Development and application of fatty acid tracers to assess the impacts of white shark cage-diving on target and non-target species

by

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Contents

Thesis summary

Marine wildlife tourism is the fastest growing sector of the tourism industry, earning billions of dollars globally, and with it, a myriad of management and conservation challenges. White shark *Carcharodon carcharias* cage-diving is particularly popular and relies on provisioning, whereby bait (tuna heads and gills) and chum (minced tuna) is used to coax sharks to within view of tourists. Owing to a number of recorded impacts on various shark species, including changes in behaviour, movement, habitat use, and activity levels, wildlife provisioning remains a contentious issue amongst managers, tourism operators, and the public alike. The objective of my thesis is to determine the effects of cage-diving on the diet of target and non-target species using biochemical tracer results obtained via new sampling and analytical methods.

Although biochemical tracers, including fatty acids (FAs), are increasingly used to investigate feeding ecology of marine megafauna, their use in this setting requires further practical development in sample collection and storage scenarios, and quantitative development to understand how to appropriately interpret FA results. Specifically, I assessed the operational limitations of using FAs in the context of white shark tissue collection, determining that muscle and sub-dermal tissue biochemistry was not directly comparable, and that 50 mg of muscle was sufficient to obtain accurate FA profiles. Following this minimum tissue quantity, I modified a biopsy probe intended for underwater use, and showed its ability to collect sufficient tissue from white sharks, both underwater and from above the water's surface. Together, these chapters provided the practical foundation to confidently apply FA analysis to samples collected from free-swimming white sharks around cage-diving vessels. Yet, analytical uncertainties remained, as our understanding of FAs generally come from controlled experiments on taxa comparatively easier to study. Chondrichthyans (sharks, rays, and chimaeras) have unique metabolic processes which may impact FA biochemical pathways, their deposition in tissues, and resulting

data interpretation. I conducted a global analysis of 106 published FA profiles of chondrichthyans, and used a series of multivariate analyses and univariate model averaging to identify which FAs could trace specific aspects of chondrichthyan ecology (i.e. different habitats, water temperatures, trophic guilds, and phylogeny). Habitat type was distinguished by five individual FAs (16:0, 18:0, 22:6ω3, 20:5ω3 and 20:4ω6), allowing these FAs to be confidently used to trace specific foraging habitats (e.g. pelagic vs. reef). These operational (sample collection) and analytical (FA tracers) advances were then applied to white sharks and non-target fishes and rays at the Neptune Islands, Australia to determine if the cage-diving industry affected their diet and nutrition. I found no evidence of dietary shifts or reduced nutritional condition attributed to tourism-exposed residency at the Neptune Islands for the white sharks, despite other work detailing changes in daily activity and habitat use. Yet, all eight non-target species including pelagic fishes, reef fishes and rays, showed dietary shifts consistent with bait and chum consumption. These results showcase how the impacts of provisioning can extend beyond the charismatic species targeted by tourism operators, and also highlight how future research and impact management necessitates an ecosystem-approach, inclusive of non-target species.

Declaration

I certify that this thesis:

1. does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and
2. to the best of my knowledge and belief, does not contain any material previously published or written by another person except where due reference is made in the text.

Signed….  .

Date……6th September, 2019……..

Author contributions, permits, and funding

The following outlines the author contributions and acknowledgments for each data chapter:

Chapter 2

Meyer, L., Pethybridge, H., Nichols, P. D., Beckmann, C., Bruce, B. D., Werry, J. M., & Huveneers, C. (2017). Assessing the Functional Limitations of Lipids and Fatty Acids for Diet Determination: The Importance of Tissue Type, Quantity, and Quality. *Frontiers in Marine Science*, *4*. doi:10.3389/fmars.2017.00369

LM, CH, CB, HP, PN, JW, BB: conceived and designed the experiments; BB, JW, CB, and CH: provided tissue samples; LM: performed the experiments, analyzed the data with the help of CH, CB, PN, and HP; LM wrote the manuscript with the advice of CH, CB, HP, PN, JW, and BB.

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Chapter 3

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LM designed the biopsy modification and study, LM, AF and CH collected samples, LM analysed the samples and results and wrote the manuscript with the advice of AF and CH.

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LM conceived the ideas and LM, HP, PN, CB, and CH designed methodology; LM collected and analysed the data and wrote the manuscript with the advice of HP, PN, CB, and CH.

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All authors designed the study. LM, CB and CH collected research material and LM, HP and CB performed the laboratory analyses. LM performed the statistical analyses and wrote the manuscript with the advice of HP, CB, BB, and CH.

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Conference presentation relevant to this thesis

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Sharks International, João Pessoa, RB, Brazil 2018, "What can fatty acids reveal about shark and ray ecology?"

Australian Marine Science Association Conference, Adelaide, SA, Australia 2018, "Assessing the effects of South Australia's cage-diving industry on target and non-target species"

Oceania Chondrichthyan Society Conference, North Stradbroke Island, QLD, Australia 2018, "Café or Buffet? Ontogenetic shifts in white shark diet and habitat use"

Indo-pacific Fish Conference, Tahiti, French Polynesia 2017, "Using fatty acid profiling to assess the dietary effects of cage-diving: target and non-target species"

Australian Section of the American Oil Chemists' Society, Barossa Valley, SA, Australia 2017, "Assessing the functional limitations of fatty acids (FAs) for diet determination"

Australian Society for Fish Biology, Hobart, TAS, Australia 2016, "Functional limitations of fatty acid profiles"

Other publications or contributions

Gallagher, A., **Meyer, L**., Pethybridge, R., Huveneers, C., Butcher, A. (In press). Physiological stress responses of white sharks (*Carcharodon carcharias*) to short-term capture: amino acids and fatty acids*. Endangered Species Research.*

May, C., **Meyer, L**., Whitmarsh, S., & Huveneers, C. (2019). Eyes on the size: Accuracy of visual length estimates of white sharks, *Carcharodon carcharias*. *Royal Society Open Science*.

Munroe, S. E. M., **Meyer, L**., & Heithaus, M. R. (2018). Dietary Biomarkers in Shark Foraging and Movement Ecology. In J. Carrier, M. Heithaus, & C. A. Simpfendorfer (Eds.), *Shark Research: Emerging Technologies and Applications for the Field and Laboratory*. Boca Raton, FL: CRC Press.

Huveneers, C., Whitmarsh, S., Thiele, M., **Meyer, L**., Fox, A., & Bradshaw, C. J. A. (2018). Effectiveness of five personal shark-bite deterrents for surfers. *PeerJ*, *6*, e5554.

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Huveneers, C., Whitmarsh, S., Thiele, M., May, C., **Meyer, L**., Fox, A., & Bradshaw, C. J. A. (2018). Response of white sharks exposed to newly developed personal shark deterrents.

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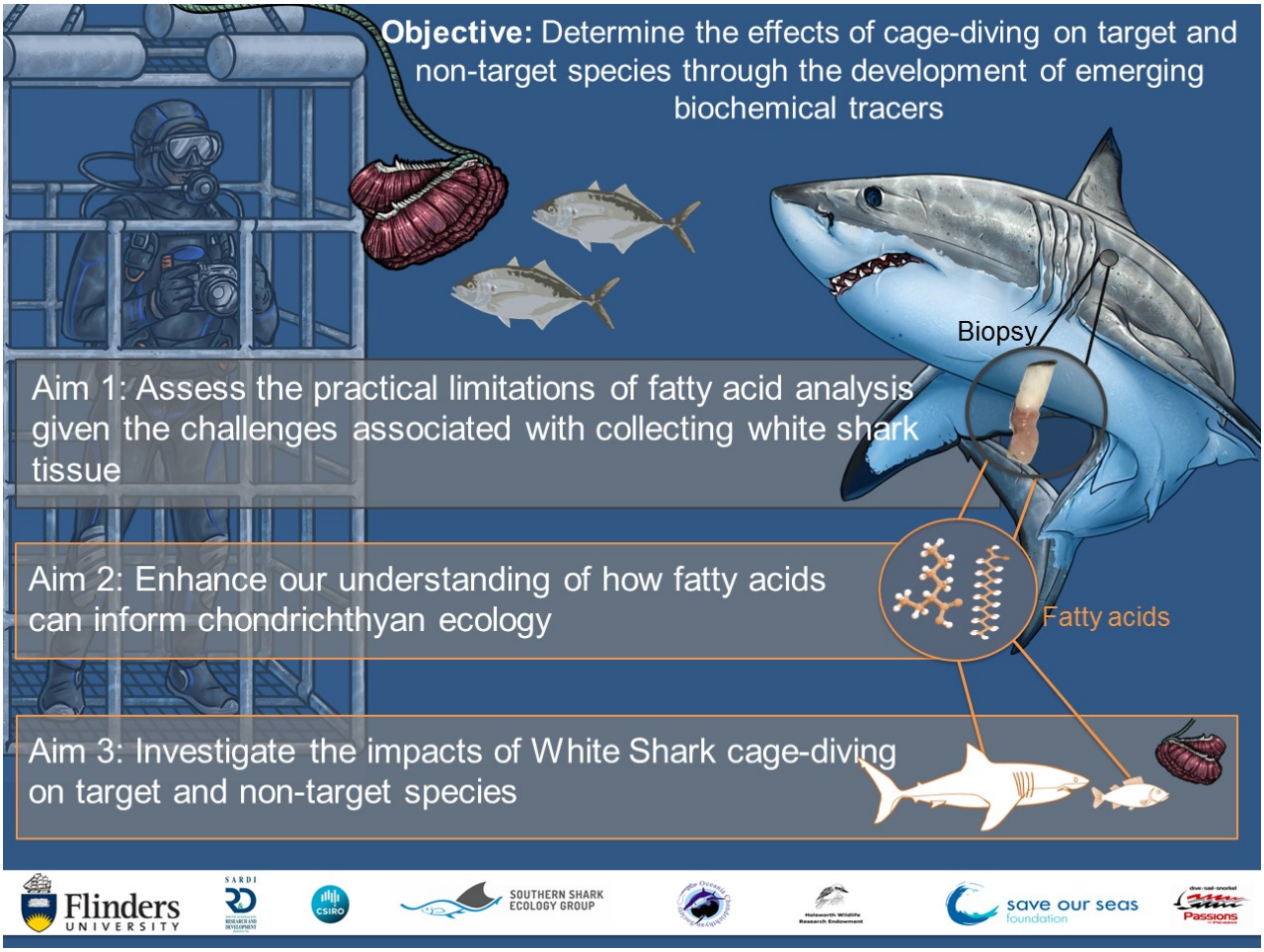
Chapter 1

General introduction



Marine wildlife tourism

Wildlife tourism is rapidly growing in popularity, diversity of locations, and targeted species (Newsome et al., 2005; Orams, 2002; Trave et al., 2017). Interacting with wildlife is already one of the top factors influencing travel decisions (Davis et al., 2001; Higginbottom, 2004), with 43% of Australia's international visitors seeking wildlife tourism experiences, amounting to 2.2 million foreign, and 2.5 million domestic participants in 2006 alone (Ballantyne et al., 2009). Estimates of global participant numbers vary between 79 and 440 million (Moorhouse et al., 2015; Trave et al., 2017), with the industry projected to double in the next 50 years (French et al., 2011). Studies exploring visitor motivation and satisfaction have revealed the importance of natural settings, education, animal welfare, and most importantly, up-close encounters (Curtin, 2005; Patroni et al., 2019; Pearce et al., 2017; Tremblay, 2002). Particularly sought after, are close encounters with large, endangered, and charismatic animals, in their natural environment (Giglio et al., 2015; Skibins et al., 2013; Tremblay, 2002). This makes operations targeting marine megafauna, i.e. sharks and marine mammals, one of the fastest growing sectors of wildlife tourism (Wearing and Neil, 2009).

The viability of wildlife tours, especially those targeting elusive animals, relies on providing reasonably constant, up-close encounters (Duffus and Dearden, 1990; Knight, 2009; Skibins et al., 2013). To aggregate target species, tour operators use a range of 'provisions' (Knight, 2009), including food (natural and unnatural prey items), baits (often tethered fish remains), and chum (minced offal, producing an inedible oil slick) (Brena et al., 2015; Patroni et al., 2018; Richards et al., 2015). Such activities are common practice in the marine environment (Bryant, 1994; Duffus and Dearden, 1990; Knight, 2009) and have been used to entice marine mammals (Bryant, 1994; Mann and Kemps, 2003), sharks (Bruce, 2015; Gallagher and Hammerschlag, 2011), rays (Semeniuk et al., 2007), and fishes (Brookhouse et al., 2014; Feitosa et al., 2012) for decades (Knight, 2009).

Despite the growing number of participants eager for up-close encounters, provisioning is highly contentious amongst tourists, managers, and scientists alike (Newsome and Rodger, 2008; Burgin and Hardiman, 2015; Richards et al., 2015; Ziegler et al., 2018). This is especially true for provisioning sharks, which are often at the forefront of species conservation and public safety concerns. Still passionately debated, supporters of shark tourism cite the unprecedented research opportunities, potential for financial and social support for conservation, and the shift in the public's largely negative perceptions of sharks (Gallagher et al., 2015; Haas et al., 2017; Macdonald et al., 2017), the latter persisting as a hurdle for global shark conservation (Friedrich et al., 2014; Neff, 2014). In contrast, opponents draw on animal welfare and public safety concerns, including provisioning induced changes to shark behaviour and residency (e.g. Bruce and Bradford, 2011), aggression (e.g. Clarke, et al., 2013), and the potential for tourism operations to condition sharks, encouraging associations between marine activities and food which could potentiate negative human-shark encounters (concerns discussed in Johnson and Kock, 2006). This debate is not limited to shark tourism, as the effects of provisioning practices on a number of marine species are the subject of ongoing research around the world (reviewed in Brena et al., 2015; Newsome and Rodger, 2008; Patroni et al., 2018; Trave et al., 2017).

Impacts on species

Provisioning wildlife to facilitate tourism encounters has a history of impacting species (Brennan et al., 1985; Cole, 1994; Orams et al., 1996). Specifically, eliciting changes in marine wildlife site occupancy (Bruce and Bradford, 2013; Brunnschweiler et al., 2014), relative and overall abundance (Brookhouse et al., 2014; Clarke et al., 2013; Meyer et al., 2009), behaviour (Brookhouse et al., 2014; Clarke et al., 2013; Mann & Kemps, 2003), activity levels (Huveneers et al., 2018b), and other physiological, behavioural, and spatial and temporal space use characteristics (recently reviewed in Brena et al., 2015; Gallagher et al., 2015; Patroni et al., 2018; Trave et al., 2017). However, the effects of provisioning on diet and nutritional condition

are relatively unexplored, despite the use of food-based attracts, popularity of directly feeding wildlife (Newsome and Rodger, 2008; Orams, 2002) and ongoing debates amongst stake holders about its potential impacts (Bruce, 2015; Gallagher et al., 2015; Richards et al., 2015). In a recent review by Brena et al., (2015), “dietary habits” were the least studied of 10 different impacts from shark tourism, with only four out of the 22 studies exploring the subject. The findings were species- and context-specific, highlighting the potential for provisioning to alter shark and ray diets (Maljković and Côté, 2011; Semeniuk et al., 2007), detrimentally impacting physiology and body condition (Semeniuk et al., 2009), or having no detectable effects (Abrantes et al., 2018). Following these context-specific responses, animal welfare concerns about the dietary and nutritional cost of different species interacting with tourism operators, has been articulated in a number of studies and reviews (e.g., Barnett et al., 2016; Brena et al., 2015; Gallagher and Huveneers, 2018).

Provisioning may also impact non-target species and ecosystems, which underpin tourism sites and support the aggregations of target species. This has the potential to impact other industries, including fishing and aquaculture, and recreational use by the public. Such impacts have been largely overlooked in research and management objectives, despite the few existing studies demonstrating shifts in non-target species behaviour and predation (Milazzo et al., 2006), movement (Rizzari et al., 2017), and parasite loads (Vignon et al., 2010) as well as ecological changes to the benthos (Wong et al., 2019) associated with local provisioning activities. In the wake of these findings, a string of recent reviews and management objectives have begun calling for the establishment of ecosystem-level studies to address the impacts of provisioning on community ecology and non-target species (Brena et al., 2015; Burgin and Hardiman, 2015; Gallagher et al., 2015; Higginbottom et al., 2003). Mirroring the scarcity of research detailing the impacts of provisioning on the diet of target species, the diet of non-target species has not been evaluated, despite observations of aggregating fishes consuming provisions at tourism sites worldwide (Gallagher and Huveneers, 2018; A. Fox pers. comm.).

White shark cage-diving in South Australia

Cage-diving with white sharks (*Carcharodon carcharias*) is particularly popular, due to their rarity, threatened conservation status, size, role as a top predator, and notoriety in popular media (Apps et al., 2016; Huveneers et al., 2017). Tours are available in Australia, Mexico, USA, South Africa, and New Zealand, often with multiple operators visiting one site simultaneously. Paralleling global growth in marine wildlife tourism, Australia's cage-diving industry operates year-round at the Neptune Islands Group (Ron and Valerie Taylor) Marine Park, hosting >10,000 passengers across three operators running 260 days per year (DEWNR, 2016) contributing $15 million annually to the regional economy (Huveneers et al., 2017). The Neptune Islands are also one of the region's Representative Marine Protected Areas, providing protection for unique offshore island habitats (DEWNR, 2012) and supporting > 130 recorded fish, marine mammal, bird, and elasmobranch species, including Australia's largest population of Long-nosed Fur Seal *Arctocephalus forsteri* and the industry's target white shark (Atlas of Living Australia, 2019).

As with much shark and ray wildlife tourism, cage-diving in South Australia relies heavily on chum (minced tuna creating an inedible oil slick) and tethered baits (Southern Bluefin Tuna *Thunnus maccoyii* heads and gills) to attract sharks within view of divers. Citing concerns about the effects of provisioning on white sharks (changes in fine-scale habitat use [Huveneers et al., 2013], residency [Bruce and Bradford, 2013], activity levels [Huveneers et al., 2018]) and other behaviour [reviewed in Bruce, 2015; discussed in Gallagher and Huveneers, 2018]) government regulations have sought to minimise such impacts on the target species (DEWNR, 2016) by limiting bait input to a maximum of 100 kg of daily bait and chum input per operator (DEWNR, 2016) and explicitly prohibiting feeding white sharks (policy 7.3, DEWNR, 2016). Australia's White Shark Recovery Plan also identifies cage-diving as a potential threat to the recovery of the species (DSEWPaC, 2013). With a rapidly growing tourism industry with hotly debated practices, centred on an ecologically vital and iconic endangered species and a growing body of

evidence suggesting provisioning induced changes, assessing the dietary and nutritional impacts of the cage-diving industry is a financial and ecological imperative (Bruce, 2015; Gallagher and Huveneers, 2018; Huveneers et al., 2018a).

Using biochemical tracers to determine diet

Several biochemical methods have evolved to complement traditional stomach content analysis, and these are growing in popularity owing to their capacity to determine detailed aspects of an organism's diet, nutritional state, and trophic level (Budge et al., 2006; Hussey et al., 2012; Munroe et al., 2018; Pethybridge et al., 2018). Specifically, fatty acid (FA) analysis is emerging as a vital tool for trophic ecologists and is used across marine and terrestrial taxa to identify spatiotemporal foraging patterns, as well as food-web dynamics and the ecological transfer of biologically essential molecules through ecosystems (Budge et al., 2006; Colombo et al., 2016; Gladyshev et al., 2017; Munroe et al., 2018; Tocher, 2003).

The utility of FAs stems from their molecular role as major components of lipids or fats, where they serve a number of essential biological functions (Tocher, 2003). For example, FAs play key roles in metabolism and buoyancy regulation (Pond and Tarling, 2011), they are essential components of membranes regulating ion balance (Glencross, 2009), and are important for immune systems (Montero et al., 2004), brain function (Masud and Tsukamoto, 1998), behaviour, and growth (Tocher, 2010) (discussed in Glencross 2009; Parrish 2013; Sargent et al. 1999; Tocher 2003). Yet, vertebrates lack the ability to synthesise a number of key FAs *de-novo* (Fraser et al., 1989; Iverson et al., 2004). Fatty acid biomodification is also energetically costly, and enzymatically limited (Iverson et al., 2004). This means that FAs generally retain their chemical structures, or undergo minimal biomodification as they are assimilated from dietary sources into vertebrate tissues (Sargent et al., 1999; Tocher, 2003).

Fatty acids have a number of diverse structures, which differ across distinct basal food web sources, allowing for foraging to be traced in consumers as these chemical structures are retained through trophic transfer. Broadly, FAs are composed of a carbon chain, with a methyl group at one end (denoted by omega, ω or n) and a carboxyl group at the other end (Sargent et al., 1999). The different carbon chain lengths (often containing 14–24 carbons), and the number and placement of double bonds has given rise to a variety of distinct FA molecules, with > 60 often detected and identified in vertebrate tissues samples (Budge et al., 2006; Parrish, 2013; Sargent et al., 1999). Based on the number of double bonds, FAs are categorised as "saturated" (SFA) with no double bonds, "monounsaturated" (MUFA) with one carbon-carbon double bond, or "polyunsaturated" (PUFA) with multiple double bonds. The placement of the first double bond (e.g. "ω3" for the first double bond between the third and the fourth carbon atom) further subdivides these groups into the ω7, ω9 and ω11 MUFAs and the ω3 and ω6 PUFAs (sometimes referred to as omega-3 and omega-6 fatty acids).

The variety of FAs, with their distinct chemical structures, originate from different producers at the base of the food web. For example, FA 20:5ω3 comes from diatoms, 18:3ω3 is produced in specific macroalgae, and 20:4ω3 originates in protozoa, red algae, and kelp (Kelly and Scheibling 2012; see Appendix Table S1 for detailed outline of origins and transfer of FAs). Abiotic factors including water temperature (Gibson et al., 1984) and freshwater input (Sargent et al. 1999) influence primary production and community composition (Lowe et al., 2014), and thus dictate which FAs are produced at the base of the food web. Biotic influences including phylogenetic differences in physiology and trophic guild can further dictate FA assimilation, especially in higher trophic level consumers (Colombo et al., 2016; Galloway and Winder, 2015; Gladyshev et al., 2017; Vasconi et al., 2015). This heterogeneity in FAs, coupled with the limited capacity for biosynthesis, allows such chemotaxonomic signatures to persist up the food web into higher order taxa, such that FAs in consumers reflect foraging dynamics, both abiotic (e.g.

habitat type and temperature) and biotic (e.g. phylogeny and ecomorphology) (Colombo et al., 2016; Gladyshev et al., 2017).

The multitude of available FA tracers and the strong physiological link between lipids and physiology, nutrition, and bioenergetics (Gallagher et al., 2017; Tocher, 2003) offers a number of opportunities not available with other biochemical tracers, such as stable isotopes (Pethybridge et al. 2018). Particularly, the abundance of FAs offers greater specificity than stable isotopes when determining prey items (e.g. McMeans et al., 2013; discussed in Budge et al., 2006), and has the capacity to detail basal linkages (Ackman, 1994; Sargent et al., 1999; Tocher, 2003). As lipids are more metabolically active than bulk protein, they reflect changes in diet and nutrition at shorter time scales than stable isotopes (weeks with FAs *vs* months-years with isotopes (Beckmann et al., 2013b), making lipid and FA analysis an ideal toolset to explore changes in feeding ecology across a short time period (Pethybridge et al., 2018). Owing to this broad applicability to detail complex foraging ecology, more than 29,000 published studies featured FA analysis for marine and aquatic taxa alone between 1990 and 2014 (Rudy et al., 2016). As many of these studies investigate the nutritional aspects of FAs in relation to human consumption, this knowledge can be applied to marine trophic ecology, enabling a better understanding of physiology and nutritional condition (Gallagher et al., 2017).

Determining if fatty acids reflect shark and ray ecology

Following advances in our understanding of biochemical tracers, including lipids, FAs, and stable isotopes, the use of such biochemical toolsets with marine megafauna is particularly promising. Given the popularity and applications of FAs in well-understood taxa like teleosts, FA tracers are being applied to complex questions about chondrichthyan (shark, ray and chimaera) ecology. These include studies on diet (McMeans et al., 2012; Pethybridge et al., 2011), habitat use (Rohner et al., 2013), ontogenetic shifts (Wai et al. 2011), quantifying trophic niche overlap (Every et al., 2017), and identifying tourism provisioning (Semeniuk et al., 2007). While FA

assimilation in chondrichthyans appears to be similar to other taxa (Beckmann et al., 2013a, 2013b), these organisms have evolved notoriously distinct physiologies and metabolic processes, relying on ketone bodies instead of lipids for energy metabolism, and using fatty, lipid rich livers as a sink for long-term energy storage (Ballantyne, 1997). Given this unique taxa-specific biochemistry and the importance of biochemical pathways and metabolism in FA assimilation from prey to predator, the uncommon physiology of chondrichthyans may impact FA deposition and usability. The appropriate use of FAs to detail shark and ray ecology therefore requires additional foundational work to further our understanding of the taxa-specific applications of this emerging toolset.

Practical limitations of sample collection

The appropriate use of FA analysis is contingent upon the collection and suitable storage of sufficient quantities of tissue. The practical limitations of which are previously unexplored in any large elasmobranchs like white sharks. Research investigating the biology and ecology of marine animals is increasingly calling for the development and use of non-lethal sampling techniques (Fossi et al., 2010; Jardine et al., 2011; Smith et al., 2018). This is especially pronounced for studies of elasmobranchs (Hammerschlag and Sulikowski, 2011; Heupel and Simpfendorfer, 2010; Marshall and Pierce, 2012), owing to their generally low abundance and high conservation concern (Dulvy et al., 2014). Minimally invasive biopsies from free-swimming sharks (Daly and Smale, 2013; Reeb and Best, 2006; Robbins, 2006) are growing in popularity, as they can obtain tissue samples for biochemical studies (e.g., Carlisle et al., 2012; Hooker et al., 2001; Hussey et al., 2012), while reducing the stress and detrimental effects of the capture and release process. When sampling free-swimming white sharks, the operational limitations of various biopsy methods extend to the amount of tissue obtained, potentially constraining the biochemical analyses that can be undertaken. With the thick epidermal layer serving as a barrier, collecting sufficient amounts of usable muscle from large

elasmobranchs can be particularly challenging. The sub-dermal layer of white sharks can be up to 3 cm thick, hindering the ability to collect the underlying muscle (Jaime-Rivera et al., 2013), the tissue most often used to study elasmobranch trophic ecology. As such, understanding the minimum quantity of different tissues necessary for accurate FA analysis is vital when considering the appropriateness of various biopsy probes, field sampling scenarios, and the applicability of the sampling method to the different available biochemical tools.

Acquiring samples of highly mobile, rare, and/or potentially dangerous megafauna is not limited to collecting biopsies from live organisms, as specimens from museums, past research, fisheries bycatch (Pethybridge et al., 2011), beach strandings (Rohner et al., 2013), and shark-control measures (Davidson et al., 2011, 2014; Pethybridge et al., 2014) provide a crucial source of potential samples for research teams. However, these samples are taken from carcasses in variable conditions, which may have spent multiple days at ambient temperature, where there is the high potential for lipid and FA degradation (Rudy et al., 2016). Additionally, field sampling often includes remote and hostile field locations (e.g., hot and humid tropics, and offshore sampling sites), with sub-optimal storage and preservation options. Unfortunately, the realities of collection opportunities challenge the use of FAs, which in other taxa oxidise when exposed to air, high temperatures, and direct sunlight, leading to tissue degradation and loss of information (Budge et al., 2006; Rudy et al., 2016). Additionally, FA studies often use tissue samples collected over a long period of time (e.g., 5 years—Davidson et al., 2011, 2014; 2 years—Rohner et al., 2013; 12 years— Pethybridge et al., 2014 and 3 years—Jaime-Rivera and Caraveo-Patiño, 2014), providing another opportunity for unchecked FA degradation throughout long periods of frozen storage. Given the challenges with acquiring samples from rare, endangered, and potentially dangerous animals, like white sharks, understanding how storage conditions impact the usability of these samples for FA analysis will enable scientists to appropriately use archived samples.

Research objectives

The overarching objective of my thesis is to determine the effects of white shark cage-diving on target and non-target species through the development of quantitative and field applications for emerging biochemical tracers. Specifically, the aims of this thesis are to:

1) Assess the practical limitations of FA analysis given the challenges associated with white shark tissue collection.
2) Enhance our understanding of the capacity of FA profiles to inform chondrichthyan ecology.
3) Investigate the dietary impacts of white shark cage-diving on target and non-target species.

To fulfil each aim, I have compiled five thesis chapters (excluding this introductory chapter [1] and a general discussion chapter [7]), each with specific goals, which link to thesis objective and aims (Figure 1.1).

Thesis structure

Chapter 1 introduces the impacts of marine wildlife tourism and outlines the gaps in understanding of the practical and analytical use of fatty acids as biochemical tracers. This chapter should serve as a brief outline, as further introductory material can be found within each following chapter.

Chapter 2 used a controlled laboratory setting to assess the operational limitations of using FAs with white shark tissue. This includes determining 1) the differences in lipid content, lipid class, and FA profiles between muscle and sub-dermal tissue; 2) the minimum tissue sample size for FA analysis; and 3) the effects of handling and freezing storage time on FA degradation. This chapter fits within aim 1 (Figure 1.1) and has been published in *Frontiers in Marine Science*.

Chapter 3 details a simple biopsy modification to collect muscle samples from free-swimming sharks. The work compares field tests from surface and underwater biopsies to determine if either obtain sufficient tissue (as per aim 1 and Chapter 2) for biochemical analyses to be run on the same tissue core (e.g. stable isotopes, FAs, and genetics), informing aim 1 (Figure 1.1). This work has been published in *Biological Conservation*.

Chapter 4 is a global analysis of published chondrichthyan FA profiles to determine the drivers of FA tracers in ecology. This analysis provides a novel understanding of the biological and ecological information that can be inferred from FA profiles and further validates the use of FAs as tracers to investigate the trophic ecology of chondrichthyans. Goals from this chapter feed into aim 2 (Figure 1.1) and this chapter had been published in *Functional Ecology*.

Chapter 5 uses lipids and FAs from biopsied tissue (following aim 1 and chapter 2 & 3) to investigate the impact of cage-diving on the foraging ecology (as per aim 2) and nutritional condition of the industry's target white shark. This work informs aim 3 and this chapter has been published in *Tourism Management*.

Chapter 6 uses multiple biochemical tracers (FA and stable isotopes) to assess the impacts of provisioning by the white shark cage-diving industry on non-target species. Findings from this chapter feed into aim 3 (Figure 1.1).

Chapter 7 discusses the major findings and synthesises the results of Chapters 2–6. It highlights the need to reclassify "provisioning" terminology in the wake of findings from Chapter 5 and 6, and uses a modified framework to identify the management implications of attracting white aharks and unintentionally provisioning other taxa.

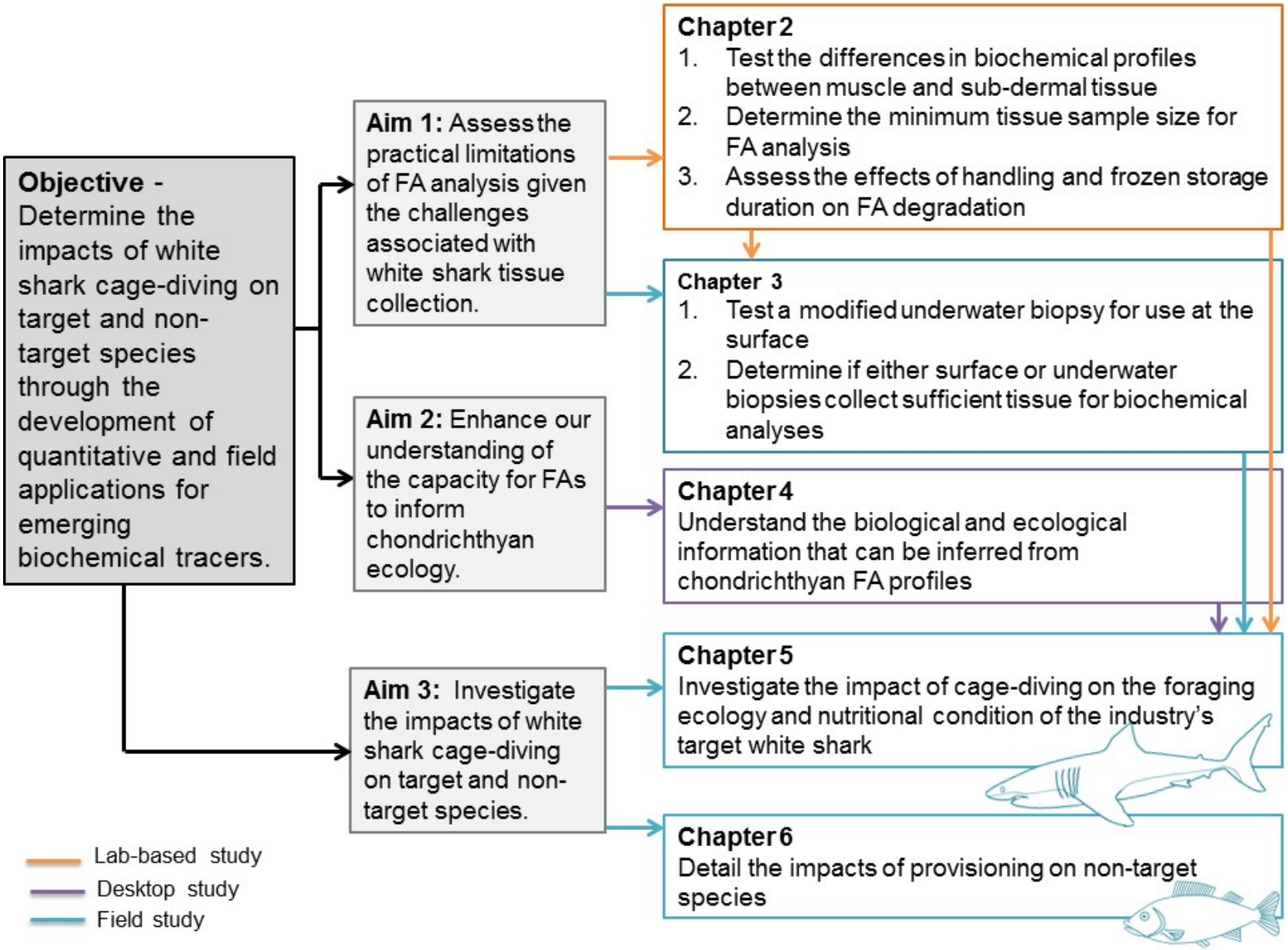


Figure 1.1 - Thesis overall objective and aims with each chapter's contribution to those aims and subsequent chapters.

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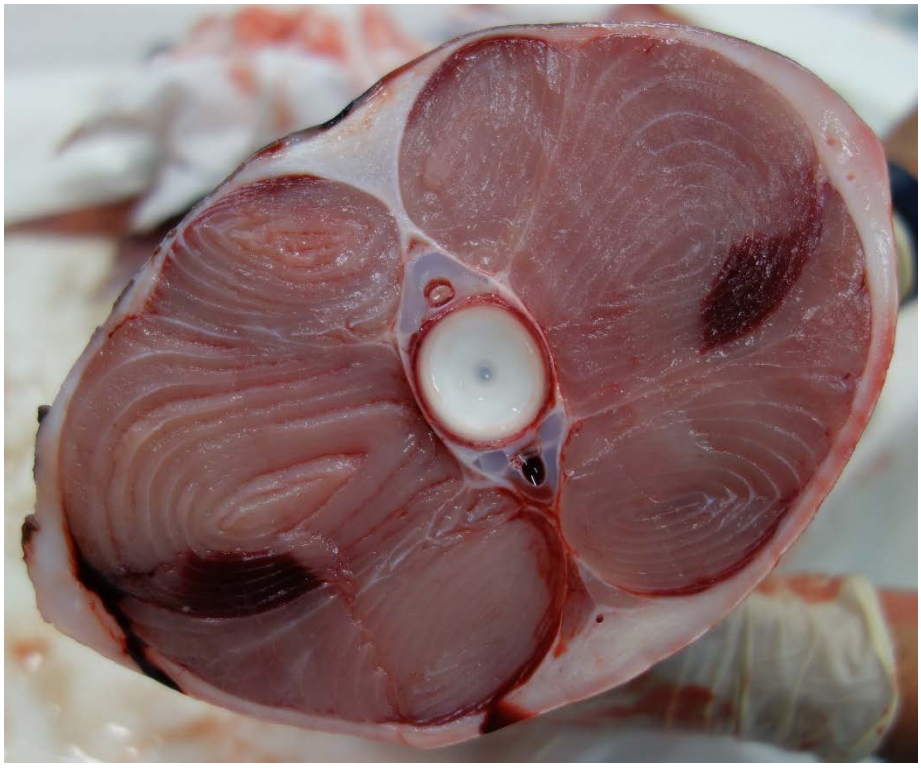
Chapter 2

Assessing the operational limitations of lipids and fatty acids for diet determination: the importance of tissue type, quantity and quality



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Abstract

Lipid and fatty acid (FA) analysis is commonly used to describe the trophic ecology of an increasing number of taxa. However, the applicability of these analyses is contingent upon the collection and storage of sufficient high quality tissue, the limitations of which are previously unexplored in elasmobranchs. Using samples from 110 white sharks, *Carcharodon carcharias*, collected throughout Australia, we investigated the importance of tissue type, sample quantity, and quality for reliable lipid class and FA analysis. We determined that muscle and sub-dermal tissue contain distinct lipid class and FA profiles, and were not directly comparable. Muscle samples as small as 12 mg dry weight (49 mg wet weight), provided reliable and consistent FA profiles, while sub-dermal tissue samples of 40 mg dry weight (186 mg wet weight) or greater were required to yield consistent profiles. This validates the suitability of minimally invasive sampling methods such as punch biopsies. The integrity of FA profiles in muscle was compromised after 24 hours at ambient temperature (~20 °C), making these degraded samples unreliable for accurate determination of dietary sources, yet sub-dermal tissue retained stable FA profiles under the same conditions, suggesting it may be a more robust tissue for trophic ecology work with potentially degraded samples. However, muscle samples archived for up to 16 years in -20°C retain their FA profiles, highlighting that tissue from museum or private collections can yield valid insights into the trophic ecology of marine elasmobranchs.

Introduction

The field of trophic ecology has seen a substantial increase in the number of available techniques and applications across aquatic and terrestrial taxa within the last half century (Layman et al., 2012, 2015; Christiansen et al., 2015; Nielsen et al., 2015; Young et al., 2015; Roslin and Majaneva, 2016). More recently, there has been a growing number of studies moving from traditional stomach-content analysis, which may provide a potentially limited view due to differences in digestibility among prey species (Hyslop, 1980), to time-integrated biochemical methods (reviewed in Traugott et al., 2013; Pethybridge et al., 2018). Lipid and fatty acid (FA) analysis is one such method growing in popularity as it has the capacity to elucidate key biological and ecological aspects, such as an organism's physiology and bioenergetics (Parrish et al., 2007; Pond and Tarling, 2011), and most often, trophic relationships (e.g., Bradshaw et al., 2003; Iverson et al., 2004; Budge et al., 2006). As per the saying "you are what you eat," certain FAs are transferred from prey to predator with minimal modification (Iverson et al., 2004; Budge et al., 2006), allowing certain functional trophic groups to be traced within a food chain. Owing to this broad applicability, more than 29,000 published studies featured FA analysis for marine and aquatic taxa alone, between 1990 and 2014 (Rudy et al., 2016).

The applicability of FA analysis is especially pertinent for threatened and iconic species for which lethal sampling, which is often used to obtain stomach contents, is not possible especially for large numbers of specimens. Instead, minimally invasive biopsy techniques are often employed to obtain tissue samples for biochemical studies (e.g. Hooker et al., 2001; Carlisle et al., 2012; Hussey et al., 2012). With the development of specialized biopsy probes (Reeb and Best, 2006; Robbins, 2006; Daly and Smale, 2013), tissue samples can be obtained from free-swimming marine organisms, reducing the stress and detrimental effects of the capture and release process, and enabling the increased use of FA analyses across a number of species, including threatened elasmobranchs (Couturier et al., 2013; Rohner et al., 2013; Every et al., 2016).

The accuracy and reliability of biochemical analyses are dependent on the methods used to collect and store samples. Sampling elasmobranchs in particular poses a series of logistical challenges, due in part to the large proportion of species considered at risk of extinction (Dulvy et al., 2014), leading to samples often being difficult and expensive to obtain. As a result, these samples are often highly valuable and one needs to understand the operational limitations of collecting and storing these tissues to maximize sampling opportunities and reliability of resulting data.

The increasing use of biopsies to collect tissues from elasmobranchs has led to constraints on the type, amount, and quality of tissue collected. Beneath the epidermis, elasmobranchs contain a deep sub-dermal layer of collagen and elastin fibers, which varies in thickness between species (Motta, 1977). The underlying physiological differences between the two tissue types (muscle, a metabolically active and protein-rich tissue vs. sub-dermal tissue, a less bioactive and largely structural tissue composed of elastin and collagen) results in distinct biochemical properties, with the potential to yield different ecological data. This is evidenced by recent isotopic studies on white sharks, *Carcharodon carcharias*, wherein muscle and sub-dermal tissue had the same ^{15}N isotopic signatures, but divergent ^{13}C signatures, which was attributed to differing tissue-specific incorporation rates (Carlisle et al., 2012; Kim et al., 2012; Jaime- Rivera et al., 2013). How these tissue-specific physiological and biochemical differences manifest in FA profiles remains poorly studied, with most elasmobranch work to date focused on the FA differences between skeletal muscle and the lipid-rich liver (e.g., Schaufler et al., 2005; Pethybridge et al., 2011; Beckmann et al., 2013), myocardial tissue (Davidson et al., 2011, 2014), and blood plasma (Ballantyne et al., 1993; McMeans et al., 2012). However, Every et al. (2016) recently showed differences in FA profiles between muscle tissue and fin clips (a mixed-tissue sample, including cartilage, connective tissue, muscle, vascularization and an outer dermal layer with denticles).

The operational limitations of various biopsy methods also extend to the amount of tissue obtained. With the thick epidermal layer serving as a barrier, collecting sufficient amounts of usable muscle from large elasmobranchs in particular, has proven challenging. The sub-dermal layer of white sharks can be up to 3 cm, hindering the ability to collect the underlying muscle (Jaime-Rivera et al., 2013). Whale sharks, *Rhincodon typus*, sampled with a biopsy probe penetrating ~2 cm yielded exclusively sub-dermal tissue (Rohner et al., 2013), whereas the ~2 cm biopsies of bull sharks, *Carcharhinus leucas* yielded 5% dermis, 40% sub-dermal and 55% muscle (Daly and Smale, 2013). These differences in the thickness of the sub-dermal layer complicate the collection of elasmobranch muscle samples. Although small amounts of tissue are sufficient for genetic (1 mg dry weight (DW), Kasajima et al., 2004) and stable isotope analysis (~10 mg DW, Jaime-Rivera et al., 2013), the minimum amount of muscle or sub-dermal tissue necessary for accurate FA analysis remains relatively unknown. Every et al. (2016) reported that FA were detectable in fin clips as small as 20 mg and muscle biopsies >10 mg dry weight, however the minimum sample amount yielding consistent results was not quantitatively assessed. Such evaluations are vital, particularly when considering the appropriateness of various biopsy probes, and the applicability of the sampling method across smaller elasmobranch species, from which removing large amounts of tissue is not feasible.

Appropriate sample acquisition, storage and tissue preservation is key when applying FA analysis techniques, as certain FAs (particularly long-chain (≥C20) polyunsaturated FAs, LC-PUFAs) oxidize when exposed to air, high temperatures, and direct sunlight, leading to tissue degradation and loss of information (Budge et al., 2006). This becomes particularly challenging when there are scarce opportunities for sampling (e.g. for highly mobile, rare, or cryptic species) and when working in remote and hostile field locations (e.g. hot and humid tropics, and offshore sampling sites). Despite the growing use of non-lethal biopsies, many FA studies use samples taken from deceased elasmobranch carcasses obtained from fisheries bycatch (Pethybridge et al., 2011), beach strandings (Rohner et al., 2013), and shark-control measures (Davidson et al.,

2011, 2014; Pethybridge et al., 2014). Given the variable condition of these carcasses, which may have spent multiple days at ambient temperature, there is the high potential for lipid and FA degradation within samples collected via these means. Additionally, FA studies often use tissue samples collected over a long period of time (e.g. 5 years—Davidson et al., 2011, 2014; 2 years—Rohner et al., 2013; 12 years— Pethybridge et al., 2014; and 3 years—Jaime-Rivera et al., 2014), providing another opportunity for unchecked FA degradation throughout these long periods of frozen storage. Several recent studies examining storage procedures have revealed significant species- and tissue-specific lipid and FA degradation over the course of several months held at −20 ˚C (e.g. Sahari et al., 2014; Paola and Isabel, 2015; Rudy et al., 2016). To date, the focus of such investigations have remained limited to highly valued commercial teleost (Roldán et al., 2005; Paola and Isabel, 2015; Rudy et al., 2016) and cephalopod species (Gullian-Klanian et al., 2017). Despite this evidence of FA degradation, it remains unassessed for the many archived elasmobranch tissues stored over the period of months to years.

Given the lack of information regarding the operational limitations and capabilities of lipid and FA biomarkers for application to highly mobile, rare or cryptic elasmobranchs, this study seeks to assess: 1) Differences in lipid content, lipid class, and FA profiles between muscle and sub-dermal tissue from white sharks; 2) The minimum muscle and sub-dermal tissue sample size required for consistent analysis of FA profiles; and 3) The effects of handling and freezing storage time on FA degradation via a controlled experiment with shark muscle tissue left at 20 ˚C for 5 days, and by comparing profiles of shark tissue stored over known periods of time at −20 ˚C, up to 16 years.

The knowledge gained from addressing these operational limitations will facilitate the more effective use of lipid and FA profiling on biopsied or potentially degraded tissues, allowing them to be employed with greater confidence in a range of ecological studies.

Methods

Sample Collection and Data Compilation

Tissue samples were collected from 110 white sharks from South Australia (SA), New South Wales (NSW), and Queensland (QLD), Australia between 2000 and 2016 (Table 2.1). Tissues were obtained through punch-biopsies of live, free-swimming white sharks from the Neptune Islands, SA, opportunistically through fisheries bycatch, the NSW Department of Primary Industries Shark Meshing Program and QLD Department of Agriculture and Fisheries Shark Control Program as part of the QLD large shark tagging research program. Samples were frozen and stored from 3 weeks to 16 years at −20 ˚C, until freeze-drying immediately prior to lipid analysis.

Table 2.1– Sample details across the three study aims, including the number of individual white sharks *Carcharodon carcharias* and the tissue and lipid parameter analysed.

	Tissue type	**Minimum tissue quantity (mg DW)**	**Degradation**		
			Ambient temperature (23 °C)	Frozen (short-term; 0-2 years at -20 °C)	Frozen (long-term; 0- 16 years at -20 °C)
Experimental parameters	Muscle vs. Sub-dermal	Muscle– 100, 50, 25, 12 Sub-dermal – 85, 40, 20, 10	Up to 4 days	Up to 2 years	Up to 16 years
Number of individuals (replicates per individual)	4(1) + 3(3)	3(3)	2(3)	55(1)^	62(1)*
Tissue analysed	Muscle Sub-dermal	Muscle Sub-dermal	Muscle Sub-dermal	Muscle	Muscle
Lipid analysis	yes	no	yes	no	no
Fatty acid analysis	yes	yes	yes	yes	yes

DW – Dry weight
^White sharks from the Neptune Islands, SA and NSW
*White sharks from NSW and QLD

Ethics Statement

In South Australia, fieldwork at the Neptune Islands was carried out in accordance with ethics permit #E398, approved by The Flinders University Animal Welfare Committee, and under DEWNR permit # Q26292. In New South Wales, tissue collection under NSW DPI Scientific Collection Permit (P07/0099-3.0 and P07/0099-4) was approved by New South Wales Department of Primary Industries (NSW DPI) Animal Research Authority (ACEC 12/07). Tissue from Queensland was obtained as part of the QLD Shark Meshing Program and QLD Department of Agriculture and Fisheries Shark Control Program as part of the QLD large shark tagging research program under fisheries permit 143005 and QLD Department of Agriculture and Fisheries Shark Animal Ethics Committee approved ethics CA 2010/11/482, CA 2013/11/737, ENV 1709 AEC.

