S., Isaacs, R. B. and Hernandez, E. L. (2019) 'Identification of shark species in commercial products using DNA barcoding', *Fisheries Research*. Elsevier, 210, pp. 81–88. doi: 10.1016/J.FISHRES.2018.10.010.

Higgins, D. (1997) 'Multiple Sequence Alignment', *Genetic Databases*. Academic Press, pp. 165–183. doi: 10.1016/B978-012101625-8/50010-4.

IUCN (2014) *A quarter of sharks and rays threatened with extinction | IUCN*, *International Union of the Conservation of Nature*.

Kumar, S. *et al.* (2018) 'MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms', *Molecular Biology and Evolution*, 35, pp. 1547–1549. doi: 10.1093/molbev/msy096.

Man, Y. B., Wu, S. C. and Wong, M. H. (2014) 'Shark fin, a symbol of wealth and good fortune may pose health risks: the case of mercury', *Environmental Geochemistry and Health*. Springer Netherlands, 36(6), pp. 1015–1027. doi: 10.1007/s10653-014-9598-3.

Milano, E. (2006) 'The New Fisheries Partnership Agreement between the European Community and the Kingdom of Morocco: Fishing too South', *Anuario Espanol de Derecho Internacional*, 22.

Moroccan Investment Development Agency (no date) *Investment Opportunities; Fishing Industry*.

Naylor, G., Fedrigo, O. and Andrés López, J. (2005) *Phylogenetic Relationships among the Major Lineages of Modern Elasmobranchs*.

Naylor, G. J. P. *et al.* (2012) 'A DNA Sequence–Based Approach To the Identification of Shark and Ray Species and Its Implications for Global Elasmobranch Diversity and Parasitology', *Bulletin of the American Museum of Natural History*. American Museum of Natural History, 367(367), pp. 1–262. doi: 10.1206/754.1.

Nei, M. (1987) 'Molecular evolutionary genetics', *Columbia University Press*, 75(3), p. 512. doi: 10.1002/ajpa.1330750317.

Nei, M. and Kumar, S. (2000) *Molecular evolution and phylogenetics*. Oxford University Press.

Pauly, D. and Le Manach, F. (2015) 'Fisheries Centre Working Paper Series Tentative adjustments of China's marine fisheries catches (1950-2010) Tentative adjustments of China's marine fisheries catches (1950-2010)'.

Plague, H. (2001) *Too many boats for too few fish | The Economist*, *The Economist*.

Queiroz, N. *et al.* (2012) 'Spatial Dynamics and Expanded Vertical Niche of Blue Sharks in Oceanographic Fronts Reveal Habitat Targets for Conservation', *PLoS ONE*. Edited by Y. Ropert-Coudert. Public Library of Science, 7(2), p. e32374. doi: 10.1371/journal.pone.0032374.

Ratnasingham, S. and Hebert, P. D. . (2007) 'BOLD: The Barcode of Life Data System www.barcodinglife.org', *Molecular Ecology Notes*. doi: 10.1111/j.1471-8286.2006.01678.x.

Roberts, C. M. (2002) 'Deep impact: the rising toll of fishing in the deep sea', *Trends in Ecology & Evolution*. Elsevier Current Trends, 17(5), pp. 242–245. doi: 10.1016/S0169-5347(02)02492-8.

Roy, C., Cury, P. and Kifani, S. (1992) 'Pelagic fish recruitment success and reproductive strategy in upwelling areas: environmental compromises', *South African Journal of Marine Science*. Taylor & Francis Group , 12(1), pp. 135–146. doi: 10.2989/02577619209504697.

Rozas, J. *et al.* (2003) 'DnaSP, DNA polymorphism analyses by the coalescent and other methods', *Bioinformatics*. Narnia, 19(18), pp. 2496–2497. doi: 10.1093/bioinformatics/btg359.

Steinke, D. *et al.* (2017) 'DNA analysis of traded shark fins and mobulid gill plates reveals a

high proportion of species of conservation concern', *Scientific Reports*. Nature Publishing Group, 7(1), p. 9505. doi: 10.1038/s41598-017-10123-5.

Steinke, D. and Hanner, R. (2011) 'The FISH-BOL collaborators' protocol', *Mitochondrial DNA*. Taylor & Francis, 22(sup1), pp. 10–14. doi: 10.3109/19401736.2010.536538.

Stevens, J. *et al.* (2000) 'The effects of fishing on sharks, rays, and chimaeras (chondrichthyans), and the implications for marine ecosystems', *ICES Journal of Marine Science*. Oxford University Press, 57(3), pp. 476–494. doi: 10.1006/jmsc.2000.0724.

Tamura, K., Nei, M. and Kumar, S. (2004) *Prospects for inferring very large phylogenies by using the neighbor-joining method*.

Techera, E. J. and Klein, N. (Natalie S. . (2014) *Sharks : conservation, governance, and management*. t_Vftr4&redir_esc=y#v=onepage&q= chimaera&f=false

The European Commission (2013) *EU Commission - Morocco Fisheries Partnership Agreement. Official Journal of the European Union*.

Vélez-Zuazo, X. and Agnarsson, I. (2011) 'Shark tales: A molecular species-level phylogeny of sharks (Selachimorpha, Chondrichthyes)', *Molecular Phylogenetics and Evolution*. Academic Press, 58(2), pp. 207–217. doi: 10.1016/J.YMPEV.2010.11.018.

Ward, P. and Myers, R. A. (2005) 'Shifts in open-ocean fish communities coinciding with the commencement of commercial fishing', *Ecology*. John Wiley & Sons, Ltd, 86(4), pp. 835–847. doi: 10.1890/03-0746.

Ward, R. D. *et al.* (2005) 'DNA barcoding Australia's fish species.', *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, 360(1462), pp. 1847–57. doi: 10.1098/rstb.2005.1716.

Ward, R. D. *et al.* (2008) 'DNA barcoding Australasian chondrichthyans: results and potential uses in conservation', *Marine and Freshwater Research*. CSIRO PUBLISHING, 59(1), p. 57. doi: 10.1071/MF07148.

Ward, R. D., Hanner, R. and Hebert, P. D. N. (2009) 'The campaign to DNA barcode all fishes, FISH-BOL', *Journal of Fish Biology*. John Wiley & Sons, Ltd (10.1111), 74(2), pp. 329–356. doi: 10.1111/j.1095-8649.2008.02080.x.

White, W. T. and Sommerville, E. (2010) 'Elasmobranchs of Tropical Marine Ecosystems', in Carrier, J. C., Musick, J. A., and Heithaus, M. R. (eds) *Sharks and Their Relatives II: Biodiversity, Adaptive Physiology, and Conservation*. 2nd edn. Boca Raton, FL: CRC Press/Taylor & Francis Group, pp. 159–240.

Worm, B. *et al.* (2013) 'Global catches, exploitation rates, and rebuilding options for sharks', *Marine Policy*. Pergamon, 40, pp. 194–204. doi: 10.1016/J.MARPOL.2012.12.034.

Zunes, S. and Mundy, J. (2010) *Western Sahara : war, nationalism, and conflict irresolution*. Syracuse University Press.

Chapter 3

Investigating proteins for the identification of shark fins and their relatives (Elasmobranchii)

S A. Hook[1], M Buckley[1*]

[1]School of Earth and Environmental Sciences, Manchester Institute of Biotechnology, University of Manchester, Manchester, M1 7DN, UK

*Corresponding Author: Michael Buckley

Author Contributions

SAH collected the samples, conducted the lab experiments, performed the analysis reported analysis and wrote the paper. MB designed the study, provided direction with the lab experiments and analysis, and reviewed the chapter prior to submission.

3.1 Abstract

Elasmobranchs, consisting of sharks, skates and rays, are currently declining, with a quarter of all species classified as threatened with extinction by The IUCN Red List. Due to overfishing and exploitation for elasmobranch products such as squalene (oil) and shark fins, it is estimated that 100 million individuals are killed every year. Once in a final state of production, elasmobranch products are difficult to identify to species level, allowing the practise of illegal, unregulated or unreported (IUU) fishing to fuel the industry. Furthermore, it is time consuming and costly to collect viable DNA from these products for molecular identification, when degraded. Here we use an adapted collagen fingerprinting by soft-ionization mass spectrometry methodology to investigate whether elasmobranch fins, processed from fresh to full desiccation, can be identified using this technique. Upon exploring a range of extraction methods tested on *Scyliorhinus canicula* we found insoluble protein extraction using guanidine-hydrochloride to be the best method of choice for the removal of non-collagenous proteins, and identified the five most abundant proteins in the fins. From these results we created a database that contained all published elasmobranch sequences for these proteins from the online National Center for Biotechnology Information (NCBI) database resource. Finally, we applied guanidine-hydrochloride insoluble protein extraction method to 27 elasmobranch species, equalling 102 samples and analysed the potential for species-specificity and characterisation in the peptide mass fingerprints. Overall, we found a greater variation of proteins than usually seen in bone, likely because of the higher abundances of the non-collagenous proteins such as actin, tubulin, myosin and tropomyosin, all which are found to contribute to the structure of muscles in other fishes. There were greater abundances of all proteins in samples which had been “naturally degraded”, rather than freeze dried. Further work on protein sequence retrieval from more species is advised in order to related and therefore understand the peptide spectra; this study limited to 27 species of a possible >1,000, with only 6 species that have partial protein sequence data available. Lastly it is advised that the extraction methods that isolate single fibres is refined which ultimately could reduce contaminating proteins.

Key words: collagen, peptide mass fingerprinting, *Scyliorhinus canicula*, shark fins

3.2 Introduction

The ability to correctly identify sharks, skate and rays (class: elamsobranchii) once processed into an end product continues to be an impediment within biological and forensic sciences. For elasmobranch identification, a multitude of genetic applications have continued to be the method of choice in cases where DNA, either partial or full, is available. It has long been recognised that specific regions of DNA, such as the cytochrome c oxidase subunit 1 (COI) gene, hold enough diversity to discriminate between species, and therefore provide the platform for DNA barcoding (Ward *et al.*, 2005; Barbuto *et al.*, 2010). DNA barcoding, in its many forms, has been a widely successful method which has dominated the field of species identification across an array of industries, such as species management and forensics (Ward *et al.*, 2005; Helyar *et al.*, 2014; Almerón-Souza *et al.*, 2018). However, conducting DNA barcoding on decayed or processed specimens, whereby the DNA has degraded, often proves difficult. The degradation of the structural properties within DNA has been extensively investigated within the literature, and generally it is stable below 100°C (Karni *et al.*, 2013). The lack of sufficient of DNA fragments in elasmobranch samples which have undergone prolonged preparation for their sale is can be a common issue for correct identification.

One of the most common food industry products in the class elasmobranchs is shark fin soup. Fins are involved in multiple intense drying stages, before prolonged boiling periods to produce shark fin soup. Fin products can be sold at any one of these processing stages which are defined as: State 1. wet (fresh, unprocessed, whole); State 2. raw (dried, whole); State 3. semi-prepared (dried, removed skin); State 4. fully prepared (individual strands of cartilaginous platelets); State 5. fin nets (separated fin needles, that have been boiled and re-dried into loose groupings); State 6. shark fin soup (ready to consume in restaurants, cans/pouches or instant soup powders) (Kreuzer, Ahmed and Lai Ka- Keong, 1989; FAO, 2019). Generally, shark fin products are most commonly marketed in the United Kingdom (UK) as the end product, shark fin soup, found in restaurants and Asian supermarkets. The largest consumer markets for such products are in East and Southeast Asia such as China, Hong Kong Special Administrative Region, Taiwan Province of China, Singapore, Malaysia and Vietnam (Dent and Clarke, 2015) and these markets continue to

rapidly grow due to the rise in higher incomes and urbanisation (Fabinyi *et al.*, 2016). Historically, shark fin consumption dates back to the Ming Dynasty (1368- 1644 AD) where the tradition began due to consume shark fin soup at high class banquets as it was regarded as an expensive cultural treasure (Rose, 1996; Clarke, Milner-Gulland and Bjorndal, 2007). Since the 1970's there has been an exponential growth in the trade market for shark fins, resulting in a current global market value of over US$1 billion (Dent and Clarke, 2015; Shea and To, 2017). Along with shark fins, there are many other elasmobranch products consumed in the Asian and global markets – including manta and devil ray gill plates (O'Malley *et al.*, 2017), shark meat (Bornatowski *et al.*, 2015) and liver oil (squalene) (Akhilesh *et al.*, 2011; Jabado *et al.*, 2018). It is estimated that 100 million sharks are killed every year for their fins (Worm *et al.*, 2013) significantly reducing global populations. As at 2014 a quarter of sharks, rays and skates (elasmobranchs) were classified as threatened with extinction by the International Union for the Conservation of Nature (IUCN) Red List of endangered species (Dulvy *et al.*, 2014; IUCN, 2014), highlighting a need for more immediate action to prevent further declines or species loss.

Recent conservation actions have resulted in species protection, especially of species with a higher threatened category, such as 'endangered' or 'critically endangered'. As of August 2019, 34 species of elasmobranchs are protected under national and international laws by the Convention on International Trade in Endangered Species (CITES) or the Conservation of Migratory species (CMS), prohibiting either fishing and landing or trade (Mundy-Taylor and Crook, 2013; CITES, 2014, 2019; CMS, 2018). This increase in protection level combined with continuous consumer demands indicates a higher probability that elasmobranch species will be caught by illegal, unregulated and unreported (IUU) methods (Bornatowski, Braga and Vitule, 2013). For example, from IUU fishing it is estimated that up to 273 million sharks are being killed every year (Worm *et al.*, 2013) and elasmobranch species sold on markets across the globe are being fraudulently mislabelled, often substituted by prohibited species a result of IUU fishing (Pazartzi *et al.*, 2019). The high number of IUU practises in the fishing industry calls for a more efficient and effective method for identifying products which have been processed.

The study of collagen (COL) peptides as a method of species identification is a proven technique used across both archaeological and modern samples in multiple forms, such as skin, bone and tissue (Nagai and Suzuki, 2000; Nomura, 2004; Buckley *et al.*, 2010). More specifically, the protein Collagen Type I (COLI) displays enough variability in the amino acid sequences between species, that the analysis of this protein has been successfully used for species identification (Buckley *et al.*, 2009, 2010, 2014; Collins *et al.*, 2010; Buckley, 2018), including in fish (Harvey, Daugnora and Buckley, 2018). The COLI protein is a tropocollagen which in essence consists of three polypeptide alpha (α) chains that entwine into a triple-helix (Kadler *et al.*, 1996). Through the successful technique of collagen peptide mass fingerprinting in archaeology as Zooarchaeology by Mass Spectrometry (ZooMS), variations between COLI sequences can be used to identify species at the fraction of the cost of traditional methods such as DNA (Buckley *et al.*, 2009, 2010, 2014; Collins *et al.*, 2010; Buckley, 2018). Overall, protein has a better rate through biochemical processing than DNA, making it a more stable molecule to analyse for species identification in samples that have undergone food processing such as boiling, as seen in shark fin soup. In order to correctly identify species through this method however, there must either be a reference of the collagen peptide sequences for the each species, or closely related reference sequences to infer the possible sequence modifications across the COLI amino acid α-chains.

The aims of this chapter are to: 1. Test the most efficient method of protein extraction for different types of elasmobranch fin tissues by using *Scyliorhinus canicula* as a test species (batch 1); 2. Use the results from batch 1 to retrieve as many proteins available for elasmobranchs as possible, creating an offline reference database for protein characterisation; 3. Apply a refined method of the most effective technique of removing the non-collagenous proteins for 27 species of elasmobranchs; 4. Use protein fingerprinting techniques to determine success of the technique in terms of its ability to distinguish between species and to characterise the peptide peaks in the fingerprints.

3.3 Methods

3.3.1 Sample collection

To complete the aims of this study, two different sets of samples were used for analysis. The first batch (batch 1) consisted of five individuals' dorsal fins from a controlled population of *S. canicula* held at the Biological Services Facility, The University of Manchester (Table 3.1). The dorsal fins were collected as a discard product from 18 month old euthanized individuals and stored frozen. The second batch (batch 2) consisted of 1 cm^2 fin samples collected from fish markets along the Atlantic coast of Morocco in 2015 and 2016. Samples were stored in RNA*later*® Sigma-Aldrich and kept at 4°C, prior to long term storage at -80°C at the Manchester Institute of Biotechnology, Manchester. All samples were collected as state 1 of fin processing; wet, fresh, unprocessed (Kreuzer, Ahmed and Lai Ka-Keong, 1989; FAO, 2019). In total, fin samples consisted of 102 shark or skate and ray (batoid) species (Supplementary Table 1). Of the 102 samples, 74 had been positively identified using COI barcoding methods (Chapter 2) and the further 28 had been identified by their morpholoy within the field upon sample collection.

Table 3.1. The methodology conditions conducted on each sample. Full description of the method can be found in the text and a flow chart detailing the method can be observed in Figure 2.

***DF* ID**	***D M***	***D D***	**FMW (mg)**	**Method**	**Analysis ID**	***PD*2 temperature (°C) + length (time, min)**	***PD*F temperature (°C) + length (time, min)**
Nov1	Fume hood	30 days	9.97	a.Is	01	95 + 60	95 + 180
				a.S	02		-
			8.04	b.Is	03	95 + 60	95 + 180
Nov3			5.34	a.Is	04	65 + 60	65 + 180
				a.S	05		-
			5.34	b.Is	06	65 + 60	65 + 180
T101	Freeze dryer	1 hour	6.11	a.Is	07	95 + 60	95 + 180
				a.S	08		-
			8.78	b.Is	09	95 + 60	95 + 180
T201			6.89	a.Is	10	65 + 60	65 + 180
				a.S	11		-
			5.1	b.Is	12	65 + 60	65 + 180
T501			14.65	a.Is	13	95 + 60	95 + 180
				a.S	14		-
			14.69	b.Is	15	95 + 60	95 + 180

***DF* ID = Individual Dorsal Fin identification tag, *D M* = desiccation method, *D D* = desiccation duration, FMW = Filament material weight, Method, a.Is = ammonium bicarbonate insoluble, a.S = ammonium bicarbonate soluble, b.Is = guanidine-hydrochloride insoluble, Analysis ID = sample identification used for results section, *PD*2 = second protein denaturation, *PD*F= final protein denaturation**

3.3.2 Sample desiccation and protein extraction

Method development was conducted on batch 1 samples. First, multiple methods of sample desiccation were conducted, in which samples were processed to state 2 (raw (dried, whole) (Kreuzer, Ahmed and Lai Ka- Keong, 1989; FAO, 2019) (Figure 3.1). These were either desiccated under semi natural conditions within a fume hood for 30 days or freeze dried using a Scanvac CoolSafe (LaboGene®) for one hour. After desiccation the skins of the samples were removed to state 3 (semi-prepared (dried, removed skin)) and strands of cartilaginous platelets (filaments) were pulled from the samples, processing samples to stage 4 (fully prepared) (Figure 3.1, Table 3.1). Sample desiccation and filament removal was conducted under clean laboratory conditions. Each sample was separated into two 1.5 ml eppendorf tubes with near equal amount of filament material, to undergo protein extraction by either (a). ammonium bicarbonate (ABC) or (b). guanidine-hydrochloride (GuHCl). Prior to both methodologies, samples were washed in 500 µl of phosphate-buffered saline (hereafter referred to as PBS) buffer and vortexed for 25 seconds before removing and disposing of the PBS buffer. This was repeated three times in total, ensuring all residual PBS had been removed at the end of the wash stage. To simulate the process state 6 preparation and to degrade all other proteins that are present within the fins that are not the desired collagen, samples were heated at 65˚C in 1000 µl of 1mM hydrochloric acid (HCl) for 60 minutes. Samples were then centrifuged at 12,400 xg for 30 seconds to create a pellet and all the HCl was removed and disposed from each sample.

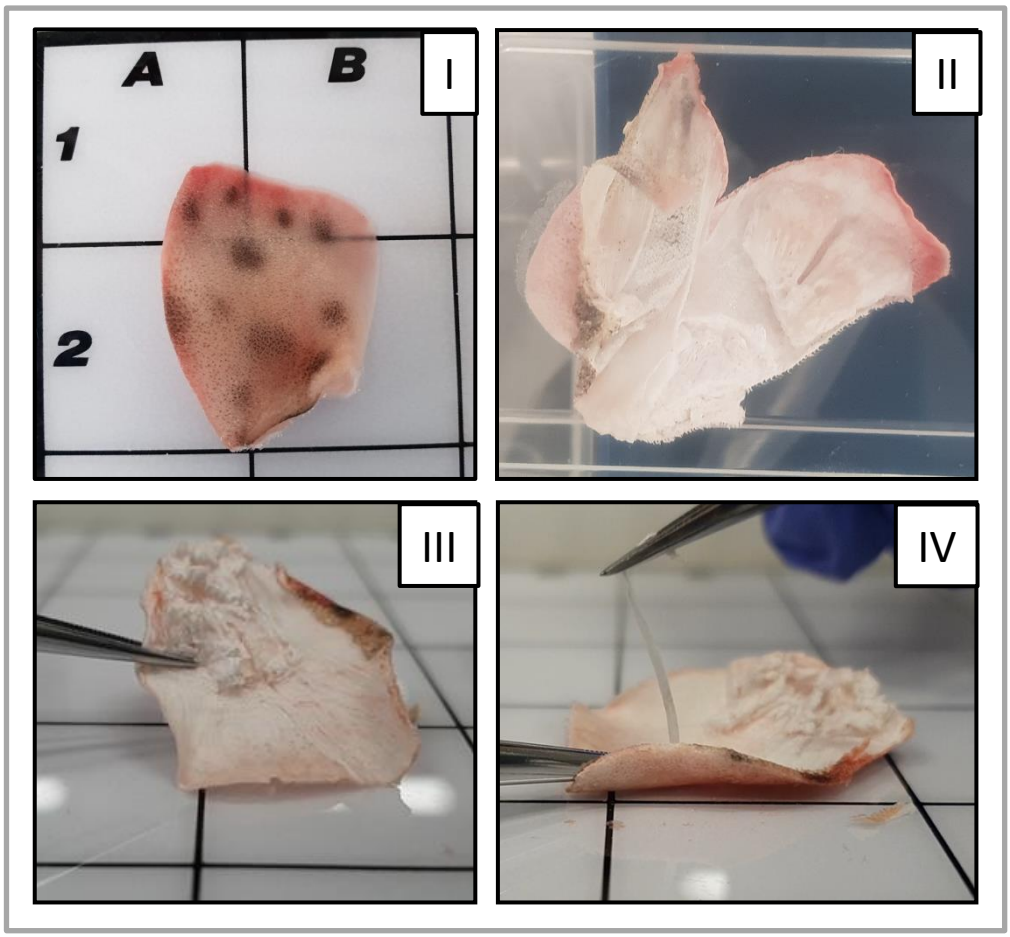

Figure 3.1. Five chronological images of sample processing from wet fins to desiccation and filament dissection. I = the fins at state 1, II = the separation of the skin from the fin and exposure of cartilaginous filaments for freeze drying, III = the fin post freeze drying, IV = the fin filaments being removed using forceps.

3.3.3 Ammonium bicarbonate (ABC) method (a)

To begin protein extraction the samples were washed in 50 mM ABC, 500 µl of PBS buffer overnight at room temperature. The PBS solution was then removed and disposed of. The samples were centrifuged for 60 seconds at 12,400 x *g* and any residual PBS solution removed and disposed of. To further reduce unwanted proteins, 300 µl of 50 mM ABC solution was added to each sample and heated at either 65˚C or 95˚C for 60 minutes to simulate the minimum and maximum temperature of shark fin soup, and of which COLI is predicted to withstand, hereafter referred to as the second protein denaturation (sample dependent, Table 3.1). Following the 60 minute heat shock, samples were cooled to room temperature. The 300 µl of 50 mM ABC solution in each sample, containing the soluble proteins, was aliquoted into a 0.5 ml eppendorf. A final protein degradation treatment where

samples were heated at either 65°C or 95˚C in 1000 µl of 1 mM hydrochloric acid (HCl) was conducted for 180 minutes prior to the reduction and alkylation step.

3.3.4 Guanidine hydrochloric acid (GuHCl) method (b)

To begin the protein extraction, samples being processed with GuHCl were left overnight at room temperature in 1,500 µl of 6M GuHCl. A second protein denaturation (*PD*2) step was then performed, whereby samples were heated to either 65˚C or 95˚C for 60 minutes (sample dependent, Table 3.1). Samples were then centrifuged at 12,400 x *g* for 60 seconds to create a pellet. The 1,500 µl of guanidine containing the soluble proteins was disposed of, leaving the insoluble proteins for analysis. To dissolve any further proteins in the insoluble pellets from the GuHCl experiments a second protein denaturation (*PD*2) was conducted whereby 500 µl of 1 mM HCl was added to each pellet and heated for one hour 65°C (sample dependent, Table 3.1). The resulting 500 µl of HCl (which contained the soluble proteins) was then added to a Vivaspin 500 membrane filter column with a 10,000 molecular weight cut off (MWCO) and centrifuged for 30 minutes at 12,400 x *g* and the flow-through disposed of. 500 µl of 50 mM ABC was then added to the Vivaspin membrane and centrifuged for 30 minutes at 12,400 x *g* to wash the column. The flow through was then disposed of and a further 500 µl of 50 mM ABC was added to each Vivaspin membrane and centrifuged again for 30 minutes at 12,400 *x g*. Samples were eluted by the addition of 100 µl of 50 mM ABC to the Vivaspin membrane using a pipette (with gentle mixing), while avoiding the pipette tip touching the membrane. The 100 µl of 50mM ABC was then pipetted out and added to 0.5 ml eppendorf. Similar to protocols for a.Is, a final protein degradation (*PD*F) treatment; where samples were heated at either 65°C or 95˚C in 1000 µl of 1mM hydrochloric acid (HCl) was conducted for 180 minutes prior to a protein reduction and alkylation step.

3.3.5 Reduction and alkylation

As the samples originated from wet fins with multiple tissue types, there was a high probability of actin, myosin, tubulin or tropomyosin being present in high quantities which could interfere with collagen protein fingerprints. We used a reduction and alkylation step after both the ABC or GuHCl methods to break the structures of proteins within the samples

for analysis. In this 4.2 µl of 100mM dithiothreital (DTT) was added to each sample and incubated at 60°C for 10 minutes to break disulphide bonds within cysteine residues. Once incubated, 8.4 µl of 100mM iodoacetamide (light sensitive) (IAM) was added to each sample and left at room temperature within a dark environment for 45 minutes to prevent reformation of any cysteine residues. Once complete 4.2 µl of 100mM DTT was added again to quench any remaining IAM and prevent it from interfering with Liquid Chromatography (LC)-Orbitrap Elite tandem MS analysis (LCMS). Finally, 2 µl of trypsin was added for overnight digestion estimated between 12 and 18 hours at 37°C.

3.3.6 Ziptipping for LC-Orbitrap Elite tandem mass spectrometry analysis

To purify and concentrate the extracted proteins within each sample, we used C18 ZipTip® protocols as defined by Buckley *et al.,* (2015). Each sample was first acidified using 1% trifluoroacetic acid (TFA), 10:100 µl. C18 ZipTips were activated in 50% acetonitrile (ACN): 0.1% TFA and then washed in 0.1% TFA. The sample proteins were then bound to the ZipTip filter by being repeatedly pushed through the filter up to ten times. Following this, the proteins were then purified in 0.1% TFA and eluted into 50% ACN + 0.1% TFA. Samples were left to dry under the fume hood for 48 hours prior to rehydration for liquid chromatography–mass spectrometry (LC-MS/MS) analysis. The LC-MS/MS analysis was conducted at the in house facilities at the Biological Mass Spectrometry core facility at The University of Manchester. Samples were rehydrated and stabilized in 5% ACN, 0.1% formic acid (FA), and blanks were loaded between groups of samples that had undergone the same protocol or that were of the same species order, to a maximum of four samples per group. Blanks were used to analyse the level of contamination between samples analysed.

3.3.7 Identification and species variation of dominant elasmobranch proteins

In order to correctly identify the dominant proteins within our samples, we began by running batch 1 against the UniProt (The UniProt Consortium, 2019). UniProt is a large online database combining the entire 560,537 human annotated proteins from Swissprot (Bairoch and Apweiler, 2000) and 167,761,270 computer annotated proteins from TrEMBL (Bairoch and Apweiler, 2000; The UniProt Consortium, 2019; UniProt, 2019). The UniProt database is therefore a full record of every available published sequence for all proteins

across all kingdoms. To identify these proteins more efficiently in future analysis of larger sample sets (batch 2), we then created a separate database (COL_Database) containing only published sequences for these five dominant proteins from elasmobranch species. To determine the variability within the COLI protein sequences collected for the reference database, we used the ClustalW full multiple alignment at 1000 bootstraps (Higgins, 1997) within BioEdit (Hall, 1999). We then used Mega X to determine the estimates of evolutionary divergence between COLI protein sequences using a pairwise distance analysis (Kumar *et al.*, 2018). We focus mainly on the α1 chain of the COLI molecule because in other species it has displayed enough variation for species identification (Buckley, 2018).

3.3.8 Batch 1 protein quantification

To determine the most appropriate method for protein extraction tested on batch 1 samples, as displayed in Figure 3.2, we used Proteome Discoverer™ V. 2.2 (ThermoFisher Scientific, Colaert *et al.*, 2011), which uses Orbitrap .mgf files to first identify and then quantify the proteins within a biological sample. We applied the Label Free Quantification (LFQ) and LFQ consensus standard processing methods (Veit *et al.*, 2016) with protein modifications of carbamidomethyl, oxidation, deamination and acetyl and ran this against both our developed COL_Database, including all collagen alpha chain sequences for fish (Harvey, Keating and Buckley, In Prep) and UniProt online, specified to *S. canicula* (The UniProt Consortium, 2019). As this was not the prime method of comparing experimental designs, no ratio steps were added within the program. We also used Progenesis QI for proteomics to view the high resolution MS data to review the visual abundance of proteins available in each sample. Using Mascot V 2.4 (Perkins *et al.*, 1999) we completed a MS/MS Ion error tolerant search against COL_Database with the same protein modifications as in Proteome Discoverer™. Using the results from Proteome Discoverer™ and Mascot analysis, we determined the best methodology which provided the largest number of collagen α1 and α2 chains with the least amount of other protein ‘contaminants’: actin, myosin, tropomyosin and tubulin. Lastly, we used matrix-assisted laser desorption ionization (MALDI) mass spectrometry (MS) with Time-of-Flight to view the fingerprint spectra in reflectron mode.

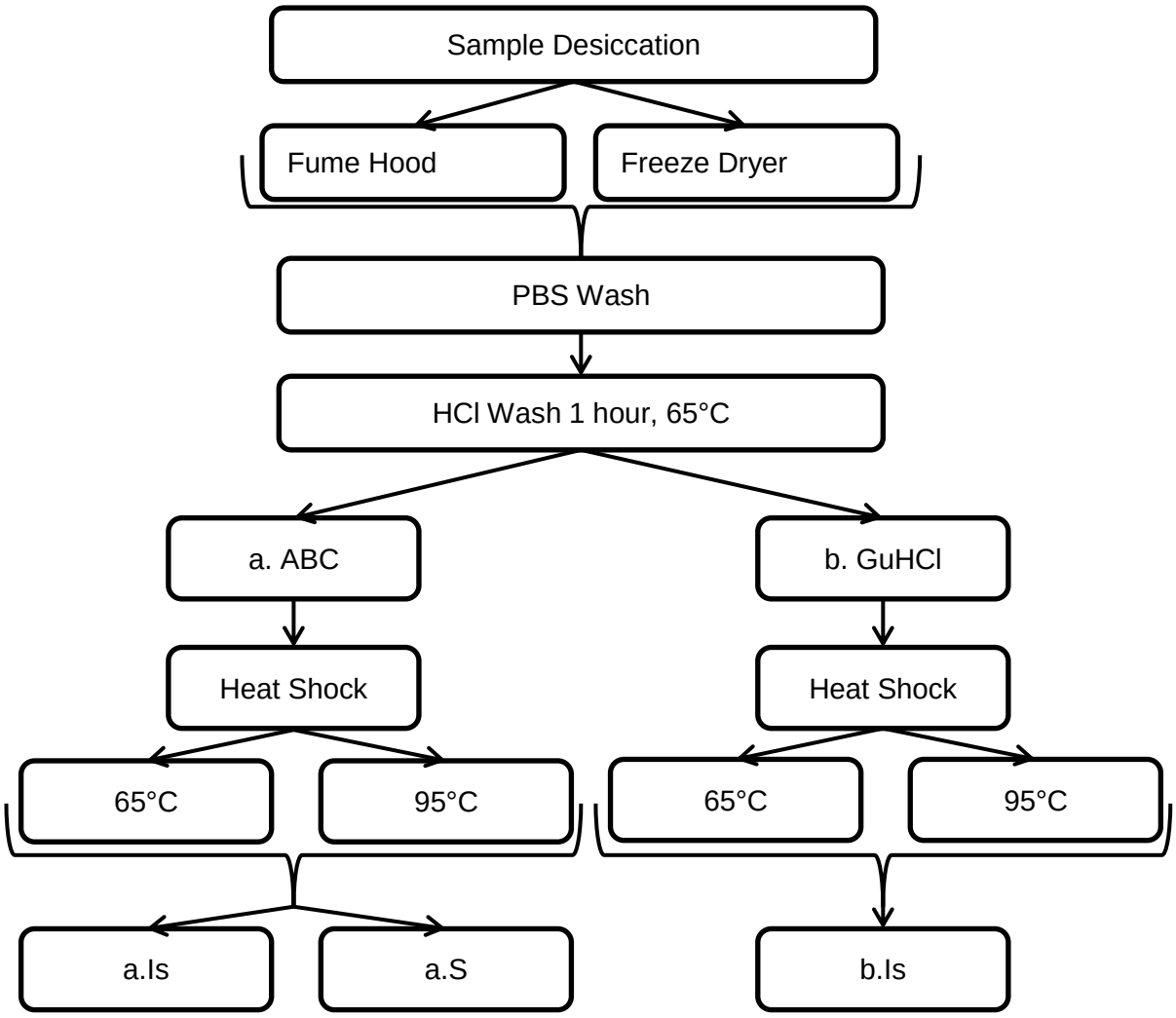

Figure 3.2. A flow chart to display the overall methodologies conducted on the batch 1 samples: a.Is = ABC insoluble, a.S = ABC soluble, b.Is = GuHCl insoluble.

3.3.9 Batch 2 sample desiccation, protein extraction and analysis

Following the results from analysis in batch 1, we conducted a one-hour freeze drying protocol and GuHCl insoluble protein extraction (b.Is). Within the b.Is methods, to further degrade unwanted proteins, samples were heated at 95˚C in 1000 µl of 1mM hydrochloric acid (HCl) for 180 minutes prior to the reduction and alkylation step. In total, 74 samples from samples which had already been positively identified to species level through COI barcoding, and 28 samples that had been identified to species level in the field were processed in the LCMS analysis. All other methods followed the same GuHCl insoluble protocols as stated above.

3.4 Results

3.4.1 Protein identification

Proteins were identified using Protein Discoverer and Mascot analysis. From the analysis provided by Protein Discoverer we found four dominant proteins, hereafter referred to as the master proteins; collagen, tubulin, tropomyosin and actin (Figure 3.3).

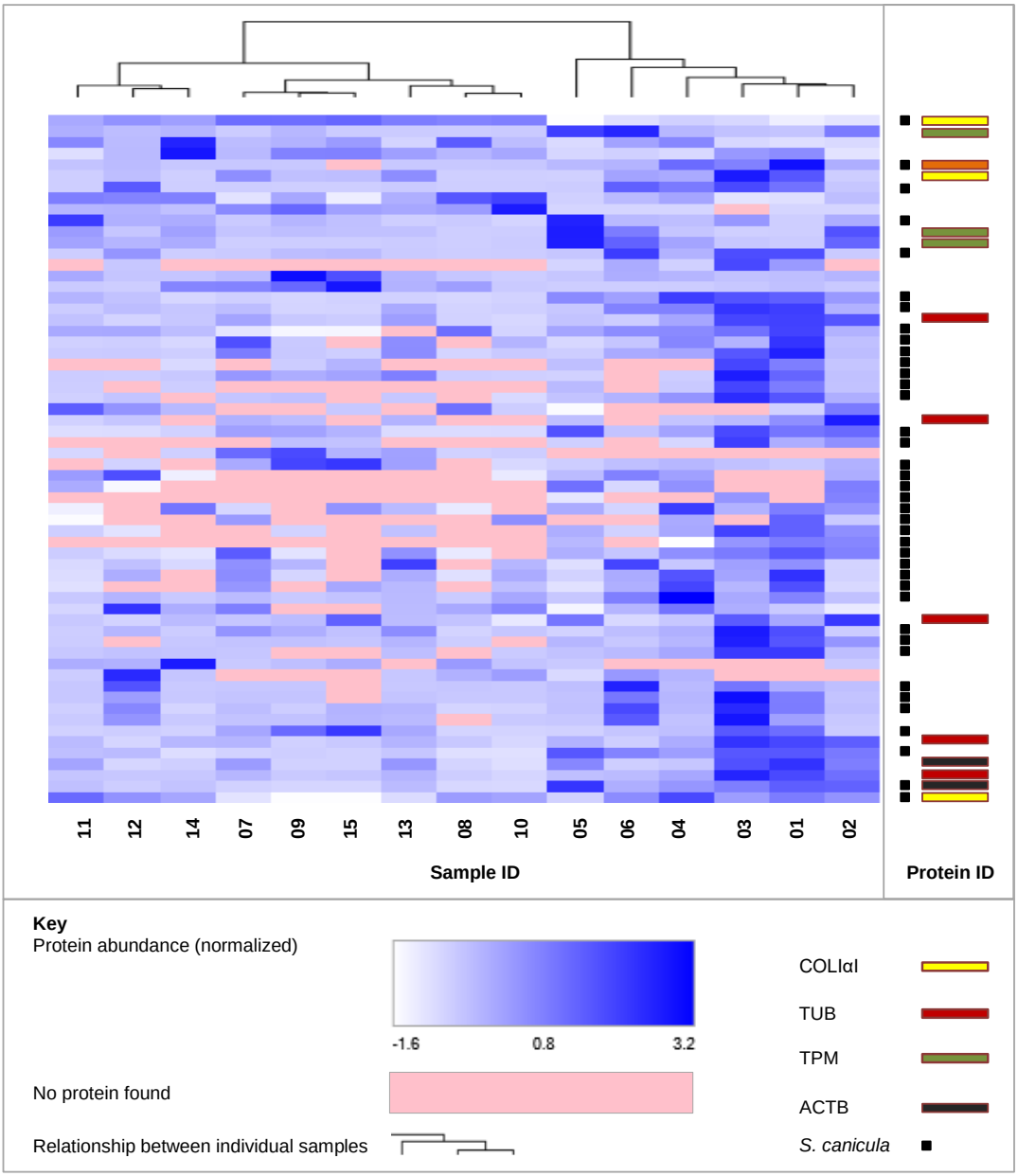

Figure 3.3. An adapted heatmap calculated in Proteome Discover v2.3.0.523 to display the normalized master protein abundances (Supplementary Table 3.2), under a Complete Linkage Methodology with the Euclidean Distance Function, Scaling after Clustering. TUB = Tubulin, TPM = Tropomyosin, ACTB = Actin,

COLIα1 = Collagen Type 1, Alpha Chain 1. Rows represent each possible peptide sequences that the samples were ran against, arranged in clusters depending on the relationships between the sequences.

The data set was normalized for the entire set of protein abundances within the samples (-1.6 = Low, 0.8 = Medium, 3.2 = High). Of the matched master proteins, 63% (39) proteins originate from *S. canicula* protein sequences available within Uniprot and our own database. Of the samples, there are three distinct cluster relationships; cluster one contains samples 11, 12 and 14, cluster two contains samples 7 to 10, 13 and 15, and cluster three contains samples 1 through to 6. Cluster three, with special attention to samples 1, 2 and 3, has the largest abundance and distribution of proteins available, indicating high quantities of other proteins, aside from collagen. Cluster three samples were all desiccated within a fume hood for 30 days prior to protein extraction, one and two were conducted under method a and also produced a relationship between samples (Table 3.1). There was a significant separation in the relationship from the complete linkage method: cluster one containing mainly a.S samples, cluster two containing mainly insoluble methods (both a.Is and b.Is) and cluster three containing all methods, with fume hood desiccation. Collagen abundances are found most predominantly in cluster two, indicating the best method for collagen extraction is using the insoluble methods.

From the Mascot analysis we found five master proteins adding myosin to the already defined master proteins from the Protein Discoverer analysis: collagen, tubulin, tropomyosin, mysoin and actin (Figure 3.4). The Mascot conducted an exhaustive search and allowed us to determine the master proteins from abundances within each sample (Supplementary Table 3.3), rather than from abundance as with Protein Discover (Supplementary Table 3.2). However abundance of myosin within this analysis was significantly lower than all other proteins, probably indicating the reason why Protein Discoverer did not define it as a master protein within the samples.

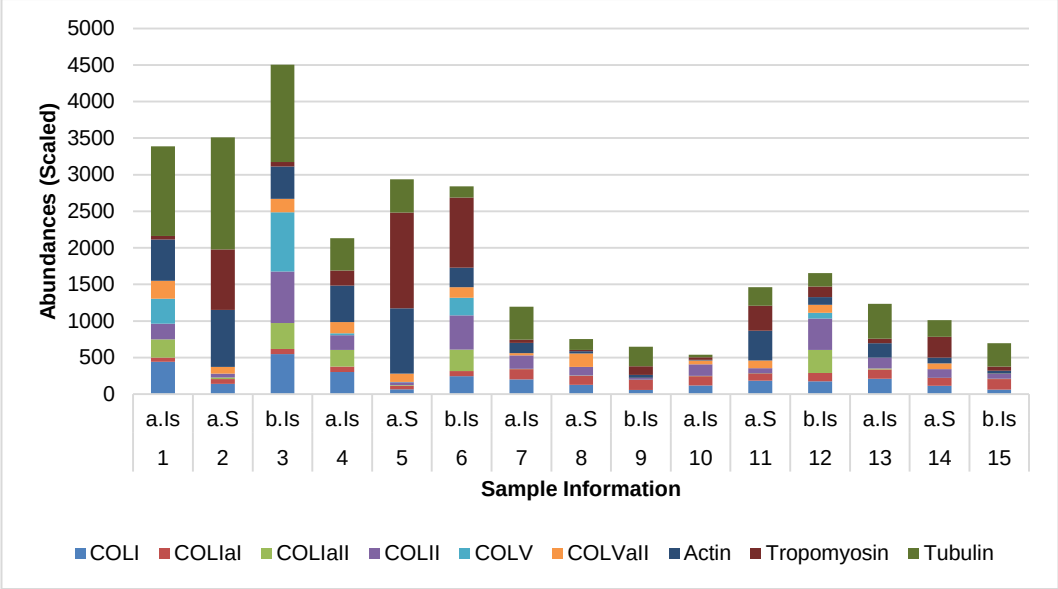

Figure 3.4. The total abundance (scaled) of each protein present within the each of the samples using Mascot Daemon analysis. Sample methodology type can be seen in Table 1. a.Is = ABC insoluble, a.S = ABC soluble, b.Is = GuHCl insoluble, TUB = Tubulin, TPM = Tropomyosin, MYS = Myosin, ACTB = Actin, COL = Collagen Type, A = alpha chain type.

From the results in Protein Discoverer we determined that the freeze dried desiccation and insoluble protein extraction produced the largest abundance of COLIαI, without the presence of non-collagenous proteins. The *t*-test analysis between the COLIαI protein abundance of methods a. and b. revealed that there was no significant difference between the two methods; mean difference = 6.17%, $t = 4.6633$, $p = 0.0625$.

3.4.2 Published elasmobranch protein identification and analysis

In total, the database contained 91 protein sequences (Supplementary Table 3.1). The majority of these sequences were partially complete and therefore contained generated peptide sequences, from chondrichthyan species, which contains elasmobranchii and Holocephali (chimaera speices).

Table 3.2. On overview of the number of protein sequences downloaded from BLAST for each master protein as defined by PD. These sequences make up the offline chondrichthyan reference database. *Of the collagen protein sequences, 68 sequences were COLI (25 =αI, 24 = α2, 15 = α2I, 4 = αV), nine sequences were COL2αI and five were COL5α2.

Protein	*Seqs#*	*Species#*	Holocephali	Shark	Batoid
Collagen*	68	6	1	3	2
Actin	6	6	1	5	0
Tubulin	6	4	1	2	1
Myosin	2	2	1	1	0
Tropomyosin	9	3	1	2	0

***Seq#,* the number of protein sequences, *Species#,* the total number of species, Holocephali, Sharks, Skates and Rays (Batoids), the number of protein sequences within each group.**

Of the COLIαI and α2 chains, there were only four species with both COLIαI and alpha 2 chains. There were no COLIα2 chain sequences for ocellate spot skate, *Okamejei kenojei.* With the use of sequences from UniProt and our LCMS analysis with error tolerance, we determined the most likely peptides for our species of interest *S. canicula*. All ambiguous positions were removed for each sequence pair (pairwise deletion option). From these sequences we found a high variability in the collagen alpha 1 and 2 chains between species (Table 3.2).

