Fatty acid profiles of a benthic chondrichthyan: captive feeding trials and ecological applications

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Summary

Fatty acid analysis is a tool for dietary investigation that complements traditional stomach content analyses. There is little known about how sharks alter dietary fatty acids following incorporation into tissues, the stability and turn-over rates of fatty acid in tissues, and whether specific fatty acids are selectively retained within tissues. Four controlled feeding experiments were used to determine the extent to which the fatty acid composition of diet is reflected in the tissues of the Port Jackson shark *Heterodontus portusjacksoni* and to improve our understanding of which fatty acid biomarkers can be used to reliably distinguish prey types. The first experiment tested the fatty acid profile of muscle and liver tissues and used two groups of sharks fed exclusive diets of prawns or squid, and an unfed control group. The liver and muscle fatty acid profiles showed significant differences between the groups fed exclusive diets and unfed control sharks, suggesting that the extent of dietary change was strong enough that both tissues could be used as indicators of diet. The different diet fed to sharks could, however, only be distinguished based on the liver fatty acid profiles, with prawn-fed sharks comparatively higher in 18:1n-7, 22:5n-3, 20:0 and 18:1n-9, while squid-fed sharks were higher in 16:0 and 22:6n-3. The lack of differences in the muscle fatty acid profiles suggesting that diet was not different enough to cause a change in the muscle fatty acid in the duration of the experiment. The second experiment further investigated liver and muscle fatty acid profile dynamics during a dietary change and assessed the fatty acid profiles of sharks fed squid for six weeks followed by prawns for an additional six weeks. This experiment showed significant differences in the liver and muscle fatty acid profiles within three weeks of a dietary switch driven by 22:6n-3 in the liver and muscle and 16:1n-7 in the liver. Higher levels of dissimilarity were observed before and after the dietary change in the muscle tissue which may indicate that dietary fatty acids are preferentially used in the muscle following a dietary change. Changes in fatty acid profiles over time and different incorporation rates between tissues were also evident when sharks were fed exclusive diets of artificial fish or poultry oil pellets for a period of 18 weeks. The fatty acid profiles from the liver and blood serum of fish oil and poultry oil fed sharks were significantly different within 12 weeks while the

muscle fatty acid profiles of fed sharks did not differ until week 18. The drivers of dissimilarity which aligned with dietary input were 14:0, 18:2n-6, 20:5n-3, 18:1n-9 and 22:6n-3 in the liver and blood serum. The fourth experiment used yolks sampled from viable egg cases and recently hatched neonates fed a known diet to determine whether the fatty acids present in high levels in yolks or in diets are reflected in the liver and muscle tissues of hatchlings. The fatty acid profiles of hatchling tissues were more similar to yolk than to diet, demonstrating the conservative transfer of fatty acid from egg yolks to hatchlings as well as the preferential retention of some fatty acids in the muscle and liver. Specifically, arachidonic acid (ARA, 20:4n-6) was preferentially retained likely as a result of eicosanoid production during growth; dietary docosapentaenoic acid (DPA, 22:5n-3) was not reflected in shark tissues and is likely catabolised for energy; docosahexaenoic acid (DHA, 22:6n-3) was reflected in tissues and was a good dietary indicator; and high proportions of saturated fatty acids were retained in the muscle at the expense of dietary polyunsaturated fatty acids.

The application of fatty acid profile analysis to investigate dietary information of wild specimens was assessed through the comparisons of fatty acid profiles and stomach contents from three locations. Fatty acid profile analysis indicated significant differences between the three locations sampled, however, stomach content analysis did not show significant differences between the two closest locations (Gulf St Vincent and Spencer Gulf). The discrepancy in the results from the two methods highlighted the ability of fatty acid profiles to complement information obtained from stomach content analysis. For example, soft-bodied prey such as molluscs which have their shells crushed and are rapidly digested, were underrepresented in the stomach content analysis, but could be readily detected in 16:1n-7 levels. Fatty acid analysis is increasingly powerful as a tool in studies of trophic ecology in marine ecosystems. This biochemical technique has become useful in deciphering spatial and temporal variability in diets, identifying predation on key species, and providing dietary information which is not always obtainable using more traditional methods.

Declaration

Statement of Originality

This thesis contains no material which has been accepted for a degree or diploma by the University or any other institution, except by way of background information and duly acknowledged in the thesis. To the best of my knowledge and belief, no material previously published or written by another person except where due acknowledgement is made in the text of the thesis. This thesis may be available for loan and limited copying in accordance to the *Copyright Act 1968*.

Crystal L. Beckmann

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Statement of Co-authorship

Chapter 1

The introduction was my own work. My supervisors, Charlie Huveneers and Jim Mitchell proofread earlier versions of this chapter.

Chapters 2–6 of this thesis have been prepared as scientific manuscripts and I am the primary author for each paper. Chapters which have been published are identified on the title page for the chapter. In all cases experimental design, field and laboratory work, data analysis and interpretation, and manuscript preparation were the primary responsibility of the candidate. However, they were carried out in collaboration with supervisors. Contributions of co-authors are outlined below.

Chapter 2 and 3

Charlie Huveneers provided advice on the experimental design. Co-authors provided information and important citations, and assisted to the drafting of the chapters by critically revising them.

Chapter 4, 5 and 6

Charlie Huveneers and David Stone provided advice on the experimental design. Co-authors provided information and important citations, and assisted to the drafting of the chapters by critically revising them.

Chapter 7

The conclusion was my own work. My supervisor, Charlie Huveneers proofread earlier versions of this chapter.

List of publications

Peer-Reviewed publications relevant to this thesis

Beckmann CL, Mitchell J, Seuront L, Stone DAJ and Huveneers C (2014) From egg to hatchling: preferential retention of fatty acid biomarkers in young-of-the-year sharks. Journal of Fish Biology. DOI: 10.1111/jfb.12451

Beckmann C, Mitchell JG, Stone DAJ and Huveneers C (2014) Inter-tissue differences in fatty acid incorporation as a result of dietary oil manipulation in Port Jackson sharks (*Heterodontus portusjacksoni)*. Lipids 49(6):577–590.

Beckmann CL, Mitchell JG, Stone DAJ, 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*. Journal of Experimental Marine Biology and Ecology 448:10–18.

Beckmann CL, Mitchell JG, Seuront L, Stone DAJ and Huveneers C (2013) Experimental Evaluation of Fatty Acid Profiles as a Technique to Determine Dietary Composition in Benthic Elasmobranchs. Physiological and Biochemical Zoology 86:266–278.

Beckmann CL., Mitchell JG, Stone DAJ and Huveneers C (in review), Stomach content and fatty acid biomarkers as indicators of spatial variations in the diet of Port Jackson shark (*Heterodontus portusjacksoni*) in southern and eastern Australia, Marine Ecology Progress Series.

Conference Presentations relevant to this thesis

Oceania Chondrichthyan Society and Australian Society for Fish Biology joint conference, Adelaide 2012, "Indicator fatty acids in multiple shark tissues and blood serum resulting from different artificial pellet diets"

Flinders University Postgraduate Conference, June 2012, "Validation of the use of Fatty Acid profiles to assess diet in sharks"

Oceania Chondrichthyan Society, Gold Coast 2011, "Validation of the use of Fatty Acid profiles to assess diet in sharks"

Flinders University Postgraduate Conference, June 2011, "Validation of the use of Fatty Acid profiles to assess diet in sharks"

Sharks International, Cairns 2010, "Using fatty acid signatures to infer diet in elasmobranchs"

Other Publications or contributions

Huveneers, C, Rogers PJ, **Beckmann C,** Semmens JM, Bruce BD and Seuront L (2013) The effects of cage-diving activities on the fine-scale swimming behaviour and space use of white sharks, Journal of Marine Biology 160(11):2863–2875

Huveneers C, Rogers PJ, Semmens J, **Beckmann C**, Kock AA, Page B and Goldsworthy S (2013) Behavioural effects of an electric field on white sharks: in situ testing of an electric deterrent. Plos One. 8(5): e62730.

Huveneers C, Rogers PJ, Semmens J, **Beckmann C**, Kock AA, Page B and Goldsworthy S.D (2012) Effects of the Shark Shield ^{TM} electric deterrent on the behaviour of white sharks (*Carcharodon carcharias*). Final report to SafeWork South Australia. Version 2. South Australian Research and Development Institute (Aquatic Sciences), Adelaide. SARDI Publication No. F2012/000123-1. SARDI Research Report Series No. 632. 61pp.

Huveneers C, Rogers PJ, **Beckmann C**, Semmens J, Bruce B, and Seuront L (2012) Effects of a cage-diving operation on the fine-scale movement of white sharks (*Carcharodon carcharias*). Final report to the Department of Environment, Water and Natural Resources Wildlife Conservation Fund and the Nature Foundation of South Australia. South Australian Research and

Development Institute (Aquatic Sciences), Adelaide. SARDI Publication No. F2012/000417-1. SARDI Research Report Series No. 657. 48pp.

Beckmann, C & Huveneers C (2011) *Notoraja sticta*. In: IUCN 2013. IUCN Red List of Threatened Species. Version 2013.1. www.iucnredlist.org.

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List of Abbreviations

ARA arachidonic acid 20:4n-6
ALA alpha-linoleic acid, 18:3n-3
ANOSIM Analysis of Similarity
ANOVA analysis of variance
CAP Canonical analysis of principal coordinates
DF degrees of freedom
DHA docosahexaenoic acid
DMA dimethyl acetal
DPA docosapentaenoic acid, 22:5n-6
EFA essential fatty acid
EPA eicosapentaenoic acid, 20:5n-3
FA fatty acid
FAME fatty acid methyl ester
FFA free fatty acid
FID Flame ionization detector
FO fish oil
FO frequency occurrence
GSV Gulf St Vincent
HUFA Highly unsaturated fatty acids
IRI Index of relative importance
LNA Linoleic acid, 18:2n-6
LPT lowest possible taxonomic level
MDS multidimensional scaling
MISA Marine innovation South Australia
MS mean squares
MUFA monounsaturated fatty acid
OLA Oleic acid, 18:1n-9
PAM 16:0
PERMANOVA permutational analysis of variance
PO Poultry oil
POA palmitoleic acid, 16:1n-7
PUFA polyunsaturated fatty acid
SARDI South Australian Research and Development Institute
SFA or SATFA saturated fatty acid
SE standard error
SG Spencer Gulf
SIMPER similarity percentage analysis
SS sum of squares
TL Total length
VAvaccenic acid, 18:1n-7
W Weight
YOY young-of-the-year

Chapter 1: General Introduction

1.1 Chondrichthyan ecology

Many chondrichthyan species (sharks, batoids, and chimaeras) are particularly vulnerable to overfishing due to their low fecundity, late maturity, and slow reproductive cycle (Baum et al. 2003; Musick et al. 2000; Stevens et al. 2000). In recent years, there has been a worldwide decline in commercially exploited fish stocks, including sharks, threatening the future of many large vertebrates (Baum and Worm 2009; Field et al. 2009; Myers and Worm 2003). Many sharks and rays are predators at, or near, the top of marine food webs and can have significant impacts on lower trophic levels (Heithaus et al. 2010; Ruppert et al. 2013; Stevens et al. 2000). For example, the depletion of large predatory sharks has been suggested to have led to the collapse of other fisheries through indirect trophic interactions, e.g. a century-old scallop fishery (Baum et al. 2003), or behaviour-mediated trophic cascade initiated by tiger sharks (Burkholder et al. 2013). It is, therefore, important to improve our knowledge of predator-prey interactions to understand how species can impact each other, and to account for these interactions within multi-species stock assessments and ecosystem-based management. The quantification of a species diet is fundamental to understanding trophic interactions. Most ecosystem models are generated to guide policy makers, relying on dietary information to assess the effects of changes in species abundance (Christensen 1995; Walters et al. 1997; Yodzis 1998) or to investigate the impact of large-scale fisheries at a broad ecosystem level (Goldsworthy et al. 2013). These models are, however, susceptible to a lack of diet composition data (Cox et al. 2002), particularly where regional, seasonal, and ontogenetic differences affect overall dietary composition (Braccini and Perez 2005).

Historically, the diet composition and feeding habits of sharks have commonly been studied through the examination of stomach contents (Cortés 1997; Hyslop 1980). As stomach content analysis can be hampered by low sample sizes, high levels of unidentifiable items, and biases towards least digestible preys and the last meal consumed, complementary methods such as molecular identification of prey (Dunne et al. 2010; Sigler et al. 2006), stable isotope analysis (Domi et al. 2005; Estrada et al. 2003; Fisk et al. 2002; Hussey et al.

2011), and fatty acid analysis (Belicka et al. 2012; McMeans et al. 2013, 2013; Pethybridge et al. 2010; Pethybridge et al. 2011a; Schaufler et al. 2005; Wai et al. 2011) have recently increased in popularity. Fatty acids have the potential to provide detailed information on consumer diets and by generating a large number of fatty acids per sample, the interpretation of fatty acid profiles can provide a greater level of complexity than possible in stable isotope analysis. Fatty acid biomarkers are characteristic of particular classes of prey and it is possible to differentiate between trophic levels. Stable isotopes also reflect food consumed and assimilated and the shifts in stable isotope ratios can be used to provide more general dietary information such as trophic position and trophic links.

1.2 What are fatty acids?

Fatty acids are components of lipids or fat, which along with proteins, are the major organic components of vertebrates (Tocher 2003). The major classes of lipids include triacylglyerols, wax esters, phosphoglycerides, sphingolipids and sterols all of which, excluding cholesterol, contain fatty acids (Sargent et al. 2002). In sharks, the muscle has a low fat content and is generally dominated by polar lipids (glycolipids and phospholipids) while the liver has a high fat content and is made up of mostly neutral lipids (triacylglcerols and wax esters) (Deprez et al. 1990, Wetherbee & Nichols 2000, Pethybridge et al. 2010). Fatty acids play key roles in metabolism as major metabolic fuels, essential components of all membranes and as gene regulators (Sargent et al. 1999). Structurally, fatty acids are composed of carbon chains, with a methyl group at one end of the molecule (omega, ω or n) and a carboxyl group at the other end (Figure 1.1). Fatty acids are either saturated (SFA) with an even number of carbon atoms, monounsaturated (MUFA) with one carbon-carbon double bond, or polyunsaturated (PUFA) with the first double bond between the third and the fourth carbon atom (n-3) or between the sixth and seventh carbon atom (n-6). These n-6 and n-3 fatty acids are also sometimes referred to as omega-3 and omega-6 fatty acids and are derived from the essential fatty acids (EFAs) linoleic acid (LA, 18:2:n-6) and α-linoleic acid (ALA, 18:3n-3), respectively (Figure 1.2). As PUFAs are produced only by

plants and phytoplankton, they are essential in the diet of all other organisms. Essential fatty acids, which are important for normal growth, development, and reproduction, need to be gained from dietary intake in the form of PUFA (Sargent et al. 1995). Furthermore, n-3 and n-6 PUFA cannot be interconverted and both are essential nutrients. Other fatty acids can be metabolised in the body through desaturation or β-oxidation. The extent of these reactions, however, varies between species and can be affected by physiological capability and available enzymes (Sargent et al. 2002).

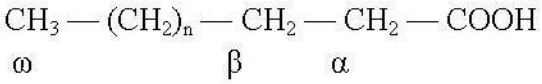

Figure 1.1 The systematic nomenclature of fatty acids illustrated with a fatty acid carbon chain. A methyl group is shown at one end on the molecules (designated omega ω) and a carboxyl group at the other end. The carbon atom next to the carboxyl group is designated as the α carbon and the subsequent one is the β carbon. Systematic nomenclature describes the methyl (ω) end and the position of double bonds from the end of the fatty acids. The letter n can be used to describe the ω position of double bonds and the systematic nomenclature for fatty acids indicates the location of double bonds with reference to the carboxyl group (Δ).

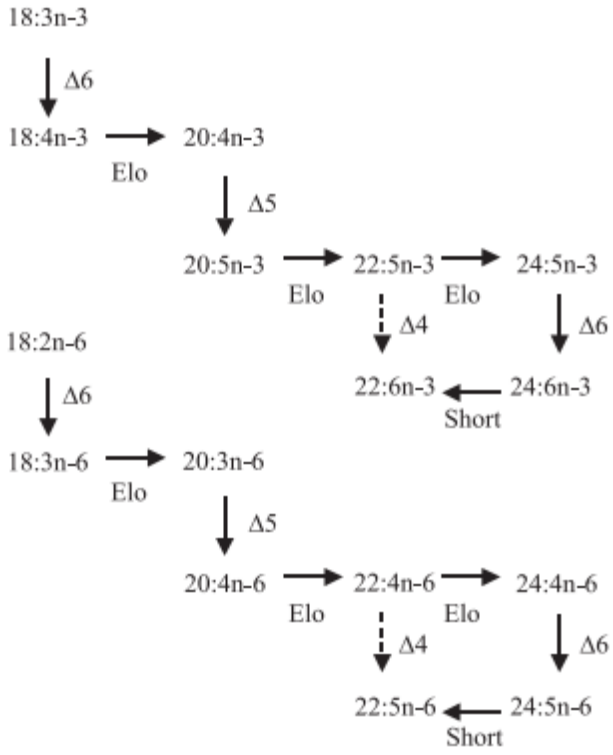

Figure 1.2 Metabolic pathways of omega-6 and omega-3 fatty acids demonstrating the conversion of dietary 18:2n-6 and 18:3n-3 to long chain, highly unsaturated fatty acids (HUFA). Solid lines represent steps that have been shown to occur in fish, while dotted lines show steps which have not been directly demonstrated in fish. Δ6, Δ5 and Δ4 represent fatty acid desaturases; Elo represents fatty acid elongases and short represents peroxisomal chain shortening. Modified from Zheng et al. (2004).

Fatty acid analysis requires an understanding of how ingested fatty acids are metabolised and deposited in various tissues of the consumer. In vertebrates, including in marine mammals, seabirds (Käkelä et al. 2009; Wang et al. 2010; Williams et al. 2009), and teleosts (Sargent et al. 1999; Tocher 2003), these processes are quite well understood. Marine vertebrates, including sharks, have the ability to synthesise some fatty acids *de novo* or through lipogenesis in the body. The process of lipogenesis is regulated by enzymes and as such the presence or absence of specific enzymes can confer the ability on an animal to manufacture a specific fatty acid. For example, as all vertebrates, including fish, lack Δ 12 and Δ 15 (omega 3) desaturases, they cannot form 18:2n-6 (LNA) or 18:3n-3 (ALA) from oleic acid, (OLA, 18:1n-9, Tocher 2003). These fatty acids must therefore be obtained through the diet and can be used as indicators of dietary transfer. Animals generally accumulate docosahexaenoic acid (DHA, 22:6n-3) rather than

docosapentaenoic acid (DPA, 22:5n-6). In addition to dietary input, DHA accumulation may reflect enzyme availability and specificity (Tocher 2003). The poor rate of conversion of ALA to eicosapentaenoic acid (EPA, 20:5n-3) and DHA in all marine fish species studied so far suggests that the fatty acid rate of conversion is affected by dietary availability (Tocher 2003). As chondrichthyan diets are lipid rich they do not rely heavily on endogenous lipid biosynthesis (Metcalf and Gemmell 2005). High concentrations of EPA and DHA, in particular those originating mainly in diatoms and flagellates, where they are transmitted intact via zooplankton to fish, have resulted in a decreased need for lipogenesis (Tocher 2003).

While other nutrients, such as proteins, are easily broken down during digestion, fatty acids are released from ingested lipid molecules and taken up by the tissue (Iverson et al. 2004). Once taken up by tissues, they are used for energy or re-esterified and stored (Iverson et al. 2004). Chondrichthyan lipid metabolism can, however, involve a diversity of storage forms and has a different metabolic organisation than other vertebrate groups (Ballantyne 1997). It has been suggested that chondrichthyans lack true adipose tissue, resulting in the liver serving as the main lipid storage site (Ballantyne 1997). The liver is very large in most sharks and in addition to fat storage for energy it can contain squalene and glyceryl ether for buoyancy purposes (Ballantyne 1997; Moyes et al. 1990; Pethybridge et al. 2010). Lipid class composition of the liver differs between families and is related to vertical habitat distribution and buoyancy needs (Pethybridge et al. 2010). Lipid class composition of the liver differs between families and is related to vertical habitat distribution and buoyancy needs (Pethybridge et al. 2010). In addition, chondrichthyans have a limited ability for β-oxidation, the process by which fatty acid molecules are broken down in the mitochondria in the first stage of fatty acid catabolism (Watson and Dickson 2001). Instead of utilizing fatty acids as fuels outside of the liver, chondrichthyans may utilise ketogenisis to provide the fuel which is distributed to other tissues for ketone body oxidation (Watson and Dickson 2001).

1.3 Fatty acid biomarkers

Fatty acid biomarkers are a useful method for determining food web relationships and dietary habits of marine species (Graeve et al. 1994). Specific fatty acids or ratios between fatty acid biomarkers can provide an indication of dietary consumption and trophic position. These fatty acid biomarkers are incorporated into consumers with relatively minor or predictable modifications (Budge et al. 2006; Dalsgaard et al. 2003; Iverson et al. 2004). For example, phytoplankton-based food webs are characterised by 16:1n-7, 18:4n-3, and 18:1n-7 (Falk-Petersen et al. 2000). Within phytoplankton based foodwebs, 16:1n-7, C16 PUFA, EPA, and ratios of >1 for 16:0/16:1n-7 can also indicate diatom-based food webs (Falk-Petersen et al. 2000; Kharlamenko et al. 1995). Dinoflagellate consumption is indicated by high proportions of 18:4n-3, 18:5n-3, and 22:6n-3 (Falk-Petersen et al. 2000), and carnivory can also be shown by low proportions of DHA, 16:1n-7, 18:1n-9, and a low ratio of 18:1n-9/18:1n-7 (Cook et al. 2000; Kharlamenko et al. 1995). Bacterial fatty acid biomarkers, often used to distinguish detrital sources, are indicated by the proportion of odd-chained and branched fatty acids, and the ratio of 18:1n-9/18:1n-7 fatty acids (Cook et al. 2010; Kharlamenko et al. 1995; Sargent et al. 1987).

When relating consumer fatty acids to diet, the ratios of polyunsaturated fatty acids are often used as indicators of diet. Phytoplankton, rich in EPA and poor in arachidonic acid (ARA, 20:4n-6), are the major source of long-chain PUFA for coastal and offshore pelagic species as well as deeper offshore demersal species (Dunstan et al. 1988). Omnivorous species, which feed on macroalgae, are considered rich in ARA, while carnivorous species are low in ARA (Dunstan et al. 1988). Carnivorous bony fish, which predominantly feed from the pelagic food web have the lowest levels of ARA (0.8–3%) and moderate to high levels of EPA (6.8–11.6%, Dunstan et al. 1988). Demersal carnivores (both cartilaginous and bony fishes) generally have intermediate values for ARA (3–7.6%) and EPA (4.2–11.3%), as they do not feed primarily from either the benthic macro-algae (ARA-rich) or the pelagic phytoplankton (ARA-poor) food webs (Dunstan et al. 1988). Demersal carnivores generally feed on invertebrates and small fish, which may use nutrients from both food webs indirectly via the detrital food chain. Additionally,

taxonomic groups can have distinct profiles. For example, cartilaginous species are considered to have low levels of EPA, cephalopods have high levels of EPA, macroalgae are generally rich in ARA and EPA, while seagrass have high levels of 18:2n-6 and 18:3n-3 (Dunstan et al. 1988).

1.4 Ecological application of fatty acid profiles in chondrichthyans

Fatty acid profiles can be obtained from various tissues, e.g., muscle, liver, blood, connective tissue, and an increasing number of studies are accounting for differences in fatty acid composition between tissues. In deepwater chondrichthyans, Pethybridge et al. (2011a) showed that the fatty acid profiles of the digestive fluid from the stomach and intestine were the most closely aligned with stomach content data. Differences in the liver and muscle fatty acid profiles were observed, with the muscle fatty acid profile being biased towards PUFA rich organisms and the liver towards MUFA rich organisms (Pethybridge et al. 2011a). A study investigating sleeper sharks, the Greenland shark (*Somniosus microcephalus*), was performed to determine within- and among-tissue variability in the muscle, liver, and blood plasma fatty acid profiles (McMeans et al. 2012). Shark liver was shown to have the most variable fatty acid profiles among individuals, retaining higher levels of long-chain MUFA such as 20:1n-9. The muscle tissue was, however, the most similar to prey fatty acid signatures and the blood plasma showed more similarities to the muscle than the liver suggesting the muscle fatty acids were of dietary origin. The timescales over which fatty acids are incorporated into different tissues sharks is, however, still unclear. Semeniuk et al.(2007) observed that the blood serum, excluding fibrinogens, of the southern stingray was reflective of long-term dietary patterns, while McMeans et al. (2012) suggested that blood plasma, including fibrinogens is a short-term dietary indicator, as this is also the case in seabirds (Käkelä et al. 2009). Experimental evaluation of the differences between tissue and blood fatty acid profiles is required to determine which tissues reflect diets and to assess the timescales over which dietary influences on fatty acid profiles can be detected.

The different tissues are known to have different physiological roles, which can influence the reliability of interpreting fatty acid profiles. The liver is the main site of lipid storage and metabolism (Bone and Roberts 1969; Medzihradsky et al. 1992), while the blood transports stored lipids to other tissues, such as the muscle, to carry out a variety of metabolic functions (Ballantyne et al. 1993). Utilising fatty acid profiles of the muscle tissue and blood serum is advantageous as the methods are not lethal. The physiological role of the muscle should, however, be considered as shark muscle generally only contains low levels of fat and is dominated by structural, cell-membrane phospholipids, rather than fat storage for energy (Jobling 2003). For example, high levels of dietary ARA have been linked with dietary intake in whale shark connective tissue and reef manta ray muscle Couturier et al. 2013). In addition to dietary input, levels of DHA, EPA and ARA in the muscle could be influenced by their involvement in the maintenance of the structures and functions of phospholipid cell membranes and their role as precursors to the paracine hormones known as eicosanoids (Sargent et al. 1999). Furthermore, accurate interpretations using the muscle and connective tissue alone are difficult without the knowledge of how dietary fatty acids are distributed to other tissues. For example, most animal species can convert LNA to ARA and while only traces of LNA (<1%) were detected in the tissues of whales sharks and manta rays (Couturier et al. 2013), the levels of this fatty acid in their liver were unknown. As the liver of chondrichthyans serves as the main lipid storage site and extrahepatic fatty acid catabolism is limited (Ballantyne 1997), the possible conversion of LNA to ARA (Figure 1.2) in the liver cannot be excluded. The fatty acid profile dynamics of shark tissues require further investigation, with a focus on essential fatty acids, fatty acid biomarkers, and metabolic pathways, to assess how fatty acid profiles of consumers reflect dietary patterns.

Fatty acid profiles have previously been used as a corroborative method to support predator-prey relationships, usually with stomach content analysis and/or stable isotopes. In chondrichthyans, fatty acid profiles have primarily been used to determine the diet of cryptic species. For example, a comprehensive study of demersal chondrichthyans in south-east Australia used fatty acid profiles to complement limited stomach content data, distinguish inter-specific differences, and detect degrees of resource overlap (Pethybridge et al. 2011a). The study

focused on inter-specific differences between sharks and chimeras, while intra-specific variation was not examined because of the limited sample sizes for most species. An investigation of the degree of predation on pinnipeds by Pacific sleeper sharks (*Somniosus pacificus*) identified a trophic relationship with planktivorous whales as indicated by high concentrations of 22:1n-11 and 20:1n-11 in their liver and muscle tissues (Schaufler et al. 2005). This was associated with the consumption of calanoid copepods, which are heavily consumed by planktivorous whales rather than pinnipeds (Schaufler et al. 2005). The diet of another species of sleeper shark, the Greenland shark, was examined by McMeans et al. (2013) using a combination of stomach contents, stable isotopes, and fatty acids with the aim to provide a more complete view of the species diet in Kongsifjorden. The Greenland sharks sampled in Kongisfjorden were found to rely heavily gadoids and seals (McMeans et al. 2013), compared to sharks sampled in Cumberland Sound, which feed predominantly on halibut and seals (McMeans et al. 2012). McMeans et al. (2013) suggest that high proportions of seal fatty acid biomarkers in the muscle and blood plasma are reflective of the ability of sharks to regularly capture and consume seals. A similar investigation of the diet of whale sharks (*Rhincodon typus)* and reef manta rays (*Manta alfredi)* demonstrated that these planktivorous feeders displayed unusually high levels of n-6 PUFA (Couturier et al. 2013). This was unexpected as direct observation of feeding suggested feeding on surface aggregations of crustaceous zooplankton, which are typically dominated by n-3 PUFA (Couturier et al. 2013). High levels of n-6 PUFA such as ARA would be expected in benthic feeders, which feed on ARA rich macroalgae (Dunstan et al. 1988). While whale sharks and manta rays feed close to the sea floor, they are unlikely to target benthic prey, but may encounter benthic zooplankton during their diel vertical migration (Couturier et al. 2013). These studies suggest chondrichthyans exhibit complex feeding ecology, which may not be detected without the use of complimentary techniques including fatty acid profile analysis. Further investigation is required at different spatial scales using a combination of stomach content and fatty acid analyses of multiple tissues to investigate how fatty acid profiles vary between regions and among demographic groups, and to examine the relationship between trends in fatty acid profiles and stomach contents.