Experimental Design

Three sets of comparative lipid and FA analyses were undertaken, each addressing one of the aims; the difference between muscle and sub-dermal tissue, minimum tissue quantity for each tissue, and the effect of tissue degradation on resulting lipid and FA profiles (Table 2.1). To investigate the difference between the muscle and sub-dermal tissue, ~300 g sections, comprising both muscle and sub-dermal tissue were collected from three deceased white sharks (a, b, and c). Lipid class and FA profiles were assessed across triplicate subsamples from these three sharks (Table 2.1) to incorporate the within-individual variability. Minimum tissue quantity was also assessed in triplicate, across the three sharks, for both muscle and sub-dermal tissue using progressively smaller samples sizes. The tissue degradation analysis was performed in three parts: (i) at ambient temperature, and (ii) short term storage at −20 ˚C (for up to 2 years), and (iii) long-term storage at −20 ˚C (for up to 16 years). The remaining portions of sharks a and b were then held at room temperature (~20 ˚C) for 4 days, and muscle and sub-dermal tissue were sub-sectioned in triplicate, every 24 h. Immediately prior to sub-sectioning, ~1 cm of the outermost edge was removed and discarded, allowing the sample to

be taken from the interior of the tissue section. This was to minimize incidentally measuring the co-occurring effects of oxygen-contact induced FA oxidation on the samples. Only *sharks* a and b underwent the ambient temperature degradation trial, as there was insufficient remaining tissue from shark c. The remaining 107 white shark muscle samples were used to assess both short- to mid-term (1 month up to 2 years) and long-term (1 month up to 16 years) FA profile degradation associated with storage at −20 ˚C (Table 2.1). Forty-five samples from the Neptune Islands, SA and 10 of the 31 samples from NSW were processed within 2 years of being obtained and thus these were assessed together for short- to mid-term degradation (1 month up to 2 years). These results were grouped into 3 months bins for statistical analysis. Sixty-two muscle samples (31 from NSW, 31 from QLD) were assessed together for long- term freezer degradation (1 month up to 16 years). This excluded the 45 Neptune Islands samples included in short-term freezer degradation analysis, limiting the potential confounding factor of collection location within long-term degradation. These long- term freezer degradation results were also grouped into bins for statistical analysis, with group 1 = 0–1 years at −20˚C, 2 = 1.1–2 years, 3 = 3–5 years, 4 = 6–10 years, 5 = 11–16 years.

Lipid Extraction

Total lipid was extracted using the modified Bligh and Dyer method (Bligh and Dyer, 1959). Briefly, samples were left overnight in a one-phase CH_2Cl_2:CH_3OH:milliQ H_2O mixture (10:20:8 mL) before the solution was broken into two phases by the addition of 10 mL CH_2Cl_2 and 10 mL of 9 g NaCL L−1 saline milliQ H_2O. The lower phase containing the lipid fraction was drained into a round bottom flask and the solvent removed using a rotary evaporator. The lipid was re-suspended in CH_2Cl_2 and transferred to a 2 mL vial and dried under N_2 gas until a constant weight was noted. The total lipid extract (TLE) was then re-suspended in 1.5 mL of CH_2Cl_2.

Lipid Content and Class Analysis

Water content, reported as percent of tissue wet weight, was determined for each sample by taking weights before and after freeze-drying at −82 ˚C for 72 h and calculating the wet to dry ratio. Similarly, the lipid content was calculated by subtracting tissue dry weight prior to lipid extraction from the weight of the resulting TLE, then multiplied by the wet to dry ratio, and reported as percent of tissue wet weight. Lipid class composition [triacylglycerols (TAG), phospholipids (PL), sterols (ST), wax esters (WE), and free fatty acids (FFA)] were measured using an Iatroscan Mark V TH10 thin layer chromatrograph coupled with a flame ion detector (TLC-FID). TLE from each sample was analyzed in triplicate. Aliquots of TLE were spotted onto chromarods and developed for 25 min in a polar solvent system [70:10:0.1 v/v/v, C6H14:$(C_2H_5)_2O$:CH_3COOH]. Rods were oven dried at 100 ˚C for 10 min and analyzed immediately. SIC-480 Scientific Software was used to identify and quantify the areas of the resulting peaks.

Fatty Acid Analysis

An aliquot of the TLE was transferred into a teflon-lined screw cap glass test tube and trans-methylated with 3 mL of CH_3OH: CH_2Cl_2:HCl (10:1:1 v/v/v) for 2 h at 80 ˚C. The tube was then cooled in a water bath, and 1 mL MilliQ H_2O was added. The resulting fatty acid methyl esters (FAME) were extracted into a 2 mL glass vial using three washes of C_6H_{14}: CH_2Cl_2 (4:1 v/v), each thoroughly mixed and then the tube centrifuged at 2,000 rpm for 5 min. The resulting FAME were dried under N_2 gas prior to the addition of 1.0 mL of C19 internal injection standard solution in preparation for gas chromatography (GC) and GC-mass spectrometry (GC-MS) analysis. Each FAME sample was injected into an Agilent Technologies 7890B GC (Palo Alto, California USA) equipped with an Equity- 1 fused silica capillary column (15 m × 0.1 mm internal diameter and 0.1 mm film thickness), a flame ionization detector, a splitless injector, and an Agilent Technologies 7683B Series auto-sampler. At an oven temperature of 120 ˚C, samples were injected in splitless mode and carried by helium gas. Oven temperature was raised to

270 ˚C at a rate of 10 ˚C per min, and then to 310 ˚C at a rate of 5 ˚C per min. Peaks were quantified using Agilent Technologies ChemStation software (Palo Alto, California USA). The identities of the peaks were confirmed using a Finnigan Thermoquest DSQ GC-MS system. All FAs were converted from chromatogram peak area to percentage of total area.

Statistical Analysis

Of the 50 total FAs detected, 21 (with averages >0.1% of total FAs across either tissue type, in quantities of 100 mg non-degraded muscle and 80 mg of non-degraded sub-dermal tissue) were used for multivariate analysis comparing the differences in profiles across factors. Statistical analysis was undertaken in PRIMER 7 (Plymouth Routines in Multivariate Ecological Research, Clarke et al., 2014) +PERMANOVA. We used Principal Coordinates Analysis (PCO) of Bray-Curtis similarity matrices calculated from the square-root transformed data to determine clustering of individual samples. To test the differences between factors we used PERMutational ANalysis Of VAriance (PERMANOVA) with Monte Carlo simulations denoted as p(MC) on the unrestricted raw values to account for the small sample sizes. PERMANOVA analyses used factors nested within shark to incorporate the triplicate samples from each individual shark. Significance was determined by $p<0.05$. Following significant ANOSIM tests, similarity percentage (SIMPER) analyses were undertaken to quantify the contribution of each parameter to the separation between the designated groups. Additionally, the sum of the saturated (SFA), monounsaturated (MUFA), total polyunsaturated fatty acids (PUFA), ω3 PUFA and the ratio of ω3 PUFA:ω6 PUFA and EPA+DHA/16:0 were calculated per replicate. We used nested (factor within shark) PERMANOVA analysis with Monte Carlo simulations to assess the response of individual lipid classes, FA values, and FA metrics (aforementioned sums and ratios). Permutational analysis of multidimensional dispersion PERMDISP denoted at p(perm) was used to determine the relative amount and statistical significance level of the dispersion within factor groups.

Results

Muscle vs. Sub-dermal Tissue

White shark muscle was high in water content 82.1 ± 1.1% wet weight (WW) with lipid content (0.6 ± 0.1% WW), and a wet/dry ratio of 4.1 ± 0.2. Sub-dermal tissue contained even lower amounts of total lipid (0.4 ± 0.2% WW), which was on average 33% less lipid than the muscle tissue. The lipid class profiles of both tissues were dominated by PL (Table 2.2) followed by ST, which were 13.5% (as % of total lipid) more abundant in sub-dermal tissue than muscle. ST contributed the greatest source of dissimilarity between the tissue types (46%) as determined by SIMPER, and when assessed individually, was the only lipid class significantly different between the tissues [p(MC) = 0.001] (Table 2.2).

Muscle tissue contained primarily PUFA 39.2 ± 8.0%, mostly consisting of 22:6ω3 (docosahexaenoic acid, DHA) and 20:4ω6 (arachidonic acid, ARA) (Table 2.2). SFA contributed 33.9 ± 4.7%, dominated by 16:0 and 18:0. MUFA contributed the remaining 21.8 ± 4.2% of the muscle tissue FA profile, nearly half of which was 18:1ω9. Sub-dermal tissue contained similar relative levels of PUFA (32.8 ± 3.5%) dominated by 20:4ω6 and 22:6ω3, and SFA (33.3 ± 3.2%) mostly 18:0 and 16:0, with MUFA (26.1 ± 2.7%) primarily consisting of 18:1ω9 (Table 2.2).

Muscle and sub-dermal tissue had distinctly different FA profiles [Nested PERMANOVA: shark p(MC) = 0.439, tissue p(MC) = 0.001, Figure 2.1]. The difference was primarily driven by high levels of 22:6ω3 in the muscle (SIMPER 17% dissimilarity contribution), followed by 18:1ω7 (8.4%), 20:4ω6 (6.2%), and i15:0 (5.6%) (Table 2.2). Sixteen of the 21 individual FAs were found to be significantly different (p(MC) < 0.05) across the two tissue types (Table 2.2), with only 16:0, 18:0, 20:1ω9, 16:3, and 22:4ω6 not significantly different between the two tissues. Muscle tissue samples showed greater dispersion than the sub-dermal tissue (p(perm) = 0.030; Figure 2.1) across the three individual sharks. However, this difference in tissue-specific dispersion was not seen within the three triplicate samples of sharks a, b, and c (Shark a p(perm) = 0.600, Shark b p(perm) = 0.456, Shark c p(perm) = 0.812).

Table 2.2 –Total lipid content, relative proportions of lipid classes and fatty acids (FA) (as percent of total lipid or FA) (mean ± standard deviation) of muscle and sub-dermal tissue (wet weight, WW) from white shark *Carcharodon carcharias.* P(MC) values were determined by Nested PERMANOVA with Monte Carlo simulation, with tissue nested within shark.

	Muscle	**Sub-dermal**	**SIMPER% contribution#**
White sharks	7	7	
n	13	13	
individual x rep.	(4x1) (3 x 3)	(4x1) (3 x 3)	
Lipid content (WW)	0.59±0.07%	0.42±0.16%	
Lipid class composition**			p(MC) = 0.01
Wax Esters (WE)	2.13±6.12	1.13±1.91	NS
Triacylglycerols (TAG)	0.66±1.04	1.42±2.22	NS
Free Fatty Acids (FFA)	4.07±10.53	1.66±1.96	NS
Sterols (ST)***	5.94±2.00	19.46±6.45	p=0.001
Phospholipids (PL)	87.21±14.52	76.33±8.13	NS
Fatty Acids***			p(MC) = 0.001
14:0***	0.36 ±0.21	0.83 ±0.48	4.18***
16:0	17.14 ±3.94	14.82 ±2.95	5.38
18:0	16.92 ±3.32	16.04 ±1.06	3.32
16:1ω7*	1.42 ±0.47	1.59 ±0.44	2.43*
17:1ω8+a17:0***	0.35 ±0.23	0.82 ±0.32	4.02***
18:1ω7***	4.00 ±2.91	4.07 ±0.50	8.39***
18:1ω9***	10.33 ±2.17	13.34 ±2.18	4.87***
20:1ω9	1.28 ±0.42	1.13 ±0.20	2.09
24:1ω7**	0.50 ±1.01	0.72 ±0.58	5.41 **
16:3	0.05 ±0.05	0.18 ±0.10	2.44
18:2ω6***	0.69 ±0.40	0.75 ±0.54	4.36***
18:4ω3*	0.10 ±0.20	0.46 ±0.41	4.54*
20:4ω3**	0.25 ±0.11	0.64 ±0.46	3.39**
20:4ω6***	11.71 ±2.23	17.06 ±1.74	6.21***
20:5ω3***	1.25 ±0.42	1.36 ±0.52	2.23***
22:4ω6	3.63 ±1.07	3.58 ±0.49	3.06
22:5ω3**	2.68 ±0.77	2.22 ±0.43	3.11**
22:5ω6***	0.96 ±0.19	0.45 ±0.09	3.54***
22:6ω3***	14.62 ±4.79	5.11 ±1.53	17***
i15:0***	0.33 ±0.15	1.22 ±0.53	5.59***
i17:0***	0.54 ±0.15	1.02 ±0.30	3.88***
∑SFA	**33.90 ±4.74**	**33.29 ±3.20**	
∑MUFA	**21.83 ±4.15**	**26.06 ±2.72**	
∑PUFA	**39.15 ±8.03**	**32.79 ±3.48**	
∑Iso-SFA	0.88±0.21	2.24±0.43	
∑Branched FA	0.16±0.09	0.47±0.12	
∑Other (<0.1%)	10.44	12.59	

SFA – saturated fatty acids, MUFA – monounsaturated fatty acids, PUFA – polyunsaturated fatty acids. The suffix i denotes branched fatty acids from the iso series. FALD- fatty aldehyde analysed as dimethyl acetal.
Data presented are for 21 components, with a cut off of 0.5%.
p(MC) indicated the p value determined by PERMANOVA run with Monte Carlo simulations.
Statistical significance determined by P(MC) - denoted by:
* p<0.05, ** p<0.01, ***p<0.001
NS - Not significant (p>0.05)

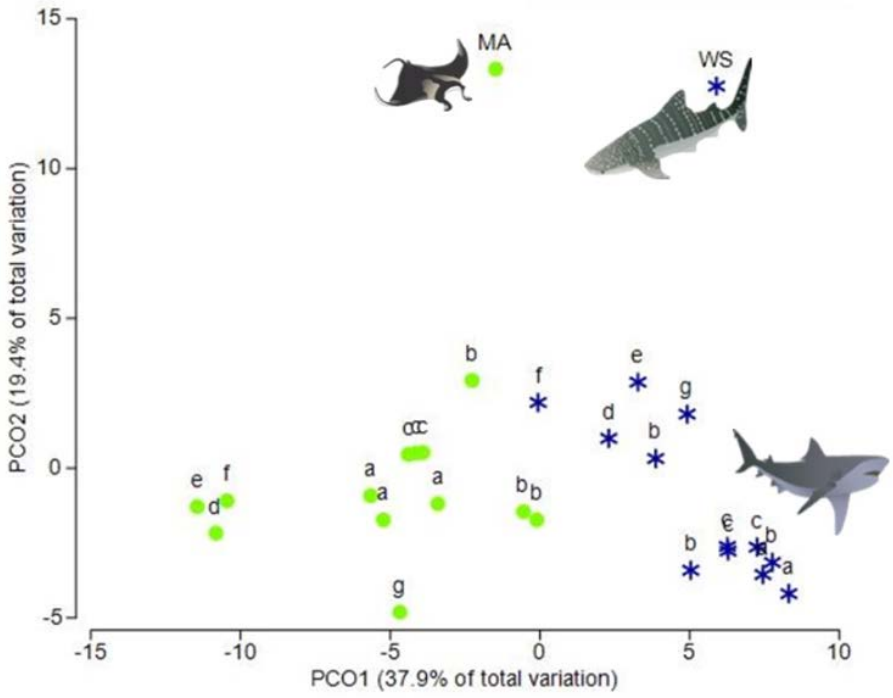

Figure 2.1 – Principal coordinates analysis (PCO) of fatty acid profiles from the muscle and sub-dermal tissue of white sharks *Carcharodon carcharias*, reef manta rays *Mobula alfredi* and whale sharks *Rhincodon typus*. Principal coordinates analysis (PCO) of fatty acid profiles of muscle (green circles) and sub-dermal tissue (blue stars) from seven white sharks, (a-g) three of which (a, b and c) were analysed in triplicate. Mean fatty acid profiles from Reef Manta Rays (MA) and whale sharks (WS) from Couturier et al. 2013 and Rohner et al. 2013 respectively are also included. Eigenvalues denote the percent of variation attributed to each axis (PCO1 and PCO2).

Minimum Sample Size

The progressively smaller muscle tissue increments (100, 50, 25, and 12 mg DW) showed no statistical difference between size groups [p(MC) = 0.28], or difference in dispersion (PERMDISP means of 5.2, 4.1, 5.3, 6.6 for the 100, 50, 25, and 12 mg samples, respectively, p > 0.05). Principal coordinates analysis showed that the clustering is not driven by tissue amount, but by individual shark (Nested PERMANOVA p(MC) = 0.28 nested within shark p(MC) = 0.001), with shark c separating from sharks a and b (Figure 2.2A).

Sub-dermal tissue increments (85, 40, 20, and 10 mg DW) revealed differing FA profiles with decreasing tissue amounts [p(MC) = 0.042 for tissue size], with the difference between the two larger (85 and 40 mg) and two smaller (20 and 10 mg) amounts driven by 18:1ω9, i15:0, 22:6ω3, and 20:4ω3 (Table 2.3). The difference in FA profiles is exacerbated by an increase in dispersion with decreasing tissue size (Figure 2.2B), particularly between the two smaller 10 and 20 mg tissue samples and the two larger 85 and 40 mg sample sizes (Table 2.3).

Table 2.3 – The differences between sub-dermal tissue sizes for white shark *Carcharodon carcharias.* p(MC) values were determined by Nested PERMANOVA with Monte Carlo simulation (three replicates nested within three sharks, n=9) and the primary fatty acids (FA) driving the significantly different groups determined by SIMPER percent contribution. FAs are listed in order of decreasing contribution. Listed PERMDISP p values indicate the significance of the differences in dispersion between the tissue sizes.

Tissue sizes	p(MC)	FA drivers	p(PERMDISP)
10, 20	0.774	NA	0.574
10, 40	0.035*	18:1ω9, i15:0, 24:1ω7	0.001***
10, 85	0.006**	i15:0, 18:1ω9, 20:4ω6	0.002**
20, 40	0.054	NA	0.019*
20, 85	0.013*	22:6ω3, 18:1ω9, i15:0	0.014*
40, 85	0.328	NA	0.905

* P<0.05
** P<0.01
***P<0.001

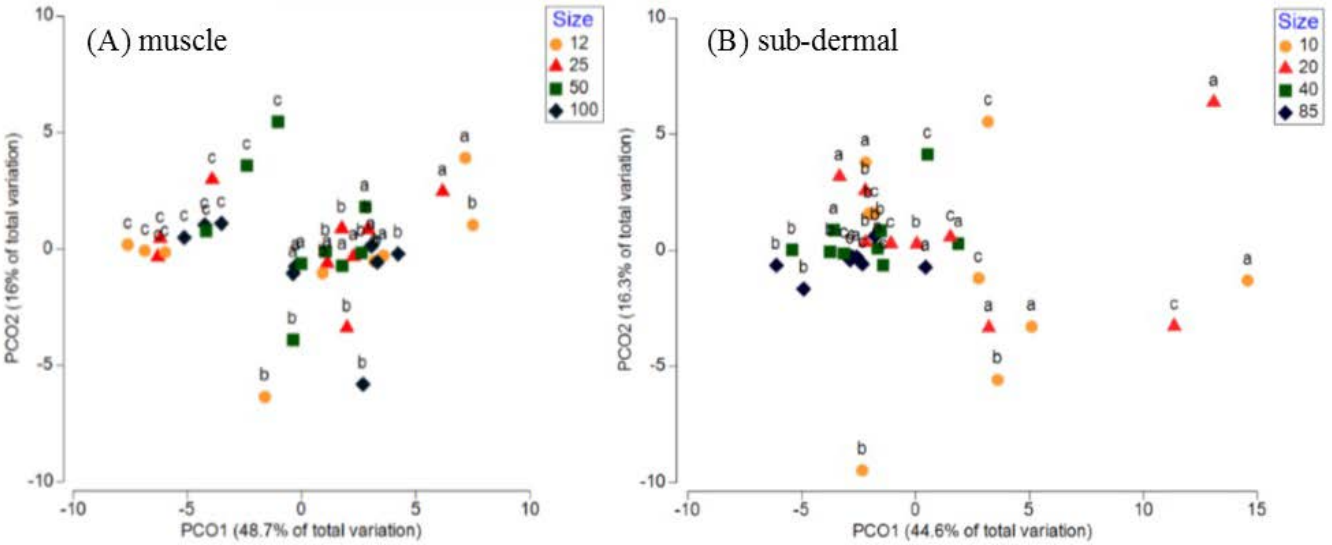

Figure 2.2 – Principal coordinates analysis (PCO) of the fatty acid profiles from white shark muscle and sub-dermal tissue across differing tissue sizes. Principal coordinates analysis (PCO) of (A) muscle, and (B) sub-dermal tissue from three white shark *Carcharodon carcharias* individuals (a, b and c), analysed in triplicate across differing tissue sizes. Eigenvalues denote the percent of variation attributed to each axis (PCO1 and PCO2).

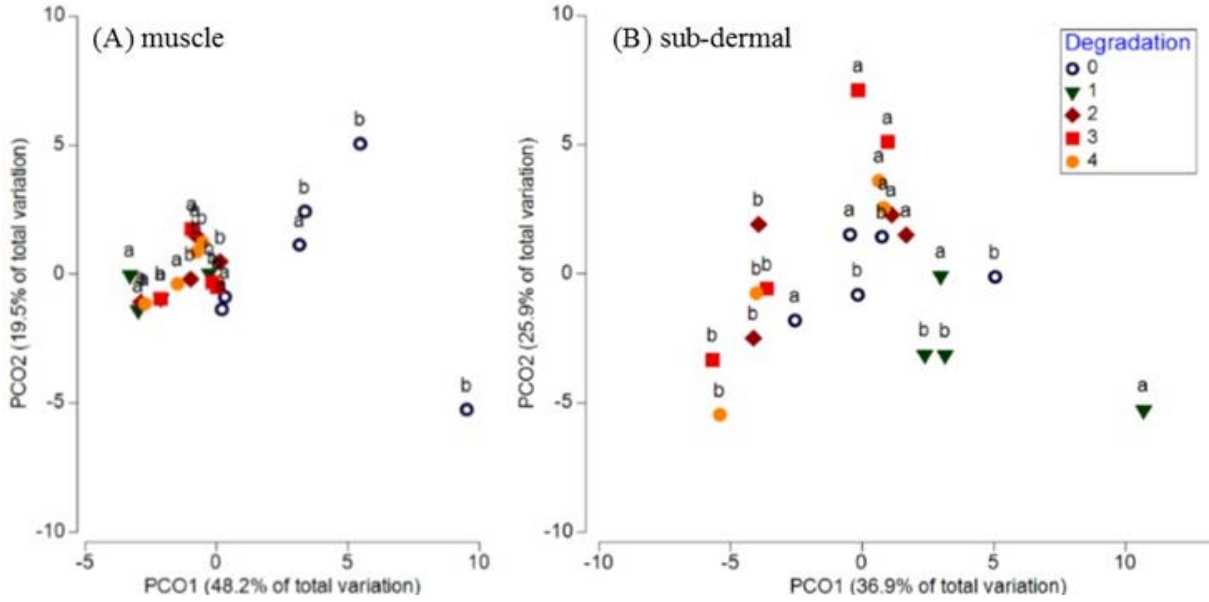

Figure 2.3 - Principal coordinates analysis (PCO) of the fatty acid profiles from white shark muscle and sub-dermal tissue across four days of degradation at 20˚C. Principal coordinates analysis (PCO) of fatty acid profiles from (A) muscle, and (B) sub-dermal tissue from two white shark *Carcharodon carcharias* individuals (a and b), analysed in triplicate across four days of degradation (0, indicating fresh tissue, 1, 2, 3 and 4 indicating the number of days left at 20 ˚C prior to analysis). Eigenvalues denote the percent of variation attributed to each axis (PCO1 and PCO2).

Lipid Class and FA Degradation at Ambient Temperature (20˚C)

The lipid class profiles from the muscle tissue showed no differences with 4 days at 20 ˚C [p(MC) = 0.127]. However, the muscle tissue showed a significant shift in FA profile over the 4 day period at 20 ˚C [p(MC) = 0.009], with significant ($p < 0.05$) differences between the fresh samples and days 1, 2, and 3 (Figure 2.3A). This was mostly driven by changes in 18:4ω3, 22:6ω3, and 18:0 (SIMPER analysis). PERMANOVA analysis of individual FA found significant differences in 18:0, total SFA, 18:4ω3, 18:2ω6, 20:5ω3, 22:6ω3, total PUFA, total ω3 PUFA, and the ω3:ω6 ratio, but not 20:5ω3+22:6ω3/16:0 (EPA+DHA/16:0). Additionally, PERMDISP analysis revealed a significant decrease in dispersion when the tissue was left at ambient temperature (p(perm) = 0.03). The mean dispersion for the fresh tissue (4.9) was significantly larger than the 2.0, 2.1, and 2.0 dispersion means for days 1, 2, and 3, respectively ($p < 0.05$), but not significantly different than the 3.0 dispersion mean at day 4 [p(perm) = 0.104]. Similar to the muscle lipid class profile, the sub-dermal tissue did not show any significant differences across the 4 day period at 20˚C (p(MC) = 0.183). There was also no discernible shift in FA profile over the 4 day period (p(MC) = 0.141; Figure 2.3B). Unlike the muscle tissue, there were no differences in the level of dispersion between the groups (overall p(perm) = 0.631).

FA Degradation of Frozen Tissue (−20 ˚C)

The FA profiles showed distinct degradation across the 24 months spent in the −20 ˚C freezer, regardless of sampling location (location p(MC) = 0.317; time in freezer nested within location p(MC) = 0.008). Within group comparisons reveal differences primarily between group 2 (3–6 months in the freezer) and all other groups, aside from group 1. Group 1 (0–3 months in the freezer) was only different to group 7 (the 19–21 month period) (Table 2.4). SIMPER analysis reveal that these differences were driven largely by 18:0, 22:6ω3, 18:2ω6, 16:0, and 18:4ω3 across the groups. Similar to the unfrozen, controlled muscle degradation trial, the total FA profile degradation manifests in changes to the level of dispersion, which decreases significantly with the amount of time spent in the freezer (p(MC) = 0.001, Table 2.4).

Table 2.4 – The differences between groups of samples combined by time spent frozen at -20°C for 55 white shark *Carcharodon carcharias samples* from the Neptune Islands, South Australia and throughout New South Wales. p(MC) values determined by Nested PERMANOVA (freezer group nested within sampling location) with Monte Carlo simulation between binned freezer groups (1 = 0-3 months) (2 = 4-6 months) (3 = 7-9 months) (4 = 10-12 months) (5 = 13-15 months)(6 = 16-18 months) (7 = 19-21 months) (8 =22-24 months), the primary fatty acids (FA) driving the significantly different groups determined by SIMPER percent contribution. FA are listed in order of decreasing contribution. Listed PERMDISP P values indicate the significance of the differences in dispersion between the groups.

Freezer group	PERMANOVA p(MC)	FA drivers	PERMDISP p(perm)
Overall	0.008**		<0.001***
1,2	0.107		<0.001***
1,4	0.075		0.168
1,6	0.150		0.041*
1,7	0.040*	18:0, 22:6ω3, 16:0	0.044*
1,8	0.322		0.097
2,4	0.010*	22:6ω3, 18:2ω6, 18:0	0.030*
2,6	<0.001***	18:0, 22:6ω3, 18:4ω3	0.097
2,7	<0.001***	18:0, 22:6ω3, 18:4ω3	0.165
2,8	0.0251*	22:6ω3, 18:0, 18:1ω9	<0.001***
4,6	0.456		0.433
4,7	0.607		0.470
4,8	0.5312		0.114
6,7	0.214		0.956
6,8	0.1757		0.005**
7,8	0.0848		0.013

* p<0.05
** p<0.01
***p<0.001

When assessing freezer-based degradation of archived samples over a long time frame (up to 16 years), there was slight discernible degradation, however, the capture location of the white sharks was more highly significant than period in the freezer (p(MC) = 0.002 vs. 0.045). For the sharks captured in NSW, none of the group level comparisons showed significant degradation (all p(MC)-values > 0.05), and within the QLD samples, only the difference between group 2 and 4 (1.1–2 years and 5.1–10 years) was significant (p(MC) = 0.041). Unlike the short-term freezer degradation and the unfrozen muscle degradation trial, there was no decrease in dispersion with the longer storage period [p(perm) = 0.620].

Discussion

Lipid class and FA analysis are increasingly used to describe the trophic ecology of a range of species, including elasmobranchs, necessitating greater understanding of the operational limitations of collection and storage methodologies. Here, we determined that muscle and sub-dermal tissue were not directly comparable, as they had tissue-specific lipid class and FA profiles. We also provide the first estimation of the minimum amount of muscle and sub-dermal tissue required to provide reliable FA profiles, which validated the suitability of minimally invasive sampling methods such as punch biopsies. Additionally, we determined that muscle tissue stored at ambient temperature was compromised after as little as 24 h, making muscle samples from beach strandings and fisheries bycatch potentially unreliable for accurate determination of dietary sources. Yet, sub-dermal tissue retained stable FA profiles under the same conditions, suggesting it may offer a more robust tissue for trophic ecology work with potentially compromised samples. However, muscle samples archived for up to 16 years in −20 ˚C retain their FA profiles, highlighting that muscle tissue from museum or private collections can yield valid insights into the trophic ecology of marine elasmobranchs. Knowledge gained from addressing these operational limitations will facilitate the more effective use of lipid and FA profiling on biopsied or potentially degraded tissues for the white shark, and in addition for other species, allowing them to be employed with greater confidence in a range of ecological studies.

Muscle vs. Sub-dermal Tissue

The lipid classes of the muscle tissue, dominated by PL (87%), were consistent with previously reported values for white sharks (92 ± 5%, Pethybridge et al., 2014) whereas the sub-dermal tissue contained higher relative levels of sterols (ST), closely resembling the profile of whale shark sub-dermal tissue (21 ± 4%, Rohner et al., 2013). Regardless of ST contribution, both tissues were dominated by PL, with relatively little contribution from the neutral lipids (triacylglycerols, wax esters, FFA) responsible for metabolic energy storage (Sargent et al., 1999). This affirms the understanding that both muscle and sub-dermal tissue contain little capacity for metabolic energy storage, unlike elasmobranch livers, which are high in lipid content and dominated by triacylglycerols (Beckmann et al., 2013; Pethybridge et al., 2014). Tissue differences across 16 of the 21 FAs (contributing >76% of total FA) are likely a reflection of divergent functions and underlying physiology. For example, 22:6ω3 and other key essential FAs including 18:2ω6, 20:4ω6 (ARA), 20:5ω3 (EPA), which serve as indicators for a range of trophic pathways differed between the two tissues. As such, the variation in FAs that accounted for the separation between muscle and sub-dermal tissue indicates that interpretation of a species' diet would be greatly affected by the tissue from which the FA profiles is derived, and thus the profiles of the different tissues are not directly comparable.

Recent studies have suggested that differences in FA profiles between muscle and sub-dermal tissue of euryhaline elasmobranches are species-specific (Every et al., 2016). However, when we include the FA profiles of manta ray muscle from Couturier et al. (2013) and whale shark sub-dermal tissue, from Rohner et al. (2013) in the PCO with our white shark samples, the manta ray and whale shark FA profiles align with the tissue-specific clusters (Figure 2.1). This suggests that the difference in FA profiles between muscle and sub-dermal tissues are not limited to white sharks, but extends to other species and across trophic levels.

The sub-dermal tissue serves as a key structural component, with a slower metabolic turnover rate than muscle (assessed in relation to divergent isotopic signatures by del Rio et al.,

2009). As such, these tissues may therefore present complementary results, reflecting diets incorporated across different time frames (Every et al., 2016). Given the opportunity to collect both tissue types through non-lethal biopsies, further investigations comparing the tissue-specific FA incorporation rates should be undertaken. Results discerning the time-frame of both tissue's FA profiles would provide the opportunity to assess multiple temporal scales of an individual's trophic history, valuable additional information when investigating individual specialization, location specific, seasonal, or ontogenetic dietary shifts.

Minimum Samples Size

Muscle biopsies of variable forms have previously been developed to collect samples for genetic and isotopic studies, e.g., punch biopsies (Robbins, 2006; Daly and Smale, 2013) or thick- gauged needles (Baker et al., 2004). Based on the ability of samples as small as 12 mg DW (= 49 mg WW) to provide consistent FA profiles, our study shows that sufficient tissue samples are collected by standard biopsy darts (e.g. Daly and Smale, 2013; Jaime-Rivera et al., 2013) including the small dart assessed by Robbins (2006) which obtained 6.6–122 mg of total tissue. Although not stated what proportion of these biopsies were muscle, the large quantity of tissue obtained (up to 122 mg WW) suggests that sufficient muscle can be collected. Furthermore, biopsy needles (14-gauge, 4 cm long, double-barreled Tru-Cut needles), designed to collect 60 mg WW of tissue from small teleosts are also sufficient to collect tissue for FA analysis (Baker et al., 2004; Logan and Lutcavage, 2010). This ability to obtain FA profiles from small amounts of muscle validates the suitability of minimally invasive sampling methods, and allows trophic ecologists to apply FA analyses to smaller elasmobranchs than previously thought without the need for lethal sampling. Additionally, multiple studies investigated the variation in muscle-derived FA profiles across different anatomical sites, and found no significant differences (Davidson et al., 2011; Pethybridge et al., 2014). Thus, these biopsy methods can be reliably used regardless of variation in sampling site, furthering the applicability of signature FA analyses. Furthermore, FA profiles can be obtained from the lipids extracted

during standard sample preparation for isotopic analysis (Marcus et al., 2017). Therefore, the minimal tissue quantities already retrieved for SIA provide researchers with the opportunity for distinct and complementary FA analyses from the same non-lethal tissue biopsies, without the need to prioritize one of the two datasets. Considering the small amount of muscle necessary, minimally invasive biopsy methods collect sufficient muscle tissue to undertake FA analysis which can be paired with existing standard sample preparation for isotopic analysis, enhancing the method's suitability for ongoing work in trophic ecology.

In contrast to muscle tissue, the FA profiles of sub-dermal tissue smaller than 40 mg DW became highly variable, indicating a minimum reliable tissue quantity of 40 mg DW (= 184 mg WW), which is more than three times the minimal requirement for muscle. This is potentially due to the difference in PL concentration between the two types of tissue of the lipid profile. Combined with the lower lipid content, the lower relative PL contribution in the sub-dermal tissue may explain the comparatively larger minimum sub-dermal tissue quantity, as the ST, which are found in higher abundance in the sub-dermal tissue, do not contribute to the FA pool. This larger minimum tissue quantity required for sub-dermal tissue compared to muscle may limit the applicability of many aforementioned non-lethal biopsy methods. For example, the biopsy method yielding the second highest tissue volume provided only 80– 172 mg WW of sub-dermal tissue (Daly and Smale, 2013), which is not sufficient for reliable FA analysis. Only the Reeb and Best's dart head (Reeb and Best, 2006) which retained an average of 0.35 cm3 of sub-dermal tissue when trialed by Jaime-Rivera et al. (2013), obtained potentially suitable tissue quantities. Furthermore, biopsies from small elasmobranchs are unlikely to yield sufficient tissue, as the thickness of the sub-dermis is greatly reduced. For example, sub-dermal tissue layers in Atlantic sharpnose shark, scalloped hammerhead and dusky smooth-hound sharks ranged 0.02–0.16 cm (Motta, 1977), compared to white sharks averaging 1.1 cm (Jaime-Rivera et al., 2013) and whale sharks exceeding 2 cm (Rohner et al., 2013).

Degradation

The consistently low levels of FFA in muscle and sub-dermal tissue throughout the degradation trial contrasts with findings across marine taxa, which highlight large increases in FFA from enzymatic hydrolysis of several non-polar lipid classes (Fernández-Reiriz et al., 1992; Kaneniwa et al., 2000; Losada et al., 2005). The difference between our findings and the pervasive trends in previous studies may be attributable to species- and taxa-specific enzymatic processes. Rudy et al. (2016) and Kaneniwa et al. (2000) hypothesized that total lipid content drove the species-specific differences in the level of observed lipid class and FA degradation amongst teleost species, with the “fatty” fish most susceptible. Compared with the six teleosts assessed in Rudy et al. (2016), white sharks were orders of magnitude leaner, with muscle containing 0.6% lipid WW and sub-dermal tissue 0.4% lipid WW (vs. 10.3–2.9% WW in teleosts). The low lipid content may explain the lack of discernible lipid class degradation across both tissues and the comparative stability in FA profiles within the sub-dermal tissue. Given the aim of determining the operational limitations of using elasmobranch specimens not immediately frozen, for example from fisheries bycatch and shark mitigation measures, our results indicate that lipid classes from muscle and sub- dermal tissues are not convoluted by degradation within a 4 day period.

The lipid-poor sub-dermal tissue also showed no discernible shift in FA profile or level of dispersion through exposure to ambient temperature for 4 days. However, the FA profiles derived from muscle tissue immediately changed, with a decrease in dispersion observed after 24 h, potentially compromising the ability to distinguish between individual samples. This advocates for exploring the use of sub-dermal tissue over muscle in situations when samples have been left at ambient temperature, and should be the subject of controlled feeding trails to assess the capacity for sub-dermal tissue to reflect diet. Our earlier findings, however, highlights that such FA profiles based on sub-dermal layers cannot be directly compared to FA profiles from muscle and that this discrepancy should be accounted for. Muscle segments stored at −20

˚C showed significant FA profile shifts in both assessment periods, highlighting concerns regarding the capacity to accurately use archived samples. Results in this study suggest that although there may be some level of FA degradation, the time frame at which this occurs and processes involved remains unclear. It is also plausible that the difference in the 3–6 months group is not driven by the time spent in the freezer, but by the influence of unassessed biotic factors (e.g. individual's state of maturity, sex, season of capture). The comparison of FA profiles from archived samples stored for 1–16 years did not provide further clarification and showed no clear differences in FA profiles. Furthermore, neither trial's FA profiles decreased in dispersion, a pattern characteristic of FA degradation in the ambient temperature trial. Regardless of the degradation that might be occurring through long-term storage, differences between locations (NSW vs. QLD) remained, further suggesting that frozen samples may retain viable and indicative FA signatures.

The shift in the relative proportions of individual FAs of the muscle tissue illustrates the complex nature of FA degradation at both 20 and −20 ˚C. Our study found that SFA, driven primarily by 18:0, can remain constant during some time periods, but also decreased drastically through other periods. The MUFA, unchanged at 20 ˚C, demonstrated some resistance to degradation, with no shifts in either individual MUFA, or the ∑MUFA. Unexpectedly, they showed variable patterns of alteration in the early month of storage, suggesting that they are prone to degradation at −20 ˚C, consistent with findings across other taxa (Table 2.5, e.g. teleosts in Rudy et al., 2016 and octopus in Gullian-Klanian et al., 2017). PUFA are more reactive owing to their numerous double-bonds and are especially prone to degradation (Refsgaard et al., 1998; Paola and Isabel, 2015; Rudy et al., 2016; Gullian-Klanian et al., 2017). However, shifts in relative levels of PUFA of white sharks, including key dietary indicators 22:6ω3 (DHA) and 20:5ω3 (EPA), were only distinguishable in the ambient temperature trial, and not in either the short- or long-term −20˚C analysis (with the exception of 18:2ω6). Additionally, the polyene index (EPA+DHA/16:0), a well-established metric for tissue

degradation, thought to be ubiquitous across taxa (Jeong et al., 1990; Paola and Isabel, 2015), showed no decrease across any trials (Table 2.5). The present study shows that white shark muscle PUFA might not show the stark degradation seen in the muscle tissue of other species. Given the relative importance of PUFA, as essential FAs and key dietary markers, these findings suggest that elasmobranch samples may retain these key FAs throughout extensive storage at −20 ˚C.

Table 2.5 – Individual fatty acid degradation, assessed by days at -20 °C, months stored at -20 °C and years stored at -20 °C. PERMNOVA P(MC) significance set at P<0.01, and denoted as either non-significant (NS) and significant (S). Month and Year data has been binned for analysis. Months at -20°C binned as: 1 = 0-3 months; 2 = 4-6 months) (3 = 7-9 months) (4 = 10-12 months) (5 = 13-15 months) (6 = 16-18 months)(7 = 19-21 months) (8 =22-24 months). Years at -20 °C binned as 1 = 0-1 years, 2 = 1.1-2years, 3 = 3-5years, 4 = 6-10 years, 5 = 11-16 years.

Fatty Acid	Days at 20 °C	Months at -20 °C	Years at -20 °C	Literature detailing the degradation potential across taxa at -20 °C
Full Profile	p(MC)=0.009	p(MC)=0.008	p(MC)=0.045	
18:0	**0 – all days**	**2-6, 2-7, 4-7**	NS	Decreased in four teleost species across 6 months (Sahari et al., 2014). Showed significant changes by freezer temperature (-20 or -80 ˚C) in 2 of 4 teleost species, but changed by time spent in the freezer in 1 of 4 teleost species (Rudy et al., 2016). Increased between 3 and 6 months in mackerel (Paola et al., 2015). Did not change over 5 months in octopus (Gullian-Klanian et al., 2016).
SFA	**0–1, 0-4**	**2-6, 2-7**	NS	Decreased in four teleosts across 6 months (Sahari et al., 2014). Increased every three months for 1 year in mackerel (Paola et al., 2015). Increased across 5 months in octopus (Gullian-Klanian et al., 2016).
16:1ω7	NS	**2-6, 2-7**	NS	Significant changes by freezer temperature (-20 or -80 ˚C) and by time spent in the freezer in 1 of 4 fish (Rudy et al., 2016). Increased, then decreased over 5 months in octopus (Gullian-Klanian et al., 2016)
20:1ω9	NS	**1-7, 2-6, 2-7, 4-7**	NS	Increased at 3 months, then decreased every three months for 1 year in mackerel (Paola et al., 2015). Decreased after 3 months in octopus (Gullian-Klanian et al., 2016).
22:1ω9	NS	**1-7, 2-6, 2-7**	NS	Decreased every three months in mackerel (Paola et al., 2015). Decreased after 3 months in octopus (Gullian-Klanian et al., 2016).
18:4ω3	**S**	**1-2, 1-6, 2-4, 2-6, 2-7, 4-6**	NS	Decreased between 6 and 9 months in mackerel (Paola et al., 2015).
18:2ω6	**0-4**	**2-4, 2-6, 4-7, 6-7**	NS	Significant changes by freezer temperature (-20 or -80 ˚C) and by time spent in the freezer in 1 of 4 fish (Rudy et al., 2016). Decreased after 1 month in mackerel (Paola et al., 2015). In octopus, it did not change over 5 months (Gullian-Klanian et al., 2016).
20:5ω3	**0-1, 0-3**	NS	NS	Decreased in Salmon at -10 ˚C and -20 ˚C (Refsgaard et al., 1998). Significant changes by freezer temperature (-20 or -80 ˚C) and by time spent in the freezer in 2 of 4 fish (Rudy et al., 2016). Decreased after 1 month, and again after 9 months in mackerel (Paola et al., 2015) Decreased across 5 months in octopus (Gullian-Klanian et al., 2016).
22:6ω3	**0-1**	NS	NS	Decreased in Salmon at -10 ˚C and -20 ˚C (Refsgaard et al., 1998). Showed significant changes by freezer temperature (-20 or -80 ˚C) in 2 of 4 fish, but changes by time spent in the freezer in 3 of 4 fish (Rudy et al., 2016). Decreased after 1 month, and again after 9 months in mackerel (Paola et al., 2015). Decreased across 5 months in octopus (Gullian-Klanian et al., 2016).
22:5ω3	**S**	NS	NS	Decreased in Salmon at -10 ˚C and -20 ˚C (Refsgaard et al., 1998). Significant changes by freezer temperature (-20 or -80 ˚C) and by time spent in the freezer in 1 of 4 fish (Rudy et al., 2016)
PUFA	**0-1, 1-3**	NS	NS	Decreased across 8 months in teleosts (Roldan et al., 2004). Decreased every three months for 1 year in mackerel (Paola et al., 2015). Was the most affected FA group in octopus across 5 months, decreasing notably in the third and fifth month, however weather or not this was significant was not noted (Gullian-Klanian et al., 2016).
PUFA -ω3	**S**	NS	NS	Decreased across fish in 8 months (Roldan et al., 2004) at -20 ˚C. Decreased in teleosts across 3 months at -12 ˚C (Polvi et al., 1991), and salmon at -10 ˚C and -20 ˚C (Refsgaard et al., 1998). Significant changes by freezer temperature (-20 or -80 ˚C) and by time spent in the freezer in 2 of 4 fish (Rudy et al., 2016). Decreased every three months for 1 year in mackerel (Paola et al., 2015).
EPA+DHA /16:0	NS	NS	NS	Determined to be a valuable indicator of lipid oxidation (Jeong et al., 1990). Decreased every three months for 1 year in mackerel (Paola et al., 2015). Did not change in a squid at ambient temperature (Phleger et al., 2006).
FAs with no significant degradation across any of the three trials - 14:0, 15:0, 16:0, 17:0, 18:1ω9, MUFA, 20:2ω6, 20:4ω6, 22:5ω6,				

Conclusion

Our findings indicate that muscle and sub-dermal tissue contain distinct FA profiles and differing individual FAs, many of which are key trophic indicators. As such, these tissues are not directly comparable. They may, however, present complementary trophic information reflecting differing time frames, providing the opportunity to garner additional information from non-lethal biopsies. The minimum tissue amount for sub-dermal tissue was 40 mg DW (184 mg WW), whereas muscle samples as small as 12 mg DW (equating to 49 mg WW) retained consistent FA profiles. This makes FA analysis an ideal tool for elucidating trophic ecology of rare or endangered elasmobranchs for which lethal sampling is inappropriate. Degradation of muscle tissue occurred within the first 24 h at ambient temperature, unlike sub-dermal tissue, which revealed no discernible degradation across 4 days. As such, the use of deceased organisms, from shark mitigation strategies, by-catch, or beach strandings should be undertaken with caution, ensuring that preservation occurs within 24 h. Muscle tissue appears to retain viable and indicative FA signatures across long periods of frozen storage (up to 16 years), advocating for the use of archived samples, especially in cases where sampling opportunities are rare or opportunistic. Overall, lipid class and FA analysis can be reliably assessed from small tissue quantities derived from minimally invasive, non- lethal biopsies, deceased elasmobranchs preserved within 24 h and archived samples, proving a robust toolset for elucidating the trophic ecology of rare and endangered wildlife.