Table 3.3. Estimates of Evolutionary Divergence between COLIαI and COLIα2 protein sequences of all chondrichthyan species known (full, partial and computer generated). The numbers of overall peptide differences between sequences are shown. The presence of n/c in the results denotes cases in which it was not possible to estimate evolutionary distances. Collagen sequences are titled with species order and group, orders alphabetically.

Collagen sequence	1	2	3	4	5	6
1. Carcharhiniformes_Scyliorhinidae _*Scyliorhinus_canicula*_G						
2. Carcharhiniformes_Scyliorhinidae _*Scyliorhinus_canicula*	67					
3. Chimaeriformes_Callorhinchidae _*Callorhinchus_milii*	26	84				
4. Orectolobiformes_Rhincodontidae _*Rhincodon_typus*	40	13	12			
5. Squaliformes_Squalidae _*Squalus_acanthias*	78	78	21	53		
6. Rajiformes_Rajidae _*Okamejei_kenojei*	91	27	90	05	n/c	

3.4.3 Batch 1 and 2 mass spectrometry fingerprint analysis

We ran each sample on a MALDI MS in order to view the final fingerprint spectra of the extracted proteins from batch 1 and 2. Through this analysis we found that while some samples provided identifying COLIαI fingerprints, there were samples which still contained contaminating proteins or had reduced number of peaks indicating a loss in all proteins (Figure 3.5). Therefore these fingerprint spectra are unsuitable for species identification.

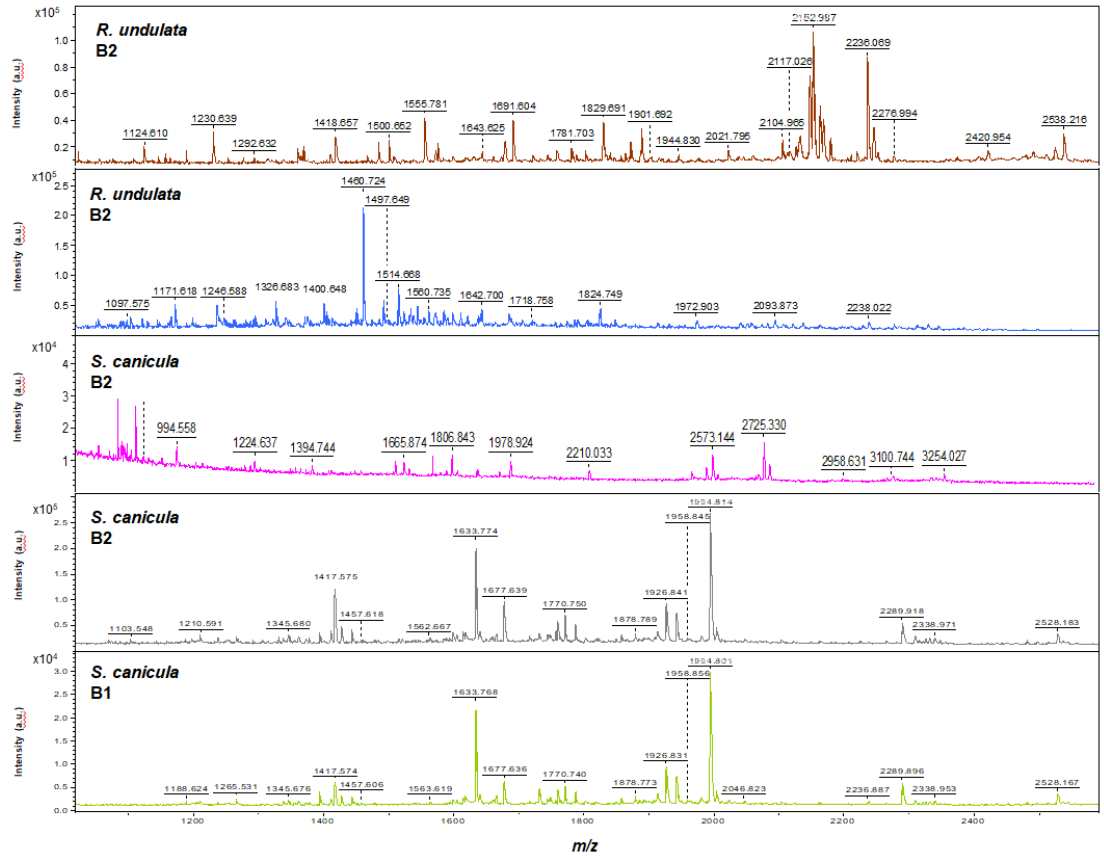

Figure 3.5. The MALDI MS spectra of the fingerprints collected in batch 1 (B1), *S. canicula* and batch 2 (B2), *S. canicula* and *R. undulata*. All samples in B2 had been genetically identified through COI barcoding. The linear spectrum was completed following zip-tip filtration. y = intensity of the ions (a.u), x = mass to charge (m/z), peaks labelled in the MALDI MS software.

As presented in Figure 3.5, although some *S. canicula* contained matching fingerprint spectra, contaminating proteins in other samples reduced the ability to observe the same fingerprints. Similarly, in *R. undulata* no fingerprints produced exact matches, as seen in Figure 3.5. In an attempt to further understand the spectra, we used the fragment sizes of each identified protein from the LC-MS/MS analysis and compared them to the peaks visualising fragment sizes in a linear mode (Figure 3.5). We found a lack of known peptide sequences for each protein identified within the samples by using Mascot Daemon. We therefore could only infer the identification of possible proteins for each peak by using the peak size as the length of the peptide sequence (Figure 3.5), and the currently available peptide sequences defined for chondrichthyans (Table 3.2). Due to the lack of prior knowledge of possible changes between amino acids, and the intensity of the data, the analysis was not completed. The number of matching fingerprints which were successfully observed on the MALDI MS can be seen in Table 3.4. In total nine species had two or more

MALDI MS fingerprints which were then compared against each other for ID for batch 2, which contained all analysed species, including new samples of *S. canicula*.

Table 3.4. The number of matching and non-matching fingerprints analysed from the MALDI MS in batch 2

Species	#TotalF	#Match	#Non-matching
Alopias superciliosus	5	0	5
Centrophorus granulosus	2	0	2
Centrophorus squamosus	2	0	2
Centroscymnus coelolepis	1	0	1
Isurus oxyrinchus	3	0	3
Raja microocellata	2	2	0
Raja undulata	2	0	2
Scyliorhinus canicula	6	2	4
Torpedo marmorata	2	2	0

#TotalF = the total number of successful fingerprints, #Matching = the number of matching fingerprints, #Non-matching = the number of non-matching fingerprints

3.5 Discussion

We conducted proteome characterisation of shark fins in order to assess the ability of collagen peptide mass fingerprinting to distinguish between different species of elasmobranchs (Buckley *et al.*, 2009). In order to analyse the samples, we successfully collated the already published sequences for protein types in chondrichthyes and found that, as seen in other species, COLIαI had a high divergence between species, making it theoretically applicable for species identification. By conducting and developing upon protein extraction from shark fin from our batch 1 *S. canicula* samples, we identified five main proteins that are present post desiccation and protein extraction; collagen, actin, tropomyosin, tubulin and myosin. Using Proteome Discoverer™ and Mascot Daemon we then quantified the difference between the developed method types in an attempt to fully eradicate all proteins with the exception of collagen and found GuHCl was best at lowering all contaminating proteins. We then used this method (GuHCl) to extract the collagen protein COLIαI from 102 fin and wing samples from 27 species, which has either been positively identified in the field, through COI barcoding or both. We used LC-MS/MS and MALDI MS to review the differences between species and found that although the method had produced unique fingerprints for each species, often contaminants still remained with the samples, as described in the testing phase, and therefore had the potential to invalidate MALDI MS

spectra, making it unreliable for correct identification at this stage. Overall, the fingerprints generated from the MALDI MS contained greater variation than expected, with only 3 species holding same MALDI MS fingerprints between samples. There were a greater number of non-matching fingerprints within each species than matching fingerprints. The method development for batch 1 *S. canicula* fins indicated that the guanidine (GuHCl) experiment was sufficient to remove contaminating proteins. However, when this method was applied to other species in batch 2 (post method development), it failed to produce replicable results (Figure 3.5, Table 3.4). For future work, more method development is required to remove these contaminating proteins from the samples in order to receive a pure COIαI MALDI MS fingerprint.

Mascot Daemon found that batch 1 samples had five master proteins; collagen, actin, tropomyosin, tubulin and myosin. Literature surrounding the main roles of these proteins in marine vertebrates is currently limited to studies on teleost fish, which evolved separately from elasmobranchs nearly 450 million years ago (Dean and Summers, 2006; Pradel *et al.*, 2009; Carrier, Musick and Heithaus., 2014; Venkatesh *et al.*, 2014). However, if adaptation of the master proteins is the same, it can be assumed that they are structural and muscle proteins (Nollet, 2009). This analysis was run against the current available protein sequences online (UniProt). Due to the extensive and often time-consuming analysis, we collated a more efficient and smaller database of the protein sequences first described in Mascot Daemon. To further analyse the composition and relatedness of the different methods in batch 1 we used Protein Discoverer. Protein Discoverer found four main master proteins within batch 1, after removing the protein myosin due to low quantifiable levels present within each sample (Figure 3.3). The analysis also matched the sample compositions to other protein sequences available that were first found using Mascot Daemon. These sequences were majorly from *S. canicula*, such as collagen Type V (Supplementary Table 3.1 and 3.2). We found that the main groups of master proteins, collagen, actin, tropomyosin, tubulin, were more likely to be extracted when samples are desiccated under simulated natural conditions. Within our dataset, cluster 3 samples were desiccated in a fume hood for 30 days maintained at room temperature and also have the highest range of proteins available. Despite the two different main methods of protein

extraction (a.Is = ammonium bicarbonate insoluble, a.S = ammonium bicarbonate soluble, b.Is = guanidine-hydrochloride insoluble), we found similar high abundances of the master proteins in all six samples of cluster 3 (Figure 3.3). For future analysis, it would be interesting to determine whether the lab conditions of natural decomposition produced similar results to the shark fin processing stages, defined from state 2 onwards. We found no significant difference between the a.Is and b.Is methods; therefore defining that no one method extracted greater quantities of COLIαI. Previously the GuHCl has been the preferred method of choice because it has shown to better remove contaminating proteins such as actin, tropomyosin, myosin and tubulin. When using ABC experiments, either soluble or insoluble, tropomyosin, tubulin and actin had the highest abundance, meaning that GuHCl was better at removing these unwanted proteins. Myosin was low in all samples, and therefore was either mostly removed during the filament extraction and/or during either ABC or GuHCl experiments.

The relative abundances of the master proteins found created new problems not previously found when analysing modern or archaeological bones. Firstly the presence of these proteins in some of the samples, especially in batch 2, created mismatching MALDI MS spectra. Secondly, due to the difference in abundances of each protein between samples, it meant that contamination was not consistent enough for true identification of MALDI spectra peaks, and therefore for species identification. In cases where consistently the same amount of master protein contamination had been eradicated, as in Figure 5, we can see however that the technique for identifying species is possible. Due to the lack of available peptide sequences for chondrichthyes, through published data sourced on UniProt, it is possible that the examined regions visualised on the MALDI MS may represent other protein that have not yet been sequenced. It is also possible that the reference sequences do not represent the entirety of the protein fragments found, as some reference sequences are either partial or inferred. In order to fully investigate the protein composition within both batch 1 and batch 2 samples, the full sequences of possible proteins are required for each investigated species or a close relative. Here, some of the data is reliant on species which diverged around 421 million years ago during the Silurian period ago, such as the ghostshark *Callorhinchus milii* (Inoue *et al.*, 2010). The high level of divergence found

between the COLIαI sequences clearly display this lack of relationship between the species due to the long period of which the groups have existed. However this divergence also shows the possible high variability between chondrichthyan species making it an excellent region to investigate for species identification. To fully evaluate the possibility of using COLIαI for species identification, future work should investigate and create more protein reference sequences, found through DNA analysis (Chapter 2). Furthermore, in order to full investigate the possibility of transferring this method to identify species in processed products, it is vital that fin morphology, structure and collagen composition is reviewed, dependent on the fin type (dorsal, pectoral, tail) and species type (benthic or pelagic). Previous reviews in to the morphology of the pectoral fins of benthic species shows how the anatomy of the fin webs allows for flexibility, bending in their environment on the sea floor (Wilga and Lauder, 2001) (Figure 3.6). Importantly for the conservation of sharks, the fin webs and filaments are the main source of produce and texture within shark fin soup. Although Wilga and Lauder (2001) described a general anatomy of the benthic dogfish shark fin, we found that *S. canicula* had a wider adapted pectoral fin shark, as seen in Figure 3.6.

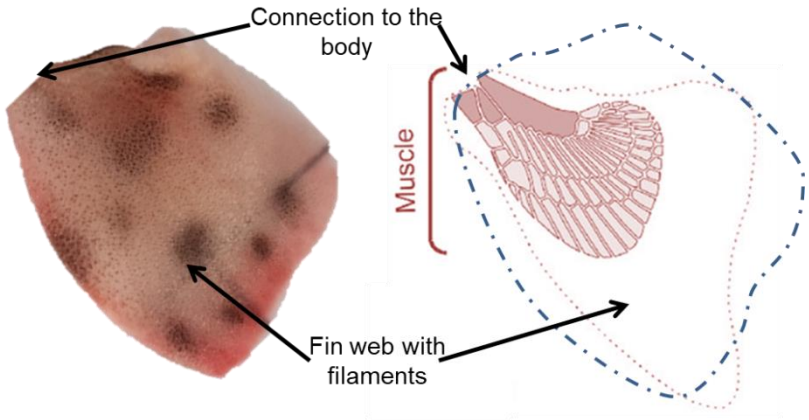

Figure 3.6. The full image of a removed small-spotted catshark S. canicula fin (left) and the skeletal structure of the pectoral fins in benthic shark species described as either bamboo, leopard or dogfish a described by Wilga and Lauder (2001) (right). Each fin is the dorsal view of the left pectoral fin. The dark blue dotted line is the outline of the S. canicula ceratotrichia into the fin web with filaments, overlayed on the red dash outline described by Wilga and Lauder (2001). The dark grey red elements are propterygium, mesopterygium, and metapterygium from anterior to posterior and light red elements are radials. The muscle insertion connects to the body and extends to the end of the third row of radials.

Our initial experimental tests were conducted on the benthic small spotted catshark *S. canicula* (batch 1). However this species are not common in processed food products. The *S. canicula* is a least concern species and the fins were waste products from other

experiments, therefore making it the perfect species to utilise to test new methods. Batch 2 contained a range of elasmobranch species, reviewing the possibility of transferring the method to other groups such as the batoids. The UK, for example, is one of many consumers of skate and ray wing, often mislabelled or unlabelled, therefore making it difficult to manage or conserve (Griffiths *et al.*, 2013). Batoids are globally more threatened than shark species, and therefore it can be argued that a greater amount of attention is needed to save this group from extinction (Dulvy *et al.*, 2014). Lastly, the adapted pectoral fins of batoids, known as the wings, contain higher amounts of mineralised cartilage, created by the encasing cartilage with COL2 proteins. Although the method specifically found that COLI has the largest diversity between known peptide sequences to identify species, the COLII region may be more prevalent in decayed samples and therefore easier to use for identification. As there were only few known protein sequences available for a maximum of six species, the use of other regions would be plausible. Specifically, future research must therefore focus on identifying the coding regions within the DNA, rather than inferring the possible protein sequences from predicted or known related species.

3.6 Acknowledgements

The authors would like to thank the Fin Fighters team, Matthew Brierley and Dr. Holly Shiels for assisting in sample collection. The authors would like to thank Lizi Johnson and Virginia Harvey for providing assistance in the lab and protein analysis.

3.7 References

Akhilesh, K. V. *et al.* (2011) 'Indian journal of marine sciences.', *Indian journal of marine sciences.* Council of Scientific & Industrial Research. Available at: http://nopr.niscair.res.in/handle/123456789/12424.

Almerón-Souza, F. *et al.* (2018) 'Molecular Identification of Shark Meat From Local Markets in Southern Brazil Based on DNA Barcoding: Evidence for Mislabeling and Trade of Endangered Species', *Frontiers in Genetics*. Frontiers, 9, p. 138. doi: 10.3389/fgene.2018.00138.

Bairoch, A. and Apweiler, R. (2000) 'The SWISS-PROT protein sequence database and its supplement TrEMBL in 2000.', *Nucleic acids research*. Oxford University Press, 28(1), pp. 45–8. doi: 10.1093/nar/28.1.45.

Barbuto, M. *et al.* (2010) 'DNA barcoding reveals fraudulent substitutions in shark seafood products: The Italian case of "palombo" (Mustelus spp.)', *Food Research International*. Elsevier Ltd, 43(1), pp. 376–381. doi: 10.1016/j.foodres.2009.10.009.

Bornatowski, H. *et al.* (2015) '"Buying a Pig in a Poke": The Problem of Elasmobranch Meat

Consumption in Southern Brazil'. doi: 10.14237/ebl.6.1.2015.451.

Bornatowski, H., Braga, R. R. and Vitule, J. R. S. (2013) 'Shark mislabeling threatens biodiversity.', *Science (New York, N.Y.)*. American Association for the Advancement of Science, 340(6135), p. 923. doi: 10.1126/science.340.6135.923-a.

Buckley, M. *et al.* (2009) 'Species identification by analysis of bone collagen using matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry', *Rapid Communications in Mass Spectrometry*. John Wiley & Sons, Ltd, 23(23), pp. 3843–3854. doi: 10.1002/rcm.4316.

Buckley, M. *et al.* (2010) 'Distinguishing between archaeological sheep and goat bones using a single collagen peptide', *Journal of Archaeological Science*. Academic Press, 37(1), pp. 13–20. doi: 10.1016/J.JAS.2009.08.020.

Buckley, M. *et al.* (2014) 'Species identification of archaeological marine mammals using collagen fingerprinting', *Journal of Archaeological Science*. Academic Press, 41, pp. 631–641. doi: 10.1016/J.JAS.2013.08.021.

Buckley, M. (2018) 'Zooarchaeology by Mass Spectrometry (ZooMS) Collagen Fingerprinting for the Species Identification of Archaeological Bone Fragments', in *Zooarchaeology in Practice*. Cham: Springer International Publishing, pp. 227–247. doi: 10.1007/978-3-319-64763-0_12.

Carrier, J. C., Musick, J. A. and Heithaus., M. R. (2014) *Biology of sharks and their relatives*. Boca Raton: CRC Press.

CITES (2014) *Casablanca Declaration & Roadmap for the Implementation of CITES Requirements in Relation to Sharks and Manta Rays*. Casablanca, Morocco.

CITES (2019) *History of CITES listing of sharks (Elasmobranchii)*, *Convention on International Trade in Endangered Species of Wild Fauna and Flora*.

Clarke, S., Milner-Gulland, E. J. and Bjorndal, T. (2007) 'Social, Economic, and Regulatory Drivers of the Shark Fin Trade', *Marine Resource Economics*. MRE Foundation, Inc., 22(3), pp. 305–327. doi: 10.1086/mre.22.3.42629561.

CMS (2018) *Memorandum of understanding on the conservation of migratory sharks*, *3rd Meeting, Monaco*.

Colaert, N. *et al.* (2011) 'thermo-msf-parser: An Open Source Java Library to Parse and Visualize Thermo Proteome Discoverer msf Files', *Journal of Proteome Research*. American Chemical Society, 10(8), pp. 3840–3843. doi: 10.1021/pr2005154.

Collins, M. *et al.* (2010) 'ZooMS: the collagen barcode and fingerprints', *Spectroscopy Europe*, 22(2).

Dean, M. N. and Summers, A. P. (2006) 'Mineralized cartilage in the skeleton of chondrichthyan fishes', *Zoology*. Urban & Fischer, 109(2), pp. 164–168. doi: 10.1016/J.ZOOL.2006.03.002.

Dent, F. and Clarke, S. (2015) 'State of the global market for shark products', *FAO Fisheries and Aquaculture Technical Paper*, (590).

Dulvy, N. K. *et al.* (2014) 'Extinction risk and conservation of the world's sharks and rays.', *eLife*. eLife Sciences Publications, Ltd, 3(e00590), p. e00590. doi: 10.7554/eLife.00590.

Fabinyi, M. *et al.* (2016) 'Aquatic product consumption patterns and perceptions among the Chinese middle class', *Regional Studies in Marine Science*. Elsevier, 7, pp. 1–9. doi: 10.1016/J.RSMA.2016.01.013.

FAO (2019) *6.2 FINS*, *The Food and Agriculture Organization*. Available at: http://www.fao.org/3/x3690e/x3690e0p.htm

Griffiths, A. M. *et al.* (2013) 'DNA barcoding unveils skate (Chondrichthyes: Rajidae) species diversity in "ray" products sold across Ireland and the UK', *PeerJ*. PeerJ Inc., 1, p. e129. doi: 10.7717/peerj.129.

Hall, T. . (1999) 'BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT'. Nucl.Acids. Symp, (41), pp. 95–98.

Harvey, V. L., Daugnora, L. and Buckley, M. (2018) 'Species identification of ancient Lithuanian fish remains using collagen fingerprinting', *Journal of Archaeological Science*. Academic Press, 98, pp. 102–111. doi: 10.1016/J.JAS.2018.07.006.

Harvey, V. L., Keating, J. N. and Buckley, M. (2019) *Collagen Type 1 sequence diversity and evolution in fishes*. University of Manchester.

Helyar, S. J. *et al.* (2014) 'Fish Product Mislabelling: Failings of Traceability in the Production Chain and Implications for Illegal, Unreported and Unregulated (IUU) Fishing', *PLoS ONE*. Edited by K. I. Stergiou. Public Library of Science, 9(6), p. e98691. doi: 10.1371/journal.pone.0098691.

Higgins, D. (1997) 'Multiple Sequence Alignment', *Genetic Databases*. Academic Press, pp. 165–183. doi: 10.1016/B978-012101625-8/50010-4.

Inoue, J. G. *et al.* (2010) 'Evolutionary Origin and Phylogeny of the Modern Holocephalans (Chondrichthyes: Chimaeriformes): A Mitogenomic Perspective', *Molecular Biology and Evolution*. Narnia, 27(11), pp. 2576–2586. doi: 10.1093/molbev/msq147.

IUCN (2014) *A quarter of sharks and rays threatened with extinction | IUCN*, *International Union of the Conservation of Nature*. Available at: https://www.iucn.org/content/quarter-sharks-and-rays-threatened-extinction

Jabado, R. W. *et al.* (2018) 'Troubled waters: Threats and extinction risk of the sharks, rays and chimaeras of the Arabian Sea and adjacent waters', *Fish and Fisheries*. John Wiley & Sons, Ltd (10.1111), 19(6), pp. 1043–1062. doi: 10.1111/faf.12311.

Kadler, K. E. *et al.* (1996) 'Collagen fibril formation.', *The Biochemical journal*. Portland Press Limited, 316 (Pt 1)(1), pp. 1–11. doi: 10.1042/bj3160001.

Karni, M. *et al.* (2013) 'Thermal Degradation of DNA', *DNA and Cell Biology*. Mary Ann Liebert, Inc. 140 Huguenot Street, 3rd Floor New Rochelle, NY 10801 USA , 32(6), pp. 298–301. doi: 10.1089/dna.2013.2056.

Kreuzer, R., Ahmed, R. and Lai Ka- Keong, E. (1989) 'Shark fins, processing and marketing in Hong Kong', *InfoFish Marketing*. Krom Pramong, 5(83).

Kumar, S. *et al.* (2018) 'MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms', *Molecular Biology and Evolution*, 35, pp. 1547–1549.

Mundy-Taylor, V. and Crook, V. (2013) *Into the deep: Implementing CITES measures for commercially-valuable sharks and manta rays*. TRAFFIC.

Nagai, T. and Suzuki, N. (2000) 'Isolation of collagen from fish waste material — skin, bone and fins', *Food Chemistry*. Elsevier, 68(3), pp. 277–281. doi: 10.1016/S0308-8146(99)00188-0.

Nollet, L. M. L. (2009) *Handbook of Seafood and Seafood Products Analysis.* CRC Press.

Nomura, Y. (2004) 'Properties and utiliztion of shark collagen', *Developments in Food Science*. Elsevier, 42, pp. 147–158. doi: 10.1016/S0167-4501(04)80017-7.

O'Malley, M. p. *et al.* (2017) 'Characterization of the trade in manta and devil ray gill plates in

China and South-east Asia through trader surveys', *Aquatic Conservation: Marine and Freshwater Ecosystems*. John Wiley & Sons, Ltd, 27(2), pp. 394–413. doi: 10.1002/aqc.2670.

Pazartzi, T. *et al.* (2019) 'High levels of mislabeling in shark meat – Investigating patterns of species utilization with DNA barcoding in Greek retailers', *Food Control*. Elsevier, 98, pp. 179–186. doi: 10.1016/J.FOODCONT.2018.11.019.

Perkins, D. N. *et al.* (1999) 'Probability-based protein identification by searching sequence databases using mass spectrometry data', *Electrophoresis*. John Wiley & Sons, Ltd, 20(18), pp. 3551–3567. doi: 10.1002/(SICI)1522-2683(19991201)20:18<3551::AID-ELPS3551>3.0.CO;2-2.

Pradel, A. *et al.* (2009) 'Skull and brain of a 300-million-year-old chimaeroid fish revealed by synchrotron holotomography.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 106(13), pp. 5224–8. doi: 10.1073/pnas.0807047106.

Rose, D. A. (1996) 'An overview of world trade in sharks and other cartilaginous fishes. A TRAFFIC Network report'. Cambridge (United Kingdom) TRAFFIC International. Available at: http://agris.fao.org/agris-search/search.do?recordID=XF2015048169 (Accessed: 21 August 2019).

Shea, K. H. and To, A. W. L. (2017) 'From boat to bowl: Patterns and dynamics of shark fin trade in Hong Kong — implications for monitoring and management', *Marine Policy*. Pergamon, 81, pp. 330–339. doi: 10.1016/J.MARPOL.2017.04.016.

The UniProt Consortium (2019) 'UniProt: a worldwide hub of protein knowledge', *Nucleic Acids Research*. Narnia, 47(D1), pp. D506–D515. doi: 10.1093/nar/gky1049.

Uniprot (2019) *Current Release Statistics < Uniprot < EMBL-EBI*. Available at: https://www.ebi.ac.uk/uniprot/TrEMBLstats.

Veit, J. *et al.* (2016) 'LFQProfiler and RNP xl: Open-Source Tools for Label-Free Quantification and Protein–RNA Cross-Linking Integrated into Proteome Discoverer', *Journal of Proteome Research*. American Chemical Society, 15(9), pp. 3441–3448. doi: 10.1021/acs.jproteome.6b00407.

Venkatesh, B. *et al.* (2014) 'Elephant shark genome provides unique insights into gnathostome evolution', *Nature*. Nature Publishing Group, 505(7482), pp. 174–179. doi: 10.1038/nature12826.

Ward, R. D. *et al.* (2005) 'DNA barcoding Australia's fish species.', *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, 360(1462), pp. 1847–57. doi: 10.1098/rstb.2005.1716.

Wilga, C. D. and Lauder, G. V. (2001) 'Functional morphology of the pectoral fins in bamboo sharks,Chiloscyllium plagiosum: Benthic vs. Pelagic station-holding', *Journal of Morphology*. John Wiley & Sons, Ltd, 249(3), pp. 195–209. doi: 10.1002/jmor.1049.

Worm, B. *et al.* (2013) 'Global catches, exploitation rates, and rebuilding options for sharks', *Marine Policy*. Pergamon, 40, pp. 194–204. doi: 10.1016/J.MARPOL.2012.12.034.

Supplementary Materials

Supplementary Table 3.1. In total fin samples consisted of 102 shark or skate and ray (batoid) species (Supplementary Table 1). Of the samples 74 had been positively identified using COI barcoding methods (Chapter 2) and the further 28 had been identified within the field upon sample collection.

Species ID (* = CO1 confirmed)	Shark/Ray	Sample Type
Aetobatus narinari	Ray	Tail Fin
*Aetomylaeus maculatus**	Ray	Tail Fin
*Aetomylaeus maculatus**	Ray	Wing
Alopias superciliosus	Shark	Dorsal Fin
Alopias superciliosus	Shark	Dorsal Fin
Alopias superciliosus	Shark	Tail Fin
Alopias superciliosus	Shark	Tail Fin
Alopias superciliosus	Shark	Pectoral Fin
Amblyraja (Raja) radiata	Ray	Tail Fin
Amblyraja (Raja) radiata	Ray	Wing
Centrophorus granulosus	Shark	Pectoral Fin
Centrophorus granulosus	Shark	Pectoral Fin
Centrophorus squamosus	Shark	Dorsal Fin
Centrophorus squamosus	Shark	Pectoral Fin
Centroscymnus coelolepis	Shark	Pectoral Fin
Deania profundorum	Shark	Tail Fin
*Galeorhinus galeus**	Shark	Tail Fin
*Galeorhinus galeus**	Shark	Pectoral Fin
*Galeus melastomus**	Shark	Dorsal Fin
*Galeus melastomus**	Shark	Pectoral Fin
Galeus melastomus/Galeus atlanticus	Shark	Tail Fin
Galeus melastomus/Galeus atlanticus	Shark	Tail Fin
Hexanchus griseus	Shark	Tail Fin
Isurus oxyrinchus	Shark	Tail Fin
Isurus oxyrinchus	Shark	Dorsal Fin
*Isurus oxyrinchus**	Shark	Dorsal Fin
*Isurus oxyrinchus**	Shark	Pectoral Fin
Leucoraja circularis	Ray	Wing
*Leucoraja naevus**	Ray	Tail Fin
*Leucoraja naevus**	Ray	Tail Fin
*Mustelus mustelus**	Shark	Tail Fin
*Mustelus mustelus**	Shark	Tail Fin
*Mustelus mustelus**	Shark	Tail Fin
*Mustelus mustelus**	Shark	Pectoral Fin
*Mustelus mustelus**	Shark	Pectoral Fin
*Mustelus mustelus**	Shark	Pectoral Fin
*Prionace glauca**	Shark	Tail Fin
*Prionace glauca**	Shark	Dorsal Fin
*Prionace glauca**	Shark	Pectoral Fin
*Prionace glauca**	Shark	Pectoral Fin
Raja brachyura	Ray	Wing
*Raja brachyura**	Ray	Wing
*Raja brachyura**	Ray	Wing
*Raja brachyura**	Ray	Tail Fin
*Raja brachyura**	Ray	Tail Fin
Raja clavata	Ray	Wing
*Raja microocellata**	Ray	Wing
*Raja microocellata**	Ray	Tail Fin
*Raja microocellata**	Ray	Tail Fin
*Raja microocellata**	Ray	Tail Fin
*Raja microocellata**	Ray	Tail Fin
*Raja microocellata**	Ray	Tail Fin
Raja miraletus	Ray	Tail Fin
Raja miraletus	Ray	Tail Fin
Raja miraletus	Ray	Wing
Raja miraletus	Ray	Wing
*Raja miraletus**	Ray	Tail Fin
*Raja miraletus**	Ray	Tail Fin
*Raja montagui**	Ray	Tail Fin
*Raja montagui**	Ray	Tail Fin

*Raja undulata**	Ray	Tail Fin
*Raja undulata**	Ray	Tail Fin
*Raja undulata**	Ray	Tail Fin
*Raja undulata**	Ray	Wing
*Raja undulata**	Ray	Wing
*Raja undulata**	Ray	Tail Fin
*Raja undulata**	Ray	Tail Fin
*Raja undulata**	Ray	Wing
*Raja undulata**	Ray	Tail Fin
*Rhinobatos rhinobatos**	Ray	Tail Fin
*Rhinobatos rhinobatos**	Shark	Tail Fin
*Scyliorhinus canicula**	Shark	Tail Fin
*Scyliorhinus canicula**	Shark	Tail Fin
*Scyliorhinus canicula**	Shark	Dorsal Fin
*Scyliorhinus canicula**	Shark	Pectoral Fin
*Scyliorhinus canicula**	Shark	Tail Fin
*Scyliorhinus canicula**	Shark	Tail Fin
*Scyliorhinus canicula**	Shark	Tail Fin
*Scyliorhinus canicula**	Shark	Tail Fin
*Scyliorhinus canicula**	Shark	Tail Fin
*Scyliorhinus canicula**	Shark	Pectoral Fin
*Scyliorhinus canicula**	Shark	Tail Fin
*Scyliorhinus canicula**	Shark	Dorsal Fin
*Scyliorhinus canicula**	Shark	Tail Fin
*Scyliorhinus canicula**	Shark	Tail Fin
*Scyliorhinus canicula**	Shark	Pectoral Fin
*Scyliorhinus canicula**	Shark	Dorsal Fin
*Scyliorhinus canicula**	Shark	Tail Fin
*Scyliorhinus canicula**	Shark	Pectoral Fin
*Scyliorhinus canicula**	Shark	Pectoral Fin
Squalus acanthias	Shark	Tail Fin
*Torpedo marmorata**	Ray	Tail Fin
*Torpedo marmorata**	Ray	Tail Fin
*Torpedo marmorata**	Ray	Wing
*Torpedo marmorata**	Ray	Tail Fin
*Torpedo marmorata**	Ray	Tail Fin
*Torpedo marmorata**	Ray	Wing
*Torpedo marmorata**	Ray	Wing
*Torpedo marmorata**	Ray	Wing
*Torpedo torpedo**	Ray	Wing
*Torpedo torpedo**	Ray	Wing
Unknown	Unknown	Fin

Supplementary Table 3.2.
The Protein Discoverer raw results, displaying protein information and abundance for each sample, corresponding to the data presented in the heatmap, Figure 3.3. FDR= Protein FDR Confidence: Combined, Acc = Accession number, PEP Score = sum of peptide score for all samples, Cov [%] = Cum of Covsergae percentage, # Pep = total number of peptide sequences, Abundance normalised (scaled) for each sample

FDR	Acc	Master Protein Description	Species	Protein	Sum PEP	Cov [%]	# Pep	Abundance (Scaled)															
								1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	
								a.ls	a.S	b.ls	a.ls	a.S	b.ls	a.ls	a.S	b.ls	a.ls	a.S	b.ls	a.ls	a.S	b.ls	
High	D0PQF7	Col1a1 (Fragment) OS=Scyliorhinus canicula OX=7830 GN=Col1a1 PE=2 SV=1	*Scyliorhinus canicula*	COLIaI	457.524	48	62	56.2	63.5	70.7	75.3	46.5	67.2	138.3	125	138	127.1	99.9	115	129	107	143	
High	M00501	CMILTR1Callorhinchidae_Callorhinchus_milii_Tropomyosin_alpha1_chain_isoform_X5	*Callorhinchus milii*	Tropomyosin	355.204	81	43	27.8	226.3	36.8	63.1	389	459.1	6.3	4.7	62.9	10.5	83.7	45.6	7.9	68.5	8.5	
High	M00503	CMILTR3Callorhinchidae_Callorhinchus_milii_Tropomyosin_alpha1_chain_isoform_X4	*Callorhinchus milii*	Tropomyosin	344.181	81	43	12.9	278	22.6	114	464	285.2	3.9	4.1	5.6	11.4	117.6	70.6	11.2	92.9	6.2	
High	M00512	SRETACCarcharhiniformes_Scyliorhinidae_Scyliorhinus_retifer_Fast_muscle_actin	*Scyliorhinus retifer*	Actin	324.792	85	34	257.2	248.5	189	140	341	90.4	17.5	6	8.6	2.7	53.3	26.7	63.2	46.9	9.1	
High	M00505	RTYPTR1Orectolobiformes_Rhincodon_typus_Tropomyosin_alpha1_chain_isoform_X7	*Rhincodon typus*	Tropomyosin	313.812	69	39	8.1	320.9	3.7	30.4	456	215.6	32	7.5	47.3	10.8	137.2	27.6	41.2	122	39.8	
High	M00583	SCANCG1Carcharhiniformes_Scyliorhinidae_Scyliorhinus_canicula_Collagen_Type_1	*Syliorhinus canicula*	COLI	250.597	15	26	149.1	124.4	113	204	57.2	131.9	37.9	114	9.4	106.9	168.7	125	45.7	104	9.3	
High	M00509	TSCMAC Triakis_Triakis_scyllium_Actin_cytoplasmic2	*Triakis scyllium*	Actin	238.118	78	29	307.2	175.2	253	50.2	160	36.9	124.9	25.4	30.4	9	108.4	21.8	133	34	30.1	
High	M00519	CMILTBCallorhinchidae_Callorhinchus_milii_Tubulin_beta	*Callorhinchus milii*	Tubulin	188.611	49	17	282.8	240.2	320	95.1	83.8	30.9	79	43.4	28.4	14.6	66.7	18.2	80.2	74.8	41.9	
High	M00520	RTYPTBOrectolobiformes_Rhincodon_typus_Tubulin_beta	*Rhincodon typus*	Tubulin	163.768	51	16	105.5	270.2	217	56.1	177	30.6	57.4	61.9	71.9	5.9	54.3	39.9	71.8	73.7	207	
High	A0A3G1ZJI6	Haptoglobin (Fragment) OS=Scyliorhinus canicula OX=7830 PE=2 SV=1	*Scyliorhinus canicula*		151.986	57	21	29.2	94.4	135	59.1	362	70.5	36.3	37.6	77.7	17.6	313.8	86.7	29.6	93.9	56.2	
High	M00515	TMATTBTorpedinidae_Torpedo_marmorata_Tubulin_alpha	*Torpedo marmorata*	Tubulin	128.762	62	18	302.1	221.8	381	104	54.8	61.9	74.3	24.2	33.8	8.4	40.7	36.6	78.1	37	41.8	
High	Q801K6	Triosephosphate isomerase (Fragment) OS=Scyliorhinus canicula OX=7830 PE=2 SV=1	*Scyliorhinus canicula*		119.124	70	15	243.3	184.7	244	120	243	167.7	29.2	7	36.7	3.6	61.8	69.4	49.6	10	30.1	
High	M00517	CMILTBCallorhinchidae_Callorhinchus_milii_Tubulin_alpha	*Callorhinchus milii*	Tubulin	113.951	57	17	220.7	528.5	108	79.6	94.1		128.6		99.8		43.5	73.7	124			
High	M00518	SACSTBSqualiformes_Squalus_acanthias_Tubulin_beta	*Squalus acanthias*	Tubulin	107.326	37	12	313.8	272.5	304	107	46.8	28.5	111.4	17	36.3	7.1	48.8	15	124	39.5	28.7	
High	D0PQH7	Sparc OS=Scyliorhinus canicula OX=7830 GN=Sparc PE=2 SV=1	*Scyliorhinus canicula*		73.639	43	10	352.8	8	355	76.7	20.9	404.6	10.2	0.3	44.9	3.5	2.3	156	9.2	2.2	53.8	
High	Q8QFS7	Beta actin (Fragment) OS=Scyliorhinus canicula OX=7830 GN=b-actin PE=2 SV=1	*Scyliorhinus canicula*	Actin	63.158	58	9		356		310	393	137.3					246	57.8				

High	M00584	SCANCG2Carcharhiniformes_Scyliorhinidae_Scyliorhinus_canicula_Collagen_Type2	*Scyliorhinus canicula*	COLIaII	61.598	15	10	246.9	23.7	357	225	8	294.1	4.6	3.5	1.4	1.5	2.7	314	12.7	1.8	3.5
High	C3SAS1	Glyceraldehyde-3-phosphate dehydrogenase (Fragment) OS=Scyliorhinus canicula OX=7830 GN=GAPDH PE=2 SV=1	*Scyliorhinus canicula*		54.916	51	3	629.5	91	244	317	52.5	71.9	7	3.9	2.8	5.4	18.6	45.8	4.3	6.1	
High	M00429	typus	*Rhincodon typus*		49.494	4	8	19.5	27.1	30.8	152	26	110.6	71.9	220	87.4	70.6	150.7	72.4	36	304	121
High	Q801K9	ATP synthase subunit alpha (Fragment) OS=Scyliorhinus canicula OX=7830 PE=2 SV=1	*Scyliorhinus canicula*		41.971	25	7	343.3	81.6	366	192	32.3	190.1	56.4	6.3	3	14.1	20.2	55.1	99	35.5	5.5
High	Q801J7	Creatine kinase (Fragment) OS=Scyliorhinus canicula OX=7830 PE=2 SV=1	*Scyliorhinus canicula*		41.399	30	7	263.5	135.9	290	339	169	119.7	16.2	2.3	4.7	9.6	76.4	45.6	12.3	2.6	13.5
High	U5NMN9	High mobility group box 3 protein OS=Scyliorhinus canicula OX=7830 GN=HMGB3 PE=2 SV=1	*Scyliorhinus canicula*		40.05	35	7	203.9	48.7	395	64.8	45.2	320.6	54.7	1	18.5	7.1	12.4	179	70.5	2.3	76.7
High	M00428	milii	*Callorhinchus milii*		35.22	3	5							441	29.2	568	46	10.1		220	36.5	149
High	Q801K5	Fructose-bisphosphate aldolase (Fragment) OS=Scyliorhinus canicula OX=7830 PE=2 SV=1	*Scyliorhinus canicula*		33.147	34	6	195.7	217.5	277	112	262	53.5	116.7	9.3	113	11.6	6.3	6.4	9.8	27	82.7
High	U5NMZ8	High mobility group box 2 protein OS=Scyliorhinus canicula OX=7830 GN=HMGB2 PE=2 SV=1	*Scyliorhinus canicula*		31.712	18	3	260.8	20.1	641	64.4	23.5	281.1	16	1.1	16.1	2.4	6.1	151	14.6	2.1	
High	A0A173FZK0	Elongation factor 1-alpha OS=Scyliorhinus canicula OX=7830 GN=Eef1a1 PE=2 SV=1	*Scyliorhinus canicula*		30.043	22	6	232.5	25.5	278	52.7	13	40	106.7	6.1	242	9.5	12.7	21.9	103	12.5	345
High	Q801H0	Ribosomal protein S4 (Fragment) OS=Scyliorhinus canicula OX=7830 PE=2 SV=1	*Scyliorhinus canicula*		25.5	16	4	307.8	23.4	426	84.1	6.9	53.4	149.6	5.6	88.5	34.8	18.6	71	164	20.7	45.6
High	D0PQF9	Col5a2 (Fragment) OS=Scyliorhinus canicula OX=7830 GN=Col5a2 PE=2 SV=1	*Scyliorhinus canicula*	COL5aII	22.654	7	4	245.1	94.8	183	149	113	146.3	27.6	187	3.4	49.9	107.1	108		80.3	5.4
High	Q801G8	40S ribosomal protein S7 (Fragment) OS=Scyliorhinus canicula OX=7830 PE=2 SV=1	*Scyliorhinus canicula*		19.787	20	3	208.6	31.5	101	585	13.7	219.8	50.3	3.1	9.8	105.5	12.6	69.8	52.7	8.6	27.9
High	O79404	Cytochrome c oxidase subunit 2 OS=Scyliorhinus canicula OX=7830 GN=MT-CO2 PE=3 SV=1	*Scyliorhinus canicula*		18.057	9	1	381.5	180.4	552	75.6	75.2	22.9	35.4	4	39.2		5.9		80	20.5	27.7
High	Q801I4	Ribosomal protein L5 (Fragment) OS=Scyliorhinus canicula OX=7830 PE=2 SV=1	*Scyliorhinus canicula*		16.275	11	2	526.6	49.2	525	135	34.9	65.7	22.9			6.5	19.1	35.5	50.9	28.8	
High	M00381	bergylta			16.266	3	5	99.9	245.3			31.7			263		106.3	293.5	203		141	116
High	M00552	OKJICG2Rajiformes_Okamejei_kenojei_Pro_Collagen_alpha_1	*Okamejei kenojei*	COLI	15.493	3	3	294.4	16.4	434	98.9	9.2	114.1	163.6	12.4	47.6	12.8	15.3	51.6	165	12.4	52.6