Fatty acid profiles have also regularly been used to assess changes in diet such as through anthropogenic changes, ontogenetic shifts, or seasonal diet variations. Anthropogenic feeding of southern stingrays (*Dasyatis americana*) was demonstrated through elevated concentrations of n-3 PUFA and decreased concentrations of n-6 PUFA in the blood serum nonesterified fatty acids (NEFAs) (Semeniuk et al. 2007). Despite no controlled experiments examining the temporal patterns in blood serum fatty acids, this study provides some justification for further experimentation as serum NEFAs represented mobilised fatty acids from storage in the liver, demonstrating long-term dietary patterns (Semeniuk et al. 2007). Ontogenetic changes were indicted in a study of Greenland sharks showing that the proportions of seal fatty acid biomarkers increased with total length, which was related to increasing dietary preference or capture ability (McMeans et al. 2013). A study on white spotted bamboo sharks (*Chiloscyllium plagiosum)* showed that this species relied on primary consumers and exhibited an ontogenetic dietary shift, with increasing animal-derived fatty acids with size, and seasonal differences (Wai et al. 2011). Similarly, large-scaled ontogenetic shifts in were shown for the longnose velvet dogfish (*Centroselachus crepidater*, Pethybridge et al. 2011a). These studies demonstrate the ability of fatty acid profiles to identify changes in diet. The diet assessment of small juveniles, particularly young-of-the-year sharks (YOY), should, however, be undertaken with caution as fatty acid profiles may be influenced by the maternal signature. Maternal yolk lipid allocations have been observed in deepwater chondrichthyans which displayed a high degree of maternal lipid investment (Pethybridge et al. 2011b). Young-of-the-year bull sharks (*Carcharhinus leucas)* were also shown to be highly enriched with $\delta^{13}C$, which is reflective of maternal diet, and 20:3n-9, which indicates an essential fatty acid deficiency as a result of inadequate or undeveloped foraging skills (Belicka et al. 2012). This suggests that the composition of neonatal animals reflects maternal diet and foraging ability, rather than neonatal diet. The timescales of fatty acid turnover in neonates requires further investigation to determine when the maternal signature is lost so that the dietary patterns of young sharks can be accurately described (Belicka et al. 2012).

Controlled feeding studies have not been undertaken on any chondrichthyan species but have been undertaken on many other taxa including;

seals (Iverson et al. 2004; Kirsch et al. 2000; Nordstrom et al. 2008), teleosts (Benedito-Palos et al. 2010; Budge et al. 2011; Kirsch et al. 1998), seabirds (Käkelä et al. 2009; Wang et al. 2010; Wang et al. 2007, 2010), and cuttlefish (Fluckiger et al. 2008). Although there is a plethora of information available about the fatty acid profiles of teleosts and other vertebrates (Sargent et al. 1987; Tocher 2003), the physiological and metabolic differences between species can limit the application of this information across taxa. Historically, many of the studies which describe the fatty acid composition of sharks (Bakes and Nichols 1995; Økland et al. 2005; Pethybridge et al. 2010; Wetherbee and Nichols 2000) focus on their use for human nutrition or potential by-product development. More recently, chondrichthyan feeding ecology has been studied using fatty acid profiles (Belicka et al. 2012; Couturier et al. 2013; McMeans et al. 2012,2013; Pethybridge et al. 2011a,b; Schaufler et al. 2005; Semeniuk et al. 2007; Wai et al. 2011), however, in comparison to studies on other taxa such as marine mammals, our knowledge is still limited.

1.5 Study organism

The Port Jackson shark *Heterodontus portusjacksoni*, a member of the horn shark family (Herteodontidae), is characterised by dark markings in the shape of a harness, a large, blunt head, bony crests above the eyes, a small mouth near the tip of the snout and, large dorsal fins with spines (Daley et al. 2002). This oviparous shark migrates inshore in the austral winter to facilitate mating and the deposition of large egg capsules within rocky crevices (Powter and Gladstone 2008). Port Jackson sharks are an abundant demersal endemic species to Australian waters inhabiting coastal reefs throughout their ranges and are most active at night when they are feeding on benthic invertebrates (Jones et al. 2010; Last and Stevens, 1994, 2009; McAuley et al. 2003; McGlaughlin and O'Gower, 1971). Port Jackson sharks have specialised durophagy including robust jaws, hypertrophied jaw adductor muscles, and moliform teeth (Powter et al 2010). Onotogenetic differences in dentition and head morphology occur and are linked to maturity

related changes in diet, with posterior moliform teeth dominating as size increases (Powter et al. 2010).

Stomach content analysis has previously revealed that the dietary compositions of southern and eastern populations of *H. portusjacksoni* are variable, with higher reliance on teleosts in the southern populations (Sommerville et al. 2011). This suggests that *H. portusjacksoni* are either responding to differences in prey availability or that their prey selectivity differ between locations (Sommerville et al. 2011). The diet of the eastern population varies by ontogenetic stage (Powter et al. 2010), which is typical of other elasmobranch species (Ebert 2002; Marshall et al. 2007). Variations in diet have been linked to changing dentition and mouth morphology and may also be explained by changing movement patterns as sharks mature (McLaughlin and O'Gower 1971; Powter et al. 2010).

The Port Jackson shark exhibits a generalist diet which can make it difficult to describe using fatty acid profiles. Generalist feeders, have less restricted diets and a larger range of prey items resulting in a mixture of the fatty acid profiles of all prey items (Bradshaw et al. 2003). The diet of generalist feeders is, however dictated by prey availability, and this allows the detection of differences in diet as a result of ecosystem differences. Stomach content analysis can therefore be used to distinguish patterns between geographical regions, which can be corroborated with fatty acid profile analysis. The Port Jackson shark was selected as the model species for this study because of its high level of availability through bycatch of the South Australian prawn fishery (Svane et al. 2008). Port Jackson sharks also have a conservation status of Least Concern (Simpfendorfer 2005) which avoids any impacts from the study on specimens collected from populations, and the species has high resilience, to capture, handling and housing (Frick et al. 2010), allowing sharks to be maintained in aquaria throughout the duration of the study with the least amount of impact.

1.6 Research Objectives

Understanding the tissue-specific differences with respect to the processing of dietary lipids is fundamental to applying fatty acid dietary tracer techniques to food web studies. While taking a biopsy of muscle tissue or a blood sample would be the preferred method for dietary analysis as it is less invasive than sampling from internal organs such as the liver. The effect of diet on liver, muscle and blood serum need to be investigated in a controlled setting to determine to what extent these tissues are reflective of diet. Specifically, this aims of this thesis are to:

- Evaluate the differences between the liver and muscle fatty acid profiles of recently captured (controls) and experimentally-fed sharks (10 weeks) to determine whether the fatty acid profiles of the diet are reflected in the fatty acid profiles of the liver and muscle tissues of *H. portusjacksoni* (Chapter 2);
- Investigate fatty acid profile dynamics of muscle and liver tissues of *H. portusjacksoni* before and after an experimental dietary change (6 weeks feeding before and after switch), with a focus on essential fatty acid, biomarkers, and metabolic pathways. This has been performed to assess whether muscle or liver fatty acid profiles of consumers can reflect recent dietary change (Chapter 3);
- Determine the effects of a known dietary lipid source on the fatty acids profile of YOY sharks (after 185 days) and test whether fatty acids are conservatively transferred from egg yolks to hatched *H. portusjacksoni* (Chapter 4). Specifically, this chapter examines whether fatty acids present in high concentrations in egg yolks or in a known diet are reflected in the liver or muscle tissues of YOY sharks, and whether there are patterns reflective of preferential retention of fatty acids in either tissue.
- Determine how the fatty acid profiles of *H. portusjacksoni* fed diets containing different fatty acid profiles, change over time (18 weeks) and compare turnover rates between blood serum, and muscle and liver tissues (Chapter 5). Specifically, this chapter investigated temporal changes in blood serum free fatty acids in relation to diet, the stability of the muscle fatty acid profile by extending the duration of the study compared to

Chapter 3, and which fatty acid are the best indicators of diet in the liver, blood serum, and muscle of Port Jackson sharks when fed a formulated pellet diet containing either fish oil or poultry oil. The longer sampling period was important to increase the likelihood of complete fatty acid turnover and allow sub sampling to detect changes over time. Furthermore, investigating the liver, muscle, and blood serum in one study was crucial to understand the temporal patterns of tissue-specific incorporation of fatty acids.

- Use the knowledge gained from Chapter 2–5 to compare dietary information obtained from stomach content and fatty acid analyses (Chapter 6). Specifically, the diet of *H. portusjacksoni* is analysed from three different regions separated by different spatial scale using a combination of stomach content and fatty acid analyses of muscle and liver tissue to investigate how fatty acid profiles vary between regions and among demographic groups, and examine the relationship between trends in fatty acid profiles and stomach contents.

1.7 Thesis Structure

This thesis is formatted in manuscript form for journal submission, each chapter addressing a specific aim. The results from Chapters 2 to 6 are published in peer-reviewed journals and have been submitted for publication, thus there is some redundancy in the introduction and methods for each chapter. In chapter 2 the liver and muscle fatty acid profiles were investigated through controlled feeding experiments. This was published in *Physiological and Biochemical Zoology* (86: 266–278, 2013). In chapter 3, the effect of dietary change on the muscle and liver tissues of Port Jackson sharks was compared and this has been published in the *Journal of Experimental Marine Biology and Ecology* (448: 10–18, 2013). In chapter 4 the focus is on the maternal input from yolk fatty acids and the effect of diet on young-of-the-year sharks and is under review at the *Journal of Fish Biology (29-7-2013).* In chapter 5 the muscle, liver and blood serum fatty acid profiles of sharks are evaluated to determine changes over time and stability of fatty acids in the different tissues. This chapter has been published the journal *Lipids* (doi:10.1007/s11745-014-3887-6). In chapter 6 the knowledge from previous chapters has been utilized to compare the dietary information obtained from stomach content and fatty acid profiles to investigate spatial and demographic differences in diet and is under review at *Marine Ecology Progress Series (6-9-2013)*. The discussion and implications of these results form Chapter 7. A single reference list has been included at the end of this thesis that includes all literature cited throughout to reduce redundancy.

Chapter 2: Experimental evaluation of fatty acid profiles as a technique to determine dietary composition in benthic elasmobranchs

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2.1 Abstract

Fatty acid analysis is a tool for dietary investigation that complements traditional stomach content analyses. Controlled feeding experiments were used to determine the extent to which the fatty acid compositions of diet are reflected in the liver and muscle tissue of the Port Jackson shark *Heterodontus portusjacksoni*. Over 10 weeks, two groups of sharks were fed prawns or squid, with distinct fatty acid profiles. The percent total fatty acids was significantly different for shark liver and muscle tissue when comparing controls to prawn- and squid-fed sharks. Compared to experimentally fed sharks, control shark muscle and liver had higher levels of 18:1n-9 and 20:2n-9. When comparing prawn- and squid-fed sharks, only liver tissue showed a significant difference in fatty acid profiles. The livers of prawn-fed sharks were comparatively higher in 18:1n-7, 22:5n-3, 20:0 and 18:1n-9 while the squid-fed sharks had higher levels of 16:0 and 22:6n-3. These fatty acids in shark liver tissue were all reflective of higher amounts in their respective dietary items, demonstrating the conservative transfer of fatty acids from diet to liver tissue. This study shows that liver and muscle fatty acid profiles can be used as indicators of dietary change through the comparison of controls and fed sharks. The timescale of this study may not have been sufficient in capturing the integration of fatty acids into muscle tissue as only liver fatty acid profiles were useful to distinguish between sharks fed different diets. These findings have important implications on sampling design where fatty acid profiles are used to infer dietary preferences.

2.2 Introduction

Accurate examination of the diet of a species can be difficult, as diet information is often only obtained by examining stomach contents (Cortés 1997). Methods such as molecular identification of prey (Dunne et al. 2010; Sigler et al. 2006), stable isotopes (Domi et al. 2005; Estrada et al. 2003; Fisk et al. 2002; Hussey et al. 2011) and fatty acid (FA) analysis (Pethybridge et al. 2011a; Pethybridge et al. 2010; Schaufler et al. 2005) have recently become more popular in chondrichthyan research. There has been limited investigation into how prey selection influences the FA profiles of different predator tissues in a controlled environment. Combining biochemical methods such as FA and stable isotope analysis is likely to give the most accurate indication of time integrated diet and may reduce the biases associated with analysing stomach contents alone. Furthermore, lethal sampling is often not possible in highly mobile, threatened and endangered species with muscle biopsy being the only way to obtain dietary information in such species.

Lipid stores and their constituent FAs are an indicator of diet (Cowey et al. 1976; Kanazawa et al. 1979) and previous studies have shown that diet influences FA profiles in consumer tissues (Fraser et al. 1989; Kirsch et al. 1998). As most FAs are not synthesised by marine vertebrates, they are usually integrated through the diet and can act as biochemical indicators of food-webs (Dalsgaard et al. 2003; Thiemann et al. 2008; Wilson et al. 2001). Long chain Polyunsaturated Fatty Acids (PUFA) have more than one double bond and are most often used as dietary indicators as they cannot be biosynthesized in sufficient quantities to ensure optimal physiological performance (Tocher and Ghioni 1999; Turner and Rooker 2005). Monounsaturated Fatty Acids (MUFA), which have one double bond, and Saturated Fatty Acids (SATFA), which do not contain double bonds, can be biosynthesised *de novo*. Dietary levels and enzyme availability can, however, affect the level to which this occurs (Tocher 2003). In most marine vertebrates, lipids are stored in the adipose tissue found in muscle or blubber (Budge et al. 2011). Sharks are different from all other vertebrates as their ability to oxidise FA (obtained from lipids) is largely confined to the liver (Ballantyne 1997; Moyes et al. 1990). As a result, shark liver is the major site of both lipid storage and

metabolism through mitochondrial and peroxisomal FA oxidation (Bone and Roberts 1969; Hallgren and Larsson 1962; Malins 1968; Medzihradsky et al. 1992). Several studies have indicated that shark tissues can accumulate dietary FA (Pethybridge et al. 2010, 2011a; Schaufler et al. 2005; Semeniuk et al. 2007; Wai et al. 2011); however, the time scales of this integration are unclear and require further investigation.

Understanding the tissue-specific differences with respect to the processing of dietary lipids is fundamental to applying FA dietary tracer techniques to food web studies. While taking a biopsy of muscle tissue would be the preferred method for dietary analysis as it is less invasive, liver tissue is known to act as a fat storage medium (Ballantyne et al. 1993; Moyes et al. 1990). As a result, the effect of diet on both liver and muscle tissue need to be investigated in a controlled setting to determine to what extent these tissues are reflective of diet. In this study, Port Jackson sharks *Heterodontus portusjacksoni* (Meyer 1793), a common benthic elasmobranch (Last and Stevens 2009) were used as the study species. The purpose of this study was (1) to investigate which FAs drove the difference between experimentally fed and wild specimens (controls), (2) to determine which FAs drove the difference between experimentally fed sharks (prawn-and squid-fed sharks), and (3) to evaluate the differences between liver and muscle FA profiles as an indication of the diet consumed.

2.3 Materials and Methods

2.3.1 Animal maintenance

Port Jackson sharks were collected during fishery-independent surveys of the South Australian Prawn Trawling Fishery in Gulf St Vincent (GSV), South Australia. Trawling was undertaken at night using standard 27-30 m double-rig demersal otter-trawl gear with two 14.6-m-wide nets and 4.5-cm diamond mesh cod-ends with trawl shots lasting for 30 min (Currie et al. 2009). Sharks were transported to the marine facilities at the South Australia Research and Development Institute, Aquatic Sciences Centre, West Beach, and maintained in 200-L plastic containers. Between 8 and 48 hours after capture, sharks were

transferred to 5,000-L tanks provided with flow through seawater, where they were housed for the duration of the project. The gender of all sharks was recorded based on the presence or absence of claspers. Sharks were also measured (total length), weighed (grams), and tagged with Hallprint dart head tags (Hallprint Pty Ltd) to allow identification of individual sharks throughout the study. To assess the natural diet of sharks, four individuals (2 males and 2 females) were euthanaized prior to any experimental feeding to act as controls. The remaining 14 sharks were kept in four tanks (three to four sharks per tank) to prevent over-crowding. The sharks in each tank were fed the same diet and each tank was held under identical conditions through a flow-through system and had an even distribution of sharks in terms of weight and length. To ensure there were no tank effects, temperature, pH and dissolved oxygen concentration were regularly recorded and showed no significant differences between the tanks (ANOVA, temperature: 21.0º–23.9ºC, $F = 0.822$, P = 0.490; pH: 7.26–7.79, $F = 0.020$, P = 0.996; dissolved oxygen: 4.82–10.45, $F = 0.188$, P = 0.904).

2.3.2 Feeding and sampling regime

Shark feeding was initiated three days after capture (December 14th 2009). Sharks were fed one of two diets, either western king prawns *Penaeus latisulcatus* (Kishinouye 1900) or squid (Southern Calamari) *Sepioteuthis australis (*Quoy & Gaimard 1832) collected from the GSV prawn trawl fishery. Sharks were fed at the same time of day to satiation three times a week for ten weeks and any uneaten food was removed from the tank approximately two hours after feeding. Prior to feeding, after five weeks of feeding and at the conclusion of the experiment, sharks were measured (total length) and weighed (grams) to track their progress. At the conclusion of the 10 week experiment on 22nd February 2010, sharks were euthanised 24 hours after feeding by spinal section and pithing, and dissected. Five to ten grams of muscle and liver tissue were collected and frozen at -20ºC until analysed. To compare the FA profiles of prawn- and squid-fed *Heterodontus portusjacksoni* with their diet source, six whole prawns and six whole squid were individually homogenised in a blender for FA analysis.

2.3.3 Lipid extraction and FA analysis

Samples were analysed by the FOODplus Fatty Acid Lab (Urrbrae, South Australia, Australia). Lipids were extracted from diet or shark tissue samples using a chloroform/methanol (2:1) method as described by Bligh and Dyer (1959). The percent lipid was calculated on a wet weight basis and the lipid was extracted with chloroform. Fatty acid methyl esters (FAME) were produced by heating the extracted lipids in 1% H_2SO_4 in methanol for 3 hrs in a 70^{o}C water bath. After cooling, distilled water was added along with 2.0 ml of *n*-heptane and was this was shaken and centrifuged allowing the phases to separate. The extracted FAME were identified based on their retention times relative to authentic lipid standards from Nu-check Prep Inc, Elysian, MN, USA using the Hewlett Packard Chemstation data system.

2.3.4 Statistical analysis

Bray-Curtis similarity matrices were calculated for square-root transformed data to test the differences between dietary items, fed sharks, and control sharks using Primer Version 6.1.13 (http://www.primer-e.com). Subsequently, analysis of similarities (ANOSIM, Clarke 1993) was performed and significant differences in FA profiles were identified using *R* values. The percent contribution of each FA to the separation between diets and fed sharks was assessed using similarity percentage (SIMPER) analysis, which measures the top 90% of contributing variables (Clarke, 1993). To complement ANOSIM, differences in FA composition between diets, tissues, and maturity status were also analysed using PERMANOVA+ version 1.0.3 (Anderson, 2001), using 9999 permutations under a reduced model and additional pair-wise tests using the square root of the pseudo-F statistic (t-test). Unlike ANOSIM, PERMANOVA is able to determine whether the interaction between the diets, tissues, and maturity status were significant. The PERMANOVA relies on comparing the observed value of a test statistic (pseudo F-ratio) against a recalculated test statistic generated from random re-ordering (permutation) of the data (Anderson, 2001). This permutation approach is a "semi-parametric" multivariate version of a univariate one-way ANOVA. The advantage

of this is the resulting test is ‘‘distribution free’’ and not constrained by many of the typical assumptions of parametric statistics. The F profiles were then depicted using non-metric multidimensional scaling (MDS) represented by two-dimensional plots. Stress values of the MDS ordination are considered good when stress is lower than 0.1 (Kruskal and Wish, 1978). ANOVA was also used to test the differences in weight and total fat percentage between fed and control sharks and diets.

2.4 Results and Discussion

The results of this study may affect future sampling designs as they indicate that dietary patterns may be detected in liver FA profiles when comparing animals feeding on different exclusive diets. Muscle FA profiles were not indicative of sharks fed exclusive diets and more research is required into the timescales of FA integration into this tissue to develop appropriate sampling designs which can be applied to studies of wild animals. Controlled experiments simulating dietary switches should be conducted in captivity and sub-samples should be taken at shorter intervals to detect dietary FAs, which are likely to be are mediated to tissues for storage or utilization in the form of energy metabolism. Extended feeding trials are required to compare changes in FA profiles over time and determine whether the FA composition of different tissues reaches a steady state or whether it is fluctuating in response to diet.

2.4.1 Size, weight and maturity

Fourteen fed sharks and four controls were analysed (Table 2.1). The weight of control sharks was not significantly different to either prawn or squid-fed sharks (ANOVA, $F < 0.001$, $P = 0.986$). The weight of prawn and squid-fed sharks, and controls was also not significantly different by week sampled (ANOVA, $F = 0.374$, $P = 0.690$) and there was no significant interaction between diet and week sampled (ANOVA, $F = 0.098$, $P = 0.906$). Squid-fed sharks gained significantly

more weight by percentage than prawn-fed sharks (ANOVA, $F = 17.016$, $P > 0.001$) and were significantly different between week sampled ($F = 4.640$, $P = 0.016$). There was, however, no significant interaction between week sampled and diet (ANOVA, $F = 1.244$, $P = 0.300$). The differences in weight gain were consistent with significantly higher levels of total fat in the squid diet (ANOVA, $F = 16.928$, $P = 0.002P = 0.002$) with 1.9% (± 0.08%) compared to 1.1% (± 0.18%) seen in prawns. Furthermore, no squid-fed sharks lost weight throughout the duration of the experiment indicating that differences in health condition and growth rate were a result of diet and not differences between individuals.

Furthermore, female sharks have been shown to store more lipids in their livers as they require sufficient nutrients for reproduction and embryo development (Bone and Moore 2008). The effect of sex-specific differences is, however, expected to be minimal in this study as animals were not sexually mature and the lipid content within shark liver has been shown to be more strongly affected by seasonal differences than sex (Jayasinghe et al. 2003). In addition, previous dietary studies on Port Jackson sharks have not detected diet differences between sexes (Powter et al. 2010). The difference between immature and sub-adult sharks was tested and stage of maturity was also not found to have an effect on FA profiles. However previous work by Wai et al. (2011). identified differences between adult and juvenile bamboo sharks *Chiloscyllium plagiosum* (Bennett 1830).

Table 2.1 Length, weight and reproductive parameters for control and fed *Heterodontus portusjacksoni*. prawn (P), squid (S), natural (N), immature (Imm.), maturing sub-adult (mat. SA).

ID	Tank	Diet	Total Length (mm)	Weight (g)			% change	Class
				Week 0	Week 5	Week 10		
1	1	P	420	450	400	500	10.00	Imm.
2	1	P	610	1450	1600	1550	6.45	Mat. SA
3	1	P	590	1350	1400	1400	3.57	Mat. SA
4	1	P	550	950	1050	1150	17.39	Mat. SA
5	4	P	650	1750	1800	1900	7.89	Mat. SA
6	4	P	490	850	850	900	5.56	Imm.
7	4	P	600	1550	1450	1450	-6.90	Mat. SA
8	2	S	580	1350	1450	1600	15.63	Mat. SA
9	2	S	660	1850	1900	2100	11.90	Mat. SA
10	2	S	460	550	550	700	21.43	Imm.
11	3	S	580	1300	1300	1400	7.14	Mat. SA
12	3	S	550	900	1100	1200	25.00	Mat. SA
13	3	S	520	750	900	1100	31.82	Mat. SA
14	3	S	580	1150	1250	1300	11.54	Mat. SA
C-1	C	N	590	1688	-	-	-	Mat. SA
C-2	C	N	450	929	-	-	-	Imm.
C-3	C	N	410	598	-	-	-	Imm.
C-4	C	N	540	1299	-	-	-	Mat. SA

Table 2.2 Statistical test of fatty acid profile differences as a result of diet (control, prawn diet, squid diet), tissue (liver, muscle) and maturity (immature, maturing sub-adult) between control *Heterodontus portusjacksoni* and fed sharks by two-way PERMANOVA.

	Df	MS	*F*	p
Diet	2	805.150	23.058	<0.001*
Tissue	1	3093.600	88.594	<0.001*
Maturity	1	31.708	0.908	0.384
Diet x tissue	2	280.550	8.034	<0.001*
Diet x maturity	2	54.579	1.563	0.179
Tissue x maturity	1	15.872	0.454	0.673
Diet x tissue x maturity	2	46.590	1.334	0.246

*P < 0.05.

2.4.2 Fatty acid composition and effects

Fatty acid analysis of diet and shark tissues identified 52 individual FA; nineteen of these had a mean of more than 0.3% (Table 2.3). Based on the Bray-Curtis similarity index, the FA compositions of Port Jackson sharks were significantly different by diet and tissue (Table 2.2). There were no significant differences when analysing sharks by FA fraction (SATFA, MUFA, PUFA) in liver (PERMANOVA pseudo-F = 1.1418, p = 0.3419 ANOSIM R = 0.183, p = 0.036) or in muscle (PERMANOVA pseudo f = 0.582, p =0.662 ANOSIM R = -0.125, P=0.982). There was a significant interaction between diet and tissue but no other significant interactions were identified (Table 2). The MDS results based on FA profiles suggest that prawn- and squid-fed sharks cannot be distinguished using muscle tissue (Figure 2.2a). Three clusters, which can be separated at the 90% similarity level, are shown on the MDS plot: group A comprised the four control sharks, group B comprised five squid-fed sharks (8, 9, 12, 13 and 14) and five prawn-fed sharks (1, 3, 5, 6 and 7) while group C comprises two squid-fed sharks (10 and 11) and two prawn-fed sharks (2 and 4). There was clear separation of the control sharks from both groups of fed sharks. However, there was no separation

between prawn- and squid-fed sharks. Significant differences were identified between muscle FA profile clusters (PERMANOVA pseudo-F = 8.9937, P > 0.001, ANOSIM R = 0.516, P = 0.050) with pairwise tests indicating that groups A and B were significantly different to group C (Table 2.2). The dietary shift in FA composition of liver tissue is more clearly visualised in the MDS scatter plot (Figure 2.2b). Five clusters, which can be separated at the 90% similarity level, have been shown on the MDS plot: group A comprises six prawn-fed sharks (1, 2, 3, 4, 6 and 7) and one squid-fed outlier (8); group B comprises three squid-fed sharks (10,12 and 14) and one prawn-fed outlier (5), group C contains 3 squid-fed sharks (9, 11, 13), group D comprises four control sharks (C-1, C-3, C-4), and group E contains another control shark (C-2) . Significant differences were identified between liver FA profile clusters (PERMANOVA pseudo-F = 22.34, P > 0.001, ANOSIM R = 0.94, P > 0.001). Pairwise tests showed that groups A and B, B and C, A and D, A and C, and B and D were significantly different (Table 2.2).There were no trends in terms of tank, or shark size, weight, or maturity that explain the outliers (Table 2.1).

Tissue-specific differences in lipid storage are indicated by differing FA profiles in relation to diet. Shark liver is metabolically different to other vertebrates as it behaves like adipose tissue (Medzihradsky et al. 1992) and is the main site of both lipid storage and metabolism (Bone and Roberts 1969; Hallgren and Larsson 1962; Malins 1968). This was demonstrated by the liver of controls containing 35.9% (±2.55%) total lipid compared to only 0.69% (±0.06%) in the muscle. While FA oxidation (one of the major processes of lipid metabolism) does not occur outside the liver (Moyes et al. 1990), the lipids stored there must be transported to other tissues to fuel a variety of metabolic functions (Ballantyne et al. 1993).