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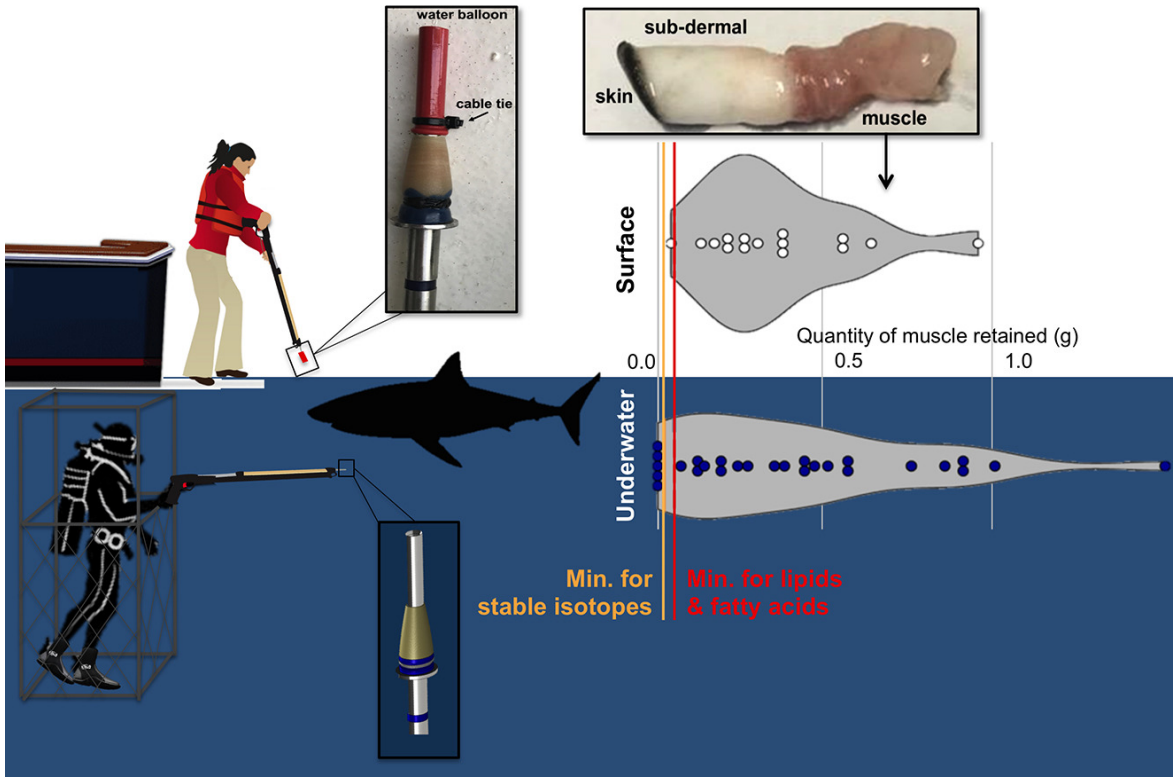
Chapter 3

Simple biopsy modification to collect muscle samples from free-swimming sharks



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Abstract

Developing and enhancing non-lethal methods for sampling species of high conservation concern, including marine megafauna, has prompted the development of numerous biopsy methods to collect tissue for biochemical analyses. However, many of these analyses require adequately-sized muscle cores for reliable results. Here, we developed and trialled a novel modification to a biopsy probe traditionally limited to underwater use, which enables sampling of free-swimming sharks from above the surface. The modified probe collected similar amounts of white shark, *Carcharodon carcharias,* muscle and sub-dermal tissue above water as the traditional underwater probe (muscle: 0.36 g vs. 0.44 g; sub-dermal tissue: 0.62 g vs. 0.44 g for surface and underwater respectively). Both methods obtained sufficient tissue for several analyses to be run on the same tissue core (e.g., stable isotopes, fatty acids, and genetics). This encourages the use of this biopsy probe, with studies assessing stock structure, trophic ecology, or physiology. The described modification adapts the probe to allow above-water deployment, providing more opportunities for effective, non-lethal sampling of free-swimming sharks.

Introduction

Research programs investigating the biology and ecology of marine animals are increasingly calling for the development and use of non-lethal sampling techniques (Fossi et al., 2010; Jardine et al., 2011; Smith et al., 2018). This is especially pronounced for studies of elasmobranchs (Heupel and Simpfendorfer, 2010; Hammershlag and Sulikowski, 2011; Marshal et al., 2012), owing to their generally low abundance and high conservation concern (Dulvy et al., 2014).

Gaining a robust understanding of diet, habitat use, population size, and stock structure is vital as it underpins appropriate conservation and management strategies (outlined in Carrier et al., 2018). Additionally, quantifying the load of natural (Meyer et al., 2016) and anthropogenic (Marsili et al., 2016; Fossi et al., 2017) toxins on elasmobranchs is increasingly important as human activity and urbanisation encroaches on a growing array of

marine habitats. Paralleling the call for non-lethal sampling, smaller tissue quantities (<1 g) can now be used in studies investigating trophic ecology (Boecklen et al., 2011; Pethybridge et al., 2018; Munroe et al., 2018; Chapter 2), population structure (Smith et al., 2018), and ecotoxicology (Marsili et al., 2016; Fossi et al., 2017). There has been a push to develop minimally invasive, *in situ* sampling devices targeting free-swimming animals (Reeb and Best 2006; Robbins 2006; Noren et al., 2012; Smith et al., 2018) as these offer alternatives to collecting tissue via lethal sampling or restraining animals, which can be logistically difficult and stressful for large species.

Advances in biopsy probe design (e.g. Reeb and Best 2006) and firing devices (including pole spears, rifles, crossbows, and spearguns) have largely been applied to sampling marine mammals from the surface (reviewed in Noren et al., 2012). Some of these designs have been adapted for underwater sampling, e.g. for elasmobranchs >1 m total length (e.g. Robbins 2006; Daly and Smale 2013). This includes the biopsy probe outlined in Daly and Smale (2013), which relies on suction to extract tissue cores. Underwater, suction is created as water is not compressible, and is thus expelled out of ventilation holes as the probe penetrates the skin. A rubber band covering the holes acts as a one-way valve (Figure 3.1A), preventing backflow into the probe, therefore creating the necessary suction to retain the tissue core as the probe is withdrawn. However, this suction mechanism does not work above the surface. Air is compressible, and the rubber band which acts as a valve underwater, creates a tight seal, preventing the air in the probe from being expelled through the ventilation holes. Upon withdrawal, the air re-expands, not creating the required suction to retain a tissue core. Obtaining adequately sized tissue cores from above the surface offers a number of practical advantages (research is not constrained by in-water limitations including communication, nitrogen accumulation, and temperature), increasing sampling opportunities. Here, we assess the effectiveness of a water-balloon adaptation to enable the use of the Reeb and Best (2006) biopsy probe (assessed in Daly and Smale 2013) to target white shark *Carcharodon carcharias* from above the water surface.

Previously, large elasmobranchs such as whale sharks *Rhinocodon typus* and white sharks have been biopsied from the surface with various probes using a hatch door system, mechanically slicing off the tissue core (described in Jaime-Rivera et al., 2013). The tissue collected has often been limited to skin and sub-dermal tissue (the thick layer of elastin and collagen underlying the skin) (e.g. Castro et al. 2007; Rohner et al., 2013; Fossi et al., 2017), however, the underlying muscle is the preferred tissue for a number of trophic analyses, e.g. stable isotopes (Hussey et al., 2012) and fatty acid analysis (Every et al., 2016). As such, the quantity of muscle retained by these biopsy devices can limit the type and number of analyses that can be conducted (Chapter 2). Responsible sampling, including maximizing the output from collection opportunities, is a financial, scientific, and ethical imperative (Heupel & Simpfendorfer, 2010). Thus, we compare the amount of sub-dermal and muscle tissue obtained from the surface-adapted and underwater biopsy probes to determine the efficacy of the water-balloon adaptation in successfully collecting sufficient tissue for multiple biochemical analyses.

Methods

The standard biopsy probe, manufactured by Rob Allen Dive Factory (www.roballen.co.za), attaches to the end of a spear and is typically fired underwater. In our study, the probe was attached to a 1.3 m steel spear, shot from a 1.1 m long Beuchat speargun powered by a 20 mm diameter elastic rubber. The probe consisted of a hollow 1 cm diameter stainless steel tube with a sharpened front edge to puncture the skin (Daly and Smale, 2013; Figure 3.1). The biopsy probe tip screwed into a ventilated base which is attached to the spear (Figure 3.1B). While underwater, this allowed the probe to puncture the skin, expelling water out of the four 3 mm holes in the base. When the biopsy probe was withdrawn, a 6 mm elastic band seals the ventilation holes, creating sufficient suction to sever the biopsy from the underlying muscle, and retain the tissue in the biopsy tip. When assembled, the tip of the probe extended 7 cm past a steel stopper ring affixed to the probe

base, preventing it from penetrating too far into the animal. A conical shaped rubber stopper, covering the proximal 3.5 cm of the probe tip limited the impact of the steel stopper ring.

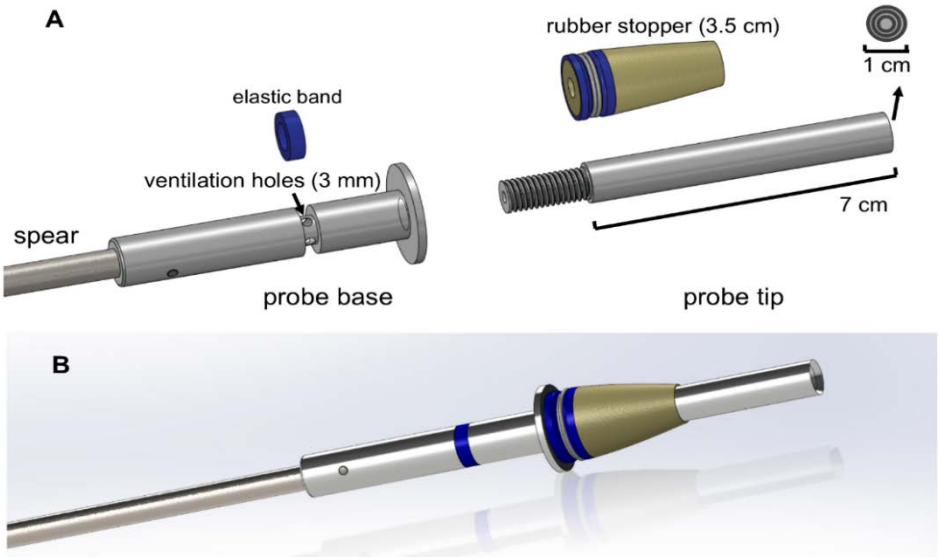

Figure 3.1 - Diagram of (A) the biopsy probe components and (B) the assembled biopsy probe.

The surface adaptation encased the tip of the biopsy probe in a water balloon (2.00 x 1.00 x 5.00 mm latex rubber water balloon) while it was submerged in water. The water balloon was emptied of any air bubbles prior to pulling it over the end of the biopsy probe tip. It was stretched tightly and secured with a small cable tie (e.g. 2.5 mm width) to the base of the tip just above the rubber stopper (Figure 3.2). The biopsy probe base was filled with water (~3–5 ml) and held vertically while the probe tip was screwed in. The air-tight water balloon prevented water from leaking out of the biopsy tip once it was removed from the bucket and screwed into the probe base, so that the entire biopsy was completely filled with water, replicating the in-water operating design. The 6 mm elastic band (Figure 3.1A) which sealed the ventilation holes prevented the water from leaking out of the adapted biopsy probe once assembled.

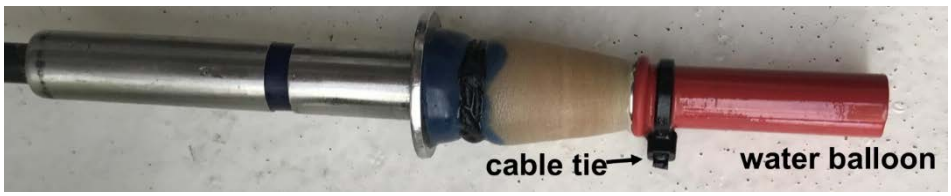

Figure 3.2 - Photograph of the biopsy probe with the surface adaptation including the water balloon (red) covering the biopsy probe tip and secured with a cable tie (98 x 2.5 mm).

Sampling

In situ sampling targeted free-swimming white sharks at the Neptune Island Group (Ron and Valerie Taylor) Marine Park, South Australia. Sampling was undertaken during standard cage-diving operations, during which sharks are attracted using a combination of berley (chum mixture of minced tuna) and bait (tuna head, tails, gills and guts) (Huveneers et al. 2017). Where possible, sharks were individually identified (Nazimi et al., 2018), sexed (based on clasper presence/absence), and sized (to the nearest 10 cm) using visual size estimates. Although not explicitly tested within this study, three personnel independently estimated the overall failure rate (percentage of events when no tissue was retained) and the rate of haemorrhaging (visible bleeding upon biopsying) for both biopsy methods. Forty-three biopsies (28 from underwater and 15 from the surface) were taken from white sharks ranging from 2.0–5.0 m estimated total length between May 2012 and January 2018 (Table 3.1).

Underwater biopsies, using the uncovered biopsy probe, were taken from the diving cages, targeting the dorsal or upper flank musculature directly below the dorsal fin. The probe was fired perpendicular to the shark from ~1.5 m distance. Above the surface, the water-balloon adapted biopsy probe was fired from the marlin board on the stern of the boat, targeting the same area. The resulting tissue cores were patted dry and the muscle was dissected from the sub-dermal tissue and skin (Figure 3.3). The skin and sub-dermal tissues were not separated (and thereafter referred to as 'sub-dermal' tissue) (Figure 3.3). Both tissue sections (muscle and sub-dermal) were weighed to the nearest 0.01 g.

Table 3.1 –Mean ± standard error tissue weight and shark morphometric data for biopsies taken underwater and from the surface.

		Tissue cores			white shark morphometrics	
Biopsy method	n	Skin and sub-dermal (g)	Muscle (g)	Cores including muscle (%)	Shark length (m)	Sex male/female [unknown]
Underwater	28	0.44 ± 0.05	0.44 ± 0.07	82	3.85 ± 0.12	19/4 [5]
Surface	15	0.62 ± 0.05	0.36 ± 0.05	100	3.26 ± 0.12	10/4 [1]

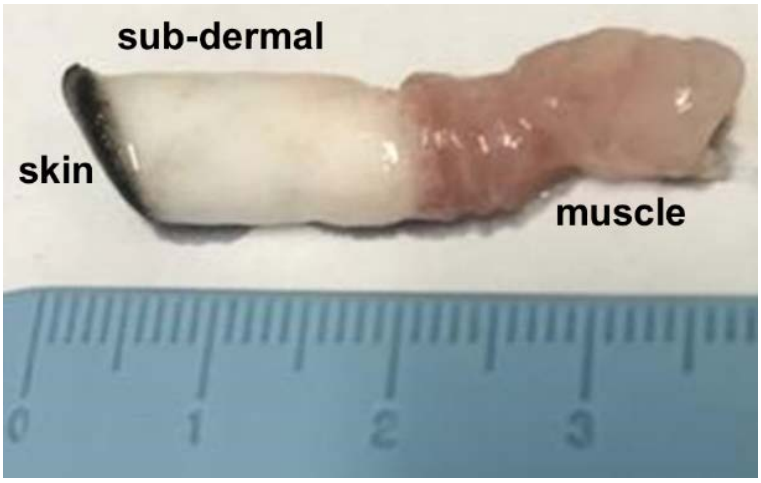

Figure 3.3 - white shark *Carcharodon carcharias* tissue core retained from the biopsy probe during surface sampling (scale shows 0–4 cm). The tissue core includes skin, sub-dermal tissue, and muscle.

Statistics

Violin plots illustrating the kernel probability density (Hintze and Nelson, 1998) were constructed to visualise the distribution of the tissue quantities of the underwater and surface biopsy methods (Figure 3.4). Each measurement (quantity of retained tissue per biopsy method) was plotted as stacked points to display how many biopsies exceeded the minimum necessary sample size for fatty acid (FA) (assessed in Chapter 2) and stable isotope (SI) analysis (quantities used in Jamie-Rivera et al., 2013). All plots were constructed by overlaying *geom* functions of the ggplot2 package, and all statistics were performed in the R statistical environment (R Development Core Team, 2016).

The difference in muscle and sub-dermal tissue quantities obtained across biopsy method (surface or underwater) and shark total length was assessed with Generalized Linear Models using the *glm* function and restricted maximum likelihood approach. Sex was excluded from the analysis due to a limited number of female sharks sampled underwater and from the surface. Following Shapiro-Wilk tests of normality and a visual inspection of the residuals for the saturated models, the GLMs were run with untransformed values. The biopsies which did not retain muscle were excluded from the analysis, as these biopsies did not penetrate the full depth of the sub-dermal tissue.

Results

The two biopsy methods retained similar quantities of muscle (surface: 0.36 ± 0.21 vs. underwater: 0.44 ± 0.37 g, p = 0.170, Figure 3.4), which was not influenced by shark total length (p = 0.131). Eighty-eight percent of total biopsies (38 of the 43 from both underwater and above the surface) contained sufficient muscle for stable isotope, genetic, fatty acid, and ecotoxicology analyses. Although not explicitly tested within this study, failure rate and haemorrhaging rate were both estimated to be approximately 10% for each biopsy method. Of the 90% of underwater biopsies which retained tissue, 82% of the tissue cores (23 out of the 28) contained muscle. Of the surface biopsies which retained tissue, all 15 cores contained muscle tissue. The minimum amount of muscle retained within the surface biopsies was 0.04 g, whereas the underwater biopsies retained a minimum 0.07 g (excluding those five which did not retain any muscle tissue, Figure 3.4). Similarly, the quantity of sub-dermal tissue was not significantly different between the two methods (surface: minimum of 0.24, mean 0.62 ± 0.21 vs. underwater: minimum of 0.18, mean 0.44 ± 0.28 g, p = 0.072, Figure 3.4), nor was it influenced by shark total length (p = 0.702).

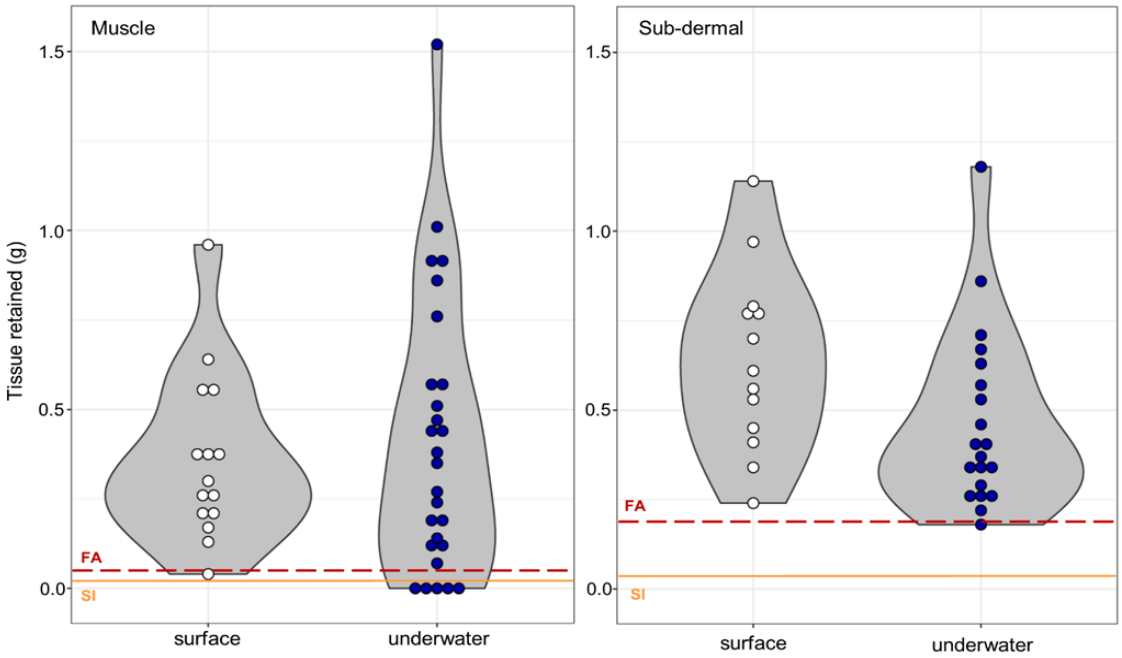

Figure 3.4 – Muscle and sub-dermal tissue quantities (g wet weight) retained with the surface and underwater biopsy methods. The grey violin plot outlines illustrate the kernel probability density, i.e. the width of the grey area represents the proportion of the data at that tissue retention quantity (Hintze and Nelson, 1998). Each point indicates the quantity of tissue retained per biopsy, with the surface denoted with white points and the underwater dark blue points. Dashed red line indicates the minimum tissue quantity for fatty acid (FA) analysis (Chapter 2) and the solid orange line, the quantity used for stable isotope (SI) analysis in Jaime-Rivera et al. (2013).

Discussion

The underwater and surface-adapted biopsy probes had the same estimated success rates (90%) and was similar to other underwater probes including the one tested by Daly and Smale (87% in Daly and Smale 2003) and both probes tested by Robbins (2006) (87% and 91%). The surface-adapted biopsy performed similarly to those tested in Jaime-Rivera et al. (2013), which had reported success rates of 80%, 95%, and 100% for the biopsy device using a trap door mechanism to retain tissue cores. As the biopsy device trialled here is approximately as effective as other underwater and surface-based biopsy devices at retaining tissue cores (which by default include skin at minimum), it is applicable to genetic and ecotoxicology studies seeking small amounts of this tissue type (e.g. Marsili et al., 2016; Fossi et al., 2017).

The underwater and surface-adapted biopsy probes retained similar quantities of muscle and sub-dermal tissue from free-swimming white sharks. Regardless of the variability in the quantity of tissue retained, both methods yielded sufficient amounts to undertake multiple analyses and investigate elasmobranch trophic ecology, ecotoxicology, biology, habitat use, and population structure. The average quantity of muscle retained for both biopsy methods far exceed the minimum amounts necessary for stable isotope (~ 0.005 g in Jaime-Rivera et al. [2013]), genetic analysis (0.003 g in Robbins [2006]), and ecotoxicology investigations (10 µg in Marsili et al. [2016] and Fossi et al. [2017]). Only one muscle-containing biopsy was insufficient for reliable fatty acid analysis (0.05 g in Chapter 2]), excluding the four biopsies which retained zero muscle. However, 70% of biopsies yielded <0.5 g of muscle, the amount used to assess organochlorines levels in white sharks (Marsili et al., 2016). Biopsies yielded sufficient skin and sub-dermal tissue for genetic, ecotoxicology, stable isotope, and fatty acid analysis in 42 of the 43 biopsies collected. Furthermore, the retention of multiple tissue types with different turnover rates enables researchers to evaluate the ecology and biology of an individual at multiple temporal scales (discussed in Boecklen et al., 2011). As such, this biopsy probe is an ideal tool to maximize sample collection

opportunities, as it retained multiple tissues in sufficient quantities for a range of biochemical analyses from free-swimming elasmobranchs both underwater and from the surface.

As both biopsies are equally effective at retaining tissue, the ability to use either enables sample collection across logistically diverse field scenarios. The surface-adapted biopsy allows collection from a vessel, eliminating the need for personnel to enter the water. This reduces a number of safety risks (and the logistical and administrative challenges of in-water activities) and extends the working time of the research team as they are not constrained by in-water limitations, including reduced communication, nitrogen accumulation, or cold temperature. This enables research to take place independently of cage-diving operations in the case of white sharks. It also expands research capabilities with a number of free-swimming elasmobranchs including those naturally frequenting the surface, such as whale sharks (*Rhincodon typus*), manta rays (*Mobula spp*.), and basking sharks (*Cetorhinus maximus*) and those encountered during marine wildlife tourism, e.g. blacktip sharks (*Carcharhinus limbatus*).

Despite well-documented increases in sub-dermal tissue thickness with shark length (Jaime-Rivera et al., 2013), we found no relationship between sub-dermal weight and shark total length. This likely stems from irregular biopsy angles and differing locations along the shark (ie. flank vs. dorsal region), highlighting the potential influence of biopsy placement, especially when seeking to penetrate through the sub-dermal tissue to obtain muscle from large elasmobranchs. Although data were not recorded, failure to obtain muscle most often occurred when biopsies were not taken perpendicular to the animal, reducing the penetration depth and the likelihood of the biopsy reaching the underlying muscle. Daly and Smale (2013) suggested that instances where biopsies failed to retain tissue were due to poor aim following avoidance movements of bull sharks *Carcharhinus leucas*. In the case of white sharks, rapid movement from attempts to consume the bait makes surface biopsy placement more challenging than from underwater, where sharks often slowly circle the cage presenting their flank (an ideal biopsy location to collect muscle). Research teams should consider where it is easiest to position themselves to provide reliable opportunities to reach the best spot on the

animal (in the case of white sharks, on the upper flank) at the most appropriate angle. The use of a band-powered speargun likely offers additional practical advantages over the pole spear. The speargun provides ample and consistent firing power, regardless of the physical strength of the user, and increasing the chances of well-placed biopsies. Unlike the biopsy poles, spearguns can be used above the surface and underwater, further increasing sample collection opportunities when paired with this adaptable biopsy probe.

While one study (Jaime-Rivera et al., 2013) obtained muscle from surface biopsies, the thick sub-dermal layer (particularly in female sharks [Pratt 1979]) has previously hindered the ability to collect underlying muscle in large elasmobranchs (e.g. white sharks [Castro et al., 2007; M Hoyas pers. comm., S Jorgensen pers. comm.] and whale sharks [Rohner et al., 2013; Fossi et al., 2017]). Despite concerns that this would limit the applicability of this biopsy probe for use with white sharks, the muscle retained within this study (mean 0.41 g) exceeded the 0.31 g total tissue obtained from bull sharks (Daly and Smale 2013) and the ~0.055 g obtained from various reef sharks (Robbins 2006) using similar underwater biopsies. Additionally, as the probe tested here was wider (1 cm diameter vs. 0.5 cm diameter in Robbins [2006] and 0.4 cm wide in Jaime-Rivera et al. [2013]), the resulting tissue cores include larger amounts of skin, the preferred tissue type for a number of ecotoxicology studies (Marsili et al., 2016; Fossi et al., 2017). Jaime-Rivera et al. (2013) demonstrated the ability to collect white shark muscle from the surface using a probe with a trap door design to retain tissue cores. However, their probe likely retained smaller muscle samples than the probe used here, as theirs was thinner (0.4 vs. 1 cm), and despite their probe being longer than ours (10.5 vs 7 cm) Jaime-Rivera et al. (2013) reported that the tissue cores did not fill the length of the 10.5 cm probe. As Jaime-Rivera et al (2013) only reported the length of retained tissues, their results are not directly comparable to the weight used here.

Furthermore, our biopsy probe had substantially fewer incidents of haemorrhaging (estimated <10% vs 100% in Jaime-Rivera et al. [2013]), and no cases of infection were observed. Although infection and the rate of haemorrhaging is not necessarily indicative of invasiveness, public perception of the impact of research and humane considerations, should

be thought out, especially when working alongside tourism operators. Sharks were often re-sighted following biopsying, and although the research team did not notice tearing or injury stemming from the cable tie, this presents an opportunity to further refine the probe adaptation. Particularly, the use of a rubber ring to secure the water balloon would eliminate the sharp cable-tie end, and should be explored to eliminate possible injuries.

Conclusion

The novel adaptation of an existing underwater biopsy probe enables its use from the surface with no significant difference in muscle or sub-dermal tissue retention. Both biopsy methods obtained ample tissue for a number of biochemical analyses to investigate trophic ecology, population structure, and ecotoxicology of chondrichthyans. With the described modification, this biopsy probe can now be used both underwater and above the surface, providing more opportunities for effective, non-lethal sampling of free-swimming sharks.

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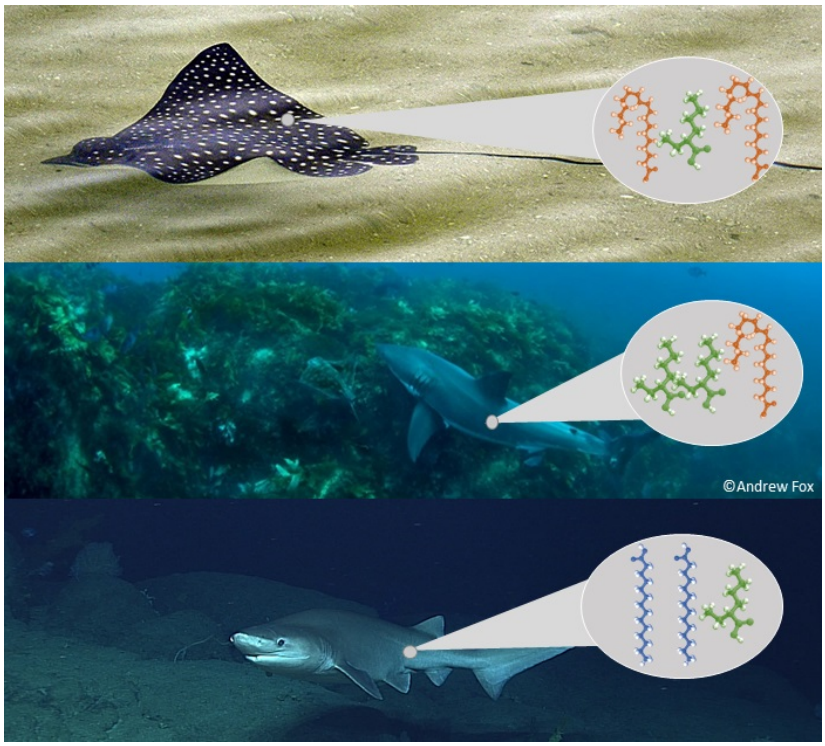
Chapter 4

Abiotic and biotic drivers of fatty acid tracers in ecology: a global analysis of chondrichthyan profiles



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Abstract

Use of fatty acid (FA) tracers is a growing tool in trophic ecology, yet FA profiles are driven by a number of abiotic and biotic parameters, making interpretation and appropriate use confusing for ecologists. We undertook a global analysis, compiling FA profiles of 106 chondrichthyan (shark, ray and chimaera) records, as a model to test the utility of FA profiles to partition *a priori* trophic guilds, phylogeny, water temperature, and habitats. Individual FAs characterising these four factors were identified, promoting the use of these FAs as ecological tracers across taxa. Habitat type was linked to five FAs: 16:0, 18:0 and biologically essential 22:6ω3 (indicative of the deep sea), 20:5ω3 (non-complex demersal and deep sea demersal) and 20:4ω6 (reef and brackish water). Temperature was a key driver of four FAs (22:5ω6, 22:4ω6, 20:1ω9 and 20:5ω3), while trophic guild and phylogeny were important drivers of two pairs of FA tracers (18:0 and 20:5ω3; 20:1ω9 and18:1ω9 respectively). This analysis provides a novel understanding of the biological and ecological information that can be inferred from FA profiles, and further validates the use of FAs as tracers to investigate the trophic ecology of chondrichthyans. Future research should prioritise *ex-situ* studies to further disentangle the influence of factors across taxa and tissue types, quantify biomodification, enabling the use of quantitative methods for diet determination and further develop 'FATscapes' to elucidate fine-scale trophic geography and climate variability. Additionally, the creation of a taxonomically inclusive FA data repository will enable further meta-analyses.

Introduction

The use of fatty acid (FA) analysis in ecology dates back to the 1960s with early research finding that marine phytoplankton produce novel, taxa-specific FAs (Ackman et al., 1968). Over the subsequent ~50 years, investigations into FA production across primary producers have detailed substantial variation in FAs across marine and terrestrial taxa, habitats, and biomes (reviewed in Colombo et al., 2016). These molecules serve essential biological functions, yet vertebrates lack the ability to synthesise a number of key FAs *de-novo* (Sargent et al., 1999). Fatty acid biomodification is also energetically costly, and enzymatically limited (Iverson et al., 2004), such that many FAs retain their chemical structures as they are assimilated from dietary sources (Sargent et al., 1999; Tocher, 2003). The variation in FA synthesis across taxa, habitats and biomes is, therefore, retained within the foodweb such that FAs in consumers reflect foraging dynamics, both abiotic (e.g. habitat type and temperature) and biotic (e.g. phylogeny and ecomorphology) (Colombo et al., 2016; Gladyshev et al., 2017).

Abiotic factors including water temperature (Gibson et al., 1984) and freshwater input (Sargent et al., 1999) influence primary production and community composition (Lowe et al., 2014), and thus dictate which FAs are produced at the base of the food web. As distinct FAs are synthesized by different producers (e.g. 20:5ω3 in diatoms and 18:3ω3 in selected macroalgae [Appendix Table S2]), such chemotaxonomic signatures may allow consumer foraging to be traced across habitats with distinct basal sources. For example, organisms foraging on rocky reefs dominated by macroalgae are high in 20:4ω6 (Alfaro et al., 2006), compared to mesopelagic feeders that are high in 20:1ω11 from consuming copepods (Pethybridge et al., 2010). However, the trophic transfer of such biomarkers is rarely assessed, particularly in controlled feeding trials, thus at times the interpretation of FA data beyond mid-trophic levels can be confusing. Fatty acid production within communities also varies with oceanographic conditions, such as nutrients, light, salinity, and sea surface temperature (Dalsgaard et al., 2003; Galloway and Winder, 2015; Leu et al., 2006; Schwenk et al., 2013).

Biotic influences, including phylogenetic differences in physiology and trophic guild can further dictate FA assimilation, especially in higher trophic level consumers (Colombo et al., 2016; Galloway and Winder, 2015; Gladyshev et al., 2017; Vasconi et al., 2015). Taxa-dependent FA interconversion through digestion and biosynthesis can occur (e.g. Iverson et al., 2004), leading to distinct taxa having inherently different FA profiles (e.g. insects vs. birds vs. mammals in Colombo et al. [2016]), with Species and Order influencing FA profiles in teleosts (Gladyshev et al., 2017; Vasconi et al., 2015). Biological factors including trophic guild (Colombo et al., 2016; Gladyshev et al., 2017; Vasconi et al., 2015), fish size (Vasconi et al., 2015), enzyme activity linked to migration potential and metabolic rate (Gladyshev et al., 2017; Hulbert, 2007), and swim speed (Gladyshev et al., 2017; Vasconi et al., 2015) can also influence FA values (Appendix Table S2).

Disentangling these potentially confounding abiotic and biotic drivers of FA tracers (compared in Colombo et al. 2016 and Gladyshev et al. 2017) makes FA data interpretation and the appropriate use of FA tracers confusing. For example, FA 20:5ω3 can be synthesized by multiple sources including diatoms, macroalgae, and other phytoplankton (Dunstan et al., 1988; Kelly and Scheibling, 2012), and is used to trace foraging in coastal foodwebs. Yet 20:5ω3 can be synthesized by some secondary consumers (e.g. polychaete worms [Olive et al., 2009]), and is preferentially retained in the muscle of fast-swimming, migratory fish (Gladyshev et al., 2017).

Despite these challenges, FA tracers are growing in popularity with marine ecologists (Pethybridge et al., 2018), owing to their ability to detail trophic ecology in finer resolution and shorter time scales (weeks to months - Beckmann et al. [2013]) than other biochemical tracers (i.e. stable isotopes and trace metals). Additionally, computing and multivariate statistical packages (e.g. PRIMER and PERMANOVA [Anderson et al., 2008; Clarke and Gorley, 2015]) have improved markedly to assist ecologists in assessing complex FA datasets. Despite this, the validity of individual tracers is largely unexplored or needs further refining for many higher level taxa.

Chondrichthyans (sharks, rays and chimaeras) exhibit complex and varied trophic ecologies, owing to their array of morphologies, habitats and foraging strategies. The use of FA tracers in chondrichthyan ecology extends to diet (McMeans et al., 2012; Pethybridge et al., 2011), habitat use (Rohner et al., 2013), ontogenetic shifts (Wai et al., 2011), and quantifying trophic niche overlap (Every et al., 2017) amongst other applications (Appendix Table S2). Although FA assimilation in chondrichthyans appears to be similar to other taxa (Beckmann et al., 2013), chondrichthyans have distinct physiologies and metabolic processes, relying on ketone bodies instead of lipids for energy metabolism, and using fatty, lipid rich livers as a sink for long-term energy storage (Ballantyne, 1997). As such, the intricacies of how biotic and abiotic factors influence FA tracers in chondrichthyans are mostly unknown.

In this study, we combined published and newly obtained FA profiles from chondrichthyans to better understand patterns and trends in FA tracers. More specifically, our study aims to: 1) determine if muscle tissue FAs can distinguish known trophic guilds, temperature of foraging grounds, habitat types, and species phylogeny; 2) assess which FA tracers are characteristic of specific levels within these factors (i.e. habitat type, temperature range, trophic guild); and 3) outline future directions for FA tracer applications in ecological studies. These results will help clarify our understanding of which abiotic and biotic factors influence the distribution of FAs. This knowledge will also better enable the assessment of diet composition and habitat use of marine consumers including chondrichthyans, which is a critical aspect of effective management and conservation programs.

Methods

Data collection

Search engines (Google Scholar, Web of Science, and Science Direct) were used to obtain peer-reviewed publications containing chondrichthyan FA profiles using the following keywords: shark, skate, ray, chimaera, elasmobranch, or chondrichthyan, and fatty acid and/or lipid with * to include plural versions. We reviewed all articles identified through the search and excluded non-relevant articles. The reference list of the resulting articles was then searched for additional citations containing appropriate FA profiles. Only those derived from muscle

tissue were used in the analysis, as they most closely resemble prey FA profiles (Beckmann et al., 2014; McMeans et al., 2012; Pethybridge et al., 2011), have less intra- and inter-individual variability (Davidson et al., 2014; Pethybridge et al., 2010), and are dominated by essential polyunsaturated FA (PUFAs), (Davidson et al., 2014; Pethybridge et al., 2010) which are the predominant FAs used to examine the influencing biotic and abiotic factors (Colombo et al., 2016; Gladyshev et al., 2017; Vasconi et al., 2015). Furthermore, liver was not assessed within this study as the FA profiles are likely heavily influenced by physiological factors (including migration, buoyancy, diet, pregnancy, energy availability [Ballantyne, 1997; Pethybridge et al., 2010b]), potentially confounding the influence of abiotic and biotic drivers of interest. Additionally, sufficient muscle for FA analysis can be taken from most chondrichthyans with sub-lethal biopsies (Chapter 2 and 3), unlike obtaining liver samples. Studies referring to unnatural diets (e.g. in captivity [Beckmann et al., 2013]) were excluded from the analysis. A list of data sources used in the study are provided in the Data sources section.

The mean of individual FA (reported as percent contribution to the overall FA profile, recorded to the nearest 0.01%) were taken for each species. Only FA profiles of the total lipid extract (TLE) (not polar/non-polar fractions) extracted using the Bligh and Dyer (1959) or Folch et al. (1957) methods, and reported as percent contribution were included.

Trophic guild, categorized as either: top predator, second-order carnivore, first-order carnivore, and herbivore were estimated based on trophic level and primary diet as listed on Fishes of Australia (http://fishesofaustralia.net.au), Fishbase (Froese and Pauly, 2017), IUCN Red List assessments (www.iucnredlist.org), and expert knowledge. Water temperature, corresponding to the season, location and depth of capture, was obtained from the study in which the record was published. When not stated in the study, temperature was estimated using the National Oceanic and Atmospheric Administration (NOAA) World Ocean Database (WOD; https://www.nodc.noaa.gov/OC5/WOD/pr_wod.html, Boyer et al., 2013) high-resolution conductivity-temperature-depth (CTD) dataset for the approximate location, depth, and season of capture. Temperature was treated as a continuous variable in the models (see below), but

was grouped into 5˚C bins and treated as a categorical factor for multivariate analysis. Species' primary habitat was categorized into: pelagic, deep sea (>200 m depth), deep sea demersal, reef (comprising rocky and coral reefs), reef demersal, brackish (estuarine environments), and non-complex demersal (demersal marine species occurring in <200 m and not associated with rocky or coral reefs). Habitat categorization was based on Fishbase listings (Froese and Pauly, 2017), IUCN Red List assessment's habitat descriptions (www.iucnredlist.org), and expert knowledge. The Order of each species was used to account for its phylogenic position (Chondrichthyan Tree of Life [www.sharksrays.org]). The factor allocations for each record, along with the species, citation and location are detailed in Appendix Table S1 and sumarised in Table 4.1.

Table 4.1 – Sample size within each factor level.

Trophic guild		**Temperature**		**Habitat**		**Phylogeny**	
Factor levels	*n*	Bin groups (°C)	*n*	Factor levels	*n*	Factor levels	*n*
Top predator	24	26-31	21	Brackish	13	Carcharhiniformes	34
Second-order carnivore	40	21-25	25	Pelagic	6	Chimaeriformes	3
First-order carnivore	38	16-20	6	Reef	21	Heterodontiformes	2
Herbivore	4	11-15	9	Reef demersal	11	Hexanchiformes	1
		6-10	28	Non-complex demersal	20	Lamniformes	7
		<6	13	Deep sea	20	Myliobatiformes	13
				Deep sea demersal	15	Orectolobiformes	3
						Rajiformes	19
						Rhinopristiformes	1
						Squaliformes	20
						Squantiniformes	3
						Carcharhiniformes	34

Statistical analysis

Only FAs reported in >50% of all of the compiled records were included in the analyses, reducing the dataset from 57 to 19 FAs.. Multivariate analyses were carried out using PRIMER v.7/PERMANOVA+ software (Anderson et al., 2008; Clarke and Gorley, 2015), while modeling was undertaken in the R statistical environment (R Development Core Team 2016).

Different levels of transformation were explored via shade plots and a square root transformation was selected to avoid over-emphasis of extreme values. Resemblance matrices were calculated using Bray-Curtis similarity measures between samples. A permutational analysis of variance (PERMANOVA) with Monte Carlo simulations (denoted as p(MC)) on the unrestricted raw values was used to test if FA profiles were affected by trophic

guild, temperature, habitat, or phylogeny (assessed independently). The interactions were not included in these PERMANOVAs to avoid overparameterisation. Canonical Analysis of Principal Coordinates (CAP) (Anderson and Willis, 2003) was then used to further discriminate between a priori groups within the factor of interest, with correlations >0.60 overlaid on the CAP ordination plots. The FAs with CAP correlation values >0.60 across two or more factors were selected for subsequent generalized linear modeling to determine which factor had the strongest influence on the FA values.

For each FA, the effect of trophic guild, temperature, habitat, and phylogeny (all fixed factors) was assessed by fitting a Generalized Linear Model using the *glm* function and restricted maximum likelihood approach. The models were fitted to a reduced dataset, eliminating the data from phylogenetic groups with less than three records (Heterodontiformes, Hexanchiformes, and Rhinopristiformes). The most appropriate statistical family, error distribution, and validity of the model were determined through an examination of the distribution of the response variable, a visual inspection of the residuals for the saturated models, and an ANOVA test between the fitted and residual values of the model. Models were fitted with either Gamma distribution with log link (for FA - 18:0, 18:1ω9, 20:1ω9, 20:4ω6 and 20:5ω3) or Gaussian with identity link (FA – 16:0, 22:4ω6, 22:5ω6 and 22:6ω3). Effect size and 95% confidence intervals for each factor level were calculated using the *allEffects* function of the '*lme4*' package (Bates et al., 2015). Effect plots were subsequently produced for the full model to visualize changes in FAs across factors (Appendix Figure S3).

To quantify the relative importance of each factor, we used a model averaging approach with importance weighting of a subset of best-fit models using the *MuMIn* package. The full model was dredged, creating a set of new models containing all possible combinations of the four factors. These were ranked on decreasing model fit, determined by a corrected Akaike's Information Criterion (AIC_c) applicable to small-sample size. All dredged models with $\Delta AICc < 4$ were used in the model averaging. The importance weights for each factor was calculated as the sum of the relative weight of each model containing the factor.

Results

The keyword search revealed >25,000 publications, 19 of which reported usable chondrichthyan muscle FA profiles. An additional nine publications were sourced from the reference lists, and one was sourced from a co-authors collection, totalling 29 publications containing 100 distinct records from 79 different species (Appendix Table S1). The data set was augmented with six unpublished records from *Bathyraja eatonii*, *Bathyraja irrasa* and *Bathyraja murrayi* from the Kergualen Plateau, and *Dasyatis brevicaudata* and *Myliobatis australis* from multiple locations in South Australia, analysed using standard FA extraction and analysis protocols (Bligh and Dyer 1959). In total, 106 distinct records were included in the analysis (Figure 4.1).

Trophic guild

Trophic guilds were distinguished by FA profiles (p(MC) = 0.001; CAP p = 0.001, Appendix Table S6), with top predators (high in 14:0) most distinct from the first-order carnivores (highest in 16:0, 16:1ω7 and 20:5ω3, Figure. S2B), with the second-order carnivores (high 22:6ω3 and low 16:1ω7) clustered in-between (Figure 4.2A). Herbivores (highest levels of 22:4ω6 and 22:5ω6) were indistinguishable from the top predators, but were distinct from the second- and first-order carnivores (Appendix Table S6, Figure 4.2A & S3). The significant CAP was driven by two SFAs (saturated fatty acids), one MUFA (monounsaturated fatty acid) and two PUFAs with correlations >0.60 (Table 4.2).

Table 4.2 – Fatty acids driving the differences between factor groups determined by CAP correlation values >0.60.

	Trophic guild	Temperature	Habitat	Phylogeny
SFA	14:0	18:0	16:0	18:0
	16:0		18:0	
MUFA	16:1ω7	18:1ω9	18:1ω9	18:1ω9
		20:1ω9	20:1ω9	20:1ω9
PUFA	20:5ω3	18:3ω3	20:4ω6	20:4ω6
	22:6ω3	20:5ω3	22:4ω6	20:5ω3
		22:4ω6	22:5ω6	22:4ω6
		22:5ω6	22:6ω3	22:6ω3
		22:6ω3		

SFA – Saturated fatty acid
MUFA – Monounsaturated fatty acid
PUFA – Polyunsaturated fatty acid

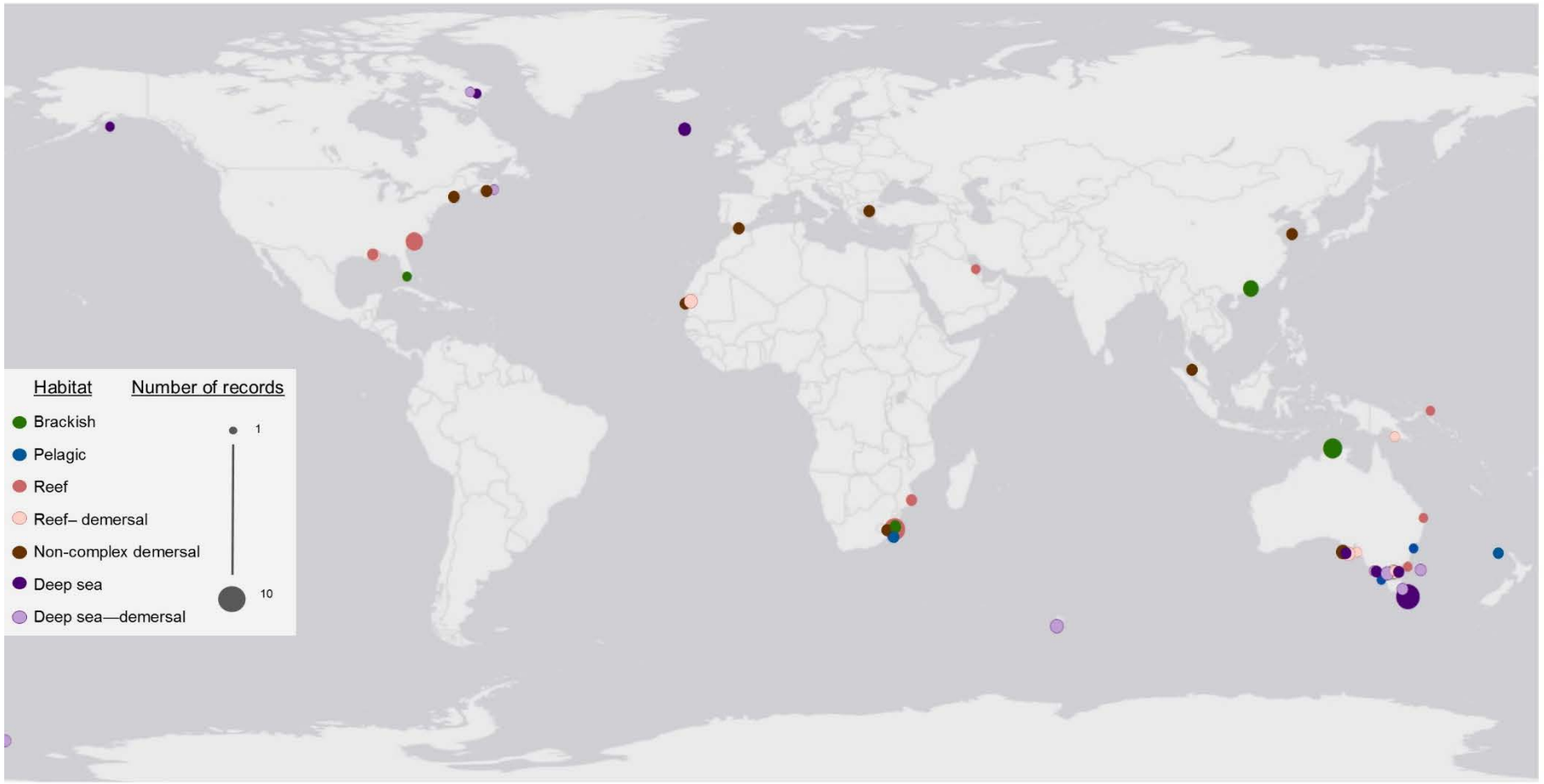

Figure 4.1 – The locations of specimen sampling for the 106 chondrichthyan muscle derived FA records, with colour denoting the habitat type and the size of the circle corresponding to the number of individual records within that location and habitat type (see Table S1 for a full list of species, locations and habitat classifications).