High	Q801I2	Ribosomal protein L7 (Fragment) OS=Scyliorhinus canicula OX=7830 PE=2 SV=1	*Scyliorhinus canicula*		15.488	22	4	394.5	60.2	92.4	200	7	48.2	330.1		40.3	89.4	23.6	27	182	5.3	
High	M00332	oculatus			15.117	3	5	138.9	22.9	123	37.3	39.2	47.3	78.4	81.7	153	102.8	29.7	78.1	114	300	154
High	Q6EE43	Ribosomal protein large P0 (Fragment) OS=Scyliorhinus canicula OX=7830 PE=2 SV=1	*Scyliorhinus canicula*		14.549	16	3	235.9	174	183	154	107	73.2	230.5	22.5	30.5		65.3	43.5	151	28.9	
High	Q801K7	Eukaryotic translation elongation factor 1 gamma (Fragment) OS=Scyliorhinus canicula OX=7830 PE=2 SV=1	*Scyliorhinus canicula*		14.099	14	3	328.8	114	428	177	63.7	102.6	56.9		71.2	10.4	32.3	61.2	53.5		
High	M00304	mexicanus			13.243	1	4	38.8	13.5	19.1	5.7	2.3	1.4	204.1	130	279	10.4	10.9	3.6	77.4	199	505
High	M00383	chrysurus			12.999	2	5															
High	M00426	armatus			12.57	2	4		15.3			11.1		12.6	214	56.6	40.3	132.8	115		769	133
High	M00346	Xiphias gladius			12.482	2	5	22.9	70.6	17	107	43.6	75.6	18.5	220	104	249.8	162.8	156	91.3	160	2.7
High	M00317	variegatus			12.241	2	4	53.6	73.1	45.7	72.5	35.8	92.4	62.1	34.2	457	18.5	100.8	37.2	75.7	37.3	304
High	Q6EE46	Chaperonin-containing subunit 8 theta (Fragment) OS=Scyliorhinus canicula OX=7830 PE=2 SV=1	*Scyliorhinus canicula*		12.17	11	2	49.1	106	67.8	90.8	39.1	72.4	122.7		366	12.8		26.2	147		400
High	Q801H1	Ribosomal protein S3 (Fragment) OS=Scyliorhinus canicula OX=7830 PE=2 SV=1	*Scyliorhinus canicula*		11.716	12	3	394	58.9	276	110	24	99.6	196.4	8.5	35.1	31.6	29	30	163	21.5	22.5
High	P35491	Parvalbumin (Fragment) OS=Scyliorhinus canicula OX=7830 PE=1 SV=1	*Scyliorhinus canicula*		11.061	36	3	94	33.8	280	50.1	35.8	556.8	9.8	11.9	10.8	3.5	8.7	398	4.6	2.7	
High	M00319	furzeri			11.005	2	4				132	15.8	222.2		141		205.5	57.1	576	71.5	78.4	
High	Q801G9	Ribosomal protein S6 (Fragment) OS=Scyliorhinus canicula OX=7830 PE=2 SV=1	*Scyliorhinus canicula*		9.207	7	1	407.2	179		248			283.5			309.3	72.6				
High	Q8QGY1	Putative Na,K-ATPase alpha 1 subunit (Fragment) OS=Scyliorhinus canicula OX=7830 GN=ATNA_SCYCA PE=2 SV=1	*Scyliorhinus canicula*		7.213	8	1	200.7	302.8	595	56.1	82.1				138						126
High	Q6EE41	Ribosomal protein S9 (Fragment) OS=Scyliorhinus canicula OX=7830 PE=2 SV=1	*Scyliorhinus canicula*		6.489	6	2	329.5	35.9	110	352	16.5	125	178.5			90.4	18.8		103		141
High	Q801J1	Ribosomal protein L15 (Fragment) OS=Scyliorhinus canicula OX=7830 PE=2 SV=1	*Scyliorhinus canicula*		6.348	11	2	360.9	32.3	126	291	31.2	112.3	174.9	4.1	25.8	112.6	12.3	108	110		
High	M00585	XENO Xenopus COL1			5.955	1	2	0.1	0.1		0.4	0.1	0.5	183.7	139	269	454.8	75.9	84.1	112	51.2	129

High	Q90YD5	GTP binding protein Rab1a (Fragment) OS=Scyliorhinus canicula OX=7830 GN=Rab1a PE=4 SV=1	*Scyliorhinus canicula*		4.41	17	1	293.1	202.9	353	153	140	71.2			69		77.7	37.4	102		
High	M00526	OKJICG1Rajiformes_Okamejei_kenojei_Pro_Collagen_alpha_1	*Okamejei kenojei*	COLI	4.325	3	2															
High	Q8QGN6	Beta-catenin (Fragment) OS=Scyliorhinus canicula OX=7830 PE=2 SV=1	*Scyliorhinus canicula*		3.968	5	1	451.7	83.6	747	46.4	125						23.3			23.6	
High	M00547	CMILCG5Callorhinchidae_Callorhinchus_milii_Collagen_alpha_2	*Callorhinchus milii*	COLII	3.837	2	1	139	8.1	593	25.5	9.7	369.7	22.6		25.3	4.8	1.5	176	52.1	4.3	68.1
High	Q6EE47	Calreticulin (Fragment) OS=Scyliorhinus canicula OX=7830 PE=2 SV=1	*Scyliorhinus canicula*		3.491	7	1		179.3		209	150	273.9				48.4	219.5	385		36.2	
High	O73928	Dystrophin (Fragment) OS=Scyliorhinus canicula OX=7830 PE=2 SV=1	*Scyliorhinus canicula*		3.198	2	1															
High	Q6EE45	Fascin (Fragment) OS=Scyliorhinus canicula OX=7830 PE=2 SV=1	*Scyliorhinus canicula*		3.112	3	1	287.9	45.6	479	13.1	3.6		154.5	14	98.4	7.5	15.1	14	219	25.6	123
High	Q9I963	Putative Na,K-ATPase beta 1 subunit (Fragment) OS=Scyliorhinus canicula OX=7830 GN=ATNB_SCYCA PE=2 SV=1	*Scyliorhinus canicula*		3.1	17	1	371.8	113.1	577		135				87					39.6	177
High	O79405	ATP synthase protein 8 OS=Scyliorhinus canicula OX=7830 GN=MT-ATP8 PE=3 SV=1	*Scyliorhinus canicula*		2.824	20	1	176.1	148.8	115	253	123	74.8	72.6	51.2			44.2		105	185	151
High	A0A1C8YZI9	Sodium/potassium-transporting ATPase subunit alpha OS=Scyliorhinus canicula OX=7830 GN=ATP4A PE=2 SV=1	*Scyliorhinus canicula*		2.7	1	1	389	369.5	340	131	271										
High	Q801H6	60S ribosomal protein L13 (Fragment) OS=Scyliorhinus canicula OX=7830 PE=2 SV=1	*Scyliorhinus canicula*		2.553	4	1	167.1	29.3	134	54.7	8.7	319.7	167.5		90.4	89.1	18.1	68.7	333	20.1	
Medium	P35490	Urotensin-2 OS=Scyliorhinus canicula OX=7830 PE=1 SV=1	*Scyliorhinus canicula*		2.481	75	1		541.5	522		437										
Medium	M00544	CMILCG2Callorhinchidae_Callorhinchus_milii_Collagen_alpha_2	*Callorhinchus milii*	COLII	2.242	1	1	79.7	42.6	110	176	29.4	101.8	164.8	116		155.5	65.8	251	95.4	111	
Low	M00569	RTYPCG8Orectolobiformes_Rhincodon_typus_Collagen_alpha_5	*Rhincodon typus*	COL5	1.453	2	1	338.3		810	29.2	5.4	238.7						78.7			

Supplementary Table 3.3.
The mascot analysis conducted on batch 1 samples, including size and percentage coverage for each protein found in the samples.

Sample Info		COL1A1		COL2A1		COL5A2		ACTB		MYS		TPM		TUB	
ID	MD	Size	C (%)	Size	C (%)	Size	C (%)	Size	C (%)	Size	C (%)	Size	C (%)	Size	C (%)
01	a.ls	4138	13.88	4200	14.09	24	0.08	2346	14.67	15	0.05	493	1.65	499	4.95
02	a.S	9618	18.57	1415	2.73	250	0.48	4959	17.18	0	0.00	1645	8.61	1311	7.27
03	b.ls	3631	11.92	4221	13.86	33	0.11	1798	11.45	16	0.10	437	1.44	508	2.88
04	a.ls	8891	18.99	4870	10.40	47	0.10	1424	5.43	18	0.07	795	4.97	329	2.33
05	a.S	5631	12.11	643	1.38	500	1.08	8986	35.80	0	0.00	3013	17.47	417	2.07
06	b.ls	6713	9.04	10474	14.11	106	0.14	1535	4.05	0	0.00	1920	7.27	207	0.49
07	a.ls	1045	13.37	478	6.11	0	0.00	218	5.39	0	0.00	79	1.01	363	9.43
08	a.S	6013	20.02	0	0.00	164	0.55	300	1.85	0	0.00	280	1.63	195	1.80
09	b.ls	410	5.71	0	0.00	0	0.00	49	0.68	15	0.21	82	1.14	91	1.71
10	a.ls	4591	16.54	0	0.00	73	0.26	79	0.28	0	0.00	563	2.03	140	0.92
11	a.S	9563	21.95	132	0.30	175	0.40	782	3.26	0	0.00	1103	7.11	224	1.76
12	b.ls	5798	10.57	7588	13.83	60	0.11	272	0.91	0	0.00	663	2.16	68	0.25
13	a.ls	1381	14.21	400	4.12	0	0.00	456	9.19	0	0.00	166	1.71	180	3.11
14	a.S	4749	14.14	152	0.45	262	0.78	993	3.78	0	0.00	1247	10.14	271	1.80
15	b.ls	465	8.90	81	1.55	0	0.00	29	0.56	0	0.00	77	1.47	119	3.54

ID = corresponding to Table 1, analysis methods, MD = corresponding to Table 1, methods, COL1A1 = Collagen type 1, alpha chain 1, COL1A2 = Collagen type 1 alpha chain 2, ACT = Actin. MYS= Myosin, TPM= Tropomyosin, TUB = Tubulin, Size = the size of the largest fragment detected, C (%) = the coverage score for each protein in each sample.

Chapter 4

Recognition Software Successfully Aids the Identification of Individual Small-Spotted Catsharks during their First Year of Life

S. A. Hook[1§], C. McMurray[1§], D. M. Ripley[1], N. Allen[2], T. Moritz[3,4], B. Grunow[5] And H. A. Shiels[1*]

[1]Faculty of Biology, Medicine and Health, University of Manchester, Core Technology Facility, Manchester, M13 9NT, U.K.

[2]Biological Services Facility, University of Manchester, Stopford Building, M13 9PT, U. K.

[3]Deutsches Meeresmuseum, Katharinenberg 14-20, 18439 Stralsund, Germany.

[4]Institut für Zoologie und Evolutionsforschung, Friedrich-Schiller-Universität Jena, Erbertsstr.1, 07743 Jena, Germany**.**

[5]Leibniz-Institute for Farm Animal Biology, Dummerstorf, Germany.

[§] These authors contributed equally to this work

* Author for correspondence: holly.shiels@manchester.ac.uk

Author contributions

HAS devised the project; CM and SAH carried out the project; BG and TM supplied the shark embryos and parentage tissue samples; and NA and DR assisted in photograph and tissue collection. CM analysed the photographic data, SAH analysed the genetic data and DR generated the statistical analyses. All authors contributed to interpretation and writing.

An updated version of this chapter can be found published in the Journal of Fish Biology. The reference is:

Hook, S.A., McMurray, C., Ripley, D.M., Allen, N., Moritz, T., Grunow, B. and Shiels, H.A., 2019. Recognition software successfully aids the identification of individual small-spotted catsharks Scyliorhinus canicula during their first year of life. *Journal of Fish Biology*, *95*(6), pp.1465-1470.

4.1 Abstract

The ability to track individual animals across time is crucial to a range of studies in biological sciences and conservation management, but invasive tagging is not always a viable option. Here, 18 captive *S. canicula* individuals were successfully identified from hatching to one year of age using the free computer photo recognition software, I^3S classic. The effect of increasing the time interval between recognition attempts on the accuracy of the software was investigated, revealing that recognition score decreases with increasing time intervals during younger (0 to 15 weeks), but not older (15 weeks onwards), sharks. Identification by I^3S was validated using genetic analyses of seven microsatellite markers, revealing a 100% success rate. Thus, this non-invasive recognition method can be used as an inexpensive and effective alternative to invasive tagging, improving animal welfare and complimenting *ex-situ* conservation methods.

Keywords: microsatellites, elasmobranchs, I^3S, conservation, management, captivity

4. 2 Introduction

Identification of individual animals is often crucial in studies of wild and captive populations in order to properly manage and conserve species (Marshall & Pierce, 2012). In fish physical tags, such as T-bar anchors and passive integrated transponders (PIT) are commonly used. However, these methods of identification can be limited by tag loss, negative effects on growth, health, and escape from predators, as well as injury or even death from tag application (Manire & Gruber, 1991; Cailliet *et al*., 1992; Feldheim *et al.,* 2002; French *et al*., 2015). Furthermore, many physical tags are too large to use on young or small individuals. Animal biometrics offers a non-invasive and economical alternative to invasive identification methods. Natural markings, scars and contours that are unique to individuals and that are maintained throughout their lives have been used for individual recognition across a variety of marine taxa including pinnipeds, cetaceans, sirenians, and elasmobranchs (MacLeod, 1998; Gubili *et al*., 2009; Wells, 2017; Pawley *et al.*, 2018). Such databases can grow large, and performing the identifications manually can become time-inefficient. Photo-recognition is therefore often used alongside invasive and non-invasive tagging methods as a tool to determine and track morphological changes, in addition to providing individual IDs (Chin *et al.,* 2015). Recognition software, such as the freeware Interactive Individual Identification System Classic (I^3S), can aid the process of manual identifications and possibly remove the need for invasive tagging. I^3S has been successfully used for individual identification in the whale shark *Rhincodon typus* (Speed *et al*., 2007; Graham and Roberts, 2007), the white shark *Carcharodon carcharias* (Andreotti *et al.*, 2016, 2018), the spotted eagle ray *Aetobatus narinari* (González-Ramos *et al.*, 2017), the ragged-tooth shark *Carcharias taurus* (van Tienhoven *et al.,* 2007), and the grey nurse shark *Carcharias taurus* (Bansemer & Bennett, 2008). I^3S software produces recognition scores derived from the distances between pairs of spots that are chosen by the user (van Tienhoven *et al.,* 2007). Using reference points, the distances between the spots are scaled to the size of the animal, so growth should not be detrimental to recognition. However, to our knowledge, biometrics and I^3S software have not been used during early development in fish.

A growing number of studies are focussing on early life-stages in fishes, however, their size and vulnerability render many standard tagging regimes inadequate. Here, the ability of I^3S to recognise individual, juvenile *S. canicula* during their first year of life was investigated. We investigate the efficacy of I^3S in identifying individuals through time by comparing matches to those generated with microsatellite marker analysis. Finally, we suggest a photography regime that maximises the efficiency of I^3S as a tool for laboratory and field use.

4.3 Materials and Methods

The study population consisted of 18 *S. canicula* (sex not determined) that arrived at The University of Manchester (UK) as embryos from the OZEANEUM, Stralsund (Germany). Once at the University of Manchester, the egg cases were transferred into 45L static seawater tanks that were maintained at 15°C and 35 ppt salinity, under a 12-hour light-dark cycle, until hatching. After hatching, the sharks were held in three 400L tanks under the same conditions as during embryogenesis.

4.3.1 Photography of the hatchlings

Photographs of the 18 individuals were taken once a week for seven weeks after hatching in small container tanks, separate to the main tank. Additional photographs were taken at weeks 12, 14, 16, 30, 32 to 34, 38, 44, and 45. The age of the hatchlings when the first photograph was taken ranged from 0 to 2 weeks for 14 individuals; the remaining 4 individuals had their first photograph at 16 weeks post-hatch. Each week of photographs was stored in a separate database containing all the individuals for the given week. A Sony Cyber-shot T300 camera and a Moto G3 phone camera were used for the photography. Using a net, the sharks were transferred individually from their holding tanks to a small transparent test tank (approximately 12cm x 20cm) containing enough water to submerge the specimen. Photographs of the dorsal side were taken, parallel to the camera lens. Afterwards, individuals were returned to their original tank. By the end of the study period, three individuals had died for unknown reasons but the data provided by these individuals for the earlier time points were not removed from the databases.

4.3.2 Data input into I^{3}S

I^{3}S requires manual input of an animal's patterns from photographs into databases. Three reference points were selected, which correct for discrepancies in angle and scale between two photos. The three reference points chosen for pattern input in this study were the anterior corners of the right and left pectoral fins where they meet the body, and the central point between the anterior corners of the pelvic fins (blue dots denoted by arrowheads; Figure 4.1a). The individual's spots were then pinpointed by the user (red dots; Figure 4.1a), creating a two-dimensional pattern which the software compares automatically to the rest of a given database by overlaying the 2D patterns (Figure 4.1b). With I^{3}S, a maximum of 30 spots can be selected. As *S. canicula* usually develop more than 30 spots, the most prominent spots on the dorsal side were pinpointed by the user.

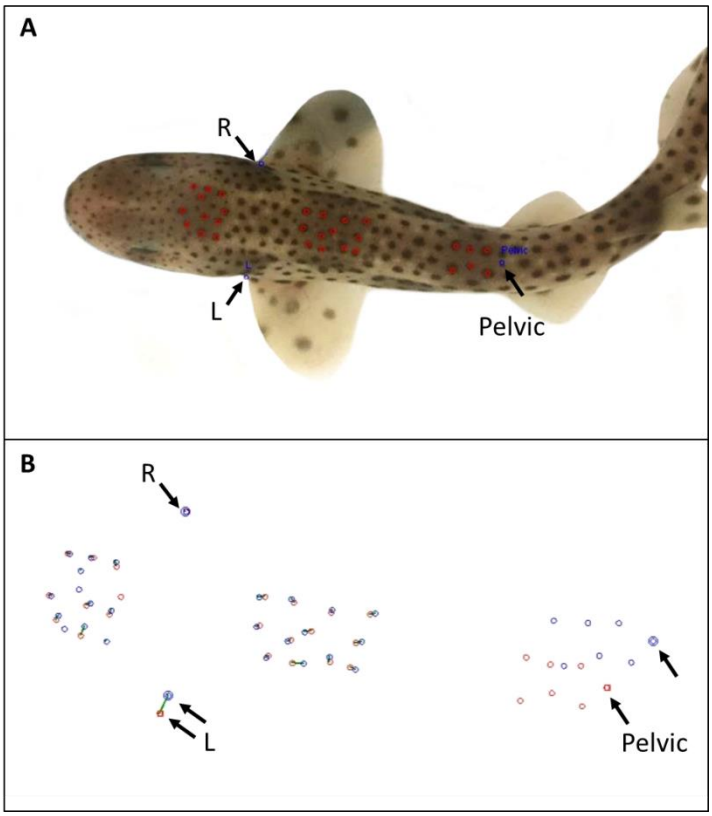

Figure 4.1. (a) Input of patterns into I^{3}S. Reference points (blue dots and arrows) for inputted patterns: the corners of the right (R) and left (L) pectoral fins and the midpoint between the pelvic fins (Pelvic). Up to 30 natural patterning spots on the hatchling are selected by the user (red dots). (b) Comparisons of patterns in I^{3}S. Two-dimensional pattern comparisons for two different individuals. Individual 1 is the catshark from (A) and its spots are shown here in blue, individual 2 (not imaged) is a separate catshark and its spots are shown in red. The same three reference regions were used for all sharks. Note the right (R) pelvic fin references points are overlaid. Where the software considers a marking to be the same between individuals, a green line joins the points. The greater the number of joined lines the closer the 2 patterns are to each other and thus the better the recognition score.

4.3.3 Data output from I^{3}S – recognition score

I^{3}S software produces a recognition score derived from the distances between pairs of spots in the images being compared (van Tienhoven *et al.,* 2007). Recognition scores demonstrate the closeness of a match between a given image and every other image in the given database. The lower the recognition score, the more similar the patterns are between images; a recognition score of '1' presents the user with the perfect match. I^{3}S produces a ranked list of potential matches for the query image against all the images in the given database. The user is then required to visually interrogate the potential matches to determine the true match. In this respect I^{3}S is a valuable aid for photographic identification but does not replace the need for visual checking by the user. Indeed, most studies employ visual confirmation of the computed matches (Speed *et al.,* 2007; van Tienhoven *et al.,* 2007; Andreotti *et al.,* 2016, 2018; González-Ramos *et al.*, 2017). However, genetic validation of matches is less commonly used (Graham & Roberts, 2007) and never, to our knowledge, during early development.

To establish how age relates to changes in the patterning of individuals, and thus how frequently photographs must be taken to track an individual over time, separate databases containing images of each animal were created for each week in I^{3}S, producing a database time series. This time-series of databases was used to compute recognition scores for each individual across pairs of weeks, with increasing time differences between them, in order to determine how the time between photographs affects the performance of the software. The recognition scores produced by I^{3}S that were associated with the correct match determined by eye were recorded.

4.3.4 Genetic analysis

Fin clips were taken from each individual at the beginning and end of the experimental period (listed as Cat01 to Cat18, and HAM43 to HAM57, respectively. Three individuals died of natural causes during the experimental period (Cat11, Cat15 and Cat18), leaving 15 individuals genetically identified at the end of the study. The entire population of potential parents (four males and three females) from the captive source population housed

at the OZEANEUM were also fin clipped and added to the sample set to allow us to account for siblingship, which affects the precision of genetic identification.

Genomic DNA from the fin clips was extracted using Bioline Islotte II Blood and Tissue kit. Samples were amplified using seven microsatellite primers (Scan02, Scan04, Scan09, Scan10, Scan12, Scan15, Scan16) (Griffiths *et al.,* 2011). The products were genotyped at the University of Manchester sequencing facility and scored using GeneMapper© v4.1 (Applied Biosystems). Alleles were checked for user error using MicroChecker v.2.2.3 (van Oosterhout *et al.,* 2004) and the genotypic fingerprints were run through the program CERVUS to determine probability of identity analysis (pID; Marshall *et al.*, 1998, Kalinowski, Taper and Marshall, 2007). Parentage analysis using the program CERVUS was conducted to determine the extent of siblingship of the offspring (i.e. full siblings, half-siblings or unrelated).

4.4 Results

The three reference points (Figure 4.1a.) were held consistent between all inputted patterns to correct for size and angle. Two-dimensional pattern comparisons of separate individuals can be visualised by I^3S and are shown in (Figure 4.1b).

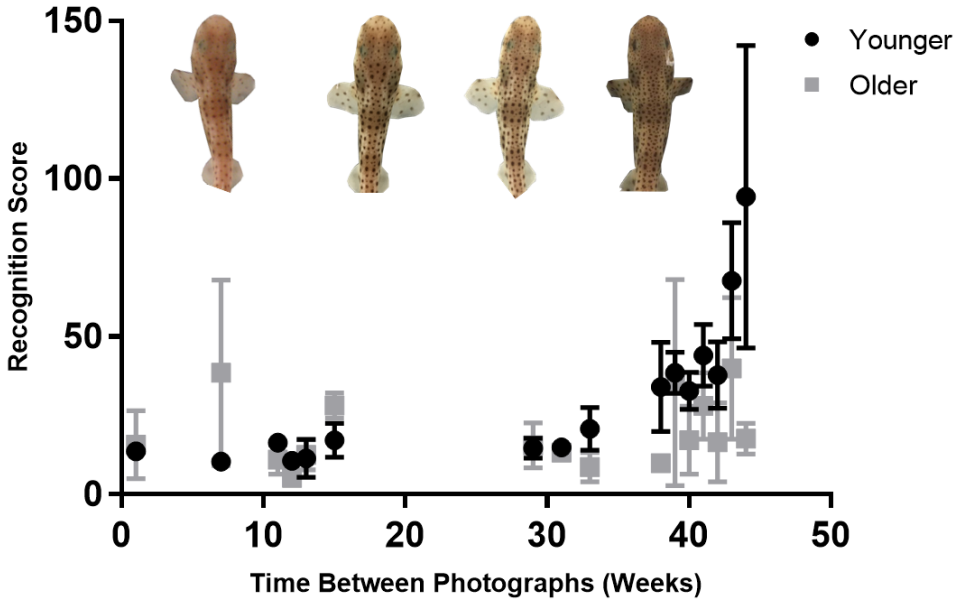

Figure 4.2. The effect of increasing time intervals between databases on recognition. The population was divided into two groups: one of older individuals (squares) and the other of younger individuals (circles). The data were tested for normality with the Shapiro-Wilk test and for correlation using Spearman's rank correlation. All statistical tests were performed using R (R Development Core Team, 2008). Photographs show the spot pattern in one shark at 16, 29, 45 and 60 weeks of age from left to right. Note: in the 60-week image the pectoral fins are angled downward.

4.4.1 The effect of photographic time interval on accuracy of photo recognition

For effective use of I^3S it is important to know how frequently photographs need to be taken for accurate identification over time. To establish how increasing the time interval between databases affects the accuracy of the software's recognition scores, and thus correct identification, we computed recognition scores for comparisons between images from the final database with matches in each of the preceding databases (Figure 4.2). For this comparison, the sample population was divided into one group of older individuals and one of younger individuals, as the rate of pattern development was suspected to vary with age. If the population had been considered as a whole, correlations between time interval and recognition score could have been confounded by age, potentially masking any relationship. Mean recognition scores and standard errors from the two groups were calculated for each database (Figure 4.2). The longer the time interval between databases, the greater the recognition scores, indicating that recognition degrades as the time interval between photographs increases (Figure 4.2b; $r_{s14} = 0.912$, $P < 0.001$, Spearman's Rank). No correlation was found in the older group between time interval and the recognition score ($r_{s14} = 0.347$, $P > 0.05$). Thus, correct identification of the younger, but not older hatchlings is more difficult with increasing periods of time between photographs.

4.4.2 Genetic validation

All seven microsatellite primers amplified each locus for all of the samples tested . Parentage analysis determined all the individuals within the source population had contributed to the photo-recognition experimental individuals (Cat01 - Cat18). As not all the individuals were full siblings, the probability of identity was calculated without the assumption of full siblingship (pID). Both probability indexes produced highly significant results, positively matching two samples, identifying them as duplicates and, therefore, the same individual. No duplicates were found within the same sampling time point, indicating an appropriate level of genetic diversity within the population for the analysis. Matching identifications were found using all seven of the microsatellite loci, with no mismatching loci.

4.5 Discussion

Through the use of microsatellite markers we show that photo recognition software can successfully identify individual small-spotted catsharks during their first year of life. The use of photo-recognition and genetic validation has been previously performed in wild great white sharks (*Carcharodon carcharias*), but only after the sharks were older than 1 year, when growth had stabilised (Graham & Roberts, 2007). Our study is the first to combine photographic recognition software and genetic analysis during early development in any elasmobranch. Our results show that photographs should be taken at intervals of one week for the first eight weeks after hatching, whilst pattern development is at its most changeable. As the shark's age and pattern prominence increases, time intervals between photographs can increase to one month without compromising recognition score.

Despite the success of this method, certain caveats should be acknowledged. Firstly, *S. canicula* is known to change colouration based on its substrate and surroundings (Visconti *et al.,* 1999). Although no such changes were observed in this study, identification in wild populations could be more difficult if the natural markings fade or change. Secondly, captive populations ensure certainty that every individual will be present in each database. It is more problematic for wild populations, where the total number of unidentified individuals is unknown. Thirdly, over long study periods, changes in body shape may pose an issue if the relative positions of the three reference points change over time. If these change, growth and scale cannot be as effectively corrected for, resulting in higher recognition scores (lower similarity). It is recommended that subsequent studies should investigate the potential effects of such changes in older *S. canicula*. If recognition success decreases, increasing the frequency of the photographs will compensate. Finally, the quality of the photograph, specifically the lighting and angle, should be consistent (Speed *et al.,* 2007).

Due to the study being conducted within captivity, true relationships between individuals were easily determined by genetic identity analysis. Previously *S. canicula* has displayed levels of multiple paternity (Griffiths *et al.,* 2012) and, therefore, parentage was necessary to correctly identify the differences between low variation and identity. Although

the majority of individuals are closely related, either as full siblings or half-siblings, no individuals had exact genotypic fingerprints or exact pattern matches.

Animal welfare is of increasing concern in captivity. Aquariums aim to reduce the number of wild-caught individuals and become more involved in captive breeding to assist conservation efforts (Smith *et al.,* 2004). Traditional identification methods such as PIT tags are less suitable at a younger ages due to the potential negative impact on animal survival, physiology or behaviour (Gibbons & Andrews, 2004). Overall this study found that *S. canicula* can be identified using natural markings from hatching to a year of life, without invasive tagging, if photographs are taken once a week for the first 8 weeks. The I^3S software therefore provides a free and reliable method for individual recognition where, beyond installing the software, no specialist equipment is required.

4.6 Acknowledgements

The authors thank M.S. Musa, G. Morrissey, D. Mortell and E. Owen for shark husbandry and B. Evans for assistance with figure preparation and M. Buckley for use of his lab for genetic analysis. The authors also thank the aquarist team of the OZEANEUM for support in catshark breeding.

4.7 References

Andreotti, S. *et al.* (2016) 'An integrated mark-recapture and genetic approach to estimate the population size of white sharks in South Africa', *Marine Ecology Progress Series*, 552, pp. 241–253. doi: 10.3354/meps11744.

Andreotti, S. *et al.* (2018) 'Semi-automated software for dorsal fin photographic identification of marine species: application to Carcharodon carcharias', *Marine Biodiversity*. Springer Berlin Heidelberg, 48(3), pp. 1655–1660. doi: 10.1007/s12526-017-0634-2.

Bansemer, C. S. and Bennett, M. B. (2008) 'Multi-year validation of photographic identification of grey nurse sharks, Carcharias taurus, and applications for non-invasive conservation research', *Marine and Freshwater Research*. CSIRO PUBLISHING, 59(4), p. 322. doi: 10.1071/MF07184.

Cailliet, G. *et al.* (1992) 'Growth and demography of the Pacific Angel Shark (Squatina californica), based upon tag returns off California', *Marine and Freshwater Research*. CSIRO PUBLISHING, 43(5), p. 1313. doi: 10.1071/MF9921313.

Chin, A., Mourier, J. and Rummer, J. L. (2015) 'Blacktip reef sharks (Carcharhinus melanopterus) show high capacity for wound healing and recovery following injury', *Conservation Physiology*. Oxford University Press, 3(1), p. cov062. doi: 10.1093/conphys/cov062.

Feldheim, K. A. *et al.* (2002) 'Genetic tagging to determine passive integrated transponder tag loss in lemon sharks', *Journal of Fish Biology*. John Wiley & Sons, Ltd (10.1111), 61(5), pp. 1309–1313. doi: 10.1111/j.1095-8649.2002.tb02474.x.

French, R. P. *et al.* (2015) 'High survivorship after catch-and-release fishing suggests physiological resilience in the endothermic shortfin mako shark (*Isurus oxyrinchus*)', *Conservation Physiology*. Oxford University Press, 3(1), p. cov044. doi: 10.1093/conphys/cov044.

Gibbons, W. J. and Andrews, K. M. (2004) 'PIT Tagging: Simple Technology at Its Best', *BioScience*. Oxford University Press, 54(5), pp. 447–454. doi: 10.1641/0006-3568(2004)054[0447:ptstai]2.0.co;2.

González-Ramos, M. S. *et al.* (2017) 'Validation of photo-identification as a mark–recapture method in the spotted eagle ray Aetobatus narinari', *Journal of Fish Biology*, 90(3), pp. 1021–1030. doi: 10.1111/jfb.13215.

Graham, R. T. and Roberts, C. M. (2007) 'Assessing the size, growth rate and structure of a seasonal population of whale sharks (Rhincodon typus Smith 1828) using conventional tagging and photo identification', *Fisheries Research*. Elsevier, 84(1), pp. 71–80. doi: 10.1016/J.FISHRES.2006.11.026.

Griffiths, A. M. *et al.* (2011) 'Characterisation of polymorphic microsatellite loci in the small-spotted catshark (Scyliorhinus canicula L.)', *Conservation Genetics Resources*. Springer Netherlands, 3(4), pp. 705–709. doi: 10.1007/s12686-011-9438-z.

Griffiths, A. M. *et al.* (2012) 'First analysis of multiple paternity in an oviparous shark, the small-spotted catshark (Scyliorhinus canicula L.)', *Journal of Heredity*. Oxford University Press, 103(2), pp. 166–173. doi: 10.1093/jhered/esr112.

Gubili, C. *et al.* (2009) 'Concordance of genetic and fin photo identification in the great white shark, Carcharodon carcharias, off Mossel Bay, South Africa', *Marine Biology*. Springer-Verlag, 156(10), pp. 2199–2207. doi: 10.1007/s00227-009-1233-y.

MacLeod, C. D. (1998) 'Intraspecific scarring in odontocete cetaceans: an indicator of male "quality" in aggressive social interactions?', *Journal of Zoology*. John Wiley & Sons, Ltd (10.1111), 244(1), pp. 71–77. doi: 10.1111/j.1469-7998.1998.tb00008.x.

Manire, C. A. and Gruber, S. H. (1991) 'Effect of M-Type Dart Tags on Field Growth of Juvenile Lemon Sharks', *Transactions of the American Fisheries Society*. Taylor & Francis Group , 120(6), pp. 776–780. doi: 10.1577/1548-8659(1991)120<0776:EOMDTO>2.3.CO;2.

Marshall, A. D. and Pierce, S. J. (2012) 'The use and abuse of photographic identification in sharks and rays', *Journal of Fish Biology*, 80(5), pp. 1361–1379. doi: 10.1111/j.1095-8649.2012.03244.x.

Marshall, T. C. *et al.* (1998) 'Statistical confidence for likelihood-based paternity inference in natural populations', *Molecular Ecology*. Blackwell Science Ltd, 7(5), pp. 639–655. doi: 10.1046/j.1365-294x.1998.00374.x.

van Oosterhout, C. *et al.* (2004) 'MICRO-CHECKER: Software for identifying and correcting genotyping errors in microsatellite data', *Molecular Ecology Notes*, 4(3), pp. 535–538. doi: 10.1111/j.1471-8286.2004.00684.x.

Pawley, M. D. M. *et al.* (2018) 'Examining the viability of dorsal fin pigmentation for individual identification of poorly-marked delphinids', *Scientific Reports*. Nature Publishing Group, 8(1), p. 12593. doi: 10.1038/s41598-018-30842-7.

Smith, M. *et al.* (2004) *Elasmobranch husbandry manual: Captive care of sharks, rays, and their relatives*, *The Elasmobranch Husbandry Manual : Captive Care of Sharks , Rays and their Relatives*.

Speed, C. W., Meekan, M. G. and Bradshaw, C. J. (2007) 'Spot the match – wildlife photo-identification using information theory', *Frontiers in Zoology*. BioMed Central, 4(1), p. 2. doi: 10.1186/1742-9994-4-2.

van Tienhoven, A. M. *et al.* (2007) 'A computer-aided program for pattern-matching of natural marks on the spotted raggedtooth shark Carcharias taurus', *Journal of Applied Ecology*. John Wiley & Sons, Ltd (10.1111), 44(2), pp. 273–280. doi: 10.1111/J.1365-2664.2006.01273.X.

Visconti, M. A. *et al.* (1999) 'Elasmobranch color change: A short review and novel data on hormone regulation', *Journal of Experimental Zoology*. John Wiley & Sons, Ltd, 284(5), pp. 485–491. doi: 10.1002/(SICI)1097-010X(19991001)284:5<485::AID-JEZ3>3.0.CO;2-5.

Wells, R. S. (2017) 'Identification Methods', in *Encyclopedia of Marine Mammals (Third Edition)*, pp. 503–509.

4.7.1 Electronic References

R Development Core Team (2008). R: A language and environment for statistical computing. R Foundation for Statistical Computing. Vienna, Austria.

Kalinowski, ST, Taper, ML & Marshall, TC (2007) Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. *Molecular Ecology* 16: 1099-1106. http://dx.doi.org/10.1111/j.1365-294x.2007.03089.x

SECTION III: SPECIES GENETICS

Chapter 5

The first investigation into wild undulate ray, *Raja undulata,* populations using DNA and non-intrusive capture mark-recapture analysis (pilot study)

S. A. Hook[1*], M. Openshaw[2], S. Openshaw[2], D. Ripley[4], A. Martin-Geary[4], M. Doggett[2, 3], J. D. Hibbitt[5], M. Buckley[1]

[1]School of Earth and Environmental Sciences, Manchester Institute of Biotechnology, University of Manchester, Manchester, M1 7DN, UK

[2] Stardis, Kingsclere, Hampshire, RG20 4SY, UK

[3]Seven Tenths Ecology Ltd, Salisbury, SP5 2BY, UK

[4]Faculty of Biology, Medicine and Health, University of Manchester, Core Technology Facility, Manchester, M13 9NT, UK

[5] SEA LIFE Programmes and Engagement, SEA LIFE Weymouth

*Corresponding Author: Samantha A. Hook, samhook1205@gmail.com

Author contributions

SAH[1] wrote the paper, collected the tissue samples, analysed the genetic and CMR results and developed the underwater technique, MO[2], SO[2] collected the capture mark-recapture data and underwater DNA samples, MD[2] collected underwater footage and assisted in CMR collection, DR[4] conducted the population estimates from CMR data, AM-G[1] completed the shark network analysis from the CMR, J-DH[5] assisted in UK sample collection, MB[1] supervised SAH[1]. All authors contributed to the review prior to submission.

5.1 Abstract

Effective methods of individual identification and genetic sampling are necessary to determine population densities and genetic health. As species decline, there is a greater need to develop non-intrusive methods to reduce the negative impacts of population assessment, such as destructive sampling whereby an individual is killed or disturbance from counting methods. The undulate ray, *Raja undulata,* is a globally endangered, but often locally-abundant species that has various protection levels throughout its range. However, there is a heavy reliance on fisheries data to understand population densities for the management of *R. undulata* despite being threatened with extinction. Here, we conduct the first genetic assessment of wild *R. undulata* using microsatellites marker analysis of 143 individuals from mucus and tissue sampling. To collect underwater mucus samples, we developed a successful non-intrusive method for resting *R. undulata* using scuba divers. Secondly, between 2012 and 2018, we conducted a capture mark-recapture study on a single sample site on the coast of Dorset, UK, by scuba divers taking images of the dorsal pattern of each undulate ray. Images were then stored according to the sampling time point (date) and processed through Wild-ID which uses a probability analysis to match each image between the sampling time points. We successfully identify 263 individuals which had been photographed equal to or more than once. The genetic results exhibited an overall high average genetic diversity (H_o = 0.66, H_e = 0.85, average alleles per locus = 19.8). Capture mark-recapture analysis demonstrated the highest number of *R. undulata* were present in autumn, and that the average estimated population size was 228 (maximum standard error ± 87). Despite a low probability of recapture (p = 0.035), the individual survivorship rate between visits was high (φ = 0.969). Finally, we use network analysis to investigate the social behaviour of *R. undulata*, demonstrating that distinct pairs of rays are present at a higher frequency than is expected by chance (95% confidence interval P ≤ 0.02), indicating a high probability of same pair migration and social interaction.

Keywords: elasmobranchs, Photo-recognition, microsatellites, Skates, *Raja undulata*, Genetic Health

5.2 Introduction

Overfishing is the main cause of the decline of shark, skate and ray (elasmobranchs) populations around the globe (Worm *et al.*, 2013; Dulvy *et al.*, 2014). However, because of the difficulties surrounding the direct observation of individuals within the marine environment, fishing data also form the main source of the fish stock assessments that influence conservation management (Beddington, Agnew and Clark, 2007). Monitoring the change in capture rates is the simplest and most common method of estimating biological population size, while sample collection from fisheries of either tissue or whole specimens has assisted in species identification and population genetics (Larson, Daly-Engel and Phillips, 2017). As of 2014, a quarter of all elasmobranchs were classified as threatened with extinction under the IUCN Red List (Dulvy *et al.*, 2014; IUCN, 2014). Thus, there is an urgent need to develop and implement better methods of population assessment.

The undulate ray, *Raja undulata,* is a globally endangered species of skate with a fragmented distribution, likely due to the available niche habitat and locations of overfishing. The species is found in the North-east Atlantic to the equator and in the Mediterranean Sea (Coelho *et al.*, 2009). In 2009, the EU enforced laws that prohibited landings in the North-east Atlantic, and placed restrictions on landings in the Mideast-Atlantic and Mediterranean (CEC, 2010). In North Africa however, high levels of illegal, unregulated and unreported (IUU) (CoC, 2015) fishing means there is little management enforced for this species. The landing restrictions within the North-east Atlantic were a debated topic due to claims that the fisheries were catching large quantities (Ellis, McCully and Brown, 2012). Independent trawler surveys were conducted to provide evidence for abundance and the results contributed towards a regional delisting of the species to Near Threatened and a gradual increase in quota sizes (Ellis, McCully and Wallis, 2015). However, despite these stock assessments relatively little is known about the movements and the connectivity of *R. undulata* between sites (Ellis, McCully and Brown, 2012).

More recently, studies have employed tagging to investigate population abundance through capture mark-recapture (CMR) (Feldheim *et al.*, 2002; Hunter *et al.*, 2005; Guttridge *et al.*, 2010). Capture mark-recapture uses tagging to identify individuals, allows record of

the presence and absence of those tagged, and movements between study areas (McCrea and Morgan, 2014). Analysis of CMR data can provide population estimates, and population viability and survivorship probabilities (McCrea and Morgan, 2014), without the need to destroy individuals. Furthermore, due to developments in recognition software (Speed, Meekan and Bradshaw, 2007), non-intrusive methods such as photographs can now be used to confidently identify individuals. Large image databases of specific regions of the fish that hold unique markings, such as patterns or marks, pigmentation, or long-standing scars, can be used to identify individuals over long periods of time. Examples include images of the dorsal fin in white sharks *Carcharodon carcharias* (Andreotti *et al.*, 2018), spot patterns on the dorsal side of the spotted eagle ray *Aetobatus narinari* (González-Ramos *et al.*, 2017) and the ventral side's natural pigmentation and spots on manta rays *Manta alfredi* and *Manta birostris* (Marshall and Pierce, 2012; Ari, 2015). Recognition software overcomes inherent sources of bias associated with invasive tagging methods such as mortality from tag application, non-reported or non-recovered tags, and tag shedding (Kohler and Turner, 2001). However, when recognition software databases reach a certain number of images, they can become over saturated and create less probable matches.

Genetic techniques have previously been used to validate the use of recognition software in elasmobranch species (Andreotti *et al.*, 2016). Furthermore, genetic sampling is a powerful tool for understanding species dynamics, separate to or alongside, CMR data. However, the collection of DNA no longer depends on invasive methods such as muscle or blood sampling, which often increase the risk of mortality or rely on the collection of fished individuals. The use of mucus sampling to collect DNA is a recently established non-intrusive technique that has proven a viable alternative for *in-situ* populations of the basking shark *Cetorhinus maximus* (Lieber *et al.*, 2013) and manta ray *Manta birostris* (Kashiwagi *et al.*, 2015), and *ex-situ* captive populations of *R. undulata* (Fox *et al.*, 2018).

Here we use 17 microsatellite markers, previously developed by Hunter *et al.* (2016) and Fox *et al.* (2018), to investigate the genetic relationships of *R. undulata* individuals obtained from the 12 sample sites across their global distribution. We analyse genetic diversity and review differences between previously prohibited fishing regions (England,

North-east Atlantic), areas with restricted quotas (mainland Europe, Mideast-Atlantic) and areas with no known quotas/high levels of IUU fishing activity (Morocco, North Africa). Secondly, with the use of CMR on a known site in England, we compare population estimates with genetic effective population size measured as effective number of breeders. Finally, we review the probability of recapture, survivorship and relationships between individuals within the single sample site of *R. undulata*, which, for most of this study, has been a protected species in the UK along its range including in and out of marine protected areas (STCEF, 2015; EU Policy EUR 27154 EN).

5.3 Methods

Global genetic analysis

5.3.1 DNA sampling

Tissue samples of *R. undulata* were collected as a by-product from fish markets in Portugal, Spain, and Morocco between 2015 and 2018 (Figure 5.1). Samples were stored in RNA*later*® at -4˚C before being transferred to -80°C at the Manchester Institute of Biotechnology.

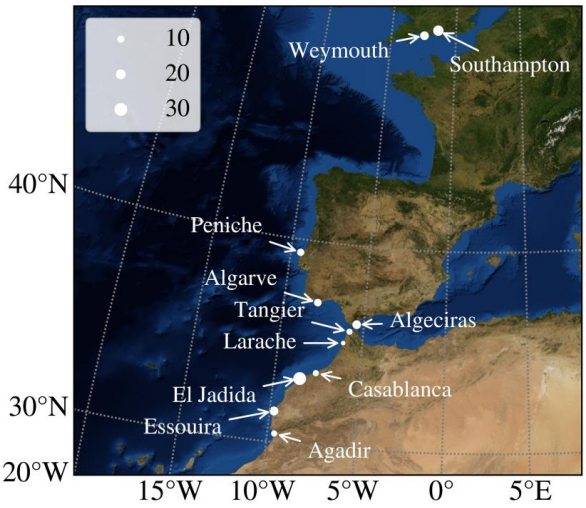

Figure 5.1. A map displaying the locations and total number of either tissue or mucus samples per site created using Python (v 2.7.16, van Rossum, 1995) from the base map library. Note, Rays' Repose was not displayed due to its close location between Weymouth and Southampton.