The utilisation of FAs outside of the liver, such as in muscle tissue, depends largely on the availability of enzymes required to facilitate oxidation (Tocher 2003; Turner and Rooker 2005). It has thus been suggested that ketone bodies may be the most important fat fuels and that there is a preference for ketone bodies rather than lipids as oxidative substrates (Ballantyne 1997; Zammit and Newsholme 1979). As a result, the majority of metabolic energy stores are likely

to be derived from lipids stored in the liver while the muscle utilises proteins (Pethybridge et al. 2010). Because of the similar FA composition in the muscle tissues of sharks fed different diets over a 10-week period, this study demonstrates that muscle may not be a suitable tissue to target when using FA profiles to investigate differences in diet.

2.4.3 Fatty acid profiles of diet fed to sharks

Prawns and squid had significantly different FA profiles (ANOSIM R = 0.989, p = 0.002, PERMANOVA pseudo-*F* = 3.976, p < 0.001), with SIMPER analysis showing diets were 25% dissimilar with PUFA being the dominant contributor to the dissimilarity. Docosahexaenoic acid (DHA, 22:6n-3), oleic acid (18:1n-9) and palmitoleic acid (16:1n-7) all contributed more than 5% to the dissimilarity observed between prawns and squid. Palmitoleic acid was 4.4% higher in prawns than in squid, and oleic acid was 6.1% higher in prawns than in squid, while DHA was 20.1% higher in squid than in prawns (Figure. 1a). Diets were also significantly different when analysed by FA class (PERMANOVA pseudo-F = 19.5, P = 0.002, ANOSIM R = 0.99 P = 0.002). Diets showed a 7% dissimilarity, which was driven by MUFA (47%) and PUFA (34%). Prawns contained 12.5% more MUFA than squid, while squid contained 4.1% more SATFA than prawns and 11.5% more PUFA than prawns.

Table 2. 3 Mean total fatty acid percentages (± standard error) of control, prawn- and squid-fed shark muscle and liver tissues, and diets fed to sharks

	Muscle						Liver						Diet			
	Control		Prawn-fed		Squid-fed		Control		Prawn-fed		Squid-fed		Prawn		Squid	
	%	±	%	±	%	±	%	±	%	±	%	±	%	±	%	±
9:00	0.00	0.00	0.09	0.06	0.11	0.07	0.00	0.00	0.01	0.00	0.00	0.00	0.34	0.16	0.07	0.03
11:00	0.04	0.00	0.06	0.04	0.09	0.06	0.02	0.00	0.01	0.00	0.00	0.00	0.25	0.11	0.07	0.03
12:00	0.01	0.00	0.00	0.00	0.00	0.00	0.04	0.01	0.10	0.01	0.04	0.01	0.03	0.00	0.03	0.00
13:00	0.03	0.01	0.00	0.00	0.00	0.00	0.03	0.00	0.03	0.00	0.02	0.00	0.13	0.05	0.04	0.01
14:00	0.41	0.05	0.34	0.02	0.44	0.03	1.19	0.01	0.79	0.10	1.19	0.14	0.96	0.09	2.43	0.32
15:00	0.21	0.01	0.23	0.03	0.23	0.02	0.84	0.06	0.80	0.04	0.76	0.03	1.38	0.17	0.92	0.03
dma 16:0	4.28	0.24	4.65	0.17	4.66	0.23	0.09	0.01	0.21	0.06	0.15	0.06	1.48	0.12	0.56	0.03
16:00	19.70	0.70	20.98	0.35	21.04	0.28	20.26	1.74	17.27	0.53	20.09	0.63	4.60	0.06	22.24	0.19
dma 18:0	0.00	0.00	0.88	0.04	0.85	0.04	0.02	0.02	2.01	0.11	1.59	0.09	0.69	0.26	1.10	0.48
18:00	8.54	0.21	8.59	0.20	8.50	0.30	6.55	0.48	0.13	0.02	0.09	0.03	10.46	0.45	7.32	0.34
20:00	0.07	0.00	0.05	0.02	0.08	0.02	0.33	0.03	6.86	0.47	6.28	0.36	0.37	0.02	0.12	0.00
22:00	0.07	0.02	0.06	0.02	0.03	0.02	0.17	0.05	0.43	0.01	0.32	0.03	0.19	0.09	0.05	0.02
24:00:00	0.01	0.01	0.23	0.16	0.23	0.15	0.07	0.01	1.88	0.21	1.56	0.24	0.07	0.03	0.07	0.03
Trans 16:1	0.26	0.16	0.31	0.07	0.33	0.05	0.00	0.00	0.43	0.04	0.30	0.05	0.30	0.06	0.18	0.06
Trans 18:1n-9	0.30	0.02	0.28	0.04	0.30	0.05	0.43	0.01	0.32	0.02	0.33	0.03	0.52	0.03	0.26	0.02
Trans 18:1n-7	0.03	0.01	0.00	0.00	0.00	0.00	0.03	0.02	0.08	0.04	0.10	0.04	0.06	0.02	0.00	0.00
Trans 18:2	0.19	0.03	0.09	0.02	0.11	0.03	0.23	0.01	0.04	0.00	0.04	0.00	0.18	0.08	0.04	0.01
15:01	0.24	0.10	0.23	0.06	0.23	0.06	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.01	0.00	0.00
16:1n-9	0.67	0.12	0.54	0.05	0.58	0.05	0.72	0.05	0.69	0.03	0.65	0.04	0.11	0.04	0.15	0.02
16:1n-7	4.02	0.49	2.76	0.23	3.14	0.22	5.17	0.89	2.88	0.29	3.02	0.26	5.35	0.26	1.00	0.19
17:01	0.77	0.24	0.64	0.03	0.63	0.04	0.88	0.09	1.02	0.06	0.79	0.05	0.47	0.21	0.23	0.10
18:1n-9	12.84	0.37	10.55	0.34	10.68	0.30	13.67	1.49	11.40	0.69	10.61	0.78	8.86	0.38	2.76	0.26

18:1n-7	6.83	0.76	6.21	0.34	6.21	0.26	7.46	0.07	8.36	0.23	7.12	0.52	3.65	0.31	1.53	0.12
19:01	0.36	0.04	0.31	0.08	0.31	0.07	0.31	0.06	0.06	0.00	0.05	0.01	0.20	0.10	0.10	0.02
20:1n-11	0.00	0.00	0.03	0.02	0.08	0.02	0.00	0.00	1.02	0.10	0.92	0.09	0.28	0.13	0.35	0.17
20:1n-9	0.00	0.00	0.36	0.04	0.44	0.03	0.11	0.07	2.38	0.12	2.35	0.10	0.62	0.13	2.02	0.07
22:1n-11	0.00	0.00	0.10	0.00	0.11	0.01	0.07	0.03	0.31	0.06	0.38	0.03	0.24	0.06	0.06	0.01
22:1n-9	0.10	0.01	0.40	0.26	0.46	0.25	0.16	0.06	0.33	0.04	0.33	0.02	0.92	0.40	0.44	0.13
10,12 18:2 cLA	0.09	0.01	0.00	0.00	0.00	0.00	0.74	0.18	0.00	0.00	0.00	0.00	0.00	0.00	0.20	0.10
18:2n-9	0.26	0.02	0.03	0.02	0.03	0.02	0.25	0.05	0.21	0.01	0.18	0.02	0.66	0.08	0.22	0.02
20:2n-9	0.49	0.04	0.03	0.02	0.04	0.02	2.14	0.17	0.02	0.00	0.02	0.00	0.05	0.02	0.02	0.01
20:3n-9	0.18	0.01	0.65	0.20	0.66	0.20	0.88	0.13	0.05	0.00	0.04	0.00	0.00	0.00	0.00	0.00
18:2n-6	0.66	0.03	0.94	0.05	0.83	0.05	0.89	0.03	1.25	0.06	1.01	0.07	1.37	0.10	0.48	0.06
18:3n-6	0.00	0.00	0.08	0.05	0.09	0.06	0.00	0.00	0.96	0.05	0.74	0.07	0.21	0.02	0.11	0.03
20:2n-6	0.27	0.07	0.20	0.02	0.19	0.01	0.25	0.06	1.19	0.05	0.89	0.07	0.81	0.07	0.39	0.03
20:3n-6	0.25	0.02	0.28	0.02	0.21	0.04	0.28	0.02	0.35	0.02	0.29	0.03	0.23	0.03	0.06	0.01
20:4n-6	11.32	1.36	12.43	0.65	10.98	0.33	3.08	0.39	3.38	0.51	2.77	0.47	7.66	0.22	2.94	0.53
16:2n-3	0.00	0.00	0.23	0.02	0.18	0.03	0.11	0.10	0.00	0.00	0.00	0.00	0.09	0.04	0.08	0.04
18:3n-3	0.16	0.01	0.10	0.00	0.10	0.00	0.55	0.06	0.00	0.00	0.00	0.00	0.52	0.09	0.26	0.07
18:4n-3	0.04	0.02	0.01	0.01	0.00	0.00	0.05	0.02	0.12	0.03	0.14	0.02	0.08	0.01	0.08	0.04
20:3n-3	0.00	0.00	0.04	0.02	0.08	0.03	0.31	0.05	0.21	0.02	0.24	0.03	0.15	0.02	0.59	0.04
20:5n-3	2.81	0.31	2.85	0.22	3.26	0.25	2.15	0.45	1.52	0.11	2.50	0.38	14.59	0.54	13.16	0.24
22:5n-3	4.83	0.29	4.58	0.29	4.46	0.25	7.06	0.47	9.52	0.31	7.95	0.47	2.31	0.20	0.91	0.10
22:6n-3	13.69	1.01	13.90	0.87	14.78	0.81	14.76	1.49	11.46	0.82	16.89	1.69	12.55	0.67	33.53	0.92
Total SATFA	33.39	1.25	36.16	1.11	36.26	1.22	29.62	2.42	30.53	1.56	32.09	1.62	30.96	1.51	35.02	1.51
TOTAL MUFA	26.80	2.40	22.90	1.63	23.67	1.48	30.89	3.06	29.43	1.76	27.08	2.05	21.90	1.31	9.44	1.31
TOTAL PUFA	34.96	3.19	36.38	2.48	35.92	2.12	32.85	3.50	30.39	2.01	33.76	3.34	52.87	2.15	52.87	2.15

Table 2.4 Pair-wise statistical tests of dietary differences in muscle and liver tissue FA profiles between control *Heterodontus portusjacksoni* and sharks fed prawn or squid by two-way factorial non-parametric multivariate analysis of variance (PERMANOVA) and analysis of similarities (ANOSIM). Sharks are grouped as A (prawn-fed sharks 1, 4, 6, and 7, squid-fed shark 8), B (squid-fed sharks 10, 12, and 14), C (squid-fed sharks 9, 11, and 13), D (control sharks C-1, C-2, and C-3) and E (control shark C-2).

		PERMANOVA		ANOSIM	
		T	p	R	P
Liver					0.003
	A, B	3.150	0.003*	0.802	*
					0.029
	B, C	2.904	0.030*	0.981	*
					0.008
	D, A	6.373	0.011*	1.000	*
					0.008
	A, C	4.746	0.009*	1.000	*
					0.029
	D, B	4.742	0.028*	1.000	*
	D, C	5.902	0.106	1.000	0.100
	D, E	2.349	0.246	1.000	0.250
	E, A	4.734	0.125	1.000	0.125
	E, B	4.375	0.198	1.000	0.200
	E, C	6.698	0.250	1.000	0.250
					0.001
	A, C	3.600	0.002*	0.864	*
Muscle	A, B	1.480	0.136	-0.010	0.436
					0.008
	C, B	5.110	0.008*	1.000	*

Note. *Indicates significant result ($p < 0.05$).

Table 2.5 Pair-wise statistical tests of dietary differences in muscle and liver tissue FA profiles between control *Heterodontus portusjacksoni* and sharks fed prawn or squid diets by two-way factorial non-parametric multivariate analysis of variance (PERMANOVA) and analysis of similarities (ANOSIM).

		PERMANOVA		ANOSIM	
		T	P	R	P
Muscle	Prawn, Squid	0.712	0.627	0.030	0.553
	Prawn, Control	2.879	0.003*	0.599	0.002*
	Squid, Control	2.960	0.003*	0.643	0.003*
Liver	Prawn, Squid	2.600	0.002*	0.109	0.039*
	Prawn, Control	7.612	0.003*	0.391	0.091
	Squid, Control	7.145	0.003*	0.461	0.118

Note. Treatments = control, squid-fed, prawn-fed; tissues = Muscle, Liver.
*Indicates significant result ($p < 0.05$)

2.4.4 Fatty acid profiles of experimentally fed sharks

PERMANOVA and ANOSIM showed that the FA profiles of muscle tissue were not significantly different between prawn- and squid-fed sharks (Table 2.5, ANOSIM R = 0.03, p = 0.553 PERMANOVA pseudo-*F* = 0.712, p = 0.627). This was in contrast to liver FA profiles, which were significantly different between prawn- and squid-fed sharks (Table 2.5, ANOSIM R = 0.109, p = 0.039 PERMANOVA pseudo-*F* = 2.6, p = 0.002). SIMPER indicated prawn- and squid-fed sharks were 14% dissimilar (Figure 2.3), which was largely driven by PUFA, contributing 50% to the total dissimilarity. This suggests that FA may not be deposited in the muscle tissue, and instead, stored in the liver. This could take place during periods of low physiological demand, when FA is stored in the liver and not transported to other tissues such as muscles. Alternatively, dietary FAs

could have also been transported to muscle tissues to fulfill a sharks immediate metabolic requirements. Dietary lipids are absorbed through the intestine packaged into lipoprotein particles (such as chlyomicrons) and transported to the cells to be stored as triacylglycerols. Therefore, it is possible that ingested fatty acids are directly transported to the muscle from the intestine to satisfy sharks immediate energy requirements. In such cases, changes in FA profiles as a result of differing dietary FAs signatures would have been detected in muscle tissue earlier than in liver tissue, and might have already equilibrated by week 10. The ability to detect changes in FA signature in various tissues may, therefore, be dependent on the sampling time following feeding. Previous work has indicated that substantial variation occurs in lipid composition among tissues, and similarities in lipid classes based on physiological function have previously been shown (Pethybridge et al. 2010). The lack of reflection of diet in muscle FA profiles may be further explained by the physiological function of muscle tissue and the fact that dietary FAs have already turned over (equilibrated) and that the muscle FA profile has reverted back to those FAs which are needed by that tissue. While there is no previous data available on the turnover rates of FA in sharks, Turner and Rooker (2005) reported a turnover rate of 1 week in the homogenised tissue of whole pelagic juvenile Cobia *Rachycentron canadum* (Linnaeus 1766).

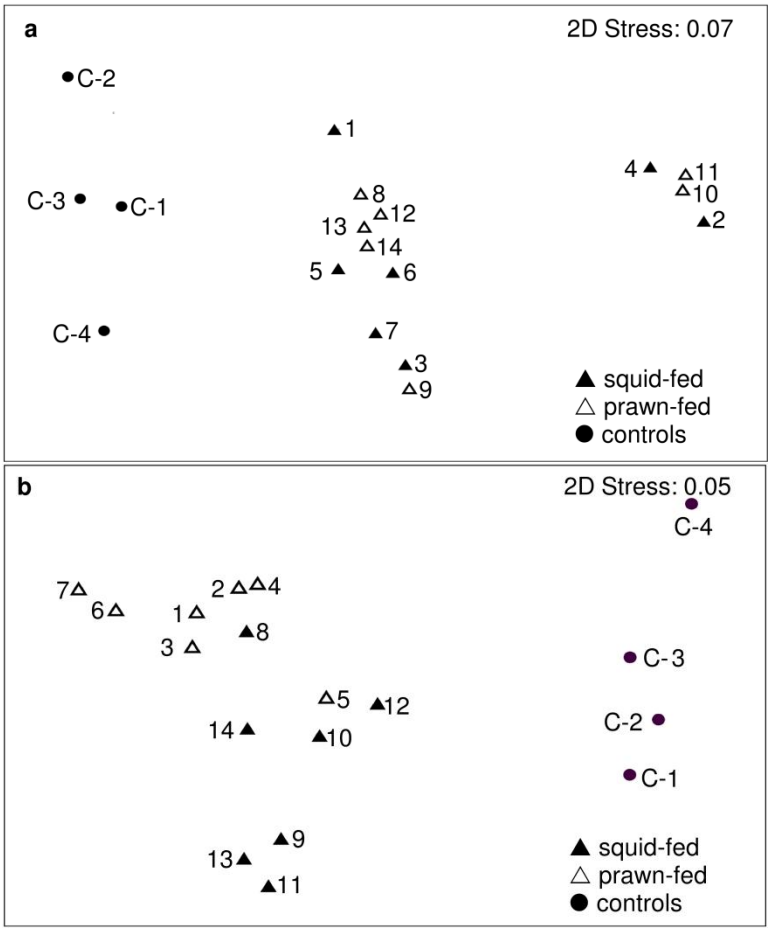

Figure 2.1 Multidimensional scaling (MDS) plot of *Heterodontus portusjacksoni* (a) muscle and (b) liver fatty acid composition of squid-fed, prawn-fed, and control sharks (after 10 weeks of feeding). Numbers represent shark identification.

Several individual FAs contributed more than 5% to the total dissimilarity of the liver FA profiles of squid- and prawn-fed sharks, including palmitic acid (16:0) and arachidic acid (20:0), DHA (22:6n-3) and docosapentaenoic acid (DPA, 22:5n-3) and oleic acid (18:1n-9) and vaccenic acid (18:1n-7). Palmitic acid was 2.8% higher in the liver of squid-fed sharks compared to prawn-fed sharks and 0.06% higher in the muscle of squid-fed sharks compared to prawn-fed sharks, DHA was 5.4% higher in the liver of squid-fed sharks compared to prawn-fed sharks and was 0.9% higher in the muscle of squid-fed sharks compared to prawn-fed sharks (Table 2.3). This was reflective of a 7.6% increase of palmitic acid and 21% increase of DHA in squid diet compared to prawn diet (Table 2.3). Compared to squid-fed sharks, prawn-fed sharks had 0.8% more oleic acid, 1.6% more DPA, 0.6% more arachidic acid, and 1.2% more vaccenic acid (Table 2.3). This was reflected in dietary items with prawns containing 6.1% more oleic acid, 1.4% more DPA, and 0.3% more arachidic acid than squid (Table 2.3).

Palmitic acid and arachidic acid are saturated FA and can both be created through *de novo* FA synthesis. Despite this, dietary levels were still shown to influence the FA composition of fed sharks, indicating that *de novo* synthesis was limited. Vaccenic acid can also be derived from palmitic acid via the metabolic pathway. Levels of vaccenic acid in the diet were, however, consistent with the levels of this FA in the liver tissue, suggesting that vaccenic acid is, regardless, a good marker of diet. DHA and DPA are omega-3 PUFA that are largely incorporated from the diet and the reduced ability of marine fish to form DHA and its metabolic precursor DPA through desaturation and elongation makes them useful as dietary tracers (Tocher 2003).

2.4.5 Fatty acid profiles of control sharks and experimentally fed sharks

The FA profiles of the muscle and liver tissues of both groups of experimentally fed sharks were significantly different from control sharks (Table 2.5). SIMPER analysis indicated the muscle FA profiles experimentally fed sharks and controls were 14% dissimilar and the liver FA profiles of fed sharks and controls were 24–26% dissimilar (Figure 2.3). The dissimilarity between groups was driven by

PUFA for muscle and SATFA for liver. Although control sharks had higher percentages of dietary derived PUFA in their muscle tissue and liver (33–35%) than fed sharks, muscle PUFA levels were still low compared to those previously reported for this species (43%, Dunstan et al. 1988) and other chondrichthyans (Supplementary Table 2.1). Values of PUFA in liver tissues also appear to vary widely across chondrichthyans species (Supplementary Table 2.1).

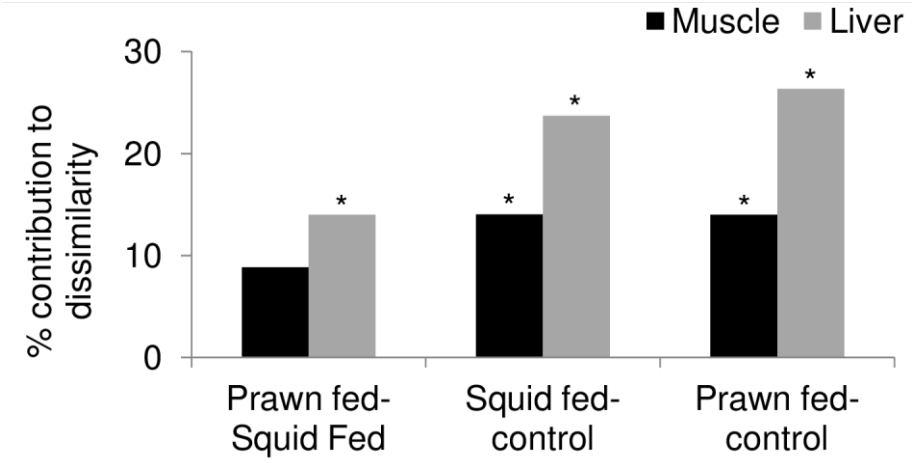

Figure 2.2 Similarity percentage analysis expressed as a percentage of dissimilarity based on a Bray-Curtis similarity matrix. Sharks fed prawns, squid, and controls are compared by tissue type (muscle and liver). An asterix represents significant differences between the total FA profiles of feeding groups; PERMANOVA and analysis of similarities values are presented in Table 2.4.

The overall dissimilarity of the muscle of fed sharks and controls was driven by octadecanal-dimethylacetal (DMA-18:0), 8,11-cis-eicosadienoic acid (20:2n-9) and eicosenoic acid (20:1n-9) in prawn-fed sharks and controls while erucic acid (22:1n-9) and mead acid (20:3n-9) contributed more than 5% to the overall dissimilarity observed between squid-fed and control sharks. The overall dissimilarity observed between the liver of fed sharks and controls was driven by arachidic acid (20:0), stearic acid (18:0), palmitic acid (16:0), DHA (22:6n-3), DPA (22:5n-3), oleic acid (18:1n-9) and eicosenoic acid (20:1n-9), which all contributed more than 5% to the dissimilarity observed between groups. The liver of control sharks had 6.4% more stearic acid, 3% more palmitic acid, 3.3% more DHA, and 2.3% more oleic acid than seen in the liver of prawn-fed sharks (c). The percentage of oleic acid was 2.3% higher in the muscle of control sharks compared

to prawn-fed sharks (Table 2.3). While the FA profile of wild diet is unknown, this suggests the natural diet had increased availability of stearic acid, palmitic acid, DHA and oleic acid compared to the experimental diet (Table 2.3). Prawn-fed sharks had 2.5% more DPA and 6.5% more arachidic acid in liver than control sharks (Table 2.3). In comparison, the DPA and arachidic acid levels in the liver of squid-fed sharks was 1.6% and 0.6% less than in prawn-fed sharks, but still 0.9% and 6% higher than in controls (Table 2.3). This was consistent with 1.4% and 0.3% increase DPA and arachidic acid in prawns compared to squid (Table 2.3). Arachidic acid was only detected at trace levels in shark muscle, while levels of DPA were relatively similar with muscle of control sharks containing 0.3% more DPA than prawn-fed sharks and 0.4% more than controls.

The liver of squid-fed sharks also contained 2.1% more DHA than the liver of controls (Table 2.3). In comparison, the liver of prawn-fed sharks contained 3.3% less DHA than controls, while squid-fed sharks contained 5.4% more DHA than prawn-fed sharks. The muscle of squid-fed sharks also contained 1.1% more DHA than controls, while the muscle of prawn-fed sharks only contained 0.2% more DHA than controls and 0.9% less DHA than squid-fed sharks (Table 2.3).Levels of DHA were 21% higher in squid compared to prawns (Table 2.3).

Compared to control sharks, experimentally fed sharks had 0.4% more eicosenoic acid (20:1n-9) in their muscle and 2.2–2.3% more in their liver (Table 2.3). The muscle and liver of experimentally fed sharks contained 0.3–0.4% and 0.2% more erucic acid (22:1n-9) than controls, respectively (Table 2.3). High levels of eicosenoic and erucic acid can be indicative of secondary consumption of zooplankton, which may indicate predation on animals such as crustaceans and squid (Phillips et al. 2003). However, these MUFA are also potential products of the elongation and desaturation of oleic acid (18:1n-9) to mead acid (20:3n-9), which is known to occur in fish and other vertebrates (Tocher 2003). Experimentally fed sharks had 0.5% more mead acid in their muscle than the muscle of controls. However, in their liver, control sharks had 0.8% more mead acid than experimentally fed sharks, which only had trace levels. Since mead acid was not present in either diet, the level of mead acid in fed sharks was either a result of the mediation from stores in the liver or as a result of biosynthesis.

Control sharks had 0.5% more 8,11-cis-eicosadienoic acid (20:2n-9) in muscle (Table 2.3) and 2.1% more in liver (Table 2.3) compared to fed sharks as a result of an experimental diet largely deficient in this FA (Table 2.3). Elevated levels of cis-eicosadienoic acid can be a result of omega-3 FA deficiencies which can result in the preferential utilisation of omega-3 and omega-6 FAs by enzymes (Caballero et al. 2002). Control sharks had no octadecanal-dimethylacetal (dma 18:0) in muscle tissue and only trace amounts in liver; while squid-fed sharks had 0.03% more dma 18:0 in their muscle than prawn-fed sharks and prawn-fed sharks contained 0.4% more than squid-fed sharks in their liver. Squid contained 0.4% more octadecanal-dimethylacetal than prawns. Plasmalogen derived DMAs, such as DMA18:0 play an important role in membrane fluidity and have previously been linked to PUFA rich diets devoid in DHA (Glick and Fischer 2010).

These FAs were indicative of changes to the relative composition of FA profiles in response to change in diet. This demonstrates the potential for the use of FA liver profile analysis to indicate dietary change. Docosahexaenoic acid and oleic acid were also among the major drivers of the dissimilarity observed between prawn and squid diet items. Furthermore, DHA is particularly useful as a dietary indicator as sharks have a reduced ability to produce it through desaturation and elongation (Tocher 2003). In comparison, previous work on deepwater shark species (Pethybridge et al. 2011a) indicated that vaccenic acid was a main predictor of prey groups in both liver and muscle tissue, while DPA, palmitic acid, and DHA were main predictors of prey in muscle tissue not in liver. Levels of dietary derived DHA in liver tissue were similar to those reported in other studies, however, levels of DHA in muscle tissue were considerably lower in this study (Table 2.3). Vaccenic acid is a potential indicator of crustaceans, benthopelagic squid and fish while DPA, DHA and palmitic acid are potential indicators of crustaceans, octopus and mesopelagic squid (Pethybridge et al. 2011a). Vaccenic acid and long chain saturated FA arachidic acid, have previously been used as biomarkers to identify the source of detritus consumed in bamboo sharks (Wai et al. 2011). High levels of oleic acid were found in shark liver (Pethybridge et al. 2010), however, this FA may be considered a product of *de novo* FA synthesis and not a result of diet (Ballantyne 1997). The differing predictors and levels of FA seen in this study are likely to be a result of the restricted diet of only one item in

the present study compared to the variety of prey items consumed in the wild. Furthermore, as a majority of the literature focuses on deepwater sharks, the trends in FA composition may be affected by the different physiological and biological parameters associated with their habitat (Pethybridge et al. 2010).

The FA profiles of control and fed sharks were significantly different when comparing muscle and liver tissue. This was in contrast to the comparison of prawn-and squid-fed sharks where only the liver FA profiles showed a significant difference. The dietary differences between controls and fed sharks were likely to be much larger than the difference between the prawn and squid diets due to the range of available food items in the wild. This suggests that the extent of the dietary change determines whether or not it can be detected in the muscle, while the liver is suitable for detecting even relatively minor dietary changes.

2.4.6 Implications time scales

Many of the FA profiles of chondrichthyans found in the literature describe the FA content of either liver (Bakes and Nichols 1995; Davidson and Cliff 2002; Davidson et al. 2011; Emokpae and Anekwe 1983; Nichols et al. 1998) or muscle tissues (Hornung et al. 1994; Wai et al. 2011). However, most studies do not make comparisons between these tissues. Comparisons between liver and muscle profiles in the dogfish *Squalus acanthias* (Linnaeus 1758) revealed differing compositions of FA with muscle containing high percentages of C20 and C22 polyenoic acids, while the liver contained high concentrations of C20 and C22 monoenic acids (Malins 1968). Although Port Jackson shark muscle tissues had higher levels of C20 polyenoic acids than liver, C22 polyenoic acids, and C20 and C22 monoenoic acids were all higher in liver tissue compared to muscle.