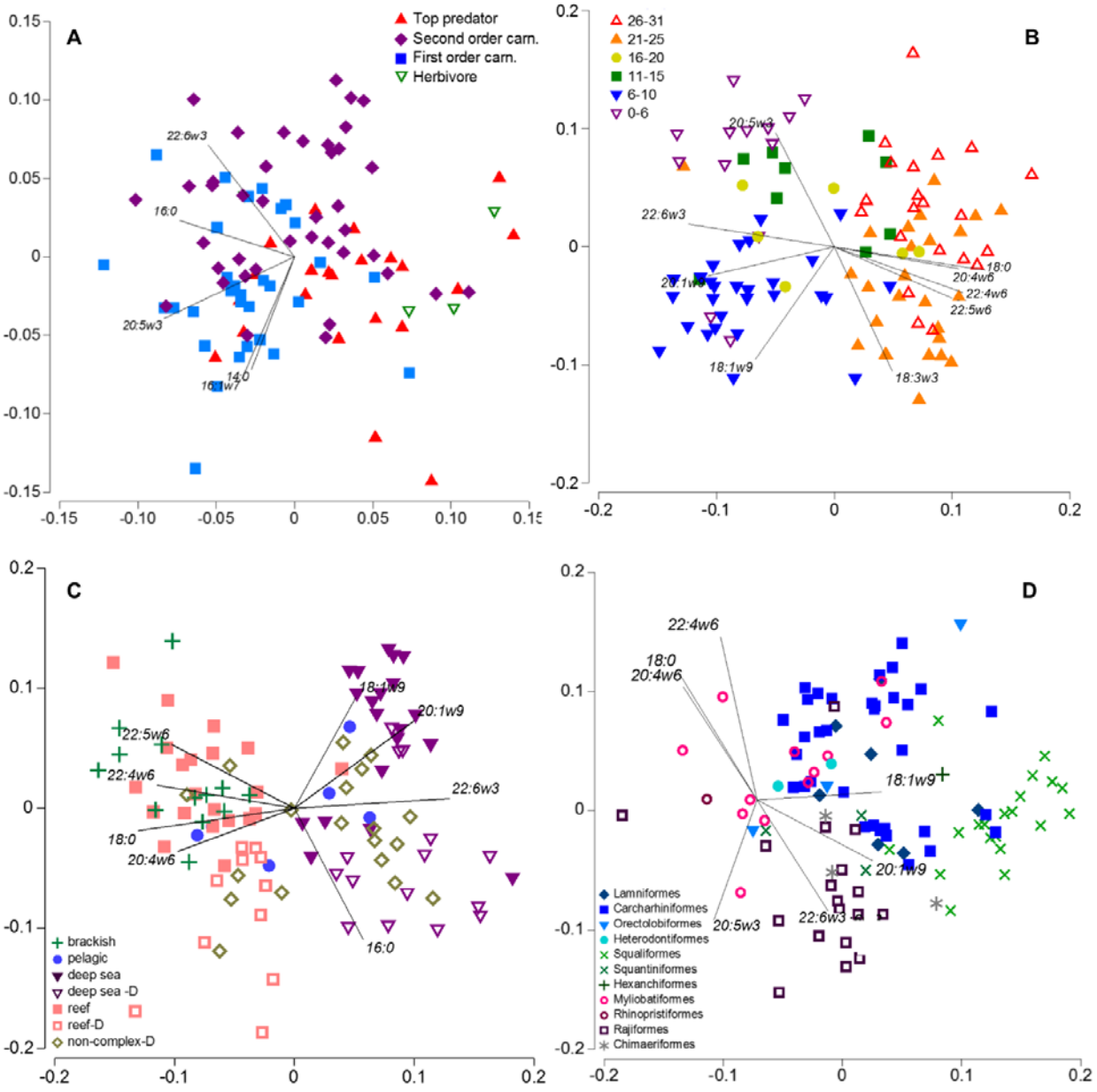

Figure 4.2 – Canonical Analysis of Principal Coordinates (CAP) of record's trophic guild (A), temperature (B), habitat (C) and phylogeny (D), with overlaid vectors of individual fatty acids with CAP correlation values >0.60. Within the habitat group analysis (C), the suffix -D indicates demersal habitat use.

Temperature

Temperature was a significant driver of FA profiles (p(MC) = 0.001, CAP p = 0.001; Appendix Table S6) with four distinct clusters (Figure 4.2B) driven predominantly by PUFAs (five with CAP correlation values >0.6) followed by SFAs and a single MUFA (Table 4.2, Appendix Figure S3). The two groups from the warmest waters were distinguished by higher levels of the SFA 18:0 (13.8% in >26˚C and 14.8% in 21–25˚C), which was conversely lowest in the cold <6˚C group (5.3%) and had the highest CAP correlation of 0.78 (Table 4.2). The warmer two groups were also high in the PUFA 22:4ω6 (3.8% in >26˚C, Appendix Figure S1I) and 22:5ω6 (2.2% in >26˚C, Appendix Figure S1H). The coldest groups were highest in 20:5ω3 (7.6% in <6˚C group, Appendix Figure S1G), 22:6ω3 (28.8% in 6-10˚C, CAP correlation = 0.75), and 20:1ω9 (3.9% in 6-10˚C, Appendix Figure S1F). 18:3ω3 and 18:1ω9 were highest in the warm (21–25˚C) and cool (6–10˚C) temperature groups and lowest in the hot (>26˚C) and cold (<6˚C) temperature extremes (Figure 4.2B).

Habitat

Habitat classifications had distinct FA profiles (PERMANOVA main test p(MC) = 0.001, CAP p = 0.001, Appendix Table S6), with five clear groupings (Figure 4.2C) driven by four PUFAs, two SFAs and MUFAs, with CAP correlations >0.6 (Appendix Table S6). 22:4ω6 (CAP correlation = 0.86) was lowest in the deep sea and deep sea demersal groups (1.3% and 1.1% respectively) and highest in the reef (4.2%) and brackish groups (3.9%) (Appendix Figure S3). Conversely, 22:6ω3 (CAP correlation = 0.81) was highest in the deep sea (30.0%) and deep sea demersal (28.8%) and lowest in the brackish (10.8%), reef demersal (14.6%), and reef (15.2%) groups (Appendix Figure S1E). SFA 18:0 (CAP correlation = 0.80) was highest in the brackish (16.7%) and reef group (14.1%, Appendix Appendix Figure S1B) unlike SFA 16:0 (CAP correlation = 0.71) which was low in the reef (15.2%) and brackish (13%) groups but high in the pelagic (20.2%) and demersal groups (reef demersal – 19.8%, and deep sea demersal 19.7%, Figure S1A). MUFAs 20:1ω9 (highest levels in the pelagic group [1.2%]) and 18:1ω9 (highest in the deep sea) and PUFAs 20:4ω6 (highest in the reef and brackish groups, Appendix Figure S1C), 20:5ω3

(high in non-complex demersal and deep sea demersal) and 22:5ω6 (high in the reef and brackish groups) also drove differences across habitats (Table 4.2).

Phylogeny

FA profiles were significantly clustered based on phylogenetic groups (PERMANOVA $p < 0.0001$, Figure 4.2D), forming four overarching clusters: (1) Carcharhiniformes, Lamniformes, and Orectolobiformes; (2) Squantiniformes, Squaliformes, and Hexanchiformes; (3) Myliobatiformes, and Rhinopristiformes; (4) Chimaeriformes and Rajiformes (Figure 4.2D, Appendix Table S6). CAP correlation values revealed that 18:0 (CAP correlation = 0.80) was a key FA driving the difference between several groups, with lowest contributions found in Chimaeriformes (5.8%) and Squaliformes (6.2%). PUFA 22:4ω6 had the second highest CAP correlation value of 0.78, and was found in higher levels in the Carcharhiniformes and Myliobatiformes (3.9 and 3.2%) than other groups. The CAP was also driven by MUFA 20:1ω9 and 18:1ω9 (CAP correlations 0.70 and 0.64 respectively) which were highest in the Squaliformes (3.9% and 17.3%), Hexanchiformes (3.4% and 15.8%) and Lamniformes (3.3% and 14.0%) (Appendix Figure S2C & S2D). PUFA 20:4ω6, 22:5ω6 and 22:6ω3 also had correlation values >0.60 (0.68, 0.64 and 0.63 respectively), with 20:4ω6 highest in Heterodontiformes (12.6%) and 22:5ω6 highest in Carcharhiniformes (1.8%). 22:6ω3 was a substantial contributor to all FA profiles, ranging from 30.1% in the Chimaeriformes and 29.0% in the Squaliformes to 10.7% in the Orectolobiformes.

Ranking of factors for individual FA tracers

Habitat was the most important explanatory factor for three (16:0, 20:4ω6 and 22:6ω3) of the nine key fatty acids, and one of the important factors for 18:0 and 20:5ω3 (Appendix Table S7). Temperature was the most important factor influencing 22:4ω6 and 22:5ω6 and was also important for 20:5ω3 (with habitat and trophic guild) and 20:1ω9 where it had the same relative importance as phylogeny. Phylogeny was the most important factor in 18:1ω9 only. Trophic guild was never the most important driving factor, but had equal importance to habitat for 18:0 and to temperature and habitat for 20:5ω3 (Appendix Table S7, Table 4.3).

Table 4.3 – Fatty acid (FA) tracers as reported in the literature (summarized from Appendix Table S2) and their sources. A colored heat map indicates the calculated relative variable importance (Appendix Table S7) with green = 1 (highest relative importance value) to red = 0 (not important).

	Recommended within the literature	Trophic guild	Temperature	Habitat	Phylogeny
Saturated Fatty Acids (SFA)					
16:0 Palmitic acid	Zooplankton[1], trophic level[2], mesopelagic, demersal vs. deep sea[3,5,6]	Second order carnivores	Warm water >21°C	Pelagic Demersal	
18:0 Stearic acid	Zooplankton[1] mesopelagic[3] demersal[3], trophic level[2]	Herbivores Second order carnivores	Warm water >21°C	Brackish Reef	Rhinopristiformes Carcharinidae
Monounsaturated Fatty Acids (MUFA)					
16:1ω7 Palmitoleic acid	Phytoplankton[6], mangroves, diatoms and bacteria[4], low trophic levels[8]	First order carnivores			
18:1ω7 Vaccenic acid	Bacteria[4], phytoplankton[6], depth[7], crustaceans, bathypelagic squid and fish[5]				
18:1ω9 Oleic acid	Macroalgae & mangroves[4], trophic level[2], carnivory[8], temperature[7], depth[7], blubber[9]		Intermediate temperatures (21-25°C and 6-10°C)	Deep sea	Squaliformes Hexanchiformes
20:1ω9 Eicosenoic acid	Copepods[4], latitude[10], temperature[11], mesopelagic fish[4], blubber[12]		Cold water <10°C	Pelagic	Squaliformes Hexanchiformes
Polyunsaturated Fatty Acids (PUFAs)					
18:3ω3 α-Linolenic acid; ALA	Terrestrial plants, macroalgae[12], mangroves[4] seagrass[4,12]		Intermediate temperature		
20:4ω6 Arachidonic acid; ARA	Diatoms[13], algae, kelp, mangroves & terrestrial plants[13], trophic position[18, 19], latitude[10], coastal habitats[13, 21], blubber[9] cartilaginous fish[14]	Top predators	Cold water <10°C	Reef Brackish	Heterodontiformes Myliobatiformes
20:5ω3 Eicosapentaenoic acid; EPA	Phytoplankton[14], diatoms, brown & red macroalgae[4,6] krill[15], trophic position[14, 19] carnivory[8], cold water[19], coastal areas, demersal habitats[14], migration[17, 20], cephalopods[14], polychaetes[18]	First order carnivore	Cold water <10°C	Non-complex demersal Deep sea demersal	Squaliformes Rajiformes
22:4ω6		Planktivore Top predator	Warm water >21°C	Reef Brackish	Lamniformes
22:5ω3 Clupanodonic acid; DPA(3)	Diatoms, algae, mangroves, terrestrial plants[13] mangroves, coastal areas[13]				
22:5ω6 Osbond acid; DPA(3)	Demersal carnivores[14], fish[14, 19], cephalopods[15]	Top predator Planktivore	Warm water >21°C	Reef Brackish	Carcharinidae Myliobatiformes
22:6ω3 Docosahexaenoic acid; DHA	Dinoflagellates zooplankton[4, 6, 16], trophic position[8, 19, 21], cold water[21], offshore migrations[17], swim speed[20], carnivory[19]	Second order carnivore	Cold water <10°C	Deep sea (water column and demersal)	Chimaeriformes Squaliformes

For those FA that were substantially correlated (CAP correlation value >0.6) to a single factor, the relative importance was assumed to be 1, and all other factors assumed to be 0.

[1]Rohner et al. 2013, [2]Schmidt-Nielsen 1997, [3]Pethybridge et al. 2010, [4]Kelly & Scheibling 2012, [5]Pethybridge et al. 2011, [6]Falk-Petersen et al. 2000, [7]Arts & Kohler, 2009, [8]Cook et al. 2000, [9]Waugh et al. 2014, [10]Gibson et al. 1984, [11]Ackman 1968, [12]Nichols et al. 1982 [13]Alfaro et al. 2006, [14]Dunstan et al. 1988 [15]Pethybridge et al. 2013,[16]Dalsgaard et al. 2003, [17]Osako et al. 2006, [18]Wai et al. 2011, [19]Colombo et al. 2016, [20]Gladyshev et al. 2017, [21]Sardenne et al. 2017.

Discussion

FA tracers are increasingly used to study marine trophic ecology, despite a lack of taxa-specific validation or adequet understanding of their main abiotic and biotic drivers. Here, we used the FA profiles from 106 chondrichthyan records to show that FA profiles can partition *a priori* trophic guilds, temperature, phylogeny, and habitats. For each factor, individual FAs were identified as drivers of FA profiles, therefore justifying and promoting their use as ecological tracers for chondrichthyans (Table 4.3). We disentangled the overlapping influence of temperature, habitat, trophic guild, and phylogeny, revealing which of these factors most affect relative changes in FAs. These findings can be used to guide the selection of FA tracers for use in trophic ecology studies. They will also aid in the interpretation of FA profiles when organisms are influenced by multiple factors simultaneously, e.g. migratory sharks foraging across different habitat types and temperatures.

Confirming FA profiles as tracers of marine ecosystems

FA profiles can trace marine abiotic (habitat and temperature) and biotic (trophic guild and phylogeny) factors in finer resolution than previously explored. Other biochemical tracers (e.g. stable isotopes) have been used to assess broad-scale trophic geography in chondrichtyans (e.g. Bird et al. [2018]). Furthermore, Colombo et al. (2016) and Gladyshev et al. (2017) undertook a similar approach, exploring variation in select PUFAs across broad taxonomic groups, biomes, and habitats. Yet using 19 FAs as a profile, we distinguished finer-scale ecology than was explored in those studies, and revealed the importance of several SFAs (16:0 and 18:0) and MUFAs (18:1ω9 and 20:1ω9) and a number of PUFAs beyond those examined previously. For example, the capacity for FA profiles to reveal small differences in habitat types (e.g. reef demersal vs. non-complex demersal, or deep sea vs. deep sea demersal) is particularly useful for detailing specific foraging grounds (listed as key research priorities for a number of chondrichthyans [Heupel et al., 2019; Huveneers et al., 2018; Shipley et al., 2017]) at a more ecologically relevant resolution than marine vs.

terrestrial or fresh/brackish vs. marine as explored in Colombo et al. [2016] and Gladyshev et al. [2017]. This advocates for the use of full FA profiles, as a robust complementary approach to other biochemical tracers such as stable isotopes, for elucidating complex ecology at fine resolution.

FA profiles clearly distinguished abiotic factors habitat and temperature, making them particularly valuable for ecologists investigating chondrichthyan foraging grounds. The substantial distinction between the deep sea and coastal (reef and brackish) habitats encourages the use of FA profiles to assess the ecology of species migrating across diverse biomes, undertaking daily diving patterns, or widely distributed species for which foraging grounds remain unknown (e.g. white shark *Carcharodon carcharias* [Huveneers et al., in review]). Furthermore, the stark partitioning of the demersal habitats indicates that FA profiles can distinguish between feeding within the water column or demersally across broad habitat types. This highlights that the intricacies of three-dimensional habitat use are encapsulated in FA profiles.

FA profiles were also able to distinguish broad trophic guilds, albeit to a lesser extent than observed for the other factors. As with other biochemical tracers (most notably nitrogen isotopes), most compounds undergo some level of biomodification following ingestion and incorporation (outlined in Munroe et al. [2018]). FAs undergo enzyme-induced biomodification in seals (Iverson 2004), fish (reviewed in Tocher [2003]), and in Port Jackson sharks (Beckmann et al., 2013). Vasconi et al. (2015) and Gladyshev et al. (2017) similarly found that FAs shifted with broad teleost trophic guilds, and Strandberg et al. (2015) detailed the selective transfer of PUFA from phytoplankton to fish. The successive enrichment of PUFA, 22:6ω3 in particular, has been documented in low trophic levels in a number of systems, including in the Mediterranean Sea (Koussoroplis et al., 2011) and freshwater food webs (Strandberg et al., 2015). This suggests that the location where an organism sits within the food web can influence FA profiles due to successive biomodification, with FAs potentially functioning similarly to stable isotopes. However, in chondrichthyans, the low trophic level herbivores were indistinguishable from the top predators, both containing high

levels of PUFAs 22:4ω6 and 22:5ω6 (indicators for zooplankton, fish and cephalopods [Rohner et al. 2013]), suggesting that the increase in PUFA within higher trophic level groups (i.e. not primary producers to primary consumers as in Standberg et al., [2015]) may be driven more by prey availability than by biomodification.

Phylogeny also affected FA profiles, but only 36% of the pairwise comparisons were significantly different, compared to 73%, 83%, and 95% for temperature, trophic guild, and habitat respectively. Additionally, six of the seven FA tracers reflecting phylogeny were also tracers for habitat or temperature, indicating that abiotic factors were more powerful drivers of FA profiles than phylogeny. This opposes findings from teleosts, where broad phylogenetic groupings (Clupeiformes, Salmoniformes, Scorpaeniformes, and Osmeriformes) accounted for 27.6% of the variation in 22:6ω3 and 20:5ω3, whereas abiotic factors (temperature and salinity) only accounted for 7.2% (Gladyshev et al., 2017). Similarly, Budge et al., (2002) found that FA profiles classified Northwest Atlantic fish and invertebrates into species with greater certainty than environmental factors like geographic location and size within a species. The influence of phylogeny on FA composition is increasingly important in low trophic groups. Phylogeny accounted for 3–4 times more variation in marine and freshwater phytoplankton and macrophyte FAs than any other environmental factor (Dalsgaard et al., 2003; Galloway and Winder, 2015; Taipale et al., 2013). This reduced importance of phylogeny in species of higher trophic level explains the difference in findings between our study and those examining primary producers, invertebrates, and low trophic level teleosts. Different taxa of primary producers synthesize different FAs underlying the importance of phylogeny outlined above. Thus, the community composition of primary producers dictates spatial FA availability. Within low trophic levels, the intermediate importance of phylogeny is likely related to differing environments and the consumption of different primary or secondary producers which is confounded by phylogeny, more than explained by it (discussed in Gladyshev et al., 2017). This trend continues as the importance of phylogeny is further reduced with high trophic level species (as per our findings), and habitat type drives FA profiles more so than phylogeny. This is due to the

differences in habitats being a product of differing basal food web FA production, driven by the distinct taxa of primary producers.

Individual FAs as tracers for accurately identifying groupings

In the wake of expanding applications in biochemical ecology, appropriate tracer selection remains crucial yet challenging. The FA tracers identified in this study generally parallel their use across biologically distinct taxa (Table 4.3, Appendix Table S2). In particular PUFAs, which are important tracers in teleosts (Gladyshev et al., 2017; Tocher, 2003) and marine mammals (Budge et al., 2004), were also found to be good tracers for habitat and temperature. For example, 22:6ω3 indicates dinoflagellate-based food webs (Dalsgaard et al., 2003; Kelly and Scheibling, 2012), cold-water (Hulbert, 2003), and high trophic position (Colombo et al., 2016; Cook et al., 2000), and in this study 22:6ω3 was highest in the deep sea habitat, cold water <10 ˚C, and second-order carnivores. Similarly, 20:4ω6, a recognized indicator for nearshore, coastal habitats, mangroves (Alfaro et al., 2006), and brown algae (Kelly and Scheibling, 2012), with a strong correlation with coastal linked $\delta^{13}C$ values across teleosts and crustaceans (Sardenne et al., 2017), was highest in the reef and brackish habitats within this study. Additionally, 20:5ω3, an indicator for cold water and demersal feeding across metabolically distinct organisms from teleosts (Pethybridge et al., 2015) to phytoplankton (Renaud et al., 2002), was high in non-complex demersal and deep sea demersal habitats, and was identified as a key tracer for temperature in particular. Despite the general alignment between our findings and previous studies, the use of individual FAs as biomarkers remains complex. As evidenced by the multiple origins for common FAs including 22:6ω3, 20:4ω6 and 20:5ω3 described above (and detailed in Appendix Table S2), FA production entangles many, often unresolved sources. This complexity is further confounded by environmental variables, secondary consumption and biological influences, including species-specific rates of bioconversion. These overlapping influences can make the use of individual FAs as biomarkers challenging and uncertain, and as such they should be interpreted with appropriate caution.

Furthermore, the consistency in tracer use is not ubiquitous across all FAs. For example, 18:1ω7, an indicator for temperature, depth, and bathypelagic environments (Pethybridge et al., 2011), was not highly correlated with any factors assessed within this analysis. Similarly, 22:5ω3, a reported tracer for coastal areas (Sargent et al., 1999), was not identified as an indicator of habitat in the present study. However 22:4ω6, a FA not commonly reported or used as a tracer in other studies, was substantially correlated with all four factors, and was particularly high in chondrichthyans from warm waters (>21 °C) and brackish and reef habitats. These tracers may reflect the unique physiology and metabolism of chondrichthyans (Ballantyne, 1997), and advocate for further taxa-specific investigations into biochemical pathways and FA metabolism.

Despite the model averaging approach revealing two phylogeny-dependant tracers (18:1ω9 and 20:1ω9), an understanding of diet input suggests ulterior drivers for these potential tracers. FAs 18:1ω9 and 20:1ω9 were measured in high levels within Squaliformes, Hexanchiforms and lamniformes, all of which are distant relatives (Chondrichthyan Tree of Life [www.sharksrays.org]). It is improbable that these particular orders independently biosynthesise or bioconvert these compounds in high quantities, as numerous studies showed direct assimilation of these MUFA from dietary sources (Iverson et al., 2004; Beckmann et al., 2013a; Beckmann, 2013b). As such, the high levels of 18:1ω9 and 20:1ω9 may stem from a common dietary source, such as blubber (McMeans et al., 2012; Waugh et al., 2014, Appendix Table S2). Given that within these three orders, the analysis included a number of species known to consume marine mammals (e.g. white shark *C. carcharias* [Huveneers et al., 2018], greenland shark *Somniosus microcephalus* [McMeans et al., 2012] and Portuguese dogfish *Centroscymnus coelopsis* [Pethybridge et al., 2011]), the influence of diet is likely the underlying cause. However, the role of MUFA biosynthesis may contribute to the levels of 18:1ω9 and 20:1ω9 in these chondrichtyhans, as 18:1ω9 is readily desaturated from 18:0 in seals (Budge et al., 2004). Such biochemical pathways warrant further research, both within and across taxa to better understand the intersection between physiology, phylogeny, and diet.

Quantifying the relative importance of multiple factors and highlighting the predominant driver does not negate the capacity for FAs to serve as tracers for multiple factors, as they are often inexorably linked. This is shown by the model averaging which revealed the inability to separate multiple factors (Appendix Table S7, Table 4.3). Given appropriate context and single factor hypotheses, some of these FA tracers are still usable indicators across multiple factors. For example, SFA 18:0, accounting for the influence of trophic guild and temperature, retained high values within the brackish, pelagic, and reef associated groups, showcasing that despite entangling multiple factors, 18:0 remains a usable tracer for habitat type (Appendix Figure S1 & S2). As such, FA tracers can effectively illuminate the trophic ecology of chondrichtyans across multiple factors of interest. This suggestion that a single tracer can inform multiple factors does not discourage the use of multiple tracers or full profiles, but simply highlights how the interpretation of FA drivers is context-specific and not limited to explaining a singular factor.

Challenges and future directions

Despite the growth in FA analysis, only 106 records from chondrichthyan muscle tissue were available, limiting the analysis. The records also highlight challenges in chondrichtyan ecology, whereby research focuses on coastal elasmobranchs, with a lack of data for pelagic species (n = 6) and uncommon taxa (Table 4.1). Additionally, the FAs were analysed in 18 different laboratories between 1984 and 2017, during which time advances in analytical techniques coupled with variations between labs may have influenced the resulting profiles. Unfortunately, a lack of data overlap prevented 'laboratory' from being included as factor in the analysis.

The confounding nature of some of the factors (e.g. the deep sea habitat and cold temperature; Appendix Table S3, S4 & S5) combined with limited available records highlights the need for controlled studies to isolate key factors (Pethybridge et al., 2018). Specifically, *ex-situ* studies should seek to determine the role of phylogeny in FA assimilation across multiple tissue types, including muscle, blood, and liver, owing to its unique function and associated physiology (Ballantyne, 1997). As explored by Hebert et al.,

(2006), the influence of trophic position necessitates further investigation to understand if the correlations between individual FAs and trophic guild are driven by successive and predicable biomodification (as with $\delta^{15}N$ [Munroe et al., 2018]) or high PUFAs in prey (i.e. Scombrid fish in Sardenne et al., 2017). Such studies assessing taxa-specific FA biomodification are warranted to enhance the quantitative capacity of FA profiling as has been achieved for other organisms (e.g. marine mammals in Iverson et al., [2004]). Additionally, the physiological effects of temperature (recommended for further exploration in Gladyshev et al. 2017) and energy expenditure (exercise in Li et al., 2016; swimming velocity in Gladyshev et al., 2017) need to be explicitly isolated from associated habitats and food webs. As with phylogeny, quantifying the influence of these factors across taxa necessitate investigating multiple tissue types, ideally paired with other tracers such as bulk $\delta^{15}N$ and $\delta^{13}C$ as well as amino acid and fatty acid specific isotopes.

Additional quantitative approaches adapted from well-understood biochemical tracers (e.g. the calculation of niche area using FAs in Every et al. 2017) should be further explored. The identification of temperature-specific FA tracers in this study advocate for the further development of 'FATscapes' (spatial contour maps of source FA tracers explored in Pethybridge et al. [2015] and temporal FATscapes with data acquired through long-term established monitoring programs) as they may be employed across taxa in the same manner as isoscapes (West et al. 2010). Following the ability of isoscapes to incorporate biochemical tracers into spatial and temporal models, and their subsequent use to discern complex movement and habitat use (e.g. Bird et al. 2018), the potential for FATscapes to elucidate fine-scale trophic geography across taxa warrants exploration. More broadly, these are particularly vital for regional-scale resource management, as a number of studies linking sea surface temperature and FA production (Budge et al., 2014; Roy, 2018) predict substantial declines in biologically essential PUFAs in response to climate change shifting primary producer communities, and subsequently, FA availability (Galloway and Winder 2015; Pethybridge et al., 2015; Hixson and Arts, 2016; Gladyshev et al., 2017).

Together, these future priorities require large spatial, temporal, and taxonomically diverse datasets, particularly for diet determination. Such work advocates for ensuring analytical consistency across labs, including cross-lab validations, the availability and use of reference material and the use of standard methods. Furthermore, such efforts favour the creation of a geographic and taxonomically inclusive FA data repository and working group, similar to the Chondrichtyan Stable Isotope Data Project (CSIDP, used in Bird et al. [2018]), the Database and Portal for Fish Stomach Records (DAPSTOM [Pinnegar 2014]) and IsoBank – a centralised repository for isotope data (Pauli et al., 2017).

Conclusion

This global analysis approach shows that FA profiles clearly distinguish different temperatures, trophic guilds, phylogenies, and habitats, further advocating the use of FA profiling as an emerging tool for trophic ecologists. The identification of new, generally applicable tracers (in particular 22:4ω6 to determine habitat associations) encourages further work assessing FA tracers across taxa and factors. Overall, this study provides a novel understanding of the biological and ecological information that can be inferred from FA profiles, and further validates the use of FAs as tracers to investigate the trophic ecology of marine consumers, including chondrichthyans. These large-scale analyses necessitate the creation of a taxonomically inclusive FA data repository to enable similar meta-analyses to further evolve the field.

Data accessibility

This analysis contains published data detailed in the Data sources and new data which has been archived and is available at DOI: 10.25957/5c871efe36f70.

Data sources

Beckmann, C. L., Mitchell, J. G., Stone, D. A. J., and Huveneers, C. (2013). A controlled feeding experiment investigating the effects of a dietary switch on muscle and liver fatty acid profiles in Port Jackson sharks *Heterodontus portusjacksoni*. *J. Exp. Mar. Bio. Ecol.* 448, 10–18. doi:10.1016/j.jembe.2013.06.009.

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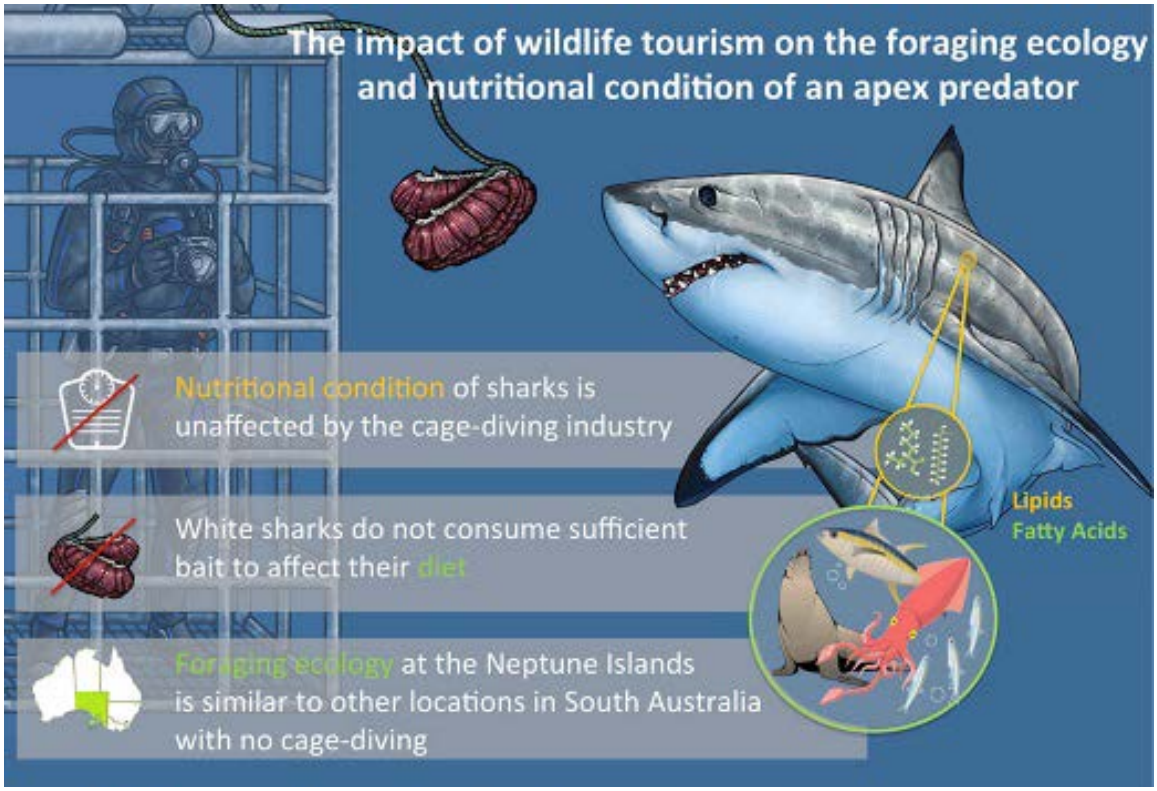
Chapter 5

Lipid analysis reveals little impact of wildlife tourism on the foraging ecology and nutritional condition of an apex predator



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Abstract

Shark and ray tourism is growing in popularity and often necessitates attractants like bait and chum to encourage close encounters. Such practices remain contentious amongst stakeholders as they may affect the species they target. We used lipid and fatty acid profiles to investigate the effects of South Australia's cage-diving industry on the diet and nutritional condition of white sharks *Carcharodon carcharias* (n = 75). We found no evidence of dietary shifts or reduced nutritional condition after a > 3 week period of tourism-exposed residency at the Neptune Islands where the cage-diving industry operates. White sharks fed on a variety of prey groups, similar to other populations around Southern Australia that are not exposed to ecotourism provisioning. These findings indicate that current cage-diving operations in South Australia do not alter white shark diet and nutritional condition where prey resources are abundant.

Introduction

Wildlife tourism is the fastest growing sector of the tourism industry (Wearing and Neil, 2009), bringing in billions of dollars globally (Wunder, 2000; Vianna et al., 2011; Huveneers et al., 2017) and with it, a myriad of management and conservation challenges (reviewed in Green and Giese, 2004; Newsome et al., 2005; Trave et al., 2017; Macdonald et al., 2017). Owing to their reputation as iconic predators, sharks are particularly popular ecotourism attractions (Gallagher and Hammerschlag, 2011; Apps et al., 2016). However their relative rarity encourages provisioning, whereby a range of attractants or direct feeding are used to coax sharks within view of tourists to ensure reliable and consistent encounters (Knight, 2009). Such practices are contentious, with polarized viewpoints from managers, tourism operators, and the public alike (Lewis and Newsome, 2003; Newsome and Rodger, 2008; Dubois and Fraser, 2013; Burgin and Hardiman, 2015; Richards et al., 2015; Ziegler et al., 2018).

Elasmobranch (shark and ray) provisioning for ecotourism encompasses numerous activities from directly feeding individuals to using noise attractants (defined in Richards et al., 2015). Such activities can elicit a range of effects on local ecosystems (Topelko and

Dearden, 2005; Shackley, 1998) and species, promoting discussion in an abundance of recent reviews (Brena et al., 2015; Gallagher et al., 2015; Trave et al., 2017; Patroni et al., 2018). Behavioural changes include shifts in site occupancy and seasonality (Bruce and Bradford, 2013; Brunnschweiler et al., 2014; Clarke et al., 2011; Rizzari et al., 2017), vertical and horizontal space use (Fitzpatrick et al., 2011; Corcoran et al., 2013; Huveneers et al., 2013), abundance (Bruce and Bradford, 2013; Clarke et al., 2013, Meyer et al., 2009), behaviour (Clua et al., 2010; Clarke et al., 2013), activity (Huveneers et al., 2018) and health and physiology (Semeniuk et al. 2007; Araujo et al., 2014; Barnett et al., 2016). However, the effects of provisioning on diet and nutritional condition are relatively unexplored. A single paper (Semeniuk et al., 2009) has detailed how provisioning negatively impacts the physiology and body condition of southern stingray *Dasyatis americana*. Changes in "dietary habits" due to provisioning was listed as the least studied of the ten ecological concepts reviewed by Brena et al., (2015), with published work on only two species noted: *D. americana* [Semeniuk et al., 2007] and Caribbean reef shark *Carcharhinus perezi* [Maljković and Côté, 2011]. Coupled with recent work on bull sharks *Carcharhinus leucas* (Abrantes et al., 2018), these three studies show differing results. The diets of *D. americana* from the Cayman Islands, and larger *C. perezi* in the Bahamas were shown to be effected by tourism provisioning (Semeniuk et al., 2007; Maljković and Côté, 2011). In contrast, there was no detectable change in the diet of *C. leucas* in Fiji (Abrantes et al., 2018). Such disparate findings advocate for context-specific studies.

Cage-diving with white sharks (*Carcharodon carcharias*) is particularly popular, due to their rarity, threatened conservation status, size, role as a top predator, and notoriety in popular media (Apps et al., 2016; Huveneers et al., 2017). White shark cage-diving occurs in Australia, Mexico, USA, South Africa, and New Zealand, often with multiple operators visiting one site simultaneously, sometimes offering multiple expeditions per day. The white-shark cage-diving industry began in the late 1970s in South Australia, where it uses tethered baits (southern bluefin tuna *Thunnus maccoyii* heads and gills), berley (minced tuna creating an inedible oil slick) and acoustics to attract sharks to the dive cages. Unlike other

elasmobranch provisioning sites (e.g. Stingray City in the Cayman Islands [Semeniuk et al., 2007] and the Bahamas [Maljković and Côté, 2011]), government regulations prohibit operators from intentionally feeding white sharks (DEWNR, 2016) thus mandating that baits are retracted prior to being consumed. However, sharks do occasionally consume the bait when operators cannot retrieve it quickly enough (Huveneers et al., 2015). This can result in the incidental consumption of a few baits, but new management regulations enacted in July 2017 (DEWNR, 2016) limit the amount of attractant operators can use, which have further reduced bait consumption (Huveneers and Lloyd, 2017). Although directly feeding sharks can alter elasmobranch's diet at wildlife tourism sites (Semeniuk et al., 2007; Maljković and Côté, 2011), the dietary effects of incidental bait consumption during cage-diving activities is currently unknown.

The time spent around cage-diving vessels changes fine-scale habitat use of white sharks (Huveneers et al., 2013) and may disrupt their natural foraging behaviour and their ability to feed on pinnipeds. Such effects have been documented in orcas (*Orcinus orca*), whereby whale watching vessels disrupted foraging activities, decreasing energy intake by 18% from lost feeding opportunities (Williams et al., 2006). Furthermore, these direct (bait consumption) and indirect (altered foraging) changes to diet may put increased pressure on shark's nutritional condition and fitness, as interacting with cage-diving increases the daily activity of white sharks (Huveneers et al., 2018). Such effects have been explored on whitetip reef sharks (*Triaenodon obesus*), whereby ecotourism activities increased energy expenditure and metabolic rate (Barnett et al., 2016), prompting inquiries about the extent and collective influence of similar effects on other species. Consumption of bait instead of natural prey can result in decreased foraging on pinnipeds with high energy yields , which could have detrimental effects on white sharks that can have high feeding requirements (Semmens et al., 2013). These concerns have been articulated in recent studies (Richards et al., 2015; Gallagher and Huveneers, 2018; Huveneers et al., 2018) and white shark cage-diving has been identified as a potential threat to the recovery of white sharks in Australia (DSEWPaC, 2013).

The use of lipids and fatty acids (FA) as dietary tracers in elasmobranchs is growing in popularity (Munroe et al., 2018; Semeniuk et al., 2007). Lipid content and the ratio of lipid classes (triacylglycerols, wax esters, phospholipids, sterols, free fatty acids) quantifies energy availability and nutritional state (Fraser, 1989; Orešič, 2009; Tocher, 2003). When energy demand exceeds intake, due to lack of 'fatty' prey items or increased activity, organisms mobilise fat stores, decreasing lipid content within tissues (Song et al., 2012) and changing the ratio of storage:structural lipid classes (Fraser, 1989; Zammit and Newsholme, 1979). Lipids can be further broken down into fatty acids (FAs), with distinct chemical structures retained from different basal food-chain production (e.g. bacteria, diatoms, dinoflagellates) (Ackman, 1994; Sargent et al., 1999; Tocher, 2003). As these compounds are passed from prey to predator with minimal modification, they can trace feeding ecology across different habitats with distinct food sources (Chapter 4). Furthermore, certain FAs are preferentially assimilated into distinct taxa-specific tissues (i.e. teleost muscle vs. marine mammal blubber vs. cephalopod mantel), providing additional insight into key prey items (Budge et al., 2006; McMeans et al., 2013; Pethybridge et al., 2013; Pethybridge et al., 2010). Fatty acids have been used in elasmobranch studies to investigate dietary shifts due to ontogeny (Belicka et al., 2012), spatial-temporal variability (Every et al., 2018; Steeves et al., 2016), and notably, provisioning during wildlife tourism operations (Semeniuk et al., 2007). As lipids are more metabolically active than bulk protein, they reflect changes in diet and nutrition at shorter time scales than stable isotopes (weeks *vs*. months-years [Beckmann et al., 2013]), making lipid and FA analysis an ideal toolset to explore changes in feeding ecology across a short time period (Pethybridge et al., 2018).

Here, we assessed the effects of South Australia's cage-diving industry on the foraging ecology of white sharks residing at the Neptune Islands. The integration period for lipids and fatty acids (Beckmann et al., 2013) allowed for the newly arrived sharks (< 3 weeks at the Neptune Islands) to serve as a control group for comparison with individuals exposed to the white shark cage-diving industry (> 3 weeks of tourism-exposed residency at the Neptune Islands). Specifically, we aim to investigate changes in 1) shark diet from incidental bait

consumption (using FA profiles and individual FAs as biomarkers for bluefin tuna consumption); and 2) altered foraging (FA profiles and markers for blubber consumption and habitat use); and 3) changes in nutritional condition (lipid content and lipid class profiles) from decreased or shifting foraging opportunities as sharks may be impacted by ongoing exposure to and interactions with provisioning-based cage-diving operations.

Methods

Sample collection

White shark muscle samples were collected from May 2012 to April 2017 at the Neptune Islands Group Marine Park, South Australia (including both North and South Neptune Islands), where free-swimming sharks were targeted opportunistically throughout the year during standard cage-diving operations. Sharks were attracted to the cage-diving vessels using a combination of attractants (bait and chum [mixture of minced bluefin tuna head, tails, gills and guts]) (DEWNR, 2016; Huveneers and Lloyd, 2017). Biopsies were taken from diving cages or from above the water's surface using a single 20 mm rubber speargun, with the end of the 1.3 m spear modified into a hollow 1 cm diameter stainless steel biopsy probe (Chapter 3), targeting the dorsal or upper flank musculature directly below the dorsal fin. Biopsies were immediately frozen (-4°C) and transported to the laboratory where white muscle tissue was dissected from the sub-dermal tissue and skin. Tissue samples were weighed and freeze dried prior to lipid extraction and analysis.

Individual sharks were identified (Nazimi et al., 2018), sexed (based on clasper presence/absence), and sized to the nearest 10 cm using visual size estimates (May et al., 2019). White sharks frequenting the Neptune Islands are identified daily by cage-diving operators, enabling to record the date each shark was first sighted, thus marking the start of their tourism-exposed residency period. All three dive operators contributed their data for this study. Telemetry was not appropriate to determine residency in this context as relatively few (n=7) biopsied sharks were tagged and tags might have not been deployed at the beginning of the period of tourist-exposed residency. The amount of interaction between sharks and operators or number of days sighted by cage-diving operators could not be reliably

quantified due to the logistical challenges of operators accurately recording this level of detail. We instead conservatively used residency at the Neptune Islands, defined as the period between first day sighted and day biopsied, acknowledging the limitation of using residency as a proxy for exposure to cage-diving operations. Where possible, sharks that had spent several weeks or more residing at the Neptune Islands, and those for which a biopsy was previously collected, were preferentially targeted. Additionally, biochemical data from eight white sharks caught at other locations throughout South Australia were also obtained (Pethybridge et al., 2014). These were included in the control group and considered not to have recently visited the Neptune Islands. *Residency* was grouped into two categories (< 3 weeks [control] and > 3 weeks [tourism-exposed] at the Neptune Islands) as shifts in FA profiles were noted within 3 weeks of a diet switch in captive Port Jackson sharks *Heterodontus portjacksoni* (Beckmann et al., 2013).

Biochemical analysis

Total lipid was extracted from freeze dried muscle samples (minimum 12 mg dry weight [DW]) using the modified Bligh and Dyer method (Bligh and Dyer, 1959; described in detail in Chapter 1). Briefly, the lipids were separated from proteins and carbohydrates using a solvent solution of dichloromethane, methanol, MilliQ water. The total lipid extract (TLE) was then dried under nitrogen and weighed prior to lipid class and FA analysis. Lipid classes [phospholipid (PL), triacylglycerol (TAG), sterols (ST), wax esters (WE) and free fatty acids (FFA)] were determined from an aliquot of the TLE using thin layer chromatography coupled with a flame ionisation detector (TLC-FID). Lipid class results were expressed as a relative proportion (percent area) of the total lipid class compounds.

Individual FAs were separated from the glycerol backbones of the polar and non-polar lipids in the TLE (not individual lipid classes) with a heated methanol, hexane, and hydrochloric acid solvent scheme. Subsequently, the FAs were identified and quantified using gas chromatography analysis using the Agilent Technologies 6890N GC (Palo Alto, California, USA) with a HP-5 cross-linked methyl silicone fused silica capillary column (50 x 0.32 mm i.d.), an FID, a splitless injector and an Agilent Technologies 7683 Series auto-

sampler. Quality checks, including the addition of internal FA standard (C23 in each sample), blank samples (each batch of 50), replicates (weekly) and gas chromatography - mas spectrophotometry checks on FAs (twice throughout the analysis) were run to ensure accurate results and appropriate laboratory protocols. FA results were expressed as a proportion of the total identified compounds. Out of the 61 fatty acids identified, only those with means >0.1% (24) were included in the subsequent statistical analyses.

Statistical analysis

We tested the influence of tourism-exposed residency (*residency* hereafter) at the Neptune Islands on white shark muscle lipid content, lipid class, and FA profiles using multivariate statistical analyses undertaken in PRIMER7 +PERMANOVA (Plymouth Routines in Multivariate Ecological Research, Clarke and Gorley, 2015). Permutational analysis of variance (PERMANOVA) main tests with Monte Carlo simulations (denoted as p(MC)) were run on Bray-Curtis similarity matrices calculated from the square-root transformed profile data to determine if *residency* significantly influenced the overall lipid content, lipid class, and FA profiles. The lipid and FA profiles of the eight sharks sampled outside of the Neptune Islands were compared (using PERMANOVAs) to the control sharks (< 3 weeks at the Neptune Isalnds). Following non-significant (lipid content p(MC)=0.847, lipid class p(MC)=0.617, FA p(MC) = 0.712) differences, these two groups were combined. PERMANOVA models testing for differences between the control (<3 weeks and sharks from outside the Neptune Islands) and tourism-exposed sharks (>3 weeks at the Neptune Islands) included sampling *season* to account for temporal variation in prey availability and FA production (Steeves et al., 2016) and *size* (total length) as a continuous covariate to account for ontogenetic diet shifts (Hussey et al., 2012b). Additionally, permutational analysis of multidimensional dispersion (PERMDISP denoted at p(perm)) was used to determine the relative amount and statistical significance of the dispersion within *residency* groups. The influence of *residency* (accounting for sampling *season* and shark *size)* was also investigated for select individual FAs (reflecting either marine mammal or teleost consumption, or pelagic foraging, Table 5.2) using Generalized Linear Mixed Effect Models

(GLMMs) fitted with gamma distribution and log link using the *glm* function and restricted maximum likelihood approach in the R statistical environment (R Core Team, 2016). Significance for all statistical tests was declared at p(MC) or p(perm) < 0.05.