Non-intrusive underwater mucus swabs from *R. undulata* were collected by a group of volunteer recreational British Sub Aqua Club (BSAC) accredited scuba divers between April 2017 and October 2018, on a site within the Studland to Portland Special Area of Conservation, Dorset, England. The site, hereafter referred to as Rays' Repose, is approximately 50 metres wide and over 200 metres from north to south, forming part of the Kimmeridge Ledges (50° 35.5' N 2° 7.5' W); a series of shallow flat ledges reaching out to sea for up to a mile. Qualified divers followed Supplementary Methods 1. to collect samples and take photographs for identification. Swab samples were taken using an autoclaved heavy-duty green scrubber (Robert Scott Ltd. Code 102450) attached to a 10 cm handle (Supplementary Methods 1.). The scrubber was gently pressed on the upper dorsal of the nearest wing and moved towards the tail end between one and three times, before placing the sample into a marked zip-locked bag (Supplementary Methods 1.). A photograph was then taken off the marked zip-lock bag to correspond to the identification image. After the dive, the green scrubber with the mucus was removed from the handle, placed into a 50 ml tube containing 40 ml of 98% ethanol and transferred onto ice. A further set of non-intrusive mucus samples were collected from recreational sea anglers in both Southampton (27 miles due east) and Weymouth (15 miles due west) in 2017. Between June and October 2017 we used the same swabbing method on non-targeted, accidental by-catch *R. undulata*, which were collected, sampled, and returned following Brownscombe *et al.,* (2017) "best angling practices guide". For each individual we collected three mucus swabs to increase the probability of successful DNA analysis. Once the samples were in a controlled environment, they were kept at -20°C before returning to the lab where they were stored at -80°C.

5.3.2 DNA extraction, amplification and genotyping

The Bioline ISOLATE II Genomic DNA Kit was used to extract DNA from the tissue samples, following the manufacturer's protocol. DNA was extracted from the swabs using an adapted method with an E.Z.N.A Mollusc DNA Kit (Omega Bio-Tek, Norcross, USA). In addition to the manufacturer protocol, we added a two-stage digestion to obtain the maximum amount of mucus from each sample. Firstly, the 0.5 cm^3 of the scrubber with the

most visible mucus was selected, together with the top layer of the remaining scrubber with any further visible mucus. The sections were added to a 1.5 ml tube and left for five minutes in a fume hood to evaporate the remaining ethanol. Secondly, the ethanol-fixed mucus that had fallen from the scrubber within the 50 ml falcon tube was centrifuged at 5000 rpm at 4˚C for 1 hour to create a mucus pellet. The ethanol was gently poured off the mucus pellet and mucus pellet was then left for five minutes in a fume hood to evaporate the residual ethanol. A total of 350 µl of ML1 lysis buffer was added to the falcon tube and vortexed for 15 seconds to re-suspend the mucus pellet into the buffer. This solution was then pipetted into the corresponding 1.5 ml tube containing the cut scrubber and visible mucus, and 25 µl of proteinase K was then added to the tube and digested at 60˚C for five hours, or 37°C overnight, to digest contaminating proteins. Once digested, the DNA was extracted following the original E.Z.N.A Mollusc DNA Kit protocol but with a single elution extended to 10 minutes at 70˚C to maximize yield. DNA extractions were quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Carlsbad, USA) to ensure yields were ≥10 ng/µl. Samples were stored at -20˚C.

A total of 17 *R. undulata* species-specific microsatellite primers were used from Hunter *et al*. (2016) and Fox *et al.* (2018) (Supplementary Table 5.1). Two different universal tails were added to the primers and Polymerase Chain Reactions (PCRs) were conducted under a three primer approach (6-FAM or HEX; Blacket *et al*., 2012) to create five multiplexes (Supplementary Table 5.1). Reaction volumes (5 µl) consisted of 0.5 µl multiplex primer mix, 1.5 µl Type-it® microsatellite master mix and 2.5 µl double-distilled H_2O added to 0.5 µl of the 10-70 ng/µl genomic DNA used for amplification. Thermal cycler conditions were as follows: initial denaturation at 5 minutes at 95˚C, 35 cycles of 30 seconds at 94˚C, 90 seconds of annealing at 60˚C and 30 seconds of extension at 72˚C, followed by 1 cycle of 30 minutes at 60˚C (Fox *et al.*, 2018). PCR products were visualized on a 1.5% agarose gel (using a Gel Green nucleic acid stain) under a UV light source to confirm successful amplification. Following successful amplification, the products were genotyped using an ABI™ 3730XL capillary sequencer at the University of Manchester DNA Sequencing Facility with GeneScan™ 500 LIZ™ dye size standard and scored using GeneMapper v.4.0 (Applied

Biosystems). Allele scores and null alleles were analysed in in Microchecker v.2.2.3 (van Oosterhout *et al.*, 2004) and Microsatellite Toolkit (97-2003) (Park, 2001).

5.3.3 Genotype statistical analysis: genetic health and structure

We investigated observed (H_o) and expected heterozygosity (H_e) as a measure of genetic diversity for each of the loci, using GenePop on the Web v4.2 (Raymond and Rousset, 1995). To identify sample location diversity levels we estimated average H_o, $H_{e,}$ number of alleles per locus, the and the number of unique alleles (private alleles) for each sampled individual using the allele frequencies estimated in GenePop on the Web, and confirmed this with Cervus v3.0.7 (Marshall *et al.*, 1998). To estimate the number of genetic populations (K) we ran STRUCTURE's (Pritchard, Wen and Falush, 2009) systematic Bayesian clustering approach, that applies Markov Chain Monte Carlo (MCMC) estimation at 10,000 repetitions to model the possible number of clusters (K = 1 to 15) at fifteen iterations (15 being the maximum number of sites sampled). This data was ran through STRUCTURE HARVESTER software (Earl and VonHoldt, 2012) which uses the Evanno method (Evanno, Regnaut and Goudet, 2005) to calculate ΔK, and CLUMPP (Jakobsson and Rosenberg, 2007) as a more accurate predictor of the cluster number. The Evanno method uses the rate of change in the log probability of the data provided from STRUCTURE to account for non-homogeneous dispersal among populations (ΔK) (Evanno, Regnaut and Goudet, 2005). CLUMPP v.1.1.2 defines the number of K by implementing three algorithms against the STRUCTURE v.2.3.4 analysis to align clusters via a membership coefficient (Jakobsson and Rosenberg, 2007).

Lastly, we calculated the effective number of breeders (N_{eb}) under the molecular co-ancestry method (Nomura, 2008) using software NeEstimator v2.1 (Do *et al.*, 2014). This method provides unbiased estimates of N_{eb} without the need for demographic information, such as age. The molecular co-ancestry method also overcomes issues found in previous methods which may not be suitable to study natural populations of endangered species, such as low number of individuals sampled (≤50) (Nomura, 2008). We analysed each sample site separately before grouping locations in the North Atlantic (Figure 5.1) to gain a

better understanding of N_{eb} in this region, which is likely linked with the single site capture mark-recapture site Rays' Repose.

Single Site Capture Mark-Recapture (CMR)

5.3.4 Dataset collection

Between April 2012 and October 2018, photographs were collected from resting *R. undulata* on Rays' Repose by the same group of scuba divers who conducted the genetic sampling. Each dive consisted of up to 3 experienced scuba divers with an average dive time of 51 ± 16.5 minutes and maximum dive depth of 17.5 ± 1.1 metres (tide dependent) (Supplementary Methods 1.).

Diving was conducted during daylight hours when the rays were found on the seabed in a resting position, consistent with ray behaviour described by Humphries et al. (2017) for other UK skate species (Humphries, Simpson and Sims, 2017). The number of individuals photographed on any dive was limited by the dive conditions (such as visibility, tides and currents), individual diver constraints and may have been influenced by individual rays becoming 'trap-shy' (avoidance of the divers). Full protocols were made to minimise ray disturbance, increasing the probability of retrieving dorsal pattern photographs (Supplementary Methods 1). Photographs of the dorsal side of the fish were taken in .jpg and RAW format with various compact and single-lens reflex (SLR) cameras. In total, 144 dives were completed on the site, collecting CMR data for 263 individuals.

5.3.5 Computer-assisted photo-ID

Each photograph had the colour removed to reduce background noise, and was converted into a standard orientation and on-screen size (20-cm x 20-cm) using Adobe® Photoshop (Figure 5.2). To assist the computer recognition program, areas of surrounding seabed were cropped from the image to leave only the ray's dorsal surface (Figure 5.2). Each formatted photograph was entered into Wild-ID v.0.9.28.17 (Bolger *et al.*, 2012). Wild-ID compares each new image and provides a numerical matching coefficient for the 20 most likely existing photos already in the dataset (Bolger *et al.*, 2012). Where the dorsal pattern was clear, the software identified images of the same ray and clearly discriminated from

other rays with a higher numerical matching coefficient. However, where the dorsal pattern was obscured, matching images were discriminated less clearly from images of the other rays, hence, the final decision for a true match was made manually by the same two users from the 20 most likely candidates identified by the software. On first capture each individual received a unique sighting number of which all future recaptures would then be associated to. The results from Wild-ID were cross-examined with a second photo-recognition software, I^3S Pattern, revealing the same exact matches between images, and thus validating the use of Wild-ID (Speed, Meekan and Bradshaw, 2007).

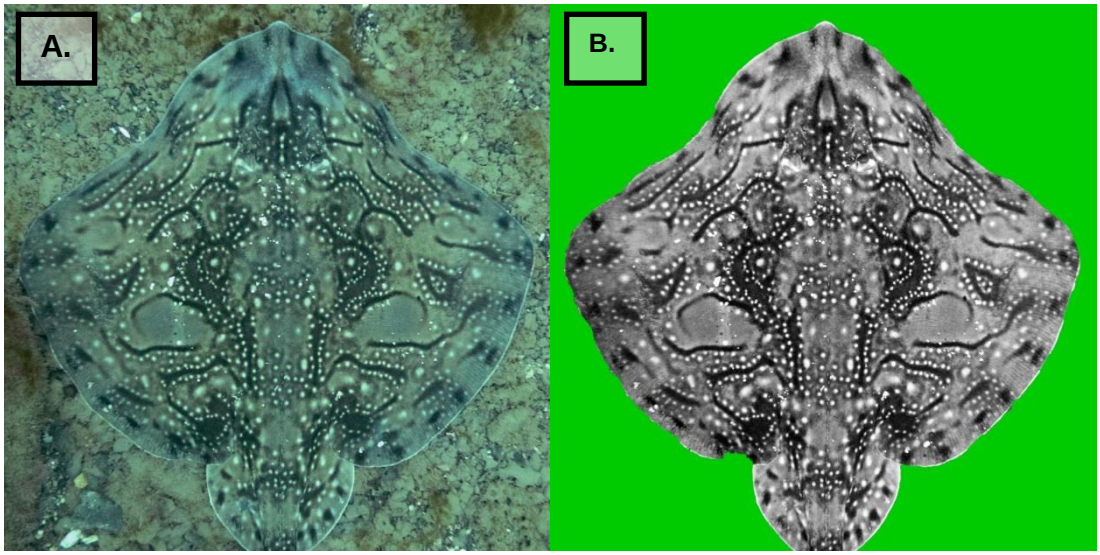

Figure 5.2. (A) The first cropped image of the dorsal pattern with the original substrate, orientated with the nose at the top of the image, and the tail at the bottom of the image. (B) Image A edited without colour and removal of the surrounding substrate. Both images are of the same individual ray ID 831, named 'Watson'.

5.3.6 Statistical analysis

In 2017, one individual from Rays' Repose was photographed by a recreational diver at Chesil Beach, Portland, Dorset (Ray ID 598), approximately 55 km, west of Rays' Repose (Openshaw and Openshaw, 2018). This indicates that the population is open, and therefore we conducted the analysis under this assumption. We used RStudio v.1.0.143 (RStudio Team, 2016) to investigate whether the seasons had an influencing factor on the number of rays captured using one-way ANOVA test of equal variances from a Welch *F*-test of unequal variances. Secondly, we created loglinear models to estimate population abundance between years in the R-package Rcapture (Baillargeon and Rivest, 2007).

To investigate an individual's probability of survival (φ) and recapture (*p*) we used a Cormack-Jolly-Seber model (CJS) in the R-package "marked" (Laake, Johnson and Conn, 2013; RStudio Team, 2016). Duration between site visits varied in length due to weather restrictions, tide, and other time constraints. We therefore were interested in the φ with the variation of time between site visits as a parameter. We also used individual sex as a parameter to determine whether there was a bias on φ and *p* estimates in separate models.

5.3.7 Network analysis

Sampling visits that occurred within a five day period were clustered together to address a potential low probability of recapture, where individuals could be present but may not be observed. The total number of clustered mark-recapture visits was 40 (original number of visits = 73). Using the clustered mark-recapture data we investigated whether the co-occurrence of observed paired individuals appearing on the site exceeded the modelled prediction which could be expected to occur at random. To model our predicted data we used the EcoSimR package v6.0 (Gotelli *et al.*, 2015) in RStudio v.1.0.143 (RStudio Team, 2016), which uses the curveball algorithm (Strona *et al.*, 2014) of matrix shuffling to generate 'random' matrices, based on the observed data, whilst maintaining row and column totals (in our case individual and time point respectively). In using the curveball algorithm rather than the more traditional sequential swap, transient effect biases are minimised and therefore the resulting matrices have demonstrably greater reliability (Strona *et al.*, 2014). We ran the EcoSimR algorithm 5 times, with 10,000 iterations each.

5.4 Results

5.4.1 Global genetic results

Genotypes for a total of 143 individuals from the 12 sites were generated using the 17 loci developed by Hunter *et al.* (2016) and Fox *et al.* (2018). Total average genetic diversity measures per locus ranged from H_o = 0.66, H_e = 0.85, and mean number of alleles per locus = 19.8 (Supplementary Table 5.1). Further to this, we investigated mean H_o, mean H_e, mean polymorphic information content (PIC), and mean number of private alleles (P_a) for each sample site (Table 5.1). We found the largest number of private alleles to be at El

Jadida (Morocco, *Pa* = 37), whilst the lowest number was found at Larache and Casablanca (Morocco, *Pa* = 0). Overall the number of private alleles for each country was: England, *Pa* = 34; Portugal, *Pa* = 19; Spain, *Pa* = 17, Morocco, *Pa* = 47.

Table 5.1. Genetic diversity levels for the samples taken at each site, measured as average observed and expected heterozygosity (Ho and He), Polymorphic Information Content (PIC), number of private alleles (Pa) and estimated effective number of breeders (Neb) and Neb at 95% confidence interval (CINeb)

Sample Site	N	H_o	H_e	PIC	P_a	N_{eb}	CIN_{eb}
Rays' Repose	16	0.141	0.296	0.242	12	-	-
Southampton	20	0.545	0.601	0.551	7	-	-
Weymouth	13	0.553	0.623	0.551	5	-	-
Peniche	8	0.539	0.688	0.597	9	-	-
Algarve	9	0.667	0.791	0.704	10	28.9 ± 80.4	80.4
Algeciras	13	0.733	0.798	0.651	17	12.5 ± 21.1	21.1
Tangier	5	0.515	0.606	0.489	1	2585.8 ± 12980.8	12980.8
Larache	2	0.441	0.461	0.294	0	-	-
Casablanca	5	0.549	0.617	0.504	0	133.2 ± 668.5	668.5
El Jadida	33	0.678	0.848	0.788	37	21.8 ± 46.1	46.1
Essaouira	14	0.706	0.808	0.721	7	-	-
Agadir	5	0.500	0.564	0.284	2	44.6 ± 224	224.0

5.4.2 Population Structure

Through the use of STRUCTURE (Pritchard, Wen and Falush, 2009) and STRUCTURE HARVESTER (Earl and VonHoldt, 2012) we found that there were six genetically distinct populations (*K*) within the sample set (Figure 5.3), and that there is a higher level of connectivity between sample locations that are geographically closer together (Figure 5.3). From the STRUCTURE results, the Evanno method and the CLUMPP analysis confirmed a *K* of 6 (*K* = 6, ΔK = 4.03, iterations = 15, Figure 5.3). Information for each loci, including null alleles, allelic richness, number of alleles per locus, observed and expected heterozygosity can be found in Supplementary Table 5.1.

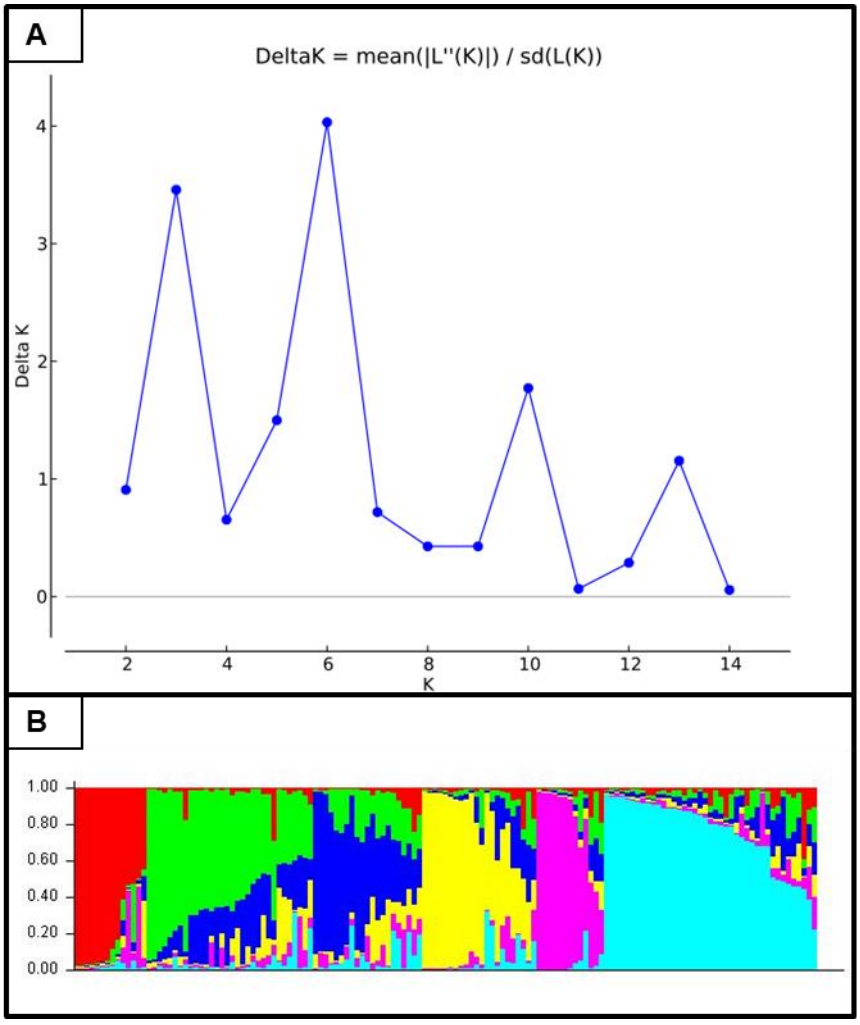

Figure 5.3. (A) The Delta K (ΔK) results from the Structure HARVESTER with use of the Evanno method (K = 6) (B) A STRUCTURE Q-Plot of the population assignment K= 6 calculated in STRUCTURE and Structure HARVESTER with use of the Evanno method and CLUMPP. STRUCTURE was ran x 10,000 Burn-in period with 10,000 x Reps.

5.4.3 Molecular co-ancestry effective number of breeders (N_{eb})

When combining the North Atlantic samples to near reach the optimal number of samples for genetic analysis (N = 49) and using the molecular co-ancestry method as defined by Nomura (2008), the estimated number of breeders as a measure of population size was unknown because the data were not sufficiently informative at a 95% confidence level. This was the case for 50% of the sample sites (Table 5.1).

Single Site Capture Mark-Recapture (CMR) Results

5.4.4 Single site capture mark-recapture

In total, we identified 263 individuals that were present on the Ray's Repose site between spring of 2012 and autumn of 2018. Of the 263 individuals, six previously

unmarked individuals were photographed on the last visit and therefore were removed from the CJS CMR model. Of the total 263 individuals, 82 were males, 173 were females, seven were juveniles and one was unknown, although these figures may be inaccurate being based only on an underwater visual assessment of maturity and gender.

5.4.5 Population distribution and estimates

The number of rays that were found varied across the seasons, dependent on the year (Figure 5.4). Overall, the highest number of rays was found in autumn and the lowest number in spring (one-way ANOVA test of equal variance p = 0.122, Welch *F*-test of unequal variances p = 0.050); months for each season were taken from the UK set dates. From the average number of individuals observed, more rays were present in summer than in any other month; average number of individuals per season was calculated from the number of rays per site visit. Population estimates for Rays' Repose fluctuated between 150 and 400 (maximum standard error +/- 125; Figure 5.4) and average estimate for the site was 228 (maximum standard error +/- 87).

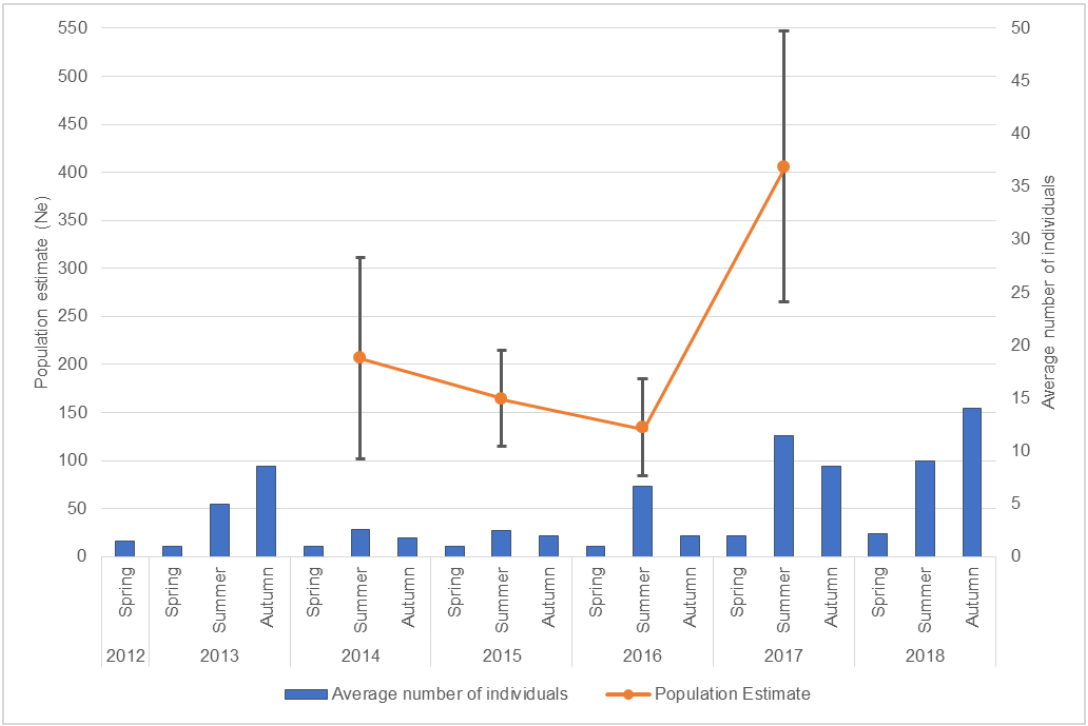

Figure 5.4. Average seasonal occurrence and population estimates of *R. undulata* at Rays' Repose between spring 2012 and autumn 2018.

5.4.6 Survival and capture probabilities

Using the CJS models under the assumption that time between visits is a variable parameter for φ and *p* we found that the overall estimate of probability of survival φ = 0.969 (standard error (SE) = 0.0054, 95% upper and lower confidence levels = 0.956 to 0.978) and capture probability *p* = 0.035, (SE = 0.004, 95% upper and lower confidence levels = 0.028 to 0.043).

5.4.7 Network analysis

When applying the curveball algorithm matrix reshuffling, the observed data exceeded both the one, and two-tailed 95% confidence intervals (p = ≤ 0.02), indicating that the observed co-occurrence of undulata rays on Rays' Repose is not random. Furthermore, we can see from the network analysis (Figure 5.5) that pair types are likely to either be female to female (19 pairs) or female to male (15 pairs) rather than male to male (6 pairs).Using probability tests we found no statistical significance between the pairing type, the possible number of pairings (NP) and the actual number of pairs (AP); female to female, NP = 300, AP = 21; female to male NP = 350, AP = 18; male to male, NP = 91, AP =3.

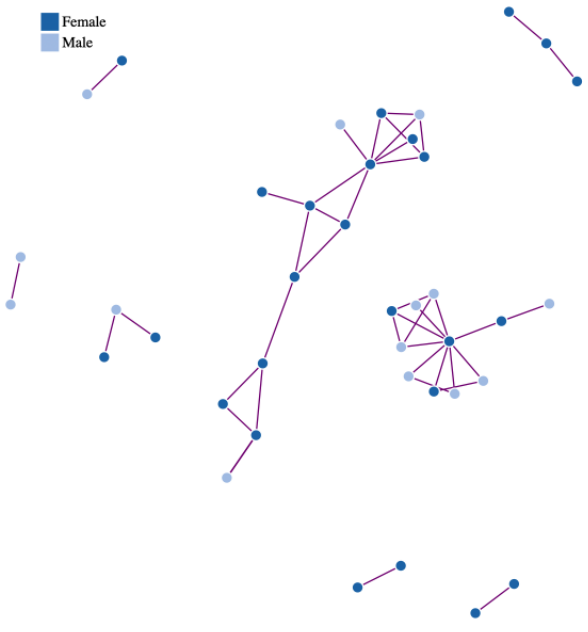

Figure 5.5. A network analysis showing calculated the networks of pairs which occurred in 2 or 3 clusters together, (EcoSimR package v6.0 (Gotelli et al., 2015), RStudio v.1.0.143 (RStudio Team, 2016), the curveball algorithm (Strona et al., 2014)). Number of retained individuals = 39, Total number of pairs = 42. (Interactive HTML available in thesis CD). Each dot is one of 39 individuals, pairs are indicated through connected branches, identified sex of each individual is represented in colours (female = dark blue, male = light blue).

5.5 Discussion

Here, we developed a successful method for non-intrusive, underwater mucus sampling to extract DNA and examine the genetic diversity of *R. undulata*. This is the first study to combine mucus samples (collected by scuba divers and recreational sea anglers) and tissue samples (collected from fish markets) to review the global genetic population structure for any elasmobranch. Lastly, this is the first 7 year capture mark-recapture (CMR) study conducted on any skate or ray (batoid) population, without the use of an invasive tagging method.

Prior to this research, similar mucus sampling techniques have been successfully used on other neotropical batoids (Lieber *et al.*, 2013; Kashiwagi *et al.*, 2015; Domingues *et al.*, 2019) and with captive individuals (Hunter, 2016). With the exception of Kashiwagi *et al.* (2015), these studies have required invasive capture methods where the individual is removed from the water to conduct non-intrusive sampling via mucus swabs (usually by fishing). Elasmobranch mortality post-capture can range depending on fishing practice such as gear type, location, fishing depth, species, and onboard conditions (Ellis, McCully Phillips and Poisson, 2017). In our sample collection, *R. undulata* were non-targeted, accidental by-catch from recreational sea anglers using rod and line, whereby mucus swabs were non-intrusively taken prior to release. Sea anglers followed the 'best angling practices guide' (Brownscombe, Chapman and Gutowsky, 2017) to increase probability of survivorship post-release. Divers used a site-specific protocol adapted from The Underwater Photographers Code of Conduct (The British Society of Underwater Photographers, no date) to safely capture images and take mucus samples with little intrusion to the rays' natural behaviour and having no known effect on mortality (Supplementary Methods 5.1). To our knowledge, this is the first study to employ mucus sampling method on resting batoids, collected by scuba divers.

We found that, despite *R. undulata* being globally endangered (Coelho *et al.*, 2009), average genetic diversity was overall high (H_o = 0.66, H_e = 0.85, average alleles per locus = 19.8). This is similar to other elasmobranch species threatened with extinction such as the small sawtooth *Pristis pectinate* (H_o = 0.85, H_e = 0.84; Chapman *et al.*, 2011), the scalloped

hammerhead *Sphyrna lewini* (H_o = 0.67, H_e = 0.72; Nance *et al.*, 2009; Green *et al.*, 2017) and the longheaded eagle ray *Aetobatus flagellum* (H_o = 0.47, H_e = 0.48; Yagishita and Yamaguchi, 2009). As elasmobranchs have long life-history traits, such as longevity, low reproductive output and late maturity, it may be that we do not observe a decrease in genetic diversity for many generations, as life spans of animals are often longer than the time span of data available. Furthermore, with the exclusion of Rays Repose', we observed little differences between populations that previously had full protection in the north-east Atlantic, and various fished populations in the mid-Atlantic. STRUCTURE defined six genetically unique clusters. Rays' Repose individuals fell into numerous clusters; however the majority of individuals were clustered within cluster 5, which also included the majority of Southampton individuals. However, this may be due to a reduced amplification rate from the underwater mucus swabs when compared to the sea angler mucus swabs. Structurally, there is almost a near north to south divide, with the majority of Morocco samples falling in clusters 1 to 3 and the majority of northern sample sites falling into clusters 4 to 6 (Figure 5.3). The fact that nearly all sample sites have individuals which fall into every cluster identified by STRUCTURE indicates a level of shared genotypes and therefore possible geneflow between regions.

The effective number of breeders could not be determined by the molecular co-ancestry method (Nomura, 2008) for 50% of sample sites because it results in an infinite (∞) estimated N_{eb} (including confidence intervals). For all sites where samples were collected by non-intrusive methods, N_{eb} was unknown because the data is not informative enough. For the tissue (invasive) samples this was the same for only three out of the nine sites and could be the product of a lack of population structure. It is interesting to note that N_{eb} could only be calculated in the more tropical regions around the Strait of Gibraltar (south Portugal and Spain) into the mid-Atlantic (Morocco). The largest estimate, with the exception of ∞, was at Tangier, Morocco (N_{eb} = 2585.8) whilst the smallest number was in Algeciras, Spain (N_{eb} = 12.5), despite the close geographical proximity between these two regions.

Despite the success of using the unique dorsal patterns for individual recognition, certain caveats should be acknowledged. The challenge of photographing rays in their wild

environment means that photographs are often lesser quality for identification purposes than what could be achieved in a controlled environment. The computer recognition process is dependent on the quality of the photographs and therefore false negatives may exist. To overcome this, we used a manual matching process to validate the identifications made within Wild.ID, which can be time-consuming. We also confirmed Wild-ID with I^3S, indicating both software were able to match individuals. As photographic equipment and recognition software improves over time, we can predict that error rates will decrease.

The frequency at which individuals are caught in CMR studies can depend on the methodology, and may either influence an over- or underestimate of the population size. For example fishing for individuals may cause them to become 'trap shy', while baiting vessels to attract individuals may cause them to become 'trap happy' (attracted to the method of CMR) (Towner *et al.*, 2013). With the exception of 2017 and 2018, individuals on Rays' Repose were only photographed, causing minimal to no disruption while rays were resting. As the *R. undulata* did not receive a known benefit from the divers, it is unlikely that they would become trap-happy; however, any minimal disruption from the divers may have encouraged individuals to move off the site (trap-shy). As divers visit on relatively few occasions (on average ≤5.6% of the year), if individuals became trap-shy, they would likely return to the site once divers had left, causing minimal disruption to their natural behaviour. This along with sampling effort, tidal conditions, length of dive and underwater visibility could be contributing factors on why the probability of recapture is low (p = 0.035). It could be argued that the level of camouflage *R. undulata* have against the seabed at Rays' Repose may have also contributed to the low probability of recapture. This camouflage is likely the reason we observed them resting for such long periods of time, and a contributing factor for such a successful survivorship (φ = 0.969). A niche habitat coupled with high levels of protection and strict landing quotas since 2009 (ICES, 2016), could have contributed to the consistent population sizes we estimated. The longest period between first and last sighting of the same ray was 2,186 days, approximately six years, highlighting the importance of continuing studies and specific sites to individual fish.

Lastly, from our CMR data collected at Rays' Repose and the network analysis performed on the data, we found the first evidence that there are possible social interactions between individual *R. undulata*. We can predict that these social interactions may influence their migratory patterns when appearing on the site, as they are non-random pairings. The observed patterns of co-occurrence of individuals highlight possible levels of social behaviour not previously explored in *R. undulata*. As the site appears to be used only for resting (defined by prolonged time of stationary activity), it can be questioned whether the networks are moving off-site together to conduct the same natural behaviours, such as feeding or mating. As the networks appear to be primarily female pairings, or female to male pairings, it can be further questioned as to whether there is an active avoidance between males. However further data and research would be required to test true significance between these pairings. Furthermore, it would be interesting to investigate movement and behaviours when individuals are on a different site as we only found evidence of resting on Rays' Repose. The overall implications of the networks signify that there are possibly more complex social behaviours than what is currently reported.

The knowledge of the site, the length of study, and the presence of rays has made this unique in its field. To replicate the CMR study elsewhere, similar conditions would have to be met. With the correct diver experience and knowledge of batoid resting sites, non-intrusive methods of CMR compete with traditional invasive tagging methods as a cheaper and more accessible tool for site-specific population assessments, assisting in conservation management across a range of species. Although the Ray's Repose site is already situated in a protected European Marine Site, the Studland to Portland Special Areas of Conservation, undulate rays are not a qualifying designated feature for that site and therefore receive no specific protection. Due to the site's topography, Rays' Repose will not likely be subjected to any bottom-destructive fishing, such as trawling (offering a level of protection) as the fishing gear would be damaged. Despite this, set net fishers do operate in the area, targeting rays and flatfish amongst others; recreational anglers also fish for rays along the coastline. At the present time, continued observation could be the best management for this population before other means such as voluntary codes of conduct or local fisheries by-laws such as bag limits or closed areas need to be considered. The

application of these developed sampling techniques to other areas or taxa will increase our knowledge and understanding of elasmobranch populations and behaviour more globally.

5.6 Acknowledgements

The authors would like to thank Jennifer Rowntree, Richard Preziozi and John Fitzpatrick for their early encouragement towards undulate ray genetics. We would also like to thank Simon Ruske and Merlin Entertainments Sea Life Aquariums for their advice and support. Lastly, we would like to thank the recreational anglers for their samples.

5.7 References

Andreotti, S. *et al.* (2016) 'An integrated mark-recapture and genetic approach to estimate the population size of white sharks in South Africa', *Marine Ecology Progress Series*, 552, pp. 241–253. doi: 10.3354/meps11744.

Andreotti, S. *et al.* (2018) 'Semi-automated software for dorsal fin photographic identification of marine species: application to *Carcharodon carcharias*', *Marine Biodiversity*. Springer Berlin Heidelberg, 48(3), pp. 1655–1660. doi: 10.1007/s12526-017-0634-2.

Ari, C. (2015) 'Long-term body pigmentation changes on a manta ray (Mobulidae)', *Biological Journal of the Linnean Society*. Oxford University Press, 114(2), pp. 406–414. doi: 10.1111/bij.12416.

Baillargeon, S. and Rivest, L.-P. (2007) *Rcapture: Loglinear Models for Capture-Recapture in R*.

Beddington, J. R., Agnew, D. J. and Clark, C. W. (2007) 'Current problems in the management of marine fisheries.', *Science (New York, N.Y.)*. American Association for the Advancement of Science, 316(5832), pp. 1713–6. doi: 10.1126/science.1137362.

Blacket, M.J., Robin, C., Good, R.T., Lee, S.F. and Miller, A.D., 2012. Universal primers for fluorescent labelling of PCR fragments—an efficient and cost-effective approach to genotyping by fluorescence. *Molecular ecology resources*, *12*(3), pp.456-463.

Bolger, D. T. *et al.* (2012) 'A computer-assisted system for photographic mark-recapture analysis', *Methods in Ecology and Evolution*. John Wiley & Sons, Ltd (10.1111), 3(5), pp. 813–822. doi: 10.1111/j.2041-210X.2012.00212.x.

Brownscombe, J. W., Chapman, J. M. and Gutowsky, L. F. G. (2017) 'Best practices for catch-and-release recreational fisheries – angling tools and tactics', *Fisheries Research*. Elsevier, 186, pp. 693–705. doi: 10.1016/J.FISHRES.2016.04.018.

CEC (2010) 'CEC (2010). Council Regulation (EU) No 23/2010 of 14 January 2010 fixing for 2010 the fishing opportunities for certain fish stocks and groups of fish stocks, applicable in EU waters and, for EU vessels, in waters where catch limitations are required', *Official Journal of the European Communities*, L21, pp. 1–20.

Chapman, D. D. *et al.* (2011) 'Genetic Diversity Despite Population Collapse in a Critically Endangered Marine Fish: The Smalltooth Sawfish (*Pristis pectinata*)', *Journal of Heredity*. Oxford University Press, 102(6), pp. 643–652. doi: 10.1093/jhered/esr098.

CoC (2015) *Compliance Committee: Working group on illegal, unreported and unregulated (IUU) fishing in the GFCM area*. Marrakech, Morocco.

Coelho, R. *et al.* (2009) *Raja undulata*, *The IUCN Red List of Threatened Species 2009: e.T161425A5420694*. IUCN Global Species Programme Red List Unit.

Do, C. *et al.* (2014) 'NeEstimator v2: re-implementation of software for the estimation of contemporary effective population size (N_e) from genetic data', *Molecular Ecology Resources*. John Wiley & Sons, Ltd (10.1111), 14(1), pp. 209–214. doi: 10.1111/1755-0998.12157.

Domingues, R. R. *et al.* (2019) 'Use of mucus as a non-invasive sampling method for DNA barcoding of stingrays and skates (batoid elasmobranchs)', *Journal of Fish Biology*, 94(3), pp. 512–516. doi: 10.1111/jfb.13919.

Dulvy, N. K. *et al.* (2014) 'Extinction risk and conservation of the world's sharks and rays.', *eLife*. eLife Sciences Publications, Ltd, 3, p. e00590. doi: 10.7554/eLife.00590.

Earl, D. A. and VonHoldt, B. M. (2012) 'STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method.' Conservation Genetics Resources, pp. 359–361. doi: 10.1007/s12686-011-9548-7.

Ellis, J. ., McCully, S. and Wallis, R. H. L. (2015) *Raja undulata European Regional Assessment*, *The IUCN Red List of Threatened Species*. International Union for Conservation of Nature - IUCN. doi: e.T161425A48909382.

Ellis, J. R., McCully Phillips, S. R. and Poisson, F. (2017) 'A review of capture and post-release mortality of elasmobranchs', *Journal of Fish Biology*. John Wiley & Sons, Ltd (10.1111), 90(3), pp. 653–722. doi: 10.1111/jfb.13197.

Ellis, J. R., McCully, S. R. and Brown, M. J. (2012) 'An overview of the biology and status of undulate ray Raja undulata in the north-east Atlantic Ocean', *Journal of Fish Biology*. John Wiley & Sons, Ltd (10.1111), 80(5), pp. 1057–1074. doi: 10.1111/j.1095-8649.2011.03211.x.

Evanno, G., Regnaut, S. and Goudet, J. (2005) 'Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study.', *Molecular Ecology*, 14(8), pp. 2611–20. doi: 10.1111/j.1365-294X.2005.02553.x.

Feldheim, K. A. *et al.* (2002) 'Genetic tagging to determine passive integrated transponder tag loss in lemon sharks', *Journal of Fish Biology*. John Wiley & Sons, Ltd (10.1111), 61(5), pp. 1309–1313. doi: 10.1111/j.1095-8649.2002.tb02474.x.

Fox, G. *et al.* (2018) 'Bespoke markers for ex-situ conservation : application , analysis and challenges in the assessment of a population of endangered undulate rays', *Jzar*, 6(2), pp. 50–56.

González-Ramos, M. S. *et al.* (2017) 'Validation of photo-identification as a mark-recapture method in the spotted eagle ray *Aetobatus narinari*', *Journal of Fish Biology*. John Wiley & Sons, Ltd (10.1111), 90(3), pp. 1021–1030. doi: 10.1111/jfb.13215.

Gotelli, N. J., Hart, E. M. and Ellison, A. M. (2015) 'EcoSimR: Null model analysis for ecological data'. doi: 10.5281/zenodo.16522.

Green, M. E. *et al.* (2017) 'Variability in multiple paternity rates for grey reef sharks (Carcharhinus amblyrhynchos) and scalloped hammerheads (*Sphyrna lewini*)', *Scientific Reports*. Nature Publishing Group, 7(1), p. 1528. doi: 10.1038/s41598-017-01416-w.

Guttridge, T. L. *et al.* (2010) 'Novel Acoustic Technology for Studying Free-Ranging Shark Social Behaviour by Recording Individuals' Interactions', *PLoS ONE*. Edited by S. J. Goldstien. Public Library of Science, 5(2), p. e9324. doi: 10.1371/journal.pone.0009324.

Humphries, N., Simpson, S. and Sims, D. (2017) 'Diel vertical migration and central place

foraging in benthic predators', *Marine Ecology Progress Series*, 582, pp. 163–180. doi: 10.3354/meps12324.

Hunter, E. *et al.* (2005) 'Migratory behaviour of the thornback ray, Raja clavata , in the southern north sea', *Journal of the Marine Biological Association of the United Kingdom*. Cambridge University Press, 85(5), pp. 1095–1105. doi: 10.1017/S0025315405012142.

Hunter, K. (2016) 'The Development of Molecular Techniques for the Conservation of Captive Elasmobranchs', pp. 0–59.

ICES (2016) *Advice on fishing opportunities, catch, and effort Celtic Seas Ecoregion*.

'ISOLATE II Genomic DNA Kit Product Manual' (no date).

IUCN (2014) *A quarter of sharks and rays threatened with extinction | IUCN*, *International Union of the Conservation of Nature*.

Jakobsson, M. and Rosenberg, N. A. (2007) 'CLUMPP: a cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure', *Bioinformatics*. Narnia, 23(14), pp. 1801–1806. doi: 10.1093/bioinformatics/btm233.

Kashiwagi, T. *et al.* (2015) 'Evaluating manta ray mucus as an alternative DNA source for population genetics study: underwater-sampling, dry-storage and PCR success', *PeerJ*. PeerJ Inc., 3, p. e1188. doi: 10.7717/peerj.1188.

Kohler, N. E. and Turner, P. A. (2001) *Shark tagging: a review of conventional methods and studies*, *Environmental Biology of Fishes*.

Laake, J. L., Johnson, D. S. and Conn, P. B. (2013) 'marked: an R package for maximum likelihood and Markov Chain Monte Carlo analysis of capture-recapture data', *Methods in Ecology and Evolution*. Edited by N. Isaac. John Wiley & Sons, Ltd (10.1111), 4(9), pp. 885–890. doi: 10.1111/2041-210X.12065.

Larson, S. E., Daly-Engel, T. S. and Phillips, N. M. (2017) 'Review of Current Conservation Genetic Analyses of Northeast Pacific Sharks', *Advances in Marine Biology*. Academic Press, 77, pp. 79–110. doi: 10.1016/BS.AMB.2017.06.005.

Lieber, L. *et al.* (2013) 'Mucus: aiding elasmobranch conservation through non-invasive genetic sampling', *Endangered Species Research*, 21(3), pp. 215–222. doi: 10.3354/esr00524.

Marshall, A. D. and Pierce, S. J. (2012) 'The use and abuse of photographic identification in sharks and rays', *Journal of Fish Biology*, 80(5), pp. 1361–1379. doi: 10.1111/j.1095-8649.2012.03244.x.

Marshall, T. C. *et al.* (1998) 'Statistical confidence for likelihood-based paternity inference in natural populations', *Molecular Ecology*. Blackwell Science Ltd, 7(5), pp. 639–655. doi: 10.1046/j.1365-294x.1998.00374.x.

McCrea, R. S. and Morgan, B. J. T. (2014) *Analysis of capture-recapture data*.

Nance, H. A., Daly-Engel, T. S. and Marko, P. B. (2009) 'New microsatellite loci for the endangered scalloped hammerhead shark, *Sphyrna lewini*', *Molecular Ecology Resources*. Blackwell Publishing Ltd, 9(3), pp. 955–957. doi: 10.1111/j.1755-0998.2008.02510.x.

Nomura, T. (2008) 'Estimation of effective number of breeders from molecular coancestry of single cohort sample', *Evolutionary Applications*. John Wiley & Sons, Ltd (10.1111), 1(3), pp. 462–474. doi: 10.1111/j.1752-4571.2008.00015.x.

van Oosterhout, C. *et al.* (2004) 'MICRO-CHECKER: Software for identifying and correcting genotyping errors in microsatellite data', *Molecular Ecology Notes*, 4(3), pp. 535–538. doi: 10.1111/j.1471-8286.2004.00684.x.

Openshaw, M. and Openshaw, S. (2018) *The Undulate Ray Project.org*. Available at: http://undulateray.uk/.

Park, S. (2001) *Trypanotolerance in West African cattle and the population genetic effects of selection. Ph.D. Thesis*. University of Dublin.

Pritchard, J. K., Wen, X. and Falush, D. (2009) *Documentation for structure software: Version 2.3*.

Raymond, M. and Rousset, F. (1995) 'GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism', *Journal of Heredity*, 86, pp. 248–249.

van Rossum, G. (1995) 'Python Software Foundation- Python Language v2.7.16'. Amsterdam: Centrum voor Wiskunde en Informatica (CWI). Available at: http://www.python.org.