A review of previous literature analysing FA profiles in elasmobranchs revealed that the FA fractions in liver tissue were significantly different between shark taxonomical orders (PERMANOVA Pseudo-F = 7.35, P > 0.001, ANOSIM R = 0.39, P = 0.004). Pair-wise tests detected differences between Squaliformes and Carcharhiniformes and Squaliformes and Lamniformes (Table 2.5 and Figure 2.4a). Although the mean FA fractions of shark muscle FA profiles also showed significant differences using ANOSIM (R = 0.18, P = 0.040), no significant difference were found using PERMANOVA (Pseudo-F = 2.82, P = 0.072). Pairwise tests detected differences between Carcharhiniformes and Orectolobiformes, Orectolobiformes and Rajiformes, Orectolobiformes and Heterodontiformes and Rajiformes and Squaliformes (Table 2.5).

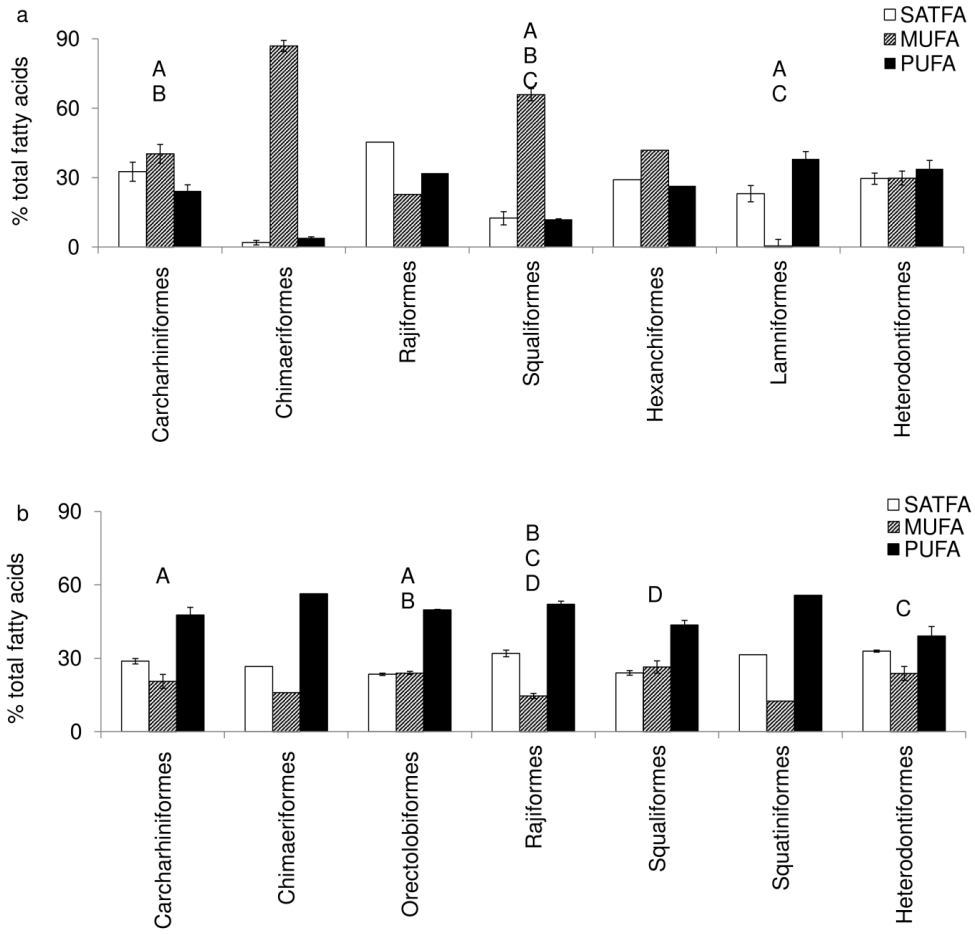

Figure 2.3 Average total fatty acid percentage of shark liver (a) and muscle (b) tissue by order. The average values for each order were calculated based on Davidson and Cliff (2002); Dunstan et al. (1988); Jayasinghe et al. (2003); Néchet et al. (2007); Nichols et al. (1998); Pethybridge et al. (2010); Schaufler et al. (2005); Wai et al. (2008). Species-specific information can be found in (Supplementary Table 2.2 and Supplementary Table 2.2) Significant differences between the fatty acid fractions of orders; PERMANOVA and analysis of similarities values are presented in Table 2.4 and are represented by the notation A, B, C, D.

Table 2.6 Pair-wise statistical tests of dietary differences in liver and muscle tissue FA fractions between by two-way factorial non-parametric MANOVA (PERMANOVA) and analysis of similarities (ANOSIM).

	PERMANOVA		ANOSIM	
Liver	T	P	R	P
Carcharhiniformes, Chimaeriformes	2.942	0.082	0.853	0.091
Carcharhiniformes, Hexanchiformes	Negative	...	-0.311	0.909
Carcharhiniformes, Lamniformes	1.457	0.117	0.252	0.035*
Carcharhiniformes, Rajiformes	0.955	0.182	0.151	0.273
Carcharhiniformes, Squaliformes	4.012	0.002*	0.474	>.001*
Chimaeriformes, Hexanchiformes	No test	...	...	...
Chimaeriformes, Lamniformes	7.563	0.163	1.000	0.167
Chimaeriformes, Rajiformes	No test	...	...	...
Chimaeriformes, Squaliformes	1.232	0.219	0.202	0.231
Hexanchiformes, Lamniformes	1.247	0.329	0.280	0.333
Hexanchiformes, Rajiformes	No test	...	...	...
Hexanchiformes, Squaliformes	1.647	0.156	0.295	0.154
Lamniformes, Rajiformes	2.376	0.170	0.840	0.167
Lamniformes, Squaliformes	4.149	>.001*	0.541	0.001*
Rajiformes, Squaliformes	2.631	0.073	0.803	0.077

Muscle	T	P	R	P
Carcharhiniformes, Chimaeriformes	0.909	0.599	0.000	0.600

Carcharhiniformes, Orectolobiformes	2.318	0.006*	0.715	0.002*
Carcharhiniformes, Rajiformes	1.631	0.143	0.241	0.171
Carcharhiniformes, Squaliformes	1.415	0.161	-0.047	0.530
Carcharhiniformes, Squatiniformes	1.229	0.401	0.167	0.200
Carcharhiniformes, Heterodontiformes	1.314	0.337	-0.036	0.600
Chimaeriformes, Orectolobiformes	3.894	0.109	1.000	0.111
Chimaeriformes, Rajiformes	1.479	0.245	0.333	0.750
Chimaeriformes, Squaliformes	1.213	0.236	0.235	0.235
Chimaeriformes, Squatiniformes	No test	...	...	...
Chimaeriformes, Heterodontiformes	2.454	0.336	1.000	0.333
Orectolobiformes, Rajiformes	6.954	0.005*	1.000	0.006*
Orectolobiformes, Squaliformes	1.161	0.272	-0.045	0.620
Orectolobiformes, Squatiniformes	5.906	0.111	1.000	0.111
Orectolobiformes, Heterodontiformes	4.850	0.024*	0.996	0.022*
Rajiformes, Squaliformes	2.416	0.031*	0.376	0.052*
Rajiformes, Squatiniformes	1.027	0.490	-0.111	0.500
Rajiformes, Heterodontiformes	3.422	0.104	0.917	0.100
Squaliformes, Squatiniformes	1.647	0.119	0.481	0.118
Squaliformes, Heterodontiformes	1.119	0.222	0.138	0.261
Squatiniformes, Heterodontiformes	2.420	0.341	1.000	0.333

Note. Average values for each order were calculated based on Davidson and Cliff (2002); Dunstan et al. (1988); Jayasinghe et al. (2003); Néchet et al. (2007); Nichols et al. (1998); Pethybridge et al. (2010); Schaufler et al. (2005); Wai et al. (2008). Species-specific information can be found in Supplementary Table 2.1 and Supplementary Table 2.2.*$P < 0.05$.

While there have been no previous controlled studies investigating FA analysis in sharks, there have been several studies using stable isotopes that have assessed tissue turnover rates and discrimination factors (Hussey et al. 2010a; Kim et al. 2012; Logan and Lutcavage 2010; Matich et al. 2010). Stable isotope tissue turnover rates for elasmobranchs have been investigated in a controlled setting for liver, whole blood, and white muscle with isotope turnover shown to be slow for shark tissues (Logan and Lutcavage 2010). Both carbon and nitrogen have been shown to incorporate faster into blood plasma than in muscle and red blood cells and the rate of incorporation of carbon into muscle is similar to patterns seen in other aquatic ectotherms (Kim et al. 2012). As a result, muscle isotope data would be unlikely to demonstrate seasonal migrations or diet switches in sharks, while liver and blood would be more likely to show shorter term movement or shifts in diet (Logan and Lutcavage 2010).

There have been very few attempts to relate descriptions of lipid profiles with dietary patterns in chondrichthyans. As seen in Port Jackson sharks, large quantities of dietary derived DHA have previously been observed in sharks (Pethybridge et al. 2010). Comparisons of pacific sleeper shark *Somniosus pacificus* (Bigelow & Schroeder 1944) tissues have also revealed distinct FA compositions with livers containing relatively high concentrations of MUFA and muscle having higher concentrations of PUFA (Schaufler et al. 2005). This differs from the results observed in Port Jackson sharks which contained high levels of PUFA in both tissues (Figure 2.4). High levels of MUFAs cetoleic acid (22:1n-11) and eicosenoic acid (20:1n-9) in sleeper sharks were linked to secondary predation on calanoid copepods through scavenging on whale blubber (Schaufler et al. 2005). Fatty acid profiles of the whale blubber were retrieved from sleeper shark stomachs and the FA profile of these and other prey items were compared to sleeper shark tissue profiles. This indicates that FA profiles are useful directly after feeding has occurred. However, if the sleeper sharks had not recently ingested the whale blubber it is not known whether the same link to copepods could be established. Fatty acid profiles have the potential to do more than indicate recent meals and experimental manipulations of diet over time can provide insights into the integration of FAs into different tissues over time.

The conservative transfer of dietary FA acids to shark tissues may provide a record of short-term diet history. Tissue-specific differences are apparent, particularly in relation to muscle and liver, and understanding differences in the timescales of FA integration is an important aspect of interpreting FA values. Fatty acid profiles are likely to be a complementary method to use in conjunction with more conventional dietary analysis techniques such as stomach contents and stable isotopes. A combination of these methods can result in unraveling trophic pathways in complex ecosystems with multiple dietary sources.

2.5 Conclusions

This study shows that liver and muscle FA profiles can be used as indicators of dietary change through the comparison of controls and fed sharks. In contrast, the similar muscle FA profiles of sharks fed different diets demonstrates that muscle may not be a suitable tissue to target when using FA profiles to investigate sharks feeding on different diets. Furthermore, the timescale of this study may not have been sufficient in capturing the integration of FA into muscle tissue as only liver FA profiles were useful to distinguish between sharks fed different diets.

We suggest that further captive experiments are required to gain further understanding into the timescales of FA integration into tissues. The diets fed to captive animals should be dramatically different in order to stimulate changes and investigate FA pathways. Furthermore, the difference between an immediate dietary change, as demonstrated by the comparison between controls and fed animals, and sharks fed exclusively different diets over time should be further investigated. The effect of diet on FA profiles in the short-term (less than ten weeks) may provide more insight into the role of muscle in energy storage and mediation. In addition, longer feeding trials (greater than ten weeks) may demonstrate how the FA profile changes over-time and particularly how long it takes for the liver FA profile to become stable in alignment with the diet, suggesting that FA stores have been exhausted and complete FA turnover has occurred.

2.6 Acknowledgements

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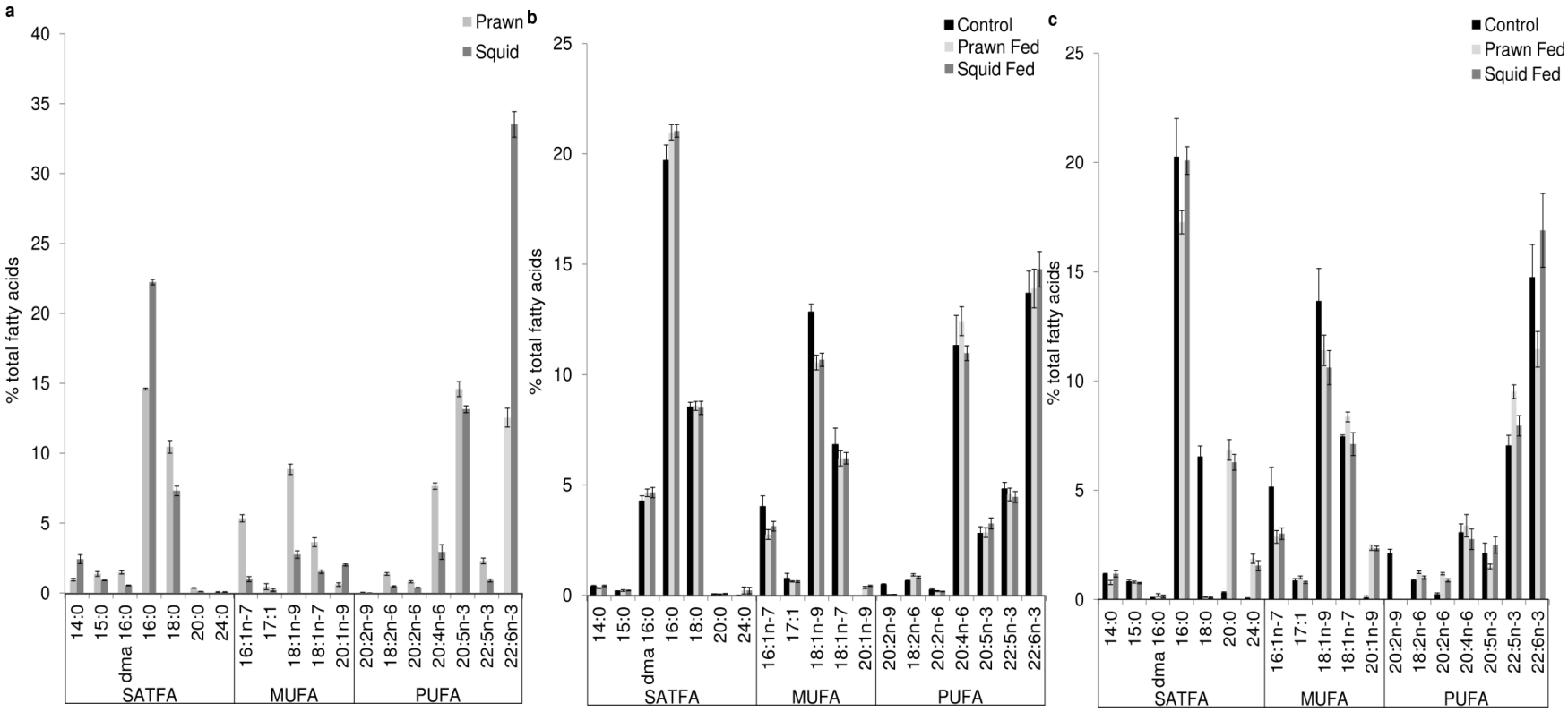
2.7 Supplementary Material



Supplementary Figure 2.1 Fatty acids (19 of 52 identified) with an overall mean of ≥0.3% of total FAs that represents the largest differences between: (a) the prey items fed to *Heterodontus portusjacksoni*, (b) the muscle profiles of sharks fed different diets and (c) the liver profiles of sharks fed different diets. Bars are means and vertical lines are standard errors.

Supplementary Table 2.1 Species-specific fatty acid percentages of liver tissue showing average and standard error values for saturated fatty acid (SATFA), monounsaturated fatty acid (MUFA), and polyunsaturated fatty acid (PUFA).

Class	Family	Species	SATFA		MUFA		PUFA		Reference
			%	±	%	±	%	±	
Carcharhiniformes	Scyliorhinidae	*Apristurus sinensis*	1.3	0.0	72.6	0.0	9.8	0.0	(Pethybridge et al. 2010)
Carcharhiniformes	Scyliorhinidae	*Figaro boardmani*	23.2	2.8	45.3	3.0	29.4	2.9	(Pethybridge et al. 2010)
Carcharhiniformes	Carcharhinidae	*Carcharhinus limbatus*	43.8	5.0	32.1	2.9	24.4	2.3	(Davidson and Cliff 2002)
Carcharhiniformes	Carcharhinidae	*Carcharhinus obscurus*	39.3	2.9	38.4	2.9	22.6	2.6	(Davidson and Cliff 2002)
Carcharhiniformes	Carcharhinidae	*Carcharhinus leucas*	41.5	3.0	40.6	2.5	18.0	2.4	(Davidson and Cliff 2002)
Carcharhiniformes	Carcharhinidae	*Carcharhinus brevipinna*	43.3	2.9	29.8	2.3	26.9	2.8	(Davidson and Cliff 2002)
Carcharhiniformes	Carcharhinidae	*Galeocerdo cuvieri*	39.0	2.5	42.8	2.9	18.2	2.1	(Davidson and Cliff 2002)
Carcharhiniformes	Sphyrnidae	*Sphyrna lewini*	36.2	3.9	43.1	2.6	20.8	2.7	(Davidson and Cliff 2002)
Carcharhiniformes	Triakidae	*Mustelus antarcticus*	31.3	3.3	29.4	3.5	36.1	2.3	(Nichols et al. 1998)
Carcharhiniformes	Triakidae	*Galeorhinus galeus*	27.3	2.4	29.4	3.5	36.8	3.3	(Nichols et al. 1998)
Carcharhiniformes mean			27.3	4.1	43.8	4.1	25.5	2.6	
Chimaeriformes	Rhinochimaeridae	*Raja pacifica*	2.0	1.0	86.9	2.4	4.0	0.5	(Pethybridge et al. 2010)
Chimaeriformes mean			15.5	1.0	86.9	2.4	15.2	0.5	
Hexanchiformes	Hexanchidae	*Notorynchus cepedianus*	29.1	0.0	41.9	0.0	26.5	0.0	(Pethybridge et al. 2010)
Hexanchiformes mean			29.1	0.0	41.9	0.0	26.5	0.0	
Lamniformes	Lamnidae	*Lamna ditropis* (male summer)	19.9	1.7	34.1	4.1	43.8	3.3	(Jayasinghe et al. 2003)

Lamniformes	Lamnidae	*Lamna ditropis (*male winter)	18.2	1.9	40.5	3.1	38.7	1.7	(Jayasinghe et al. 2003)
Lamniformes	Lamnidae	*Lamna ditropis* (female summer)	20.1	2.2	34.9	2.2	44.0	2.5	(Jayasinghe et al. 2003)
Lamniformes	Lamnidae	*Lamna ditropis* (winter)	20.2	0.8	39.7	2.3	37.6	1.7	(Jayasinghe et al. 2003)
Lamniformes	Odontaspididae	*Carcharias taurus*	37.1	4.2	36.4	2.5	26.6	2.4	(Davidson and Cliff 2002)
Lamniformes mean			32.6	3.5	37.5	1.3	29.2	3.2	
Rajiformes	Dasyatidae	*Himantura bleekeri*	45.3	-	22.8	-	31.9	-	(Néchet et al. 2007)
Rajiformes mean			45.3	-	22.8	-	31.9	-	
Squaliformes	Squalidae	*Squalus acanthias*	23.5	-	49.7	-	24.3	-	(Nichols et al. 1998)
Squaliformes	Somniosidae	*Somniosus pacificus*	16.2	0.6	70.2	0.7	13.6	0.4	(Schaufler et al. 2005)
Squaliformes	Centrophoridae	*Deania calcea*	0.9	2.7	63.0	4.4	8.1	1.3	(Pethybridge et al. 2010)
Squaliformes	Centrophoridae	*Centrophorus zeehani*	26.7	1.7	57.9	2.5	12.1	1.3	(Pethybridge et al. 2010)
Squaliformes	Chimaeridae	*Chimaera lignaria*	0.3	1.9	76.8	3.7	6.0	0.6	(Pethybridge et al. 2010)
Squaliformes	Dalatiidae	*Dalatias licha*	0.9	0.9	70.9	2.6	11.6	1.8	(Pethybridge et al. 2010)
Squaliformes	Etmopteridae	*Etmopterus baxteri*	16.2	1.9	76.9	3.1	4.6	1.1	(Pethybridge et al. 2010)
Squaliformes	Somniosidae	*Centroselachus crepidater* (adult)	17.9	1.8	71.6	4.9	8.4	1.3	(Pethybridge et al. 2010)
Squaliformes	Somniosidae	*Centroscymnus coelolepis*	0.6	0.0	60.0	0.0	20.2	0.0	(Pethybridge et al. 2010)
Squaliformes	Somniosidae	*Proscymnodon plunketi*	0.2	1.9	81.8	3.1	3.9	0.5	(Pethybridge et al. 2010)
Squaliformes	Squalidae	*Squalus megalops*	15.8	2.3	58.8	2.7	15.8	0.8	(Pethybridge et al. 2010)
Squaliformes	Squalidae	*Squalus acanthis*	23.1	3.4	57.3	4.2	14.7	2.2	(Pethybridge et al. 2010)
Squaliformes	Squalidae	*Squalus chloroculus*	20.4	3.3	61.6	5.5	13.0	2.7	(Pethybridge et al. 2010)
Squaliformes mean			22.7	2.9	49.7	2.4	22.1	1.4	
Heterodontiformes	Heterodontidae	*Heterodontus portusjacksoni* (control)	29.6	2.4	29.9	3.0	33.8	3.8	Present study

Supplementary Table 2.2 Species-specific fatty acid percentages of muscle tissue showing average and standard error values for saturated fatty acid (SATFA), monounsaturated fatty acid (MUFA), and polyunsaturated fatty acid (PUFA).

Class	Family	Species	SATFA		MUFA		PUFA		Reference
			%	±	%	±	%	±	
Carcharhiniformes	Scyliorhinidae	*Apristurus sinensis*	26.7	-	24.5	-	45.0	-	(Pethybridge et al. 2010)
Carcharhiniformes	Scyliorhinidae	*Figaro boardmani*	27.3	3.2	20.9	4.3	45.8	4.7	(Pethybridge et al. 2010)
Carcharhiniformes	Scyliorhinidae	*Asymbolus analis*	29.7	-	12.6	-	57.1	-	(Dunstan et al. 1988)
Carcharhiniformes	Scyliorhinidae	*Cephaloscyllium sp.*	31.6	-	24.5	-	42.6	-	(Dunstan et al. 1988)
Carcharhiniformes mean			27.4	1.1	22.9	2.8	46.5	3.2	
Chimaeriformes	Chimaeridae	*Hydrolagus ogilbyi*	26.6	-	16.0	-	56.4	-	(Dunstan et al. 1988)
Chimaeriformes mean			27.3	-	19.8	-	51.0	-	
Orectolobiformes	Hemiscylliidae	*Chiloscyllium plagiosum* (summer juvenile)	24.7	1.7	22.0	2.4	50.2	3.0	(Wai et al. 2011)
Orectolobiformes	Hemiscylliidae	*Chiloscyllium plagiosum* (summer adult)	24.3	1.7	25.4	2.0	47.9	2.9	(Wai et al. 2011)
Orectolobiformes	Hemiscylliidae	*Chiloscyllium plagiosum* (winter juvenile)	23.1	2.4	25.3	1.6	48.4	2.3	(Wai et al. 2011)
Orectolobiformes	Hemiscylliidae	*Chiloscyllium plagiosum* (winter adult)	21.9	1.6	24.6	2.2	50.9	1.8	(Wai et al. 2011)
Orectolobiformes	Hemiscylliidae	*Chiloscyllium plagiosum* (inner estuary Juvenile)	26.0	1.3	21.1	1.9	49.9	2.7	(Wai et al. 2011)
Orectolobiformes	Hemiscylliidae	*Chiloscyllium plagiosum* (inner estuary adult)	22.8	1.8	26.1	2.4	48.6	3.4	(Wai et al. 2011)
Orectolobiformes	Hemiscylliidae	*Chiloscyllium plagiosum* (outer estuary juvenile)	22.4	0.9	25.0	2.6	49.5	2.7	(Wai et al. 2011)
Orectolobiformes	Hemiscylliidae	*Chiloscyllium plagiosum* (outer estuary adult)	22.5	0.6	22.8	2.1	52.8	3.0	(Wai et al. 2011)
Orectolobiformes mean			23.5	0.5	24.0	0.6	49.8	0.6	
Rajiformes	Urolophidae	*Urolophus bucculentus*	33.8	-	16.0	-	49.4	-	(Dunstan et al. 1988)
Rajiformes	Rajidae	*Spiniraja whitleyi*	29.4	-	15.3	-	53.1	-	(Dunstan et al. 1988)
Rajiformes	Rajidae	*Raja nasuta*	32.8	-	12.6	-	53.6	-	(Dunstan et al. 1988)
Rajiformes mean			32.0	1.3	14.6	1.0	52.0	1.3	
Squaliformes	Squalidae	*Squalus megalops*	29.0	-	13.6	-	57.3	-	(Dunstan et al. 1988)

Squaliformes	Somniosidae	*Somniosus pacificus*	17.4	2.0	59.5	0.8	23.0	0.5	(Schaufler et al. 2005)
Squaliformes	Etmopteridae	*Etmopterus baxteri*	20.2	2.5	30.6	4.2	39.7	4.6	(Pethybridge et al. 2010)
Squaliformes	Somniosidae	*Centroselachus crepidater* (adult)	24.8	4.9	25.0	3.2	45.5	3.1	(Pethybridge et al. 2010)
Squaliformes	Somniosidae	*Centroselachus crepidater* (juvenile)	20.9	1.9	19.5	1.2	53.5	2.0	(Pethybridge et al. 2010)
Squaliformes	Somniosidae	*Centroscymnus coelolepis*	21.3	-	28.8	-	41.7	-	(Pethybridge et al. 2010)
Squaliformes	Somniosidae	*Centroscymnus owstoni*	24.7	-	21.9	-	44.7	-	(Pethybridge et al. 2010)
Squaliformes	Somniosidae	*Proscymnodon plunketi*	26.5	2.1	21.7	1.4	46.0	1.8	(Pethybridge et al. 2010)
Squaliformes	Centrophoridae	*Deania calcea*	23.9	1.2	21.7	1.6	48.1	2.6	(Pethybridge et al. 2010)
Squaliformes	Dalatiidae	*Dalatias licha*	26.2	2.9	30.5	3.2	38.0	2.3	(Pethybridge et al. 2010)
Squaliformes	Chimaeridae	*Chimaera lignaria*	23.8	3.1	24.5	5.4	46.3	0.7	(Pethybridge et al. 2010)
Squaliformes	Chimaeridae	*Chimaera fulva*	25.5	-	26.0	-	41.6	-	(Pethybridge et al. 2010)
Squaliformes	Centrophoridae	*Centrophorus zeehani*	31.3	1.3	21.9	1.2	42.3	0.9	(Pethybridge et al. 2010)
Squaliformes	Squalidae	*Squalus megalops*	27.7	1.0	23.4	1.2	44.0	0.8	(Pethybridge et al. 2010)
Squaliformes	Squalidae	*Squalus acanthis*	18.4	3.2	34.5	4.6	34.0	5.1	(Pethybridge et al. 2010)
Squaliformes	Squalidae	*Squalus chloroculus*	23.3	3.4	20.4	3.8	50.9	4.0	(Pethybridge et al. 2010)
Squaliformes mean			26.5	0.9	23.4	2.5	46.1	2.0	
Squatiniformes	Squatinidae	*Squatina australis*	31.4	-	12.6	-	55.7	-	(Dunstan et al. 1988)
Squatiniformes mean			31.4	-	12.6	-	55.7	-	
Heterodontiformes	Heterodontidae	*Heterodontus portusjacksoni* (control)	33.4	1.3	26.7	2.4	35.2	3.2	Present Study
Heterodontiformes	Heterodontidae	*Heterodontus portusjacksoni*	32.5	-	21.0	-	43.0	-	(Dunstan et al. 1988)
Heterodontiformes mean			32.9	0.4	23.8	2.8	39.1	3.9	

Chapter 3: A controlled feeding experiment investigating the effects of a dietary switch on muscle and liver fatty acid profiles in Port Jackson sharks *Heterodontus portusjacksoni*

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3.1 Abstract

Fatty acid analysis is increasingly being used as a tool to investigate the diet of sharks and rays. Quantifying the diet of sharks using fatty acid profiles, however, requires an understanding of the effects that fluctuating diet has on the biochemical pathways in different tissues, and of the time scales of fatty acid integration. The effects of dietary changes on the muscle and liver fatty acid profiles were investigated in a controlled feeding experiment with Port Jackson sharks (*Heterodontus portusjacksoni*) fed exclusively on squid then prawns for six weeks each. Multivariate analysis indicated shark muscle and liver fatty acid profiles were 9.8% and 7.7% dissimilar after dietary change. This suggests that dietary fatty acids are preferentially utilised by muscle after dietary change and that muscle fatty acid profiles are a stronger indicator of dietary changes occurring within three weeks of sampling than the fatty acid profiles of the liver. This should be carefully considered when determining the type of tissue and frequency of sampling needed to undertake chondrichthyan dietary studies based on fatty acid profile analysis. Some fatty acid biomarkers and their metabolic processes are also identified and described, but further work is required to enable quantitative assessment of a species diet. Data presented here gives justification towards more complex experiments to investigate metabolic and fatty acid tissue incorporation rates.