As the 3-week threshold determined by Beckmann et al., (2013) used captive Port Jackson sharks, it is uncertain whether this threshold is directly applicable to white sharks in a natural setting. Furthermore, Port Jackson sharks were not sampled prior to 3 weeks, so the turnover rate may in fact be quicker. As such, all PERMANOVA and GLMM analyses were repeated with *residency* groups < 1, 1–2, 2–3, and < 3 weeks; and < 2 weeks (control) and < 2 weeks (tourism-exposed); and CAPs were run on these categorical *residency* groups along with the CAPs of *residency* (days) as a continuous factor as reported below. Similarly, all GLMMs were run with *residency* as a continuous (days) or categorical (grouped by week, and 2 week threshold as above). None of the alternative groupings altered our findings and results from the < 3 week and < 3 week *residency* groupings are presented (Figure 5.1). To visualise and quantify shifts in lipid class and FA profiles across *residency*, a Canonical Analysis of Principal Coordinates (CAP) (Anderson and Willis, 2003) was run against *residency* (in days) as a continuous covariate.

Results

Seventy-five white sharks (26 females, 46 males and 3 unknown) ranging 1.8–5.5 m total length (mean ± standard deviation: 3.5 ± 0.7 m) were sampled in South Australia, 67 of which were biopsied at the Neptune Islands and eight sampled as bycatch from various locations in South Australia. Most (34%) were sampled in spring, followed by autumn (27%), summer (23%), and least in winter (16%). These sharks were sighted and identified by the cage-diving operators at the Neptune Islands from 0 to 62 days prior to sampling (5.0 ± 12.2 days), the majority (n = 61) of which had spent less than one week interacting with the cage-diving vessels. Of those that remained at the Neptune Islands for more than a week, five sharks were sampled between 1–2 weeks of arriving, two between 2–3 weeks, and the remaining eight sharks were sampled after more than three weeks of interacting with the cage-diving vessels. Three sharks (two females; S-66, S-63, and one male; S-72) were

sampled twice throughout their residency (in any one sampling year). S-63, a 4.1 m female was initially biopsied 14 days after being first sighted, and again after 62 days (the longest period of time after which a shark was sampled). S-66 (4.7 m) was biopsied after three days and 56 days later, while S-72 (3.8 m) was sampled on the first day he was sighted and 35 days later.

Fatty Acids

The FA profiles (composed of 21 FAs, not grouped into PUFA, SFA, and MUFAs, Table 5.1) showed no discernible shift with *residency* (CAP p = 0.639, p(MC) = 0.834, Figure 5.1A & S4A); accounting for sampling *season* (p(MC) = 0.06), and shark *size* p(MC) = 0.082). There was also no change in FA profile dispersion between the two *residency* categories (control vs. tourism-exposed PERMDISP p = 0.356, Figure 5.1A and S4A). Similarly, none of the three FA groups or seven individual FAs indicative of bluefin tuna (bait) consumption (16:0, 18:0, 22:6ω3, PUFAs [Nichols et al., 1998; Meyer unpublished data]), blubber consumption (18:1ω9, 20:1ω9, 20:4ω6, MUFAs [Bradshaw et al., 2003; Budge et al., 2006; Waugh et al., 2014]) or pelagic foraging (16:0, 22:6w3, PUFAs [Gladyshev et al., 2017; Parrish et al., 2014; Pethybridge et al., 2010; Chapter 4]) were influenced by *residency* (Table 5.2). The three repeat sampled individual sharks had variable changes in FA profiles, as S-66 and S-63's profiles shifted from positive to negative along the Y axis (CAP1), while S-72 shifted in the opposing direction. Individual indicator FAs and FA groups (PUFAs, MUFAs, and SFAs) also showed no change in relation to *residency* (Table 5.2). Similarly, individual FAs indicative of pelagic foraging, blubber, or bluefin tuna (bait) consumption shifted inconsistently between the three resampled individuals (Table 5.3), further suggesting a lack of industry-induced shifts in foraging, diet, and habitat use.

Table 5.1 - Total lipid content (n = 65) and relative proportions of lipid classes (n = 27) and fatty acids (n = 78) (as mean precent ± standard deviation of total lipid or FA) of muscle from white shark *Carcharodon carcharias*

Lipid content	28.0±7.4
Lipid class	
TAG	1.01±2.45
FFA	2.49±6.89
ST	6.79±3.11
PL	89.63±7.93
Fatty Acid	
14:0	0.48±0.35
16:0	17.96±4.54
17:0	0.53±0.23
18:0	14.38±6.64
22:0	0.16±0.25
∑SFA	33.53±7.23
16:1ω7	1.44±1.27
17:1ω8^	0.59±0.27
18:1ω9	18.67±5.16
20:1ω9	1.44±0.61
20:1ω7	0.19±0.12
22:1ω9	0.37±0.27
22:1ω7	0.18±0.23
24:1ω9	1.01±1.70
∑MUFA	23.89±6.62
18:4ω3	0.20±0.24
18:2ω6	0.31±0.30
20:4ω6	10.75±3.11
20:5ω3	1.09±1.01
20:3ω6	0.24±0.41
20:4ω3	0.15±0.10
20:2ω6	0.21±0.10
22:5ω6	0.92±0.37
22:6ω3	16.88±7.84
22:4ω6	3.53±1.50
22:5ω3	2.37±0.97
∑PUFA	36.66±12.78

TAG - triacylglycerols; FFA – free fatty acids; ST – sterols; PL – phospholipids; SFA - saturated fatty acids; MUFA - monounsaturated fatty acids; PUFA - polyunsaturated fatty acids. ^ coellute with a17:0.

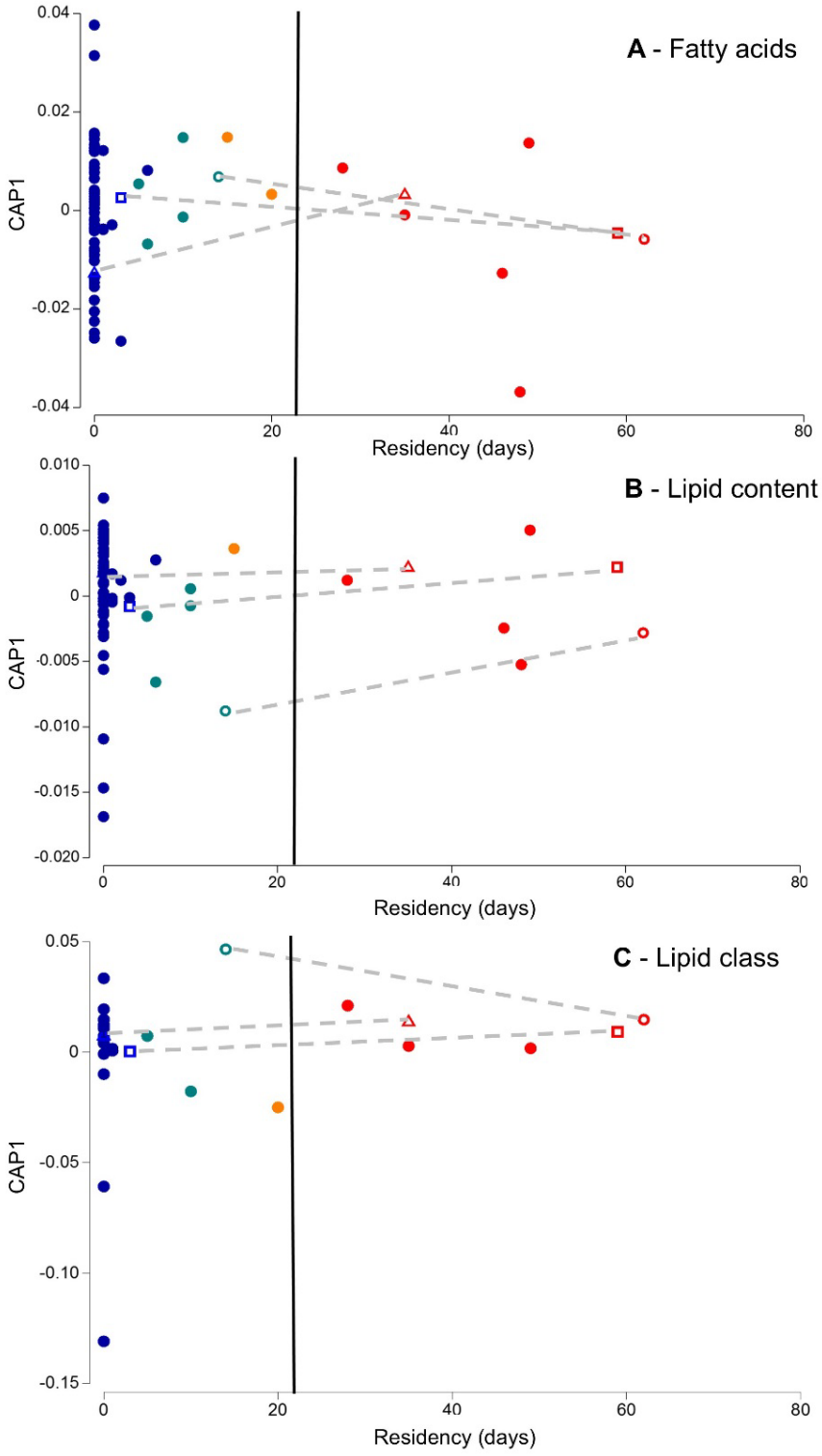

Figure 5.1 - Canonical Analysis of Principal Coordinates (CAP) of white shark *Carcharodon carcharias* muscle **A** – Fatty acid profile, **B** – Lipid content, and **C** – Lipid class profile plotted against residency (days) at the Neptune Islands. Dark blue symbols indicate individual sharks which have spent <1 week at the Neptune Islands, green 1–2 weeks, orange 2–3 weeks and red >3 weeks. The black vertical line demarcates the 3 week biochemical integration period for lipids and fatty acids (Beckmann et al., 2013a), such that data on the left represents control sharks and data on the right, tourism-exposed sharks. Open circles indicate results from S-63, open squares from S-66, and open triangles from S-72. The dashed grey line shows the magnitude and direction of shift between two samples taken from three individual sharks.

Table 5.2 – The influence of *residency* (<3 weeks [control] vs. <3 weeks [tourism-exposed]) at the Neptune Islands, sampling *season,* and shark *size* on individual fatty acids and fatty acid groups, determined by linear mixed effect models fitted with a gamma distribution and log link. **Bold** indicates statistical significance determined as $p < 0.05$.

Fatty acid		Effect	Standard error	t-value	p-value	Diet indicator
16:0	**Intercept**	**3.00**	**0.15**	**19.64**	**<0.001**	Mesopelagic fish[1]
	Residency	0.14	0.08	1.72	0.09	Pelagic foraging[2]
	Spring	0.03	0.07	0.49	0.62	Tuna[8]
	Summer	0.11	0.08	1.48	0.14	Bait[9]
	Winter	-0.02	0.08	-0.25	0.80	
	Size	-0.02	0.04	-0.62	0.54	
18:0	**Intercept**	**2.46**	**0.33**	**7.46**	**<0.001**	Reef foraging[2]
	Residency	-0.05	0.18	-0.27	0.79	Tuna[8]
	Spring	-0.16	0.14	-1.11	0.27	
	Summer	-0.31	0.16	-1.90	0.06	
	Winter	-0.32	0.18	-1.81	0.08	
	Size	0.12	0.09	1.42	0.16	
18:1ω9	**Intercept**	**2.63**	**0.19**	**13.70**	**<0.001**	Blubber consumption[3]
	Residency	0.02	0.11	0.20	0.84	
	Spring	0.04	0.08	0.49	0.63	
	Summer	0.04	0.09	0.39	0.70	
	Winter	-0.07	0.10	-0.73	0.47	
	Size	0.10	0.05	1.97	0.05	
20:1ω9	Intercept	0.33	0.30	1.08	0.28	Blubber consumption[4]
	Residency	0.07	0.17	0.42	0.68	
	Spring	-0.05	0.13	-0.42	0.68	
	Summer	-0.17	0.15	-1.12	0.27	
	Winter	-0.32	0.16	-1.97	0.05	
	Size	0.05	0.08	0.60	0.55	
20:4ω6	**Intercept**	**2.60**	**0.20**	**13.30**	**<0.001**	Reef foraging[2]
	Residency	-0.01	0.11	-0.11	0.92	Blubber consumption[3]
	Spring	-0.02	0.08	-0.28	0.78	
	Summer	-0.09	0.10	-0.89	0.38	
	Winter	0.18	0.10	1.70	0.10	
	Size	-0.05	0.05	-0.95	0.35	
20:5ω3	Intercept	0.22	0.42	0.52	0.61	Demersal foraging[2]
	Residency	-0.15	0.23	-0.64	0.52	
	Spring	0.10	0.18	0.52	0.60	
	Summer	0.13	0.21	0.63	0.53	
	Winter	0.34	0.22	1.52	0.13	
	Size	-0.08	0.11	-0.72	0.46	
22:6ω3	**Intercept**	**3.44**	**0.32**	**10.91**	**<0.001**	Offshore migrations[5]
	Residency	-0.06	0.17	-0.34	0.73	Deep sea foraging[2]
	Spring	0.01	0.14	0.05	0.96	Tuna[8]
	Summer	0.04	0.16	0.29	0.78	
	Winter	0.24	0.17	0.43	0.16	
	Size	**-0.18**	**0.08**	**-2.16**	**0.03**	
SFA	**Intercept**	**3.46**	**0.15**	**23.47**	**<0.001**	Preferentially metabolised during migrations[7]
	Residency	0.07	0.08	0.82	0.41	
	Spring	-0.08	0.06	-1.32	0.19	
	Summer	-0.09	0.07	-1.24	0.22	
	Winter	-0.15	0.08	-1.92	0.06	
	Size	0.03	0.04	0.89	0.38	
MUFA	**Intercept**	**2.82**	**0.19**	**14.91**	**< 0.001**	Preferentially metabolised during migrations[7]
	Residency	0.01	0.10	0.14	0.89	
	Spring	0.03	0.08	0.37	0.71	Blubber consumption[3,4]
	Summer	0.07	0.09	0.77	0.44	
	Winter	-0.09	0.10	-0.93	0.36	
	Size	0.10	0.05	1.96	0.05	
PUFA	**Intercept**	**3.97**	**0.25**	**16.36**	**<0.001**	Preferentially retained during migrations[7]
	Residency	-0.03	0.13	-0.20	0.84	Tuna[8]
	Spring	-0.01	0.11	-0.09	0.93	
	Summer	-0.02	0.12	-0.14	0.89	
	Winter	0.19	0.13	1.47	0.15	
	Size	-0.11	0.06	-1.80	0.08	

[1]Pethybridge et al., 2010, [2]Chapter 4, [3]Waugh et al., 2014, [4]Bradshaw et al., 2003, [5]Colombo et al., 2016, [6]Alfaro et al., 2006, [7]Osako et al., 2006, [8]Nichols et al., 1998, [9]Meyer et al., unpublished data, [10]Gladyshev et al., 2017

Lipid content

White shark muscle lipid content was highly variable, ranging from 12.5 to 50.1 mg/g dry muscle (28.0 ± 7.4) (Figure 5.1B) and was not influenced by *residency* (CAP p = 0.452, PERMANOVA p(MC) = 0.895, Figure 5.1B), accounting for *season* (p(MC)= 0.756) and *size* (p(MC) = 0.744). All three resampled sharks increased in lipid content between sampling (Table 5.3, Figure 5.1B).

Lipid class

White shark muscle was dominated by phospholipids (89.63 ±7.93), with little relative contribution from sterols, free fatty acids, or triacylglycerols (Table 5.1). *Residency,* accounting for *season* (p(MC)=0.575), and *size* (p(MC)=0.644) had no effect on the lipid class profiles (CAP p =0.731, p(MC) = 0.573, Figure 5.1C & S4B). The three resampled individuals did not show any trends in lipid class throughout *residency,* as minimal and inconsistent shifts were detected in TAG and FFA (Table 5.3). ST and PL showed greater shifts across *residency* (difference > 7% each), however these changes were similarly inconsistent (Table 5.3).

Table 5.3 – Mean relative (%) changes in muscle lipid content and lipid class components for three resampled White sharks at the Neptune Islands.

Shark ID	Days within residency individuals were biopsied	Lipid content	Lipid class (change in % of total profile)		Fatty acids (change in % of total profile)	
S-63	14–63	+ 55%	TAG	- 0.03	16:0	+ 0.42
			FFA	+ 0.11	18:1ω9	+ 0.23
			ST	+ 5.23	20:4ω6	+ 0.50
			PL	- 5.33	22:6ω3	+ 0.31
					PUFA	+ 5.62
S-66	3–59	+ 25%	TAG	- 0.21	16:0	+ 0.09
			FFA	- 0.60	18:1ω9	+ 0.27
			ST	+ 4.40	20:4ω6	- 0.10
			PL	- 3.11	22:6w3	- 0.66
					PUFA	- 7.48
S-72	0–34	+ 3%	TAG	+ 0.02	16:0	- 0.57
			FFA	- 0.05	18:1ω9	- 0.77
			ST	- 7.35	20:4ω6	+ 0.76
			PL	+ 7.21	22:6ω3	+ 1.23
					PUFA	+ 16.8

Discussion

Shark- and ray-based tourism is growing in popularity worldwide (Gallagher and Hammerschlag, 2011), but provisioning remains contentious amongst scientists, managers, and tourists (Burgin and Hardiman, 2015; Newsome and Rodger, 2008). Using lipid content, lipid class, and FA profiles, we found no evidence of nutritional or dietary shifts as sharks reside around cage-diving operators at the Neptune Islands Group Marine Park. Many of the biochemical markers were highly variable among individuals, but showed no consistent increase or decrease with tourism-exposed residency. The lack of shift in FAs indicative of marine mammal or tuna consumption, or pelagic foraging suggest that white sharks have a similar diet at the Neptune Islands than in other areas, foraging on a variety of prey and not solely on pinnipeds.

The lack of dietary shifts towards a bluefin tuna (bait) based diet may be attributed to industry management strategies (DEWNR, 2016), prohibiting intentional feeding sharks and limiting the amount of bait that can be used by operators. The small number of baits consumed by sharks were not sufficient to elicit a measurable shift in overall diet or increase in tuna markers FAs 16:0, 18:0, 22:6ω3 and ∑PUFAs. Unlike findings from directly provisioned stingrays in the Cayman Islands (Semeniuk et al., 2007) and reef sharks in the Bahamas (Maljković and Côté, 2011), we found no shift in diet at the community or individual level using comparable biochemical approaches, similar to work from bull sharks in Fiji (Abrantes et al., 2018). Furthermore, our sampling strategy (detailed in Chapter 3) inherently targeted the boldest individuals that came within a few meters of the cages, and interacted with the industry most regularly, as they provided us with greater opportunity to obtain a biopsy. Our sampling was, therefore, well-suited to detect changes in bold individuals, if the effects of the industry was limited to bold sharks, as observed in reef sharks (Maljković and Côté, 2011) and noted at other white shark cage-diving sites, e.g. South Africa (Johnson and Kock, 2006; Laroche et al., 2007). However, as no changes were detected, even in a shark that visited the Neptune Islands over a period of 63 days, the use of bait at the Neptune Islands, does not appear to measurably effect the sharks' diet.

The provisioning attracts a number of animals, including birds, teleosts and other chondrichthyans, some of which are potential white shark prey items (Hussey et al., 2012; Malcolm et al., 2001; Pethybridge et al., 2014) (e.g. yellowtail kingfish *Seriola lalandi*, bronze whalers *Carcharhinus brachyurus*, and rays). However, the shark's unaltered diet negates concerns that large groups of teleosts, encouraged by presence of bait and chum, create additional feeding opportunities around the cage-diving operators. For example, a switch from pinnipeds to teleosts would manifest altered FA profiles, and be particularly apparent with increased teleost indicators (FA 22:6ω3) and decreased marine mammal indicators (i.e. 18:1ω9, 20:1ω9, 20:4ω6), which was not seen here. Additionally, dive operators and scientists have yet to witness attempted predation on any of the species attracted by the bait and chum, despite close proximity and apparent ease of capture (pers. com. A. Fox and A. Wright). This combination of observation and dietary biomarkers negates the hypotheses that provisioning creates additional or unnatural foraging opportunities for white sharks around cage-diving operations.

Despite the lack of direct provisioning, a number of studies have found that interacting with the cage-diving industry elicits changes in white shark swimming behaviour (Laroche et al., 2007; Bruce and Bradford 2013; Huveneers et al., 2013) and increases daily activity (Huveneers et al., 2018), prompting concerns about the indirect effects on white shark nutrition. Lipid content and lipid class profiles (revealing nutritional condition), however, remained unchanged with residency, suggesting no detectable effect on nutrition, despite increased activity from interacting with cage-diving vessels and in light of the species' notoriously high feeding requirements (Semmems et al; 2013). As white sharks are highly mobile, high-energy ambush predators, the increase in daily activity associated with interacting with the industry may not be costly enough to deplete the lipid stores of these naturally active sharks. Instead, all three resampled sharks showed an increase in lipid content through residency (+3%, +25% and +55%), despite the group comparison (Lipid content PERMANVOAs comparing control and tourism-exposed sharks, n = 65) showing no difference. This disparity in results could be a reflection of the high variability in lipid content

(mean ± SD 28.0±7.4 mg/g), which may be masking an underlying increase not detectable in the grouped analysis of 65 individuals. Such an increase in lipid content corroborates that white sharks at the Neptune Islands forage on locally abundant prey items, such as energy rich pinnipeds (Figure 5.2A) and teleosts (including tunas), and are unperturbed by exposure to the cage-diving industry. Alternatively, the increase in lipid content in three individuals is a product of chance in a small sample size, and lipid content is unchanged with residency. This still supports that cage-diving does not negatively affect the nutritional condition of white sharks through extended exposure to ecotourism. However, as we were unable to quantify the level of interaction with dive operators, instead using residency at the Neptune Islands as a proxy, further investigations comparing lipid content, lipid class, and other markers with clearly quantified levels of interaction with the industry warrants investigation and may reveal different results.

Figure 5.2 – White sharks *Carcharodon carcharias* at the Neptune Islands, South Australia. A – White shark with pinniped entrails trailing from the mouth. B – Shark with fresh wounds under the bottom jaw, presumably from longnosed furseal *Arctocephalus forsteria* teeth. Photographs by Andrew Fox.

As white sharks linger around cage-diving sites, with increased local residency (Bruce and Bradford 2013) and altered fine-scale swimming patterns (Huveneers et al., 2013), the need to investigate industry-induced disruptions to natural foraging patterns have been highlighted (Dubois and Fraser, 2013; Gallagher and Huveneers, 2018). As the FA profiles and levels of individual FA tracers were not detectably different, it indicates that the diet of white sharks at the Neptune Islands includes prey in similar proportions to other regions frequented by white sharks prior to visiting the Neptune Islands. Specifically, the unchanged proportions of marine mammal indicators (FAs 20:5ω3, 18:1ω9, 20:1ω9, 20:4ω6 and 22:5ω3) highlight that despite the cage-diving industry operating at the Neptune Islands, sharks are consuming pinnipeds in similar quantities as elsewhere. This is corroborated by the frequent observation of sharks with protruding stomachs (Figure 5.2A), presumably from pinniped consumption, and fresh wounds from predation attempts on pinnipeds (Figure 5.2B, A. Fox and A. Wright pers. comm.), highlighting that they remain a key food source for sharks around the Neptune Islands. In South Africa, cage-diving operations elicited changes in white shark swimming behaviour (Laroche et al., 2006), similar to those documented in South Australia (Bruce and Bradford 2013), yet predation pressure on the seals remained unaffected (Laroche, 2006; Laroche et al., 2007). This was attributed to relatively few sharks showing interest in the cage-diving vessels, while the majority continue to forage unaffected. The effects of South Australia industry may be similar and limited to a few individuals, with most sharks being transient (Nazimi et al., 2018) and having short interactions with operators.

These findings provide the first insights into the nutritional effects of white shark cage-diving, a need highlighted in scientific literature (Gallagher and Huveneers, 2018; Huveneers et al., 2018) and in management strategies (DEWNR 2012). Australia's white shark recovery plan (DSEWPaC, 2013) and the Neptune Islands Marine Park management plan (DEWNR, 2012) specifically mention the importance of investigating the impacts of wildlife tourism, as regional managers need to balance ecology, protected species conservation, industry, economics, and the ecosystem functionality and conservation

capacity of the Neptune Islands as a marine park. The lack of dietary effects from tourism operations indicate that current management strategies are adequately protecting the nutritional health of the industry's focal species, a key factor in Dubous and Fraser (2013) framework for assessing wildlife provisioning acceptability. This helps ensure the long-term sustainability of white shark-cage diving, while contributing towards a socially acceptable license for the industry to operate.

Furthermore, as the diet and nutrition of white sharks at the Neptune Islands does not differ from elsewhere in southern Australia, this marine park is likely one of many regionally-important foraging grounds. Hypotheses that white sharks aggregate around this marine park solely to predate upon pinnipeds may overestimate the significance of this group of long-nosed fur seals (*Arctocephalus forsteri*), understating the value of other pinniped-rich foraging grounds, which warrant investigation (DSEWPaC, 2013; objective 7 – identify and protect critical white shark habitat, with an emphasis on key foraging areas). Additionally, the dietary importance of pinnipeds may be overstated, driven by the relative ease of observing breaching predation attempts (Hammerschlag et al., 2006; Martin et al., 2005) and that most known white shark aggregations are in the vicinity of pinniped colonies, despite the abundance of cetaceans and teleosts in white shark gut content (Hussey et al., 2012b). Understanding the relative importance of different prey items, in the context of key foraging grounds, requires further research extending outside cage-diving locations. Such insight informs species-specific and regional management strategies, ensuring the protection of one of Australia's most iconic marine species.

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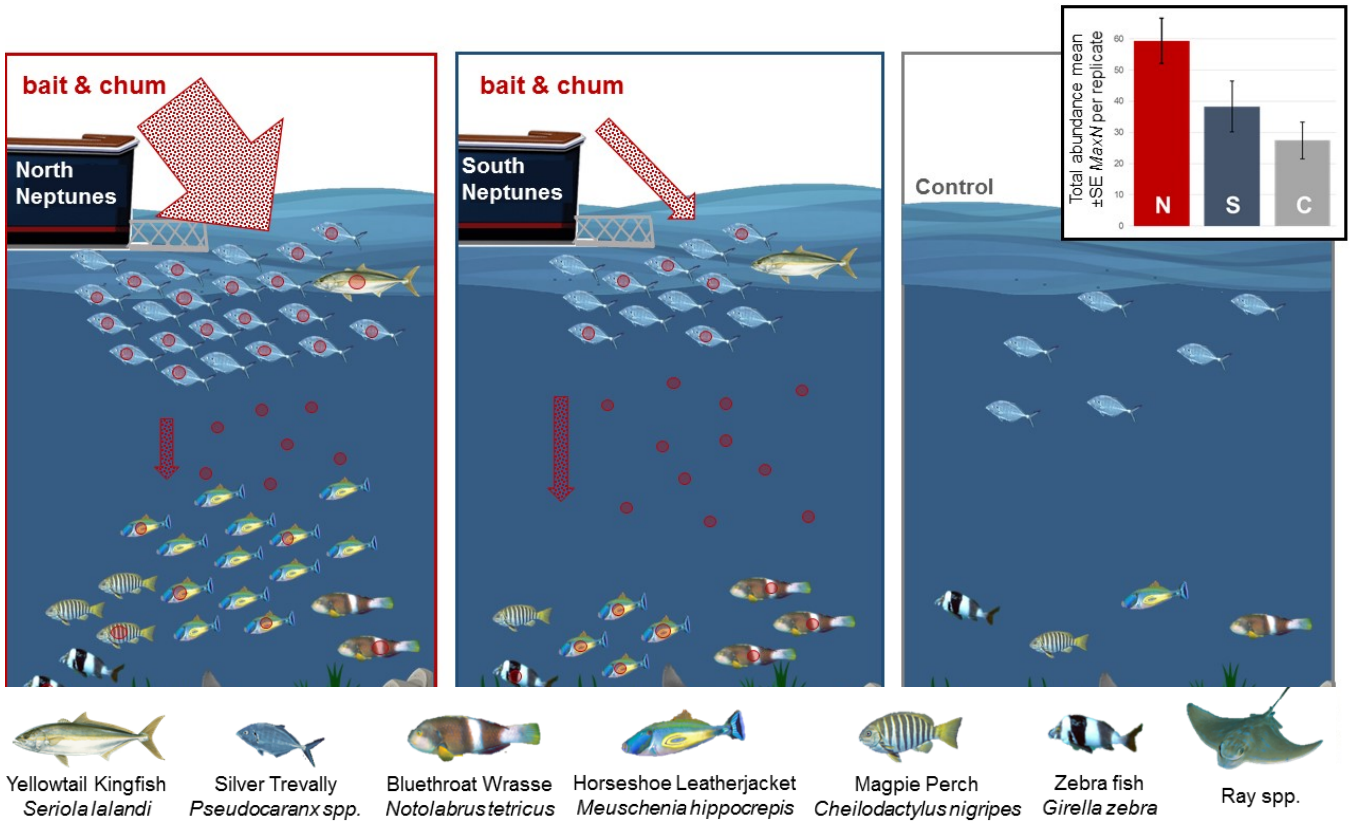
Chapter 6

Unintentional provisioning: the effects of white shark cage-diving bait and chum on non-target species



Graphical abstract highlighting the main findings of the chapter. See Figure 6.6.

Abstract

Marine wildlife tourism is growing in popularity, with corresponding growth in studies examining its impacts. Yet, these studies focus nearly exclusively on the industry's effects on target species, overlooking a myriad of non-target organisms that may also be affected. Here, I assessed the effects of bait and chum input from the white shark cage-diving industry on the diet of eight non-target species from different functional groups (pelagic fishes, reef fishes, and rays). These effects were investigated across two sites with different intensity of wildlife tourism (cage-diving operators go to North Neptune Islands ~8.5 times more often than South Neptune Islands), and compared to a control site with no wildlife tourism. Stomach content, fatty acid profiles, and nitrogen stable isotope values revealed site-specific diets for all eight species, consistent with the consumption of bait and chum at both cage-diving sites. However, these dietary shifts were incongruent with the extent of bait and chum input between North and South Neptune Islands. Pelagic fishes had a stepwise increase in bait and chum consumption mirroring input from the cage-diving industry, yet reef fish showed the opposite, with generally higher consumption at South Neptunes compared to North Neptunes, highlighting the complexity of the effect of provisioning on non-target species. This may be attributed to differences in consumer abundance, as North Neptunes is home to 1.6 times the number of individual consumers compared to South Neptunes, spreading the resource subsidy across more individuals. Furthermore, silver trevally *Pseudocaranx* spp. dominated North Neptune Islands and may consume most of the bait and chum at the surface, preventing it from reaching benthic species and therefore moderating the impact of the cage-diving industry at this site. Results detailing which non-target species diets are impacted by the cage-diving industry enables the application of a management framework to assess the acceptability of wildlife feeding at the Neptune Islands. Following these findings, an ecosystem-approach inclusive of non-target species is recommended to ensure appropriate management of wildlife tourism and associated provisioning.

Introduction

Wildlife tourism, particularly activities targeting large and charismatic animals, is rapidly growing in popularity, diversity of locations, and target species (Newsome et al., 2005; Orams, 2002; Trave et al., 2017). However, the relative scarcity of charismatic marine megafauna often requires “provisioning” (defined in Richards et al., 2015), whereby an attractant, typically food-related, is used to aggregate target species and ensure consistent, up-close encounters for tourists (Knight, 2009). Such activities are common practice (Bryant, 1994; Duffus and Dearden, 1990; Knight, 2009) and have been used to attract marine mammals (Mann and Kemps, 2003), sharks (Bruce, 2015), rays (Semeniuk et al., 2007), and fish (Brookhouse et al., 2014; Feitosa et al., 2012) for decades. Such practices can elicit a multitude of effects on target species (reviewed in Brena et al., 2015; Hammerschlag et al., 2012; Patroni et al., 2018; Trave et al., 2017).

Of 44 papers assessing the effects of wildlife provisioning reviewed in Trave et al., (2017), only three examined non-target species or ecosystem-wide impacts (Milazzo et al., 2006; Turner and Ruhl, 2007; Vignon et al., 2010). None of these three studies investigated diet, despite observations of non-target species consuming bait and chum across a number of tourism sites, including dives with sharks in the Bahamas and white shark cage-diving worldwide (Gallagher and Huveneers, 2018). Examples from the terrestrial environment highlight the extent to which non-target species can be unintentionally provisioned, as they consume the majority (up to 98% [Inslerman et al., 2006]) of feed intended for game wildlife (Donalty et al., 2003) with substantial effects, sometimes mirroring the effects on target species (i.e. increase in abundance [Donalty et al., 2003; Feitosa et al., 2012; Selva et al., 2014] and others reviewed in Milner et al., 2014). Following such examples, the need for more inclusive research assessing the effects of resource subsidies (provisioning in this case) on non-target species has been highlighted in recent reviews (Gallagher et al., 2015; Gallagher and Huveneers, 2018) and management frameworks (Higginbottom et al., 2003).

Cage-diving with white sharks (*Carcharodon carcharias*) is particularly popular (Huveneers et al., 2017), with tours available in Australia, Mexico, USA, South Africa, and New Zealand, often with multiple operators visiting one site simultaneously. As with most shark and ray tourism, cage-diving at the Neptune Islands Group Marine Park in South Australia relies on chum (minced Southern bluefin tuna *Thunnus maccoyii* creating an inedible oil slick) and tethered baits (*T. maccoyii* heads and gills) to attract sharks within view of the divers. Citing concerns about the effects of provisioning practices on *C. carcharias* (reviewed in Bruce, 2015; discussed in Gallagher and Huveneers, 2018; Chapter 5), the South Australian government has limited licences to two operators using provisioning and one using sound. Conditions include: 10 operation days a fortnight (totalling 260 days a year), 85% of which occur at North Neptune Islands (chronic bait and chum input) *vs.* 15% at South Neptunes (pulse bait and chum input) (C. Huveneers, unpublished data), a maximum of 100 kg of bait and chum input per day and operator (DEWNR, 2016), and the prohibition of shark feeding (policy 7.3, DEWNR, 2016). The marine park also provides protection for unique offshore island habitats (DEWNR, 2012), supporting > 130 recorded fish, marine mammal, bird, and elasmobranch species (Atlas of Living Australia, 2019). While bait consumption by sharks is prohibited and monitored, no regulations manage how much bait and chum (also referred to as provisions) are consumed by fish, which are regularly observed feeding on bait and chum (Figure 6.1). Despite evidence that provisioning does not alter the diet or nutrition of the target *C. carcharias* at the Neptunes (Chapter 5), the dietary effects of cage-diving on non-target species remains unquantified.

Figure 6.1 – Aerial view of silver trevally *Pseudocaranx spp.* consuming a tethered bait (southern bluefin tuna, gills and guts) from the stern of a White Shark cage-diving boat at North Neptune Islands, South Australia.

A variety of methods have been used to determine the diet of wild animals, including stomach content analysis and biochemical tracers. Stomach content analysis has been popular since the 1960s (Baker et al., 2014; Cortés, 1997; Hynes, 1950), providing a direct snapshot of recent meals. However, empty stomachs, relative absence of easily digestible prey, and the presence of unidentifiable items has given rise to more time-integrated dietary biomarker methods such as fatty acid (FA) and stable isotope (SI) analyses (Hussey et al., 2012; Munroe et al., 2018; Pethybridge et al., 2018; Chapter 4). Both biomarker approaches use the same underlying premises: different basal food-chain production creates distinct biochemical signatures, which are then passed up the food-web with minimal or predictable modification. As such, these approaches are used to assess consumer habitat use, diet, and trophic level across a range of taxa (e.g. Gladyshev et al., 2017; Hussey et al., 2012; Chapter 4). These three trophic indicators (stomach content analysis, FAs, and SIs) can be used together to assess changes over time. Stomach content reveals the most recent feeding events, while FAs and SIs require the biochemical signatures to be incorporated from prey into the lipids or proteins respectively. Lipids are more metabolically active than bulk protein, and therefore reflect changes in diet more rapidly (weeks *vs* months for lipids and protein, respectively) (Beckmann et al., 2013; Buchheister and Latour, 2010). Collectively, this makes these three trophic indicators ideal to assess immediate, recent, and long-term changes (Pethybridge et al., 2018; Tocher, 2003).

Secondary to altering diets, provisioning has the potential to shape species assemblages. Baited Remote Underwater Video Stations (BRUVS) are becoming a popular non-extractive method to quantitatively compare fish assemblages (reviewed in Whitmarsh et al., 2017). Their use investigating anthropogenic stressors, including fishing or aquaculture (e.g. Tanner and Williams, 2015; Whitmarsh, 2018) and urbanisation (Vargas‐Fonseca et al., 2016), to name a few, makes them an ideal tool to gain an understanding of relative fish abundance between sites affected by tourism provisioning. BRUVS can be modified to assess both benthic and pelagic fish assemblages (Clarke et al., 2019); they are non-extractive, and do not require researchers to enter the water. Thus, they are well-suited

to the Neptune Islands, as a marine protected area with distinct benthic and pelagic fish assemblages, which is also home to Australia's largest aggregation of adult *C. carcharias*, preventing other traditional monitoring methods (e.g. netting, long-lining, diver fish-surveying, manta tows).

Here, I assessed the effects of provisioning from South Australia's white shark cage-diving industry on non-target species using stomach content analysis, biochemical tracers, and BRUVS. Specifically, I investigated: 1) which non-target species consume provisions, and if this is linked to functional groups (pelagic fishes, reef fishes, rays); 2) how effect varies with intensity of bait and chum input, given a difference in magnitude at two nearby cage-diving sites (North Neptune Islands is visited approximately 8.5 times more often than South Neptune Islands), compared to control locations without wildlife tourism; 3) how the relative abundance of fishes and rays influences the effects of periodic (South Neptune Islands) *vs.* chronic (North Neptune Islands) bait and chum input. These results were then used to assess the acceptability of provisioning non-target species at the Neptune Islands, using a management framework developed by Dubois and Fraser (2013).

Methods

Sampling sites and collection

Sample collection to assess bait and chum consumption occurred at the Neptune Islands Group (Ron and Valerie Taylor) Marine Park, 70 km south of Port Lincoln South Australia. The park includes two sets of offshore island groups, North Neptune Islands 35.2342**°** S, 136.0656**°** E (herein referred to as North Neptunes), and 11 km away, South Neptune Islands S 35.3375° S, E 136.1199° E (herein referred to as South Neptunes). North Neptunes is the primary cage-diving location, due to the comparative proximity to Port Lincoln and the availability of sheltered anchoring locations in variable weather conditions. As such, the cage-diving operators spend ~85% of the 260 days a year at North Neptunes, with the remaining ~15% at South Neptunes (C. Huveneers, unpublished data). Control samples were collected from several Islands with similar habitat and exposure throughout South Australia (Dangerous Reef, 34.8156° S, 136.2125° E which is a sanctuary 'no-take'

zone, and Liguanea Island 34.9892° S, 136.6220° E, Buffalo Reef 34.7240° S, 136.4664° E, and Kangaroo Island 35.5847° S, 137.666° E, which are open to fishing). Dangerous Reef and Liguanea Island were also cage-diving sites in the 1980s, but were closed to the industry in 2002 (Robbins et al., 2015).

Table 6.1 – Sampling regime for each species by functional group (blue – pelagic fishes, green – reef fishes, brown – rays). Trophic analyses are abbreviated as FA (fatty acid), SI (stable isotope), and SC (stomach content). The samples size indicates the total number of samples collected across all locations, with N (North Neptunes), S (South Neptunes), and C (Control) indicating the number of samples collected at that site.

	Species	Sample size (N, S, C)	Control location(s)	Collection date	Analyses
Pelagic fishes	Yellowtail kingfish *Seriola lalandi*	11 (4, 5, 3)	Gulf Saint Vincent*	Apr. 2016	FA, SI
	Silver trevally *Pseudocaranx spp.*	104 (38, 32, 34)	Buffalo Reef* Langton Island*	Apr 2016	SC, FA, SI
Reef fishes	Bluethroat wrasse *Notolabrus tetricus*	49 (16, 13, 20)	Liguanea Island Dangerous Reef	Jan – Feb 2016	SC, FA, SI
	Horseshoe leatherjacket *Meuschenia hippocrepis*	21 (5, 5, 11)	Liguanea Island Dangerous Reef	Jan – Feb 2016	SC, FA, SI
	Magpie perch *Cheilodactylus nigripes*	16 (5, 6, 5)	Dangerous Reef	Feb 2016	SC, FA, SI
	Zebra fish *Girella zebra*	15 (8, 1, 6)	Dangerous Reef	Feb 2016	SC, FA, SI
Rays	Eagle ray *Myliobatis tenuicaudatus*	10 (2, 3, 5)	Gulf Saint Vincent*	Feb – May 2017	FA, SI
	Smooth ray *Bathytoshia brevicaudata*	7 (3, 4, 0)	NA	Apr 2016 – May 2017	FA, SI

Between October 2016 and June 2018, six fish species (yellowtail kingfish *Seriola lalandi*, silver trevally *Pseudocaranx spp*., bluethroat wrasse *Notolabrus tetricus*, horseshoe leatherjacket *Meuschenia hippocrepis*, magpie perch *Cheilodactylus nigripes,* zebrafish *Girella zebra*) and two ray species (eagle ray *Myliobatis tenuicaudatus* and smooth ray *Bathytoshia brevicaudata*) were sampled at North Neptunes, South Neptunes, and at least one control site (Table 6.1). These species were classified as either pelagic fishes, reef fishes, or rays according to their predominant foraging habitat (Gomon et al., 2008) or taxonomy. Fish were caught from research and cage-diving vessels using rod and reel, handlines, and a speargun while cage-diving. Whole fish were measured and dissected on-board the vessels, with muscle samples and stomachs collected for biochemical and

stomach content analysis respectively. Free-swimming rays were biopsied at North Neptunes and South Neptunes using a speargun (detailed in Chapter 3), while control samples were collected at a fish market from rays caught within the Gulf of St Vincent, SA (160–210 km from the Neptune Islands). *Seriola lalandi* muscle samples were donated from a recreational fishing charter operating around Buffalo Reef (Table 6.1). All samples for each fish species were collected within a one month period to avoid confounding possible seasonal changes in diet or FA profiles (Pethybridge et al., 2015). All tissue was immediately frozen (-4˚C) in the field and maintained at -20 ˚C during transportation. At the laboratory, all frozen muscle samples were further dissected (discarding the outer layer to avoid any potential contamination from the field dissection), and a sub-sample freeze-dried prior to biochemical analysis.

Stomach content analysis

Prey remains were identified to the lowest taxonomical level. Most prey items were either crushed or heavily digested making prey identification difficult and leading to prey remains being grouped into functional level (teleost remains [including bait and chum], squid, crustacean, echinoderm, shell, algae, and sponge material), and weighed to the nearest 0.001 g. I attempted to dissociate ingested bait and chum *vs*. other teleost remains, but could not do so with sufficient confidence and therefore combined them into teleost remains.

Fatty acid analysis

Fatty acids were extracted from freeze-dried muscle samples (minimum 12 mg dry weight [DW]) using the modified Bligh and Dyer method (Bligh and Dyer, 1959); described in detail in Chapter 2. Briefly, the lipids were separated from proteins and carbohydrates using a solvent solution of dichloromethane, methanol, MilliQ water. Individual FAs were separated from the glycerol backbones of the polar and non-polar lipids in the total lipid extract with a heated methanol, dichloromethane, and hydrochloric acid treatment. Subsequently, the FAs were extracted, and then analysed including identification and quantification by gas chromatography using an Agilent Technologies 6890N GC (Palo Alto, California, USA) fitted with a HP-5 cross-linked methyl silicone fused silica capillary column (50 x 0.32 mm i.d.), an

FID, a split/splitless injector operated in splitless mode and an Agilent Technologies 7683 Series auto-sampler. FA results were expressed as a proportion (percent of total FA) of the total identified compounds. Out of the 61 fatty acids identified, only those with means >0.1% (28) were included in the subsequent statistical analyses.

Stable isotope analysis

As lipid removal is recommended prior to isotopic analysis (Sotiropoulos et al., 2004; Sweeting et al., 2006), I used the same sample from which the lipids were removed for FA analysis (detailed above). Specifically, the freeze-dried sample was taken out of the dichloromethane, methanol, MilliQ water solvent mixture and dried in an oven (40 ˚C for 48 hours) to remove excess solvent prior to isotopic analysis. Dried samples were then ground and weighed (~ 0.5 mg) into tin cups for analysis. Samples were analysed using a Carlo Erba NA1500 CNS analyser interfaced via a Conflo II to a Finnigan Mat Delta S isotope ratio mass spectrometer, operating in the continuous flow mode. Combustion and oxidation occurred at 1,090 ˚C and reduction at 650˚C. Results are presented and analysed in δ notation:$\delta = \frac{R_{sample}}{R_{standard}} - 1$ where R = $^{15}N/^{14}N$. Of the SI results, only $\delta^{15}N$ data was assessed in the subsequent analysis, as it reflects trophic position (Peterson & Fry, 1987) and thus best reveals feeding on bait and chum with comparatively high δ15N levels (L. Meyer, unpublished data). The $\delta^{13}C$ SI values were excluded as they primarily reflect habitat use (Peterson and Fry, 1987), which is less suited to determine changes in trophic level associated with an altered diet.

Baited Remote Underwater Video Stations (BRUVS)

Fish assemblages were observed and quantified at North Neptunes and South Neptunes and Dangerous Reef (control site) using BRUVS as detailed in Whitmarsh (2018). Briefly, benthic (*n* = 12) and pelagic (*n* = 6, deployed approximately 5 m below the surface) BRUVS were deployed at each of the three locations (North Neptunes, South Neptunes, and Dangerous Reef serving as a control site) over a 7-day period in January 2017, to quantify fish assemblages on the sea floor and within the water column respectively (Table S8). Benthic BRUVS consisted of a GoPro Hero 3+ Silver edition camera fixed within a metal

frame with a bait arm. Pelagic BRUVS consisted of a wooden board with the GoPro Hero 3+ Silver camera and bait arm set below the surface and anchored to the seafloor with weights to prevent the BRUVS from drifting (Clarke et al., 2019). Cameras recorded in 1080p at 60 fps with a wide field of view. All mesh bait bags were filled with 500 g of minced sardines, BRUVS were deployed for 60 min and all stations were spaced ≥ 250 m apart (as per Clarke et al., 2019; Whitmarsh et al., 2017). Videos were analysed using SeaGIS *EventMeasure* software (SeaGIS Pty. Ltd., Bacchus Marsh, Victoria, Australia; www.seagis.com.au/event.html), where fish and rays were identified to the species or genus level (Gomon et al., 2008) and counted using *MaxN* as the relative abundance measure. *MaxN* is the maximum number of individual fish (for each species or taxon) observed in a single frame throughout the duration of the deployment. The abundance of only the eight species of interest, for which I collected specimens for stomach content and biochemical analysis, were compared between locations (see statistical analysis section). To understand how the diet of individual species was impacted by overall fish abundance, I pooled all 62 identified teleosts and chondrichthyans *MaxN* values into a single total abundance measure per-replicate, to be compared between sites.