RStudio Team (2016) 'RStudio: Integrated Development for R. RStudio'. Boston, MA. Available at: http://www.rstudio.com/.

Scientific, Technical and Economic Committee for Fisheries (STECF) (2015) – Possible by-catch provisions for undulate ray in ICES areas VIIde, VIIIab and IX (STECF-15-03). 2015. Publications Office of the European Union, Luxembourg, EUR 27154 EN, JRC 95199, 17 pp.

Speed, C. W., Meekan, M. G. and Bradshaw, C. J. (2007) 'Spot the match – wildlife photo-identification using information theory', *Frontiers in Zoology*. BioMed Central, 4(1), p. 2. doi: 10.1186/1742-9994-4-2.

Strona, G. *et al.* (2014) 'A fast and unbiased procedure to randomize ecological binary matrices with fixed row and column totals', *Nature Communications*. Nature Publishing Group, 5(1), p. 4114. doi: 10.1038/ncomms5114.

The British Society of Underwater Photographers (no date) 'The Underwater Photographers Code of Conduct'. BSoUP, pp. 1–2.

Towner, A. V *et al.* (2013) 'Gauging the Threat: The First Population Estimate for White Sharks in South Africa Using Photo Identification and Automated Software', *PLoS ONE*, 8(6), p. 66035. doi: 10.1371/journal.pone.0066035.

Worm, B. *et al.* (2013) 'Global catches, exploitation rates, and rebuilding options for sharks', *Marine Policy*. Pergamon, 40, pp. 194–204. doi: 10.1016/J.MARPOL.2012.12.034.

Yagishita, N. and Yamaguchi, A. (2009) 'Isolation and characterization of eight microsatellite loci from the longheaded eagle ray, *Aetobatus flagellum* (Elasmobranchii, Myliobatidae)', *Molecular Ecology Resources*. John Wiley & Sons, Ltd (10.1111), 9(3), pp. 1034–1036. doi: 10.1111/j.1755-0998.2009.02568.x.

Supplementary Materials

Supplementary Methods 1

Diving Protocol: Rays' Reposes

Introduction

This protocol is supplementary to 'The Underwater Photographers Code of Conduct' for the collection of *Raja undulata* photographs and DNA samples. All divers should have, as a minimum, a Sports Diver qualification under the British Sub Aqua Club (BSAC) or equivalent diving qualification, valid diving insurance and medical certification as represented by a current BSAC or equivalent organisation membership. Divers are additionally expected to have completed a minimum of 50 temperate sea dives prior to joining the project to ensure experience. All divers must abide by their qualification, 'The Underwater Photographers Code of Conduct' (http://www.bsoup.org/Code.php) and this protocol. Failure to do so will result in early termination of the dive and no further diving for the project. When DNA samples are being collected, buddy pairs will contain a photographer (diver one) and a sample collector (diver two). In all other diving, either one or both divers are expected to have a camera for images. All sampling (whether photographic or DNA) comes second to diver safety. The aim of the protocol is to minimise disturbance caused to the *R. undulata* and other marine life when diving in their natural environment. All divers should be sufficiently experienced to maintain good buoyancy control and be aware of their surroundings at all time. In the ideal encounter the *R. undulata* will remain in its original position after the divers have moved away.

Materials and Methods

All divers will be expected to carry a delayed surface marker buoy (DSMB) as standard for Sports Diver or above. A camera and multiple DNA sampling kits will be carried by the divers. On the location of a resting R. undulata, divers will follow these steps:

1. **Divers will slowly approach the resting *R. undulata***

Most rays can be approached slowly, however if the individual begins to swim away, divers should not pursue them for any distance. Once an individual starts to swim away, it is

unlikely it will allow you (as the diver) close enough to take a detailed photograph or DNA samples. Only one diver should approach at any time, and the second diver should remain 4 to 5 metres away. Rays are often partially buried or have quantities of shale and stone on their dorsal side. Do not attempt to uncover them, as they will swim away. If a ray raises the centre of its body, identified by a lifting of the spine from the ground, it has become disturbed by the divers' presence and is preparing to swim away, divers should move away immediately. Often the ray will settle and remain, providing you are not too close.

2. **Diver one will take the photograph**

Photographs for ID need to be taken from above; the ray will be less sensitive if the diver is higher in the water column. If possible, the ID picture should be taken from 2 to 3 metres above the seabed but this is dependent on the camera equipment. Limit the time should be spent photographing a single ray, especially if using strobes or powerful lights. Once the image has been collected, diver one should then move to approximately 5 metres from the resting ray to avoid further disturbance.

3. **Diver two will take the DNA sample**

DNA sampling should take place once an ID image has been collected. When collecting mucus samples for analysis, first take the ID photograph, move away and prepare the swab for sampling, then move back to the ray and take the sample, move away again. Seal the sample in the pre-labelled sample bag and photograph the sample bag label to provide identification for the ray photograph. The ideal encounter is when the ray does not swim away but remains in its original position, after sampling and when the divers move away.

Supplementary Video 5.1

Please see the provided disk located at the back of the thesis.

NERC SCIENCE OF THE ENVIRONMENT

THE UNDULATE RAY PROJECT

The undulate ray sampling protocol

MATT DOGGETT NATURE · PHOTOGRAPHY · STORIES

MANCHESTER 1824 The University of Manchester

1. Sterilized scrubbers
2. Handles
3. Zip Ties
4. Individually marked sampling bags
5. 50 ml eppendorf tubes with 100% ethanol
6. Recording sheet, pencil & permanent marker
7. Scissors & forceps

1 Locate the ray and take a photograph for identification purposes – ensuring all areas of the pattern are within shot.

2 Use the scrubber, attached to the handle with the zip tie to collect mucus sample, taking an image of the bag to ensure sample can be matched to ID photograph

3 Once on the surface, cut the scrubber from the handle, and use the forceps to transfer the scrubber sample to a 50ml eppendorf (100% ethanol).

4 Using the permanent marker, write the corresponding to the ID number on the tube and store on ice until transferred to a secure -20°C freezer.

For longer storage samples should be kept at -80°C.

Supplementary Table 5.1
The 17 polymorphic microsatellite loci for R. undulata. RM: Repeat Motif, AR: Allele Range, PM: primer mix; N: number of samples per locus; Ar: allele richness/number of alleles per locus; NA: estimated null allele frequency; Ho: observed heterozygosity; He: expected heterozygosityHunter, 2016; Fox et al., 2018).

Loci	RM	*AR*	PM	Primers		A_r	N	H_o	H_e	NA
Ru_pp02	AAG	347 - 419	1	F:	CTAGTTATTGCTCAGCGGTCCCTGTTCTCCTGCTCTCCATTACC	27	123	0.715	0.872	0.0973
	AGG			R:	CTCTCCCTATAGCTCAGGCCTTCGG					
Ru_pp08	AGGTG	351 - 415	1	F:	TGTAAAACGACGGCCAGTTGAGGAATTCATTGCCACAAACTGC	36	116	0.724	0.951	0.1327
				R:	TCCTCTCACATAACCCTGTGTATGCC					
Ru_pp14	AGGC	277 - 313	1	F:	CTAGTTATTGCTCAGCGGTACCTCGAAACCGCCATTAAGAATCC	23	127	0.843	0.888	0.0253
				R:	CTGCATGTTATCGAGCAATCAGTCG					
Ru_pp09	ATAG	209 - 385	2	F:	TGTAAAACGACGGCCAGTTCTTTGCTCCTACCGGTTCTTCTCG	34	113	0.938	0.942	0.0005
				R:	CAGAACAAGGCTTGGTGGTCTTGG					
Ru_pp20	ACAG	374 - 407	2	F:	CTAGTTATTGCTCAGCGGTGGACACTTGACACAGCTTTGGTCTCC	20	119	0.882	0.907	0.0115
				R:	GGGAGTTACCTTCATGGTGAGACAGG					
Ru_v2_pp02	TTGTG	301 – 331	2	F:	CTAGTTATTGCTCAGCGGTCCCACTCTGGGTCATGGTGCC	16	121	0.669	0.734	0.0381
				R:	AGGCATGTGCATGTCTGTGGG					
Ru_pp13	ACAG	317 - 373	3	F:	TGTAAAACGACGGCCAGTCATTCTTAACAGGGCAGCTACTTGTGG	9	18	0.333	0.883	0.4359
				R:	AAAGATTGGTAGGAAGATGGATCGG					
Ru_v2_pp04	ATCT	335 – 395	3	F:	CTAGTTATTGCTCAGCGGTTGCACTGTTCTTGTTTAATCCCAGCC	19	48	0.833	0.865	0.0059
				R:	CTAATCCACGTGGTGCTGACTGGC					
Ru_v2_pp06	TCTG	209 – 268	3	F:	CTAGTTATTGCTCAGCGGTTGCTTATTGTCCAATAGACACAAATCCCC	29	84	0.202	0.95	0.6480
				R:	CTTCACAATTTGCAACTCCTCTGCC					
Ru_v2_pp07	AATG	402 – 448	4	F:	TGTAAAACGACGGCCAGTGCGGAGGTACGAAGGGAAGGG	14	21	0.524	0.886	0.2527
				R:	CCCAATCGAGATTCTACCAAACAGCC					
Ru_v2_pp09	ACCT	270 – 315	4	F:	CTAGTTATTGCTCAGCGGTCTTTTATTGGTGTGCTGCAAATGGG	11	21	0.238	0.816	0.5475
				R:	TAGATTGGTGGATGGAGTCAGTCGG					
Ru_v2_pp12	ATGT	350 – 462	4	F:	CTAGTTATTGCTCAGCGGTCCACATGCCTTTCCATACAAATGGG	22	37	0.811	0.926	0.0571
				R:	ACAGGAGAACGGTGTGTAAACTGGC					
Ru_pp21	AAT	373 - 388	4	F:	TGTAAAACGACGGCCAGTCATGACTGGGGCTAGAAGGTGTTGC	11	44	0.636	0.733	0.0594
				R:	GTTAGAGCAGTCCGCCATGAAGGG					
Ru_v2_pp13	ATC	314 – 346	5	F:	TGTAAAACGACGGCCAGTCTGACCGTGGGAGAACAAAGAGCG	13	105	0.724	0.766	0.0198
				R:	GAAGAGGAGACTGAAAGCTGATTTGGC					
Ru_v2_pp19	ATT	303 – 362	5	F:	CTAGTTATTGCTCAGCGGTCAGAAGTGAGAATGAATGCTGGCG	24	109	0.872	0.92	0.0245
				R:	CTTGTGGGAGCGGTCTTTTATGGG					
Ru_v2_pp20	AGG	252 – 279	5	F:	CTAGTTATTGCTCAGCGGTTTACTGTCCGCAGGCCAACCC	11	107	0.486	0.58	0.0763
				R:	AGTTGTGTGATCTGTCTTGTGGCCG					
Ru_pp03	ACT	412 - 463	5	F:	TGTAAAACGACGGCCAGTCATTCACAACTGCAGTCCAATGTCC	17	107	0.738	0.847	0.0636
	GCC			R:	TCTGCTGTCAAGCTGTTGTGTCAGG					

Chapter 6

The application of genetics for the *ex-situ* conservation and management of elasmobranchs (sharks, rays and skates)

S. A. Hook[1]*, A. Verissimo[7], B. Gimeno Molina[6], J. D. Hibitt[3], T. Moritz [4,5], M. Buckley[1], H. A. Shiels[2]

[1]School of Earth and Environmental Sciences, Manchester Institute of Biotechnology, The University of Manchester, M1 7DN, UK

[2] Faculty of Biology, Medicine and Health, The University of Manchester, M13 9NT, UK

[3]Merlin Animal Welfare Development (MAWD), Sea Life Weymouth

[4]Deutsches Meeresmuseum, Katharinenberg 14-20, 18439 Stralsund, Germany

[5]Institut für Spezielle Zoologie und Evolutionsbiologie, Friedrich-Schiller-Universität Jena, 07743 Jena, Germany

[6]Universidad Politécnica de Valencia. Camino de Vera s/n 46022Valencia, Spain

[7]The Centre in Biodiversity and Genetic Resources, The University of Porto, 4485-661 Vairão, Portugal

Corresponding author: Samantha A. Hook, samhook1205@gmail.com

Author contributions

SAH[1] wrote the paper, conducted the lab work and analyzed the genetic results. AV[7] provided the lab and supervision for work conducted on *S. canicula* samples. BGM[6] assisted in the lab work and analysis for *C. melanopterus*. J-DH[3] provided the samples for *C. melanopterus.* TM[4,5] provided the parentage samples and embryos for *S. canicula.* MB[1] provided the lab for *C. melanopterus*. HAS[2] provided the housing for *S. canicula* embryos and created the idea. All authors contributed to the review prior to submission.

6.1 Abstract

Without the assistance of breeding programs and cooperation of multiple aquaria, individual collections within zoos and aquariums could be too small to be much value to long-term conservation. Aquariums often aim to establish *ex-situ* breeding programmes to produce successful viable populations. This requires populations to be healthy and capable of self-sustaining amongst several aquaria. The blacktip reef shark *Carcharhinus melanopterus* and the small-spotted catshark *Scyliorhinus canicula* are two charismatic species currently held in aquariums across the globe. Both species are successfully reproducing in captivity and has the potential to become a demographic and genetic backup to wild populations, and reduce the need to acquire individuals from the wild. Here, we conducted the first research on the application of microsatellite markers to assist in the studbook management of these two species, displaying a cost-effective and simple method which aquariums can transfer to other shark and batoid (elasmobranch) species. We identified the first possible case of multiple paternity in *C. melanopterus*, increasing the necessity to correctly identify parentage. We found no signs of inbreeding in either species, despite management having little studbook records to reduce these effects (*C. melanopterus* F_{IS} = 0.0547; *S. canicula* F_{IS} =0.0519). Both species however displayed possible signs of outbreeding with a reduction in F_{IS} values when divided between wild and captive-bred individuals. Lastly, we defined 39 species that currently have sufficient microsatellites which could assist aquariums to achieve their goals in captive management, conservation, research and education.

Keywords: *Carcharhinus melanopterus, Scyliorhinus canicula*, dogfish, blacktip reef shark, elasmobranchs, captivity, microsatellite, studbooks, conservation

6.2 Introduction

Sharks, along with skates and rays (batoids), are a distinct group within the chondrichthyes (cartilaginous fishes) and are housed in most public aquariums worldwide. Nearly 700 million people visit aquariums across the globe every year, with sharks as a leading attraction (Janse *et al.*, 2017). Despite their popularity, wild populations of sharks and batoids face massive declines from overexploitation and climate change. As of 2014, 25% of all sharks and batoids were classified as threatened with extinction by The IUCN Red List of Endangered Species, hereafter referred to as The IUCN Red List (Dulvy *et al.*, 2014). In order to recover species loss, zoos and aquariums have increased their research and public education efforts to become more conservation focussed. In addition to research and public engagement (Schwan, Grajal and Lewalter, 2014), one of the primary means for *ex-situ* conservation within zoos and aquariums is captive breeding (Conde *et al.*, 2013). Zoo/Aquarium captive breeding programs have aided reintroductions across a range of endangered taxa, for example the Green and Golden Bell Frog *Litoria aurea* (Daly *et al.*, 2008), the Przewalski's horse *Equus ferus przewalskiior* (Xia *et al.*, 2014) and most famously the California condor *Gymnogyps californianus* (Toone and Wallace, 1994). For sharks, captive breeding can be problematic due to their life-history traits, such as late sexual maturity, low fecundity and various reproductive modes. Captive breeding efforts have even resulted in a number of unwanted consequences including fatality of mating individuals, stillborn pups, abnormal retention of pups (over-gestation; Henningsen *et al.*, 2004) and cannibalism (Hibbitt, Rees and Brown, 2017). As sharks and batoids undergo large population declines in the wild, the urgency for aquariums to successfully breed healthy viable captive populations and to understand genetic relationships within the captive breeding group is growing.

Genetic analysis can be used to effectively manage captive elasmobranch populations and understand relationships between elusive individuals. Although next-generation sequencing (NGS) techniques are revolutionising the field of population genetics, they remain too costly to be applied to practical conservation (Puckett, 2017). The use of microsatellites is still the method of choice for many conservation genetics studies due to the high number of polymorphisms in populations and the variety of outputs available from

microsatellite application, such as parentage, kinship, population structure, inbreeding coefficients and gene mapping (Vieira *et al.*, 2016). For species of sharks and batoids where multiple paternity or parthenogenesis is found, it is especially important to apply conservation genetics to their captive management. Of the 102 captive elasmobranch species defined by Janse *et al.*, (2017) (Supplementary Table 6.1), microsatellite markers have been successfully used in 39 (Figure 6.1), and multiple paternities have been found in 14 species. However, there is no record of the application of genetics to the conservation of sharks held in captivity with the exception of defining parthenogenesis (Feldheim *et al.*, 2017).

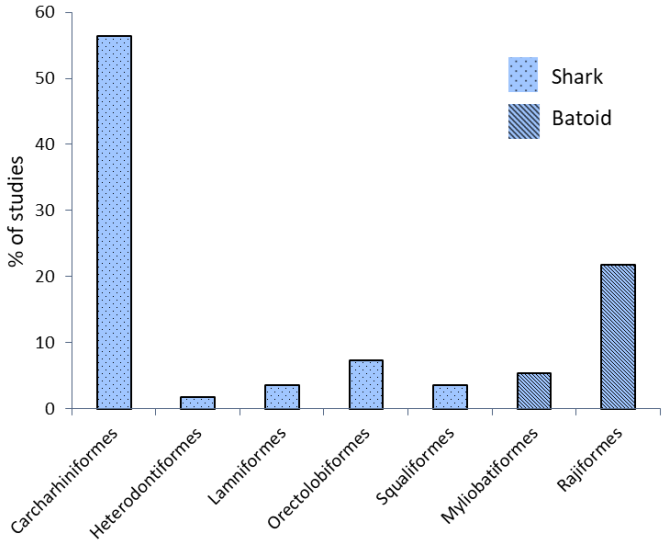

Figure 6.1. The percentage of microsatellite studies published (n = 55) for the different orders of elasmobranchs which are routinely kept in captivity (n = 39). Data was extracted from a literature review we conducted, using the species described as held within captivity by Janse et al. (2019)(full literature review can be found in the Supplementary Table 6.1).

The blacktip reef shark *Carcharhinus melanopterus* (Quoy & Gaimard, 1824) and the small-spotted catshark *Scyliorhinus canicula* (Linnaeus, 1758) are two of the most common sharks on display in aquaria worldwide (Hibbitt, Rees and Brown, 2017). Both species have microsatellite primers developed and genetic analysis for the purpose of *in-situ* conservation (Griffiths *et al.*, 2011; Mourier and Planes, 2013). The reproductive traits of the two species are very different and this has contributed to a higher captive breeding success in *S. canicula* when compared to *C. melanopterus*: *S. canicula* is an oviparous species laying a pair of eggs every 2-3 weeks (29-62 eggs per year; Ellis *et al.*, 2009; Griffiths *et al.*, 2012), whereas *C. melanopterus* is a viviparous placental species give birth to 2-4 live pups

per year (Compagno, 1984; Chin *et al.*, 2013). Due to the vulnerability of captive *C. melanopterus* and an increase in captive-bred offspring, studbooks have been implemented to inform on husbandry techniques, which has produced a higher number of live offspring (Hibbitt, Rees and Brown, 2017). Studbooks provide a useful record of observed relationships between individuals held in captivity, which assists in future exchange of individuals to reduce the probability of inbreeding. The *S. canicula* is classified as least concern by The IUCN Red List, with no studbook records, despite a high reproductive output in captivity. The application of studbooks on captive populations of *S. canicula* without genetic information would be difficult in aquaria as the species can display multiple paternity (Griffiths *et al.*, 2012), and it is often difficult to identify individuals without the use of tagging or photographs (Hook *et al.* 2019). Due to the small captive population sizes and the ability to identify individuals from morphological features, maternity is rarely disputed in *C. melanopterus*. However in populations with two or more paternal candidates, genetic markers are necessary to determine full parentage.

Here we test the use of already developed microsatellite markers on two species of captive shark, *C. melanopterus* and *S. canicula,* for captive management within aquariums. We investigate the genetic diversity between parents and offspring, and review the level of inbreeding or outbreeding that is occurring within the captive populations. Finally we review the relatedness between individuals of the same species to create family trees for management and educational purposes.

6.3 Materials and Methods

6.3.1 Sample collection

Tissue samples from 14 individual *C. melanopterus* were collected from LEGOLAND Deutschland Freizeitpark, Günzburg by members of the aquarium team. The 14 individuals consisted of two litters totalling nine offspring and their maternal parent (M1), and a litter of four offspring from a second unsampled mother (M2). All individuals had died within seven days of birth, while M1 died from natural causes. Individuals of the *C. melanopterus* were removed from the tanks within 12 hours of death and frozen in long term -20˚C storage. To collect tissue samples, 1 cm cubed of tissue was taken from the least decomposed areas of

the body (typically the inner flesh). Samples were stored in 100% RNALater and sent to the University of Manchester Institute of Biotechnology labs for processing. A total of 65 individual *S. canicula* eggs were successfully developed and hatched at the Biological Services Facility (BSF) at the University of Manchester from a captive source population at the OZEANEUM (Stralsund, Germany). Once hatched fin clips of equal to or less than 10mg were taken from 16 offspring and stored in 100% RNALater. Additionally, the seven potential parents were fin-clipped at the source population and stored in 100% RNALater prior to transportation to the University of Manchester. All samples were stored at -20˚C before DNA extraction.

6.3.2 DNA extraction

Genomic DNA was isolated from *C. melanopterus* and *S. canicula* samples using an QIAGEN® DNeasy® Blood and Tissue kit where the incubation and digestion step was conducted overnight. Samples were diluted to 20-50 ng/µl of genomic DNA for amplification. Extracted DNA samples of *S. canicula* were transported to the Research Centre in Biodiversity and Genetic Resources (CIBIO) Porto, Portugal for amplification whereas the *C. melanopterus* loci were amplified at the University of Manchester, Institute of Biotechnology, Manchester, UK.

6.3.3 *Carcharhinus melanopterus* DNA amplification

Here we used twelve microsatellite loci primers, developed by Keeny and Heist (2003) and Feldheim *et al.* (2001, 2002; Supplementary Table 6.2). Forward primers were altered with TAG regions for FAM and HEX proprietary dyes (Culley *et al.*, 2013) (Supplementary Table 6.2). PCR reactions consisted of 2.5 µL of ddH_2O, 1.5 µL of Type-it® Microsatellite 127PCR kit (Qiagen) and 0.5 µL of primer mix (0.75 µl of 100 mM Forward Primer, 2.5 µL of 100 mM Reverse Primer, 1 µL of 100 mM stock proprietary dye made to 50 µL with ddH_2O) and 1 µl of genomic DNA (20 ng-70 ng/µL). Microsatellites were amplified under the adapted thermal conditions of Keeney *et al*. (2003), Vignaud *et al*. (2013) and Mourier & Planes (2013): initial denaturation at 95 ^{o}C for 4 min, 25 cycles of 1 min at 95 ^{o}C, 30 s at 57/63 ^{o}C, and 30 s at 70 ^{o}C, and at 70 ^{o}C for 4 min for final extension; held at 4 ^{o}C. If

DNA yields prior to amplification were lower than 20 ng/µL, the number of cycles was increased from 25 to 35.

6.3.4 *Scyliorhinus canicula* DNA amplification

A total of 11 species-specific primers were used for DNA amplification developed by Griffiths et al., (2011) (Supplementary Table 6.2). Proprietary tags of FAM, VIC or NED were added to each primer, and dyes were added to the primer cocktail for successful genotyping. PCR reactions consisted of 1 µL of genomic DNA (20-70 ng/µl), 1 µL of the Primer cocktail, 3 µL of ddH_2O and 5 µL of QIAGEN Multiplex PCR Kit. The primer cocktail contained 11 tailed forward primers, 11 complimentary reverse primers (Table 6.1), and three tail dyes; FAM, VIC and NED. The DNA was amplified in a thermal cycler under the following conditions: initial denaturation cycle at 95 °C for 15 min, followed by 35 cycles of 94 °C for 30 s, 60 °C for 90 s and 72 °C for 45 s, finalised by one cycle at 72 °C for 30 minutes; held at 4°C.

6.3.5 Gel electrophoresis, genotyping and allele scoring

Products were viewed on a 2% agarose gel electrophoresis before sending for genotyping. The PCR products for *C. melanopterus* which displayed positive bands were multiplexed together post PCR, with dilution rates estimated from the band strength: bands at a high intensity were diluted to a 1 in 20, whereas bands at a low intensity were not diluted. PCR products were genotyped on an ABI™ 3730XL capillary sequencer and size scoring was completed using GeneScan™ 500 LIZ™ dye Size Standard. Genotypes were viewed and scored on GeneMapper version 4.0 using pre-determined allele size scoring from the wild type genotypes of both species (Griffiths *et al.*, 2012; Mourier and Planes, 2013).

6.3.6 Statistical Analysis

Microchecker Version v2.2.3 (van Oosterhout *et al.*, 2004) was used to establish the scoring error due to stuttering, and also to assess the evidence of large allele drop out or the presence of null alleles. Cervus v3.0.7 (Marshall *et al.*, 1998) was used to determine the expected and observed heterozygosity values. The relatedness between individuals within

the population was estimated using software Relatedness v4.2 (Goodnight and Queller, 1995), based on the programmed algorithm designed by Queller and Goodnight (1989). The program COLONY v2.0.6.4 (Jones and Wang, 2010) was used to reconstruct the pedigrees (including sibship and parentage) due to the high resolution of the full-likelihood approach when compared to other methods (Harrison *et al.*, 2013; Taylor *et al.*, 2015) and the flexibility of parameters when setting an analysis. As 100% of the individuals was sampled, no algorithm for novel assignment was necessary. COLONY parameters were set depending on species life-history traits: *S. canicula* was set to female and male polygamy with inbreeding in a diploid species; *C. melanopterus* was set to female monogamy and male polygamy with inbreeding in a diploid species. A genotyping error of 0.01 was specified due to its lower importance in assignment accuracy and low allelic diversity (Amos *et al.*, 2006; Harrison *et al.*, 2013). Weir's *F* inbreeding coefficient was used to calculate the level of inbreeding within the population using GenePop on the Web. To maintain a healthy captive population with the resources available at the BSF Manchester, 16 of the original 65 individuals we kept more than four months after hatching. Therefore infographics were created to display pedigree analysis on the remaining 16 individuals. Microsatellite marker Cli107 was removed from the *C. melanopterus* analysis due to low numbers of successfully amplified samples.

6.4 Results

6.4.1 Genetic diversity and inbreeding coefficient

Of the samples collected (Table 6.1) 7% (n = 1) of *C. melanopterus* and 10% (n = 7) of *S. canicula* originated from the source populations (wild-caught). As only one sample in *C. melanopterus* originated from the source population, the ability to analyse the differences between the source and the captive-bred population could not be conducted. However, despite the low population size, there was no significant difference in genetic diversity levels between the captive population and previously studied wild populations of both species; *C. melanopterus* wild H_o = 0.572, H_e = 0.581 (Mourier and Planes, 2013), *S. canicula* wild H_o = 0.648, H_e = 0.648 (Griffiths *et al.*, 2011, 2012). The *C. melanopterus* displayed a lower F_{IS} value than that found in wild populations (F_{IS} = 0.078, Mourier and Planes, 2013) indicating

that the population is outbreeding. By contrast, the *S. canicula* source population produced a higher F_{IS} value than the captive population, indicating a reduced level of inbreeding between the two generations; the mean number of alleles (N_a) in *S. canicula* was higher than in the wild population by Griffith *et al*., 2011 (N_a = 7.7) than this source population (N_a = 3.545).

Table 6.1. Genetic diversity and inbreeding coefficients for the *C. melanopterus* captive and total population and the *S. canicula* source, captive and total population. Number of captive individuals (*Ncap*) number of founding individuals for the captive population *(NF*), number of individuals sampled (*N*), number of primers (*#P*), observed heterozygosity (H_o), expected heterozygosity *(*H_e), inbreeding coefficient (F_{IS}), mean number of alleles (N_a).

	***Ncap* alive**	***NF***	***Ncap* dead**	***N***	***#P***	H_o	H_e	F_{IS}	N_a
C. melanopterus									
Captive	3	4	14	13	11	0.594	0.544	0.0296	
Total	3	4	14	14	11	0.536	0.566	0.0547	3.818
S. canicula									
Source	7	7	0	7	11	0.571	0.579	0.0702	
Captive	16	7	49	65	11	0.524	0.523	-0.0093	
Total	16	7	49	72	11	0.529	0.526	0.0519	3.545

The *S. canicula* had a large reproductive output (65) with a near equal distribution between males and females from the seven founding source individuals. Father 1 (F1) is the most dominating genetic male within the offspring, as this individual has bred with all females. There is a high probability that sperm storage and multiple paternity also had roles to play within this population (Griffiths *et al.*, 2012). Due to the rearing conditions of the eggs, and mixing of individuals after hatching to reduce effects from different tanks (tank effects), it is impossible to exactly state the level of multiple paternities as egg pairs were separated.

6.4.2 Relatedness and pedigree reconstruction

Due to the regulations of sample collection for non-urgent, non-medical purposes, only tissue from dead *C. melanopterus* could be collected resulting in a low number of samples for this species (Table 6.2). COLONY revealed an overall sire reproductive skew and therefore that reproductive success in *S. canicula* was most dominant in individual F1 (0.59), followed by F3 (0.26) and finally F2 (0.15) (Figure 6.2). The analysis also found that four individuals of *S. canicula* could not be maternally allocated in eight computations of

substructure parent pair probability (p = 0.0106): 50% sired by F1 (average probability = 0.9874), 50% sired by F2 (average probability = 1).

Table 6.2. Genetic analysis of the probability of parentage for the offspring of *C. melanopterus* and *S. canicula*

Female ID	Colony				
	Number of offspring	Number of Sires	Sire ID	Sire skew	Probability
C. melanopterus					
M1	9	1	#1	9 (1.00)	0.9223
M2	4	2	#2	2 (0.50)	0.9447
			#3	2 (0.50)	0.554
S. canicula					
M1	26	3	F1	10 (0.38)	0.9955
			F2	7 (0.27)	0.8556
			F3	9 (0.35)	0.9996
M2	5	1	F1	5 (1.00)	0.9513
M3	3	3	F1	1 (0.33)	0.9578
			F2	1 (0.33)	1
			F3	1 (0.33)	1
M4	24	3	F1	18 (0.75)	0.9999
			F2	1 (0.04)	1
			F3	5 (0.21)	0.9749

Data calculated with COLONY and Gerud. Female ID: Mother = M; Sire ID: Father genotype sampled = F, Simulated Father Genotype = #; Sire Skew: reproductive skew of the individuals on a decimal basis; Probability= the average probability for the sire reproductive skew.

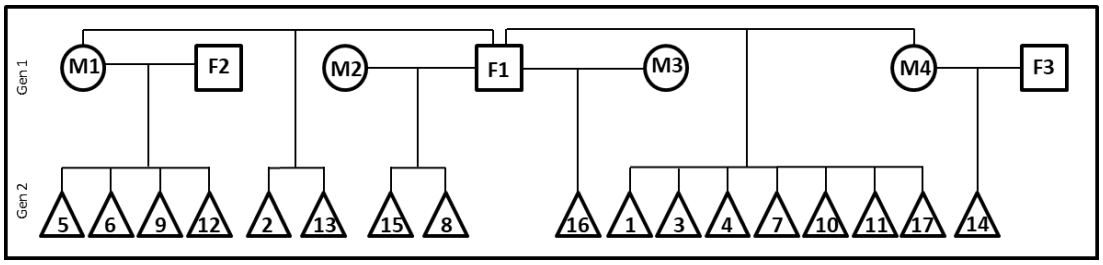

Figure 6.2. Infographic of pedigree reconstruction calculated in COLONY (p = 0.0363) for the 16 live individuals held within captivity at the BSF, The University of Manchester and their source population from the OZEANEUM, Germany, across two generations (Gen1 and Gen2); circles represent females, squares represent males and triangles represent unknown sex.

The average probability that correct maternal assignment of the nine offspring of *C. melanopterus* to M1 is 0.9223. As no paternal genotypes were present, COLONY used a pairwise likelihood methodology to determine assignment to offspring, including average probability of individuals to determine possible multiple paternity. Further computations of substructure parent probability found a possibility that only one male has sired M2 offspring (p = 0.0186), however the average probability is much lower than produced through pairwise

likelihood methodology. The expected heterozygosity values for *S. canicula* are normally distributed under Sharpiro-Wilk W test (captive population: $p = 0.02985$, Griffith et al 2011: $p = 0.01033$). There is no significant difference between the captive and wild population expected heterozygosity or the observed heterozygosity (one-way ANOVA, $p = 0.4154$).

6.5 Discussion

Here, we provide the first use of genetics to review the breeding structure and genetic diversity of the small-spotted catshark *S. canicula* and the blacktip reef shark *C. melanopterus* in captivity. We found that there was no inbreeding between generations despite a lack of studbook management. We have also revealed the first case of multiple paternity in the *C. melanopterus*. These results provide insight into genetic stocks, pedigrees and the future application of studbooks to help aquariums meet their aims of: 1) reducing their reliance on wild-caught specimens; 2) increasing captive population management; and 3) playing more of a roll in conservation.

Historically zoos and aquariums lacked in their ability to keep accurate records of breeding and in seemingly complete studbooks, neonates and stillbirths have gone unreported (Thornhill, 1993). Therefore, pedigrees are rarely complete; limiting the use of captive populations for conservation and future reintroductions. As aquariums have very few captive-bred individuals, studbooks could be more accurately applied improving their roll in management and conservation. In the case of *C. melanopterus,* currently all births within captivity are recorded, whether stillborn, neonate or live (Hibbitt, Rees and Brown, 2017). As *S. canicula* is an oviparous species, individual eggs are often destroyed before viability is determined and in many situations, the number of egg cases retrieved and destroyed goes unreported. Currently there are fewer numbers of *C. melanopterus* in captivity when compared with *S. canicula*. This is primarily due to their greater husbandry needs, such as limiting group numbers and large tanks space.

We found no evidence of inbreeding events of offspring within *C. melanopterus* ($F_{IS} = 0.0296$), however possible multiple paternity within the second mother (M2) for her 4 offspring was observed. As samples were not collected from the potential fathers, paternal genotypes could not be used for the analysis, resulting in a lower probability of the sire

reproductive skew (average p = 0.7494) when compared to Mother 1 offspring which displayed no possible multiple paternity. Multiple paternity in *C. melanopterus* has not been found in the wild, despite higher number of mothers and pups being tested. These results could be facultative; similar to the parthenogenesis observed in captive elasmobranchs, such as the swellshark shark, *Cephaloscyllium ventriosum* (Feldheim *et al.*, 2017).

Blacktip reef sharks *C. melanopterus* display low levels of migration and mixing patterns in wild populations between residing areas (Papastamatiou *et al.*, 2010), with one report on a single island in the French Polynesia showing that females tend to be more philopatric than males, at least in some areas (Mourier and Planes, 2013). Due to low levels of migration, *C. melanopterus* display high levels of inbreeding and a reduced effective population size (Mourier and Planes, 2013), however, contradictory evidence from Chin *et al.* (2013) suggests that migration movements may be more variable in this species. The captive population of *C. melanopterus* displayed a lower level of inbreeding than wild populations suggesting that the founding population of individuals could be from genetically distant regions across their habitat range, decreasing the level of F_{IS} observed. The variability in migration patterns and the possibility of large-scale movements between multiple populations of the species, may help maintain genetic diversity levels and adaptability (Chin *et al.*, 2013). To ensure both *S. canicula* and *C. melanopterus* remain at this level, and are not subjected to either inbreeding or outbreeding, it would be advised that, if offspring were raised to maturity, either the individuals should be separated from the same parents or that offspring should be exchanged, emulating migration to other (captive) populations.

The Hardy-Weinberg Equilibrium presumes that the population is randomly mating and therefore gene frequencies remain consistent between generations (Raymond and Rousset, 1995). However, it can be questioned whether random mating is possible in captivity as the population is isolated from other individuals. The population size, number of sexually mature individuals and various husbandry-related factors (such as tank size, temperature control and so on) all play a role in the availability for individuals to reproduce (Koob, 2004; Smith *et al.*, 2004). The parametric tests between the wild and captive

populations in both *S. canicula* and *C. melanopterus* produced no significant difference of expected and observed heterozygosity. Therefore captive breeding appears to show no significant effect on the level of genetic diversity within the populations. The inbreeding coefficient (F_{IS}) shows outbreeding of the *S. canicula* in the current offspring when compared to the source population, reducing the overall population inbreeding coefficient. This indicates that the parents of the offspring share very little genetic resemblance and therefore are less related.

Isolated populations which have no immigration or emigration for many generations, such as those in captivity, can succumb to genetic problems. As a group, elasmobranchs have late maturation (Dulvy *et al.*, 2014) and the effects of inbreeding from isolation in captivity may not been observed for many generations. Under the advisory bodies such as the European Association of Zoos and Aquariums (EAZA), animal exchange is encouraged and in some cases facilitated (EAZA, 2017). If genetics are not studied and monitored throughout captive populations, inbreeding or outbreeding may not be detected. It therefore can be argued that it is important to gather genetic information and individual identification from the entire captive population, rather than analysing individual aquaria populations. A development from this study could be the application of genetics for the full studbook management across all institutions that house *C. melanopterus,* which is currently being coordinated by Sea Life (Hibbitt, Rees and Brown, 2017). Programs such as this however, can be limited by aquaria resources, aquaria and research institution participation, and regional licenses for the collection of tissue samples. When applying studbooks or any conservation management, there is often a bias in effort and resources towards species classified with a higher International Union of the Conservation of Nature Red List category, or species which are defined as charismatic to the public (Trimble and van Aarde, 2010).

At any one time, an aquarium in Europe can house up to 340 individual elasmobranchs across 20 different species (Janse *et al.*, 2017). On average each captive species listed in Supplementary Table 6.1 have 12 define microsatellite primers. There is a significant relationship between the year of publication and number of microsatellite primers (generalized linear regression model, $p = 0.043$), multivariate linear regression model ($p =$

0.050), suggesting that more recent publication use more microsatellite markers per species. Therefore depending on the age of the study and resources available (often at cost to the aquarium), genetics can be actively applied to multiple captive elasmobranchs to aid studbooks. Thishighlights the requirement for new microsatellite research in other orders of elasmobranchs in wild populations. In cases where captive species do not have species-specific microsatellites, it is also possible to gain genetic information from the use of microsatellites for species of the same genus. For example, *Pristis pristis* and *Pristis zijsron* are two species that are critically endangered and microsatellites have been defined for the closely related *Pristis pectinate* (Feldheim *et al.*, 2010). It has been shown that in these species have displayed high genetic similarity on mitochondrial DNA and therefore it could be possible to transfer microsatellite markers between these species (Phillips *et al.*, 2011). Certain programs require source population allelic frequencies for analysis of captive-bred individuals; such as Cervus which to identifies parentage and breeding coefficients. To utilize current microsatellite markers (Supplementary Table 6.1) for captive management in other species, aquaria need to review the current numbers of individuals held within captivity and levels of successful breeding before collecting samples. If husbandry techniques cannot improve the conditions for individuals to successfully breed, the species in question would eventually need to be resourced from the wild, therefore removing the requirement of genetic analysis.

Overall, for breeding populations, genetic research within aquariums can assist the management in captivity, to help to reach their conservation and management based aims. However there is still a high need for wild populations of sharks and batoids to be studied, to then apply knowledge to captive management.

6.6 Acknowledgements

We would like to acknowledge the CASE partner Merlin Animal Welfare Development for providing samples and NERC doctoral Training Program for funding this project. I would like to thank Bianka Gronsow for assisting in the sample collection of adults for the *S. canicula* species.

6.7 References

Amos, W. *et al.* (2006) 'Automated binning of microsatellite alleles: problems and solutions', *Molecular Ecology Notes*. Blackwell Publishing Ltd, 7(1), pp. 10–14. doi: 10.1111/j.1471-8286.2006.01560.x.

Chin, A. *et al.* (2013) 'Ontogenetic movements of juvenile blacktip reef sharks: Evidence of dispersal and connectivity between coastal habitats and coral reefs', *Aquatic Conservation: Marine and Freshwater Ecosystems*, 23(3), pp. 468–474. doi: 10.1002/aqc.2349.

Chin, Andrew *et al.* (2013) 'Validated age, growth and reproductive biology of Carcharhinus melanopterus, a widely distributed and exploited reef shark', *Marine and Freshwater Research*. CSIRO PUBLISHING, 64(10), p. 965. doi: 10.1071/MF13017.

Compagno, L. J. (1984) *Sharks of the world / 2. Carcharhiniformes.* Food and Agriculture Organization of the United Nations.

Conde, D. A. *et al.* (2013) 'Zoos through the Lens of the IUCN Red List: A Global Metapopulation Approach to Support Conservation Breeding Programs', *PLoS ONE*. Edited by M. Hayward. Public Library of Science, 8(12), p. e80311. doi: 10.1371/journal.pone.0080311.

Culley, T. M. *et al.* (2013) 'An Efficient Technique for Primer Development and Application that Integrates Fluorescent Labeling and Multiplex PCR', *Applications in Plant Sciences*. Botanical Society of America, 1(10), p. 1300027. doi: 10.3732/apps.1300027.

Daly, G. *et al.* (2008) 'Reintroduction of the Green and Golden Bell Frog *Litoria aurea* to Pambula on the south coast of New South Wales', *Australian Zoologist*. Royal Zoological Society of NSW , 34(3), pp. 261–270. doi: 10.7882/AZ.2008.003.

Dulvy, N. K. *et al.* (2014) 'Extinction risk and conservation of the world's sharks and rays.', *eLife*. eLife Sciences Publications, Ltd, 3, p. e00590. doi: 10.7554/eLife.00590.

EAZA (2017) *EAZA Population Management Manual*.

Ellis, J. *et al.* (2009) *Scyliorhinus canicula*, *The IUCN Red List of Threatened Species 2009: e.T161399A5415204*.

Feldheim, K. A. *et al.* (2010) 'Genetic tools to support the conservation of the endangered smalltooth sawfish, *Pristis pectinata*', *Conservation Genetics Resources*. Springer Netherlands, 2(1), pp. 105–113. doi: 10.1007/s12686-010-9175-8.

Feldheim, K. A. *et al.* (2017) 'Multiple births by a captive swellshark *Cephaloscyllium ventriosum via* facultative parthenogenesis', *Journal of Fish Biology*. Blackwell Publishing Ltd, 90(3), pp. 1047–1053. doi: 10.1111/jfb.13202.

Goodnight, K. F. and Queller, D. Q. (1995) 'Relatedness'. Houston, Texas: Goodnight Software.

Griffiths, A. M. *et al.* (2011) 'Characterisation of polymorphic microsatellite loci in the small-spotted catshark (*Scyliorhinus canicula* L.)', *Conservation Genetics Resources*. Springer Netherlands, 3(4), pp. 705–709. doi: 10.1007/s12686-011-9438-z.

Griffiths, A. M. *et al.* (2012) 'First analysis of multiple paternity in an oviparous shark, the small-spotted catshark (*Scyliorhinus canicula* L.)', *Journal of Heredity*. Oxford University Press, 103(2), pp. 166–173. doi: 10.1093/jhered/esr112.

Harrison, H. B. *et al.* (2013) 'Relative accuracy of three common methods of parentage analysis in natural populations', *Molecular Ecology*, 22(4), pp. 1158–1170. doi: 10.1111/mec.12138.

Henningsen, A. D. *et al.* (2004) 'Reproduction, Embryonic Development, and Reproductive Physiology of Elasmobranchs', in Smith, M. et al. (eds) *Elasmobranch Husbandry Manual: Captive Care of Sharks, Rays, and their Relatives*. Ohio Biological Survey, pp. 227–236.

Hibbitt, J.-D., Rees, E. and Brown, C. (2017) 'Blacktip reef shark reproduction and neonate survivorship in public aquaria', in Smith, M. et al. (eds) *The Elasmobranch Husbandry Manual II : Recent Advances in the Care of Sharks , Rays and their Relatives Editors*. Ohio Biological Survey, pp. 443–450.

Janse, M. *et al.* (2017) 'Sustainable species management of the elasmobranch populations within European aquariums: a conservation challenge', *Journal of Zoo and Aquarium Research*, 5(4), pp. 172–181.

Jones, O. R. and Wang, J. (2010) 'COLONY: a program for parentage and sibship inference from multilocus genotype data', *Molecular Ecology Resources*. Blackwell Publishing Ltd, 10(3), pp. 551–555. doi: 10.1111/j.1755-0998.2009.02787.x.

Koob, T. J. (2004) 'Elasmobranchs in the Public Aquarium: 1860 to 1930', in Smith, M. et al. (eds) *Elasmobranch Husbandry Manual: Captive Care of Sharks, Rays, and their Relatives*. Ohio Biological Survey, pp. 1–14.