Keywords: controlled feeding experiment, fatty acid, Port Jackson shark, feeding ecology, diet

3.2 Introduction

The combination of increasing global fishing effort and the relatively slow growth and reproductive rates of chondrichthyans has resulted in the decline of populations around the world (Musick et al. 2000; Worm et al. 2013). The possible effects of declines in shark population on food web structures are often explored using ecosystem models (Ferretti et al. 2010). Ecosystem models require detailed dietary information, with the quality of the model output largely depending on the available diet descriptions (Plagányi and Butterworth 2004). Detailed diet information has been lacking for chondrichthyans because the collection of a large number of stomach contents can be logistically difficult (Pethybridge et al. 2011a). The difficulty in gathering stomach contents and the ambiguity of those stomach contents resulting from difficulty in identifying prey items, have driven the development of alternative methods of determining shark diet composition, such as fatty acid (FA) profiling (Beckmann et al. 2013a; Belicka et al. 2012; McMeans et al. 2012; Pethybridge et al. 2011a; Pethybridge et al. 2010; Schaufler et al. 2005; Wai et al. 2011).

Previous studies have described the FA composition of shark tissues (Bakes and Nichols 1995; Økland et al. 2005; Pethybridge et al. 2010; Wetherbee and Nichols 2000). However, most focus on human nutrition and by-product development for economic gain with limited work using FA profile analysis to assess diet in sharks using the muscle and liver (Pethybridge et al. 2011a). A previous study on sleeper sharks *Somniosus pacificus* identified a trophic relationship with planktivorous whales (Schaufler et al. 2005). Recently, a comprehensive dietary study of demersal chondrichthyans in south-east Australia showed that FA profiles and multivariate analysis can complement limited stomach content data by distinguishing inter-specific differences and detecting degrees of resource overlap (Pethybridge et al. 2011a). Fatty acid profiles in combination with stable isotope analysis were also used to show that white spotted bamboo sharks *Chiloscyllium plagiosum* rely on primary consumers and exhibited an ontogenetic dietary shift with increasing animal-derived FA with size (Wai et al. 2011).

Trophic pathways in a food web have distinct FA profiles (Kharlamenko et al. 2001; Mfilinge et al. 2005; Wai et al. 2011) and specific FA act as biomarkers that are conservatively transferred to high trophic levels (Fraser et al. 1989; Graeve et al. 1994; Lee et al. 1971; St John and Lund 1996). In teleosts, marine mammals, and birds, this has allowed for the inference of diet (Baylis et al. 2009; Käkelä et al. 2010), which in turn has driven the analysis of FA metabolic pathways. Fatty acids of carbon chain-length 14 or greater remain relatively unchanged following digestion, and as a result are passed into the circulatory system and taken up by tissues intact (Iverson et al. 2004). The extent to which FAs are oxidised for energy, reduced to FA alcohols, elongated to longer chain FA, or desaturated can affect the viability of FA as biochemical tracers (Henderson 1996). Inferring diet in sharks using FA profiles, therefore, requires an understanding of the effects that fluctuating diet has on biochemical pathways in different tissues and of the time scales of FA integration.

To our knowledge, only one study (Beckmann et al. 2013a) has examined the effect of diet on shark FA profiles in a controlled setting. In (Beckmann et al. 2013a), two groups of Port Jackson sharks, *Heterodontus portusjacksoni* (Meyer 1793), were shown to have significantly different liver FA profiles after being fed differing diets for 10 weeks. These experimentally fed sharks were only sampled before and after feeding for ten weeks on two different diets and no sub-samples were collected at shorter intervals. Sharks were found to be significantly different in both liver and muscle tissue when compared to wild sharks used as controls (Beckmann et al. 2013a). Muscle FA profiles did not differ by diet, which suggests that the time scales of FA integration differ by tissue and warrant further investigation and sub-sampling of sharks fed earlier than ten weeks after dietary change. The experimental design of the current study investigated dietary changes in a controlled setting with sharks fed exclusively on squid then prawns for six weeks each, and included sub-sampling the sharks every three weeks. The aim of this study was to investigate FA profile dynamics of the muscle and liver tissues before and after a dietary change, with a focus on essential FA, biomarkers, and metabolic pathways.

3.3 Methods

3.3.1 Experimental design and animal maintenance

Port Jackson sharks were collected during fishery-independent surveys of the South Australian Prawn Trawling Fishery in Gulf St Vincent (GSV), South Australia. Trawling was undertaken at night using standard 27–30 m double-rig demersal otter-trawl gear with two 14.6 m-wide nets and 4.5 cm diamond mesh cod-ends with trawl shots lasting for 30 min (Dixon et al. 2011). Sharks were transported to the marine facilities at SARDI Aquatic Sciences Centre, West Beach and maintained in 200-litre plastic containers. Between 8 and 48 h after capture, sharks were transferred to 5,000-litre tanks provided with flow-through seawater, where they were housed for the duration of the project. The gender of each shark was identified using the presence or absence of claspers. Sharks were measured (total length, TL and clasper length), weighed, and tagged with Hallprint plastic dart head identification tags (Hallprint Pty Ltd). Shark maturity was assessed according to Powter et al. (2010). Four individuals (two males and two females) were collected from the field and euthanized prior to any experimental feeding to act as controls. The remaining 21 sharks were then assigned to one of four tanks. Where possible each tank was evenly distributed in terms of shark weight and length. Temperature, pH, and dissolved oxygen concentration were regularly recorded and were within the expected normal range (temperature 20.4–21.3°C, salinity 32.8–40.3, pH 8.06–8.47, dissolved oxygen 9.22–9.8 ppm).

3.3.2 Food and feeding regime

Commencing one day after capture, March 12th 2010, sharks were fed southern calamari *Sepioteuthis australis (*Quoy & Gaimard 1832) at the same time of day at 10% of their combined body weight, or until satiated, three times a week for six weeks. The diet was switched to western king prawns *Penaeus latisulcatus* (Kishinouye 1900) at week 6. Any uneaten food was removed from the tank approximately 2 h after feeding. Dietary items were collected from the GSV prawn trawl fishery. All sharks were weighed and measured at weeks 3, 6, 9, and 12. Four sharks were sacrificed 24 h after feeding at each of weeks 3, 6 and 9, with the

remaining nine sharks sacrificed at week 12. Sharks were sacrificed by pithing and spinal section. Following euthanasia, each shark was dissected and 5–10 g of the muscle and liver tissues was collected and frozen at -20ºC until analysed. The FA profiles of prawn-fed and squid-fed sharks were compared with that of whole samples of prawns and squid, which were homogenised in a blender.

3.3.3 Lipid extraction and fatty acid analysis

Samples were analysed by the FOODplus Fatty Acid Lab (Urrbrae, South Australia, Australia). Lipids were extracted from diet or shark tissue samples using a chloroform/methanol (2:1) method as described by Bligh and Dyer (1959). The percent lipid was calculated on a wet weight basis and the lipid was extracted into chloroform. Fatty Acid Methyl Esters (FAME) were produced by heating the extracted lipids in 1% H_2SO_4 in methanol for 3 h in a 70^{o}C water bath. After cooling, 750 µl distilled water was added along with 2.0 ml of *n*-heptane and the solution was centrifuged allowing the phases to separate. The extracted FAMEs were separated and quantified using a gas chromatograph (Palo Alto, CA, USA) to determine fatty acid composition. Samples were run on a gas chromatograph with a flame ionization detector (FID) and an external standard was used with approximately 46 different FAME types. Additional FAs were identified by the relative locations of other peaks in human blood. Gas chromatography-mass spectrometry results were calibrated against an external standard with areas converted into to normalised percentages.

3.3.4 Data analysis

Bray–Curtis similarity matrices were calculated for square-root transformed data to test the differences between dietary items, fed sharks, and control sharks using Primer Version 6.1.13 (http://www.primer-e.com). The average sample dissimilarity and percent contribution of each fatty acid to the separation between diets and fed sharks were assessed using Similarity Percentage (SIMPER) analysis

which measures the top 90% of contributing variables (Clarke 1993). Differences in fatty acid composition between diets, tissues and maturity status were also analysed using a Permutational Multivariate Analysis Of Variance, PERMANOVA+ version 1.0.3 (Anderson 2001), using 9999 unrestricted permutations and additional pair-wise tests using the square root of the pseudo-F statistic (t-test). Constrained Canonical Analysis of Principal coordinates (CAP) was used to provide a graphical representation of the differences between fed and control shark FA. As CAP analysis is a constrained ordination with groupings known a priori, the 'leave-one-out' approach gives a reasonable and unbiased measure of how distinct groups are in multivariate space (Anderson and Willis 2003). Two sample t-tests and ANOVA were also employed to test the differences in weight, and lipid content between fed and control sharks. Where data is expressed as mean values, standard error of the mean was used.

3.4 Results

3.4.1 Shark size, weight, and lipid content

Twenty-five sharks were analysed, including four control sharks (Table 3.1). The PERMANOVA model showed that maturity had no significant effect on FA profiles and there was no interaction between maturity and tissue or treatment (Table 3.2). The weight of control and fed sharks was not significantly different (ANOVA F =0.221, P = 0.953). Significant differences were detected in percent weight change between sampling intervals (ANOVA F = 20.320, P > 0.001, post hoc tests available in Supplementary Table 3. 1). The lipid content of prawn- and squid-fed sharks and controls was also significantly different in the liver (ANOVA F = 4.132, P = 0.013) and muscle (ANOVA F = 6.213, P = 0.002, post hoc tests available in Supplementary Table 3. 2.

Table 3.1 Length, weight, and reproductive status for control and fed *Heterodontus portusjacksoni*. F=Female, M=Male, TL= Total Length.

Diet/wk	ID	Tank	Sex	Maturity	TL (mm)	Weight (grams)			
						Wk 0	Wk 3	Wk 6	Wk 9
Controls	C-1	-	M	Sub-adult	590	1688	-	-	-
	C-2	-	M	Immature	450	929	-	-	-
	C-3	-	F	Immature	410	598	-	-	-
	C-4	-	F	Sub-adult	540	1299	-	-	-
Squid wk 3	166	1	F	Immature	425	550	650	-	-
	186	2	F	Immature	270	700	750	-	-
	182	2	M	Sub-adult	590	1400	1500	-	-
	190	4	M	Immature	500	850	900	-	-
Squid wk 6	S168	1	M	Sub-adult	515	1000	1000	1150	-
	S191	2	M	Sub-adult	510	700	750	950	-
	S181	3	F	Immature	470	750	800	900	-
	S187	4	F	Immature	400	500	550	700	-
Prawn wk 9	S176	2	F	Immature	430	550	600	800	800
	S180	3	M	Sub-adult	550	900	1050	1200	1350
	S185	3	M	Sub-adult	610	1200	1300	1400	1400
	S193	4	F	Immature	440	750	800	900	950
Prawn wk 12	S170	1	M	Immature	460	650	650	650	750
	S189	1	M	Sub-adult	560	450	500	600	600
	S165	2	F	Sub-adult	550	1050	1100	1300	1200
	S167	2	F	Immature	500	800	850	1050	1050
	S188	3	F	Immature	420	500	450	650	600
	S183	3	F	Immature	470	900	950	1200	1100
	S171	4	M	Sub-adult	510	900	1050	1250	1250
	S184	4	M	Sub-adult	660	1850	2000	2200	2100
	S192	4	F	Immature	470	700	750	900	950

Table 3.2 Two-way non-parametric multivariate analysis of variance (PERMANOVA) results of fatty acid profile differences between tissue (liver, muscle), treatment (control at week 0, squid-fed at weeks 3 and 6, prawn-fed at weeks 9 and 12) and maturity (immature, sub-adult) of *Heterodontus portusjacksoni*. *Indicates significant result ($p < 0.05$)

Source	Df	MS	F	P
Tissue	1	3428.50	139.19	0.001*
Treatment	4	422.70	17.11	<0.001*
Maturity	1	14.04	0.57	0.604
Tissue x Treatment	4	67.38	2.58	0.012*
Tissue x Maturity	1	1.49	0.06	0.988
Treatment x Maturity	4	29.29	1.18	0.293
Treatment x tissue x maturity	4	18.13	0.73	0.688

3.4.2 Fatty acid profiles and differences between treatments

Fatty acid analysis of diets and shark tissues identified 50 individual FAs. Based on the Bray–Curtis similarity index, the PERMANOVA model showed that FA compositions in Port Jackson sharks were significantly different by treatment (diet and week sampled, F = 17.11, P = 0.001) and tissue (F = 139.19, P < 0.001) and that the interaction between diet and tissue was also significant (F = 2.58.11, P = 0.012, (Table 3.2).

The CAP results based on the FA profiles suggest that control and experimentally fed sharks can be distinguished using the muscle and liver tissues (Figure 3.1). Strong associations between the multivariate data 'cloud' and dietary differences were indicated by the reasonably large size of the canonical correlations. The liver FA biplot shows clear separation of squid- and prawn-fed sharks sampled at weeks 3 and 9, however, there is some overlap of squid-fed sharks sampled at weeks 6 and prawn-fed sharks sampled at week 12 (Figure 3.1a). The muscle FA biplot shows that squid-fed sharks are clearly separated from prawn-fed sharks (Figure 3.1b). In both liver and muscle tissues, there was no separation between sharks fed the same diet for 3 or 6 weeks. Individual distributions were correlated with diet and week sampled for muscle (CAP, first canonical correlation = 2.045, P < 0.001) and liver (CAP, first canonical

correlation =1.661 P < 0.001). Cross validation of the CAP model (*via* leave-one-out allocation of observations to groups) showed that sharks were correctly classified to diet by week sampled 60% of the time for muscle and 64% of the time for liver based solely on FA% data. Correct classification increased to 100% for both tissues when week sampled was removed and diet was the only classifier used (muscle CAP, first canonical correlation = 0.993; P < 0.001, liver CAP, first canonical correlation = 1.858; P < 0.001).

3.4.3 Prey fatty acid profiles

The percent total lipid of squid (1.9% ± 0.1) and prawns (1.1% ± 0.2) was significantly different (ANOVA F = 16.928, P = 0.002). Squid and prawns also had significantly different FA profiles (PERMANOVA F = 18.01, P = 0.002) with SIMPER analysis showing a 24.5% average dissimilarity. Prawns contained higher levels of 16:1n-7and 18:1n-9 compared to squid, and 22:6n-3 and 20:4n-6 were higher in squid compared to prawns (Table 3.3).

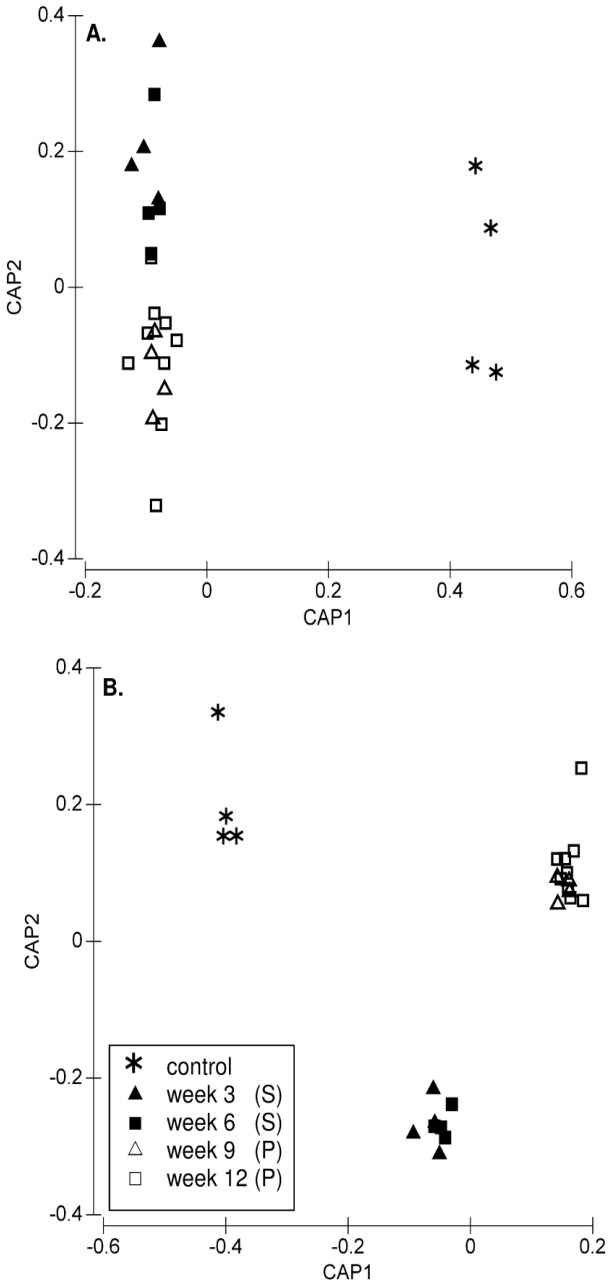

Figure 3.1 Canonical analysis of principal coordinates (CAP) plot from shark (A) liver and (B) muscle fatty acid profiles compared by diet and week sampled as well as controls.

Table 3.3 Mean total fatty acid percentages (± standard error) of squid and prawn diets fed to *Heterodontus portusjacksoni*.

	Squid		Prawn	
16:00	22.24	± 0.19	14.60	± 0.06
dma 18:0	1.10	± 0.48	0.69	± 0.26
18:00	7.32	± 0.34	10.46	± 0.45
24:00:00	0.54	± 0.18	0.65	± 0.23
Trans 18:1n-7	0.00	± 0.00	0.06	± 0.02
16:1n-7	1.00	± 0.19	5.35	± 0.26
18:1n-9	2.76	± 0.26	8.86	± 0.38
18:1n-7	1.53	± 0.12	3.65	± 0.31
19:01	0.10	± 0.02	0.20	± 0.10
20:1n-11	0.35	± 0.17	0.28	± 0.13
20:1n-9	2.02	± 0.07	0.62	± 0.13
22:1n-11	0.06	± 0.01	0.24	± 0.06
18:2n-9	0.22	± 0.02	0.66	± 0.08
20:2n-9	0.02	± 0.01	0.05	± 0.02
20:3n-9	0.00	± 0.00	0.00	± 0.00
18:2n-6	0.48	± 0.06	1.37	± 0.10
18:3n-6	0.11	± 0.03	0.21	± 0.02
20:4n-6	2.94	± 0.53	7.66	± 0.22
16:2n-3	0.08	± 0.04	0.09	± 0.04
18:3n-3	0.26	± 0.07	0.52	± 0.09
20:5n-3	13.16	± 0.24	14.59	± 0.54
22:5n-3	0.91	± 0.10	2.31	± 0.20
22:6n-3	33.53	± 0.92	12.55	± 0.67
Total SATFA	35.02	± 1.51	30.96	± 1.51
Total MUFA	9.44	± 1.31	21.90	± 1.31
Total PUFA	52.87	± 2.15	52.87	± 2.15

3.4.4 Liver fatty acid profiles

PERMANOVA showed significant differences in the FA profiles of shark liver tissue sampled at different weeks ($F = 21.92$, $P > 0.001$). Pairwise tests showed significant differences between the liver FA profiles of control sharks and sharks before and after the dietary switch (Table 3.4). PERMANOVA also detected a difference between the liver FA profiles of sharks fed different diets and sampled at weeks 6 and 12 and weeks 3 and 9 (Table 3.4). SIMPER analysis identified up to 18.2% average dissimilarity in the liver FA profiles of controls and fed sharks and 7.7% average dissimilarity between prawn- and squid-fed sharks sampled before and after the dietary shift (Figure 3.3). At all weeks sampled, shark liver FA profiles had higher levels of 18:3n-6, 20:1n-11, 20:1n-9 and 24:0 compared to the liver of control sharks (Table 3.5). The liver FA profiles of control sharks had higher levels of 20:2n-9, and 20:3n-9 compared to the liver of fed sharks (Table 3.5) and this pattern was reflective of low levels of these FAs in squid and prawns (Table 3.4).

Table 3.4 Pairwise statistical tests of dietary differences in liver and muscle tissue fatty acid profiles between control *Heterodontus portusjacksoni* and sharks fed prawn or squid diets by two-way factorial non-parametric multivariate analysis of variance (PERMANOVA) . Treatments = control, week 3 (S, squid-fed), week 6 (S), week 9 (P, prawn-fed), week 12 (P). *Indicates significant result ($p < 0.05$)

Treatment	Liver PERMANOVA		Muscle PERMANOVA	
	t	P (perm)	*t*	P (perm)
Control – wk 3 (S)	4.493	0.029*	3.200	0.029*
Control – wk 6 (S)	4.790	0.029*	3.501	0.031*
Control – wk 9 (P)	4.333	0.024*	4.969	0.025*
Control – wk 12 (P)	5.196	0.001*	5.126	0.005*
Wk 3 (S) – wk 6 (S)	1.025	0.395	1.0767	0.302
Wk 3 (S) – wk 9 (P)	1.876	0.069*	3.449	0.036*
Wk 3 (S) – wk 12 (P)	1.915	0.013*	3.565	0.002*
Wk 6 (S) – wk 9 (P)	1.771	0.035*	3.481	0.026*
Wk 6 (S) - wk 12 (P)	1.792	0.014*	3.686	0.002*
Wk 9 (P) - wk 12 (P)	0.551	0.914	0.670	0.742

Higher levels of trans 18:1n-7 were seen in squid-fed sharks, compared to prawn-fed sharks, where only trace levels were observed (Table 3.5). This pattern was reflective of diet as prawns and squid contained similar levels of trans 18:1n-7 (Table 3.3) . 22:6n-3 was, however, seen in higher levels in the liver of squid-fed sharks than in prawn-fed sharks (Table 3.5) and this pattern was consistent with diet (Table 3.3). Conversely, 16:1n-7 and oleic acid 18:1n-9 were seen in higher levels in the liver of prawn-fed sharks compared to the liver of squid-fed sharks (Table 3.5) and this was reflective of diet (Table 3.3). Furthermore the ratio of 16:1n-7 to 16:0 increased slightly from squid-fed sharks at week 6 to prawn-fed sharks sampled at week 9 (Figure 3.3a), this was despite a higher dietary ratio in prawns at 0.37 ± 0.05 compared to 0.05 ± 0.01 in squid. The 20:3n-9 to ARA ratio was near zero in controls and showed an increasing trend up to week 6 (Figure 3.3c), this was despite an identical dietary ratio as a result of no dietary input of 20:3n-9 in either diet. The ARA (20:4n-6)/EPA ratio was highest in controls and remained relatively stable through from week 3 to week 12 (Figure 3.3b). This was despite a higher ratio in prawns with 0.53 ± 0.02 compared to squid with 0.23 ± 0.04. A higher n-3/n-6 ratio was also seen in controls compared to fed sharks (Figure 3.3b). A slight increase occurred in the ratio of n-3/n-6 in prawn fed sharks which was not related to the dietary ratio where prawns contained 2.93 ± 0.10 compared to squid with 13.60 ± 2.15 (Figure 3.3a).

3.4.5 Muscle fatty acid profiles

PERMANOVA showed a significant difference between the FA profiles of shark muscle tissue sampled at different weeks ($F = 12.84$, $P > 0.001$). Pairwise tests indicated significant differences between control sharks and the muscle FA profiles of sharks sampled at all other weeks (Table 3.4). Similar to shark liver FA profiles, significant differences were also observed before and after the dietary switch. SIMPER analysis identified 15.6% average dissimilarity between control muscle FA profiles and the FA profiles of all sharks sampled throughout the duration of the study and a 9.8% average dissimilarity when comparing sharks before and after the dietary switch (Figure 3.2). At all weeks sampled levels of

20:1n-9 and DMA 18:0 were higher in the muscle of fed sharks than in the muscle of controls **(**Table 3.5**)**. The muscle of prawn-fed sharks had higher levels of 16:2n-3, 18:3n-6, and 24:0 compared to only trace amounts detected in controls (Table 3.5).

Levels of 24:0 increased when prawn diet commenced, however, no change occurred to levels of 16:2n-3 and 18:3n-6 in the diet. 20:3n-9 and 18:2n-9 were both seen in higher levels in the muscle of fed sharks than control sharks **(**Table 3.5**).** For 20:3n-9, this pattern is not consistent with diet as it was not detected in squid or prawns (Table 3.3). Higher levels of 18:2n-9 were detected in prawns than in squid and this was consistent with higher levels in the muscle of prawn-fed sharks compared to only trace amounts seen in squid-fed sharks (Table 3.5). 20:2n-9 was seen in higher levels in the muscle of control sharks than squid-fed sharks (Table 3.5). 20:2n-9 was not detected in prawn-fed sharks and both diets only contained trace amounts (Table 3.5)

Levels of 19:1 were higher in the muscle of control and squid-fed sharks compared to the muscle of prawn-fed sharks where none was detected (Table 3.5). This is inconsistent with diet as prawns contained slightly higher levels of 19:1 than squid (Table 3.3). The muscle of control and squid-fed sharks sampled at week 3 also had higher levels of 20:4n-6 than squid-fed sharks (week 6) and prawn-fed sharks (Table 3.5) which was again inconsistent with diet as prawns contained higher levels of 20:4n-6 than squid (Table 3.3). The 16:1n.-7:16:0 ratio increased from week 3 to week 9 (Table 3.3c) and this was consistent with a slightly higher ratio in prawns (0.37 ± 0.02) compared to squid (0.23 ± 0.04). The 20:3n-9 to ARA (20:4n-6) ratio was higher in control sharks compared to fed sharks (Table 3.3c) and the stable levels seen in fed shark liver were reflective of identical ratios in prawn (0.00 ± 0.00) and squid diets (0.00 ± 0.00). The n-6:n-3 ratio was highest in control sharks however it remained relatively constant from weeks 3 to 12 (Table 3.3d), this was despite a higher ratio in squid (13.60 ± 0.10) compared to prawns (2.93 ±0.10). The ARA:EPA ratio decreased from weeks 3 to 6 followed by an increase up to similar levels as seen in controls at week 12 (Table 3.3d). This is consistent with diet as prawns had a higher ratio (0.53 ± 0.02) compared to squid (0.23 ± 0.04).

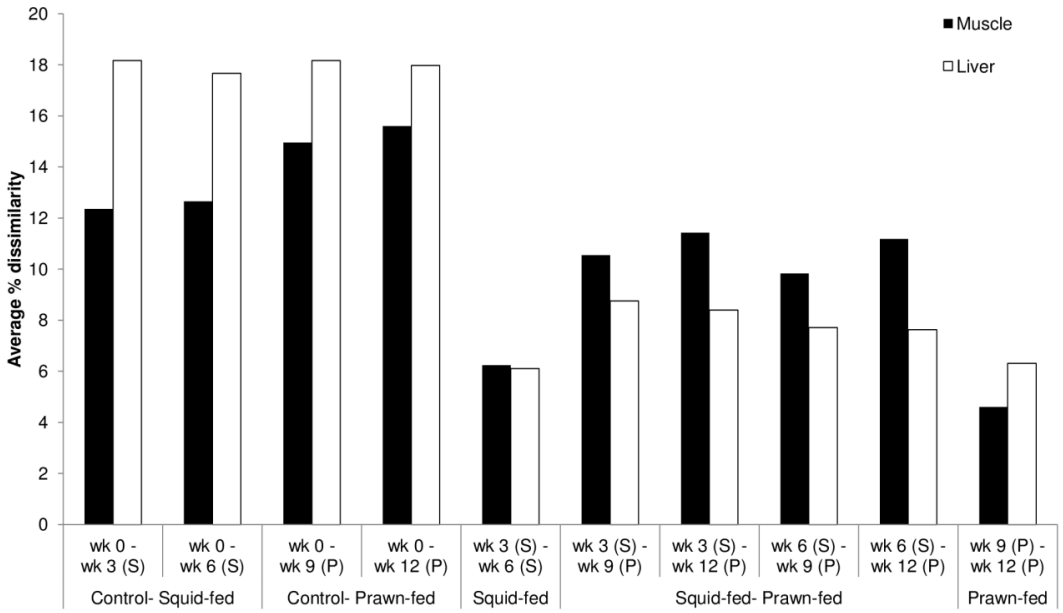

Figure 3.2 Similarity percentage (SIMPER) analysis of fatty acid percentages from the liver and muscle of *Heterodontus portusjacksoni* expressed as a percentage of average dissimilarity based on a Bray-Curtis similarity matrix. Sharks fed prawns, squid and control compared by tissue (muscle, liver).