Statistical analysis

Multivariate and univariate statistical analyses in PRIMER7 +PERMANOVA (Plymouth Routines in Multivariate Ecological Research [Clarke and Gorley, 2015]) were used to assess if and how the quantity of teleost remains within fish stomach content, FA profiles, δ15N values, and species and total abundance differed with location as a fixed factor with three levels (North Neptunes, South Neptunes, and control sites). Fatty acid profiles were assessed using multivariate analysis. PERMutational ANalysis Of VAriance (PERMANOVA) with Monte Carlo simulations (denoted as p(MC)) were run on Bray-Curtis similarity matrices calculated from the square-root transformed profile data to determine if locations were significantly distinct ($p(MC) < 0.05$). Following significant PERMANOVAs, SIMilarity PERcentage analysis (SIMPER) was used to determine which individual FAs were driving pairwise dissimilarities. A Canonical analysis of principal coordinates (CAP) (Anderson and

Willis, 2003) was calculated from the Bray-Curtis similarity matrix of the FA profiles of each individual species with the FA profiles of the bait and chum. This was used to visualise the difference in a species' FA profiles between locations, with specific FAs with CAP correlations >0.70 overlaid on the ordination plots to visualise which FAs were driving the differences between locations.

The quantity of teleost remains within fish stomach content, $\delta^{15}N$ values, and species abundances were assessed as univariate data using the same PERMANOVA design as described above, with location as the single fixed factor containing three levels. Euclidean distance was used instead of Bray-Curtis similarity for these PERMANOVAs, as it is better suited to univariate data and the large numbers of true 0s (not a lack of data) for the species abundance.

Results

Pelagic fishes

The diet of pelagic fishes varied between locations, with distinct FA profiles occurring between North Neptunes compared to South Neptunes and the control site (Table 6.2, Figure 6.2A & B). Both fishes were characterised by high relative levels of FAs 18:1ω9, 20:5ω3 and low levels of 22:6ω3 and 20:4ω6 at North Neptunes, followed by intermediate levels of these FAs in the *Pseudocaranx spp*. from South Neptunes relative to Control (Table 6.2, Figure 6.2A & B). Bait and chum FA profiles were also rich in 18:1ω9 and relatively low in 22:6ω3 and 20:4ω6, consistent with the tuna aquaculture industry (fed pilchards high in 18:1ω9 and low in 22:6ω3 and 20:4ω6), which supplies the tuna-derived bait and chum for the cage-diving operators. *Pseudocaranx spp*. stomachs contained more than double the amount of teleosts remains at the cage-diving locations compared to the control site (Figure 6.3), while stomach content was not available for *S. lalandi. Pseudocaranx spp*. had comparatively enriched δ15N at North Neptunes, followed by South Neptunes, both of which were higher than the control site (Figure 6.3), while *S. lalandi* $\delta^{15}N$ was indistinguishable across locations (Figure 6.3).

Table 6.2 – PERMANOVA with Monte Carlo Simulation p(MC) results for non-target species comparing locations. Habitat types denoted by colour (blue – pelagic, green – reef, brown – rays). SIMPER results for fatty acid (FA) profile data reporting the top 3 FAs driving significantly different locations.

Species	Teleost remains	p(MC)	FA profile p(MC)		SIMPER drivers	Stable isotope $\delta^{15}N$ p(MC)	
Seriola lalandi			**North – South**	**0.037**	18:1ω9, 22:6ω3, 20:5ω3	North – South	NS
	NA		**North - Control**	**0.039**	22:6ω3, 18:1ω9, 18:1ω7	North - Control	NS
			South - Control	0.666		South - Control	NS
Pseudocaranx spp.	North – South	0.399	**North – South**	**0.025**	22:6ω3, 18:1ω9, 16:0	**North – South**	**0.001**
	North - Control	**0.028**	**North - Control**	**0.002**	22:6ω3, 18:1ω9, 20:4ω6	**North - Control**	**0.001**
	South - Control	0.004	**South - Control**	**0.022**	22:6ω3, 18:1ω9, 20:4ω6	**South - Control**	**0.003**
Notolabrus tetricus	**North – South**	**0.029**	**North – South**	**0.045**	20:4ω6, 22:6ω3, 18:1ω9	North – South	0.181
	North - Control	0.522	**North - Control**	**0.008**	20:4ω6, 22:6ω3, 18:1ω9	**North - Control**	**0.001**
	South - Control	0.189	**South - Control**	**0.003**	22:6ω3, 20:4ω6, 22:5ω6	**South - Control**	**0.001**
Meuschenia hippocrepis	**North – South**	**0.031**	**North – South**	**0.036**	22:6ω3, 18:1ω9, 18:1ω7	**North – South**	**0.04**
	North - Control	0.386	North - Control	0.492		**North - Control**	**0.033**
	South - Control	**0.001**	**South - Control**	**0.028**	18.1ω9, 22:6ω3, 20:4ω6	**South - Control**	**0.002**
Cheilodactylus nigripes	**North – South**	**0.004**	North – South	NS		North – South	0.09
	North - Control	0.931	North - Control	NS		**North - Control**	**0.009**
	South - Control	**0.001**	South - Control	NS		**South - Control**	**0.001**
Girella zebra	North – South	NA	North – South	NA		North – South	0.502
	North - Control	0.708	**North - Control**	**0.001**	22:6ω3, 20:5ω3, 22:5ω3	**North - Control**	**0.001**
	South - Control	NA	South - Control	NA		South - Control	NA
Rays			North – South	NS		North – South	0.714
	NA		North - Control	NS		**North - Control**	**0.014**
			South - Control	NS		**South - Control**	**0.037**

Bold indicates significant differences (p(MC) <0.05). NS indicates the p(MC) main test was > 0.05, and NA indicates no samples were available for the given analysis.

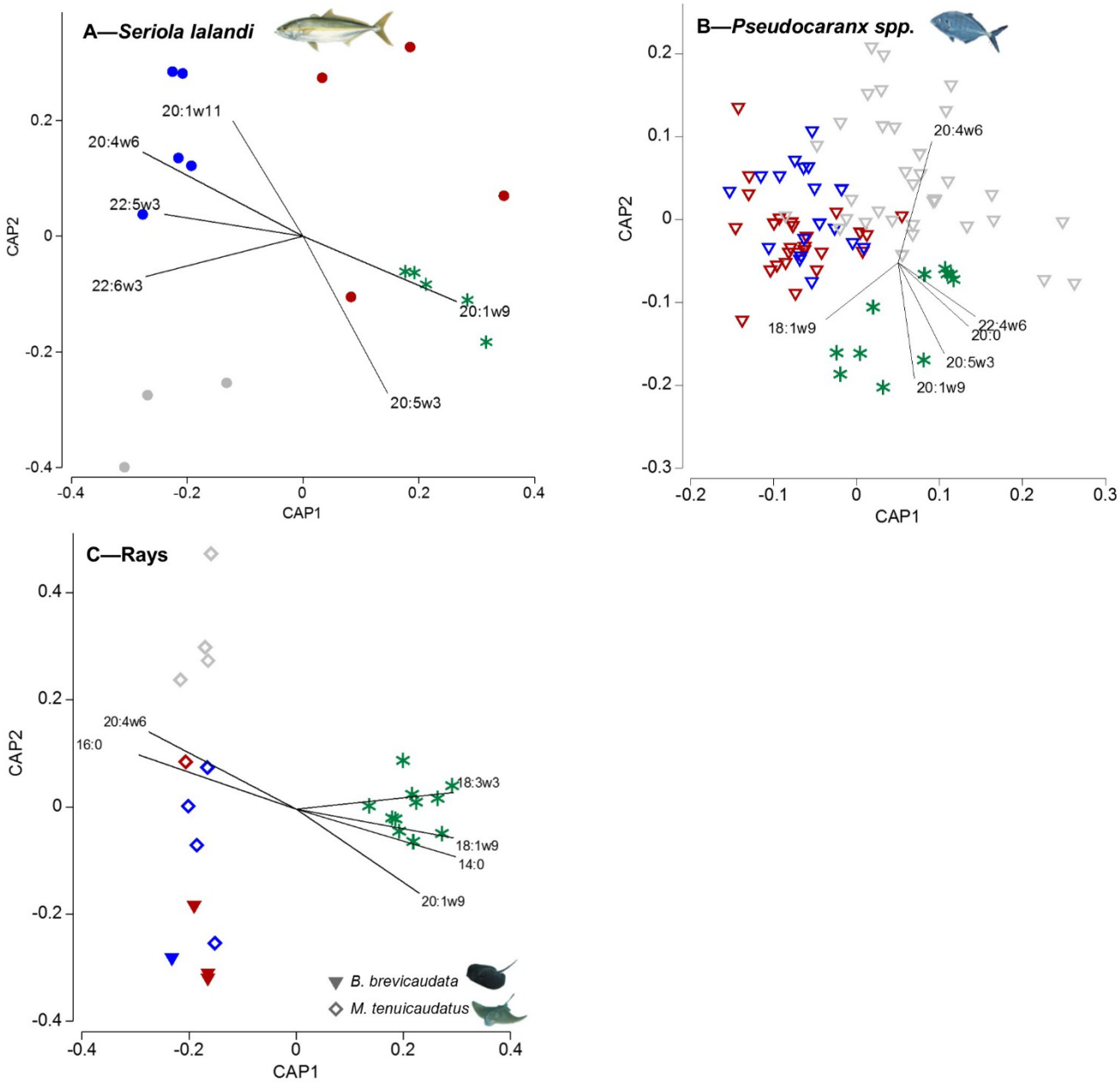


Figure 6.2 - Canonical Analysis of Principal Coordinates (CAP) of fatty acid profiles from individual fishes and rays with overlaid vectors of individual fatty acids with CAP correlation values >0.70. Across all species, red represents North Neptunes, blue South Neptunes, and light grey control sites. Green stars are bait and chum.

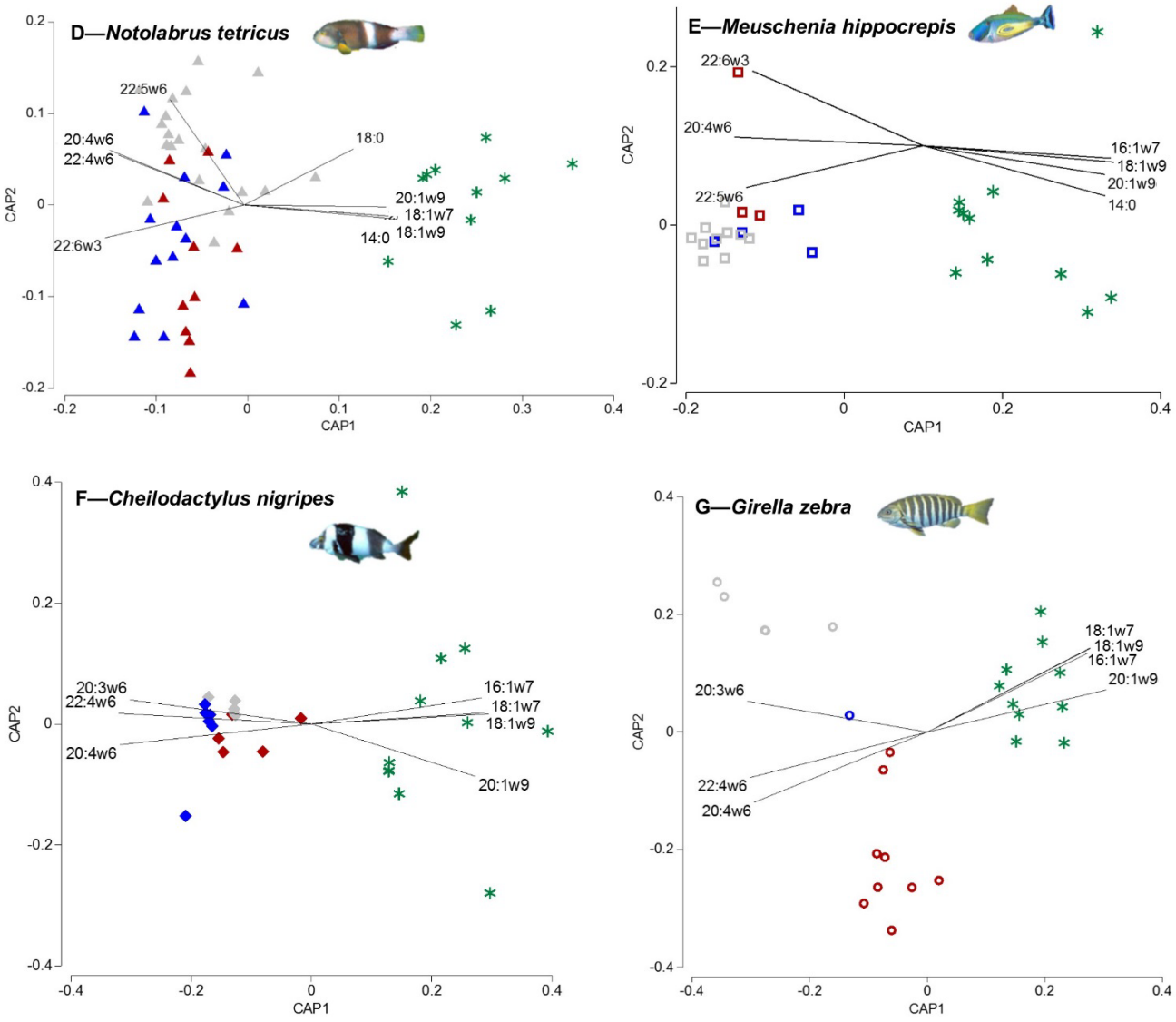


Figure 6.2 continued - Canonical Analysis of Principal Coordinates (CAP) of fatty acid profiles from individual fishes and rays with overlaid vectors of individual fatty acids with CAP correlation values >0.70. Across all species, red represents North Neptunes, blue South Neptunes, and light grey control sites. Green stars are bait and chum.

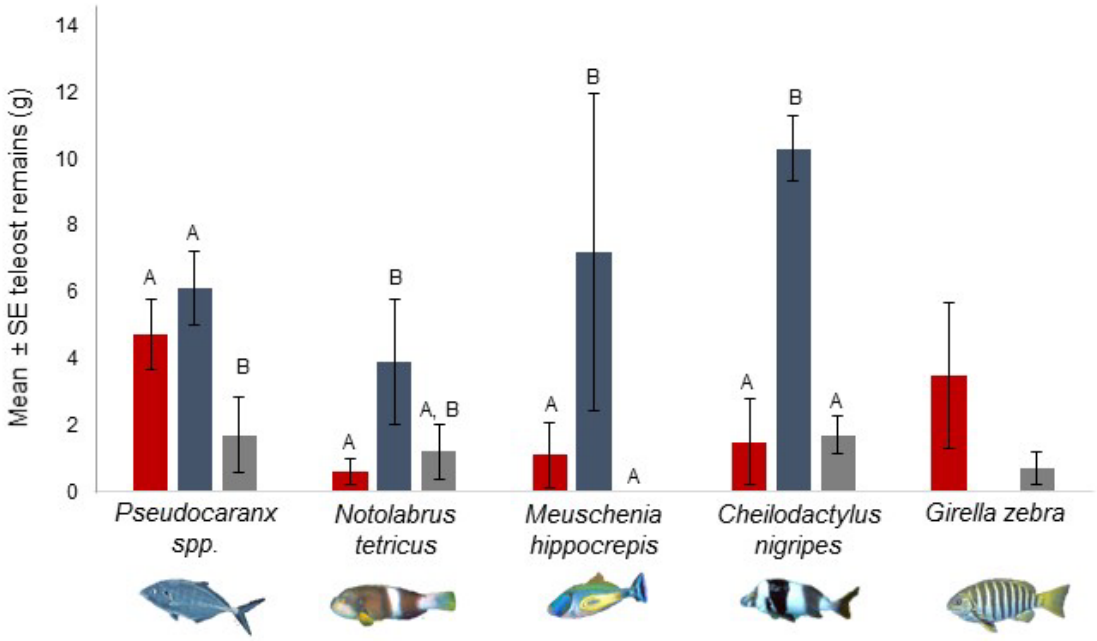


Figure 6.3 - Mean ±SE teleost remains (g) from fish stomach content at each location (red = North Neptune, blue = South Neptune, grey = control sites). Letters indicate a significant difference (p(MC)<0.05) between locations for that species.

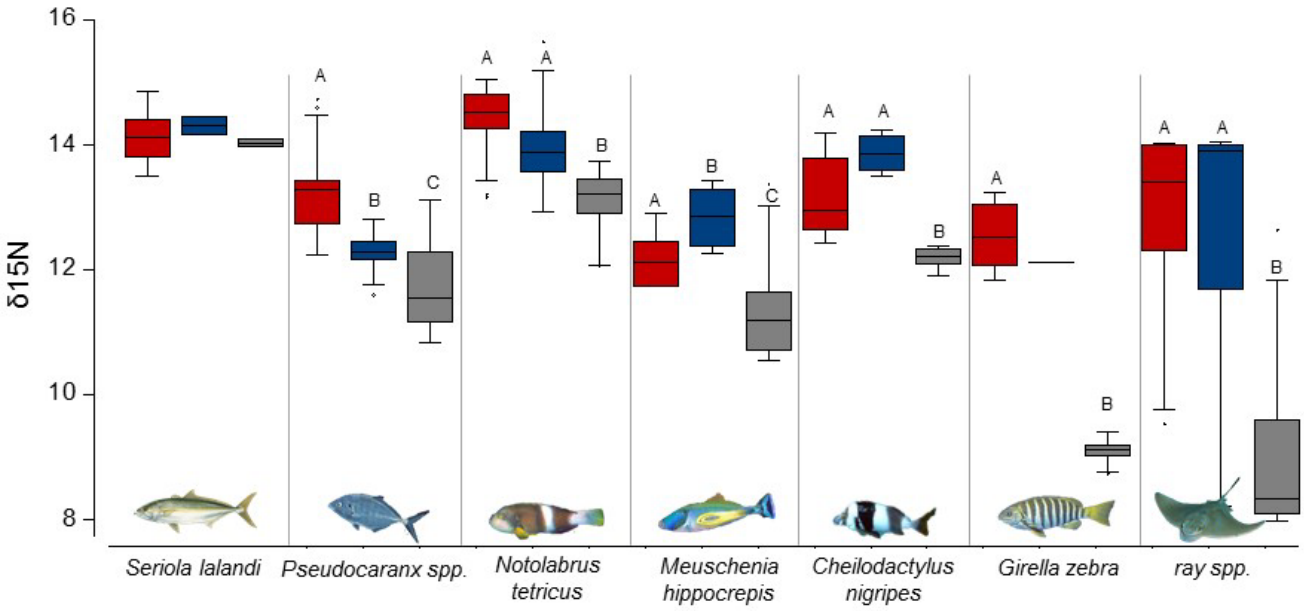


Figure 6.4 - Mean ±SE $\delta^{15}N$ for each species by location (red = North Neptune, blue = South Neptune, and grey = control sites). Letters indicate a significant difference (p(MC)<0.05) between locations for that species.

Reef fishes

Reef fish diet was most distinct at South Neptunes compared to control sites, with less pronounced differences at North Neptunes (Table 6.2, Figure 6.2, 6.3 & 6.4). *Meuschenia hippocrepis* from South Neptunes had the highest $\delta^{15}N$ values (Figure 6.4), FA profiles characterised by relatively high levels of 18:1ω9 and low levels of 22:6ω3 and 20:4ω6, consistent with the tuna-derived bait and chum profiles (Table 6.2, Figure 6.2E) and contained >6.5 times the amount of teleost remains in their stomachs compared to North Neptunes and Control (Figure 6.3). Similarly, *N. tetricus* FA profiles were location-specific, driven by the bait and chum indicator FAs (Figure 6.2D). Stomach content analysis showed *N. tetricus*, *M. hippocrepis*, and *C. nigripes* all contained >4.5 times as much teleost remains at South Neptunes compared to North Neptunes and the control site (Figure 6.3). *Notolabrus tetricus*, *C. nigripes*, and *G. zebra* all had enriched δ15N at both cage-diving sites compared to the control site (Figure 6.4).

Girella zebra, which were largely absent at South Neptunes (*n* = 1), had distinct FA profiles at North Neptunes compared to the control site (Figure 6.2G). According to SIMPER, the location specific differences were driven by high 22:6ω3, low 20:5ω3 and 22:5ω3, the opposing trends as found in *Pseudocaranx spp.*, *S. lalandi*, *N. tetricus*, and *M. hippocrepis*. However, the CAP highlights that location specific differences are separated along CAP2, while CAP1 placed North Neptunes closer than the control site to the bait and chum profiles (Figure 6.2G). *Girella zebra* had enriched $\delta^{15}N$ at both cage-diving sites compared to the control site (Figure 6.4), while the amount of teleost remains was not significantly different between North Neptunes and the control site (Figure 6.3).

Rays

Rays had enriched $\delta^{15}N$ at both cage-diving sites compared to the control site (Figure 6.4), but no location-specific differences in FA profiles (Table 6.2, Figure 6.2C). As the samples from the cage-diving locations were taken from sub-lethal biopsies (described in Chapter 3), no stomach content samples were available to compare short-term diet across sites.

Species Abundance

North Neptunes had 1.6 times more individuals (total abundance *MaxN*) than South Neptunes, and 2.2 times more than the control site (Figure 6.6 inset, p(MC) main test for location = 0.012, all pairwise p(MC) <0.05). *Pseudocaranx spp.* were dominant at all locations, with double the abundance of the next highest species (*M. hippocrepis*; Figure 6.5). These two species were twice and three times as abundant at North Neptunes compared to South Neptunes, and 3.7 and 12.8 times more abundant at North Neptunes compared to the control site respectively (Figure 6.5, p(MC) main test = 0.002 and 0.001 respectively, all pairwise p(MC) <0.05). Similarly, *C. nigripes* and *G. zebra* were more abundant at North Neptunes than the other sites (p(MC) pairwise test North Neptunes and Control = 0.003 and 0.001), although they were less numerous (mean abundance 0.82 ± 0.73 and 2.59 ± 4.50 respectively) than the *Pseudocaranx spp*. and *M. hippocrepis* (22.47 ± 19.04 and 13.59 ± 8.55) at North Neptunes. *Notolabrus tetricus* and ray abundance was indistinguishable between locations (p(MC) main test = 0.11 and 0.24, respectively).

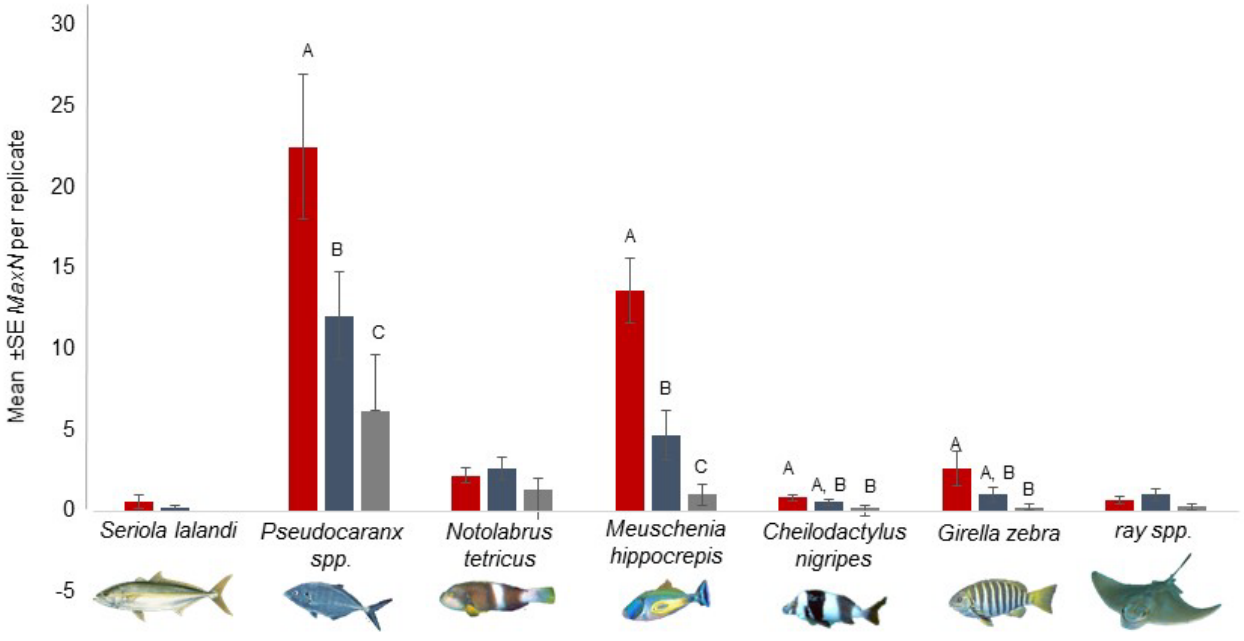


Figure 6.5 - Mean ±SE species *MaxN* per replicate for each location (red = North Neptune, blue = South Neptune, and grey = control sites). Letters indicate a significant difference (p(MC)<0.05) between locations for that species.

Discussion

Marine wildlife tourism provisioning has been shown to affect target species (Hammerschlag et al., 2012; Patroni et al., 2018; Trave et al., 2017), yet the impacts on less charismatic non-target species often go unassessed. Here, I assessed the effects of cage-diving bait and chum input on the diet of eight non-target species from different functional groups (pelagic fishes, reef fishes, and rays). Stomach content, FA profiles, and $\delta^{15}N$ values revealed site-specific diets for all eight species, although diet differences between these sites were inconsistent with the amount of bait and chum used. Pelagic fishes had a stepwise increase in bait and chum consumption mirroring input from the cage-diving industry (more frequent at North Neptunes than South Neptunes), but the diet of benthic fishes suggests the consumption of more provisions at South Neptunes than North Neptunes, highlighting the complexity of the effect of provisioning on non-target species.

Effects of provisions on non-target species

No previous studies have investigated dietary changes in non-target species feeding on tourism provisions, despite observations of such activities occurring at sites around the world. The biochemical profiles and stomach content of *Pseudocaranx spp.* were consistent with the amount of provisions used at the Neptune Islands, with the highest consumption of bait and chum at North Neptunes followed by South Neptunes, and none used at control sites. *Pseudocaranx spp.* often aggregate around cage-diving vessels and are regularly seen feeding on provisions (L. Meyer, C. Huveneers, A. Fox, per. obs., Figure 6.1), explaining the site-specific diet observed and confirming that the methods used in the present study are suitable to detect changes in diet linked to the consumption of bait and chum. Similarly, *S. lalandi* are often sighted around cage-diving vessels and feeding on baits, and their FA profiles suggest feeding on provisions at North Neptunes. However, δ15N values of *S. lalandi* failed to show differences between sites. This is likely due to this species being highly mobile with tag-recapture studies showing movements of up 100 km between captures (Gillanders, Ferrell, & Andrew, 2001). Isotopic signatures reflect diet

across several months compared to several weeks for FA profiles (Lane et al., 2006; Phillips and Eldridge, 2006). As such, the indistinguishable δ15N values across sites may reflect feeding over vast distances across several months prior to sampling, while the FA profiles showcase comparatively recent feeding on provisions at North Neptunes. Furthermore, the difference in $\delta^{15}N$ values between the bait and chum (farmed *T. maccoyii*) and *S. lalandi*'s natural prey (small fish, squid, and crustaceans [Gomon et al., 2008]) may be insufficient to elicit detectable $\delta^{15}N$ enrichment.

While pelagic fishes are regularly seen around cage-diving vessels, it is more difficult to observe whether reef fishes and rays feed on provisions, as they cannot be seen from the surface. Biochemical profiles and stomach content revealed that all six benthic species had altered diets at one or both cage-diving sites, showcasing that the effects of wildlife tourism can extend beyond the target species and conspicuous non-target organisms. *Notolabrus tetricus* had short-term differences in diet (FAs) across all locations and long-term differences ($\delta^{15}N$) between cage-diving sites and controls. Biochemical profiles of *M. hippocrepis* and *C. nigripes* showed they are substantial consumers of provisions at South Neptunes, which is consistent with their roles as trophic generalists and opportunistic scavengers. *Meuschenia spp*. are notoriously voracious scavengers, attracted by fish-based bait, caught as by-catch in the commercial rock-lobster fishery (Rodgers et al., 2013), and seen swarming and preying on live octopi (Roff, 2019). As with the other reef fishes, *G. zebra* diet was distinct at North Neptunes compared to the control sites. This was, however, driven by different FAs than in the other fish, with *G. zebra* having similar levels of the FAs 18:1ω9 and 20:4ω6 (indicative of bait and chum consumption) between North Neptunes and control, while other species had higher levels of these FAs at the cage-diving locations. The difference in *G. zebra* diet between North Neptunes and the control sites may be attributed to natural differences between locations rather than provisioning input. This is supported by the stomach contents, which did not contain large amounts of teleost in *G. zebra* from North Neptunes.

As with the pelagic and reef fishes, rays demonstrated long-term shifts in diet owing to bait and chum consumption, which aligns with previous work from the same site (Rizzari et al., 2017). Radio-acoustic positioning of *B. brevicaudata* found the presence of cage-diving operators changed fine-scale space use, as rays spent more time in close proximity to the operators (Rizzari et al., 2017). Paired with our findings of provisioned diet, it is clear that non-target species, such as rays, are not only attracted to the cage-diving vessels (Rizzari et al., 2017), but sufficiently forage on these provisions to noticeably alter their diet. Similar shifts in diet have been documented where rays are the target species for tourist operations and are directly fed (at Stingray City, Cayman Islands [Semeniuk et al., 2007, 2009], and where they are incidentally provisioned from recreational anglers discarding fish waste (Pini-Fitzsimmons et al., 2018). This highlights that marine wildlife tourism provisioning can impact species' diets, regardless of whether they are intentionally or unintentionally fed.

Magnitude and regularity of provisioning

Despite the substantial difference in bait and chum input between the sites (North Neptunes is visited approximately 8.5 times more often than South Neptunes), the difference in species abundance, and thus competition for available resources, reduces the effects of the industry at North Neptunes (Figure 6.6). The diet of pelagic fish mirrored provisioning effort, with more bait and chum consumption occurring at North Neptunes than South Neptunes. Reef fishes, however, showed the opposing trend, with relatively more provision consumption at South Neptunes. This may be attributed to their relative abundance at these two sites. North Neptunes had 1.6 times more individuals (across all 62 identified species) than South Neptunes, and most notably, almost twice the abundance of *Pseudocaranx spp.* (Figure 6.5). The large volume of bait and chum at North Neptunes is eagerly consumed by pelagic fish on the surface, only leaving a small amount of chum to reach the substrate ~30 m below (C. Huveneers, L. Meyer, and A. Fox pers. obs.) (Figure 6.6). What bait and chum does trickle through is divided between the large number of scavengers, such that each individual reef fish consumes less provisions than fishes at South Neptunes. Furthermore, the difference in both individual and total abundance

between the sites may be underestimated by the sampling strategy, as *MaxN* is a conservative approach to fish counts due to potential saturation of the screen in BRUVS (more individuals occur within a field of view than can be counted, i.e. individuals are obscured by one another) (Schobernd et al., 2013; Stobart et al., 2015).

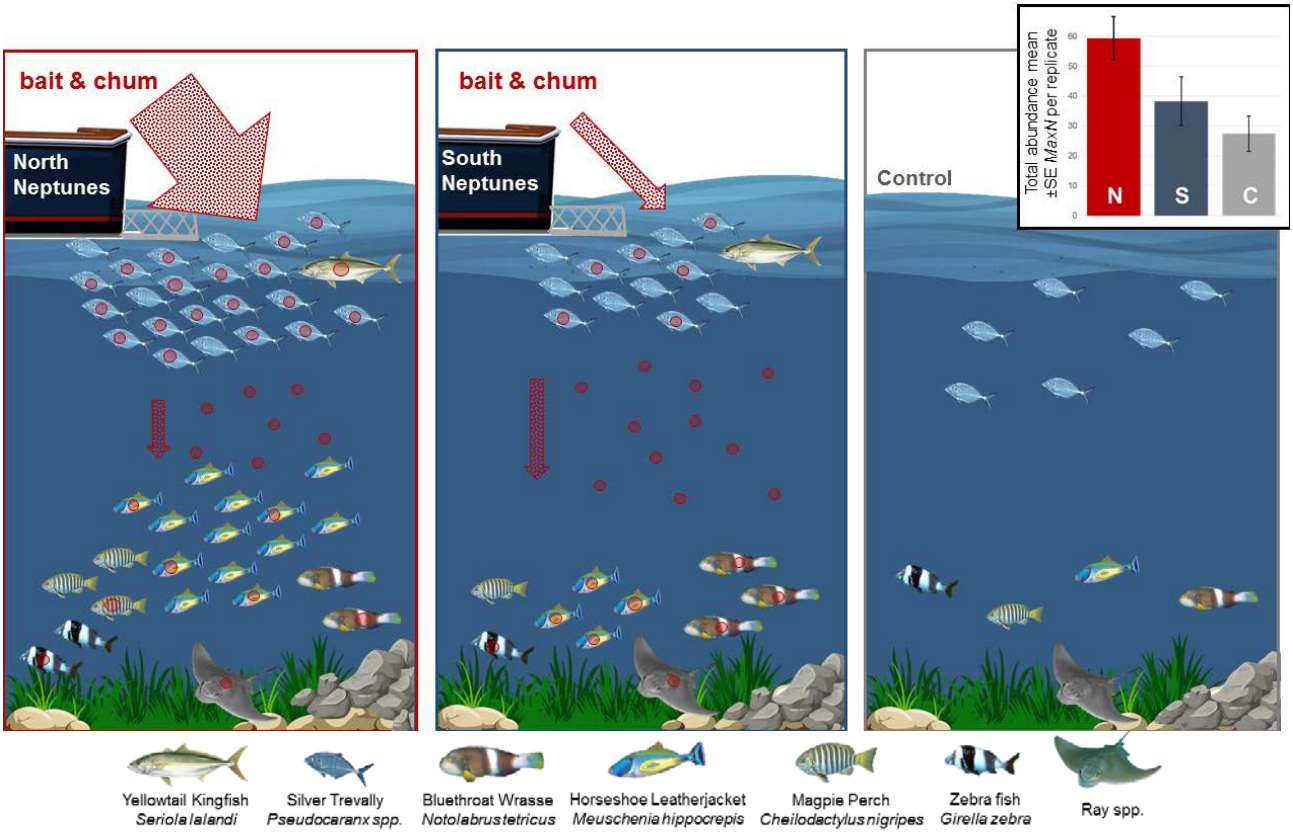


Figure 6.6 – Conceptual diagram of cage-diving provisioning input at North Neptune and South Neptune Islands. Arrow thickness indicates the magnitude of bait and chum use at each location. The number of each fish spp. in each location pane is equivalent to the mean species *MaxN* (rounded to the nearest whole fish). Red circles within individual fish indicate the consumption of provisions based on fatty acid and stable isotope data. Inset – Mean ± SE total abundance of 62 teleost and benthic chondrichthyan species at North Neptunes (N), South Neptunes (S) and Control (C).

Pseudocaranx spp. are overwhelmingly abundant around cage-diving vessels (Figure 6.1 & 6.5) and consume substantial provisions at North Neptunes in particular, while potentially limiting the quantity of the bait and chum that are available to other species. As such, they may fill a unique niche whereby their abundance and role as pelagic scavengers enable them to buffer or mitigate point-source effects or resource pulses, thus acting as "eco-moderators". This role may be similar to keystone species, i.e. species whose effect is disproportionately large relative to their abundance (Mills et al., 1993; Power et al., 1996),

such as apex-predators in systems with meso-predator overabundance (Wallach et al., 2015) or primary producers when a single species structures food-webs (e.g. Terborgh 1986). However, for a species to be considered an eco-moderator, it must mediate or reduce resource pulses from affecting other species or ecosystems rather than simply having a disproportionately large overall effect, relative to abundance (defined in Mills et al., 1993; Power et al., 1996). Eco-moderation has been described in other taxa and habitats. In the Northeast Pacific Ocean, pinnipeds seasonally aggregate to consume migrating salmon, reducing upstream resources by nearly 65% (Naughton et al., 2011). Similarly, marsh fish shunt resource pulses from cohabitating estuarine fish (Boucek and Rehage, 2013), while the burrowing crab *Neohelice granulate* engineers intertidal ecosystems to regulate the consumption of polychaetes by fire ants (Garcia et al., 2011). Such instances of eco-moderation of natural and anthropogenic resource pulses (such as tourism-related provisioning) highlight how marine consumers adapt to fluxing food-webs. The dynamic nature of such trophic roles warrants ongoing research and incorporation into local and regional management to assess the presence of eco-moderators and their ability to moderate natural and anthropogenic changes in resource availability.

Management implications

Managing wildlife tourism requires balancing the complex trade-offs between animal welfare, area and species conservation, visitor satisfaction, and benefits to the regional economy (Catlin et al., 2011; Duffus and Dearden, 1990; Reynolds and Braithwaite, 2001) to evaluate industry sustainability (e.g. Rodger et al., 2011) or mitigating negative impacts (e.g. Higginbottom et al., 2003). Dubois and Fraser (2013) developed a management framework to structure assessments of wildlife feeding acceptability, based on the feasibility to monitor and control provisioning, and the effects on conservation and animal welfare (Table 6.3). Since the different functional groups of non-target species at the Neptune Islands consume enough provisions to alter their diet, the Dubois and Fraser feeding acceptability framework can be applied here to facilitate a pragmatic evaluation of bait and chum use by the white shark cage-diving industry.

Table 6.3 - Wildlife feeding acceptability framework: evaluated by the ability to be controlled, the effects on conservation, and impacts on animal welfare. Framework from Dubois and Fraser, 2013 and applied to pelagic fishes, reef fishes, and rays at the Neptune Islands Marine Park, South Australia.

Factors	Pelagic	Reef	Rays
Ability to be controlled			
Feasible to regulate/monitor/intervene	**+ +**	**+ +**	**+ +**
	bait and chum input is limited to the tightly regulated cage-diving industry[1]		
Safe for the public	**+ +**	**+ / -** *M. hippocrepis* can be aggressive[2]	**+ +**
Effects on conservation			
Contributes to saving endangered species		**+/ unknown**	
Does not facilitate illegal fishing	**- / unknown**	**unknown**	**unknown**
Contributes to public education		**+ / unknown**	
Contributes to understanding the species	**+ +** currently providing opportunities for research[3,4,5]	**+** provides potential research opportunities[5]	**+** provides potential research opportunities[5]
Provides economic benefits	**+ +** observable from three operators	**+** observable from one operator	**+** observable from one operator
	The industry contributes $15 M to the regional economy[11]		
Contributes to population survival	**+ / unknown** potential given sufficient provisioning[9,10]	**unknown**	**unknown**
Animal welfare			
Does not facilitate disease	**- / unknown** aggregations potentiate higher parasite load and disease[8, 15]	**- / unknown** aggregations potentiate higher parasite load and disease,[8, 15]	**+/ unknown** unlikely due to low population density
Effects relatively few animals	**- -** effects large aggregations	**-** location dependent	**+** relatively low abundance
Does not cause physiological stress to animal	**+ / - unknown** unnatural diets can cause physiological benefits[9,10] or deficits[12]	**+ / - unknown** unnatural diets can cause physiological benefits[9,10] or deficits[12]	**unknown**
Does not cause physical harm to animal	**- / unknown** aggressive competition can cause injury[13, 14, 15]	**- / unknown** aggressive competition can cause injury[13, 14, 15]	**unknown**
Affects only a small portion of lifespan	**- / unknown** provisioning may increase residency in transient fishes[16]	**-** generally resident across lifespan	**- / unknown** may increase residency[17]
Does not disrupt natural foraging	**- -**	**-** location dependent	**-**

Items are rated high (+ +), somewhat high (+), somewhat low (-) or low (- -), I – indirectly, and not applicable (N/A) based on general knowledge of the literature. Blue indicated knowledge gained directly from this work, and orange indicates key areas of future research.

[1]DEWNR, 2016; [2]A. Fox pers. obs.; [3]Clarke et al., unpublished data; [4]Dennis et al., unpublished data; [5]Whitmarsh 2018; [6]DEWNR, 2012; [8]Vignon et al., 2010; [9]López-Bao et al. 2010 [10]Dunkley and Cattet 2003; [11]Huveneers et al., 2017; [12]Semeniuk et al., 2009; [13]Clua et al., 2010; [14]Newsome et al., 2004; [15]Brookhouse et al., 2013; [16]Bruce and Bradford, 2013; [17]Rizzari et al., 2017

The use of provisions by cage-diving operators is already regulated and feeding fishes and rays does not pose a risk to public safety (Table 6.3). The industry is small (three operators, two of which use bait and chum) and closely monitored, with regional adaptive management strategies minimising impacts through temporal closures, limited licences, and restricted bait and chum input (DEWNR, 2012; DEWNR, 2016). The existing management infrastructure makes the input of provisions at the Neptune Islands feasible to control, highlighted by recent precautionary intervention in July 2017 which has further limited bait and chum use after the sampling for this study was undertaken.

The effects of provisioning on conservation are generally positive, but less clear. Research and socio-economic benefits are indirect, while impacts on biological conservation are poorly understood (Table 6.3). Provisioning underpins the industry, which provides research opportunities for both target and non-target species, contributing to public education (Apps et al., 2018), and the regional economy (Huveneers et al., 2017). The role of the industry in preventing illegal fishing at the Neptune Islands, of which North Neptunes is a 'no-take' sanctuary zone, is potentially mixed. Although the presence of the operators reduce the incentive to illegally fish and provides a level of compliance monitoring, the public schedule allows illegal fishers to know when operators will be absent. The provisioned pelagic fishes at North Neptunes are more abundant (Figure 6.5) and larger than at South Neptunes and control sites (L. Meyer and J. Dennis unpublished data), which may be an incentive for illegal fishing activity.

The impacts of provisioning on biological conservation and animal welfare at the Neptune Islands are variable and uncertain (Table 6.3). The role of supplemental food on population survival has a history of mixed results across marine and terrestrial ecosystems. Terrestrial species management has a number of prescribed feeding programs, which support recovering species (e.g. bearded vulture [Oro et al. 2008], and Iberian lynx [López-Bao et al. 2010]) and mitigate the effects of food as a limiting factor (Martin, 1987; Sullivan et al., 1983). Conversely, provisioned stingrays in the Caribbean had physiological deficits owing to the consumption of unnatural, nutritionally deficient baits (Semeniuk et al. 2009).

As the tuna used as bait and chum at the Neptune Islands offers a nutritionally rich food source, which is consumed predominantly by the pelagic fishes, it may have physiological benefits for these species, contributing to the growth of individuals and the population. However, these aggregations may also facilitate disease, large parasite loads (Brookhouse et al., 2014; Vignon et al., 2010), and aggressive inter- and intra-species competition, causing physical harm or injury (Brookhouse et al., 2014; Clua et al., 2010; Newsome et al., 2004). These impacts affect large portions of the lifespan of resident reef fishes and rays, and they may be exacerbated should the provisioning extend the residency of the transient pelagic fishes as has been documented in rays (Rizzari et al., 2017) and *C. carcharias* from the Neptune Islands (Bruce and Bradford 2013).

Food-conditioned wildlife may become dependent on unreliable food sources (discussed in Orams 2002), such that the periodic nature of provisioning at South Neptunes may have different impacts on animal welfare than the consistent provisioning at North Neptunes. The presence of *Pseudocaranx spp*. in such high density may have additional flow on impacts not assessed in the Dubois and Fraser (2013) framework, including as a source of excess nitrogen enrichment shaping the benthos (Turner and Ruhl, 2007; Wong et al., 2019) or causing trophic cascades following unnatural foraging. However, they may also provide a key prey source for local pinnipeds, species whose conservation is highlighted in the Marine Park Management Plan (DEWNR, 2012). Ongoing research should explore how provisioning impacts species' abundance, diversity, trophic links, and the health of local and regional ecosystems to inform effective management.

Conclusion

This work is the first insight into the dietary effects of tourism-associated provisioning on non-target species, following recommendations for such work detailed in numerous reviews (e.g. Gallagher and Huveneers, 2018; Patroni et al., 2018; Trave et al., 2017) and tourism management frameworks (Higginbottom et al., 2003). Eight non-target species had altered diets owing to the consumption of bait and chum from the white shark

cage-diving industry at the Neptune Islands, South Australia. Findings that some species were more impacted at South Neptunes, where bait and chum are used less frequently than at North Neptunes, highlights that community responses to resource fluxes are complex, indirect, and non-linear (Bentley et al., 2012; Marcarelli et al., 2011; Yang et al., 2010). Additionally, the impacts from bait and chum input may have been moderated by the high abundance of pelagic scavengers *Pseudocaranx* spp. at North Neptunes, where they potentially act as eco-moderators. This ecosystem role is not explicitly detailed in the literature, with only few similar examples. However, it should be further explored as managers of marine wildlife tourism, and other anthropogenic resource pulses, look to protect the ecological function and resilience of areas like the Neptune Islands (DEWNR, 2012). The use of the feeding acceptability framework (Dubois and Fraser, 2013) highlights that the sum of the effects of the cage-diving industry may not necessarily be negative or deemed unacceptable, as there is the propensity to over-report negative impacts (Bateman and Fleming, 2017). Determining whether diets are altered from consuming bait and chum, only confirms that a number of non-target species are affected, which should provide the impetus for subsequent work assessing benefits or deficits in animal welfare from this resource subsidy (detailed in Table 6.3).

Findings detailing impacts beyond target species highlight the need for ongoing wildlife tourism research and management to include broad considerations of species and ecosystems. Although not explored in this study, potential impacts on the ecosystem which uphold the tourism industry are not to be overlooked (Higginbottom et al., 2003; Lim and McAleer, 2005). The Neptune Islands are part of South Australia's network of Marine Parks, providing protection for unique offshore island habitats (DEWNR, 2012), and as such appropriate management is imperative and necessitates an ecosystem-approach, inclusive of these non-target species. The complexity of such impacts makes use of this ecosystem-approach challenging, as there are species- and location-specific, potentially warranting different management strategies for North Neptunes and South Neptunes. Several management frameworks (e.g. Catlin et al., 2011; Dubois and Fraser, 2013; Higginbottom

et al., 2003) also highlight the necessity of multi-disciplinary approaches to investigate the extent of such effects. Specifically, ongoing work should combine physiology, abundance, community composition, fine-scale (vicinity of dive operators) and large-scale (immigration and emigration) movement patterns, and ecosystem modelling to gain holistic insights into the effects of provisioning, measures usually limited to understanding target species. By undertaking a comprehensive assessment of the effect of the industry on the diet of non-target species, this study provides the first step towards a broad, eco-system approach to understanding and managing the effects of wildlife tourism.