Marshall, T. C. *et al.* (1998) 'Statistical confidence for likelihood-based paternity inference in natural populations', *Molecular Ecology*. Blackwell Science Ltd, 7(5), pp. 639–655. doi: 10.1046/j.1365-294x.1998.00374.x.

Mourier, J. and Planes, S. (2013) 'Direct genetic evidence for reproductive philopatry and associated fine-scale migrations in female blacktip reef sharks (*Carcharhinus melanopterus*) in French Polynesia', *Molecular Ecology*, 22(1), pp. 201–214. doi: 10.1111/mec.12103.

van Oosterhout, C. *et al.* (2004) 'MICRO-CHECKER: Software for identifying and correcting genotyping errors in microsatellite data', *Molecular Ecology Notes*, 4(3), pp. 535–538. doi: 10.1111/j.1471-8286.2004.00684.x.

Papastamatiou, Y. P. *et al.* (2010) 'Long-term movement patterns and trophic ecology of blacktip reef sharks (*Carcharhinus melanopterus*) at Palmyra Atoll', *Journal of Experimental Marine Biology and Ecology*. Elsevier, 386(1–2), pp. 94–102. doi: 10.1016/J.JEMBE.2010.02.009.

Phillips, N. M. *et al.* (2011) 'Population genetic structure and genetic diversity of three critically endangered Pristis sawfishes in Australian waters', *Marine Biology*. Springer-Verlag, 158(4), pp. 903–915. doi: 10.1007/s00227-010-1617-z.

Puckett, E. E. (2017) 'Variability in total project and per sample genotyping costs under varying study designs including with microsatellites or SNPs to answer conservation genetic questions', *Conservation Genetics Resources*. Springer Netherlands, 9(2), pp. 289–304. doi: 10.1007/s12686-016-0643-7.

Queller, D. C. and Goodnight, K. F. (1989) 'Estimating relatedness using genetic markers', *Evolution*, 43(2), pp. 258–275. doi: 10.1111/j.1558-5646.1989.tb04226.x.

Raymond, M. and Rousset, F. (1995) 'GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism', *Journal of Heredity*, 86, pp. 248–249.

Rousset, F. (2008) 'Genepop'007: a complete reimplementation of the Genepop software for Windows and Linux', *Molecular Ecol Resources*, 8, pp. 103–106.

Schwan, S., Grajal, A. and Lewalter, D. (2014) 'Understanding and Engagement in Places of Science Experience: Science Museums, Science Centers, Zoos, and Aquariums', *Educational Psychologist*. Routledge, 49(2), pp. 70–85. doi: 10.1080/00461520.2014.917588.

Smith, M. *et al.* (2004) *Elasmobranch husbandry manual: Captive care of sharks, rays, and their relatives*, *The Elasmobranch Husbandry Manual : Captive Care of Sharks , Rays and their Relatives*.

Taylor, H. R. *et al.* (2015) 'Valid estimates of individual inbreeding coefficients from marker-based pedigrees are not feasible in wild populations with low allelic diversity', *Conservation Genetics*. Springer Netherlands, 16(4), pp. 901–913. doi: 10.1007/s10592-015-0709-1.

Thornhill, N. W. (1993) *The Natural history of inbreeding and outbreeding : theoretical and empirical perspectives*. University of Chicago Press.

Toone, W. D. and Wallace, M. P. (1994) 'The extinction in the wild and reintroduction of the California condor (Gymnogyps californianus)', in *Creative Conservation*. Dordrecht: Springer Netherlands, pp. 411–419. doi: 10.1007/978-94-011-0721-1_23.

Trimble, M. J. and van Aarde, R. J. (2010) 'Species Inequality in Scientific Study', *Conservation Biology*. John Wiley & Sons, Ltd (10.1111), 24(3), pp. 886–890. doi: 10.1111/j.1523-1739.2010.01453.x.

Vieira, M. L. C. *et al.* (2016) 'Microsatellite markers: what they mean and why they are so useful.', *Genetics and molecular biology*. Sociedade Brasileira de Genética, 39(3), pp. 312–28. doi: 10.1590/1678-4685-GMB-2016-0027.

Xia, C. *et al.* (2014) 'Reintroduction of Przewalski's horse (*Equus ferus przewalskii*) in Xinjiang, China: The status and experience', *Biological Conservation*. Elsevier, 177, pp. 142–147. doi: 10.1016/J.BIOCON.2014.06.021.

Supplementary Materials

Supplementary Table 6.1
Table of genetic diversity and multiple paternity statuses from microsatellite marker publications of elasmobranch species found in captivity in Europe to date. Data includes IUCN Red List Assessment; vulnerability category (Cat), population trend (Pop Trend: D (decreasing), I(increasing), S(stable), U(unknown)), and whether IUCN status needs updating (Req. Updates); multiple paternity (MP) presence and reference (Reference); number of primers (#P), species-specific (SS), number of individuals (N), observed heterozygosity (Ho), expected heterozygosity (He) and reference for one or more genetic studies. Genetic diversity levels are from wild populations.

	IUCN			**MP**	**Microsatellite information**					
Species	**Cat**	**Pop Trend**	**Req. Updates**	**Presence, Reference**	**#P**	**SS**	***N***	**H_o**	**H_e**	**Reference**
Carcharhinus acronotus	NT	D	No	-	37	Yes	32	0.491	0.508 / 0.666	Giresi *et al.*, 2012 / Portnoy, Hollenbeck, Belcher, *et al.*, 2014
Carcharhinus amblyrhynchos	NT	U	Yes	Yes, Green *et al.*, 2017	15	Yes	60	0.779	0.782	Momigliano *et al.*, 2014; Green *et al.*, 2017
Carcharhinus leucas	NT	U	Yes	-	5		136	0.830	0.840	Karl *et al.*, 2011
Carcharhinus melanopterus	NT	D	Yes	-	17	No	264	0.572	0.581	Mourier and Planes, 2013
Carcharhinus plumbeus	V	D	No	Yes, Daly-Engel *et al.*, 2006	6	No	69	0.538	0.573	Daly-Engel *et al.*, 2006
Negaprion acutidens	V	D	Yes	-	16	No	85	0.621	0.637	Mourier and Planes, 2013
Negaprion brevirostris	NT	U	Yes	Yes, Feldheim, Gruber and Ashley, 2009	9	Yes	80	0.730	0.810	Schultz *et al.*, 2008
Prionace glauca	LC	U	Yes	No, Ovenden *et al.*, 2009; Taguchi *et al.*, 2013; King *et al.*, 2015	31	Mix	842	0.474/ 0.618/ 0.670	0.580 / 0.602 / 0.733	Ovenden *et al.*, 2009 / King *et al.*, 2015 / Taguchi *et al.*, 2013
Triaenodon obesus	NT	U	Yes	-	24	No	2	-	-	Portnoy, Hollenbeck, Johnston, *et al.*, 2014
Cephaloscyllium ventriosum	LC	U	No	-	12	Yes	6	-	-	Feldheim *et al.*, 2017
Poroderma africanum	NT	U	Yes	-	12	No	1	-	-	Maduna *et al.*, 2014
Poroderma pantherinum	DD	U	Yes	-	10	No	4	-	-	Maduna *et al.*, 2014

Scyliorhinus canicula	LC	S	No	Yes, Griffiths *et al.*, 2012	17	Yes	77	0.648	0.648	Griffiths *et al.*, 2011, 2012
Scyliorhinus stellaris	NT	U	Yes	-	11	No	8	0.427	0.413	Griffiths *et al.*, 2011; Hook *et al,.* 2019
Sphyrna lewini	E	U	No	Yes, Rossouw, Wintner and Bester-Van Der Merwe, 2016; Green *et al.*, 2017	18	Mix	133	0.667	0.724	Nance, Daly-Engel and Marko, 2009; Ovenden *et al.*, 2009
Sphyrna tiburo	LC	S	No	Yes, Chapman *et al.*, 2004	4	Yes	119	0.654	0.686	Chapman *et al.*, 2004
Galeorhinus galeus	V	D	Yes	Yes, Hernández *et al.*, 2014	12	Mix	124	0.680	0.720	Chabot and Nigenda, 2011; Bitalo *et al.*, 2015
Mustelus asterias	LC	U	Yes	Yes, Farrell *et al.*, 2011	5	No	122	0.698	0.655	Boomer and Stow, 2010; Farrell *et al.*, 2014
Mustelus californicus	LC	U	No	-	7	No	-	-	-	Chabot and Nigenda, 2011
Mustelus mustelus	V	D	Yes	Yes, Rossouw, Wintner and Bester-Van Der Merwe, 2016	12	Mix	105	0.682	0.534	Bitalo *et al.*, 2015
Triakis semifasciata	LC	U	No	Yes, Nosal, Lewallen and Burton, 2013	4 / 15	Mix		0.858 / 0.545	0.851	Nosal, Lewallen and Burton, 2013 / (Larson, Tinnemore and Ameniya, 2009
Heterodontus portusjacksoni	LC	S	No	-	12	Yes	39	0.614	0.660	Clark *et al.*, 2017; Clark et al. 2017
Carcharias taurus	V	U	Yes	Yes, Townsend *et al.*, 201)	9		212	0.652	0.734	Feldheim *et al.*, 2007; Ahonen, Harcourt and Stow, 2009; Townsend *et al.*, 2015
Aetobatus narinari	NT	D	Yes	Yes, Janse, Kappe and Van Kuijk, 2013	10	Yes	30	0.754	0.732	Sellas *et al.*, 2011
Bathytoshia brevicaudata	LC	S	No	-	11	Yes	1	0.560	0.537	Le Port *et al.*, 2016
Dasyatis thetidis	LC	S	No	-	10		2			Le Port *et al.*, 2016
Ginglymostoma cirratum	DD	U	Yes	Yes (Saville *et al.*, 2002)	9	Yes	29	0.548	0.538	Heist *et al.*, 2003
Chiloscyllium plagiosum	NT	U	Yes	-	12	Yes	34	0.620	0.693	Ding *et al.*, 2009
Orectolobus maculatus	LC	U	No	-	2	No	150	-	-	Corrigan *et al.*, 2008
Stegostoma fasciatum	E	D	No	-	14	Yes	138	0.768	0.742	Dudgeon *et al.*, 2006
Potamotrygon falkneri	DD	U	Yes	-	6	No	30	-	-	Cruz, 2013; Cruz *et al.*, 2015
Potamotrygon motoro	DD	U	Yes	-	10	Yes	34	0.412	0.374	Cruz, 2013; Cruz *et al.*, 2015

Leucoraja naevus	LC	U	No	-	10	No	23	0.542	0.516	El Nagar *et al.*, 2010
Raja asterias	NT	D	No	-	8	No	185	-	-	El Nagar *et al.*, 2010; Gerotto, 2013
Raja clavata	NT	D	Yes	Yes, Chevolot *et al.*, 2007	4	Mix	1375	0.659	0.680	Chevolot *et al.*, 2007; El Nagar *et al.*, 2010
Raja montagui	LC	S	No	-	10	No	23	0.588	0.661	El Nagar *et al.*, 2010
Raja undulata	E	D	Yes	-	17	Yes	108	0.683	0.714	Fox *et al.,* 2018
Urolophus halleri	LC	S	No	-	7	Yes	300	-	0.884	Plank *et al.*, 2010
Squalus acanthias	V	D	No	Yes, Verissimo *et al.*, 2011	8	Yes	474	0.590	0.686	McCauley *et al.*, 2004; Verissimo *et al.*, 2011

Chapter 7

Twins! Microsatellite analysis of two embryos within one egg case in oviparous elasmobranchs

S. A. Hook[1,2], S. M. Musa[1,3], D. M. Ripley[1], J. D. Hibbitt[4], B. Grunow[5], T. Moritz [6,7], H. A. Shiels[1,*]

[1]Faculty of Biology, Medicine and Health, University of Manchester, Manchester, UK

[2]School of Earth and Environmental Sciences, University of Manchester, Manchester, UK

[3]School of Environmental and Natural Resource Sciences, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, Selangor, Malaysia

[4] SEA LIFE Programmes and Engagement, SEA LIFE Weymouth

[5]Leibniz-Institute of Farm Animal Biology, Dummerstorf, Germany

[6]Deutsches Meeresmuseum, Stralsund, Germany

[7]Institut für Zoologie und Evolutionsforschung, Friedrich-Schiller-Universität Jena, Germany

*Corresponding author email: holly.shiels@manchester.ac.uk

Author contributions

HAS devised the project; SAH, carried out molecular lab work, completed genetic data analysis and drafted the manuscript; SMM calculated embryo size, collected photographs; DR reared *S. canicula,* collected tissue samples and edited photographs; BG and TM supplied the *S. canicula* embryos and parentage tissue samples; JDH supplied information on *R. undulata* specimen. SAH, SMM, DR and HAS wrote the paper with input from all authors who have agreed on the final version.

An updated version of this chapter can be found published in PLOS ONE. The reference is:

Hook, S.A, Musa, S.M, Ripley, D.M, Hibbitt, J-D, Grunow, B, Moritz, T., Shiels, H. A. (2019). Twins! Microsatellite analysis of two embroys within one egg case in oviparous elasmobranchs. PLoS ONE 14(12): e0224397.

7.1 Abstract

Elasmobranchs display various reproductive modes, which have been key to their evolutionary success. In recent decades there has been a rise in the number of reported cases of foetal abnormalities including fertilised, double-embryos held within one egg capsule, hereafter referred to as twins. Previously, the occurrences of twin egg cases have been reported in two batoid and one shark species. We report the first cases of twins in three species of oviparous elasmobranchs: the undulate ray (*Raja undulata*), the nursehound (*Scyliorhinus stellaris*), and the small-spotted catshark (*Scyliorhinus canicula*). We investigated the genetic relationships between the twins in *S. stellaris*, and *S. canicula* using microsatellite markers. Whilst the *S. stellaris* twins displayed the same genotypes, we found that the *S. canicula* twin individuals arose through heteropaternal superfecundation. This is the first reported incidence of such a paternity in elasmobranchs. The relationship between environmental change and reproductive strategy in elasmobranchs is unclear and further research is needed to determine its effect on the prevalence and mechanisms of formation of elasmobranch twins.

Keywords: *Raja undulata*, *Scyliorhinus stellaris, Scyliorhinus canicula*, reproduction, captivity, heteropaternal superfecundation

7.2 Introduction

Elasmobranchs comprise almost 1,200 species (Compagno, 2005; Naylor *et al.*, 2012) of sharks and batoids (guitarfishes, sawfishes, skates and rays) (Naylor, Fedrigo and Andrés López, 2005) that display complex reproductive modes, characterised by low numbers of offspring that are born, or hatched, as active, fully-formed individuals (Carrier, Musick and Heithaus, 2004). All extant elasmobranchs employ internal fertilisation, with unique organ systems that increase the efficiency and likelihood of fertilisation, whilst minimising sperm wastage and the predation of unfertilised eggs (Carrier, Musick and Heithaus, 2004; Henningsen *et al.*, 2004). Their diversity of reproductive traits is suggested to be a major selective advantage that has contributed to the group's success (Carrier, Musick and Heithaus, 2012). However, these reproductive traits, coupled with slow growth, long gestation times, and late sexual maturity, have also increased the susceptibility of elasmobranchs to extinction in the current era of overexploitation and climate change (Dulvy *et al.*, 2017).

Elasmobranchs display distinct reproductive modes: oviparity (egg-laying), and viviparity (yolk sac, histotrophic, oophagic) and placental viviparity (Carrier, Musick and Heithaus, 2004; Hamlett *et al.*, 2005). Approximately 43% of elasmobranchs, predominantly the skates and benthic sharks, are oviparous (Compagno, 1990; Last *et al.*, 2016). The female reproductive organs of nearly all oviparous elasmobranchs consists of paired ovaries that each secrete oocytes into individual reproductive tracts (uteri) (Dodd, 1983; Ellis and Shackley, 1997; Coelho and Erzini, 2006; Serra-Pereira *et al.*, 2011). Each uterus comprises of oviducal (shelling) glands and muscular regions, before joining to form one lower uterus to release the fully formed eggs into the environment via the cloaca (Dodd, 1983; Ellis and Shackley, 1997; Coelho and Erzini, 2006; Serra-Pereira *et al.*, 2011). Typically, a single embryo is found within each egg case. Twin egg cases are rare, being reported in the oviparous skates *Leucoraja erinacea* (Richards, Merriman and Calhoun, 1963), *Sympterygia bonapartii* (Jañez and Sueiro, 2009) and one viviparous (yolk sac) shark species *Mustelus asterias* (Farrell, Mariani and Clarke, 2010). Double vitellogenic oocytes have also been observed in *Sympterygia acuta* (Mabragaña *et al.*, 2015). Twin egg cases are only a common feature in the oviparious batoid species *Beringraja binoculata* (Ebert and Davis,

2007), and *Beringraja pulchra* (Ishiyama, 1958; Hitz, 1964; Kang *et al.*, 2013; Howard, 2017). For this reason, Ishihara *et al.,* (2012) proposed a new genus for these species, “Beringraja”(Jeong *et al.*, 2012).

Here we report two individuals formed in the same egg capsule in the undulate ray (*Raja undulata*), and fertilized double-embryo egg cases in the nursehound (*Scyliorhinus stellaris*) and the small-spotted catshark (*Scyliorhinus canicula*), hereafter referring to fraternal double-embryos as twins. Uniquely, our study employed microsatellite analysis to understand the reproductive origins of the double-embryos in *S. stellaris* and *S. canicula*.

7.3 Methods

7.3.1 Sample collection

On the 6th of September 2013 an egg case containing two embryos from *R. undulata* was laid by a wild-caught mother, within a clutch of unknown size, at the SEA LIFE aquarium Weymouth, UK. The *S. stellaris* egg cases were laid in captivity by a wild-caught population at the Native Marine Centre (Portland, UK). The source population of *S. stellaris* individuals had either deposited eggs in captivity after copulation in the wild, or after copulation in the captive environment with other wild-caught individuals. The egg cases from *S. canicula* were from a captive breeding population held at the Deutsches Meeresmuseum (Stralsund, Germany), made up of a source population of both captive and wild individuals.

The egg cases from both shark species were sent to the University of Manchester, UK, at approximately 4 weeks, and 1 week, post-laying, for *S. canicula* and *S. stellaris*, respectively (Ballard, Mellinger and Lechenault, 1993; Musa, Czachur and Shiels, 2018). In Manchester, the embryos were held in 55L seawater tanks at 15˚C, dissolved oxygen > 95 %, and 35ppt salinity, in a 12-hour light-dark cycle, until hatching. To ensure the nitrogenous waste contents was maintained at safe levels for the developing sharks, nitrate, nitrite and ammonia were routinely monitored, and water changes were carried out three times a week. The *S. stellaris* and *S. canicula* egg cases were photographed alongside a ruler, using a Canon PowerShot G16 camera, and the size of the egg cases, embryos, and external yolk

sacs were measured using ImageJ (*ImageJ*). The volumes of the external yolk sacs were calculated using the formula for an ellipsoid.

7.3.2 DNA extraction, amplification and analysis

The *S. stellaris* and *S. canicula* embryos were fin-clipped post-hatch and the tissues stored in 98% ethanol for DNA extraction. A further 6 captive offspring *S. stellaris* samples were added to the dataset to investigate polymorphisms within the species. In *S. canicula,* the potential parents (fathers = 7, mothers = 11), 60 potential siblings, and the twin individuals from the captive breeding program were fin-clipped to analyse parentage (in total n = 80). Samples were extracted using the Bioline Isolate II Genomic kit with an extended digestion time of 10 minutes to maximise the genomic DNA yield. Genomic DNA (20-70ng/µl), was amplified with one primer cocktail containing 5mM of the three tail dyes (FAM, VIC and NED), 5mM of each forward microsatellite marker, and 10mM of each reverse microsatellite loci (Griffiths *et al.*, 2011). The 11 microsatellite primers and thermal cycling conditions were selected from Griffiths *et al.* [20]: Scan02, Scan03, Scan04, Scan05, Scan06, Scan09, Scan10, Scan12, Scan12, Scan15 and Scan16. PCR reactions consisted of 1µl of genomic DNA, 1µl of the primer cocktail, 3µl of ddH2O, and 5µl of QIAGEN Multiplex PCR Kit (*QIAGEN Multiplex PCR Kit*, 2019). The products were genotyped using an ABI™ 3730XL capillary sequencer with GeneScan™ 500 LIZ™ dye Size Standard and scored using GeneMapper v.4.0 (Applied Biosystems). Allele scores were checked for user error in Microchecker v.2.2.3 (van Oosterhout *et al.*, 2004).

GenePop (v 4.2) (Michel Raymond and Rousset, 1995) was used to calculate observed heterozygosity (H_o), expected heterozygosity (H_e) and number of alleles per locus (N_a). Cervus (Marshall *et al.*, 1998) was used to calculate polymorphism information content (PIC) and frequency of null alleles F(Null) (29). Parentage analysis for all offspring of *S. canicula* was determined using a full-likelihood and pair-likelihood-score combined (FPLS) method in Colony (Jones and Wang, 2010) and using a parent-pair log-likelihood ratio (LOD) analysis in Cervus (Marshall *et al.*, 1998). Colony analysis was conducted under the assumption of female and male polygamy without inbreeding or clones. The simulation program within Cervus was used to produce 10,000 offspring and parental genotypes from

allele frequencies taken from the North-Atlantic sampled by Gubili *et al.,* (Gubili *et al.*, 2014)to generate statistically significant LOD scores at a confidence level of 95%. Microsatellite markers for both *S. stellaris* and *S. canicula* that displayed PIC values ≥0.500 were displayed for the twins and six individuals to visually highlight similarities and differences in the genotypes.

7.4 Results

7.4.1 Undulate Ray, *Raja undulata*

The *R. undulata* twin embryo egg case length and width (excluding horns) was 58mm and 35mm respectively. While there was no reported difference at the time, these measurements show the egg case length to be slightly shorter when compared to other egg cases in the same clutch and those typical for the species (80.4 ± 4.4mm) (Caldeira, 2006; Luer *et al.*, 2007; Gordon, Hood and Ellis, 2016). During incubation the egg case was kept with others of the same clutch in 2500L natural seawater and maintained at 16.5˚C ± 1.8˚C with a dissolved oxygen of >95% and a salinity of 35ppt. Appropriate life support systems were also in place to ensure the nitrogenous waste contents were maintained at safe levels for the developing egg cases. On the 23rd of April 2014 the egg case displayed signs of being unviable and so was opened, revealing two small dead juveniles (Figure 7.1, A1). One juvenile was smaller and exhibited the early signs of decay with no evidence of a yolk sack while the larger juvenile was in the final stages of yolk sack absorption. It is unknown if the individuals were attached to a single yolk, or whether the egg consisted of two separate yolks. The wingspan of the larger individual within the twin egg case was 4 cm (Figure 7.1, A2, A3), whereas a fully developed, healthy individual which hatched 8 days later from the same clutch had a wingspan of 9 cm (not shown).

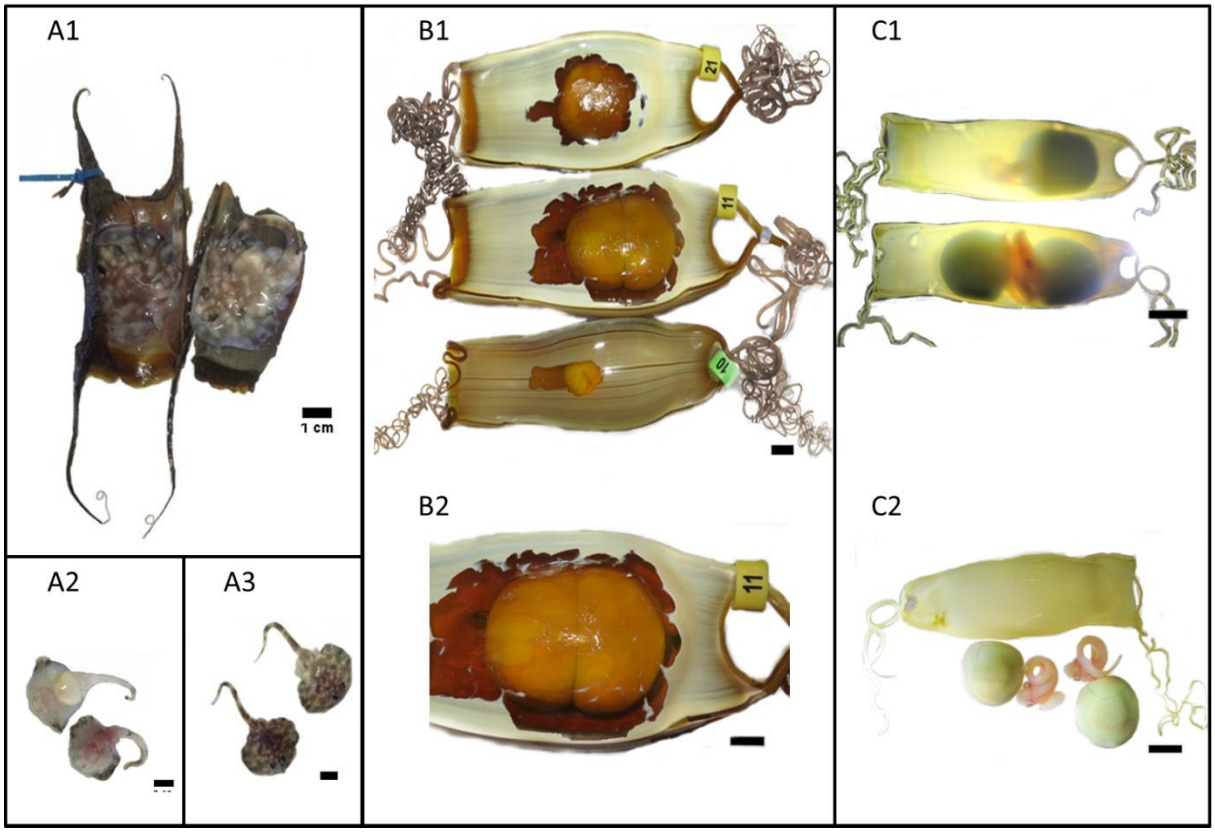

Figure 7.1. (A1) Two *R. undulata* juveniles in one, splayed open, egg case. (A2) The ventral surface and (A3) the dorsal surface of the two *R. undulata* twins. (B1) The *S. stellaris* twin egg case (middle) next to its paired-egg case and another egg (bottom). (B2) Closer view of the twined egg case. (C1) *S. canicula* twin embryos (bottom) next to a non-twin sibling (top). (C2) *S. canicula* twins after removal from their egg case. The black scale bars are 1cm in length.

7.4.2 Nursehound, *Scyliorhinus stellaris*

The *S. stellaris* twin egg case was larger than its paired egg case (i.e. the case laid at the same time as the twin egg case from the other oviduct, Figure 7.1, B1). The twin egg case, excluding the tendrils, was 12.25 cm long, 5.65 cm wide and 3.70 cm deep. The average size of *S. stellaris* egg cases from the same cohort was 11.58 ± 0.1 cm, 4.32 ± 0.05 cm, 2.88 ± 0.06 cm in length (excluding the tendrils), width, and depth respectively (mean ± SEM, n = 12). At 12 weeks of development the external yolk sacs of the *S. stellaris* twins had a combined volume of 37.17 cm^3 (Figure 7.1, B2), more than twice that of a single yolk sac from a typical *S. stellaris* embryo reared under the same conditions (15.91 ± 0.93 cm^3, mean ± SEM, n = 12). The twins survived for 12 weeks and developed to stage 20 defined by Ballard *et al.* (1993), and stage 3 defined by Musa *et al.* (2018), with total body lengths of 0.70 cm and 0.80 cm.

Genetic analysis of the *S. stellaris* twins revealed identical genotypes on all 9 successfully amplified loci; individuals did not amplify with Scan06 and Scan16. The PIC was ≥0.500 for 4 of the 11 microsatellites for all samples (Table 7.1). As the individuals were developed from two yolks, rather than being monovular (i.e. two individuals with a single yolk), the monozygosity in the genotypes probably emanates from a lack of species-specific loci, and therefore a loss of interrogated diversity between the twins. Of the markers used Scan02, Scan09, Scan10, Scan13, and Scan15 displayed the highest H_o and H_e levels for the greatest number of individuals (87.5% to 100% of the sample size) and N_a for each was equal to or above 4 (Table 7.2). Overall, average genetic diversity for all eleven markers was H_o = 0.427 and H_e = 0.413 (Table 7.2).

Table 7.1. Genotypic information gathered from each microsatellite locus for the twin individuals (DY1, DY2) and a further randomly selected 6 possible siblings (SIB1-SIB6) of *S. stellaris* and *S. canicula* to display the genotype variance. Microsatellites with a polymorphism information content (PIC) value equal to or higher than 0.500 were used to display genotypes. Genotypes are displayed in base pairs (BP)

S. stellaris								
ID	***Scan02***		***Scan 09***		***Scan10***		***Scan15***	
DY1	135	137	131	133	268	276	250	252
DY2	135	137	131	133	268	276	250	252
SIB1	125	137	-	-	268	274	248	250
SIB2	125	139	133	133	274	274	248	250
SIB3	125	139	133	133	266	274	250	250
SIB4	137	139	133	137	268	268	248	250
SIB5	123	137	131	133	268	276	250	252
SIB6	133	141	129	131	274	276	258	260

S. canicula												
ID	***Scan02***		***Scan04***		***Scan06***		***Scan12***		***Scan15***		***Scan16***	
DY1	132	136	257	265	233	237	119	121	254	256	283	285
DY2	136	142	257	257	227	237	119	121	254	260	283	283
SIB1	132	134	257	267	233	237	119	121	254	256	283	285
SIB2	136	140	257	265	227	227	117	119	258	260	281	287
SIB3	132	144	257	265	227	233	119	121	256	260	279	281
SIB4	132	132	257	265	229	237	117	119	256	258	283	285
SIB5	132	132	265	265	227	229	117	119	254	258	283	285
SIB6	132	134	257	263	229	237	119	119	256	258	283	285

Table 7.2. Microsatellite information gathered from each locus for the entire population studied (including the twins) for *S. stellaris* and *S. canicula*. N% = Percentage of individuals scored, Na = numbers of alleles, H e= expected heterozygosity, HO = observed heterozygosity.

Loci information		***S. stellaris***				***S. canicula***			
Locus	**Tail dye**	***N%***	***Na***	***H_e***	***H_o***	***N%***	***Na***	***H_e***	***H_o***
Scan 02	NED	100	7	1.000	0.858	100	6	0.745	0.738

Scan 03	FAM	63	3	0.200	0.378	96	4	0.513	0.182
Scan 04	VIC	50	3	0.250	0.607	98	5	0.689	0.474
Scan 05	NED	100	2	0.125	0.125	100	4	0.543	0.450
Scan 06	FAM	38	1	0.000	0.000	100	9	0.730	0.713
Scan 09	VIC	88	4	0.714	0.626	100	2	0.025	0.000
Scan 10	NED	100	4	0.750	0.742	98	5	0.585	0.526
Scan 12	FAM	100	2	0.125	0.125	100	6	0.666	0.638
Scan 13	VIC	100	4	0.500	0.517	96	4	0.382	0.429
Scan 15	FAM	100	5	0.875	0.717	98	5	0.739	0.859
Scan 16	VIC	38	1	0.000	0.000	98	6	0.730	0.756

7.4.3 Small-spotted catshark, *Scyliorhinus canicula*

The twin *S. canicula* egg case (Figure 7.1, C1) was 6.52 cm in length (excluding the tendrils), 2.21 cm in width, and 1.57 cm in depth, making it slightly larger than the single embryo egg cases from the same clutch (6.33 ± 0.04 cm, 2.18 ± 0.05 cm, 1.36 ± 0.02 cm in length, width and depth respectively, mean ± SEM, n = 11). The lengths of the *S. canicula* twins at approximately 9 weeks post-laying were 4.67 cm and 4.69 cm, whilst the external yolk sac volumes measured 2.63 cm^3 and 2.75 cm^3. The lengths and key morphological features suggest that the twins reached somewhere between stages 28 and 32 of the Ballard *et al.* (1993) (Ballard, Mellinger and Lechenault, 1993) developmental scale, and stage 4 of the Musa *et al.* (2018) (Musa, Czachur and Shiels, 2018) developmental scale. Due to concern for their well-being, the egg case containing the *S. canicula* twins was opened and the embryos (Figure 7.1, C2) were transferred to individual artificial egg cases with larger dimensions and continued their development at 15˚C. Both animals survived with good health to hatch.

The average genetic diversity for all microsatellites was H_o = 0.524 and H_e = 0.577 (Table 7.2). The PIC was ≥0.500 on 6 of the 11 microsatellites (Table 7.1). Parentage analysis suggested that the twins derived from different paternities. Cervus parent pair non-exclusion probabilities all equal to or less than 1.30E-03 and Colony probability index of parent pairs were between 0.516 and 1.000 accurate (Table 7.2). Cervus gave more conclusive results in parentage due to the simulations for the log-likelihood ratio. These results suggest heteropaternal superfecundation (individuals from separate paternities, and

therefore products of two distinct copulatory events) for the twins (DY1 and DY2) in *S. canicula* (Table 7.3).

Table 7.3. Results of parentage assignment from Cervus and Colony for *S.canicula*. Trio log-likelihood ratio (Trio LOD score) is the probability of relationship between the offspring, mother and father. Probability Index= probability of family clusters. DY = Twin individuals, SI = possible sibling individuals

	Cervus			Colony		
Offspring ID	***Candidate mother ID***	***Candidate father ID***	***Trio LOD score***	***Candidate Mother***	***Candidate Father***	***Probability Index***
DY1	MotherB6	FatherB1	7.92E+00	MotherB6	FatherB1	1
DY2	MotherB6	FatherB2	2.70E+00	MotherB6	FatherB2	0.512
SIB1	MotherB6	FatherB1	3.49E+00	MotherB6	FatherB1	0.512
SIB2	MotherB3	FatherB1	6.69E+00	MotherB3	FatherB1	1
SIB3	MotherB3	FatherB1	4.81E+00	MotherB3	FatherB1	1
SIB4	MotherB5	FatherB4	2.33E+00	B2	*	1
SIB5	MotherB5	FatherB1	1.75E+00	B2	*	1
SIB6	MotherB5	FatherB4	3.01E+00	B2	*	1

7.5 Discussion

Here we report the first incidence of an egg case containing two embryos in the oviparous *Raja* elasmobranch, the undulate ray (*R. undulata*). We also add two new species of oviparous benthic sharks (*S. stellaris and S. canicula*) to the list of elasmobranchii twin eggs, and provide the first genetic evidence of heteropaternal superfecundation in *S. canicula*.

The *S. canicula* and *S. stellaris* eggs all had two yolk sacs, indicating that two oocytes were released into the same oviducal gland for shelling in a single egg case. Genetic analysis revealed that the *S. canicula* twins were from heteropaternal superfecundation, meaning that each oocyte was fertilized by a different male, and thus suggesting sperm storage within the oviducal gland. Previous findings showed that females isolated from males for up to two years can produce fertile eggs (Dodd, 1983), displaying longevity of the sperm and sperm storage which could account for the heteropaternal superfecundation reported here, if the female only mated with one individual during ovulation.

The mechanisms of double-embryo formation in the three oviparous elasmobranch species cannot be fully elucidated until development is tracked from ovary secretion, through

the oviducal gland, to deposition. However, our findings are the first reported cases of shark twins in captive environments and provide the first evidence of heteropaternal superfecundation in a species of oviparous elasmobranch. The evolution of twin egg cases as a method of reproductive biology may have implications on the success of the species, if such individuals are unlikely to survive. However, if viable, increasing the number of individuals per reproductive output by producing twin egg cases would be advantageous, especially in a group facing massive declines. Overall we found that there are an increasing number of reports on the occurrence of reproductive mutations such as double-embryo egg cases and conjoined individuals. This could be accounted to higher number of individuals reporting twin occurrences. The captive species which produce twin egg cases usually display high reproductive performance and plasticity (Jañez *et al.*, 2018), although without human input, twin egg cases typically do not succeed to hatch (Mabragaña *et al.*, 2015). Considering the significant stress on wild populations of sharks and rays, further research is needed to understand and identify the mechanisms producing, and consequences of, elasmobranch twins.

7.6 Acknowledgements

We would like to thank Amy Callaghan and Native Marine Centre who supplied the greater spotted catshark *S. stellaris* egg cases and the Shiels Lab members from the University of Manchester for their continued support and ideas. Lastly we would like to thank Dr. Michael Buckley and Dr. Ana Verissimo for their continuous guidance.

7.7 References

Ballard, W. W., Mellinger, J. and Lechenault, H. (1993) 'A series of normal stages for development of *Scyliorhinus canicula*, the lesser spotted dogfish(Chondrichthyes: Scyliorhinidae)', *Journal of Experimental Zoology*. John Wiley & Sons, Ltd, 267(3), pp. 318–336. doi: 10.1002/jez.1402670309.

Caldeira, B. F. (2006) 'Morfologia e biometria do desenvolvimento embrionário da raia *Sympterygia acuta* Garman, 1877 (Elasmobranchiil; Rajidae)'.

Carrier, J. C., Musick, J. A. and Heithaus, M. R. (2012) *Biology of sharks and their relatives*. Taylor & Francis Group.

Carrier, J., Musick, J. and Heithaus, M. (2004) *Biology of Sharks and Biology of Marine Birds*.

Coelho, R. and Erzini, K. (2006) 'Reproductive aspects of the undulate ray, Raja undulata, from the south coast of Portugal', *Fisheries Research*. Elsevier, 81(1), pp. 80–85. doi: 10.1016/J.FISHRES.2006.05.017.

Compagno, L. J. V. (1990) 'Alternative life-history styles of cartilaginous fishes in time and space', *Environmental Biology of Fishes*. Kluwer Academic Publishers, 28(1–4), pp. 33–75. doi: 10.1007/BF00751027.

Compagno, L. J. V. (2005) 'Checklist of living Chondrichthyes', *Reproductive biology and phylogeny of Chondrichthyes : sharks, batoids and chimaeras*. Science Publishers, pp. 503–548.

Dodd, J. M. (1983) *Reproduction in Cartilaginous Fishes (Chondrichthyes)*, *Fish Physiology*. Academic Press.

Dulvy, N. K. *et al.* (2017) 'Challenges and Priorities in Shark and Ray Conservation', *Current Biology*. Cell Press, 27(11), pp. R565–R572. doi: 10.1016/J.CUB.2017.04.038.

Ebert, D. A. and Davis, C. D. (2007) 'Descriptions of skate egg cases (Chondrichthyes: Rajiformes: Rajoidei) from the eastern North Pacific', *Zootaxa*, 1393, pp. 1–18. doi: 10.11646/zootaxa.1393.1.1.

Ellis, J. R. and Shackley, S. E. (1997) 'The reproductive biology of *Scyliorhinus canicula* in the Bristol Channel, U.K.', *Journal of Fish Biology*. Wiley/Blackwell (10.1111), 51(2), pp. 361–372. doi: 10.1111/j.1095-8649.1997.tb01672.x.

Farrell, E. D., Mariani, S. and Clarke, M. W. (2010) 'Reproductive biology of the starry smooth-hound shark *Mustelus asterias*: geographic variation and implications for sustainable exploitation', *Journal of Fish Biology*. Wiley/Blackwell (10.1111), 77(7), pp. 1505–1525. doi: 10.1111/j.1095-8649.2010.02771.x.

Gordon, C. A., Hood, A. R. and Ellis, J. R. (2016) 'Descriptions and revised key to the eggcases of the skates (Rajiformes: Rajidae) and catsharks (Carcharhiniformes: Scyliorhinidae) of the British Isles.', *Zootaxa*, 4150(3), pp. 255–80.

Griffiths, A. M. *et al.* (2011) 'Characterisation of polymorphic microsatellite loci in the small-spotted catshark (*Scyliorhinus canicula* L.)', *Conservation Genetics Resources*. Springer Netherlands, 3(4), pp. 705–709. doi: 10.1007/s12686-011-9438-z.

Gubili, C. *et al.* (2014) 'A tale of two seas: contrasting patterns of population structure in the small-spotted catshark across Europe', *Royal Society Open Science*. The Royal Society Publishing, 1(3), p. 140175. doi: 10.1098/rsos.140175.

Hamlett, W. *et al.* (2005) 'Oviducal glands in chondrichthyans', in Hamlett, W. (ed.) *Reproductive biology and phylogeny of chondrichthyes: sharks, batoid and chimaeras*. Enfield: Science Publishers Inc, pp. 301–336.

Henningsen, A. D. *et al.* (2004) 'Reproduction, Embryonic Development, and Reproductive Physiology of Elasmobranchs', in Smith, M. et al. (eds) *Elasmobranch Husbandry Manual: Captive Care of Sharks, Rays, and their Relatives*. Ohio Biological Survey, pp. 227–236.

Hitz, C. R. (1964) 'Observations on Egg Cases of the Big Skate (*Raja binoculata* Girard) Found in Oregon Coastal Waters', *Journal of the Fisheries Research Board of Canada*. NRC Research Press Ottawa, Canada , 21(4), pp. 851–854. doi: 10.1139/f64-076.

Howard, M. . (2017) 'Fecundity, egg capsule size and neonate morphometrics of big skate, *Beringraja binoculata* (Girard, 1855)', in Smith, M. et al. (eds) *The Elasmobranch Husbandry Manual II: Recent Advances in the Care of Sharks, Rays and their Relatives. Special Publication of the Ohio Biological Survey.* Ohio Biological Survey, p. 451.

ImageJ (no date). Available at: https://imagej.nih.gov/ij/.

Ishiyama, R. (1958) 'Studies on the rajid fishes (Rajidae) found in the waters around Japan', *J Shimonoseki Coll Fish*, 7, pp. 191–394.

'ISOLATE II Genomic DNA Kit Product Manual'. Available at: http://www.bioline.com/downloads/dl/file/id/873/isolateii_genomic_pm_web.pdf (Accessed: 5 September 2017).

Jañez, J. A. *et al.* (2018) 'Growth and reproduction in captivity unveils remarkable life-history plasticity in the smallnose fanskate, *Sympterygia bonapartii* (Chondrichthyes: Rajiformes)', *Neotropical Ichthyology*, 16(4). doi: 10.1590/1982-0224-20180013.

Jañez, J. A. and Sueiro, M. C. (2009) *Scientific Note Oviposition rate of the fanskate Sympterygia bonapartii (Elasmobranchii, Rajidae) (Müller & Henle, 1841) held in captivity*, *Pan-American Journal of Aquatic Sciences*.

Jeong, C.-H. *et al.* (2012) 'The Comparative Morphology of Skate Egg Capsules (Chondrichthyes: Elasmobranchii: Rajiformes)', *Bull. Kanagawa prefect. Mus. Nat. Sci*, (41), pp. 17–33.

Jones, O. R. and Wang, J. (2010) 'COLONY: a program for parentage and sibship inference from multilocus genotype data', *Molecular Ecology Resources*. Blackwell Publishing Ltd, 10(3), pp. 551–555. doi: 10.1111/j.1755-0998.2009.02787.x.

Kang, H.-W. *et al.* (2013) 'Spawning Characteristics and Artificial Hatching of Female Mottled Skate, Beringraja pulchra in the West Coast of Korea.', *Development & reproduction*. Korean Society of Developmental Biology, 17(3), pp. 247–55. doi: 10.12717/DR.2013.17.3.247.

Last, P. *et al.* (2016) *Rays of the World*. 1st Edition. Australia: CSIRO Publishing.

Luer, C. A. *et al.* (2007) 'Normal embryonic development in the clearnose skate, *Raja eglanteria*, with experimental observations on artificial insemination', in *Biology of Skates*. Dordrecht: Springer Netherlands, pp. 133–149. doi: 10.1007/978-1-4020-9703-4_9.

Mabragaña, E. *et al.* (2015) 'Seasonal Reproductive Biology of the Bignose Fanskate *Sympterygia acuta* (Chondrichthyes, Rajidae)', *Estuaries and Coasts*. Springer US, 38(5), pp. 1466–1476. doi: 10.1007/s12237-014-9888-0.

Marshall, T. C. *et al.* (1998) 'Statistical confidence for likelihood-based paternity inference in natural populations', *Molecular Ecology*. Blackwell Science Ltd, 7(5), pp. 639–655. doi: 10.1046/j.1365-294x.1998.00374.x.

Musa, S. M., Czachur, M. V and Shiels, H. A. (2018) 'Oviparous elasmobranch development inside the egg case in 7 key stages.', *PloS one*. Public Library of Science, 13(11), p. e0206984. doi: 10.1371/journal.pone.0206984.

Naylor, G., Fedrigo, O. and Andrés López, J. (2005) *Phylogenetic Relationships among the Major Lineages of Modern Elasmobranchs*.

Naylor, G. J. P. *et al.* (2012) 'A DNA Sequence–Based Approach To the Identification of Shark and Ray Species and Its Implications for Global Elasmobranch Diversity and Parasitology', *Bulletin of the American Museum of Natural History*. American Museum of Natural History, 367(367), pp. 1–262. doi: 10.1206/754.1.

van Oosterhout, C. *et al.* (2004) 'MICRO-CHECKER: Software for identifying and correcting genotyping errors in microsatellite data', *Molecular Ecology Notes*, 4(3), pp. 535–538. doi: 10.1111/j.1471-8286.2004.00684.x.