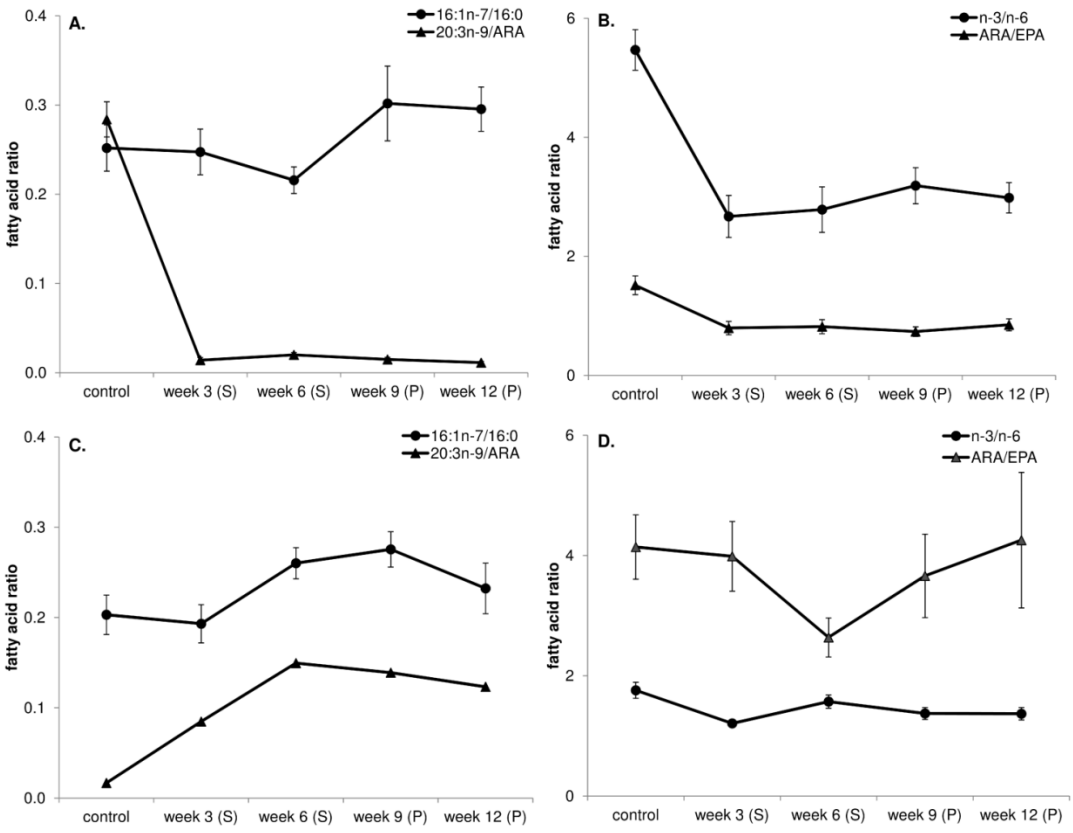

Figure 3.3 Ratios of polyunsaturated fatty acids and n-3:n-6 fatty acids in (a), (b) liver and (c), (d) muscle tissues of *Heterodontus portusjacksoni*. Lines are ratios and vertical lines are standard error (SE)

Table 3.5 Mean total fatty acid percentages (+/- standard error) of control, squid-fed and prawn-fed *Heterodontus portusjacksoni* liver and muscle tissues.

	Muscle										Liver									
	Control		Squid				Prawn				Control		Squid				Prawn			
	Week 0		Week 3		Week 6		Week 9		Week 12		Week 0		Week 3		Week 6		Week 9		Week 12	
16:0	19.7	±1.4	19.8	±0.5	20.0	±0.3	20.2	±0.1	19.6	±0.2	20.2	±1.7	20.3	±1.4	21.5	±0.6	22.3	±	21.4	±0.7
dma 18:0	0.00	±0.0	0.84	±0.1	0.87	±0.0	0.86	±0.0	1.01	±0.0	0.02	±0.0	0.06	±0.0	0.09	±0.0	0.06	±	0.05	±0.0
18:0	8.54	±0.4	7.87	±0.1	7.03	±0.2	7.84	±0.0	8.58	±0.2	6.55	±0.4	6.76	±0.7	5.68	±0.2	5.07	±	5.39	±0.2
24:0	0.01	±0.0	0.00	±0.0	0.00	±0.0	0.88	±0.0	0.94	±0.0	0.07	±0.0	1.17	±0.0	1.06	±0.0	0.97	±	1.12	±0.1
Trans18:1n-	0.03	±0.0	0.00	±0.0	0.00	±0.0	0.00	±0.0	0.00	±0.0	0.03	±0.0	0.20	±0.0	0.19	±0.0	0.00	±	0.00	±0.0
16:1n-7	4.02	±0.9	3.81	±0.3	5.24	±0.4	5.58	±0.4	4.57	±0.5	5.17	±0.8	5.09	±0.7	4.63	±0.2	6.88	±	6.48	±0.7
18:1n-9	12.8	±0.7	11.0	±0.5	12.4	±0.3	12.8	±0.4	12.7	±0.4	13.6	±1.4	12.3	±0.6	12.5	±0.3	15.2	±	14.0	±0.7
18:1n-7	6.83	±1.5	6.64	±0.5	6.87	±0.3	6.99	±0.2	6.84	±0.3	7.46	±0.0	5.85	±0.3	6.47	±0.5	6.23	±	6.45	±0.4
19:1	0.36	±0.0	0.5	±0.0	0.47	±0.0	0.00	±0.0	0.00	±0.0	0.31	±0.0	0.05	±0.0	0.05	±0.0	0.04	±	0.04	±0.0
20:1n-11	0.00	±0.0	0.07	±0.0	0.08	±0.0	0.1	±0.0	0.11	±0.0	0.00	±0.0	1.31	±0.1	0.85	±0.0	0.75	±	0.78	±0.0
20:1n-9	0.00	±0.0	0.37	±0.0	0.43	±0.0	0.57	±0.0	0.52	±0.0	0.11	±0.0	1.69	±0.1	2.14	±0.2	1.86	±	1.77	±0.1
22:1n-11	0.00	±0.0	0.15	±0.0	0.15	±0.0	0.01	±0.0	0.00	±0.0	0.07	±0.0	0.44	±0.0	0.47	±0.0	0.01	±	0.00	±0.0
18:2n-9	0.26	±0.0	0.00	±0.0	0.00	±0.0	0.28	±0.0	0.31	±0.0	0.25	±0.0	0.17	±0.0	0.15	±0.0	0.16	±	0.14	±0.0
20:2n-9	0.49	±0.0	0.08	±0.2	0.12	±0.0	0.00	±0.0	0.00	±0.0	2.14	±0.1	0.01	±0.0	0.01	±0.0	0.00	±	0.00	±0.0
20:3n-9	0.18	±0.0	0.98	±0.2	1.31	±0.3	1.22	±0.3	1.08	±0.1	0.88	±0.1	0.04	±0.0	0.04	±0.0	0.03	±	0.02	±0.0
18:2n-6	0.66	±0.0	0.85	±0.0	0.74	±0.0	0.83	±0.0	0.93	±0.0	0.89	±0.0	1.25	±0.2	1.09	±0.0	0.98	±	1.21	±0.1
18:3n-6	0.00	±0.0	0.00	±0.0	0.00	±0.0	0.41	±0.0	0.43	±0.0	0.00	±0.0	0.58	±0.0	0.65	±0.0	0.60	±	0.61	±0.0

20:4n-6	11.3	±2.72	12.0	±0.76	9.59	±0.86	9.44	±0.74	10.3	±1.13	3.08	±0.39	2.86	±0.69	2.29	±0.25	1.82	±	2.11	±0.1
16:2n-3	0.00	±0.00	0.17	±0.02	0.17	±0.01	0.40	±0.02	0.47	±0.04	0.11	±0.10	0.23	±0.02	0.25	±0.02	0.35	±	0.36	±0.0
18:3n-3	0.16	±0.02	0.12	±0.02	0.08	±0.01	0.09	±0.01	0.11	±0.01	0.55	±0.06	0.48	±0.12	0.38	±0.07	0.37	±	0.52	±0.0
20:5n-3	2.81	±0.62	3.17	±0.36	3.71	±0.23	2.72	±0.25	2.88	±0.24	2.15	±0.45	3.46	±0.40	3.01	±0.56	2.62	±	2.68	±0.2
22:5n-3	4.83	±0.59	4.75	±0.31	4.56	±0.55	4.07	±0.20	4.12	±0.13	7.06	±0.47	6.19	±0.59	7.07	±0.57	6.90	±	7.11	±0.3
22:6n-3	13.6	±2.02	13.2	±0.82	13.6	±0.33	12.0	±0.79	11.8	±0.51	14.7	±1.49	15.1	±0.62	15.7	±1.29	14.5	±	14.5	±0.8
Total	25.6	±	24.4	±	27.7	±	28.0	±	26.6	±	26.9	±	28.9	±	29.2	±	33.1	±	31.6	±1.1
Total PUFA	40.4	±3.48	40.4	±1.21	38.1	±1.35	35.2	±1.07	36.0	±1.17	42.9	±1.46	40.8	±1.46	41.6	±1.51	36.3	±3.44	41.5	±1.1
Total	33.2	±1.41	34.3	±0.57	33.2	±0.35	34.9	±0.24	35.3	±0.38	28.5	±0.93	32.3	±1.15	31.7	±0.72	31.6	±1.13	31.3	±0.4

3.5 Discussion

Our controlled experiment provided a unique opportunity to study tissue-specific incorporation of dietary-derived FA over time. This study demonstrates differing incorporation rates between muscle and liver FAs in sharks. The FA profiles of the liver and muscle have been (Beckmann et al. 2013a) demonstrated as accurate indicators of dietary change, with the muscle being a stronger indicator of recent dietary change than the liver. These findings indicate that dietary FAs are preferentially utilised in the muscle tissue following a dietary change and that changes to FA profiles occur quickly following dietary change resulting in sampling intervals needing to be suitably timed to detect these changes. While previous studies have demonstrated inter-tissue differences in FA profiles and their linkages to different dietary components (McMeans et al. 2012; Pethybridge et al. 2010), this is the first controlled study in chondrichthyans to report tissue specific incorporation of dietary-derived FA over time.

A previous controlled feeding study demonstrated significant differences in the liver FA profiles of sharks fed different experimental diets (Beckmann et al. 2013a). The reason that no significant difference was found between the muscle tissues of sharks fed different experimental diets after ten weeks in the previous study was likely due to the physiological function of muscle, which has low levels of fat and derives metabolic energy mainly from proteins (Pethybridge et al. 2010). After ten weeks of feeding, it was hypothesised that dietary FA such as 22:6n-3 had already turned over (equilibrated) and that the muscle FA profiles had already reverted back to those FA required for function (Beckmann et al. 2013a). This hypothesis is supported by the current study as differences in FA composition were detected in both liver and muscle tissues within three weeks following an experimental dietary switch. Furthermore, this study showed that the FA profiles of both tissues were not significantly different when diet remained unchanged. This finding has implications for the study of intraspecific dietary differences such as ontogenetic shifts as FA profiles of sharks fed the same diet showed no significant difference in FA concentrations despite differing sizes and sex ranges. Recent work by McMeans et al. (2012) suggested that the muscle FA profile of

chondrichthyans is proportionally the most similar to prey, suggesting direct incorporation of most FA from the diet. Our findings indicate that the muscle is the most indicative of recent dietary change given higher levels of dissimilarity between FA profiles before and after the dietary switch. This is supported by Pethybridge et al. (2011a) who suggests that the PUFAs which characterise muscle tissue are preferentially diverted to the muscle to meet physiological requirements. While the muscle has been successfully used as an indicator of dietary change in this study, a previous study indicated that the liver is in fact more indicative of long-term dietary patterns (Beckmann et al. 2013a). This is likely a result of the liver being a major site of both lipid storage and metabolism in sharks and the limited ability of sharks to oxidise FA outside of the liver (Ballantyne 1997; Moyes et al. 1990).

3.5.1 Essential fatty acids

The liver and muscle FA profiles from sharks fed a squid-based diet prior to the dietary switch consistently had higher levels of dietary derived 22:6n-3 than prawn-fed sharks following the dietary switch, and this result is supported by the previous 10-week trial (Beckmann et al. 2013a). 22:6n-3 was among the major drivers of the difference between prawn and squid diet items and is particularly useful as a dietary indicator because sharks have a reduced ability to form it through desaturation and elongation (Tocher 2003). In addition, DMA 18:0 was seen in higher levels in fed sharks than controls and may be linked to PUFA rich diets devoid in 22:6n-3 (Glick and Fischer 2010). This was demonstrated by a 37% reduction in dietary 22:6n-3 with the introduction of prawns into the diet and a corresponding increase in DMA 18:0 levels. While there is much in the literature regarding the metabolic pathways of FA in vertebrates, questions remain about the ability of sharks to elongate and desaturate different FAs. For example, 20:4n-6 is usually only considered an essential FA when there is a deficiency in 18:2n-6 or if there is an inability to convert 18:2n-6 to 20:4n-6. As vertebrates lack Δ12 and Δ15 desaturases, they cannot form 18:2n-6 and18:3n-3 from 18:1n-9 (Tocher 2003). Despite low dietary input, 18:3n-6 increased in the muscle of fed sharks

following the dietary switch to prawns. Many vertebrates do have the ability to create 20:4n-6 from 18:2n-6 and 18:3n-6 is an intermediate of this process (Sargent et al. 1999). The lack of 18:3n-6 in control shark tissues suggests that there was a limited need to produce 20:4n-6 from 18:2n-6 when consuming the wild diet. This is likely due to a surplus of 20:4n-6 in available prey items such as molluscs, which prey on macroalgae, which can be inferred by the FA biomarker 20:4n-6 (Wai et al. 2011). Dietary studies of Port Jackson sharks that indicated molluscs, which consume mainly macroalgae, were one of the most abundant prey types (Powter et al. 2010). This is supported by the lower levels of 20:4n-6 in fed sharks compared to control sharks, which may be the reason 20:4n-6 synthesis was triggered in the fed sharks. Previously, 18:2n-6 has been utilised as a biomarker of fresh plant material, which indicates secondary predation on herbivorous species (Wai et al. 2011). Our findings indicate that inputs of 18:2n-6 are affected by synthesis of 20:4n-6 and this could be detected by monitoring 18:3n-6 levels.

Polyunsaturated FA can be converted to eicosanoids, which are a range of highly active compounds with carbon chain lengths of 20 formed in small amounts by most tissues and are involved in a variety of physiological functions (Henderson 1996; Sargent et al. 1999). The major precursor of eicosanoids is 20:4n-6, whereas 20:5n-3 is less biologically active and can inhibit the formation of 20:4n-6 (Sargent et al. 1999). The ratios of n-6:n-3 PUFA and long-chain PUFA 20:5n-3, 20:4n-6 and 22:6n-3 are often examined to determine whether eicosanoid actions are elevated indicating a response to stress (Sargent et al. 1999) . Excess levels of n-6 PUFA can inhibit the production of n-3 PUFA, which are essential for cellular processes (Sargent et al. 1999). Both control and fed sharks had higher levels of n-3 PUFA than n-6 FA. Control sharks also had elevated levels of 20:2n-9 which can occur due to deficiencies in n-3 FA (Caballero et al. 2002). Deficient levels of n-3 PUFA can cause the activation of Δ6 and Δ5 desaturases, which can allow the preferential utilisation of n-3 and n-6 PUFA by enzymes (Caballero et al. 2002). Control sharks had higher ARA:EPA ratios than fed sharks in the liver. Increased ratios of ARA:EPA may indicate enhanced eicosanoid actions such as inflammatory response, however, the optimal ratio of these FAs in terms of health is unknown (Sargent et al. 1999).

3.5.2 Fatty acid biomarkers

20:1n-9, an indicator of copepods (Phillips et al. 2003), was seen in higher levels in the liver and muscle of fed sharks compared to control sharks. The reason for the deficiency of this FA in control sharks may demonstrate either a lack of copepod dietary sources or the preferential utilisation of this FA for energy. Most of the sharks sampled were immature or sub-adults, therefore, a higher percentage of copepod markers would be expected in adult sharks due to ontogenetic changes in diet resulting in increased predation on teleost fish, which consume copepods (Powter et al. 2010).

Elevated ratios of 16:1n-7/16:0 have previously been suggested as indicators of diatom based food webs (St John and Lund 1996). Prawn-fed sharks had consistently higher levels of 16:1n-7 in the liver, which differs to a previous study showing increased levels of 18:1n-7 and 22:5n-3 as the top contributors to the difference between prawn- and squid-fed sharks (Beckmann et al. 2013a). This can, however, be explained by the metabolic pathway of 16:1n-7, which is converted to 18:1n-7 while 22:5n-3 is an intermediate of 22:6n-3 metabolism.

Higher levels of LC-SFA such as 24:0 can also be indicative of the transfer of terrestrial organic matter (Budge et al. 2001; Wai et al. 2011). The liver and muscle tissues of sharks fed experimental diets had higher levels of 24:0 than those of control sharks. This would normally be seen as a result of the consumption of omnivorous and suspension or deposit feeding organisms such as polychaetes (Wai et al. 2011) suggesting that wild diet was in fact deficient in 24:0 compared to sharks that were fed squid. This is unexpected as juvenile Port Jackson sharks are known to consume mainly soft-bodied prey such as polychaete worms (McLaughlin and O'Gower 1971; Powter et al. 2010). As a result, the control sharks would have been expected to contain higher levels of 24:0 than squid- or prawn-fed sharks.

Trans 18:1n-7 has been suggested as a useful biomarker as trans FA are readily incorporated into adipose tissue and cannot be endogenously synthesised

by carnivores (Chen et al. 1995; Ohlrogge et al. 1981; Thiemann et al. 2008). Trans 18:1n-7, detected at higher levels in livers of squid-fed sharks than controls or prawn-fed sharks, is the predominant trans FA isomer in ruminant FA (Ledoux et al. 2007; Parodi 1976). Trans 18:1n-7 was not present in the squid fed to sharks. As it cannot be manufactured, it is likely that this FA has accumulated from prior consumption.

3.5.3 Fatty acid synthesis

Dietary FAs which are consumed in excess of the immediate energy requirements of an animal are generally deposited and stored largely intact. There is, however, potential during metabolism and transportation for FA to be modified (Iverson 2009). High levels of essential FA, including long-chain n-3 and n-6 PUFA, exist in the natural diets of marine predators, and as a result modification is likely to be limited and FA stored intact (Ackman 1980). Despite these limitations, there is still potential for FA to follow biochemical pathways of which sharks may be either pre-disposed to or may be activated by deficiencies in the diet. Specific, FA such as 20:3n-9 (mead acid) can be used as indicators of essential fatty acid (EFA) deficiencies (Csengeri 1996). Levels of mead acid increased in the muscle of squid-fed sharks despite no dietary input suggesting the desaturation and elongation of 18:1n-9 to 20:3n-9 (Cook 1996). Despite this, 18:1n-9 was an indicator of prawn-fed sharks and has previously been indicated as a biomarker of brown algae (Alfaro et al. 2006). Levels of 18:1n-9 can, however, be affected by inadequate levels of 18:2n-6 and 18:3n-3, as 18:1n-9 is elongated and desaturated to 20:3n-9 (Curtis-Prior 2004). Higher levels of 18:2n-6 and 18:3n-3 were present in prawn diet compared to squid and as a result the ratio of 20:3n-9 to 20:4n-6 increased in squid-fed sharks due to a deficient levels of 20:4n-6 and increasing levels of 20:3n-9. The 20:3n-9/20:4n-6 ratio can be used as a biochemical marker for EFA deficiency (Holman 1977). 20:3n-9 was preferentially stored in the muscle where dietary input was low, however, in control sharks, preferential storage occurred in the liver. As 20:3n-9 can also be accumulated due to EFA

deficiencies (Seiliez et al. 2003; Tocher 2003; Wang et al. 2010); this result suggests that accumulation is tissue specific.

Accumulation of 18:2n-9, an intermediate in the synthesis of 20:3n-9, was also seen in the liver and muscle of control sharks and muscle of prawn-fed sharks. This accumulation is consistent with diet but can also suggest a deficiency in FA desaturation/elongation (Seiliez et al. 2003). Additionally, high levels of 20:1n-11 and 22:1n-11 were seen in fed sharks compared to control sharks for both tissues with the exception of the liver of prawn-fed sharks. 20:1n-11 is the product of the partial chain-shortening of 22:1n-11 and is typically found in higher trophic level predators (Bremer and Norum 1982; Cooper et al. 2006, 2009; Norseth and Christophersen 1978). Higher levels of 20:1n-11 in the liver tissue of fed sharks were consistent with diet, however, levels of 22:1n-11in fed sharks were not consistent with diet. This may be explained by preferential utilisation of 18:0 and 18:1n-9, which can be metabolised to 20:1n-9. These interactions are important to recognise as 22:1n-11 and 20:1n-9 are derived from the corresponding FA alcohols in wax esters of copepods and this is likely to indicate secondary predation by squid (Phillips et al. 2003), and hence a step in the trophic pathway.

3.5.4 Implications and significance

Sharks that were fed different diets showed clear prey-related FA profiles in the muscle and liver tissues. Prominent FAs which were typical for the mollusc and crustacean species given as food were shown to increase in the Port Jackson shark tissues within three weeks after commencement of the study. Differentiation between dietary FAs and those that are affected by metabolism and biosynthesis was possible to some extent in this study. Several FAs were identified as top contributors to the difference between weeks. However, the elevated levels of these FAs were not always consistent with the diet being fed at the time and tracing their biochemical pathways generally allowed the source of FA to be identified.

The aim of this study was to investigate FA profile dynamics before and after a dietary change. Our results indicate that dietary FA are preferentially utilised and accumulated by muscle tissue following a dietary change. This is an important consideration when determining experimental design as muscle FA profiles are a stronger indicator of recent dietary change than liver. While the different metabolic capacities of chondrichthyan tissues are still poorly understood, previous studies have shown that FA profiles can be consistent with stomach contents information (Pethybridge et al. 2011a). This is supported by a strong link between dietary change and muscle FA profiles.

The management of ecosystems relies on the accurate assessment of trophic relationships and consumer diets. Alternative approaches of dietary analysis such as FA profiling and the use of FA biomarkers can be used to reduce the biased associated with traditional approaches, such as stomach content analysis. Here, we have demonstrated the use of individual FA biomarkers to identify changes in diet. While the use of FA profiles does not generally provide quantitative information about the diet of a species, FA biomarkers can be used to determine predominant dietary groups and dietary changes through comparison of FA profiles. Quantitative assessment of a species diet can be performed through a statistical model, as developed by Iverson et al.(2004). This approach, however, requires complete FA turnover time to be calculated to understand the effects of metabolism on the FA stored in consumers (Iverson 2009). To develop quantitative models, more extensive manipulations of diet in a controlled environment are required to understand the rates of metabolism and FA tissue incorporation.

3.6 Acknowledgments

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3.7 Supplementary Material

Supplementary Table 3.1 Games-Howell post hoc tests comparing sampling intervals for weight change (%) in liver and muscle tissue fatty acid profiles between control *Heterodontus portusjacksoni* and sharks fed prawn or squid diets observed in a two-way ANOVA . Treatments = control, week 3 (S, squid-fed), week 6 (S), week 9 (P, prawn-fed), week 12 (P). *Indicates significant result ($p < 0.05$).

Period of weight change compared		P
week 0 – week 3 (S)	week 3 (S) – week 6 (S)	0.006*
	week 6 (S) – week 9 (P)	0.262
	week 9 (P) – week 12 (P)	0.998
	week 0- week 6 (S)	0.000*
	week 0 – week 12 (P)	0.003*
	Week 6 (S) – week 12 (P)	1.000
week 3 (S) – week 6 (S)	week 0 – week 3 (S)	0.006*
	week 6 (S) – week 9 (P)	<0.001*
	week 9 (P) – week 12 (P	0.002
	week 0 – week 6 (S)	0.514
	week 0 – week 12 (P)	0.204
	week 6 (S) – week 12 (P)	0.02*
week 6 (S) – week 9 (P)	week 0 – week 3 (S)	0.262
	week 3 (S) – week 6 (S)	<0.001*
	week 9 (P) – week 12 (P	0.292
	week 0 – week 6 (S)	<0.001*
	week 0 – week 12 (P)	<0.001*
	week 6 (S) – week 12 (P)	0.774
week 9 (P) – week 12 (P	week 0 – week 3 (S)	0.998
	week 3 (S) – week 6 (S)	0.002*
	week 6 (S) – week 9 (P)	0.292
	week 0 – week 6 (S)	<0.001*
	week 0 – week 12 (P)	0.003*
	week 6 (S) – week 12 (P)	1.000
week 0 – week 6 (S)	week 0 – week 3 (S)	<0.001*
	week 3 (S) – week 6 (S)	0.514
	week 6 (S) – week 9 (P)	<0.001*
	week 9 (P) – week 12 (P	<0.001*
	week 0 – week 12 (P)	0.942
	week 6 (S) – week 12 (P)	<0.001*
week 0 – week 12 (P)	week 0 – week 3 (S)	0.003*
	week 3 (S) – week 6 (S)	0.204
	week 6 (S) – week 9 (P)	<0.001*
	week 9 (P) – week 12 (P	0.003*
	week 0 – week 6 (S)	0.942
	week 6 (S) – week 12 (P)	0.002*
week 6 (S) – week 12 (P)	week 0 – week 3 (S)	1.000
	week 3 (S) – week 6 (S)	0.020*
	week 6 (S) – week 9 (P)	0.774
	week 9 (P) – week 12 (P	1.000
	week 0 – week 6 (S)	<0.001*
	week 0 – week 12 (P)	0.002*

Supplementary Table 3.2 Tukey post hoc tests comparing sampling intervals for total lipid (%) in liver and muscle tissue fatty acid profiles between control *Heterodontus portusjacksoni* and sharks fed prawn or squid diets observed in a two-way ANOVA . Treatments = control, week 3 (S, squid-fed), week 6 (S), week 9 (P, prawn-fed), week 12 (P). *Indicates significant result ($p < 0.05$)

Treatments compared		Muscle	Liver
		P	P
control	week 3 (S)	0.967	0.183
	week 6 (S)	0.890	1.000
	week 9 (P)	0.006*	0.975
	week 12 (P)	0.757	0.707
week 3 (S)	Control	0.967	0.183
	week 6 (S)	0.546	0.188
	week 9 (P)	0.001*	0.060
	week 12 (P)	0.330	0.006*
week 6 (S)	control	0.890	1.000
	week 3 (S)	0.546	0.188
	week 9 (P)	0.045*	0.972
	week 12 (P)	1.000	0.695
week 9 (P)	control	0.006*	0.975
	week 3 (S)	0.001*	0.060
	week 6 (S)	0.045*	0.972
	week 12 (P)	0.018*	0.976
week 12 (P)	control	0.757	0.707
	week 3 (S)	0.330	0.006*
	week 6 (S)	1.000	0.695
	week 9 (P)	0.018	0.976

Chapter 4: From egg to hatchling: preferential retention of fatty acid biomarkers in young-of-the-year Port Jackson sharks *Heterodontus portusjacksoni*

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4.1 Abstract

The muscle and liver fatty acid composition of young-of-the-year (YOY) sharks were investigated to determine the effects of a known dietary lipid source versus maternal input as demonstrated by egg yolk fatty acid profiles. Ten Port Jackson shark *Heterodontus portusjacksoni* egg yolks were collected *in situ* and compared to four hatched sharks fed a known diet in a controlled feeding experiment of 185 days. This study demonstrated that fatty acids are likely conservatively transferred from egg yolks to YOY, while diet did not have a large effect on the fatty acid composition of shark liver or muscle. Specifically, arachidonic acid (ARA, 20:4n-6) was preferentially retained likely as a result of eicosanoid production during growth; dietary docosapentaenoic acid (DPA, 22:5n-3) was not reflected in shark tissues and is likely catabolised for energy; docosahexaenoic acid (DHA, 22:6n-3) was reflected in tissues and was a good dietary indicator; and high proportions of saturated fatty acids were retained in the muscle at the expense of dietary polyunsaturated fatty acids.