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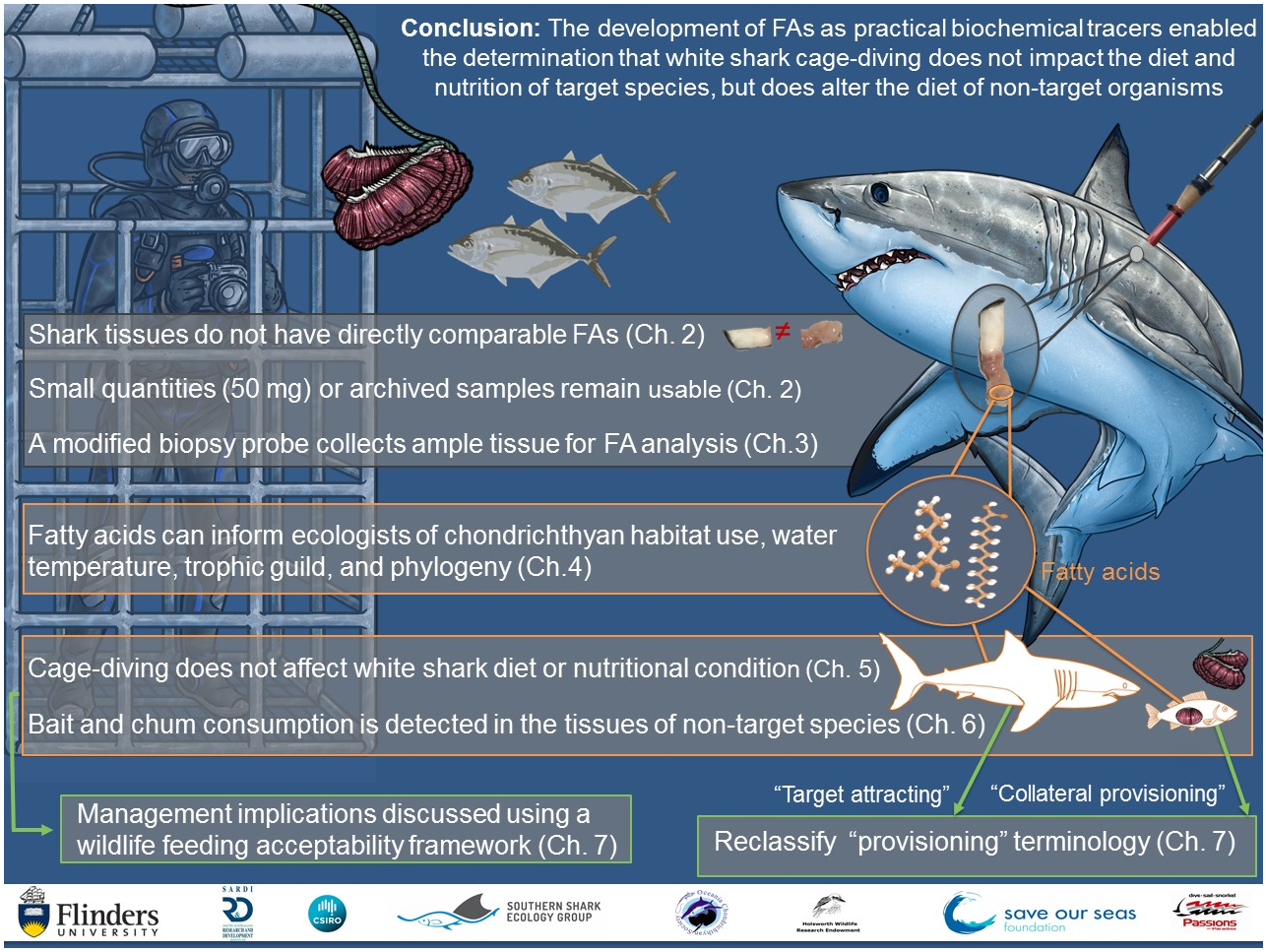
Chapter 7

General discussion



Overview

Provisioning associated with shark and ray tourism is a long-standing practice across numerous regions and species, with a range of reported impacts (reviewed in Brena et al., 2015; Hammerschlag et al., 2012; Trave et al., 2017). The effects of the tourism industry on the diet and nutritional condition of these species has been historically overlooked, partly due to practical and analytical constraints limiting the application and interpretation of emerging biochemical methods including fatty acid (FA) analysis. The objective of this thesis was to determine the effects of cage-diving on the diet of target white sharks and non-target fishes and rays using biochemical tracers obtained via new sampling and analytical methods.

Small quantities of white shark muscle were collected from free-swimming sharks using a modified biopsy probe from above and below the water's surface. This study verified that these small samples yield reliable FA profiles, which can reveal diet and habitat use in more detail than previously explored in chondrichthyans. The application of practical (biopsy devices) and statistical advances to further understand fatty acid tracersenabled the determination that white shark diet and nutritional condition was not impacted by the cage-diving industry. However, bait and chum was being consumed by a number of non-target species including pelagic fishes, reef fishes, and rays, which highlights the need for a more inclusive, ecosystem approach to wildlife tourism research and management.

The key findings of this study include:

- White shark muscle and sub-dermal tissue biochemistry is not directly comparable, and 50 mg of muscle is sufficient to obtain accurate FA profiles (Chapter 2);
- A biopsy probe intended for underwater use can be modified to collect sufficient muscle tissue from free-swimming white sharks above and below the water's surface (Chapter 3);

- Fatty acids can trace aspects of chondrichthyan ecology (i.e. individual or species associations to different habitats, water temperatures, trophic guilds, and phylogeny) with greater specificity than previously described (Chapter 4);
- White sharks exhibited no evidence of dietary shifts or reduced nutritional condition attributed to tourism-exposed residency around the cage-diving industry (Chapter 5); and
- Eight non-target species including pelagic fishes, reef fishes, and rays showed dietary shifts consistent with the consumption of bait and chum from the white shark cage-diving industry (Chapter 6).

The use of FAs in marine megafauna ecology

Practical advances

Biochemical tracers, including FAs, are increasingly used to investigate the feeding ecology of marine megafauna (Budge et al., 2006; Munroe et al., 2018; Pethybridge et al., 2018). However, their use in the setting of my studies required further practical development given the challenges of collecting samples from free-swimming white sharks. This included determining the most suitable tissue type and minimum tissue quantity (Chapter 2) to facilitate sub-lethal sample acquisition (Chapter 3) from a species of high conservation concern.

The ability to acquire suitable tissue from large elasmobranchs is limited by a deep sub-dermal layer of collagen and elastin fibres, which can be > 3 cm thick in white sharks (Jaime-Rivera et al., 2013; Motta, 1977). Coupled with notoriously tough skin, these layers have limited the collection of underlying muscle tissue without lethal sampling, which is inappropriate for many megafauna, especially endangered and protected species (Gales et al., 2009; Hammerschlag and Sulikowski, 2011). This presented an opportunity to explore the use of skin and sub-dermal tissue often retained in sub-lethal biopsies (e.g. Jaime-Rivera et al., 2013; Robbins, 2006; Rohner et al., 2013). I determined that the FA profiles of

the sub-dermal tissue and muscle were not directly comparable (Chapter 2). Few studies have explored chondrichthyan sub-dermal FA profiles (Couturier et al., 2013; Every et al., 2016), while controlled (Beckmann et al., 2013a, 2013b) and applied (e.g. McMeans et al., 2012; Pethybridge et al., 2014) studies had validated the use of muscle derived FA profiles. I found that muscle contained higher levels of PUFAs (Chapter 2) which are relatively well-studied, essential FAs commonly used to trace foraging ecology in a number of taxa (e.g. Colombo et al., 2016; Gladyshev et al., 2017; Appendix Table S2; Chapter 4). This result encourages the use of muscle tissue as the most suitable sample, as long as it can be obtained in sufficient quantities for reliable analysis (0.49 mg wet weight (WW) muscle, as determined in Chapter 2).

There is a push to develop minimally invasive, *in situ* sampling devices to target free-swimming animals (Noren and Mocklin, 2012; Reeb and Best, 2006; Robbins, 2006), as these offer alternatives to collecting tissue via lethal sampling or restraining animals, which can be logistically difficult and stressful for large species. Stable isotope analysis (~10 mg dry weight [DW]; Jaime-Rivera et al., 2013), genetics (1 mg [DW]; Smith et al., 2018), direct FA quantification (10 mg DW; Parrish et al., 2015), and full lipid and FA analysis (12 mg DW; Chapter 2) require very small quantities of muscle tissue, making these biochemical tools ideal to use with samples collected from sub-lethal biopsy methods. Quantifying the minimum tissue quantity for FA analysis (Chapter 2) provided the foundations to determine the suitability of sub-lethal biopsies to sample white sharks, given the challenges of accessing muscle underlying the sub-dermal tissue. The modification of a biopsy probe, which collected ample muscle tissue from a vessel and underwater (Chapter 3) enables sample collection across logistically diverse field operations. Using a biopsy probe from a vessel eliminates the need for research personnel to enter the water, thereby decreasing the safety risk and the logistical and physical challenges of in-water activities. Furthermore, it presents opportunities to sample a wider range of megafauna, which are potentially dangerous (i.e. large sharks), or unreliable to encounter underwater (e.g. basking sharks *Cetorhinus maximus* and many marine mammals).

The ability to take sub-lethal biopsies, with ample tissue acquisition from free-swimming animals, facilitates the application of biochemical tools across a wider array of species, without the stress and detrimental effects of the capture and release process (Dapp et al., 2016; Marshall et al., 2012; Wilson et al., 2014). This is especially valuable for a number of sharks with particularly high capture-induced mortality, including spinner (*Carcharhinus brevipinna*), blacktip (*C. limbatus*), bigeye thresher (*Alopias superciliosus*), silky (*C. falciformis*), night (*C. signatus*), and hammerhead (*Sphyrna spp.*) sharks (Butcher et al., 2015; Gallagher et al., 2014b, 2014a). The logistical challenges and physiological cost of capturing marine mammals for research also advocates for the continued refinement of sub-lethal sampling techniques (discussed in Hunt et al., 2013; Noren and Mocklin, 2012). The adapted biopsy probe detailed in Chapter 3 was not explicitly assessed for use with other taxa, but it may provide a suitable device to obtain skin and blubber from free-swimming marine mammals, both of which are useful tissues for biochemical studies (Beck et al., 2005; Bradshaw et al., 2003; Budge et al., 2006).

Analytical advances

Following chapters exploring practical (Chapter 3) and operational (Chapter 2) limitations of using FAs with white sharks, the unique metabolism of this taxa (Ballantyne, 1997) necessitated additional research, to facilitate the application of FAs tracers to questions of shark ecology (Chapter 4). Previously, the large-scale, comparative work identifying biochemical tracers was done on other taxa, such as teleosts (e.g., Colombo et al., 2016; Gladyshev et al., 2017; Vasconi et al., 2015). Yet, FA tracers have been used by shark and ray ecologists for decades (Dunstan et al., 1988; Gooch et al., 1987; Lytle and Lytle, 1994), presuming that FA metabolism, and assimilation into shark and ray muscle, mirrors that of the well-studied teleosts. However, the distinctive metabolism of sharks and rays made the utility of specific tracers largely unconfirmed (but see Beckmann et al., 2013a), and the somewhat haphazard application prompted a review of FAs use in chondrichthyan ecology (Appendix Table S2). The confusion that arose during this initial review, highlighted a clear need to quantitatively explore FA use, and identify taxa-specific

tracers which could be confidently applied by trophic ecologists. The resulting global analysis (Chapter 4) provided a detailed understanding of the biological and ecological information that can be inferred from FA profiles, with the identification of individual tracers that can detail habitat use in finer detail than previously explored. This enhances the capacity of this biochemical method to be confidently applied to studies investigating consumer foraging ecology, including the assessment of the effects of wildlife tourism on the diet of white sharks (as in Chapter 5). Furthermore, the statistical design of this study, including multivariate analyses and univariate modelling of FA profiles from wild animals, provides a model for similar work on other taxa which may be too challenging for captive studies. The assembled dataset also provides a means by which to quantitatively compare sharks and rays with poorly described foraging ecology (e.g., cookiecutter sharks *Isistius brasiliensis* [A. Carlisle and L. Meyer, unpublished data])., to those whose ecologies are better understood (data from Chapter 4). Specifically, FA profiles from understudied groups, species, or life history stages, can be analysed against the *a priori* groupings from Chapter 4, and SIMPER dissimilarity scores or CAPs can reveal previously unresolved foraging ecology.

Future directions

FA profiling is regularly applied to a number of different tissue types in sharks, rays, and other species. This often includes blood (e.g. McMeans et al., 2012; Semeniuk et al., 2007; Tierney et al., 2008), liver in sharks and rays (e.g. Davidson and Cliff, 2002; Pethybridge et al., 2014; Schaufler et al., 2005), and blubber in marine mammals (Beck et al., 2005; Budge et al., 2004). Several studies suggest muscle most closely resembles prey FA profiles (Beckmann et al., 2014; McMeans et al., 2012; Pethybridge et al., 2011). However, future work similar to Beckmann et al., (2013b) should use *in-situ* studies and replicate Chapter 4 with other tissue types to describe the use and limitations of these tissues, and assess their comparability. Furthermore, ongoing work should explore the role of physiological factors, including migration, buoyancy, pregnancy, capture stress, and energy availability on the FA

profiles of these different tissues, furthering their use as physiological indicators (Gallagher et al., 2017; Pethybridge et al., 2010; Wood et al., 2010).

As discussed in Chapter 4, FA profiling is gaining popularity, and can be readily analyzed in many laboratories around the world, using internationally accepted methods (Bligh and Dyer, 1959; Folch et al., 1957; Parrish et al., 2015). The growth in international collaborations pooling results from different locations (as in Chapter 4), advocates for ensuring analytical consistency across laboratories, including cross-laboratory validations and the availability and use of reference material. This standardisation and associated procedures would be further benefit global-scale studies, which are increasingly popular across taxa (e.g., Colombo et al., 2016; Gladyshev et al., 2017; Vasconi et al., 2015). Such work would underpin a geographic and taxonomically inclusive FA data repository and working group (discussed in Chapter 4). This would facilitate further advances in the utility of FAs, including supporting a number of specific future research directions, such as the development of 'FATscapes' (spatial contour maps of source FA tracers first proposed and explored in Pethybridge et al. [2015)]) and compiling datasets suitable for quantitative FA analysis (Iverson, 2009).

The impacts of wildlife tourism

The impacts of wildlife tourism provisioning has been extensively reviewed in the literature (e.g. Patroni et al., 2018; Richards et al., 2015; Trave et al., 2017), with studies exploring changes in movement, habitat use, abundance, and behavior of target species. Investigating the dietary effects of wildlife tourism, as I explored in this thesis (Chapter 5 and 6), has been largely overlooked, with only a few recent studies employing biochemical tracers to assess these impacts (Abrantes et al., 2018; Semeniuk et al., 2007, 2009). The advances in sample acquisition and FA tracer identification (Chapters 2, 3 and 4) facilitated the determination that the cage-diving industry in South Australia elicited no shifts in white shark diet or reduced nutritional condition (Chapter 5). These findings highlight that current management strategies (DEWNR), 2016) are adequately protecting the nutritional health of

the industry's focal species, a key animal welfare concern used to assess the acceptability of wildlife feeding (Dubois and Fraser, 2013; Table 7.1).

The use of individual FA tracers (identified in Chapter 4) to identify foraging in specific habitats, furthered the capacity to assess if interacting with the cage-diving industry impacted the ecological role of white sharks at the Neptune Islands. The lack of dietary shifts determined in Chapter 5 suggest preserved natural foraging patterns at the Neptune Islands (Chapter 5), similar to the finding that white shark predation pressure on seals remained unaffected at a popular cage-diving local in South Africa (Laroche, 2006; Laroche et al., 2007). These findings indicate white sharks are continuing to fill their natural ecological role as top predators in these regions, despite the level of interaction with cage-diving operators. Predator abundance and foraging is vital for maintaining healthy ecosystems (Hammerschlag et al., 2019; Heupel et al., 2014), yet preserving these roles is overlooked when assessing the impacts of wildfire tourism. Given the increasing popularity of top predator tourism around the world, expanding studies to include exploring the effects of tourism on the ecological roles of these species should be a management imperative. This key area of future research is encouraged by the number of emerging approaches in wildlife ecology, including FA analysis (Chapter 2, 3, and 4), which can be used in concert with other methods to reveal tourism-associated shifts in predator ecology (Carrier et al., 2018; Hammerschlag, 2019).

Tourism provisioning has the potential to affect a number of species beyond the charismatic megafauna, which is often the target of wildlife tourism operators and the bulk of the scientific literature. Of 44 papers assessing the impact of wildlife provisioning reviewed in Trave et al., (2017), only three examined non-target species or ecosystem-wide impacts (Milazzo et al., 2006; Turner and Ruhl, 2007; Vignon et al., 2010), none of which explored changes to the organism's diet. Yet, all of the eight non-target species assessed in Chapter 6 had diets consistent with the consumption of bait and chum (Chapter 6). This finding was not entirely unexpected, as it confirmed decades of observations of non-target species eating provisions at the Neptune Islands (A. Fox and A. Wright pers. comm.), as

seen at a number of marine tourism sites around the world (Gallagher and Huveneers, 2018). Studies detailing the extent that non-target species consume provisions are exclusive to terrestrial environments. These species have been found to be the primary consumer of feed intended for game wildlife (Donalty et al., 2003; Inslerman et al., 2006), with effects mirroring those detailed in target species (i.e. increase in abundance [Donalty et al., 2003; Feitosa et al., 2012; Selva et al., 2014]). In the wake of mounting empirical and observational evidence that wildlife tourism provisioning has the potential to impact non-target species (e.g. Inslerman et al., 2006; Rizzari et al., 2017; Vignon et al., 2010; Chapter 6), research and management must become more inclusive of non-target species and ecosystem services.

Refining provisioning terminology

Categorising the activities and impacts of wildlife tourism is made particularly challenging by the lack of specificity and the exclusion of non-target species when using the term “provisioning”. This umbrella term is used interchangeably with “feeding”, “baiting”, “attracting”, “artificial provisioning”, and “luring” in the scientific literature and amongst tourism operators, with no clear definition (see Brena et al., 2015; Richards et al., 2015). For example, the applicability of the term to describe cage-diving at the Neptune Islands is unresolved, and its’ current use is potentially misleading. The industry’s use of bait and chum is labelled as provisioning, leading to the presumption that sharks are fed (e.g. Brena et al., 2015), despite regulations preventing direct feeding (DEWNR, 2016) and research showing diet is not impacted by cage-diving activities (Chapter 5), while the term also overlooks the unintentional feeding of non-target species (described in Chapter 6). Similarly, manta rays (*Manta birostris*) in Hawaii are attracted to divers by aggregating zooplankton (their primary prey item) using lights, but the mantas are not directly fed anything by the dive operators (Osada, 2010). Whether or not these activities should be deemed “provisioning” is unclear and confusing. The challenge in deciding how to categorise the use of bait and chum by South Australia’s cage-diving industry reveals the issues with using the

term “provisioning”, which has become the industry standard used by scientists, managers, tourism operators and the public.

This inherent lack of specificity, and the exclusion of unintentional impacts, makes the use of “provisioning” to communicate wildlife tourism practices problematic. The absence of terminology to detail effects beyond target species, excludes the non-target species and broader ecosystem from research and management narratives. As such, the term “provisioning” necessitates reclassification, with clearly defined categories that reflect: 1) the nature of the attractant (edible or inedible); 2) the intention of the activity if using edible attractants (intentional or unintentional provisioning); and 3) which species are affected by the activity (target or non-target species) (Figure 7.1).

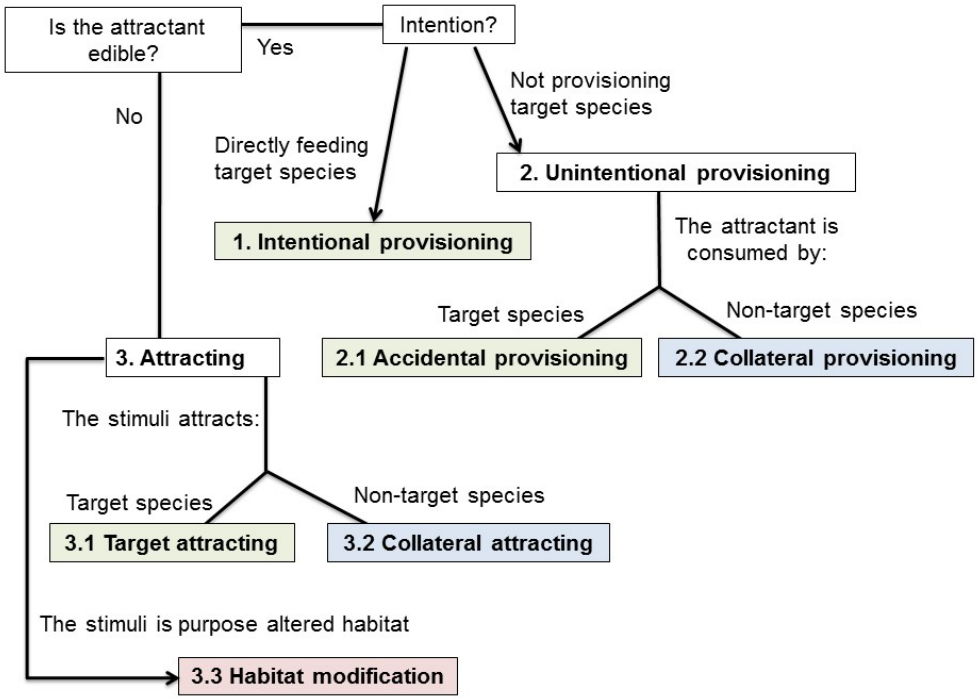


Figure 7.1 – Provisioning terminology decision framework with categories indicating the nature of the attractant, the intention of the activity, and which species groups are affected. Green indicates target species, blue non-target species, and red indicated habitat as an attractant.

Following the provisioning terminology framework detailed in Figure 7.1, South Australia’s cage-diving industry includes multiple categories of activity. The industry uses a combination of edible (bait and chum) and inedible attractants (DEWNR, 2016), with one operator classified as (3.1) *Target attracting*, given their use of an inedible stimuli (i.e.

sound) to attract target white sharks. The use of bait and chum, which is not eaten by the white sharks, but is consumed by non-target species categorises this activity as (2.2) *Collateral provisioning*. This activity could have also been classified as (2.1) *Accidental provisioning* if the FA profiles in Chapter 5 had revealed changes to the diet of white sharks.

Reclassifying provisioning terminology to detail the industry's multiple activities, and include both intentional and unintentional attracting and provisioning, enables a more transparent and accurate discourse about wildlife tourism. Furthermore, the inclusion of non-target species and habitat terminology encourages holistic assessments of these industries, as these species warrant the same consideration as the target-species. With the specific categories detailed in Figure 7.1, research and management teams can better detail and directly compare wildlife tourism activities and impacts, promoting transparency across the industry and clarity when communicating wildlife tourism practices to the public (Ballantyne et al., 2009; Curtin, 2005; Richards et al., 2015; Ziegler et al., 2018).

Management implications

Tourism management can employ a number of frameworks to evaluate industry sustainability (e.g. Catlin et al., 2011; Rodger et al., 2011) or mitigate negative impacts (e.g. Higginbottom et al., 2003). These frameworks conceptually balance the complex trade-offs between animal welfare, conservation, tourist satisfaction, research opportunities, and economic benefits (Catlin et al., 2011; Duffus and Dearden, 1990; Reynolds and Braithwaite, 2001). A framework assessing the acceptability of wildlife feeding was developed by Dubois and Fraser (2013), and has been applied to wild dolphins (Patroni et al., 2019), rays (Pini-Fitzsimmons et al., 2018), and bears (Penteriani et al., 2017), and used to discuss the impacts of intentional provisioning in a number of reviews (e.g. Murray et al., 2016; Patroni et al., 2018; Richards et al., 2015). This framework examines the net positive and negative effects on animal welfare and conservation, while including pragmatic considerations such as economic benefits and the feasibility to manage the industry and mitigate impacts (Dubois and Fraser, 2013). As stated in Higginbottom et al., (2003) and

discussed above and in Chapter 6, herein tourism management should include a consideration of the broader effects of intentional and unintentional provisioning on ecosystems. I have added this aspect to Dubois and Fraser (2013) framework to more inclusively asses of bait and chum use at the Neptune Islands, specifically examining attracting target white sharks and unintentionally provisioning non-target fishes and rays (Table 7.1). In addition to the direct positive and negative effects used in the original framework and in Chapter 6, I have included indirect effects, to accommodate the breadth of impacts the industry has on the region and the different groups of species. I have used this modified framework to identify gaps in knowledge, and highlight key areas of future research to facilitate more informed industry management.

The feasibility to manage and control white shark cage-diving in South Australia is generally high (Table 7.1), as this is a small (three operators), highly-regulated industry, with a history of compliance with temporal closures and restrictions on bait and chum use, and a number of ongoing monitoring programs (DEWNR, 2012; DEWNR, 2016). However, managing and monitoring non-target species has been relatively absent (but see Whitmarsh, 2018), and preventing unintentional provisioning would be challenging without further restrictions being placed on the industry. Public safety is not at risk from the unintentional provisioning of fishes and rays, however, attracting white sharks to dive operators has raised concerns from the public about encouraging negative human-shark interactions outside of cage-diving locations, following the hypothesis that sharks associate boats or humans with bait (discussed in Gallagher and Huveneers, 2018; Johnson and Kock, 2006). This is not explored in my thesis, but warrants research and empirical evidence to aid managers in determining the acceptability of actively attracting white sharks (Johnson and Kock, 2006).

Table 7.1 – Modified Dubois and Fraser (2013) framework assessing the acceptability of attracting target white sharks and provisioning non-target fishes and rays, evaluated by their ability to be controlled and their effects on conservation, animal welfare, and ecosystem impacts.

Factors	Target	Non-target
Ability to be controlled		
Feasible to regulate/monitor/intervene	**+ +**	**+**
	Bait and chum input is limited to the tightly regulated cage-diving industry[1]	
Safe for the public	**+ / unknown** conditioning improbable[25]	**+ +**
Conservation		
Contributes to understanding the species	**+ +** history of providing opportunities for research[19, 20, 21, 23, 24]	**+** currently providing opportunities for research [3,4,5]
Contributes to saving endangered species	**+**	**+ Indirect**
Contributes to population survival	**+ Indirect**	**+ / unknown** potential given sufficient provisioning[9,10]
Does not facilitate illegal fishing	**unknown**	**unknown**
Contributes to public education	**+ +** [22, 23]	**+ Indirect**
Provides economic benefits	**+ +**	**+ Indirect**
	The industry contributes $15 M to the regional economy[11]	
Animal welfare		
Effects relatively few animals	**+** [16]	**- -** effects large aggregations
Does not cause physiological stress to animal	**+**	**+ / - unknown** unnatural diets can cause physiological benefits[9,10] or deficits[12]
Does not cause physical harm to animal	**- / unknown** aggressive competition and cage-diving can cause injury[13,]	**- / unknown** aggressive competition can cause injury[13, 14, 15]
Does not facilitate disease	**- / unknown** aggregations potentiate higher parasite load and disease[8, 15]	**- - / unknown** aggregations potentiate higher parasite load and disease,[8, 15]
Does not disrupt natural foraging	**+ +**	**-** location dependent
Ecosystem impacts		
Does not disrupt ecosystem services	**+ / unknown**	**- / unknown** change in diet and abundance
Does not impact habitat or competitively exclude species	**unknown**	**- / unknown**

Items are rated high (+ +), somewhat high (+), somewhat low (-) or low (- -), and not applicable (N/A) based on previous studies when available or expert opinion. Green highlight indicates indirect effects; blue indicates knowledge gained directly from this PhD, and orange denotes key areas of future research.

[1]DEWNR, 2016; [2]L Meyer and A Fox pers. obs.; [3]Clarke et al., unpublished data; [4]Dennis et al., unpublished data; [5]Whitmarsh, 2018; [6]DEWNR, 2012; [8]Vignon et al., 2010; [9]López-Bao et al., 2010 [10]Dunkley and Cattet 2003; [11]Huveneers et al., 2017; [12]Semeniuk et al., 2009; [13]Clua et al., 2010; [14]Newsome et al., 2004; [15]Brookhouse et al., 2014; [16]Bruce and Bradford, 2013; [17]Rizzari et al., 2017; [18] Huveneers et al., 2013; [19]Rogers and Huveneers, 2016; [20]Watanabe et al., 2019; [21]Huveneers et al., 2016; [22]Apps et al., 2016; [23]Apps et al., 2018; [24]May et al., 2019 [25]Johnson and Kock, 2006

The cage-diving industry offers a number of direct benefits to the conservation of the target species (discussed in Macdonald et al., 2017; Table 7.1), specifically in the form of opportunities for education (Apps et al., 2018), research (Huveneers et al., 2018; May et al., 2019), and economic benefits to the regional economy (Huveneers et al., 2017). Despite the Neptune Islands being a marine park and hosting a range of native flora and fauna (ALA, 2019), viewing non-target species is a secondary benefit for tourists travelling to the region. As such, most of the conservation benefits for non-target species (e.g. contributes to research) and from them (e.g. economic and educational) are indirect effects from the industry. The contribution of the industry to the survival of white sharks and non-target species (exclusive of conservation education and awareness), is less clear. The industry financially and socially supports the Neptune Islands as a marine park, providing healthy habitat and abundant prey items for white sharks (DEWNR, 2012), as per Australia's White Shark Recovery Plan (DSEWPaC, 2013b). The protection of these islands also provides suitable habitat and protection for the non-target species, while the resource subsidy (chum and bait) may be supporting large aggregations of fishes, which are not limited by the traditional constraints of food availability (discussed in Martin, 1987; Sullivan et al., 1983; Chapter 6).

The impacts of the industry on animal welfare may be potentially negative and largely unknown, especially for those species that aggregate in larger schools which can foster parasites and disease and where aggressive competition can cause injury (Brookhouse et al., 2014; Newsome et al., 2004; Vignon et al., 2010). White sharks are relatively rare, and only a few regularly interact with cage-diving operators despite more being detected in the area (Laroche et al., 2007; Rogers et al., 2014). Additionally, the average residency of white sharks at the Neptune Islands is only nine days (Bruce and Bradford, 2013). This means that the industry has the potential to affect only few individuals over few days a year, with the relatively low abundance limiting the susceptibility of these species to disease and some physical harm (although even a few conspecifics can encourage aggressive behaviour around baits [A. Fox and A. Wright, pers. comm.]).

The trophic role of the white sharks at the Neptune Islands is not measurably impacted by the cage-diving industry (Chapter 5). Although the increase in the abundance and residency (Bruce and Bradford, 2013) of these top predators with high feeding requirements (Semmens et al., 2013) may put some trophic stress on this ecosystem. Ongoing studies revealing non-trophic structuring of food webs (Laundré et al., 2014) suggest that presence alone elicits changes in prey behaviour and food webs dynamics. This should be quantitatively explored at the Neptune Islands in conjunction with pinniped, shark, and ray abundance and behaviour. Such studies should pay careful consideration to the role of these species as meso-predators, not exclusively prey items, to understand the flow-on effects of the shifts in the abundance and presence of the industry's target white sharks (Table 7.1). Similarly, the impacts on the trophic role and ecosystem value of non-target species (discussed in Chapter 6) are likely more pronounced than on the white sharks. However, the capacity for these lower-order species to structure ecosystems is less understood, and should be explored together with the meso- and top-predators.

The modified Dubois and Fraser (2013) framework illustrates that the impacts of the white shark cage-diving industry at the Neptune Islands, South Australia differ between target and non-target species. While existing adaptive management (DEWNR, 2012; DEWNR, 2016; DSEWPaC, 2013) has curtailed the direct impacts on white shark welfare, while maintaining the benefits to conservation, the welfare of the non-target species has not been adequately considered. The effects on ecosystem services across all species remains largely unknown and the impacts at the intersection of trophic ecology, predator and prey behaviour, abundance, community composition, and ecosystem health are largely unexplored. This encourages the use of ecosystem modelling to disentangle complex interactions and explore how different bait and chum input scenarios might affect this regionally important marine protected area.

Conclusion

My thesis had the objective to determine the effects of cage-diving on the diet of target white sharks and non-target species using biochemical tracer results obtained via new sampling and analytical methods (Figure 7.2). This was achieved by: 1) assessing the practical limitations of FA analysis given the challenges associated with white shark tissue collection (Chapters 2 & 3); 2) detailing the capacity to use FA profiles to inform chondrichthyan ecology (Chapter 4); and 3) investigating the impacts of white shark cage-diving on target and non-target species (Chapters 5 & 6). Generally, my thesis encourages the use of biochemical tracers in marine megafauna ecology and furthers our understanding of the impacts of wildlife tourism provisioning on both a target and non-target species. The work detailing practical advances are broadly applicable to other elasmobranchs (Chapter 2) and megafauna in general (Chapter 3), contributing to our capacity to study the ecology and biology of rare, endangered, and challenging to sample species. Further, the global analysis of chondricththyan FA profiles identified specific tracers to detail fine-scale ecology (Chapter 4), which promotes the enhanced use of this biochemical tracer approach to detail complex foraging in these understudied and ecologically essential taxa. While these findings supported the assessment of wildlife tourism at the Neptune Islands (Figure 7.2), their broader contribution to ecology will enable a multitude of studies beyond the scope of wildlife tourism or white shark ecology. The determination that white shark diet and nutritional condition was not affected by the cage-diving industry answered long-standing questions from scientists (Gallagher and Huveneers, 2018), managers (DEWNR, 2016; DSEWPaC, 2013), tourism operators, and the public about the impact of the industry. Detailing the effects on non-target species provided new insight into how the industry impacts these less charismatic species, which mandate similar considerations to the target organisms (Figure 7.2). This furthers our understanding of wildlife tourism, and highlights the need for a more inclusive, ecosystem approach to research and management.

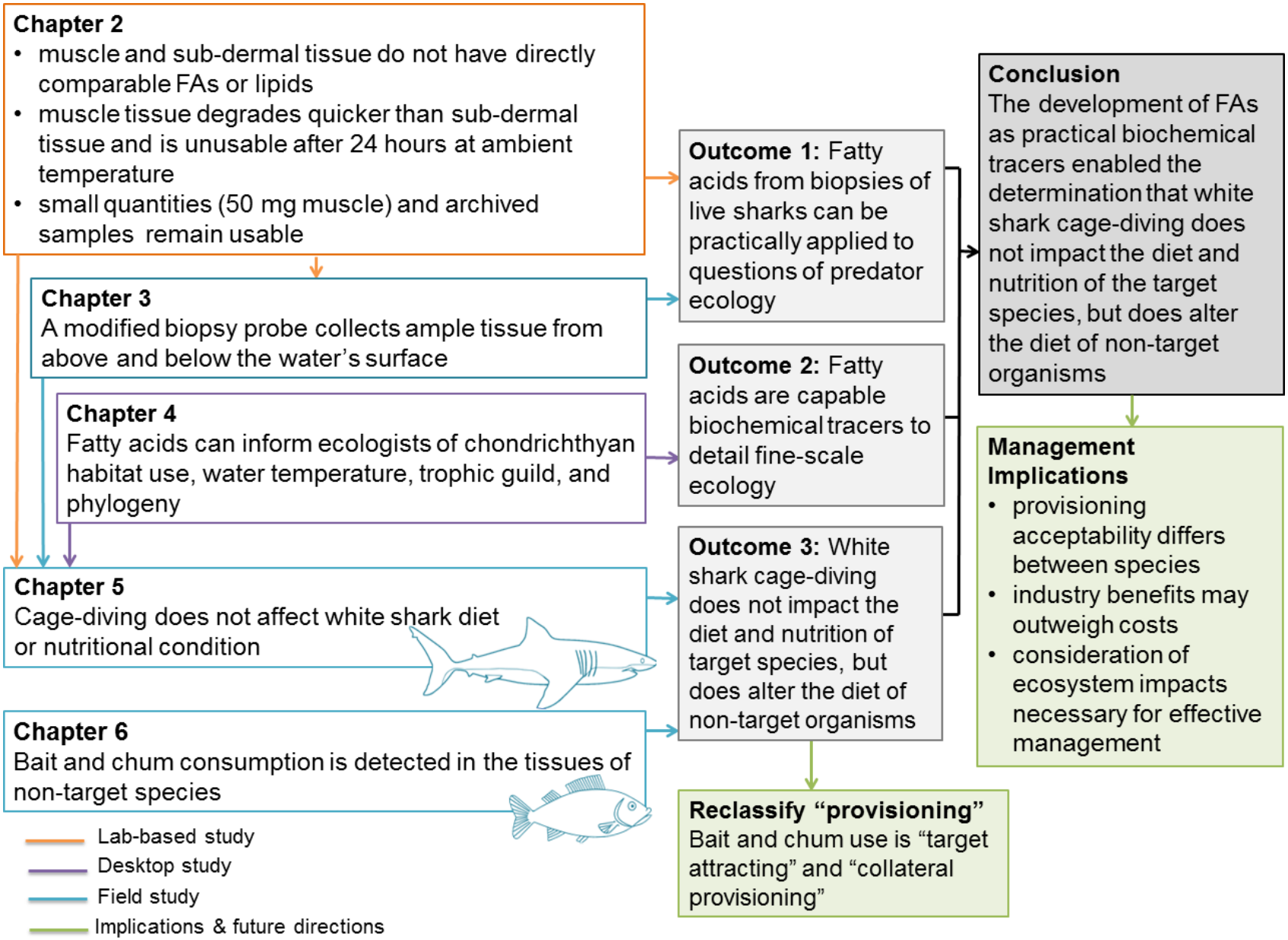


Figure 7.2 - Thesis overall conclusion and outcomes with each chapter's contribution.

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Appendix

Table S1 – Metadata and factor allocations for 106 records of chondrichthyan fatty acid profiles.

				Factor level allocations			
Common name (location)	***scientific name***	**Citation**	**Location**	**Temperature (°C)**	**Trophic guild**	**Habitat**	**Phylogeny**
White shark (AUS)	*Carcharodon carcharias*	Pethybridge et al., 2014	Australia	25	Top predator	pelagic	Lamniformes
Basking shark	*Cetorhinus maximus*	Pethybridge et al., 2014	Australia	12	Herbivore	pelagic	Lamniformes
Portuguese dogfish	*Centroscymnus coelolepis*	Økland et al., 2005	UK	4	Top predator	deep sea	Squaliformes
Black dogfish	*Centroscyllium fabricii*	Økland et al., 2005	UK	9	First order carnivore	deep sea	Squaliformes
Leafscale gulper shark	*Centrophorus squamosus*	Økland et al., 2005	UK	4	Second order carnivore	deep sea	Squaliformes
Whale shark	*Rhincodon typus*	Rohner et al., 2013	Mozambique	24	Herbivore	reef	Orectolobiformes
Tiger shark (SA)	*Galeocerdo cuvier*	Davidson et al., 2011	South Africa	25	Top predator	reef	Carcharhiniformes
Spinner shark	*Carcharhinus brevipinna*	Davidson et al., 2011	South Africa	25	Second order carnivore	reef	Carcharhiniformes
Smooth Hammerhead	*Sphyrna zygaena*	Davidson et al., 2014	South Africa	25	Top predator	reef	Carcharhiniformes
Scalloped Hammerhead (SA)	*Sphyrna lewini*	Davidson et al., 2014	South Africa	25	Top predator	reef	Carcharhiniformes
Spotted eagle ray	*Aetobatus laticeps*	Davidson et al., 2011	South Africa	25	First order carnivore	reef- D	Myliobatiformes
Honeycomb stingray	*Himantura uarnak*	Davidson et al., 2011	South Africa	25	First order carnivore	non-complex- D	Myliobatiformes
Sand tiger	*Carcharias taurus*	Davidson et al., 2011	South Africa	25	Top predator	reef	Lamniformes
Shortfin mako	*Isurus oxyrinchus*	Davidson et al., 2011	South Africa	25	Top predator	pelagic	Lamniformes
Pigeye shark (SA)	*Carcharhinus amboinensis*	Davidson et al., 2011	South Africa	25	Top predator	brackish	Carcharhiniformes
White shark (SA)	*Carcharodon carcharias*	Davidson et al., 2011	South Africa	25	Top predator	pelagic	Lamniformes
Dusky	*Carcharhinus obscurus*	Davidson et al., 2011	South Africa	25	Second order carnivore	reef	Carcharhiniformes
Bronze whaler	*Carcharhinus brachyurus*	Davidson et al., 2011	South Africa	25	Top predator	reef	Carcharhiniformes
African angelshark	*Squatina africana*	Davidson et al., 2011	South Africa	25	Second order carnivore	non-complex-D	Squantiniformes
Bull shark (SA)	*Carcharhinus leucas*	Davidson et al., 2011	South Africa	25	Top predator	brackish	Carcharhiniformes
Blacktip (SA)	*Carcharhinus limbatus*	Davidson et al., 2011	South Africa	25	Second order carnivore	reef	Carcharhiniformes
Greenland shark	*Somniosus microcephalus*	McMeans et al., 2012	Canada	6	Top predator	deep sea	Squaliformes
Arctic skate	*Amblyraja hyperborea*	McMeans et al., 2012	Canada	1	Second order carnivore	deep sea - D	Rajiformes
Pacific sleeper shark	*Somniosus pacificus*	McMeans et al., 2012	Gulf of Alaska	6	Top predator	deep sea	Squaliformes
New Zealand lanternshark	*Etmopterus baxteri*	Pethybridge et al., 2010	South east Australia	6	Second order carnivore	deep sea	Squaliformes
Longnose velvet dogfish -ad	*Centroselachus crepidater*	Pethybridge et al., 2010	South east Australia	6	Second order carnivore	deep sea	Squaliformes

Longnose velvet dogfish - jv	*Centroselachus crepidater*	Pethybridge et al., 2010	South east Australia	6	Second order carnivore	deep sea	Squaliformes
Portuguese dogfish (AUS)	*Centroscymnus coelopsis*	Pethybridge et al., 2010	South east Australia	6	Top predator	deep sea	Squaliformes
Roughskin dogfish	*Centroscymnus owstoni*	Pethybridge et al., 2010	South east Australia	6	Second order carnivore	deep sea - D	Squaliformes
Plunket shark	*Proscymnodon plunketi*	Pethybridge et al., 2010	South east Australia	6	Second order carnivore	deep sea	Squaliformes
Australian sawtail catshark	*Figaro boardmani*	Pethybridge et al., 2010	South east Australia	6	Second order carnivore	deep sea - D	Carcharhiniformes
Birdbeak dogfish	*Deania calcea*	Pethybridge et al., 2010	South east Australia	6	Second order carnivore	deep sea	Squaliformes
Kitefin shark	*Dalatias licha*	Pethybridge et al., 2010	South east Australia	6	Top predator	deep sea	Squaliformes
Southern chimaera	*Chimaera fulva*	Pethybridge et al., 2010	South east Australia	6	First order carnivore	deep sea	Chimaeriformes
South China catshark	*Apristurus sinensis*	Pethybridge et al., 2010	South east Australia	6	First order carnivore	deep sea - D	Carcharhiniformes
Southern dogfish	*Centrophorus zeehaani*	Pethybridge et al., 2010	South east Australia	6	Second order carnivore	deep sea	Squaliformes
Piked dogfish (TAS AUS)	*Squalus megalops*	Pethybridge et al., 2010	South east Australia	6	Second order carnivore	deep sea	Squaliformes
Spiny dogfish (TAS AUS)	*Squalus acanthias*	Pethybridge et al., 2010	South east Australia	6	Second order carnivore	deep sea	Squaliformes
Greeneye spurdog	*Squalus chloroculus*	Pethybridge et al., 2010	South east Australia	6	Second order carnivore	deep sea	Squaliformes
Port jackson shark (GSV AUS)	*Heterodontus portusjacksoni*	Beckmann et al., 2013	South Australia, Australia	19	First order carnivore	reef-D	Heterodontiformes
Reef Manta (AUS)	*Manta alfredi (Mobula alfredi)*	Couturier et al., 2013	Queensland, Australia	23	Herbivore	reef	Myliobatiformes
Reef Manta (MOZ)	*Manta alfredi (Mobula alfredi)*	Courier et al., 2013	Mozambique	25	Herbivore	reef	Myliobatiformes
Sandy-backed stingaree	*Urolophus bucculentus*	Dunstan et al., 1988	Australia	6	First order carnivore	deep sea - D	Rajiformes
Melbourne skate	*Spiniraja whitleyi*	Dunstan et al., 1988	Australia	6	Second order carnivore	non-complex-D	Rajiformes
Long-snouted Skate	*Zearaja nasutus*	Dunstan et al., 1988	Australia	6	Second order carnivore	deep sea - D	Rajiformes
Port jackson shark (BS AUS)	*Heterodontus portusjacksoni*	Dunstan et al., 1988	Australia	6	First order carnivore	reef-D	Heterodontiformes
Angel Shark (BS AUS)	*Squatina australis*	Dunstan et al., 1988	Australia	6	Second order carnivore	non-complex-D	Squantiniformes
Rusty catshark	*Asymbolus analis*	Dunstan et al., 1988	Australia	6	Second order carnivore	non-complex-D	Carcharhiniformes
Draughtboard shark	*Cephaloscyllium isabellum*	Dunstan et al., 1988	Australia	6	Second order carnivore	deep sea	Carcharhiniformes
Piked Dogfish (BS AUS)	*Squalus megalops*	Dunstan et al., 1988	Australia	6	Second order carnivore	deep sea	Squaliformes
Ogilby's ghost Shark	*Hydrolagus ogilbyi*	Dunstan et al., 1988	Australia	6	First order carnivore	deep sea - D	Chimaeriformes
Pale-edged stingray	*Telatrygon zugei*	Gibson et al., 1984	Malaysia	28	First order carnivore	non-complex-D	Myliobatiformes
Blue-spotted stingray	*Neotrygon kuhlii*	Hansel et al., 1993	Papua New Guinea	28	First order carnivore	reef-D	Myliobatiformes
Spadenose shark - jv	*Scoliodon laticaudus*	Wai et al., 2012	Hong Kong	28	First order carnivore	brackish	Carcharhiniformes
Spadenose shark - ad	*Scoliodon laticaudus*	Wai et al., 2012	Hong Kong	28	First order carnivore	brackish	Carcharhiniformes
Bamboo shark - jv	*Chiloscyllium plagiosum*	Wai et al., 2011	Hong Kong	28	First order carnivore	brackish	Orectolobiformes

Bamboo shark - ad	*Chiloscyllium plagiosum*	Wai et al., 2011	Hong Kong	28	First order carnivore	brackish	Orectolobiformes
Bull shark (FL)	*Carcharhinus leucas*	Belicka, et al., 2012	Florida, USA	31	Top predator	brackish	Carcharhiniformes
Thornback ray	*Raja clavata*	Colakoglu et al., 2011	Aegean Sea, Turkey	14	First order carnivore	non-complex-D	Rajiformes
Spiny dogfish (AG)	*Squalus acanthias*	Colakoglu et al., 2011	Aegean Sea, Turkey	6	Second order carnivore	non-complex-D	Squaliformes
Marbled stingray	*Dasyatis marmorata*	El Kebir et al., 2007	Maritias	27	First order carnivore	non-complex-D	Myliobatiformes
Lusitanian cownose ray	*Rhinoptera marginata*	El Kabir et al., 2007	Maritias	27	First order carnivore	reef-D	Rajiformes
Blackchin guitarfish	*Rhinobatos cemiculus*	El Kabir et al., 2007	Maritias	27	Second order carnivore	reef-D	Rhinopristiformes
Pigeye shark (AUS)	*Carcharhinus amboinensis*	Every et al., 2017	Northern Territory, AUS	25	Top predator	brackish	Carcharhiniformes
Bull shark (AUS)	*Carcharhinus leucas*	Every et al., 2017	Northern Territory, AUS	25	Top predator	brackish	Carcharhiniformes
Northern river shark	*Glyphis garricki*	Every et al., 2017	Northern Territory, AUS	25	Second order carnivore	brackish	Carcharhiniformes
Speartooth shark	*Glyphis gliphis*	Every et al., 2017	Northern Territory, AUS	25	Second order carnivore	brackish	Carcharhiniformes
Freshwater whipray	Urogymnus dalyensis	Every et al., 2017	Northern Territory, AUS	25	First order carnivore	brackish	Myliobatiformes
Australian sharpnose shark	*Rhizoprionodon taylori*	Every et al., 2017	Northern Territory, AUS	25	Second order carnivore	brackish	Carcharhiniformes
Piked dogfish (FRDC AUS)	*Squalus megalops*	Nichols et al., 2002	Australia	15	Second order carnivore	deep sea - D	Squaliformes
Skate	*Raja sp.*	Nichols et al., 2002	Australia		First order carnivore	deep sea - D	Rajiformes
Eaton's skate (KER)	*Bathyraja eatonii*	Unpublished data	Kerguelen plateau	2	First order carnivore	deep sea - D	Rajiformes
Kerguelen Sandpaper Skate	*Bathyraja irrasa*	Unpublished data	Kerguelen plateau	2	First order carnivore	deep sea - D	Rajiformes
Murray's skate	*Bathyraja murrayi*	Unpublished data	Kerguelen plateau	*3*	First order carnivore	deep sea - D	Rajiformes
McCain's skate (ROS)	*Bathyraja maccaini*	Jo et al., 2013	Ross Sea, Antarctica	-2	First order carnivore	deep sea - D	Rajiformes
Eaton's skate (ROS)	*Bathyraja eatonii*	Jo et al. 2013	Ross Sea, Antarctica	-2	First order carnivore	deep sea - D	Rajiformes
Eagel ray (NEP)	*Myliobatis australis*	Unpublished data	South Australia	14	First order carnivore	reef-D	Myliobatiformes
Smooth ray	*Dasyatis brevicaudata*	Unpublished data	South Australia	14	Second order carnivore	reef-D	Myliobatiformes
Eagle ray (GSV)	*Myliobatis australis*	Unpublished data	South Australia	19	First order carnivore	reef-D	Myliobatiformes
Smooth skate	*Raja senta*	Budge et al., 2002	Scotian Shelf, Canada	5	First order carnivore	deep sea - D	Rajiformes
Thorny skate (CAN)	*Raja radiata*	Budge et al., 2002	Scotian Shelf, Canada	5	First order carnivore	non-complex-D	Rajiformes
Winter skate (CAN)	*Raja ocellata*	Budge et al., 2002	Scotian Shelf, Canada	5	First order carnivore	non-complex-D	Rajiformes
Small-spotted catshark (winter)	*Scyliorhinus canicula*	Garcia-Moreno et al., 2013	West Alboran Sea	15	First order carnivore	non-complex-D	Carcharhiniformes
Small-spotted catshark (summer)	*Scyliorhinus canicula*	Garcia-Moreno et al. 2013	West Alboran Sea	19	First order carnivore	non-complex-D	Carcharhiniformes
Angel shark (FRDC AUS)	*Squatina australis*	Nichols et al., 2002	Tasmania, Australia	14	Second order carnivore	non-complex-D	Squantiniformes
Whitecheek shark	*Carcharhinus dussumieri*	Nichols et al., 2002	Persian gulf	26	Second order carnivore	reef	Carcharhiniformes

Spottail shark	*Carcharhinus sorrah*	Nichols et al., 2002	Indo-West Pacific	28	Second order carnivore	reef	Carcharhiniformes
Broadnose shark	*Notorynchus cepedianus*	Nichols et al., 2002	Australia	17	Top predator	reef	Hexanchiformes
Gummy shark	*Mustelus antarcticus*	Nichols et al., 2002	Southern Australia	13	Second order carnivore	non-complex-D	Carcharhiniformes
School shark	*Galeorhinus galeus*	Nichols et al., 2002	Australia	18	Second order carnivore	non-complex-D	Carcharhiniformes
Elephant fish	Callorhinchus milii	Nichols et al., 2002	Southern Australia	16	First order carnivore	deep sea	Chimaeriformes
Ocellate spot skate	*Okamejei kenojei*	Jeong et al., 1998	Tongyeong, Korea	13	Second order carnivore	non-complex-D	Rajiformes
Electric ray	*Narke japonica*	Jeong et al., 1998	Tongyeong, Korea	23	First order carnivore	non-complex-D	Rajiformes
Atlantic stingray	*Dasyatis sabina*	Lytle & Lytle, 1994	Gulf of Mexico	30	First order carnivore	reef-D	Myliobatiformes
Southern stingray	*Hypanus americanus*	Lytle & Lytle 1994	Gulf of Mexico	30	First order carnivore	reef-D	Myliobatiformes
Atlantic sharpnose shark (MEX)	*Rhizoprionodon terraenovae*	Lytle & Lytle 1994	Gulf of Mexico	30	Second order carnivore	reef	Carcharhiniformes
Blacktip shark (MEX)	*Carcharhinus limbatus*	Lytle & Lytle 1994	Gulf of Mexico	30	Second order carnivore	reef	Carcharhiniformes
Atlantic sharpnose shark (USA)	*Rhizoprionodon terraenovae*	Gooch et al., 1987	South Carolina, USA	27	Second order carnivore	reef	Carcharhiniformes
Sandbar shark	*Carcharhinus plumbeus*	Gooch et al., 1987	South Carolina, USA	27	Top predator	reef	Carcharhiniformes
Lemon shark	*Negaprion brevirostris*	Gooch et al., 1987	South Carolina, USA	27	Second order carnivore	reef	Carcharhiniformes
Scalloped hammerhead (USA)	*Sphyrna lewini*	Gooch et al., 1987	South Carolina, USA	27	Top predator	reef	Carcharhiniformes
Tiger shark (USA)	*Galeocerdo cuvier*	Gooch et al., 1987	South Carolina, USA	27	Top predator	reef	Carcharhiniformes
Winter skate (USA)	*Raja ocellata*	Krzynowek & Panunzio, 1989	Massachusets, USA	5	First order carnivore	non-complex-D	Rajiformes
Thorny skate (USA)	*Amblyraja radiata*	Krzynowek & Panunzio, 1989	Massachusets, USA	5	First order carnivore	non-complex-D	Rajiformes
Shortfin mako	*Isurus oxyrinchus*	Vlieg et al., 1993	New Zealand	16	Top predator	pelagic	Lamniformes
Poorbeagle	*Lamna nasus*	Vlieg et al., 1993	New Zealand	13	Top predator	pelagic	Lamniformes

Habitat allocations include – D to indicate demersal feeding.