QIAGEN Multiplex PCR Kit (2019). Available at: https://www.qiagen.com/gb/shop/pcr/end-point-pcr-enzymes-and-kits/regular-pcr/qiagen-multiplex-pcr-kit/.

Raymond, Michel and Rousset, F. (1995) 'An exact test for population differentiation', *Evolution*. John Wiley & Sons, Ltd (10.1111), 49(6), pp. 1280–1283. doi: 10.1111/j.1558-5646.1995.tb04456.x.

Raymond, M and Rousset, F. (1995) 'GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism', *Journal of Heredity*, 86, pp. 248–249.

Richards, S., Merriman, D. and Calhoun, L. (1963) 'The biology of the little skate, Raja erinaecea Mitchell', *Studies on the marine resources of southern New England. IX. . Bulletin of the Bingham Oceanographic Collection*, 18(3), pp. 5–67.

Serra-Pereira, B. *et al.* (2011) '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', *Ivone Figueiredo & Leonel Serrano Gordo*. Taylor & Francis Group , 3(1), pp. 160–175. doi: 10.1080/19425120.2011.555707.

SECTION IV: DISCUSSION AND CONCLUSIONS

8. Discussion and Conclusions

8.1 Impact summary

Sharks, rays and skate (elasmobranchs) are among many species currently facing global mass extinction due to man-made events (Stachowicz *et al.*, 1999; Cardinale *et al.*, 2012; Hooper *et al.*, 2012). As of 2014, a quarter of all elasmobranchs were classified as threatened with extinction under The IUCN Red List, and more information is required to analyse extinction risk for their closest relative within the class of chondrichthyes, the group holocephali (chimaeras) (Dulvy *et al.*, 2014). A limited amount of information is present throughout most elasmobranch species, and often this information is required to make appropriate assessments for management and conservation (Dulvy *et al.*, 2017). Here, the thesis reports on the development and application of techniques to review species biology and population structure for management and conservation.

Throughout the thesis, there has been an emphasis on using samples which are either a discard product or are collected non-intrusively. This ensures the studies have not contributed to the decline of wild populations. In achieving this, a new technique for mucus containing DNA collection using scuba divers was developed for the undulate ray *Raja undulata,* with evidence from other work that this could be applied to more species (Kashiwagi *et al.*, 2015; Hunter, 2016; Fox *et al.*, 2018). The method development of a new technique of species identification using proteins was explored with elasmobranch discard products in Chapter 3. The aims of this were to eventually apply the method to degraded products such as shark fin soup, as it is estimated a 100 million sharks are killed for their fins annually (Worm *et al.*, 2013). This method development was a second chapter on the same set of samples, increasing the purpose of a product already defined as a discard. The samples for chapter 2 and 3 are the first to be collected in Morocco and chapter 2 reviews the true impact of chondrichthyan fishing in a previously unexplored region by identifying species genetically and reviewing genetic diversity. The methods of individual identification developed and applied in chapters 4 and 5 offer alternatives to invasive tagging of fragile

elasmobranch populations, as invasive approaches have been found to adversely affect populations they are trying to study and conserve.

By applying genetic techniques on captive populations, the thesis has also discovered new reproductive traits and defined family relationships, which were used to manage captive populations of two shark species: the small-spotted catshark *Scyliorhinus canicula* and the blacktip reef shark *Carcharhinus melanopterus*. Additionally, by applying microsatellite analysis to wild populations of *R. undulata*, chapter 5 has defined the different populations and their genetic diversity across their habitat range, which ultimately will influence the management of fisheries due to the high levels of fragmentation found between the areas studied. Similarly, the application of capture mark-recapture techniques on the single site of *R. undulata* helps further the understanding into the relationships and movements between individuals, so that better management for conservation can be applied, especially in this species which is globally classified as endangered.

As elasmobranchs continue to decline, it is imperative that further studies are not only conducted but applied in order to conserve this group for the future. The outcomes of this thesis have generated a deeper understanding of the biology, connectivity and utilisation of elasmobranch populations in both *in-situ* and *ex-situ* sites. Ultimately it is the responsibility of scientists and governments to continue the research into elasmobranch biology and population structure, to influence management, conservation and social change.

8.2 Overview

The central aim of the thesis was to investigate some of the key areas of elasmobranch research that had not yet been fully scrutinised in order to create a better understanding of species populations for conservation and management. The literature review defined four key issues which had insufficient data required for general conservation and management. The key issues were defined as:

1. General biology of certain species of elasmobranchs
2. Population dynamics including genetic diversity, connectivity, number of individuals and regional threats for certain species

3. Regional management implemented by governments; inadequate funds, technical ability and politics inadvertently assisting IUU fishing
4. *Ex-situ* conservation management

Although these key issues can be considered as extensive work that may even be beyond the current limits of scientific technology, this thesis presented new information and novel techniques which increase the current level of knowledge within each subject area. Structurally, the results of this thesis were presented in two distinct sections, the first relating to applying methods for species identification for chondrichthyan conservation and management, and the second to population dynamics for endangered species of rays and captive species of sharks. To ensure the real-world applications of the research, most of the research chapters have incorporated at least one collaborator who has utilised the information produced. The thesis has capitalized on the increased support from conservation organisations as well as the public, to successfully investigate those key issues from the literature that were considered.

8.3 General biology of certain species of elasmobranchs

Chapter 7 reported the first occurrence of twins in one species of oviparous batoid, the undulate ray *Raja undulata,* and two species of oviparous benthic sharks *Scyliorhinus stellaris* and *Scyliorhinus canicula*, adding information of the reproductive biology of these three elasmobranch species. To further understand the single occurrence within our studies, the chapter describes the first exploration into the paternity of twin individuals and found the first genetic evidence of heteropaternal superfecundation in *S. canicula*, defined as two individuals in the same egg having different paternities. Previously, multiple paternity had been identified in litters of *S. canicula* (Griffiths *et al.*, 2012), however the results presented here show that multiple paternity can occur in the same uterus of this oviparous shark.

Within other species of viviparous and ovoviviparous elasmobranchs, observations of reproductive biology in captive environments have also led to significant contributions to further understanding reproduction. For example, from ultrasounds conducted on pregnant captive tawny nurse sharks, *Nebrius ferrugineus,* the first reliable evidence of active embryonic locomotion in live-bearing vertebrates was found for possible predation on sibling

eggs in the uteri (Tomita *et al.*, 2018). Similarly, the discovery of parthenogenesis in elasmobranchs was due to observations conducted in captivity, such as in the zebra shark, *Stegostoma fasciatum,* (Robinson *et al.*, 2011) and white-spotted bamboo shark, *Chiloscyllium plagiosum* (Feldheim *et al.*, 2010). Essentially, without the ability to observe and investigate the biology and behaviour of elasmobranchs within captivity, it would be difficult to collect further information on general reproductive biology. Fundamentally, scientific understanding is the result of observational work, which leads to further investigation. Early era naturalists used observational data to advance science, which today is vastly improved by advances in technology such as the ability to investigate genetics (Sagarin and Pauchard, 2010). Observational data is often the foundation for testing hypothesis and investigation of twins in oviparous elasmobranchs follows this same principle. Although observational data can be used as a single entity to study species, these approaches are strengthened when combined with experimental investigations that can isolate fine scale biological mechanisms (Sagarin and Pauchard, 2010). As the field of conservation continues to expand into a dynamic study area, it is important to continue to observe and investigate even the most basic of findings, particularly if the observation has not been previously reported or if new technologies can advance the understanding of the observation. Future work for conservation and management may depend upon information on general reproductive biology, especially if for example, more twin eggs are observed, or if populations decline and there is a greater reliance on captive bred individuals.

To gain further information on general reproductive biology using microsatellite analysis, the parentage of three litters of captive-bred blacktip reef sharks *Carcharhinus melanopterus* was investigated as part of Chapter 6. Similar to the research conducted on the twin elasmobranchs *S. canicula* and *S. stellaris*, microsatellite analysis was performed to investigate a simple hypothesis derived from observational data. This analysis found conclusive evidence of multiple paternity in one of three litters. Despite previous extensive research in the French Polynesia, no evidence of multiple paternity had been found in *C. melanopterus* prior to the results presented in Chapter 6 (Mourier and Planes, 2013; Hibbitt, Rees and Brown, 2017). As *ex-situ* management, such as the application of studbooks, is increasing, findings of new multiple paternities are important not only for the knowledge of

general biology but to help achieve healthy captive populations. If reproductive traits such as multiple paternity are not known with captive species, it is possible that individuals may inbreed or outbreed. One of the major goals for captive breeding programmes through studbook management is to conserve the genetic variability within populations, which can be lost through both inbreeding and outbreeding effects (Thornhill, 1993; Witzenberger and Hochkirch, 2011). The true number of species which can exhibit multiple paternity or other reproductive traits such as parthenogenesis is yet to be explored. Furthermore, there has yet to be conclusive studies into the evolutionary divergence of multiple paternity, however it is thought that the two of more paternities per offspring cluster increases genetic variation and therefore offspring survivability (Farrell *et al.*, 2014; Corrigan, Kacev and Werry, 2015; Rossouw, Wintner and Bester-Van Der Merwe, 2016). Overall however, there is still a large amount regarding general elasmobranch biology yet to be discovered. Without knowledge of general elasmobranch biology or ecology, such as breeding grounds and nursery habitat, or information regarding development to adulthood, defined as reaching sexual maturity, it is difficult to make proper assessments for population management and conservation.

To increase knowledge of morphological development in the early stages of *S. canicula* post-hatch, and to develop the method of identifying individuals without the use of invasive techniques, spot pattern recognition technology was explored. Chapter 3 reports that up to six months old spot patterns stabilised with age (post-hatch). The technique was also validated with the use of microsatellite markers. Chapter 3 represents the first study where spot pattern recognition has been applied to individuals in early stages of development and the first genetic analysis which has proven spot pattern recognition is a valid technique for individual identification. Photo recognition for individual identification in CMR studies has previously been applied in young to mature adult elasmobranchs such as white sharks, *Carcharodon carcharias* (Gubili *et al.*, 2009) and sand tiger sharks, *Carcharias taurus* (van Tienhoven *et al.*, 2007). Traditional methods of capturing individuals through fishing, and releasing with an invasive tag has become less popular as results from previous studies found that the method can lead to rates of post-release mortality or signs of captive stress such as increased swimming activity (Ellis, McCully Phillips and Poisson, 2017). Although the first development of our technique still relied upon the use of removing

individuals from their main tank and placing them in a smaller tank for photographing. Future work should expand the abilities in this non-intrusive method, such as automatic recognition software or training individuals to enter small holding tanks with food incentives, so no handling is involved.

8.4 Population dynamics

By confirming that pattern recognition is a successful method for individual identification in Chapter 4, a technique was used on wild population of undulate rays *Raja undulata* for Chapter 5. Photographs were taken by divers on a single-site on the south coast of the UK over a 7-year period, rendering this as the first long-term study completed on a wild set of undulate rays in the UK, with the use of photo recognition. The individual identification allowed for a full CMR study to be completed for the seven years, including reviewing population size, survivorship and social behaviour. Despite a low probability of recapture ($p = 0.035$), believed to be limited by the sampling through non-intrusive photographs collected by scuba divers, the individual survivorship rate between visits was high ($n = 263$, $\varphi = 0.969$). Using a network analysis to investigate the social behaviour of the *R. undulata*, distinct pairs of rays were present at a higher frequency than is expected by chance (95% confidence interval $P \leq 0.02$), indicating a high probability of same pair migration and social interaction.

The investigation into the population dynamics and connectivity of *R. undulata* using invasive and non-intrusive sampling was originally the main aim of Chapter 5. Samples were collected across their distribution except for France, due to limitations including accessing viable sites and samples. Similar to the UK, France fisheries regions within the English Channel , prohibited the landing of *R. undulata* species under EU laws (CEC, 2010; Ellis, McCully and Brown, 2012). The genetic information from this region could hold key information regarding individuals crossing the English Chanel, or the migration patterns from the English Chanel to the North Atlantic, along the west coast of France, into the North and West coastal regions of Portugal. Secondly, although sampling efforts were conducted in the Mediterranean, including the coastal town of Valencia, Spain and the Tyrrhenian Sea, including Palermo, Sicily and Rome, mainland Italy, no samples from true identified *R.*

undulata could be collected. The *R. undulata* is known to be sporadically fragmented through the Mediterranean with no confirmed knowledge of its area of occupancy, due to limited fishing records and IUU fishing (Stéphan *et al.*, 2014; Ellis and McCully, 2016). These fragmented populations within a suspected threshold region of 2,000 km^2 were classified as near threatened in 2016 by The IUCN Red List (Ellis and McCully, 2016). The absence of viable fishing records and the lack of information regarding any possible breeding activity combined with high fragmentation between populations (Ellis and McCully, 2016), limited our ability to accurately locate samples in the Mediterranean for DNA analysis.

Despite the lack of samples from this region, it was evident that there were six distinct population differences between the North-east and South-east of the Atlantic. As a benthic species, with prior knowledge of population fragmentation between regions, it was expected distinct populations would be observed. For conservation, microsatellite analysis as a method has further assisted in understanding population differentiation for management purposes. Microsatellite analysis often proves a highly desirable method in conservation as it more cost-effective than other techniques, such as single-nucleotide polymorphism analysis (SNPs). However in other studies, SNPs have helped further understand that within distinct populations defined by microsatellites, there may be more discrepancies which could affect population management (Manuzzi *et al.*, 2019). Besides population structure, genetic health also revealed, overall, high average genetic diversity: observed heterozygosity = 0.66, expected heterozygosity = 0.85, average alleles per locus = 19.8. High genetic diversity in species threatened with extinction is common within elasmobranchs (as found in the literature review and Appendix 1) and no study has yet reviewed if changes of genetic diversity can be observed between generations. However, our investigation into undulate rays (Chapter 5) was the first study conducted into differences in genetic diversity between protected and non-protected regions. Here we found no difference between the heterozygosity levels as a measure of diversity. From the literature review in Chapter 1, body size was found to be the main contributor to extinction risk, and that, despite many reviews of genetic diversity, heterozygosity levels do not correlate with extinction risk. The undulate ray *R. undulata* is classed as medium to large-bodied ray that is considered of higher value, however is primarily caught and retained as bycatch in demersal fisheries

(Ellis, McCully and Brown, 2012). It is possible that the fragmentation of the populations described through the microsatellite analysis coupled with the limiting life-history traits of medium to large-bodied batoids, such as an average of three generations within 45 years, is contributing to their susceptibility of exploitation (Coelho *et al.*, 2009; Ellis, McCully and Brown, 2012). These coexisting events and characteristics could possibly increase their local IUCN Red List categories again, if not correctly managed and conserved. Furthermore it is important to distinguish breeding populations, as well as natural behaviours such as migrations and possible pairings in order to fully understand population connectivity and health. As explained, the use of the CMR study on the single-site over seven years reveals migrations and relationships between individuals. Yet additional information is required on breeding and egg-laying sites, and seasonal migration patterns, such as where individuals may migrate to when not present on the resting site studied in Chapter 5.

8.5 Regional management of elasmobranchs

Over such an expansive habitat range, it is often important to understand the roles which each species and orders play, in order to correctly manage and conserve them. During this thesis period *R. undulata,* along with other elasmobranch species, had different changes in their protection levels. This included, but was not limited, to their regional and global IUCN Red List assessment and listings within Appendices defined by The Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), The Convention of Migratory Species of Wild Animals (CMS) and the Working Group on Elasmobranch Fishes (WGEF) run by the International Council for the Exploration of the Sea (ICES). For *R. undulata* The IUCN Red List moved the regional assessment from Endangered to Near Threatened, which eventually was passed through to government policy and allowed a specific weightof the species to be landed in the UK by the end of this thesis period. Similarly, some species landed and sampled during the Moroccan investigations in 2015 and 2016, were listed species in CITES Appendix II in 2017 and 2019, prohibiting all landing and trade (CITES, 2019a).

Prior to the research presented in Chapter 2, little was known about the elasmobranch diversity and fisheries in Morocco, despite the country participating in

assessment and agreement of species-specific protections. To investigate IUU fishing of chondrichthyans in Morocco, two field investigations were conducted in 2015 and 2016, where observation data and DNA samples were collected. This fieldwork found that fishing and trade of chondrichthyans increased between years and in most of the markets there was a lack of observed management which normally records catch. Infrastructure to manage and report on catches is often more prevalent within economically richer countries, yet Morocco is a large exporter to the EU, and a participating member of the international trade agreements such as those implemented by CITES. Morocco also aims to increase their fishing activity and therefore economic value (Milano, 2006). Fundamentally, Chapter 2 used DNA barcoding of the COI gene region of mitochondrial DNA and reference material available on the BOLD for species identification and regional haplotype assignment of these samples collected in Morocco. Additionally, Chapter 2 reported on the taxonomic relationships between individuals using phylogenetic analysis. In total, one chimaera, 13 sharks and 11 batoids from 113 chondrichthyan samples were identified based on a ≥ 95% sequence genetic similarity criterion on the COI barcode, with an average evolutionary divergence between the species of 0.29%. Of these species 29% were classified as threatened with extinction, 20% were data deficient and 3% were not evaluated by The IUCN Red List of Endangered Species. In 2017 the big-eyed thresher shark, *Alopias superciliosus* was listed in appendix II of CITES prohibiting all international fishing and trade (CITES, 2016) and in August 2019 the short-fin mako *Isurus oxyrinchus* was also added to this appendix (CITES, 2019a). Decisions for such listings are based on scientific data and fisheries assessments, often presented by organisations such as WGEF, CITES and CMS (WGEF, 2018; CITES, 2019b; CMS, 2019). The policy and law of elasmobranch fishing depends on species and regional stock assessments, however if regional assessments are not completed, either from government or through scientific studies, it is difficult to accurately assess population stability. The level of IUU fishing will also increase, if governments do not provide sufficient management infrastructure, or even basic information regarding international agreements and law.

For the regional management of fishes in general, DNA barcoding has proven the most appropriate method of choice to identify possible IUU fishing and fraudulent species

(Miller and Mariani, 2010; Helyar *et al.*, 2014; Vandamme *et al.*, 2016) and to report on enforcement (Mariani *et al.*, 2015). Specifically in elasmobranchs, DNA barcoding techniques have most recently identified endangered spiny dogfish, *Squalus acanthias*, being fraudulently sold in UK under the title of “rock salmon” in takeaway and fishmongers, as well as imported shark fins found in wholesalers, originating from endangered scalloped hammerhead sharks, *Sphyrna lewini* (Hobbs *et al.*, 2019). The trade of fish products that are mislabelled, either through absent information or fraudulent activity, is often a result of some sort of IUU fishing practise or a lack of knowledge regarding species identification (human error). Once a species has been landed under IUU practises, it is often then mislabelled as a legal species in order to produce a sale and profit. In the United States of America for example, it was estimated that a third of all fish sold is illegal (Pramod *et al.*, 2014). High levels of IUU fishing and trade often occurs in both economically rich and poor regions, however the economic structure within richer countries can often assist in the management and restrictions of IUU fishing and illegal trade. In accordance with the UK and EU Fish Labelling Regulations (2013), all fish products must be labelled with the commercial and scientific name of the fish, the production method including gear type and the catchment area regarding the FAO sub-area or division, treatments, additives and a best before date (European Commission, 2016; GOV.UK, 2017). However, despite regular trade inspection and legal enforcement, illegal endangered species are often prevalent on the UK market. Once processed it has proven difficult to identify the species using DNA. Most recently, Hellberg *et al.* (2019) created a novel mini DNA barcoding method on the COI gene, which enabled them to identify species in shark cartilage pills, shark jerky, and shark fin soup. This method is one of the first to overcome the challenge identifying degraded or highly fragmented samples (Hellberg, Isaacs and Hernandez, 2019).

Chapter 3 attempted to investigate alternative methods to DNA for species identification in elasmobranchs, by examining proteins and the possible variation between their fingerprints. Samples collected for the investigations presented in Chapter 2 were also used for Chapter 3. These shark fins, batoid wings and batoid tail fins were experimentally degraded as part of the method development in Chapter 3. The methods focused on collagen type I alpha I (COLIαI), as previously in bone techniques it has proven the most

resilient to decay through time and food processing (Bae *et al.*, 2008; Collins *et al.*, 2010; Harvey, Daugnora and Buckley, 2018). Although the research did not quite achieve its preferred target of successfully extracting only the COLIαI from any type of sample (fresh to processed), further information was found on the protein composition in fins. Firstly, by reviewing the already published peptide sequences it was found that the COLIαI had sufficient variation for species identification. These results, however, were limited to only five known species of chondrichthyan currently available on SwissProt and UniProt. By utilising waste fin products of *S. canicula* to test the different types of protein extraction, it was identified that for this sample type, GuHCl was the most successful method of collagen extraction. By analysing the LC-Orbitrap Elite tandem mass spectrometry results through Mascot Daemon, first against the entire UniProt database and second against our filtered reference database of 105 chondrichthyan protein sequences, the five main (master) proteins were defined as collagen, actin, tubulin, tropomyosin and myosin. These master proteins existing within fins post desiccation and acidic washing; however collagen provided the highest coverage per sample.

Proteins of chondrichthyans have evolved between species over time, however despite this divergence shown in Chapter 3, certain matches for other species were found when analysing the samples against all available peptide sequences using UniProt. It is therefore difficult to exclude the possibility that there were other master proteins present within the samples. Within these results, it is also important to note that in the majority of samples, COL2αI was the second type of collagen present. This was not surprising as the skeletal structure of chondrichthyans comprises of cartilage encases in COL2 proteins. However the volumes of this protein throughout the skeletal form may differ depending on the region, and the density of the bone. Furthermore, the fin compositions such as the basal and radial elements differ amongst taxa and fin type, defined as pectoral, dorsal, anal or tail fins (Tomita *et al.*, 2014; Da Silva and De Carvalho, 2015; da Silva, Vaz and de Carvalho, 2015). Generally, the morphology and molecular composition of the different types of fins may influence the coverage of the protein types found (Da Silva and De Carvalho, 2015). This could be a reason why such variations were found in the spectra of species, especially in those that had been identified genetically as the same species. Furthermore, the method

development was conducted on a species with fairly flexibly pectoral fins in comparison to fast-swimming pelagic sharks that are highly targeted for shark fin products such as the Carcharhinidae family (Verlecar *et al.*, 2007), and therefore the methods might not be fully transferable to other species. However it is unclear as to how these morphological and molecular differences affect the end products of shark fin soup, as it is often described as tasteless (Verlecar *et al.*, 2007; Dent and Clarke, 2015; Hellberg, Isaacs and Hernandez, 2019). However in order of replicable methodologies and successful application for future analysis, consistent sample type to review the same protein sequence is important.

The level of divergence found between the COLIαI sequences and the protein coverage within each sample however indicated that only partial matches could be made using the current amount of information available. Therefore in order to fully apply the method to chondrichthyan species, future work would need to be conducted in the sequencing of DNA and messenger RNA (mRNA) in order to determine the translated proteins available. In order to complete this, the mRNA extracted from the hearts of the same *S. canicula* individuals has been sequenced in full as part of an ongoing project in which the author is contributing to. The quantities of the different types of mRNA between individuals raised in different climatic environments will be analysed as part of the research conducted by PhD student Daniel Ripley. The raw sequences are readily available thanks to the collaboration, leaving scope for future development of collagen sequence research within *S. canicula*.

Granted, the development of protein extraction did provide a useful tool to describe the proteins present within elasmobranch wings and fins, it did not necessarily display an alternative method for identifying elasmobranch species, when compared to DNA. Techniques to sequence DNA from processed or desiccated samples, ancient DNA (aDNA) and environmental DNA (eDNA) are emerging fields that have successfully been applied in elasmobranch research. The combination of sample use for Chapter 2 and 3 utilised important samples of chondrichthyan species which through the duration of this PhD became increasingly more threatened and therefore more protected. Generally, the high number of species threatened with extinction that were being fished within Morocco created

questions as to whether the practical conservation management in the field to educate individuals on species protections could be implemented, or if species will continue decline, turning conservation focus to *ex-situ* management.

8.6 *Ex-situ* conservation management

The largest group of *ex-situ* management facilities are zoos and aquariums, which historically kept animals for entertainment purposes. Today, zoos and aquariums are becoming centred on conservation by using *ex-situ* management strategies in order to create viable captive populations. It is hoped that these captive populations, if managed correctly, could support or rebuild wild populations if they become endangered or extinct. Although new studbook records to manage current breeding stocks have been implemented in some species, often there little to no information for older individuals. This can lead to individuals inbreeding or outbreeding, having an adverse effect on the concept of zoos and aquariums being used as a genetic back-up.

In order to investigate the breeding relationships of elasmobranchs as a method for studbooks and *ex-situ* conservation, microsatellites were applied to captive populations of small-spotted catshark *S. canicula* and the blacktip reef shark *C. melanopterus* in Chapter 6. The analysis from Chapter 6 found that the populations have remained genetically healthy between generations with no inbreeding, despite little management, providing an insight into genetic stocks and pedigrees. Secondly, a full review was conducted on the already developed microsatellites for current species held within captivity, as defined by Janse *et al.*, (2017). This information examines the future application of management strategies to help aquariums meet their aims of: 1) reducing their reliance on wild-caught specimens; 2) increasing captive population management; and 3) playing more of a roll in conservation by maintaining a healthy population for possible reintroductions. Although this was the first study to be conducted on captive management of sharks, with end user application, other investigations similar to this Chapter were presented for captive populations *R. undulata* in Europe and the UK by Hunter (2016) and Fox *et al.* (2018).

As Chapter 6 had a collaboration with Sea Life aquariums, Europe and Meeresmuseum, Germany, this information has been used to directly inform management

and therefore breeding strategies. For example, the Meeresmuseum created a second tank exhibit to separate individuals of close relation. Similarly, the information provided to Sea Life was passed to higher management and began a full genetic analysis of the entire *C. melanopterus* population across both their facilities and partner facilities under the umbrella organisation of the European Association of Zoos and Aquariums (EAZA). My collaboration with the non-government organisation Fin Fighters and BBC production director Matthew Brierly produced a one and half hour documentary into the decline of sharks. Similar media coverage, including BBC documentaries “Beach Live: Jurassic Coast Revealed” and “Blue Planet UK” and the award of commendation presented by the Duke of Cambridge was also obtained for Chapter 5, due to collaborations between The Undulate Ray Project and the thesis author.

8.7 Overall conclusions

Throughout this thesis, I have successfully investigated aspects of the key issues defined in elasmobranch biology, population analysis, and in *ex-situ* and *in-situ* conservation management by implementing novel techniques examining both species identification and population dynamics. Following the structure of the thesis, the main outcomes of the thesis which contributed novel methods or new information were:

1. Conducting the first investigations into elasmobranch fisheries along the Atlantic coast of Morocco and contributing new COI barcodes for this previously unstudied region.
2. Establishing a new protein extraction method for elasmobranch fin products to determine species identification.
3. Successfully validating a known method of individual identification using genetics, by adapting technique to use on post-hatch individuals of *Scyliorhinus canicula.*
4. Creating a novel method for swabbing DNA from underwater resting rays *Raja undulata*, collected by scuba divers, and applying the microsatellite analysis to infer the population connectivity across the Atlantic and in the Strait of Gibraltar. Here we

were the first to use photo based capture mark-recapture to analyse the population size and relationships between individuals on a single site.

5. Investigating the possible use of microsatellites in captivity as a method of *ex-situ* conservation and management and applying these techniques in two captive-bred populations, *Scyliorhinus canicula* and *Carcharhinus melanopterus*.
6. Discovering twin individuals in three oviparous elasmobranchs that had yet to be added to the literature and applying microsatellite analysis to find heteropaternal fecundation in *Scyliorhinus canicula*.

This research has furthermore been implemented due to the collaborative aspect most chapters have maintained throughout the PhD. The opportunities to create such collaborations have ensured the thesis has remained within the requirements necessary to improve knowledge and create pioneering methods for the overall goal of conserving elasmobranch species.

8.6 References

Bae, I. *et al.* (2008) 'Biochemical properties of acid-soluble collagens extracted from the skins of underutilised fishes', *Food Chemistry*. Elsevier, 108(1), pp. 49–54. doi: 10.1016/J.FOODCHEM.2007.10.039.

Cardinale, B. J. *et al.* (2012) 'Biodiversity loss and its impact on humanity', *Nature*. Nature Publishing Group, 486(7401), pp. 59–67. doi: 10.1038/nature11148.

CEC (2010) 'CEC (2010). Council Regulation (EU) No 23/2010 of 14 January 2010 fixing for 2010 the fishing opportunities for certain fish stocks and groups of fish stocks, applicable in EU waters and, for EU vessels, in waters where catch limitations are required and ', *Official Journal of the European Communities*, L21, pp. 1–20.

CITES (2016) *Consideration of proposal for amendment of Appendices I and II*. Available at: https://cites.org/sites/default/files/eng/cop/17/prop/060216/E-CoP17-Prop-43.pdf

CITES (2019a) 'Consideration of Proposals for Amendment of Appendices I and II', in *CoP18 Prop. 44*. Colombo, Sri Lanka. Available at: https://cites.org/sites/default/files/eng/cop/18/prop/19032019/E-CoP18-Prop-44.pdf.

CITES (2019b) *How CITES works | CITES*, *Convention on International Trade in Endangered Species of Wild Fauna and Flora*. Available at: https://www.cites.org/eng/disc/how.php.

CMS (2019) *Appendix I & II of CMS*, *Convention on the Conservation of Migratory Species of Wild Animals*.

Coelho, R. *et al.* (2009) *Raja undulata. The IUCN Red List of Threatened Species*, *e.T161425A5420694.* doi: http://dx.doi.org/10.2305/IUCN.UK.2009-2.RLTS.T161425A5420694.en.

Collins, M. *et al.* (2010) 'ZooMS: the collagen barcode and fingerprints', *Spectroscopy*

Europe, 22(2).

Corrigan, S., Kacev, D. and Werry, J. (2015) 'A case of genetic polyandry in the shortfin mako *Isurus oxyrinchus*', *Journal of Fish Biology*. John Wiley & Sons, Ltd (10.1111), 87(3), pp. 794–798. doi: 10.1111/jfb.12743.

Dent, F. and Clarke, S. (2015) *State of the global market for shark products Technical Coordinator-Sharks and Bycatch ABNJ (Common Oceans) Tuna Project Western and Central Pacific Fisheries Commission Pohnpei, The Federated States of Micronesia*. Rome.

Dulvy, N. K. *et al.* (2014) 'Extinction risk and conservation of the world's sharks and rays.', *eLife*. eLife Sciences Publications, Ltd, 3(e00590), p. e00590. doi: 10.7554/eLife.00590.

Dulvy, N. K. *et al.* (2017) 'Challenges and Priorities in Shark and Ray Conservation', *Current Biology*. Cell Press, 27(11), pp. R565–R572. doi: 10.1016/J.CUB.2017.04.038.

Ellis, J. R., McCully Phillips, S. R. and Poisson, F. (2017) 'A review of capture and post-release mortality of elasmobranchs', *Journal of Fish Biology*. John Wiley & Sons, Ltd (10.1111), 90(3), pp. 653–722. doi: 10.1111/jfb.13197.

Ellis, J. R. and McCully, S. (2016) *Raja undulata. The IUCN Red List of Threatened Species*. doi: e.T161425A16527741.

Ellis, J. R., McCully, S. R. and Brown, M. J. (2012) 'An overview of the biology and status of undulate ray Raja undulata in the north-east Atlantic Ocean', *Journal of Fish Biology*. John Wiley & Sons, Ltd (10.1111), 80(5), pp. 1057–1074. doi: 10.1111/j.1095-8649.2011.03211.x.

European Commission (2016) *REPORT FROM THE COMMISSION TO THE EUROPEAN PARLIAMENT AND THE COUNCIL*. EU.

Farrell, E. D. *et al.* (2014) 'Multiple paternity in the starry smooth-hound shark *Mustelus asterias* (Carcharhiniformes: Triakidae)', *Biological Journal of the Linnean Society*, 111(1), pp. 119–125. doi: 10.1111/bij.12179.

Feldheim, K. A. *et al.* (2010) 'Shark Virgin Birth Produces Multiple, Viable Offspring', *Journal of Heredity*. Narnia, 101(3), pp. 374–377. doi: 10.1093/jhered/esp129.

Fox, G. *et al.* (2018) 'Bespoke markers for ex-situ conservation : application , analysis and challenges in the assessment of a population of endangered undulate rays', *Jzar*, 6(2), pp. 50–56.

GOV.UK (2017) *Food labelling: country of origin*, *Department for Environment, Food & Rural Affairs Guidance Document*. Available at: https://www.gov.uk/guidance/food-labelling-country-of-origin.

Griffiths, A. M. *et al.* (2012) 'First analysis of multiple paternity in an oviparous shark, the small-spotted catshark (Scyliorhinus canicula L.)', *Journal of Heredity*. Oxford University Press, 103(2), pp. 166–173. doi: 10.1093/jhered/esr112.

Gubili, C. *et al.* (2009) 'Concordance of genetic and fin photo identification in the great white shark, Carcharodon carcharias, off Mossel Bay, South Africa', *Marine Biology*. Springer-Verlag, 156(10), pp. 2199–2207. doi: 10.1007/s00227-009-1233-y.

Harvey, V. L., Daugnora, L. and Buckley, M. (2018) 'Species identification of ancient Lithuanian fish remains using collagen fingerprinting', *Journal of Archaeological Science*. Academic Press, 98, pp. 102–111. doi: 10.1016/J.JAS.2018.07.006.

Hellberg, R. S., Isaacs, R. B. and Hernandez, E. L. (2019) 'Identification of shark species in commercial products using DNA barcoding', *Fisheries Research*. Elsevier, 210, pp.

81–88. doi: 10.1016/J.FISHRES.2018.10.010.

Helyar, S. J. *et al.* (2014) 'Fish Product Mislabelling: Failings of Traceability in the Production Chain and Implications for Illegal, Unreported and Unregulated (IUU) Fishing', *PLoS ONE*. Edited by K. I. Stergiou. Public Library of Science, 9(6), p. e98691. doi: 10.1371/journal.pone.0098691.

Hibbitt, J.-D., Rees, E. and Brown, C. (2017) 'Blacktip reef shark reproduction and neonate survivorship in public aquaria', in Smith, M. et al. (eds) *The Elasmobranch Husbandry Manual II : Recent Advances in the Care of Sharks , Rays and their Relatives Editors*. Ohio Biological Survey, pp. 443–450.

Hobbs, C. A. D. *et al.* (2019) 'Using DNA Barcoding to Investigate Patterns of Species Utilisation in UK Shark Products Reveals Threatened Species on Sale', *Scientific Reports*. Nature Publishing Group, 9(1), p. 1028. doi: 10.1038/s41598-018-38270-3.

Hooper, D. U. *et al.* (2012) 'A global synthesis reveals biodiversity loss as a major driver of ecosystem change', *Nature*. Nature Publishing Group, 486(7401), pp. 105–108. doi: 10.1038/nature11118.

Hunter, K. (2016) 'The Development of Molecular Techniques for the Conservation of Captive Elasmobranchs', pp. 0–59.

Janse, M. *et al.* (2017) 'Sustainable species management of the elasmobranch populations within European aquariums: a conservation challenge', *Journal of Zoo and Aquarium Research*, 5(4), pp. 172–181.

Kashiwagi, T. *et al.* (2015) 'Evaluating manta ray mucus as an alternative DNA source for population genetics study: underwater-sampling, dry-storage and PCR success', *PeerJ*. PeerJ Inc., 3, p. e1188. doi: 10.7717/peerj.1188.

Manuzzi, A. *et al.* (2019) 'Population genomics and phylogeography of a benthic coastal shark (*Scyliorhinus canicula*) using 2b-RAD single nucleotide polymorphisms', *Biological Journal of the Linnean Society*. Narnia, 126(2), pp. 289–303. doi: 10.1093/biolinnean/bly185.

Mariani, S. *et al.* (2015) 'Low mislabeling rates indicate marked improvements in European seafood market operations', *Frontiers in Ecology and the Environment*. John Wiley & Sons, Ltd, 13(10), pp. 536–540. doi: 10.1890/150119.

Milano, E. (2006) 'The New Fisheries Partnership Agreement between the European Community and the Kingdom of Morocco: Fishing too South', *Anuario Espanol de Derecho Internacional*, 22.

Miller, D. D. and Mariani, S. (2010) 'Smoke, mirrors, and mislabeled cod: poor transparency in the European seafood industry', *Frontiers in Ecology and the Environment*. John Wiley & Sons, Ltd, 8(10), pp. 517–521. doi: 10.1890/090212.

Mourier, J. and Planes, S. (2013) 'Direct genetic evidence for reproductive philopatry and associated fine-scale migrations in female blacktip reef sharks (Carcharhinus melanopterus) in French Polynesia', *Molecular Ecology*. John Wiley & Sons, Ltd (10.1111), 22(1), pp. 201–214. doi: 10.1111/mec.12103.

Pramod, G. *et al.* (2014) 'Estimates of illegal and unreported fish in seafood imports to the USA', *Marine Policy*. Pergamon, 48, pp. 102–113. doi: 10.1016/J.MARPOL.2014.03.019.

Robinson, D. P. *et al.* (2011) 'Annually recurring parthenogenesis in a zebra shark Stegostoma fasciatum', *Journal of Fish Biology*. John Wiley & Sons, Ltd (10.1111), 79(5), pp. 1376–1382. doi: 10.1111/j.1095-8649.2011.03110.x.

Rossouw, C., Wintner, S. P. and Bester-Van Der Merwe, A. E. (2016) 'Assessing multiple

paternity in three commercially exploited shark species: *Mustelus mustelus* , *Carcharhinus obscurus* and *Sphyrna lewini*', *Journal of Fish Biology*. Blackwell Publishing Ltd, 89(2), pp. 1125–1141. doi: 10.1111/jfb.12996.

Sagarin, R. and Pauchard, A. (2010) 'Observational approaches in ecology open new ground in a changing world', *Frontiers in Ecology and the Environment*. John Wiley & Sons, Ltd, 8(7), pp. 379–386. doi: 10.1890/090001.

Da Silva, J. P. C. B. and De Carvalho, M. R. (2015) 'Morphology and phylogenetic significance of the pectoral articular region in elasmobranchs (Chondrichthyes)', *Zoological Journal of the Linnean Society*. Narnia, 175(3), pp. 525–568. doi: 10.1111/zoj.12287.

da Silva, J. P. C. B., Vaz, D. F. B. and de Carvalho, M. R. (2015) 'Systematic Implications of the Anterior Pectoral Basals in Squaliform Sharks (Chondrichthyes: Elasmobranchii)', *Copeia*. The American Society of Ichthyologists and Herpetologists 810 East 10th Street, P.O. Box 1897, Lawrence, Kansas 66044 , 103(4), pp. 874–885. doi: 10.1643/CI-14-138.

Stachowicz, J. J. *et al.* (1999) 'Species Diversity and Invasion Resistance in a Marine Ecosystem', *Science*. American Association for the Advancement of Science, 286(5444), pp. 1577–1579. doi: 10.1126/science.286.5444.1577.

Stéphan, E. *et al.* (2014) 'Length at maturity , conversion factors , movement patterns and population genetic structure of undulate ray (Raja undulata) along the French Atlantic and English Channel coasts : preliminary results .', (June), pp. 1–16.

Thornhill, N. W. (1993) *The Natural history of inbreeding and outbreeding : theoretical and empirical perspectives*. University of Chicago Press.

van Tienhoven, A. M. *et al.* (2007) 'A computer-aided program for pattern-matching of natural marks on the spotted raggedtooth shark Carcharias taurus', *Journal of Applied Ecology*. John Wiley & Sons, Ltd (10.1111), 44(2), pp. 273–280. doi: 10.1111/J.1365-2664.2006.01273.X.

Tomita, T. *et al.* (2014) 'Pectoral Fin of the Megamouth Shark: Skeletal and Muscular Systems, Skin Histology, and Functional Morphology', *PLoS ONE*. Edited by P. E. Witten. Public Library of Science, 9(1), p. e86205. doi: 10.1371/journal.pone.0086205.

Tomita, T. *et al.* (2018) 'Locomotion is not a privilege after birth: Ultrasound images of viviparous shark embryos swimming from one uterus to the other', *Ethology*. Edited by R. Bshary. John Wiley & Sons, Ltd (10.1111), 125(2), p. eth.12828. doi: 10.1111/eth.12828.

Vandamme, S. G. *et al.* (2016) 'Sushi barcoding in the UK: another kettle of fish', *PeerJ*. PeerJ Inc., 4, p. e1891. doi: 10.7717/peerj.1891.

Verlecar, X. N. *et al.* (2007) 'Shark hunting - An indiscriminate trade endangering elasmobranchs to extinction'. Indian Academy of Sciences. Available at: http://drs.nio.org/drs/handle/2264/602?show=full.

WGEF (2018) *Report of the Working Group on Elasmobranch Fishes (WGEF)*. Lisbon, Portugal. Available at: http://ices.dk/sites/pub/Publication Reports/Expert Group Report/acom/2018/WGEF/02 WGEF Report 2018_Executive_Summary.pdf

Witzenberger, K. A. and Hochkirch, A. (2011) 'Ex situ conservation genetics: a review of molecular studies on the genetic consequences of captive breeding programmes for endangered animal species', *Biodiversity and Conservation*. Springer Netherlands, 20(9), pp. 1843–1861. doi: 10.1007/s10531-011-0074-4.

Worm, B. *et al.* (2013) 'Global catches, exploitation rates, and rebuilding options for sharks',

Marine Policy. Pergamon, 40, pp. 194–204. doi: 10.1016/J.MARPOL.2012.12.034.

SECTION V: APPENDICES

Appendix 1. Does IUCN status predict genetic diversity in elasmobranchs?

S. A. Hook[1], J. Rowntree[2], R. Preziossi[2], J. Fitzpatrick[3]

[1]School of Earth and Environmental Sciences, Manchester Institute of Biotechnology, University of Manchester, Manchester, M1 7DN, UK

[2] The Manchester Metropolitan University

[3]Stockholm University

Author contributions

SAH[1] wrote the paper, collected the data and conducted the statistical analysis. JF[3] developed the concept, provided guidance on the data analysis and write-up. JR[2] and RP[2] supervised SAH for the duration of this work (first year of the PhD). All authors contributed to the review.

Abstract

There is a growing concern for the conservation of sharks and rays (elasmobranchs). Around a quarter of all elasmobranchs are classified as threatened with extinction by the IUCN Red List. Elasmobranchs are being killed at alarming rates, with an estimated 100 million elasmobranchs being fished every year. Many authors argue that the population declines of elasmobranchs exhibit the same genetic diversity pattern as other groups i.e. diversity is reduced as species become more threatened. However few studies have seriously addressed the question. To fully investigate this, comparisons were conducted between 1) life history traits as a predictor to IUCN Red List category, 2) genetic diversity levels as a response to IUCN Red List category, 3) life history traits as a predictor for genetic diversity. Data from 63 species across 21 families and 7 orders were collected from 129 publications released until the 31st of January 2016. Genetic diversity measures included expected heterozygosity (*He*) and observed heterozygosity (*Ho*) values from nuclear DNA and haplotype diversity (*h*), nucleotide diversity (π) and haplotype number (H) from mitochondrial DNA were analysed. Contrary to expectations, it was found that

measures of nuclear genetic diversity did not relate to IUCN status. However body size had a positive relationship with IUCN Red List category (P = 0.0102) and total haplotype number (P = 8.09E-07). Lastly there was no significant difference observed between the expected heterozygosity values of threatened (average *He* = 0.6631) and non-threatened elasmobranchs (average *He* = 0.6395). The results contradict previous findings that genetic diversity is reduced in threatened taxa when compared with non-threatened taxa. This identifies the gap in knowledge and highlights how further research is needed to fully understand the implications of genetic diversity levels in elasmobranchs.

Introduction

Biodiversity loss is one of the most critical environmental problems that threaten the natural world (Myers *et al*., 2000). The claim that we are facing our sixth mass extinction is heavily supported by current extinction rates which are thought to be at least 100 times faster than any pre-human background rate (Pimms *et al*., 1995; Barnosky *et al.,* 2011; Ceballos *et al.,* 2015). Observations strongly suggest that humans are the cause of the sixth mass extinction (Myers, 1990; Pimms *et al.,* 1995; Leakey and Lewin, 1997; Dirzo and Raven, 2003; Dulvy *et al*., 2014) through habitat fragmentation, climate change, introduction of non-native species, transmission of pathogens and overexploitation of resources (Dirzo and Raven, 2003; Barnoksy, 2010; Hoffmann *et al*., 2010; Barnosky *et al*., 2011; Dulvy *et al*., 2014; Ceballos *et al*., 2015) Due to these high extinction rates and a lack of detailed knowledge about extant biodiversity, there is a high probability that a species will become extinct before it is even discovered (Mora *et al*., 2011). In addition conservation efforts to protect species have not slowed the rate of biodiversity loss (Butchart *et al*., 2010).