Key words: chondrichthyan, controlled feeding experiment, diet; lipid, oviparous, Port Jackson shark.

4.2 Introduction

Fatty acid analysis is increasingly being applied to study the feeding ecology of sharks (Schaufler et al. 2005; Pethybridge et al. 2010, 2011b; Wai et al. 2011; McMeans et al. 2012, 2013). The fatty acid profile of any organism, including sharks, is dependent on its original fatty acid composition and the cumulative intake of dietary fatty acids, but is also affected by various metabolic processes associated with growth and maturation (Robin et al. 2003). Consequently, the interpretation of fatty acid profiles is hampered by these factors and controlled experiments are required to investigate which fatty acids are preferentially retained through the various metabolic processes. Oviparous chondrichthyans, such as the Port Jackson shark *Heterodontus portusjacksoni* (Meyer 1793), provide an ideal model to compare fatty acid profiles of egg yolks to young-of-the-year (YOY) sharks fed a known diet. As *H. portusjacksoni* develops externally without maternal care, all organic requirements are supplied in the egg through the yolk (Rodda & Seymour, 2008). This allows a more thorough understanding of which fatty acids are deposited directly or indirectly as a result of diet, and which are deposited as a result of biosynthesis.

Shifts in fatty acid composition occur following hatching as a result of the preferential retention and utilisation of specific fatty acids and possible fatty acids synthesis (Heming and Buddington, 1988). In this context, the aims of this study were to demonstrate whether fatty acids were conservatively transferred from egg yolks to YOY *H. portusjacksoni* and also to determine the effect of a known dietary lipid source on the fatty acids profile of YOY sharks. Specifically, the aim was to determine whether the fatty acids present in high concentrations in egg yolks, or in the diet, were reflected in the liver and muscle tissues of YOY sharks fed a known diet for 185 days, and whether there were patterns reflective of preferential retention of fatty acids in either tissue.

4.3 Methods

Sixteen *H. portusjacksoni* egg cases were collected at Lassiter's Reef (35° 30' 38.997" S 138° 13' 0.886" E) off Second Valley, South Australia during October 2010. *Heterodontus portusjacksoni* reach sexual maturity at 11–14 years of age for females and 8–10 years of age for males (McLaughlin and O'Gower, 1971), and have an 18-month period between vitellogenesis and ovulation (Tovar-Ávila *et al*., 2007). Egg cases remain pliable for approximately two weeks after laying before they harden (Rodda & Seymour, 2008). Of the collected egg cases, 11 were identified as freshly laid due to their clean, soft, pliable casings and olive green colour (Rodda & Seymour, 2008). The remaining five egg cases contained full-term embryos. Ten of the fresh eggs and four of the hard eggs were viable and used in the analysis. Additional eggs or full-term embryos were not collected from additional sites or on additional trips to avoid spatio-temporal variations confounding the results. The likelihood of the egg cases originating from the same female was minimal because of the number of eggs found at the location exceeded the annual 10–18 eggs laid per female (Gomon et al. 1994; Last and Stevens, 2009; McLaughlin and O'Gower, 1971) and that egg cases were collected from different areas within the site. Egg cases were transported in aerated ambient seawater in an insulated ice box to aquarium facilities at Flinders University, South Australia, Australia. The ten fresh egg cases were sacrificed to analyse yolks for initial fatty acid concentrations. The remaining four egg cases with developed embryos visible inside were maintained in aquaria with ambient recirculating seawater at approximately 18° C and 38–40 ppt salinity until hatching, which occurred within seven to eight days following collection. The recirculating aquarium systems consisted of four 32-litre plastic containers, provided with oxygenated seawater. The tanks were connected to a 32-litre sump containing a mechanical filter with standard coarse grade sponge and "bio-balls" as a biological filtration media. Fifty percent of the water was changed weekly and sharks were fed cockles**,** *Donax deltoides* (Lamarck 1818), three times per week to satiation. Cockles were all obtained from the Coorong region, South Australia and maintained at -18° C throughout the study. Port Jackson sharks are known to consume molluscs, including bivalves, which may contribute up to 2.6% by weight to the diet of juvenile sharks (Powter et al. 2010). Bivalves have not been

detected in the stomach content of adult and subadult sharks, however, high levels of unidentifiable items (~35%) is common, which could possibly include bivalves (Powter et al. 2010). Water parameters including pH 7.9-8.4, ammonia (NH_3/NH_4^+) <0.5 mg/L, nitrite (NO_2^-) <1.0 mg/L and nitrate (NO_3^+)<20 mg/L, were monitored using an aquarium (API master, Chalfont, PA, USA) test kit and sharks were periodically monitored for length and weight gain. All research was conducted under Flinders University Animal Welfare permit E301.

At the conclusion of the experiment, following 185 days in captivity, sharks were sacrificed 24 hours after feeding by pithing and spinal dissection. Prior to feeding, the hatched sharks measured 195.0 mm L_T (± 5.8 mm) and weighed 44.7 g (± 3.7g). At the conclusion of the experiment, sharks measured 241.2 mm L_T (± 8.5 mm) and weighed 96.2 g (± 9.7 g). Whole livers and 5–10 g of muscle were collected from the ventral flanks of each shark and frozen at -20ºC until analysed. Six whole cockles, ten whole egg yolks, and muscle and liver tissue samples from each shark were individually homogenised and analysed for fatty acids by the FOODplus Fatty Acid Lab, Urrbrae, South Australia, Australia. Lipids were extracted from diet or shark tissue samples using a chloroform/methanol (2:1) extraction method as described by Bligh and Dyer (1959). Fatty acids were calculated on a wet weight basis and expressed as µg g^{-1} and as a percent of total fatty acids identified. Fatty Acid Methyl Esters (FAME) were extracted from the tissues and diets, separated and quantified using a Hewlett-Packard 6890 gas chromatograph (Palo Alto, CA, USA) equipped with a flame ionization detection (FID) following the method in Beckmann et al. (2013a). An external standard Nu-Chek Prep Inc, Elysian, MN, USA was used with 46 different FAME types. Additional fatty acids were identified by the relative locations of other peaks in human blood.

Bray–Curtis similarity matrices were calculated for square-root transformed fatty acid data expressed as µg g^{-1} to test the differences between dietary items (cockles), YOY sharks, and egg yolks using Primer Version 6.1.13 (www.primer-e.com). The percentage contribution of each fatty acid to the separation between diets and fed sharks was assessed using similarity percentage (SIMPER) (Clarke, 1993). The similarity of groups was represented by

hierarchical cluster analyses based on group-average linking. Differences in fatty acid composition were also analysed using PERMANOVA+ version 1.0.3 (Anderson, 2001), with additional pairwise tests conducted using the square root of the PERMANOVA test statistic (a multivariate pseudo *t*-test) under a reduced model using 9999 permutations.

4.4 Results and Discussion

Following hatching, neonates were unreceptive to offers of food for approximately two weeks. Fasting directly after hatching has previously been observed in young dogfish (Wrisez et al. 1993), and is likely related to internal yolk stores still present in neonates at hatching (Rodda & Seymour, 2008). Egg yolks contained the highest proprtions of total lipid, followed by YOY shark liver and muscle, and cockles (Supplementary Table 4.1).

The cluster dendogram showeds high variability in the fatty acid profiles of the egg yolks with two clusters of eggs (Figure 4.1) and SIMPER analysis indicated 16.4% dissimilarity between the clusters. Docosahexaenoic acid (22:6n-3, DHA) was the major driver of dissimilarity contributing 14.1% to the average dissimilarity between the two clusters. One cluster (referred to as DHA deficient eggs) had a mean DHA concentration of 2, 180.9 µg g^{-1} compared to the other cluster (referred to as viable yolks), which displayed a mean DHA concentration of 11, 663.6 µg g^{-1} corresponding to a 12.8% difference in DHA proportions between the two clusters (Supplementary Table 4.1 and 4.2). In addition, DHA deficient eggs had significantly different fatty acid profiles to viable yolks (PERMANOVA t = 3.2, P = 0.007). Although DHA is an essential dietary fatty acid, it is unclear whether it is used as an energy source or converted to other physiologically important compounds, such as prostoglandins, during early development. Low concentrations of DHA are, however, of concern, as this essential fatty acid is required for the growth and functional development of the brain and the nervous system (Bell et al. 1995; Horrocks & Yeo, 1999). The DHA deficient egg yolks were, therefore, considered to be potentially unviable and removed from further statistical analyses.

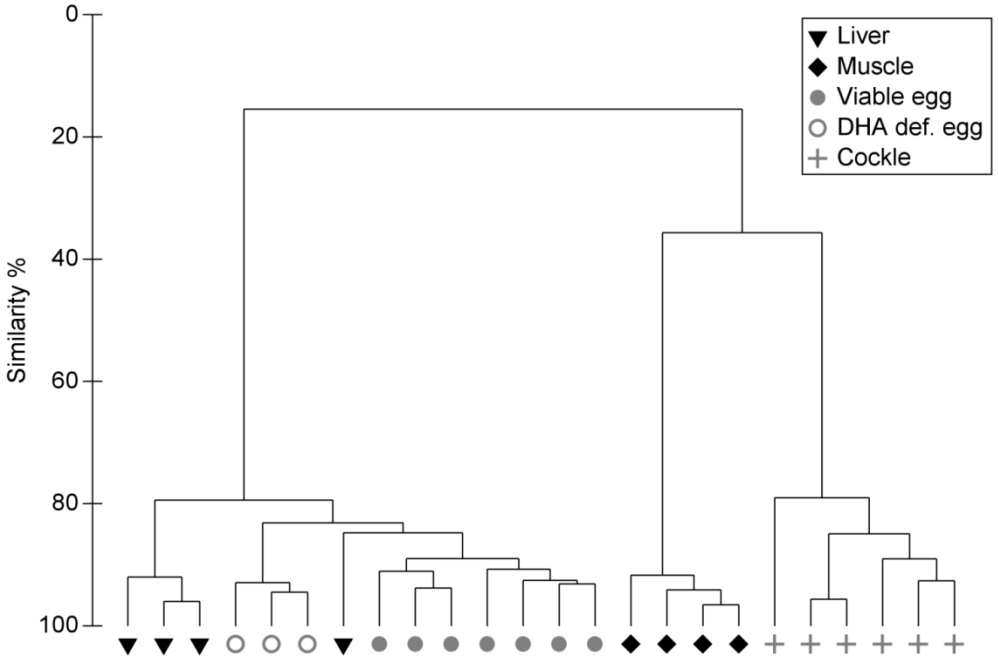

Figure 4.1 Cluster dendogram of fatty acid profiles of *Heterodontus portusjacksoni* egg yolks collected in 2010 at Lassiters Reef, South Australia, and the muscle and liver of YOY sharks hatched and fed cockles *Donax deltoides* for 185 days. Samples clusted by group average of ranked Bray–Curtis similarity index based on square root transformed data expressed as µg/g (wet weight).

Table 4.1 PERMANOVA comparing fatty acid composition (µg g^{-1}) in *Heterodontus portusjacksoni* egg yolks collected in 2010 at Lassiters Reef, South Australia, and the muscle and liver of YOY sharks hatched and fed cockles *Donax deltoides* for 185 days. The average dissimilarity of groups was measured using SIMPER. * Significant difference P < 0.05

	PERMANOVA		SIMPER
	t	P	Average Dissimilarity %
Groups compared			
Viable egg yolks, Cockle	10.1	0.004*	92.5
Liver, Cockle	13.1	0.004*	91.8
Viable egg yolks, Muscle	18.1	0.004*	73.5
Liver, Muscle	13.8	0.029*	70.0
Cockle, Muscle	17.4	<0.001*	64.4
Viable egg yolks, Liver	3.0	0.003*	17.6

Results showed significant differences in the fatty acid profiles (PERMANOVA df = 3, pseudo-F = 183.4, P < 0.001) and pairwise tests disclosed significant differences in the fatty acid profiles of the liver, muscle, egg yolks, and cockles (Table 4.1). The 92.5% average dissimilarity between the composition of fatty acids in viable egg yolks and cockles was driven by 16:0, DHA, arachidonic acid (ARA, 20:4n-6), 18:1n-9, docosapentaenoic acid (DPA, 22:5n-3), 18:0 and 18:1n-7, which contributed a combined 28.3% to the average dissimilarity. The concentation of all these fatty acids was higher in viable egg yolks than in cockles (Supplementary Table 4.2). The proportions of 18:0 were higher in the liver, while 22:6n-3 were higher in cockles compared to egg yolks (Supplementary Table 4.1). The fatty acid profiles of shark liver were more similar to viable egg yolks than to their cockle diet. This was demonstrated by the low levels of dissimilarity between the fatty acid profiles of egg yolks and liver with 17.6% compared to egg yolks and cockles with 92.5% dissimilarity (Table 4.1).The composition of muscle fatty acids and viable egg yolks showed a higher level of dissimilarity at 73.5% compared to muscle and cockles with 64.4% (Table 4.1). This was likely a result of the high levels of lipid observed in egg yolks and liver tissue compared to muscle and cockles. High lipid levels in egg yolks (16.1%) and shark liver (11.9%) compared to muscle (0.8%) and cockles (0.8%) corresponded to higher oveall levels of fatty acids (Supplementrary Table 4.1). While proportions of fatty acids may be similar, the absolute amount of fatty acids varied considerably.

While egg yolks contained the highest concentration of ARA, the muscle contained the highest proportion of ARA (Supplementary Table 4.1 and 4.2). ARA is regarded as the major source of precursers of eicosanoids including prostaglandins, which are important during larval growth and development (Bell et al. 1994; Sargent et al. 1999). High levels of eicosapentaenoic acid (20:5n-3, EPA) relative to ARA are normally retained in the membrane phosphoglycerides of fish and EPA competitively inhibits the production and efficiacy of eicosanoids derived from ARA (Lands, 1989). In this study, higher concentrations and proportions of ARA compared to EPA were detected in the liver and muscle (Supplementary Table 4.1 and 4.2). The YOY sharks examined in the present study grew rapidly, suggesting high levels of eicosanoid production are normal for

growth and development. Hence, studies of fish sustaining the eggs and newly hatched larvae indicate that ARA concentrations are up to seven times higher than in the normal body lipids of metamorposed fish, indicating the high biological importance of ARA (Falk-Petersen et al. 1989; Tocher, 2003). Preferential retention of ARA has previously been reported in the muscle and plasma of Greenland sharks *Somniosus microcephalus* (McMeans et al. 2012). Physiological compounds such as eicosanoids, which are associated with the release of ARA from membrane phospholipids, are important during larval development even at low physiological concentrations (McPhail et al. 1984; Smith, 1989; Koven et al. 2001). While dietary concentrations of ARA may influence fatty acid compostion of tissues, particularly in the muscle, it appears that the physiological role of ARA may make it unsuitable as an indicator of diet.

The dissimilarity between egg yolks and muscle, and egg yolks and cockles was also driven by DPA. Higher proportions and concentrations of DPA were measured in egg yolks than in muscle tissue, cockles, and liver tissue (Supplementary Table 4.1). Lower DPA levels in tissues compared to egg yolks suggest that this fatty acid is catabolised for energy and not retained in tissues. DPA is predominantly supplied in the diet to marine fish, however, marine fish have a limited ability to synthesise DPA from other fatty acid precursors via desaturation and elongation (Tocher, 2003). The liver of the shark that grew the most during the experiment had high concentrations of DPA, 16:0, 16:1n-7, 18:0, 18:1n-9, 18:1n-7, 20:2n-9, and DHA. This resulted in the liver of this shark being more similar to viable egg yolks than to the liver of the remaining three sharks (Figure 4.1).

The dissimilarity in fatty acid profiles between egg yolks and muscle tissues and egg yolks and liver was driven by the concentrations of 16:0, ARA, 18:1n-9 and 18:1n-7. In addition, differences in the egg yolk and muscle tissue fatty acid profiles were driven by DHA, 22:5n-3 and 18:0. Except for 18:0 and DHA, all of the aftorementioned fatty acids had higher concentrations and proportions in egg yolks than in liver or muscle. The proportions of DHA observed in the liver were higher than in the egg yolks and in the cockles, suggesting some dietary input. Previous controlled experiments have indicated that

high proportions of DHA are reflected in the liver and muscle fatty acid profiles (Beckmann 2013a). The level of DHA within *in utero* embryos has also been shown to increase throughout embryonic development (Pethybridge et al. 2011b). This indicates that developing embryos require high concentrations of DHA, which are being used for anabolic processes as fast as it is being taken up. This is likely due to the role of DHA in developing visual and neural tissues, which account for a relatively great proportion of total body mass in larval stages (Sargent et al. 2002). The reliance of young sharks on maternal resources has previously been demonstrated in dusky sharks *Carcharhinus obscurus*, where tissue concentrations of essential fatty acids increased once the initial period of rapid growth had passed (Hussey et al. 2010b). Higher proportions of 18:0 were also observed in the muscle than in egg yolks and higher concentrations in the liver than in egg yolks. As 18:0 can be synthesised *de novo* (Tocher, 2003) it is difficult to determine whether high proportions of 18:0 observed in cockles are reflected in tissues.

The fatty acid profiles of the liver of YOY sharks was most dissimilar to the fatty acid profile of the cockle diet. In comparison, the cockle and muscle fatty acid profiles grouped more closely. The 91.8% average dissimilarity between the concentrations of fatty acids observed in cockles and liver and the 64.4% dissimilarity between cockles and muscle was driven by DHA, 16:0, 18:0 and 18:1n-9. In addition, the dissimilarity between cockles and muscle was driven by 18:1n-7. Higher concentrations of the aforementioned fatty acids occurred in the liver than in the muscle or cockles. The muscle, however, contained higher proportions of 16:0 than the liver and cockles. Overall, higher proportions of SFA were observed in the muscle compared to the liver, cockles and egg yolk. This is consistent with previous studies where shark muscle was typified by high proportions of saturated fatty acids, as well as high relative levels of PUFA (Pethybridge et al. 2010). Cockles contained the highest proportions of DHA followed by the liver, while viable egg yolks contained the highest proportions of 18:1n-9 followed by the muscle. The muscle contained the highest proportions of 18:1n-7 followed by viable egg yolks, liver and cockles.

4.5 Conclusion

This study indicated that fatty acids are conservatively transferred from egg yolks to YOY sharks. The fatty acid profiles of shark liver were more similar to viable egg yolks than to their cockle diet, indicating that diet was not having a large effect on the fatty acid composition of shark liver. Muscle fatty acid and viable egg yolk fatty acids showed a higher level of dissimilarity than muscle and cockles, however, this was correlated with lipid levels rather than direct dietary effects. High concentrations of ARA in egg yolks were reflected in the liver and proportionally in the muscle and preferential retention of ARA may be indicative of eicosanoid production which is normal for growth and development in young fish. High proportions and concentrations of DPA were measured in egg yolks but were not, however, reflected in shark tissues, suggesting that DPA is catabolised for energy. Higher proportions of DHA were observed in the liver than in egg yolks, suggesting some dietary input. High proportions of SFA were detected in the muscle and this may be indicative of the domination of cell-membrane phospholipids resulting in this tissue being less responsive to dietary change than would be expected in the storage fats (Jobling, 2003). Combined with rapid growth and development, the fatty acid profiles of YOY sharks are very difficult to relate to diet and are more likely reflective of maternal inputs and indicative of limited foraging ability during this time. This was a short-term study and although sample size was relatively small, the variability within each group was also small and this suggests that the low sample size is unlikely to have affected reliability of the results. Further research assessing the changes in the fatty acid profiles through different life history stages is needed to investigate the effects of metabolic rate related to reproduction. Furthermore, investigations and hatching of DHA deficient eggs could reveal whether eggs are in fact viable and what effect low levels of DHA have on shark development.

4.6 Supplementary Material

Supplementary Table 4.1 Selected Fatty acid proportions expressed as a percent of total fatty acids identified in *Heterodontus portusjacksoni* egg yolks collected in 2010 at Lassiters Reef, South Australia and YOY sharks hatched and fed cockles *Donax deltoides* for 185 days. Means ± standard deviation are expressed as a percent of total fatty acids detected. Docosahexaenoic acid (DHA) deficient eggs had significantly different fatty acid profiles to viable eggs (PERMANOVA t = 3.2, P = 0.007) and displayed a mean DHA proportion of 12.8% lower than viable eggs.

	Viable eggs (n=7)		DHA def. Egg (n=3)		Cockle (n=6)		Liver (n=4)		Muscle (n=4)	
	Mean	±	Mean	±	Mean	±	Mean	±	Mean	±
Lipid (% ww)	16.1	2.4	17.6	1.8	0.8	0.2	11.9	3.9	0.8	0.1
14:0	0.7	0.1	1.3	0.5	2.9	1.8	1.0	0.1	0.5	0.0
15:0	0.7	0.2	0.4	0.1	1.4	0.3	1.0	0.1	0.3	0.0
DMA 16:0	0.6	0.1	0.5	0.2	0.4	0.1	0.2	0.1	3.5	0.7
16:0	19.8	1.0	22.5	0.5	18.8	0.6	15.5	1.0	20.7	0.6
Trans 16:1	0.5	0.3	0.1	0.1	0.0	0.0	0.1	0.0	0.2	0.1
16:1n-9	0.5	0.1	0.5	0.1	0.0	0.0	0.6	0.0	0.5	0.0
16:1n-7	4.0	0.9	4.4	0.5	5.4	2.3	5.2	0.3	4.7	1.3
17:1	0.6	0.1	0.5	0.1	0.3	0.1	1.2	0.2	0.5	0.1
18:0	6.6	0.6	7.2	0.8	7.8	1.6	10.3	1.1	8.4	0.6
Trans 18:1n-9	0.7	0.3	0.5	0.1	0.2	0.1	0.5	0.0	0.3	0.1
18:1n-9	9.7	1.5	12.7	1.3	2.6	0.3	6.4	0.5	8.5	0.4
18:1n-7	6.1	0.5	9.4	0.7	1.8	0.7	5.0	1.7	6.3	0.5
18:2n-6	0.8	0.2	0.5	0.1	0.9	0.2	0.7	0.0	0.4	0.1

19:1	0.5	0.1	0.3	0.0	0.2	0.3	0.8	0.1	0.4	0.1
18:3n-3	0.2	0.1	0.3	0.1	0.8	0.2	0.5	0.1	0.1	0.0
10,12 18:2 cLA	0.9	0.7	1.1	0.1	2.0	1.0	0.8	0.1	0.1	0.0
20:1n-9	0.1	0.1	0.0	0.0	1.6	0.2	0.0	0.0	0.0	0.0
20:2n-9	2.5	0.8	2.9	0.3	0.2	0.1	2.3	0.3	0.7	0.1
20:2n-6	0.2	0.1	0.5	0.0	1.3	0.2	0.0	0.0	0.0	0.0
20:3n-9	0.9	0.2	0.2	0.1	0.1	0.1	1.3	0.2	0.3	0.1
20:3n-6	0.3	0.1	0.3	0.1	0.4	0.1	0.5	0.0	0.2	0.0
20:4n-6 ARA	10.8	1.8	12.2	0.5	4.0	0.6	6.5	1.7	15.9	2.1
20:4 n-3	0.4	0.2	0.2	0.0	0.9	0.1	0.8	0.1	0.1	0.0
20:5n-3EPA	3.1	0.7	1.8	0.5	13.4	0.9	5.4	0.9	2.9	0.5
22:3 n-6	0.2	0.1	0.0	0.0	0.5	0.1	0.9	0.1	0.2	0.0
22:4n-6+22:3n-3	4.0	1.7	6.6	0.9	1.1	0.2	4.0	1.1	3.3	1.2
22:4n-3	1.0	0.2	0.2	0.1	1.2	0.2	1.8	0.4	0.9	0.3
22:5n-3DPA	6.8	0.8	9.2	1.4	4.0	1.2	5.4	1.0	3.5	0.9
22:6n-3DHA	15.1	2.2	2.3	0.7	23.0	4.1	18.2	1.3	15.0	3.0
∑SFA	29.0	0.3	32.2	0.6	32.0	0.5	28.7	0.4	33.8	0.7
∑MUFA	23.9	0.5	29.8	1.1	15.1	1.7	21.4	0.9	22.2	0.8
∑PUFA	46.7	0.6	37.5	0.7	52.7	2.1	49.6	0.8	43.9	0.4

Dma, dimethyl acetal; cLA, conjugated Linoleic acid; ARA, arachidonic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid, DHA, docosahexaenoic acid; SFA, saturated fatty acid, MUFA, monounsaturated fatty acid, PUFA polyunsaturated fatty acid, FA fatty acid

Supplementary Table 4.2 Selected Fatty acid concentrations expressed as µg g^{-1}(wet weight basis) in *Heterodontus portusjacksoni* egg yolks collected in 2010 at Lassiters Reef, South Australia and YOY sharks hatched and fed cockles *Donax deltoides* for 185 days. Means ± standard deviation are expressed in µg g^{-1} (wet weight) of total fatty acid detected. Docosahexaenoic acid (DHA) deficient eggs had significantly different fatty acid profiles to viable eggs (PERMANOVA t = 3.2, P = 0.007) and displayed a mean DHA concentration of 9,482.7 µg g^{-1} lower than viable eggs.

	Viable eggs (n=7)		DHA def. Egg (n=3)		Cockle (n=6)		Liver (n=4)		Muscle (n=4)	
	Mean	±	Mean	±	Mean	±	Mean	±	Mean	±
14:0	537.7	157.2	1208.4	493.8	4.3	3.4	556.9	148.1	10.7	1.5
15:0	562.6	148.5	340.9	39.9	1.9	0.9	555.1	144.6	7.1	0.9
DMA 16:0	479.1	61.1	453.0	180.5	0.5	0.1	118.5	11.9	79.0	20.0
16:0	15207.7	2334.2	21255.5	789.4	25.3	8.6	8781.5	3887.6	460.9	51.2
Trans 16:1	428.1	273.8	77.0	86.9	0.0	0.0	26.7	18.2	4.9	1.5
16:1n-9	356.6	69.2	489.1	97.7	0.0	0.0	315.0	124.7	12.1	1.5
16:1n-7	3036.6	893.4	4153.1	540.0	7.8	5.0	2846.5	933.7	105.9	33.2
17:1	481.5	74.5	423.9	56.4	0.4	0.1	642.5	233.7	10.4	2.1
18:0	5104.8	1064.8	6820.2	688.5	10.1	3.1	5571.3	1382.1	187.4	14.1
Trans 18:1n-9	567.9	224.7	490.2	123.7	0.3	0.1	250.1	88.5	7.2	1.6
18:1n-9	7476.4	1717.8	11939.2	1077.3	3.5	1.3	3603.0	1559.7	188.9	23.0
18:1n-7	4709.6	841.0	8892.3	615.7	2.6	1.7	3054.4	2279.2	139.4	6.2
18:2n-6	603.7	171.2	449.3	47.2	1.2	0.7	408.0	129.5	9.7	0.9
19:1	411.8	138.9	282.8	36.1	0.4	0.6	458.9	138.6	9.1	1.8
18:3n-3	150.5	70.8	237.1	68.4	1.1	0.6	253.6	84.0	2.4	0.6
10,12 18:2 cLA	696.2	490.3	1059.8	146.8	2.9	2.2	440.7	178.9	2.7	0.6
20:1n-9	42.8	73.2	0.0	0.0	2.1	0.8	0.0	0.0	0.0	0.0

20:2n-9	1928.6	585.1	2724.3	309.8	0.3	0.2	1309.3	677.3	14.3	2.0
20:2n-6	133.2	48.9	487.6	34.7	1.9	0.8	0.0	0.0	0.0	0.0
20:3n-9	663.6	142.9	200.8	53.7	0.1	0.1	726.0	194.4	6.6	1.6
20:3n-6	193.5	66.2	278.0	45.4	0.6	0.3	297.5	122.7	4.7	0.1
20:4n-6 ARA	8262.4	1729.1	11533.9	555.2	5.2	1.5	3393.4	287.7	355.5	74.3
20:4 n-3	309.4	181.4	159.4	35.4	1.3	0.6	466.0	190.6	3.2	0.5
20:5n-3 EPA	2388.0	723.5	1690.5	490.4	18.2	7.2	2920.4	842.4	64.9	7.3
22:3 n-6	127.1	64.6	29.9	25.9	0.7	0.2	478.9	193.3	4.4	0.7
22:4n-6+22:3n-3	3034.0	1130.8	6247.2	814.9	1.4	0.4	2258.7	1176.4	74.4	31.1
22:4n-3	792.7	208.3	172.7	64.7	1.5	0.5	1050.0	634.7	19.0	4.9
22:5n-3 DPA	5178.2	796.3	8660.8	1212.7	5.0	1.4	3172.8	1842.6	78.8	24.5
22:6n-3 DHA	11663.6	2759.1	2180.9	668.2	30.1	9.7	9995.1	3055.4	331.4	62.7
∑SFA	22237.5	3515.0	30434.6	1410.2	43.1	14.9	15934.4	5688.8	753.0	79.8
∑MUFA	18614.1	3127.9	28545.4	1557.2	21.4	11.3	12408.0	5775.5	499.0	64.2
∑PUFA	35951.3	5387.7	35479.7	1105.2	70.0	22.7	27480.7	9201.9	977.6	91.7
∑FA	76802.9	11729.7	94459.7	1435.5	134.5	46.5	55823.1	20635.8	2229.6	217.9

Dma, dimethyl acetal; cLA, conjugated Linoleic acid; ARA, arachidonic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid, DHA, docosahexaenoic acid; SFA, saturated fatty acid, MUFA, monounsaturated fatty acid, PUFA polyunsaturated fatty acid, FA fatty acid

Chapter 5: Inter-tissue differences in fatty acid incorporation as a result of dietary oil manipulation in Port Jackson sharks (*Heterodontus portusjacksoni*)

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5.1 Abstract

Fatty acid profile analysis is a tool for dietary investigation that may complement traditional stomach contents analysis. While recent studies have shown that the liver of sharks fed different diets have differing fatty acid profiles, the degree to which diet is reflected in shark blood serum and muscle tissue is still poorly understood. An 18-week controlled feeding experiment was undertaken using captive Port Jackson sharks (*Heterodontus portusjacksoni*). Sharks were fed exclusive diets of artificial pellets treated with fish or poultry oil and sampled every six weeks. The fatty acid profiles from liver, blood serum, and muscle were affected differently, with the period from which significant differences were observed varying by tissue and diet type. The total fatty acid profiles of fish oil and poultry oil fed sharks were significantly different from week 12 onwards in the liver and blood serum, but significant differences were only observed by week 18 in the muscle tissue of sharks fed different diets. The drivers of dissimilarity which aligned with dietary input were 14:0, 18:2n-6, 20:5n-3, 18:1n-9 and 22:6n-3 in the liver and blood serum. Dietary fatty acids accumulated more consistently in the liver than in the blood plasma or muscle, likely due to its role as the central organ for fat processing and storage. Blood serum and muscle fatty acid profiles were influenced by diet, but fluctuated over-time. The low level of correlation between diet and muscle FA profiles is likely a result of low levels of fat (<1%) in the muscle and the domination of structural, cell-membrane phospholipids in shark muscle tissue. Our findings describe inter-tissue differences in the incorporation of fatty acid from diet to consumer, which should be taken into account when interpreting dietary patterns from fatty acid profiles.