Table S2 – Expanded table of fatty acid (FA) tracers as recommended in the literature alongside their current use within studies of chondrichthyan trophic ecology.

Fatty Acid	Bio-indicator	Pathways	Use within chondrichthyan trophic ecology
14:0 Myristic acid	Proteobacteria, Diatoms/Prymnesiophytes (Dalsgaard et al., 2003; Bergé & Barnathan, 2005).	Centric diatoms in a nearshore mid-latitude environment (Parrish et al., 1995).	
16:0 Palmitic acid	Zooplankton (Rohner et al., 2013) Trophic position (Schmidt-Nielsen, 1997)	Found in high levels in mesopelagic squid, fish & crustaceans (Pethybridge et al., 2010). Prone to some level of biosynthesis, but is also highly indicative of differences in various prey (Iverson et al., 1993; Budge et al., 2006 Beckmann et al., 2013), especially when corrected with calibration coefficients (Iverson et al., 2004).	Key FA in differentiating demersal and deep sea elasmobranch species (Pethybridge et al., 2011; Pethybridge et al., 2014). Distinguished captive Port Jackson shark *Heterodontus portusjacksoni* feed squid vs. prawn diets (Beckmann, et al., 2013). Indicated high zooplankton in whale sharks (Rohner et al., 2013).
17:0 Margaric acid	Bacteria (Dalsgaard et al., 2003)	a17:0 and i17:0 (both odd-chain branched FAs) high in bacteria and found in greater abundance within estuaries (Wai et al., 2011).	a17:0 and i17:0 used to identify bacterial input in estuarine spadenosed shark *Scoliodon laticaudus* and bamboo sharks *Chiloscyllium plagiosum* (Wai et al., 2011; 2012).
18:0 Stearic acid		High in mesopelagic squid, fish, crustaceans (Pethybridge et al., 2010). Precursor FA 18:1ω9 (Dalsgaard et al., 2003). Readily bio-converted, but remains indicative of differences in various prey (Iverson 1993; Budge et al., 2006; Beckmann et al., 2013), especially when corrected with calibration coefficients (Iverson et al., 2004).	Reflects prey (halibut and ringed seals) in Greenland shark *Somniosus microcephalus* (McMeans et al., 2012). High levels in demersal sharks (Pethybridge et al., 2010). Differentiated deep sea elasmobranch species (Pethybridge et al., 2014).
20:0 Arachidic acid	Detritus (Wai et al., 2011)	Greater amounts in terrestrial detritus vs. macro algal detritus (Wai et al., 2011)	Paired with other long-chain saturated fatty acids to reflect detritus in bamboo and spadenose sharks (Wai et al., 2011; 2012).
24:0 Lignoceric acid	Mangroves & terrestrial plants (Budge et al., 2001; Wai et al. 2011; Joseph et al., 2012)	Greater amounts in terrestrial detritus vs. macro algal detritus (Wai et al., 2011)	Reflected detritus in spadenosed shark *Scoliodon laticaudus* and bamboo sharks *Chiloscyllium plagiosum* (Wai et al., 2011; 2012).
SFA Saturated Fatty Acids		Variation in SFAs (specifically 16:0 and 18:0) amongst tissue types according to rates of cellular metabolism (Tocher, 2003).	Dominated Whale Shark *Rhincodon typus* profile (Couturier et al., 2013; Rohner et al., 2013). Reflected ontogenetic changes in estuarine sharks (Wai et al., 2012), likely due to differences in bacterial and detrital input (Wai et al., 2012).

16:1ω7 Palmitoleic acid	Phytoplankton based food web (Falk-Petersen et al., 2000) Mangroves, diatoms and bacteria (Ackman et al., 1968; Kelly & Scheibling, 2012) Low levels indicative of carnivory (Cook, et al., 2000; Kamenev, 1995)	High quantities in coastal herbivores (Graeve et al., 1994; Wai et al., 2011). High levels in blubber (Waugh et al., 2014), particularly from odontocetes (Ackman, 1989) and sea lions (Beck et al., 2005). High in squid (Pethybridge et al., 2010) Questionable indicator as there is some capacity for biosynthesis (in the case of seals [Iverson et al., 2004]), however remains highly indicative of differences in various prey items (Iverson, 1993; Iverson et al., 2002).	Found in lower proportions in shark vs. prey profiles (halibut and seal vs Greenland shark [McMeans et al., 2012]) suggesting selective catabolism or elongation to 18:1ω7 (Tocher, 2003). Responsible for grouping of Australian white sharks (Pethybridge et al., 2014)
16:1ω9	Diatoms		Highlighted ontogenetic variation in diet in estuarine sharks (Wai et al., 2011, 2012).
18:1ω7 Vaccenic acid	Bacteria (Kelly and Scheibling 2012), phytoplankton-based food web (Falk-Petersen et al., 2000).	Indicator for crustaceans, bathypelagic squid and fish (Pethybridge et al., 2011). Can be metabolized from 16:1ω7 (palmitic acid), however remains a reliable marker of diet (Beckmann et al., 2013).	Found to reflect prey (halibut and ringed seals) in greenland shark (McMeans et al., 2012), Port Jackson sharks (Beckman et al., 2013) and reflected ontogenetic change and capture location in estuarine sharks (Wai et al., 2011; 2012).
18:1ω9 Oleic acid	Macroalgae, mangroves, (Lewis, 1967; Kelly & Scheibling, 2012) brown algae (Alfaro et al., 2006). Temperature (Arts & Kohler, 2009; Velansky & Kostetsky, 2008) Reflects trophic position and carnivory (Kamenev, 1995; Schmidt-Nielsen, 1997; Cook et al., 2000). Levels increase with water depth (Lewis 1967).	High levels in mesopelagic squid, fish and crustaceans (Pethybridge et al., 2010). High in blubber (Waugh et al., 2014). Some capacity for biosynthesis in predators (e.g. seals [Iverson et al., 2004]) as it can be elongated and desaturated to 20:3ω9 (Curtis-Prior, 2004), but remains indicative of differences in various prey (Iverson et al. 2001). Hard to distinguish between 18:1ω11 (Iverson et al., 2004), although the latter FA is generally only a very minor FA.	Surprisingly high levels in whale sharks (Rohner et al., 2013); different in muscle vs fin in euryhaline sharks (Every et al., 2016). Found in higher levels in young-of-the-year (YOY) bull sharks than in immature/mature sharks (Belicka et al., 2012), which was attributed to either persistent maternal signatures and depleted essential fatty acids.
20:1ω9 Eicosenoic acid	Copepods (Dahl et al., 2000; Kelly & Scheibling, 2012; Phillips et al., 2003;) Latitude (Gibson et al., 1984) Temperature (Ackman, 1982)	Indicates secondary zooplankton consumption via animals such as crustaceans and squid (Pethybridge et al., 2010; Phillips et al, 2003). Considered major constituent in cold-water fish lipids (Ackman 1982), seen in low levels in Australian tropical and sub-tropical fish (Gibson 1984). High concentrations in fin whale blubber (Ackman 1989) specifically minke whales (Møller et al., 2003), however lower in odontocetes (Ackman, 1989), harbor seals (Iverson, et al., 1997) and sea lions (Beck et al., 2005) in northern gulf of Alaska.	Responsible for distinguishing different shark species (Pethybridge et al., 2014). Positively correlated with total length of bamboo sharks, separated juvenile vs. adults (Wai et al., 2011)

20:1ω11 Gadoleic	Copepods (Kelly & Scheibling, 2012; Saito et al., 1997; Schaufler et al., 2005) Latitude (Gibson 1984)	High in copepod-consuming, mesopelagic fish and squid (Pethybridge et al., 2010) Found in high levels in foraging fish species in the Gulf of Alaska (Iverson et al., 2002). Considered major constituent in cold-water fish lipids (Ackman 1982), low levels in Australian tropical and sub-tropical fish (Gibson 1984).	When paired with 20:1ω9, positively correlated with total length of bamboo sharks, separated juvenile vs. adults (Wai et al., 2011).
22:1ω9 Erucic	Zooplankton (George and Parrish, 2015)	Important dietary indicator in seals (Iverson et al., 2004), indicate secondary zooplankton consumption via animals such as crustaceans and squid (Phillips et al., 2003). High levels in squid (Pethybridge et al., 2010).	
22:1ω11 Docosenoic	Zooplankton (George and Parrish, 2015), specifically copepods (Dahl et al., 2000).	Important dietary indicator in seals (Iverson et al., 2004). Found in high levels in foraging fish species in the Gulf of Alaska. High concentrations in fin whale blubber (Ackman 1989) specifically minke whales (Moller et al., 2003). May exhibit reduced deposition (Bremer and Norum, 1982).	
MUFA Monounsaturated Fatty Acids	MUFA composition used to distinguish carnivory and herbivory (Drazen et al., 2008).	Long-chain MUFAs are generally consistent with copepods or higher predator food chain (Phillips et al., 2001).	Higher levels in cartilaginous fish (Dunstan et al., 1988). Significantly different for spadenose sharks from inner and outer pearl river estuary in Hong Kong (Wai et al., 2012)
18:2ω6 Linoleic; LA	Mangrove, seagrass & macroalgae (Nichols et al., 1982; Kelly & Scheibling 2012).	Essential for growth, development and cellular function (Le et al., 2009). Cannot be synthesised by vertebratsd, and thus must be obtained through diet alone (Tocher 2003).	Reflects prey (halibut and ringed seals) in Greenland shark (McMeans et al., 2013); found in relatively high abundance in estuarine sharks from northern Australia (Every et al., 2016). Positively correlated with total length of estuarine spadenose shark showcasing ontogenetic variation in diet (Wai et al., 2012). Significantly different for spadenose sharks from the inner and outer sections of the pearl river estuary in Hong Kong (Wai et al., 2012)
18:3ω3 □-linolenic; ALA	Mangrove, seagrass & macroalgae (Nichols et al., 1982 Kelly & Scheibling 2012)	Essential for growth, development and cellular function (Le et al., 2009). Cannot be synthesised by vertebrates, and thus must be obtained through diet alone (Tocher 2003).	Reflected inter-annual and location differences in Greenland sharks (Steeves et al., 2016), attributed to proportionally higher levels in halibut from one of the two locations (McMeans et al., 2013). Negatively correlated with total length of estuarine bamboo sharks and sympatric spadenose sharks, showcasing ontogenetic variation in diet (Wai et al., 2011, 2012), Significantly different for spadenose sharks from the inner and outer sections of the pearl river estuary in Hong Kong (Wai et al., 2012)
20:3ω9	Essential fatty acid depletion (Le et al., 2009)	The ω9 FAs are synthesized only in the absence of sufficient ω3 and ω6 fatty acids, which have a competitive advantage over the ω9s for enzymatic conversion (Le et al., 2009). Found in high abundance in cartilage (Adkisson 4th et al., 1991).	Found in high levels in YOY bull sharks, indicating essential fatty acid deficiency due to inadequate foraging skills and/or limited prey (Belicka et al., 2012).

20:4ω6 Arachidonic; ARA,	Protozoa, microeukaryotes, red algae, kelp, diatoms, algae, mangroves & terrestrial plants (Sargent et al., 1999; Alfero et al 2006) macroalgae (Dunstan et al., 1988). Demersal zooplankton, benthopelagic orgnaisms (Copeman and Parrish, 2003) Low in phytoplankton (Dunstan et al., 1988) Coralline algae and coral mucus (van Duyl et al., 2011) Low in carnivores (Dunstan et al., 1988).	High in benthic herbivores & omnivores vs. low levels in pelagic teleosts and cephalopods (Dunstan et al., 1988). High in Blubber (Waugh et al., 2012) May not be a reliable indicator of diet as it can be affected by fluctuations in 18:2ω6 (Beckmann et al., 2014).	Higher levels in cartilaginous fish (Dunstan et al., 1988). High levels reported in Port Jackson sharks as a result of predation on sea urchins and snails (Dalsgaard et al. 2003). Responsible for separating different shark species (Pethybridge et al., 2014). Interspecific variation between species found in estuarine sharks from northern Australia (Every et al., 2016). Positively correlated with total length of estuarine bamboo sharks and sympatric spadenose sharks, showcasing ontogenetic variation in diet (Wai et al., 2011, 2012). Lower levels in YOY bull sharks compared with immature/mature sharks (Belicka et al 2012), attributed to either persistent maternal signatures and depleted essential fatty acids. Significantly different for spadenose sharks from an inner and outer pearl river estuary in Hong Kong (Wai et al., 2012)
20:5ω3 Eicosapentaenoic; EPA	Diatoms, brown and red macroalgae (Kelly & Scheibling 2012, (Falk-Petersen et al., 2000) and Phytoplankton (Dunstan et al., 1988). High levels in Krill (Pethybridge et al., 2013). Moderate to high levels in carnivores (Dunstan et al., 1988). High levels in migratory fish (Bell et al., 1986)	The major source of long-chain PUFA for coastal and offshore pelagic species as well as deeper offshore demersal species (see reviews by Sargent et al., 1999). Observed in high quantities in coastal herbivores indicating the assimilation of benthic microalgae and plant material associated with ingesting fine sediments (Graeve et al., 1994; Viso & Marty, 1993; Wai et al., 2011). High levels in polychaetes (Wai et al., 2011), zooplankton, cephalopods (Pethybridge et al., 2010). Pelagic feeding fish have moderate to high levels of EPA (6.8-11.6%; Dunstan et al., 1988). Elevated levels can indicate amphipod consumption (Pethybridge et al., 2013). Characteristically high in migratory fish (Bell et al., 1986) due to SFA and MUFA metabolism during energetically demanding migrations (Saito et al., 1997), particularly off shore migrations (Osako et al., 2006). The high EPA contents in the muscle of Clupeiformes and some Salmoniformes attributed to adaptations for fast continuous swimming (Gladyshev et al., 2017). Within both individual algal species and whole algal assemblages, high light conditions generally lower algal PUFAs, especially EPA, by causing oxidative damage (Cashman et al., 2013). Inorganic nutrients decreased DHA, EPA, and the ratio of ω3 to ω6 PUFAs even though they increased the shorter chain ω3 PUFA ALA.	Higher proportions in cold water elasmobranchs (Semeniuk et al., 2007). Positively correlated with total length of estuarine bamboo sharks and sympatric spadenose sharks, showcasing ontogenetic variation in diet (Wai et al., 2011, 2012).

20:4ω3 Eicosatetraenoic	Fungi, protozoa, algae (Kelly & Scheibling 2012), diatoms, algae, mangroves and terrestrial plants (Alfero et al 2006; Sargent et al., 1999)		Found in relatively high abundance in estuarine sharks from Northern Territory waters, Australia (Every et al., 2016)
22:5ω3 Clupanodonic; DPA	Diatoms, algae, mangroves, terrestrial plants (Alfero et al 2006; Sargent et al., 1999)	Potential indicator of crustaceans, octopuses and mesopelagic squid (Pethybridge et al., 2013). Relatively high in seal blubber (McMeans et al., 2013). Some level of biosynthesis in the case of seals (Ackman et al. 1988, Iverson et al., 2004).	Higher levels in cartilaginous fish (Dunstan et al., 1988). Found to reflect prey (halibut and ringed seals) in greenland sharks (McMeans et al., 2012) Significantly different for spadenose sharks from an inner and outer pearl river estuary in Hong Kong (Wai et al., 2012)
22:5ω6 Osbond; ω6-DPA	Pelagic vs. demersal carnivores (Dunstan et al., 1988)	High levels in fish and cephalopods (Graeve et al., 1994; Couturier et al., 2013).	Lower amounts in pelagic carnivores relative to demersal carnivores (Dunstan et al., 1988).
22:6ω3 Docosahexaenoic; DHA	Dinoflagellates & zooplankton (Kelly & Scheibling 2012; Falk-Petersen et al. 2000; Alfaro et al., 2006, Dalsgaard et al., 2003). Migratory fish (Osako et al.,2006) Low values in carnivores (Cook et al., 2000; Kharlamenko et al., 1995)	High levels in mesopelagic squid, fish and crustaceans (Budge et al., 2002; Pethybridge et al., 2010;). 9% higher in squid than prawns (Beckmann et al., 2013) DHA is biomagnified and preferentially retained at higher trophic levels (Strandberg et al., 2015). Inorganic nutrients decreased DHA, EPA, and the ratio of ω3 to ω6 PUFAs even though they increased the shorter chain ω3 PUFA ALA (Twining et al., 2016) Characteristically high in migratory fish (Bell et al., 1986) due to SFA and MUFA metabolism during energetically demanding migrations (Watanabe et al.,1995; Saito et al.,1997), particularly off shore migrations (Osako et al.,2006). Considered “pacemakers” for animal metabolism, as muscle DHA correlated with metabolic rate (Hulbert, 2007). Acts as a performance enhancing agent activating enzymes which are key in the lipid fuel pathway in migratory birds (Weber, 2011). The high DHA contents in the muscle of Clupeiformes and some Salmoniformes attributed to adaptations for fast continuous swimming (Gladyshev et al., 2017). See review by Valentune et al., (2004).	Higher proportions in cold water elasmobranchs (Semeniuk et al., 2007). Key in separating Port Jackson sharks fed differing diets (Beckman et al., 2013). Positively correlated with total length of estuarine bamboo sharks and sympatric spadenose sharks, showcasing ontogenetic variation in diet (Wai et al., 2011, 2012). Higher in deep-sea condrichtyans than whale sharks (Rohner et al., 2013). Lower levels in YOY bull sharks compared with immature/mature sharks (Belicka et al 2012), attributed to either persistent maternal signatures and depleted essential fatty acids. Significantly different for spadenose sharks from an inner and outer pearl river estuary in Hong Kong (Wai et al., 2012)
PUFA Polyunsaturated Fatty Acids	Highly variable as indicators due to the difference in ω3 and ω6 production at the base of distinct food webs.	Most often used as dietary indicators as they cannot be biosynthesized in sufficient quantities to ensure optimal physiological performance (Turner & Rooker, 2005)	Major source of FAs in coastal and offshore pelagic species and deeper offshore demersal species (Dunstan et al., 1988).

Not included within the table - 19:0, 21:0, 22:0, 16:1ω9, 16:1ω11, 17:1, 18:1ω5, 20:1ω7, 22:1ω7, 18:2ω4, 18:3ω6, 18:4ω3, 20:2ω6, 20:3ω6

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Table S3 – Count of trophic guild for each phylogenetic order.

	Trophic Guild			
Phylogeny	**Herbivore**	**First order carnivore**	**Second order carnivore**	**Top predator**
Carcharhiniformes	5		17	12
Chimaeriformes	3			
Heterodontiformes	2			
Hexanchiformes				1
Lamniformes		1		6
Myliobatiformes	10	2	1	
Orectolobiformes	2	1		
Rajiformes	15		4	
Rhinopristiformes			1	
Squaliformes	1		14	5
Squantiniformes			3	

Table S4 – Count of habitat type for each phylogenetic order group.

	Habitat type						
Phylogeny	**Brackish**	**Pelagic**	**Reef**	**Reef-D**	**Non-complex -D**	**Deep sea**	**Deep sea - D**
Carcharhiniformes	10		16		5	1	2
Chimaeriformes						2	1
Heterodontiformes				2			
Hexanchiformes			1				
Lamniformes		6	1				
Myliobatiformes	1		2	7	3		
Orectolobiformes	2		1				
Rajiformes				1	8		10
Rhinopristiformes				1			
Squaliformes					1	17	2
Squantiniformes					3		

- D indicates demersal habitats

Table S5 – Count of trophic guild for each habitat group.

	Trophic Guild			
Habitat type	**Herbivore**	**First order carnivore**	**Second order carnivore**	**Top predator**
brackish		5	3	5
pelagic	1			5
reef	3		9	9
reef-D		9	2	
non-complex-D		11	9	
deep sea		3	12	5
deep sea - D		10	5	

Table S6 – PERMANOVA with Monte Carlo simulation P values comparing levels within factors. Within the habitat group analysis, the suffix -D indicates demersal habitat use. Bold indicates statistical significance p(MC) <0.05.

Trophic Guild		Temperature (°C)		Habitat		Phylogeny	
Main test	**0.001**	**Main test**	**0.001**	**Main test**	**0.001**	**Main test**	**0.001**
Top predator – Second order carviore	**0.001**	**26-31, 21-25**	**0.011**	**Pelagic, Deep sea**	**0.005**	**Lamniformes, Squaliformes**	**0.004**
Top predator – First order carviore	**0.001**	26-31, 16-20	0.052	**Pelagic, Deep sea-D**	**0.007**	Lamniformes, Orectolobiformes	0.059
Top predator – Herbivore	0.199	**26-31, 11-15**	**0.008**	**Pelagic, Non-complex-D**	**0.041**	Lamniformes, Carcharhiniformes	0.187
Second order carnivore – First order carnivore	**0.006**	**26-31, 6-10**	**0.001**	**Pelagic, Reef**	**0.047**	**Lamniformes, Myliobatiformes**	**0.021**
Second order carnivore – Herbivore	**0.019**	**26-31, 0-6**	**0.001**	**Pelagic, Reef-D**	**0.044**	Lamniformes, Squantiniformes	0.230
First order carnivore – Herbivore	**0.013**	**21-25, 16-20**	**0.008**	**Pelagic, Brackish**	**0.012**	**Lamniformes, Rajiformes**	**0.009**
		21-25, 11-15	**0.001**	**Deep sea, Deep sea-D**	**0.020**	Lamniformes, Chimaeriformes	0.073
		21-25, 6-10	**0.001**	**Deep sea, Reef**	**0.001**	**Squaliformes, Orectolobiformes**	**0.005**
		21-25, 0-6	**0.001**	**Deep sea, Reef-D**	**0.001**	**Squaliformes, Carcharhiniformes**	**0.001**
		16-20, 11-15	0.873	**Deep sea, Non-complex-D**	**0.004**	**Squaliformes, Myliobatiformes**	**0.001**
		16-20, 6-10	0.328	**Deep sea, Brackish**	**0.001**	Squaliformes, Squantiniformes	0.151
		16-20, 0-6	**0.014**	**Deep sea-D, Brackish**	**0.001**	**Squaliformes, Rajiformes**	**0.003**
		11-15, 6-10	0.051	**Deep sea-D, Reef**	**0.001**	Squaliformes, Chimaeriformes	0.245
		11-15, 0-6	**0.029**	**Deep sea-D, Reef-D**	**0.002**	Orectolobiformes, Carcharhiniformes	0.167
		6-10, 0-6	**0.005**	**Deep sea-D, Non-complex-D**	**0.006**	Orectolobiformes, Myliobatiformes	0.107
				Reef, Reef-D	**0.028**	Orectolobiformes, Squantiniformes	0.164
				Reef, Brackish	0.165	**Orectolobiformes, Rajiformes**	**0.012**
				Reef, Non-complex-D	**0.001**	Orectolobiformes, Chimaeriformes	0.089
				Reef-D, Brackish	**0.005**	**Carcharhiniformes, Myliobatiformes**	**0.014**
				Reef-D, Non-complex-D	**0.001**	Carcharhiniformes, Squantiniformes	0.148
				Brackish, Non-complex-D	**0.001**	**Carcharhiniformes, Rajiformes**	**0.001**
						Carcharhiniformes, Chimaeriformes	**0.040**
						Myliobatiformes, Squantiniformes	0.208
						Myliobatiformes, Rajiformes	**0.001**
						Myliobatiformes, Chimaeriformes	**0.017**
						Squantiniformes, Rajiformes	0.238
						Squantiniformes, Chimaeriformes	0.414
						Rajiformes, Chimaeriformes	0.187

Table S7 – The relative variable importance across each FA determined using a full glm model averaging approach (using the *MuMIn* package) with importance weighting of a subset of best-fit models assessed using AIC.

Fatty Acid	Factors	Relative variable importance	
		Importance	N containing models
16:0	Trophic guild	0.31	2
	Temperature	0.60	2
	Habitat	**1.00**	**4**
	Phylogeny	NA	0
18:0	**Trophic guild**	**1.00**	**2**
	Temperature	0.73	1
	Habitat	**1.00**	**2**
	Phylogeny	NA	0
18:1ω9	Trophic group	NA	0
	Temperature	0.25	1
	Habitat	0.23	1
	Phylogeny	**0.77**	**2**
20:1ω9	Trophic guild	NA	0
	Temperature	**1.00**	**1**
	Habitat	NA	0
	Phylogeny	**1.00**	**1**
20:4ω6	Trophic guild	0.77	2
	Temperature	0.23	2
	Habitat	**1.00**	3
	Phylogeny	NA	0
20:5ω3	**Trophic guild**	**1.00**	**1**
	Temperature	**1.00**	**1**
	Habitat	**1.00**	**1**
	Phylogeny	NA	0
22:4ω6	Trophic guild	0.46	1
	Temperature	**0.90**	**2**
	Habitat	0.10	1
	Phylogeny	NA	0
22:5ω6	Trophic guild	0.29	1
	Temperature	**1.00**	**2**
	Habitat	NA	0
	Phylogeny	NA	0
22:6ω3	Trophic guild	0.42	3
	Temperature	0.32	3
	Habitat	**0.92**	**4**
	Phylogeny	NA	0

Table S8 - Summary of BRUVS replicates for each location and habitat type observed on the video footage from Whitmarsh 2018.

Location	Habitat classification	Number of replicates	Average number of species/replicate	Average of the total MaxN/replicate
North Neptunes	Reef	9	17	99
	Sand	2	12	90
	Seagrass	0	-	-
	Pelagic	6	5	87
	Total	**17**		
South Neptunes	Reef	5	18	111
	Sand	6	9	93
	Seagrass	0	-	-
	Pelagic	6	1	14
	Total	**17**		
Dangerous Reef (Control)	Reef	1	22	63
	Sand	7	6	90
	Seagrass	4	15	60
	Pelagic	6	2	23
	Total	**18**		

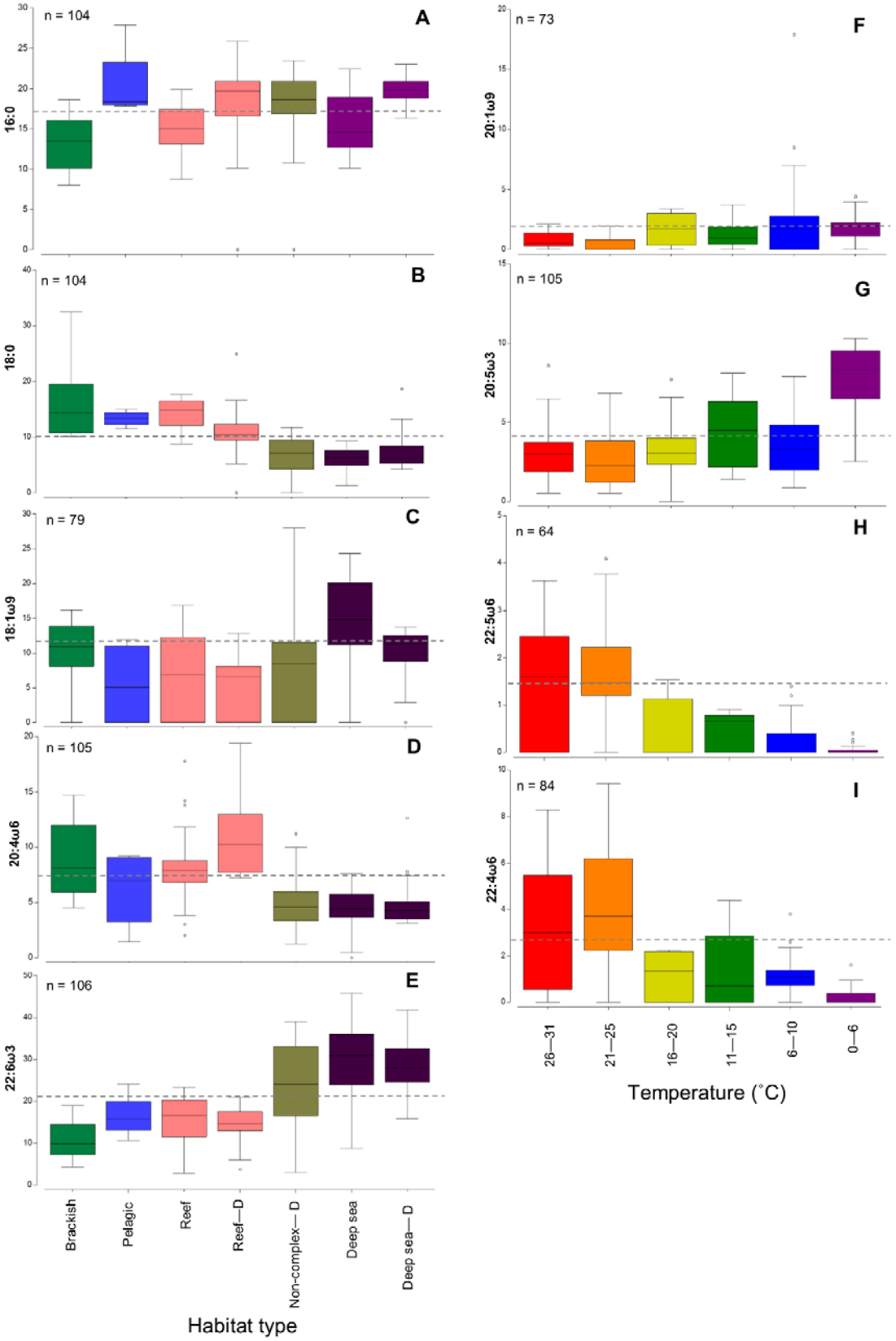

Fig 6ω3(E) across the seven habitat types (D- indicates demersal habitat use) and 20:1ω9(F), 20:5ω3(G), 22:5ω6(H) and 22:4ω6(I) across water temperature groups. The grey line indicates the mean FA value across all records in which it was reported. The factors plotted for each FA presented here are those with the highest relative variable importance determined using the full glm model averaging approach (Table S7).

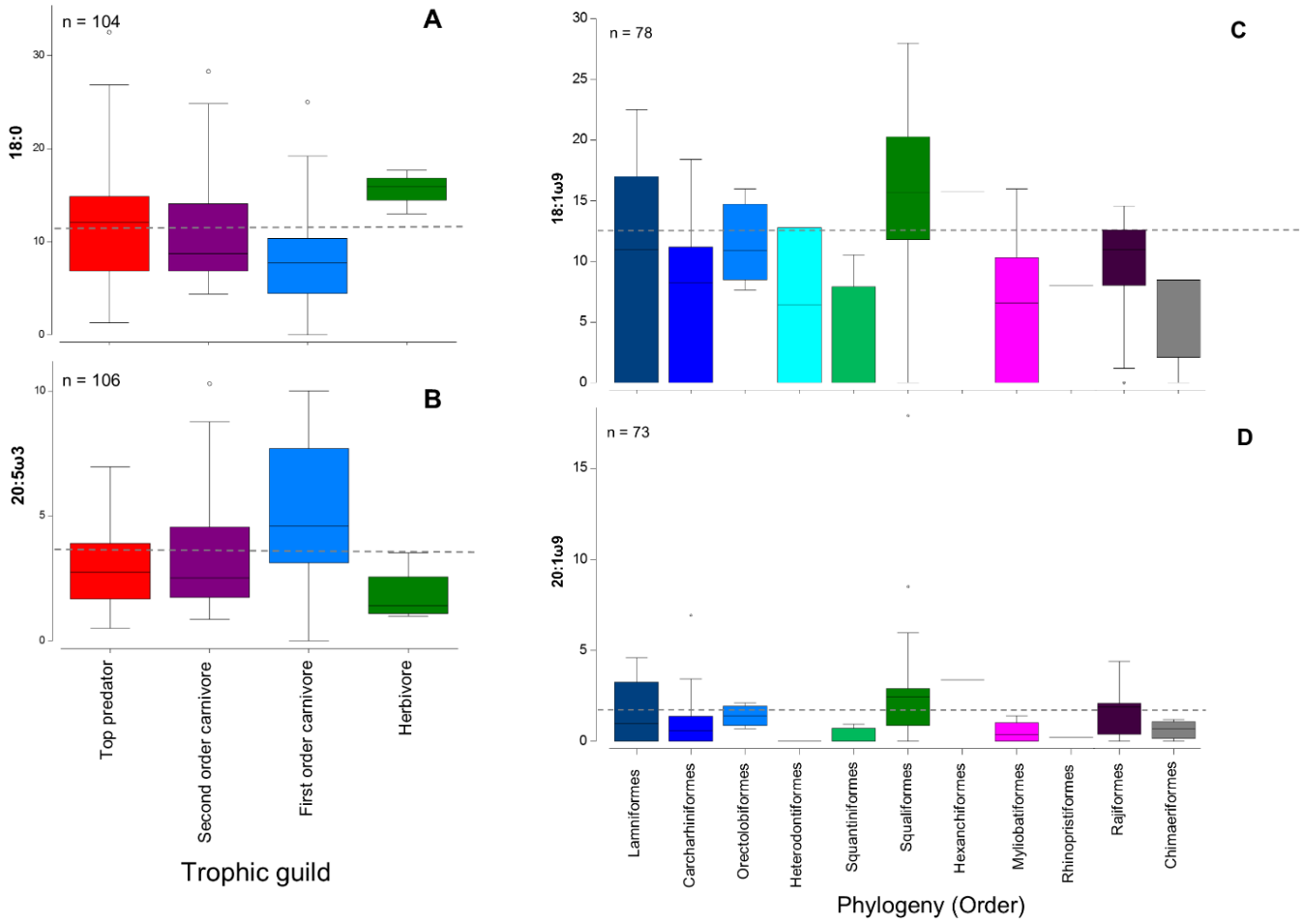

Figure S2 – Boxplots of untransformed fatty acid values for 18:0(A) and 20:5ω3(B) across trophic guild and 18:1ω9(C) and 20:1ω9(D) across phylogenetic groups. The grey line indicates the mean FA value across all records in which it was reported. The factors plotted for each FA presented here are those with the highest relative variable importance determined using the full glm model averaging approach (Table S7).

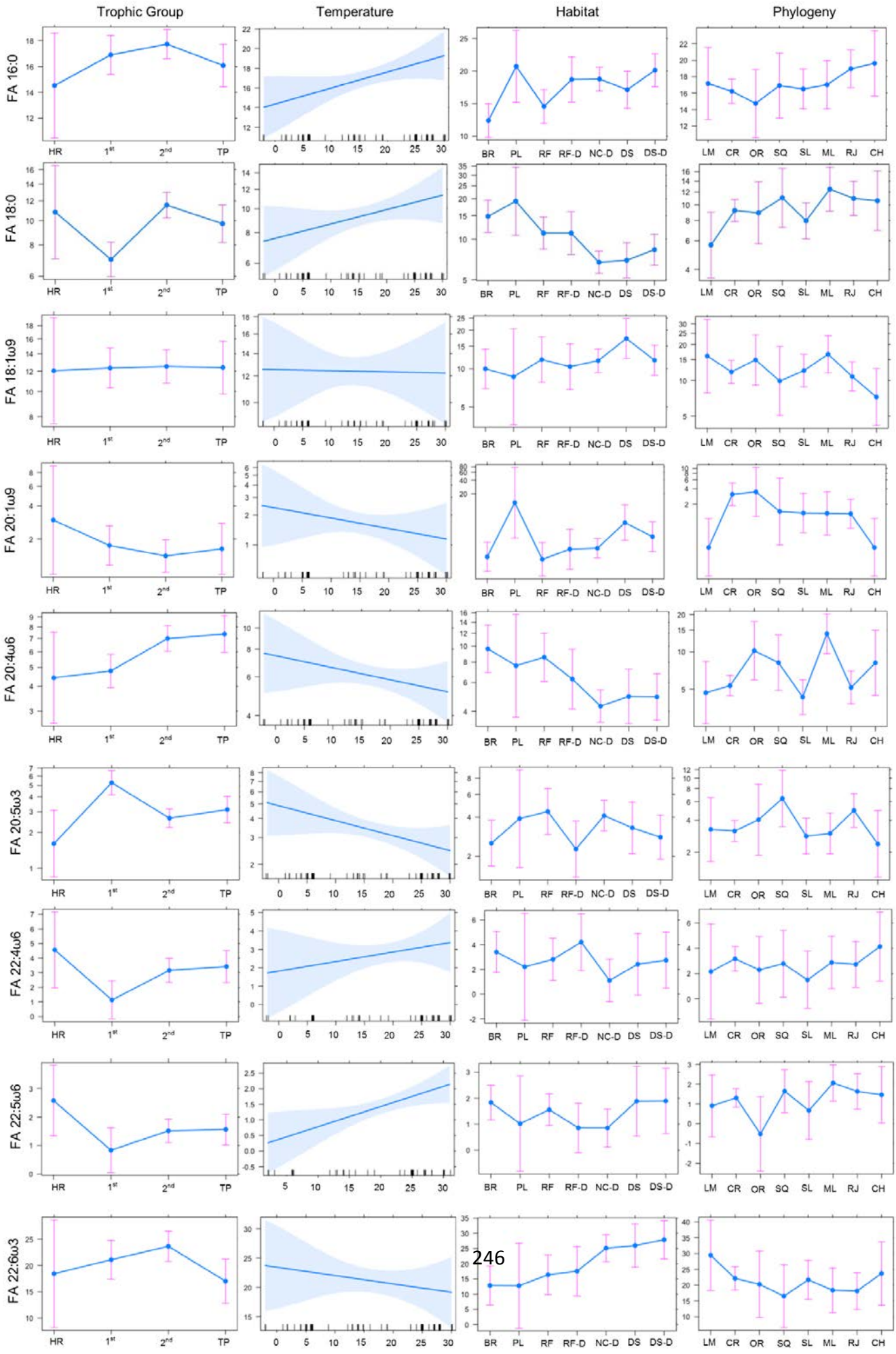
Trophic Group
Temperature
Habitat
Phylogeny
FA 16:0
FA 18:0
FA 18:1ω9
FA 20:1ω9
FA 20:4ω6
FA 20:5ω3
FA 22:4ω6
FA 22:5ω6
FA 22:6ω3
HR
1st
2nd
TP
BR
PL
RF
RF-D
NC-D
DS
DS-D
LM
CR
OR
SQ
SL
ML
RJ
CH

Figure S3 – Effect plots for fatty acid (FA) % compositions derived from the generalised linear model containing all four factors; trophic group, temperature, habitat and phylogeny. The figure was constructed by plotting the allEffects function of the model using the 'Lme4' package (Bates et al., 2015). Within the trophic guild factor, the factor levels are denoted as HR – Herbivore, 1st – First order carnivore, 2nd – Second order carnivore, TP – Top predator. Temperature (˚C) intervals are displayed along the x-axis within column 2. Habitat factor levels are abbreviated as BR – Brackish, PL – Pelagic, RF – Reef, RF-D – Reef demersal, NC-D – non-complex demersal, DS – Deep sea, DS-D – deep sea demersal. Phylogenetic factor levels are abbreviated as LM- Lamniformes, CR – Carcharhiniformes, OR – Orectolobiformes, SQ – Squantiniformes, SL-Squaliformes, ML – Myliobatiformes, RJ – Rajiformes, CH – Chimaeriformes.

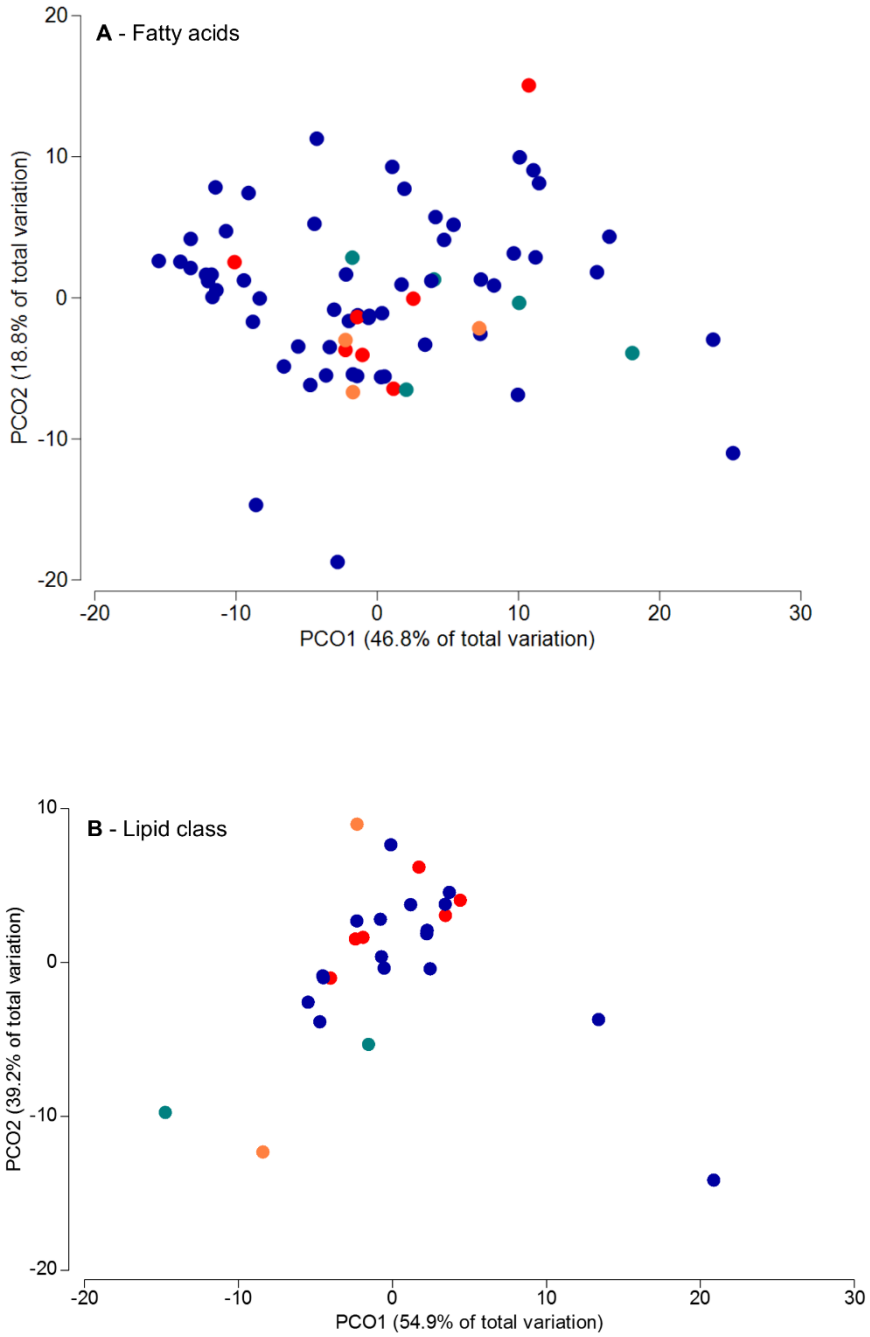

Figure S4 – Principle Coordinate Analysiws of white shark *Carcharodon carcharias* muscle **A** – Fatty acid profiles, and **B** – Lipid class profiles. Dark blue symbols indicate individual sharks which have spent <1 week at the Neptune Islands, green 1–2 weeks, orange 2–3 weeks and red >3 weeks.