Roughly one billion people worldwide rely on fish as their primary source of food (Engelhaupt, 2007) and around 60% of all commercially important fish stocks are overexploited, depleted or recovering (FAO, 2012). The impacts of fishing pressures on ocean predators such as sharks and rays (elasmobranchs) are largely unknown (Dulvy *et al*., 2008). Elasmobranch fishing is currently a great concern worldwide due to their low productivity in relation to teleost fish (Stevens *et al*., 2000). Accidental by-catch and illegal, unreported and unregulated (IUU) fishing contributes to 50% of global elasmobranch catch

(FAO, 1999; Gilman *et al*., 2005; Pinsky *et al*., 2013; Stevens *et al*., 2000). In addition, up to 100 million individual sharks (1.4 metric tons) are killed each year for their meat and fins (Pinsky *et al*., 2013). As of 2014, roughly a quarter of the world's elasmobranchs were classed as threatened with extinction (IUCN, 2014).

Evolutionary traits, which have previously contributed to the success of elasmobranchs, now threaten their existence (Snelson Jr. *et al*., 2008). The majority of elasmobranch life history traits fall under the K-selection theory, which characterises such species as having slower development/growth, lower resource thresholds, late reproduction, larger body sizes, later attainment of sexual maturity, longer life spans, low fecundity and longer gestation periods (MacArthur and Wilson, 1967; Pianka, 1970; Audzijonyte *et al.,* 2016). The relationships between elasmobranch life history traits related to K-Selection and extinction risk have previously been analysed by Dulvy *et al.* (2014). Body size, minimum depth and depth range were found to display positive correlations with extinction risk (IUCN category; Dulvy *et al.,* 2014). In other marine fishes empirical evidence suggests that maturation and body size are the best predictors of extinction risk (Reynolds *et al*., 2005). It is assumed that maximum body size is a predictor of IUCN status and therefore extinction risk because of the relationship between body size and natural rate of shark and ray population increase (Dulvy *et al.,* 2014)

Body size is also an indicator of extinction risk in mammals, as impacts from intrinsic and environmental factors sharply increase above a body mass of 3 kilograms (kg; Cardillo *et al*., 2005). Just under half of all sharks have a fork body length over 100cm and weight over 3kg (Shiffman, 2016). For example the blue shark *Prionace glauca* which is the most heavily fished species of shark (No Limits, 2016) that reaches average sexual maturity at 220cm (Fishbase, 2016) or a body mass of roughly 0.09872 tonnes (Fish Weights, 2016) has suffered an estimated 60% population decline over 15 years (Baum *et al*., 2003). Despite this decline the blue shark is classified as Near Threatened by the IUCN Red List (Stevens, 2009).

The application of genetic theory and techniques to conserve any species or ecosystem is known as conservation genetics, and is a fairly modern concept that is

gradually becoming more prevalent in biodiversity management (Smith *et al*., 1991; Spielman *et al*., 2004b). Through conservation genetics, levels of genetic diversity within and among populations can be assessed and related to environmental pressures such as habitat fragmentation, overfishing and climate change. Genetic diversity is most commonly defined as a representation of the essential raw material necessary for a species to evolve and adapt to changing environments (Frankham *et al*., 2004). Species existing in large populations typically have extensive genetic diversity and are more resilient to extinction pressures. In contrast species that exist in small populations often have reduced genetic diversity and can be more prone extinction (England *et al*., 2003), likely due to inbreeding effects (Charlesworth *et al*., 2003; Spielman *et al*., 2004).

Very little research has been conducted into the genetic diversity and the risk of extinction of elasmobranchs. In a previous study Spielman *et al*. (2004b) found that genetic diversity was lower in 77% of 170 threatened taxa when compared to related non-threatened taxa. However, only 35 species of Poikilotherms including fish, amphibians and reptiles were analysed in this data set with no reference to elasmobranch genetic diversity (Spielman *et al*., 2004b). Prior to this study it was hypothesized by Lande (1988) that species were driven to extinction before effects on genetic diversity was observed (Lande, 1988). The aim of this study is to investigate and analyse the relationship between genetic diversity and IUCN Red List status for elasmobranchs. If elasmobranchs follow a similar pattern to the taxa analysed in Spielman *et al.* (2004b) there should be significant differences between genetic diversity of threatened and non-threatened species. In addition, it was examined if K- selection life history traits (such as body size and late maturity; Reynolds *et al*., 2005, Dulvy *et al.,* 2014) predict extinction risk and/or genetic diversity in elasmobranchs. Here it evaluates if IUCN categories are related with life history traits. In addition both the relationship between genetic diversity and IUCN categories and the relationship between genetic diversity and life history traits was examined.

Methods

Data Collection

Sources of Genetic Diversity

Research into the number of publications with information on the genetic diversity of elasmobranchs was conducted through numerous scholar databases. The three main databases used for this search were the Web of Science (http://apps.webofknowledge.com/), Google Scholar (https://scholar.google.co.uk/) and Shark References (http://shark-references.com/). In order to find publications containing genetic diversity measures from either mitochondrial or nuclear DNA, the following searched terms were used alone or in combination in all three databases: 'Genetic Diversity'; 'Microsatellites'; 'Shark'; 'Ray'; 'Population analysis'; 'Mitochondrial'; 'Nuclear'.

Information regarding the region from which samples were collected, the number of samples tested and the measures of genetic diversity used were recorded. Estimates of genetic diversity were either derived from small or whole regions of mitochondrial DNA (haplotype diversity, nucleotide diversity, haplotype number) or nuclear DNA (observed heterozygosity and expected heterozygosity). There were a small number of publications which recorded nuclear DNA genetic diversity levels through other methods such as Amplified Fragment Length Polymorphism (AFLP's). Due to the limited number of these publications they were removed from the analysis. If multiple estimates of genetic diversity were available for the same species (i.e. from different studies of the same species), the combined weighted average of each measure was used.

IUCN and Life History Data

The conservation status of each species was taken from the IUCN Red List of Threatened Species (http://www.iucnredlist.org/ accessed between 1st December 2015 and 31st of January 2016; Table 1). Additional information regarding the year of assessment and current population trends was also recorded. Data regarding life history traits, range and environmental data for each species was extracted from the IUCN Red List (http://www.iucnredlist.org/search), Fish Base (http://www.fishbase.se/search.php) and

FLMNH Ichthyology Department (http://www.flmnh.ufl.edu/fish/). Life history data consisted of maximum age (years), total length (cm), maximum adult size (cm), age of sexual maturity (years), length at first maturity (L_m), total gestation period (months), average brood size, size of young at birth and breeding method. Where traits differed between males and females this information was recorded separately. Range and environmental data included: regional differences in life history traits within each species, Food and Agriculture Organisation (FAO) marine fishing areas, the average lowest depth observed (m) and climate. IUCN Red List categories were converted into continuous 5 point scale: 1 is least concern (LC), 2 is near threatened (NT), 3 is vulnerable (VU), 4 is endangered (EN) and 5 is critically endangered (CE). Any species that were classified as data deficient by the IUCN Red List were removed from the analysis, as the relationship between genetic diversity and extinction risk could not be assessed. Data were also divided into two broad categories: threatened with extinction and non-threatened categories. These correspond to the IUCN Red List definitions, whereby least concern and near threatened were classified as non-threatened species while vulnerable, endangered and critically endangered species were classified as species threatened with extinction. Life history traits for gestation, female and male age of sexual maturity (years) and length at sexual maturity was a taken as at the lowest recorded value.

Analysis

Data were analysed in three ways. Firstly the relationship between each life history trait and the IUCN continuous data was examined using separate linear regression models (Table 2). A multiple regression model was then used to analyse all life history traits and the IUCN continuous data set (Table 2). Secondly I looked at the relationship between the IUCN continuous data and the genetic diversity levels where IUCN status was the predictor variable and genetic diversity was the response variable (Table 3). Although the analysis was initially conducted on both observed and expect heterozygosity values, expected heterozygosity levels show no bias from the sample size and therefore are more reliable measure of genetic diversity. Lastly we investigated the relationship between life history traits and asked whether single or multiple traits best predict genetic diversity levels (Table 4). All statistical analyses were carried out in R v2.3.0. (R Core Team, 2016) with multiple

packages: car, effects and plotrix. Single and multiple regression models were used to conduct T-tests, collect F-statistics and levels of significance (P-values).

Results

Genetic diversity summary

A total of 63 species representing 21 families and 7 orders of elasmobranchs from 129 publications contained data for mitochondrial DNA genetic diversity levels (representing 16, 424 individual elasmobranchs), microsatellite genetic diversity levels (representing 13,714 individual elasmobranchs), or both (Table 1) and have an IUCN status that either fell into least concern (LC), near threatened (NT), vulnerable (VU), endangered (EN) or critically endangered (CE). Heterozygosity values were available for a total of 46 species and estimates of mitochondrial genetic diversity for a total of 37 species. Information on genetic diversity was available for a further 10 species, but these fell into the data deficient category and were therefore stricken form the dataset. A total of 20 species had both heterozygosity and mitochondrial genetic diversity levels. Data was normally distributed.

Table 1: An overview of the data extracted and analyzed from the IUCN Red List and current publications into genetic diversity of nuclear and mitochondrial DNA. nDNA studies are taken from microsatellite studies only.

Species	mtDNA Studies	nDNA Studies	*Pop*	*N*	*N* H	H	*h*	π	H_o	H_e	IUCN	TWE or NNT	Trend
Aetobatus flagellum	0	1	1	24	-	-	-	-	0.4648	0.4808	EN	TWE	D
Aetobatus narinari	2	1	8	763	50	10	0.6834	0.00562	0.726	0.739	NT	NNT	D
Alopias pelagicus	1	1	9	618	19	19	0.4195	0.116	0.6747	0.729	VU	TWE	D
Carcharhinus acronotus	0	1	1	32	-	-	-	-	0.4911	0.5068	NT	NNT	D
Carcharhinus brevipinna	1	0	2	430	37	37	0.677	0.0013	-	-	NT	NNT	U
Carcharhinus falciformis	0	1	1	53	-	-	-	-	0.5516	0.7088	NT	NNT	D
Carcharhinus leucas	1	2	3	249	14	14	0.76	0.0028	0.612	0.6189	NT	NNT	U
Carcharhinus limbatus	3	1	14	1058	69	17	0.636	0.00231	0.5	0.5	NT	NNT	U
Carcharhinus longimanus	0	1	1	28	-	-	-	-	0.5415	0.7001	VU	TWE	D
Carcharhinus melanopterus	0	1	1	264	-	-	-	-	0.5721	0.581	NT	NNT	D
Carcharhinus obscurus	2	1	10	308	32	16	0.72	0.005	0.623	0.732	VU	TWE	D
Carcharhinus plumbeus	1	1	10	398	67	67	0.959	0.00475	0.5378	0.5725	VU	TWE	D
Carcharhinus sorrah	1	2	3	177	12	12	0.6	0.003	0.51879	0.54384	NT	NNT	U
Carcharhinus tilstoni	0	1	1	79	-	-	-	-	0.64874	0.40766	LC	NNT	S
Carcharias taurus	2	3	9	609	27	3	0.47875	0.00187	0.7258	0.7857	VU	TWE	U
Carcharodon carcharias	6	6	12	1319	118	17	0.69928	0.00655	0.6489	0.6354	VU	TWE	U
Centroscymnus coelolepis	0	1	1	211	-	-	-	-	0.77	0.77	NT	NNT	U
Centroselachus crepidater	0	1	2	20	-	-	-	-	0.5109	0.5145	LC	NNT	U
Cetorhinus maximus	2	0	6	674	133	7	0.57705	0.0018	-	-	VU	TWE	D
Chiloscyllium plagiosum	0	1	1	34	-	-	-	-	0.62	0.693	NT	NNT	U

Dasyatis akajei	1	0	3	107	28	28	0.9393	0.0069	-	-	NT	NNT	U
Dipturus batis	1	1	2	144	15	8	0.455	0.00093	0.3164	0.38818	CE	TWE	D
Galeorhinus galeus	1	2	7	285	38	38	0.92	0.0071	0.6356	0.6642	VU	TWE	D
Glyphis glyphis	1	0	3	93	12	12	0.76	0.00019	-	-	EN	TWE	D
Hexanchus griseus	0	2	2	467	-	-	-	-	0.477	0.749	NT	NNT	U
Isurus oxyrinchus	0	3	5	647	-	-	-	-	0.7384	0.7818	VU	TWE	D
Leucoraja naevus	0	1	1	17	-	-	-	-	0.542	0.516	LC	NNT	U
Manta alfredi	0	1	1	60	-	-	-	-	0.4767	0.48	VU	TWE	D
Mustelus antarcticus	0	2	2	357	-	-	-	-	0.6841	0.6806	LC	NNT	S
Mustelus asterias	0	1	1	127	-	-	-	-	0.745	0.735	LC	NNT	U
Mustelus canis	0	1	1	91	-	-	-	-	0.5014	0.5245	NT	NNT	U
Mustelus henlei	0	1	1	213	-	-	-	-	0.7825	0.695	LC	NNT	U
Mustelus lenticulatus	0	1	1	75	-	-	-	-	0.614	0.621	LC	NNT	I
Mustelus mustelus	0	2	6	125	-	-	-	-	0.6363	0.5153	VU	TWE	D
Mustelus schmitti	1	0	2	198	22	11	0.226	0.0015	-	-	EN	TWE	D
Negaprion acutidens	1	2	7	156	4	4	0.28	0.0006	0.6007	0.6533	VU	TWE	D
Negaprion brevirostris	1	3	2	1876	11	11	0.78	0.0059	0.7571	0.7933	NT	NNT	U
Prionace glauca	1	4	10	1022	16	16	0.92	0.0054	0.6038	0.613	NT	NNT	U
Pristis clavata	1	0	1	73	15	15	0.489	0.0062	-	-	EN	TWE	D
Pristis microdon	1	0	1	149	18	18	0.65	0.0044	-	-	CE	TWE	D
Pristis pectinata	0	2	2	167	-	-	-	-	0.8486	0.8384	CE	TWE	D
Pristis zijsron	1	1	3	109	9	9	0.555	0.0036	0.81	0.8384	CE	TWE	D
Raja asterias	1	0	3	18	2	2	0.29	0.0092	-	-	NT	NNT	D
Raja clavata	3	4	24	1934	61	20	0.6163	0.00541	0.6594	0.6804	NT	NNT	D
Raja maderensis	1	0	2	37	4	4	0.482	0.00134	-	-	VU	TWE	D
Raja miraletus	1	0	3	18	2	2	0.17	0.0031	-	-	LC	NNT	S
Raja montagui	0	1	1	23	-	-	-	-	0.588	0.661	LC	NNT	S

Raja radulta	1	0	2	3	3	3	1	0.00914	-	-	EN	TWE	D
Raja undulata	0	1	9	108	-	-	-	-	0.683	0.7138	EN	TWE	D
Rhincodon typus	3	2	35	1254	273	25	0.9929	0.01125	0.6009	0.6227	VU	TWE	D
Rhinobatos productus	1	0	4	64	17	17	0.767	0.119	-	-	NT	NNT	U
Rhizoprionodon porosus	2	0	3	385	75	19	0.66	0.0029	-	-	LC	NNT	S
Rhizoprionodon terraenovae	1	0	2	80	24	12	0.762	0.00315	-	-	LC	NNT	U
Scyliorhinus canicula	0	1	1	150	-	-	-	-	0.6484	0.648	LC	NNT	S
Somniosus microcephalus	1	0	1	16	7	7	0.775	0.0022	-	-	NT	NNT	U
Sphyrna lewini	2	2	18	451	33	17	0.7075	0.0114	0.6671	0.7236	EN	TWE	U
Sphyrna tiburo	0	1	1	119	-	-	-	-	0.654	0.686	LC	NNT	U
Sphyrna tudes	1	0	1	55	6	3	0.1385	0.000335	-	-	VU	TWE	D
Squalus acanthias	1	3	5	909	103	103	0.839	0.0086	0.515	0.6557	VU	TWE	D
Squatina californica	0	1	1	3	-	-	-	-	0.631	0.59	NT	NNT	U
Stegostoma fasciatum	1	1	2	75	8	8	0.72	0.14	0.7679	0.7516	VU	TWE	D
Triaenodon obesus	1	0	2	310	15	15	0.55	0.00213	-	-	NT	NNT	U
Triakis semifasciata	0	1	1	471	-	-	-	-	0.85825	0.851	LC	NNT	U

mtDNA Studies, number of mitochondrial studies; nDNA Studies, number of nuclear DNA studies; Pop, Total number of populations studied; *N*, number of individuals analysed; *N*H, number of haplotypes; H, average number of haplotypes found; *h,* haplotype diversity; *π*, nucleotide diversity; *Ho*, observed heterozygosity; *He*, expected heterozygosity; IUCN, the species IUCN Red List category (LC, Least Concern; NT, Near Threatened; VU, Vulnerable; E, Endangered; CR, Critically Endangered); TWE or NNT, Threatened with Extinction or Non-threatened; Trends, species population trend (U, unknown; D, decreasing; S, stable, I, increasing).

Life history predictors of extinction risk

Male maturation age, female maturation age and minimum reproductive cycle was removed from the full model due to the low sample number in each category. There was a significant relationship between IUCN category and body size in both the separate linear regression model and the multiple linear regression model (Table 2). This is a positive significant relationship between IUCN status and life history traits in a model controlled for the underlying relationship between sample number and life history traits (full model; Table 2).

Table 2: Regression models of the relationships between life history traits of elasmobranchs and IUCN categories (5 point scale).

Predictor Variable	*N*	β	β Std. Error	*t*	*F*	*P*
Body Size	44	0.0013994	0.00052	2.689	7.2300	0.0102
Offspring Number	41	-0.0082200	0.00654	-1.257	1.5810	0.2161
Min Reproductive Cycle	23	0.0436900	0.02420	1.805	3.2580	0.0854
Male Maturation Age	27	-0.0010620	0.00338	-0.314	0.0985	0.7563
Female Maturation Age	27	0.0299300	0.04088	0.732	0.5360	0.4709
Gestation Period	37	0.0197800	0.05334	0.371	0.1375	0.7130
Maximum Depth	44	-0.0002875	0.00026	-1.104	1.2200	0.2757
Full Model						
Body Size	28	5.03E-03	9.39900	5.354	28.6607	0.0000
Offspring Number	28	2.14E-03	7.33400	0.291	0.0847	0.7730
Gestation Period	28	-1.94E-02	5.68300	-0.341	0.1166	0.7350
Maximum Depth	28	-5.49E-05	8.14000	-0.067	0.0046	0.9470
Overall	33				8.2490	0.0002

***N*, is the number of individuals analysed; β, estimated coefficient; β Std. Error, the estimated coefficient stand error; *t*, t-test value, representing the angle of the slope; *F*, F-value between the two means of the model; *P*, P-values showing whether or not significance has been found. Bold P Values are significant values (*P* = ≤ 0.05). Maximum Depth, metres; Gestation Period, months; Maturation Age, years; Reproductive Cycle, months; Body Size, centimetres.**

IUCN status as a predictor to genetic diversity

There was no relationship between IUCN status and genetic diversity measures in a model that controlled for the underlying relationship between sample number and genetic diversity estimates (Table 3). Standard error was highest in total haplotype number, however was reduced significantly when IUCN status was compared with mean haplotype number.

Table 3: Separate linear regression models for the relationship between predictor IUCN categories (5 point scale) and response values of genetic diversities.

Response variables	*N*	β	β Std. Error	*t*	*F*	*P*
Mitochondrial Sample Number	35	-0.00048	0.000821	-0.582	0.3393	0.5640
Nuclear Sample Number	43	-0.00023	0.000478	-0.481	0.2316	0.6330
Total Haplotype Number	35	-1.72600	8.215000	-0.210	0.0442	0.8348
Mean Haplotype Number	35	-0.10740	3.031000	-0.035	0.0013	0.9719
Haplotype Diversity	35	-0.01393	0.036040	-0.387	0.1495	0.7010
Nucleotide Diversity	35	-0.00075	0.005378	-0.139	0.0194	0.8900
Observed Heterozygosity	44	-0.00456	0.015040	-0.303	0.0919	0.7630
Expected Heterozygosity	44	0.01051	0.015140	0.694	0.4821	0.4910

***N*, is the number of individuals analysed; β, estimated coefficient; β Std. Error, the estimated coefficient stand error; *t*, t-test value, representing the angle of the slope; *F*, F-value between the two means of the model; *P*, P-values showing whether or not significance has been found. Bold P Values are significant values (*P* = ≤ 0.05).**

Life history traits as a predictor of genetic diversity levels

There was a strong relationship between the body size and total number of haplotypes found and the total number of mitochondrial samples (Table 4). Total number of haplotypes had significant positive relationships with 4 out of 7 different life history traits.

Relationships between each life history trait

Body size was positively related with minimum reproductive cycle (months); however showed no significant relationship with any other life history trait measured. Minimum reproductive cycle had a positive relationship with female maturation age (years) and gestation period (months). Maximum depth (metres) was only significantly related with female maturation age. Gestation was significantly related with offspring number, and male and female maturation ages were also positively related.

Table 4: Separate linear regression models to display the relationship between life history traits (predictor value) and genetic diversity measures (response values).

Predictor Value	**Response Value**	***N***	**β**	**β Std. Error**	***t***	***F***	***P***
Body Size	Mitochondrial Sample Number	34	0.23696	0.089270	2.654	7.0460	**0.0120**
Body Size	Nuclear Sample Number	41	0.23420	0.180200	1.3000	1.6888	0.2010
Body Size	Total Haplotype Number	34	0.09803	0.016280	6.0210	36.2500	**8.09E-07**
Body Size	Mean Haplotype Number	34	-0.00176	0.008624	-0.2040	0.0415	0.8397
Body Size	Haplotype Diversity	34	1.36800	9.977000	1.3710	1.8787	0.1790
Body Size	Nucleotide Diversity	34	-7.16100	1.526000	-0.4690	0.2202	0.6419
Body Size	Observed Heterozygosity	42	3.50E-05	5.66E-05	0.6110	0.3730	0.5446
Body Size	Expected Heterozygosity	42	6.13E-05	5.70E-05	1.0760	1.1571	0.2882
Offspring Number	Mitochondrial Sample Number	20	1.95600	1.630000	1.2000	1.4400	0.2442
Offspring Number	Nuclear Sample Number	39	3.98800	2.114000	1.8870	3.5611	0.0666
Offspring Number	Total Haplotype Number	20	0.25370	0.160300	1.5830	2.5060	0.1291
Offspring Number	Mean Haplotype Number	20	0.04608	0.120120	0.3840	0.1472	0.7053
Offspring Number	Haplotype Diversity	20	0.000686	0.001989	0.3450	0.1187	0.7340
Offspring Number	Nucleotide Diversity	20	-0.00017	0.000290	-0.5840	0.3414	0.5655
Offspring Number	Observed Heterozygosity	39	-0.0003	0.000689	-0.4310	0.1859	0.6688
Offspring Number	Expected Heterozygosity	39	0.000181	0.000699	0.2590	0.0671	0.7970
Minimum Reproductive Cycle	Mitochondrial Sample Number	17	14.792	5.834000	2.5360	6.4300	0.2133
Minimum Reproductive Cycle	Nuclear Sample Number	21	5.59	6.205000	0.9010	0.8118	0.3778
Minimum Reproductive Cycle	Total Haplotype Number	17	2.0573	0.998700	2.0600	4.2440	**0.0541**
Minimum Reproductive Cycle	Mean Haplotype Number	17	0.3103	0.469100	0.6610	0.4375	0.5172
Minimum Reproductive Cycle	Haplotype Diversity	17	0.001014	0.007040	0.1440	0.2080	0.8871
Minimum Reproductive Cycle	Nucleotide Diversity	17	-0.00133	0.000762	-1.7450	3.0453	0.0990
Minimum Reproductive Cycle	Observed Heterozygosity	21	0.001711	0.002770	0.6180	0.3815	0.5434

Minimum Reproductive Cycle	Expected Heterozygosity	21	0.003963	0.002961	1.3380	1.7904	0.1952
Male Maturation Age	Mitochondrial Sample Number	21	6.575	10.601000	0.6200	0.3846	0.5418
Male Maturation Age	Nuclear Sample Number	25	-0.315	1.015000	-0.3100	0.0964	0.7588
Male Maturation Age	Total Haplotype Number	21	1.904	1.651000	1.1540	1.3312	0.2616
Male Maturation Age	Mean Haplotype Number	21	0.8218	0.650100	1.2640	1.5981	0.2200
Male Maturation Age	Haplotype Diversity	21	0.01	0.009990	1.0010	1.0026	0.3281
Male Maturation Age	Nucleotide Diversity	21	6.055	1.595000	0.0380	0.0014	0.9701
Male Maturation Age	Observed Heterozygosity	25	-8.69E-05	3.25E-04	-0.2670	0.0715	0.7913
Male Maturation Age	Expected Heterozygosity	25	-7.70E-05	3.430-04	-0.2240	0.0504	0.8243
Female Maturation Age	Mitochondrial Sample Number	21	9.019	8.662000	1.0410	1.0841	0.3096
Female Maturation Age	Nuclear Sample Number	25	7.832	13.282000	0.5900	0.3477	0.5607
Female Maturation Age	Total Haplotype Number	21	2.19	1.327000	1.6500	2.7216	0.1139
Female Maturation Age	Mean Haplotype Number	21	0.773	0.527000	1.4670	2.1517	0.1572
Female Maturation Age	Haplotype Diversity	21	0.011674	0.007926	1.4730	2.1693	0.1556
Female Maturation Age	Nucleotide Diversity	21	-0.00028	0.001318	-0.2140	0.0457	0.8329
Female Maturation Age	Observed Heterozygosity	25	-0.0002	0.003837	-0.0530	0.0028	0.9585
Female Maturation Age	Expected Heterozygosity	25	0.002877	0.004277	0.6730	0.4525	0.5073
Gestation Period	Mitochondrial Sample Number	26	14.27	10.940000	1.3050	1.7033	0.2033
Gestation Period	Nuclear Sample Number	35	-3.105	18.271000	-0.1700	0.0289	0.8660
Gestation Period	Total Haplotype Number	26	3.9682	1.682900	2.3580	5.5598	**0.0262**
Gestation Period	Mean Haplotype Number	26	2.385	1.001000	2.3830	5.6785	0.2477
Gestation Period	Haplotype Diversity	26	0.008382	0.010859	0.7720	0.5958	0.4471
Gestation Period	Nucleotide Diversity	26	0.000875	0.001143	0.7660	0.5862	0.4508
Gestation Period	Observed Heterozygosity	35	0.001331	0.005292	0.2510	0.0632	0.8029
Gestation Period	Expected Heterozygosity	35	0.002974	0.005448	0.5460	0.2979	0.5886

Maximum Depth	Mitochondrial Sample Number	34	0.2194	0.102600	2.1370	4.5678	**0.0399**
Maximum Depth	Nuclear Sample Number	41	-0.00636	0.086729	-0.0730	0.0054	0.9419
Maximum Depth	Total Haplotype Number	34	0.07215	0.022850	3.1570	9.9674	**0.0033**
Maximum Depth	Mean Haplotype Number	34	-0.00014	0.009606	-0.0140	0.0002	0.9887
Maximum Depth	Haplotype Diversity	34	9.31E-05	1.13E-04	0.8240	0.6787	0.4158
Maximum Depth	Nucleotide Diversity	34	-1.64E-05	1.68E-05	-0.9780	0.9574	0.3348
Maximum Depth	Observed Heterozygosity	42	-1.21E-05	2.63E-05	-0.4600	0.2113	0.6481
Maximum Depth	Expected Heterozygosity	42	1.54E-05	2.64E-05	0.5820	0.3387	0.5637

***N*, the number of individuals analysed; β, estimated coefficient; β Std. Error, the estimated coefficient stand error; *t*, t-test value, representing the angle of the slope; *F*, F-value between the two means of the model; *P*, P-values showing whether or not significance has been found. Bold P Values are significant values (*P* = ≤ 0.05). Maximum Depth, metres; Gestation Period, months; Maturation Age, years; Reproductive Cycle, months; Body Size, centimetres.**

Table 5: Separate liner regression models between different life history traits analyzed. T values are displayed with P values in brackets.

	Body Size	**Offspring #**	**Repro Cycle**	**M Maturation**	**F Maturation**	**Gestation**	**Max Depth**
Body Size	-						
Offspring #	-1.3490(0.1839)	-					
Repro Cycle	2.3110(**0.0287**)	-1.6330(0.1154)	-				
M Maturation	0.1830(0.8560)	-0.3930(0.6970)	-0.4060(0.6890)	-			
F Maturation	0.1830(0.8560)	0.1400(0.8899)	3.4760(**0.0024**)	2.1340(**0.0402**)	-		
Gestation	0.5600(0.5790)	-3.5830(**0.0009**)	2.3730(**0.0250**)	0.1720(0.8650)	2.6070(**0.0139**)	-	
Max Depth	0.9800(0.3313)	1.2270(0.2261)	0.8890(0.3820)	-0.2880(0.7750)	2.7110(**0.0103**)	1.2450 (0.2200)	-

N: 63, Offspring #, offspring number, Repro Cycle, minimum reproductive cycle; M Maturation, male maturation age (years), F Maturation, female maturation age (years); Gestation, gestation period (months); Max Depth, maximum depth (m). Bold P Values are significant values (P = ≤ 0.05).

Discussion

We found there was no significant relationship between IUCN Red List category (extinction risk) and genetic diversity in elasmobranchs. It appears with the current information gathered that high levels of genetic diversity are maintained in threatened species of elasmobranchs. These findings conflict with the research conducted by Spielman *et al.* (2004), in which genetic diversity is lower in taxon that are more threatened. There was a significant relationship between IUCN category and body size as seen by Reynolds *et al*. (2005) and Dulvy *et al.* (2014), however there was no relationship between age of maturity and IUCN categories which conflicts with their findings. Despite the low species sample number (N = 63) in relation to total number of elasmobranchs (N = approximately 1000), the analysis covered a larger number of families (N =21) and orders (N= 7) in which correlations did not meet the expectations that genetic diversity would be lower in threatened species.

Our findings suggest that the larger you are the more threatened you are likely to be, however there is no relationship between body size and nuclear genetic diversity. We do find that there is a positive relationship between total number of haplotypes and body size; however it is very possible that this is due to the number of samples in each species. A higher number of samples will often produce more haplotypes, therefore when considering the mean number of haplotypes as a response to body size there is actually no relationship present. Although we did not find a direct relationship between maturation of males or females and body size as seen previously in other taxa (Reynolds *et al*., 2005), body size and reproductive cycle was significantly positively related. It would be expected that the larger a species is, the longer a reproductive cycle would take (Hoenig *et al.,* 1990), for example the shortfin mako *Isurus oxyrinchus* reaches a total length of 396 cm and has a reproductive cycle up to 36 months (Mollet *et al.,* 2000) while the narrownose shark *Mustelus schmitti* reaches a total length of 101 cm and has a maximum reproductive cycle of 12 months (Menni *et al*., 1986). Interestingly, as well as body size, female maturation age (years) had a significant relationship with reproductive cycle and other life history traits such as gestation period, maximum depth and male maturation age. The relationship between gestation period and female maturation age suggests that the later a female matures, the longer the gestation period is, which support the findings of other research (Branstetter,

1987; Hoenig *et al*., 1990; Cortés, 2000). The lack of a relationship between body size and female or male maturation age is surprising as previous studies have shown that these two traits exhibit a strong positive correlation. Cortés (2000) stated that sexual maturity occurs in 75% of maximum body size of elasmobranchs. Our results are probably due to the limited number of species (*N* = 63 compared to Cortés' (2000) *N* = 164). Although females tend to mature earlier than males it is expected there would be a strong significant relationship between the two life history traits.

A full model conducted with life history traits (body size, offspring number, gestation period and maximum depth) and IUCN status suggest that life history traits are a predictor of IUCN Red List category, although body size is the driving trait for this relationship. Body size does have a positive correlation with minimum reproductive cycle length, however this was stricken from the full model analysis due to sample number. Future work to delve into the relationship between elasmobranch life history traits and IUCN Red List category would require higher number of species for a greater analysis.

If elasmobranchs were to exhibit low genetic diversity due to population declines we would expect to observe this in the data. A caveat within our data set is that there are a low number of genetic diversity levels for species classified as critically endangered. It could be argued that the results are due to a low overall species number as numerous studies were stricken from the analysis due to their poor primer numbers, sample numbers or what appears to be invalid data (such as heterozygosity levels over the maximum of 1.000). Nonetheless we would expect to see a significant difference between the diversity levels between species threatened with extinction (overall elasmobranch average *He* = 0.6631) and non-threatened (overall elasmobranch average *He* = 0.6395) which we do not. Evidence of population declines, such as bottlenecks, would usually be observed through the genetic diversity levels often for multiple generations depending on the extremity of the declines or bottlenecks. Chapman *et al*. (2011) hypothesized that the high genetic diversity in a critically endangered species smalltooth sawfish *Pristis pectinata* was due to; 1) the longevity of the species which would slow genetic loss; 2) the current population size at its lowest is still large enough to maintain genetic diversity; and 3) life history traits and reproductive

behaviour of elasmobranchs reducing the susceptibility loss of genetic diversity from inbreeding or bottlenecks. Within this study the three Chapman *et al.* (2011) hypotheses were investigated, with the exception of reproductive behaviour. Body size the only significant predictor for IUCN category however there was no significant relationships found between genetic diversity and life history traits.

In conclusion we therefore propose the following hypothesis; 1) body size is a predictor for all elasmobranch IUCN categories, in that the larger an elasmobranch species is, the more likely the species is classified as threatened; 2) genetic diversity is maintained in threatened species of elasmobranchs due to behaviour such as migration or reproductive behaviour; 3) low genetic diversity is not observed in threatened elasmobranchs because either the bottleneck has not yet taken effect due to longevity, maturation ages and dates of which samples were taken.

Further work is need to 1) delve into allelic richness as a measure of genetic diversity in microsatellites; 2) the differences between the genetic diversity measures and threatened and non-threatened elasmobranchs as taxa, and 3) finally the differences in genetic diversity levels found in the same species (20 species total) by either mitochondrial or nuclear DNA. Preliminary results for 2) show that the average expected heterozygosity in threatened with extinction species was 0.6631 and the expected heterozygosity in non-threatened species was 0.6395.

References

Allendorf, F. W., Berry, O., & Ryman, N., 2014. So long to genetic diversity, and thanks for all the fish. *Molecular ecology*, *23*(1), 23-25.

Audzijonyte, A., Fulton, E., Haddon, M., Helidoniotis, F., Hobday, A.J., Kuparinen, A., Morrongiello, J., Smith, A.D., Upston, J. and Waples, R.S., 2016. Trends and management implications of human- influenced life- history changes in marine ectotherms. *Fish and Fisheries*.

Barnosky, A.D., 2010. *Heatstroke: nature in an age of global warming*. Island Press.

Barnosky, A.D., Matzke, N., Tomiya, S., Wogan, G.O., Swartz, B., Quental, T.B., Marshall, C., McGuire, J.L., Lindsey, E.L., Maguire, K.C. and Mersey, B., 2011. Has the Earth/'s sixth mass extinction already arrived?. *Nature*,*471*(7336), pp.51-57.

Baum, J.K., Myers, R.A., Kehler, D.G., Worm, B., Harley, S.J. and Doherty, P.A., 2003. Collapse and conservation of shark populations in the Northwest Atlantic. *Science*, *299*(5605), pp.389-392.

Branstetter, S., 1987. Age, growth and reproductive biology of the silky shark, Carcharhinus falciformis, and the scalloped hammerhead, Sphyrna lewini, from the northwestern Gulf of Mexico. *Environmental Biology of Fishes*, *19*(3), pp.161-173.

Butchart, S.H., Walpole, M., Collen, B., Van Strien, A., Scharlemann, J.P., Almond, R.E., Baillie, J.E., Bomhard, B., Brown, C., Bruno, J. and Carpenter, K.E., 2010. Global biodiversity: indicators of recent declines.*Science*, *328*(5982), pp.1164-1168.

Cardillo, M., Mace, G.M., Jones, K.E., Bielby, J., Bininda-Emonds, O.R., Sechrest, W., Orme, C.D.L. and Purvis, A., 2005. Multiple causes of high extinction risk in large mammal species. *Science*, *309*(5738), pp.1239-1241.

Ceballos, G., Ehrlich, P.R., Barnosky, A.D., García, A., Pringle, R.M. and Palmer, T.M., 2015. Accelerated modern human–induced species losses: Entering the sixth mass extinction. *Science Advances*, *1*(5), p.e1400253.

Charlesworth, D., 2003. Effects of inbreeding on the genetic diversity of populations. *Philosophical Transactions of the Royal Society of London B: Biological Sciences*, *358*(1434), pp.1051-1070.

Cortés, E., 2000. Life history patterns and correlations in sharks. *Reviews in Fisheries Science*, *8*(4), pp.299-344.

Dirzo, R. and Raven, P.H., 2003. Global state of biodiversity and loss.*Annual Review of Environment and Resources*, *28*(1), pp.137-167.

Dulvy, N.K., Baum J.K., Clarke, S. et al., 2008. You can swim but you can't hide: the global status and conservation of oceanic pelagic sharks. *Aquatic Conservation* 18, 459-482

Dulvy, N.K., Fowler, S.L., Musick, J.A., Cavanagh, R.D., Kyne, P.M., Harrison, L.R., Carlson, J.K., Davidson, L.N., Fordham, S.V., Francis, M.P. and Pollock, C.M., 2014. Extinction risk and conservation of the world's sharks and rays. *Elife*, *3*, p.e00590.

Engelhaupt, E., 2007. *Farming the Deep Blue Sea*. *Environment of Science Technology*, 41 (12), pp 4188–4191

England, P.R., Osler, G.H., Woodworth, L.M., Montgomery, M.E., Briscoe, D.A. and Frankham, R., 2003. Effects of intense versus diffuse population bottlenecks on microsatellite genetic diversity and evolutionary potential.*Conservation Genetics*, *4*(5), pp.595-604.

FAO, 1999. *The State of World Fisheries and Aquaculture 1999*. Food and Agriculture Organization of the United Nations. Rome

FAO, 2012. *The State of World Fisheries and Aquaculture 2012.* Food and Agriculture Organization of the United Nations, Rome.

Fish Weights. 2016. *Fish Weight Calculator.* [ONLINE] Available at: http://fishweights.net/. [Accessed 5 July 2016]

Fishbase. 2016*. Prionace glauca (Linnaeus, 1758) Blue shark*. [ONLINE] Available at: http://www.fishbase.org/Summary/SpeciesSummary.php?ID=898&AT=blue+shark. [Accessed 5 July 2016].

Frankham, R., Ballou, J.D., and Bricoe, D.A., 2004. A Primer of Conservation Genetics. Cambridge, United Kingdom: Cambridge University Press. 20-175.

Gilman, E., Brothers, N. and Kobayashi, D.R., 2005. Principles and approaches to abate seabird by- catch in longline fisheries. *Fish and Fisheries*, *6*(1), pp.35-49.

Hoenig, J.M. and Gruber, S.H., 1990. Life-history patterns in the elasmobranchs: implications for fisheries management. *NOAA Technical Report NMFS*, *90*(1), p.16.

Hoffmann, M., Hilton-Taylor, C., Angulo, A., Böhm, M., Brooks, T.M., Butchart, S.H., Carpenter, K.E., Chanson, J., Collen, B., Cox, N.A. and Darwall, W.R., 2010. The impact of conservation on the status of the world's vertebrates. *science*, *330*(6010), pp.1503-1509.

IUCN, 2014. *A quarter of sharks and rays threatened with extinction.* [ONLINE] Available at: http://www.iucn.org/?14311/A-quarter-sharks-and-rays-threatened-with-extinction [Accessed 07 December 15].

Lande, R., 1988. Genetics and demography in biological conservation. *Science(Washington)*, *241*(4872), pp.1455-1460.

Leakey, R. and Lewin, R., 1997. The Sixth Extinction: Patterns of Life and the Future of Humankind. *Journal of Leisure Research*, *29*(4), p.476.

MacArthur, R. H., and Wilson, E.O., 1967. The theory of island biogeography. Princeton Univ. Press, Princeton, N.J. 203 p.

Menni, R.C., Cousseau, M.B. and Gosztonyi, A.R., 1986. Sobre la biología de los tiburones costeros de la provincia de Buenos Aires.[On the biology of coastal sharks of the Province of Buenos Aires, Argentina]. In *Anales de la Sociedad Científica Argentina* (Vol. 213, pp. 3-26). Sociedad Científica Argentina, Buenos Aires. AR.

Mollet, H.F., Cliff, G., Pratt Jr, H.L. and Stevens, J., 2000. Reproductive biology of the female shortfin mako, Isurus oxyrinchus Rafinesque, 1810, with comments on the embryonic development of lamnoids. *Fishery Bulletin*, (2).

Mora, C., Tittensor, D.P., Adl, S., Simpson, A.G. and Worm, B., 2011. How many species are there on Earth and in the ocean?. *PLoS Biol*, *9*(8), p.e1001127.

Myers, N., 1990. Mass extinctions: what can the past tell us about the present and the future?. *Palaeogeography, Palaeoclimatology, Palaeoecology*, *82*(1-2), pp.175-185.

Myers, N., Mittermeier, R.A., Mittermeier, C.G., Da Fonseca, G.A. and Kent, J., 2000. Biodiversity hotspots for conservation priorities. *Nature*, *403*(6772), pp.853-858.

Myers, R.A., Hutchings, J.A. and Barrowman, N.J., 1997. Why do fish stocks collapse? The example of cod in Atlantic Canada. *Ecological applications*, *7*(1), pp.91-106.

No Limits. 2016. *Blue Shark*. [ONLINE] Available at: http://www.nolimitsnofuture.org/sharks/blue-shark/. [Accessed 5 July 2016].

Pianka, E.R., 1970. On r-and K-selection. *The American Naturalist*,*104*(940), pp.592-597.

Pimm, S.L., Russell, G.J., Gittleman, J.L. and Brooks, T.M., 1995. The future of biodiversity. *Science-AAAS-Weekly Paper Edition*, *269*(5222), pp.347-349.

Pimms, S.l., Russell, J.L., Gittleman, and Brooks, T.M., 1995. The Future of Biodiversity. *Science* 269, 347-350

Piñeiro, Carmen, et al., 2003. Proteomics as a tool for the investigation of seafood and other marine products. *Journal of proteome research,* 2.2, 127-135.

Pinsky M.L. and Palumbi S.R., 2014. Meta-analysis reveals lower genetic diversity in overfished populations.*Molecular Ecology*, 23, 29–39.

R Core Team., 2016. *R: A language and environment for statistical computing*. R Foundation for Statistical Computing, Vienna, Austria. URL [Available at: https://www.R-project.org/]

Reynolds, J.D., Dulvy, N.K., Goodwin, N.B. and Hutchings, J.A., 2005. Biology of extinction risk in marine fishes. *Proceedings of the Royal Society of London B: Biological Sciences*, *272*(1579), pp.2337-2344.

Safina, C. and Klinger, D.H., 2008. Collapse of bluefin tuna in the western Atlantic. *Conservation Biology*, *22*(2), pp.243-246.

Shiffman, D. 2016. *Sharks(Euselachii)by The Ocean Portal Team; Reviewed by David Shiffman, University of Miami.* [ONLINE] Available at: http://ocean.si.edu/sharks. [Accessed 5 July 2016].

Smith, P.J., Francis, R.I.C.C. and McVeagh, M., 1991. Loss of genetic diversity due to fishing pressure. *Fisheries Research*, *10*(3-4), pp.309-316.

Spielman, D., Brook, B.W. and Frankham, R., 2004 (b). Most species are not driven to extinction before genetic factors impact them. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(42), pp.15261-15264.

Spielman, D., Brook, B.W., Briscoe, D.A. and Frankham, R., 2004. Does inbreeding and loss of genetic diversity decrease disease resistance?.*Conservation Genetics*, *5*(4), pp.439-448.

Stevens, J. 2009. *Prionace glauca*. The IUCN Red List of Threatened Species 2009: e.T39381A10222811. http://dx.doi.org/10.2305/IUCN.UK.2009-2.RLTS.T39381A10222811.en.Downloaded on 05 July 2016.

Stevens, J.D., Bonfil, R., Dulvy, N.K. and Walker, P.A., 2000. The effects of fishing on sharks, rays, and chimaeras (chondrichthyans), and the implications for marine ecosystems. *ICES Journal of Marine Science: Journal du Conseil*, *57*(3), pp.476-494.

Webb, T. J., & Mindel, B. L., 2015. Global patterns of extinction risk in marine and non-marine systems. *Current Biology*, *25*(4), 506-511.

Worm, B., Davis, B., Kettemer, L., Ward-Paige, C. A., Chapman, D., Heithaus, M. R., Kessel, S.T. & Gruber, S. H., 2013. Global catches, exploitation rates, and rebuilding options for sharks. *Marine Policy*, 40, 194-204.