Key Words: captive feeding trial, chondrichthyan, lipid, free fatty acid, turnover, metabolism, essential fatty acid

5.2 Introduction

Predator-prey relationships are an important component to understand the dynamics of marine ecosystems (Baum and Worm 2009). Chondrichthyans (sharks, batoids, and chimaeras) can act as regulators of these ecosystems, affecting the size, abundance, and variety of their prey (Baum and Worm 2009; Heithaus et al. 2008; Myers et al. 2007). There is a lack of dietary information for many shark species, which is exacerbated by the diet of many species varying ontogenetically as well as spatio-temporally (Grubbs 2009). Most previous dietary studies have investigated diet through stomach contents analysis, but biochemical methods, such as stable isotopes and fatty acids (FA), are becoming increasingly common (Hussey et al. 2011; McMeans et al. 2012; Pethybridge et al. 2011a). Several studies using stable isotopes have investigated tissue turnover rates and discrimination factors (Hussey et al. 2010a; Kim et al. 2012; Logan and Lutcavage 2010; Matich et al. 2010). There has, however, only been limited investigation into how consumed prey affects the FA profiles of different chondrichthyan tissues in a controlled environment (Beckmann et al. 2013a,b).

In most marine vertebrates, lipids are stored in the adipose tissue found in muscle or blubber (Budge et al. 2011). Sharks are different from all other vertebrates as their ability to oxidise FA (obtained from lipids) is largely confined to the liver (Ballantyne 1997; Moyes et al. 1990). As a result, shark liver is the major site of both lipid storage and metabolism through mitochondrial and peroxisomal FA oxidation (Bone and Roberts 1969; Hallgren and Larsson 1962; Malins 1968; Medzihradsky et al. 1992). The energy stored in the liver is transported to other tissues such as the muscle, via the blood, to fuel a variety of metabolic functions (Ballantyne et al. 1993). The FA required by the different tissues can vary depending on physiological state and dietary availability. Shark muscle generally only contains low levels of fat and is dominated by structural, cell-membrane phospholipids, rather than fat storage for energy (Jobling 2003). Furthermore, shark muscle tissue is biased towards PUFA rich dietary sources, while the liver can more accurately demonstrate the range of prey items consumed (Pethybridge et al. 2011a). As dietary surpluses or deficits of FA can affect the extent of processes such as FA oxidation and elongation, the products and

precursors to these reactions need to be considered alongside the FA commonly used as bioindicators.

In chondrichthyans, the profiles of blood serum FA have not previously been reported in a controlled setting and are an important component to take into consideration due to the role of blood in FA transport to tissue for oxidation (Henderson and Tocher 1987). The main lipid transport form in teleosts is free fatty acids (FFA), which are usually carried to tissues by serum albumin, where they are used in *β*-oxidation (Metcalf and Gemmell 2005; Richieri et al. 1993). It has been suggested that in chondrichthyans, FA are converted in the liver to ketone bodies through *b*-oxidation and ketogenesis and that a carrier protein such as albumin is not required (Watson and Dickson 2001). A limited amount of *β*-oxidation still appears to occur in the liver (Watson and Dickson 2001), which necessitates FA and some FA transport in the form of FFA in the blood serum. Blood serum is believed to be a short-term indicator of diet, as seen in sea birds (Käkelä et al. 2009), and may prove useful in determining changes, which might not be apparent in other tissues with slower turnover rates (McMeans et al. 2012). For example, previous research has indicated that blood serum FA profiling can distinguish between southern stingrays *Dasyatis americana* (Hildebrand & Schroeder 1928) fed different diets (Semeniuk et al. 2007).

Previously, controlled feeding experiments have been used to determine the extent to which the FA compositions of diet are reflected in the liver and muscle tissue of the Port Jackson shark *Heterodontus portusjacksoni* (Myer 1793). Captive sharks fed exclusively different diets over ten weeks showed significant differences in liver but not muscle FA profiles (Beckmann et al. 2013a). The prawn diet contained higher proportions of 16:1n-7 an 18:1n-9 compared to squid, while 20:4n-6 was higher in squid when compared to prawns (Beckmann et al. 2013a). While the differences in exclusive diets were not detected in the muscle FA profile after 10 weeks, sampling at a smaller temporal scale showed that liver and muscle FA profiles detected a dietary switch within three weeks of feeding (Beckmann et al. 2013b). The possible reason that no significant difference was found between the FA profiles of muscle of sharks fed different experimental diets after 10 weeks may have been due to the physiological function of muscle tissue,

which has low levels of fat and derives metabolic energy mainly from proteins (Pethybridge et al. 2010). Following dietary changes, elevated levels of dietary FA, such as 22:6n-3, can be detected in the muscle and the FA profile quickly reverts back to those FA required for function (Beckmann et al. 2013a). This indicates that liver FA profiles can be useful for detecting different diets in sharks, but that muscle FA are more reflective of changes occurring on a shorter temporal scale (e.g., three weeks), as may be seen in migratory species consuming different prey items throughout their migratory route. Fatty acid profiles have previously been used as a corroborative method to support predator-prey relationships, usually with stomach content analysis and/or stable isotopes. The use of stomach contents is limited in many shark species due to sample size limitations, a high number of empty stomachs and/or unidentifiable prey items. The use of FA profile analysis can strengthen dietary analysis by providing information about food web relationships and dietary habits. Furthermore, specific fatty acids or ratios between fatty acid biomarkers can provide an indication of dietary consumption and trophic position.

The aim of this study was to build on the findings from previous experiments (Beckmann et al. 2013a,b) and determine how the FA profiles of sharks fed diets containing different FA profiles, changed over time. Previous experiments examined FA profiles following exclusive diets after 10 weeks of feeding (Beckmann et al. 2013a) and after 12 weeks of feeding, with a dietary switch occurring at week 6 (Beckmann et al. 2013b). The current experiment examines a time series of two groups of sharks fed different diets over 18 weeks, with subsamples taken at 6 weekly intervals. The serum and tissues analysed were selected because of their role in lipid storage and transport. Muscle tissue and blood serum were also selected because their ability to be sampled using non-lethal techniques. The six weekly sampling intervals allowed us to investigate and compare incorporation rates between tissues and serum. Specifically, the aims of this study were to: (1) investigate temporal changes in blood serum FFA in relation to diet, (2) investigate the stability of the muscle FA profile by extending the duration of the feeding study, and (3) investigate which FA are the best indicators of diet in the liver, blood serum, and muscle of Port Jackson sharks when fed a formulated pellet diet containing either fish oil or poultry oil.

5.3 Materials and Methods

5.3.1 Animal maintenance

The Port Jackson shark is an abundant demersal endemic species to Australian waters (Last and Stevens 2009). This Port Jackson shark was selected as the model species for this study because of its high level of availability through bycatch of the South Australian prawn fishery (Svane et al. 2008). Port Jackson sharks also have a conservation status of Least Concern (Simpfendorfer 2005) which avoids any impacts from the study on specimens collected from populations, and the species has high resilience, to capture, handling and housing (Frick et al. 2010), allowing sharks to be maintained in aquaria throughout the duration of the study with the least amount of impact.

Port Jackson sharks were collected during fishery-independent surveys of the South Australian Prawn Trawling Fishery in Gulf St Vincent, South Australia. Trawling was undertaken at night in December 2010 using a demersal otter-trawl of 27–30 m length with trawl shots lasting for 30 minutes (Dixon et al. 2011). Sharks were transported to the pool farm marine facilities at SARDI Aquatic Sciences Centre, West Beach in 200-litre plastic containers with battery-powered aeration to maintain oxygenation. Water exchanges were made between each trawl shot with a hose left running when no fishing was taking place to provide a constant supply of fresh seawater. Between 8 and 48 hours after capture, sharks were transferred to and housed within 5,000-litre tanks provided with aerated ambient temperature flow through seawater for the duration of the project. The gender of all sharks was determined using the presence or absence of claspers. Sharks were also measured (total length), weighed, and tagged with Hallprint dart head tags (Hallprint Pty Ltd, Hindmarsh Valley, South Australia, Australia). To assess the FA profile of sharks which fed on their natural diet, four sharks (2 male and 2 female) were sacrificed prior to any experimental feeding and tissue samples were collected for FA profile analysis. The remaining 45 sharks were kept in four tanks, 11–12 sharks per tank to prevent over-crowding. Two replicate tanks were allocated to each dietary treatment. Each tank had an even distribution of sharks in terms of weight and length, and was held under identical conditions in a flow-through system. Three sharks from each tank were sub-sampled at weeks 6, 12,

and the remaining 17 sharks were sampled at week 18. Four mortalities (not treatment related) occurred during the study and these sharks were not included in the analysis.

5.3.2 Food and feeding regime

Shark feeding was initiated four days after capture (December 10th 2010). Sharks were fed one of two diets, artificial pellets containing either fish oil (FO) or poultry oil (PO), at the same time of day to satiation three times a week for eighteen weeks. For the first two weeks sharks were fed at 1.2% of their body weight, this was increased to 1.5% at week three and remained at 2.3% from week 4–18. Any uneaten food was removed from the tank approximately two hours after feeding, and the amount food remaining was visually accounted for. The two experimental diets were made from the same basal formulation (Supplementary Table 5.1) and contained 45% crude protein and 25% crude lipid on a dry weight basis (Bowyer et al. 2012). In all diets, 5% lipid was provided from the residual oil in the fish meal and other dietary ingredients, while the other 20% lipid was achieved through separate additions of fish oil, or poultry oil to the diets. As a result, the two test diets were diet 1: 100% FO, diet 2: 100% PO. The diets were formulated to satisfy the nutritional requirements of a carnivorous marine fish and to supply sufficient n-3 long chain (LC)-PUFA for normal growth and development (NRC 2012). The diets were produced at the SARDI Australasian Experimental Stockfeed Extrusion Centre (Roseworthy, Adelaide, Australia) as cooked, extruded, slow sinking 3 mm pellets. After extrusion, feed pellets were vacuum infused with the oils. Enough feed for approximately one week was maintained at 4°C, otherwise feeds were kept frozen (−20°C) until used. The fatty acid profiles of the experimental oils and diets are shown in Table 5.1.

Samples of liver, blood serum and muscle were collected at week 0, 6, 12, and 18. Twelve sharks (six per diet) were sampled at week 6 and 12, while the 17 remaining sharks were sampled at week 18 (seven fed FO and ten fed PO). Three to five ml of blood was extracted using a hypodermic needle from the caudal vein. Blood was refrigerated and allowed to coagulate overnight prior to extracting

serum via centrifugation. Following blood sampling, each shark was measured and weighed prior to being sacrificed by pithing and spinal dissection. Sharks were then dissected and five to ten grams of muscle and liver tissue were collected and frozen at -20ºC until analysed. Dietary FA of fish oil and poultry oil treated diet pellets were analysed from three individual samples to compare the FA profile of the dietary source to that of the liver, blood serum, and muscle of fed sharks.

Two commonly applied condition indices were used to evaluate the condition of sharks during the study, a somatic measure, hepatosomatic index (HSI) and a morphometric measure, condition factor (CF) using the following equations:

$$HSI = [M_{TL}(kg)/M_{TB}(kg)] \times 100 \quad (1)$$

and

$$CF = [M_{TB\,(kg)}/ TL(cm)^{3}_{]} \times 10^{5} \quad (2)$$

Where M_{TL} equals the total mass of both liver lobes and M_{TB} is the total body mass of each shark.

5.3.3 Lipid extraction and FA analysis

Samples were analysed for FA by the FOODplus Fatty Acid Lab (Urrbrae, South Australia, Australia) according to the methods described in our previous paper (Beckmann et al. 2013a). Briefly, lipids were extracted from diet or shark tissue samples using a chloroform/methanol (2:1) method as described by (Bligh and Dyer 1959). For the analysis of free fatty acids in the blood serum, samples were thawed at room temperature and centrifuged for 2 minutes at 13,000 revolutions per minute. The percent lipid was calculated on a wet weight basis and the lipid was extracted with chloroform. Fatty Acid Methyl Esters (FAME) were produced by heating the extracted lipids in 1% H_2SO_4 in methanol for 3 hrs in a 70^{o}C water bath. After cooling, 750 µl distilled water was added along with 2.0 ml of *n*-heptane and the solution was centrifuged allowing the phases to separate. The

extracted FAME were separated and quantified using a gas chromatograph (Palo Alto, CA, USA) to determine fatty acid composition. Samples were run on a gas chromatograph with a flame ionization detector (FID) and an external standard was used with approximately 46 different FAME types. Additional FA were identified by the relative locations of other peaks in human blood. Data is presented as a percentage of the total FA, with concentration values (µg/g) available in the supplementary matreial.

Table 5.1 Mean fatty acid composition (mol % of total FA ± standard deviation) of fish oil- and poultry oil-treated pellets. Minor FAs (≤1%) are not shown.

	Fish oil-pellet		Poultry oil-pellet	
	Mean	±	Mean	±
14:0	11.1	0.2	1.8	0.0
16:0	27.6	0.7	24.2	0.1
16:1n-7	9.3	0.2	5.9	0.0
18:0	5.1	0.1	6.0	0.0
18:1n-9	12.5	0.2	37	0.0
18:1n-7	3.3	0.1	2.4	0.0
18:2n-6	3.5	0.2	12.5	0.0
18:3n-3	0.7	0.0	1.8	0.0
20:1n-11	1.6	0.1	0.2	0.0
20:5n-3	9.9	0.9	0.9	0.0
22:5n-3	1.2	0.1	0.3	0.0
22:6n-3	6.6	0.5	2.2	0.0
∑ SFA	45.6	1.0	32.9	0.1
∑ PUFA	30.6	0.5	48.4	0.0
∑ MUFA	23.8	1.5	18.7	0.1
∑ n-3	18.6	1.5	5.3	0.1
∑ n-6	4.9	0.1	13.3	0.1
∑ n-9	13.9	0.2	38.4	0.1
Total lipid (%)	23.2	0.0	23.1	0.0

Minor FA (≤1 % are not shown)

5.3.4 Statistical analysis

Euclidian distance matrices were calculated for square-root transformed liver, muscle and blood serum FA percentages to test the differences between fed sharks,

and between control and fed sharks using Primer Version 6.1.13 (http://www.primer-e.com). Differences in FA composition were then analysed based on Euclidian distance matrices using a Permutational Multivariate Analysis Of Variance, PERMANOVA+ version 1.0.3 (Anderson 2001), using 9999 unrestricted permutation. PERMANOVA relies on comparing the observed value of a test statistic (pseudo F-ratio) against a recalculated test statistic generated from random re-ordering (permutation) of the data (Anderson 2001). This permutation approach is a semi-parametric multivariate version of a univariate one-way ANOVA. The advantage of this is the resulting test is distribution free and not constrained by many of the typical assumptions of parametric statistics. A two-way analysis was used to compare the fatty acid composition of experimentally fed sharks for differences between weeks sampled and tanks nested within diet. Following a lack of significant difference between tanks (see results), a one-way analysis was performed with weeks sampled and diet as one seven-level factor to allow for comparisons within and between diet groups and weeks sampled. Additional pair-wise tests were then conducted using the square root of the pseudo-F statistic (t-test). The percent contribution of each fatty acid to the separation between diets and fed sharks was assessed using Similarity Percentage (SIMPER) analysis, based on the Euclidean distance matrices, which measures the top 90% contributing variables (Clarke 1993). Individual FA which contributed more than 5% to the average dissimilarity between diets and fed sharks were considered major drivers of differences and were discussed in more detail. To reduce table sizes, only those FA which were identified as major drivers of dissimilarity or those FA that were present average levels $\geq 1\%$ are presented. Unfed controls were also compared to fed sharks, however, the drivers of the differences between controls and fed sharks are not discussed in detail as this is reported in our previous studies (Beckmann et al. 2013a,b).Where PERMANOVA showed significant differences, Constrained Canonical Analysis of Principal Coordinates (CAP) were used to calculate eigenvectors (δ), which are the Principal Coordinate Analysis (PCA) axes that allow a graphical representation of fed and control shark differences in two dimensions.

5.4 Results

5.4.1 Shark, length, and weight of sharks

Prior to the commencement of the experiment, sharks fed PO-pellets measured 44.8 ± 7.8 cm and weighed 0.7 ± 0.3 kg and sharks fed FO-pellets measured 44.8 ± 6.4 cm and weighed 0.7 ± 0.5 kg. At week 6, PO-fed sharks measured 49.8 ± 8.5 cm and weighed 0.9 ± 0.6 kg and FO-fed sharks measured 45 ± 4.3 cm and weighed 0.6 ± 0.2 kg. At the end week 18, PO-fed sharks measured 53.6 ± 10.7 cm and weighed 0.9 ± 0.6 kg, and FO-fed sharks measured 46.2 cm and weighed 0.8 kg ± 0.2 kg.

Condition factor decreased slightly over time in both treatments, prior to the commencement of the experiment CF was 0.78 ± 0.21 in PO-fed sharks and 0.78 ± 0.19 in FO-fed sharks and by week 18 both FO- and PO-fed sharks had a CF of 0.65 ± 0.08. The HSI was higher in controls at 6.91 ± 0.81 than in sharks sampled at week 6 or 12 but a large increase in HSI was seen in both diets at week 18, PO-fed sharks measuring 14.04 ± 2.46 and FO-fed sharks 12.77 ± 2.93.

Sharks were not observed consuming food in the first week of feeding. In the second week of the experiment, sharks were observed feeding in two of the four tanks, one from each dietary treatment, however, only ¼ of the food provided was consumed. Food consumption increased over time in both treatments and by week two, 25% of all food was being consumed, this increased to 50% by week 4, 75% by week 6 and 95–100% in the remaining weeks.

5.4.2 FA profiles of test diets

Up to 48 different FA were detected in fish oil and poultry oil treated pellets (Table 1). SIMPER identified a 24.4% difference between FO and PO treated pellets. 20:5n-3, 18:1n-9, 22:6n-3, 18:2n-6, and 14:0 were the main drivers of the difference between diets. 20:5n-3, 22:6n-3, and 14:0 were 9.0%, 4.4%, and 9.3%

higher in FO-pellets than in PO-pellets, respectively. In contrast, 18:1n-9 and 18:2n-6 were 24.6% and 9.0% higher in PO-pellets than in FO-pellets (Table 5.1).

5.4.3 FA profiles and differences between treatments

Fifty-nine FA were identified in the shark tissues, with 29 present more than at trace levels (>0.5%, Table 5.2, 5.3, and 5.4). A two-way PERMANOVA indicated that the FA profiles of experimentally fed Port Jackson sharks fed were significantly different by diet (liver: $F_1 = 6.2$, $P = 0.035$) and week sampled (liver: $F_2 = 9.8$, $P = <0.001$, muscle: $F_2 = 9.8$, $P < 0.001$, blood serum: $F_2 = 3.7$, $P = 0.010$), and there was an interaction between diet and week sampled in the liver ($F_2 = 3.1$, $P = 0.030$). There were, however, no significant tank effects (Table 5.5). Tank was then removed as a factor and diet was grouped by week sampled and also compared with controls. Significant differences between diets grouped by week sampled were indicated in all tissues (liver: $F_{6,} = 6.5$, $P < 0.001$, muscle: $F_6 = 5.1$, $P < 0.001$, blood serum: $F_6 = 3.4$, $P < 0.001$). Pairwise tests revealed significant differences between FO and PO fed sharks were detected at week 12 and 18 in the liver and blood serum and at week 18 in the muscle (Table 5.6). Significant differences between FO-fed sharks sampled at weeks 6 and 12 were detected in all tissues, significant differences between FO-fed sharks sampled at week 12 and 18 were seen in the liver and muscle, and significant differences between FO-fed sharks sampled at weeks 6 and 18 were seen in the muscle and blood serum (Table 5.6). Significant differences in PO-fed sharks were detected between weeks 6 and 12 in the liver and blood serum, between weeks 12 and 18 in the liver and muscle, and between weeks 6 and 18 in all tissues (Table 5.6). Significant differences between controls and FO-fed sharks were detected in the liver and muscle at week 12 and in the muscle at week 18 (Table 5.6). Control and PO-fed sharks showed significant differences in muscle FA profiles between week 6 and week 12, while all tissues showed significant differences between control and PO-fed sharks at week 12 and 18 (Table 5.6).

The CAP results suggest that sharks feeding on different diets can be distinguished using the FA profiles of the liver, and muscle. The first two

canonical axes clearly separated feeding groups by sampling interval with the liver FA providing the most correct allocations to diet grouped by week sampled at 86.7%, followed by the muscle at 82.2%, and the blood serum at 67.4% (Figure 5.1). The differences in diet over time were indicated by the reasonably large size of the canonical correlations between individual FA and the CAP axes (muscle: $\delta 1 = 0.995$, $\delta 2 = 0.982$; liver: $\delta 1 = 0.999$, $\delta 2 = 0.998$; and blood serum: $\delta 1 = 0.958$, $\delta 2 = 0.948$). In the muscle biplot, allocation success increased over time in FO-fed sharks from 50% at week 6 to 83.33% at week 12 and 100% by 18, however, PO-fed sharks had higher allocation success at week 6 with 83.3% followed by a decrease to 66.7% by week 12 and a return to 100% by week 18 (Figure 5.1a). In the liver FA biplot, successful allocation to dietary group increased over time with 66.7% in FO-fed sharks at week 6, and 100% by week 12 and 18 (Figure 5.1b). The liver of PO-fed sharks showed 83.3% successful allocation at week 6 and 12 and increased to 90% at week 18 (Figure 5.1b). In the blood serum, FO-fed sharks showed 66.7% correct allocations at week 6 and 12, with an increase to 85.7% at week 18. The blood serum of PO-fed sharks also showed 66.7% correct allocation at week 6, decreasing to 40% at week 12, and returning to 60% by week 18 (Figure 5.1c).

5.4.4 Major drivers of differences between sharks: FA with higher concentrations in FO-pellet diet

Levels of 20:5n-3 and 22:6n-3, were major contributors to the average dissimilarity between FO and PO-pellets and to the dissimilarity between FO- and PO-fed sharks. Specifically, 20:5n-3 was a major contributor to the dissimilarity between FO-and PO-fed sharks in the liver, blood serum, and muscle fatty acid profiles while 22:6n-3 drove the difference in FA profiles in the liver and blood serum (Table 5.7). Higher proportions of 20:5n-3 were observed in FO-fed sharks, where a 4.5% difference occurred in the liver by week 18 (Table 5.2). By week 12 in the blood serum, FO-fed sharks contained 14.4% more 20:5n-3 than PO-fed sharks and this difference decreased slightly to 12.5% by week 18 (Table 5.3). In the muscle, higher levels of variation occurred, with FO-fed sharks showing

increased proportions of 20:5n-3 at weeks 6 and 18, but with PO-fed sharks showing equivalent amounts of 20:5n-3 with FO-fed sharks at week 18 (Table 5.4). Similarly, proportions of 22:6n-3 were higher in the liver of FO-fed sharks by week 18, where a 7.4% difference was observed (Table 5.2). Although differences were apparent between 22:6n-3 levels in FO- and PO-fed shark blood serum and muscle, high levels of variation were observed between individuals (Table 5.3).

High dietary levels of 14:0 were also reflected in the blood serum of FO-fed sharks, with levels increasing over time in FO-fed sharks to a peak difference of 2.3% by week 18 (Table 5.3). Similar trends in 14:0 levels were observed in the liver and muscle tissues, but 14:0 was only identified as a major driver of dissimilarity at weeks 12 and 18 in the blood serum (Table 5.7). In the blood serum, 16:1n-7 was also higher in FO-fed sharks at week 12 (2.5%), A high level of variation between individuals was, however, observed in muscle tissue and blood serum (Table 5.3 and 5.4).

5.4.5 Major drivers of differences between sharks: FA with higher concentrations in PO-pellet diet

Higher levels of n-9 FA in the PO-pellet diet were reflected in the blood serum (Table 5.3) and liver of PO-fed sharks (Table 5.2) and 18:1n-9 was a driver of the dissimilarity between PO- and FO-fed sharks in all tissues sampled (Table 5.7). Variation between individuals was observed at week 6 and 12, but by week 18 PO-fed sharks had 17.7% and 7.8% more 18:1n-9 in the liver and by blood serum (Table 5.2 and 5.3). This was consistent with diet as PO-pellets contained 3 times more 18:1n-9 than FO-pellets (Table 5.1). In the muscle, 18:1n-9 was a driver of dissimilarity between FO- and PO-fed sharks at week 12, however, high levels of variation between sharks fed the FO diet was observed (Table 5.4).

One of the major drivers of the dissimilarity between PO- and FO-fed sharks in the liver, blood serum and muscle was 18:2n-6 (Table 5.7). The proportion of 18:2n-6 increased over time in all tissues of PO-fed sharks and a peak difference of 6.5% was observed at week 6 in the liver. In the blood serum

and muscle, higher levels of 18:2n-6 were observed in PO-fed sharks by week 18, with differences of 4.2% and 1.1%, respectively (Table 5.2 and 5.3). High levels of variation were, however, observed in the muscle of sharks at all weeks and in the blood serum of FO-fed sharks, particularly at week 6 and 12 (Table 5.3).

Table 5.2 Mean FA composition (mol% of total FA, ± standard deviation) of Port Jackson shark H. portusjacksoni liver tissue sampled prior to feeding any experimental diet (control), and after feeding at 6, 12 and 18 weeks on poultry oil (PO) or fish oil (FO) pellet diet. Minor FAs (≤1%) are not shown.

	Control		FO						PO					
	Week 0 (n = 4)		Week 6 (n = 6)		Week 12 (n = 6)		Week 18 (n = 7)		Week 6 (n = 6)		Week 12 (n = 6)		Week 18 (n = 10)	
	Mean	±	Mean	±	Mean	±	Mean	±	Mean	±	Mean	±	Mean	±
14:0	1.5	0.0	1.2	0.5	2.3	1.9	2.8	1.7	1.1	0.5	0.8	0.3	0.9	0.2
15:0	1.0	0.2	1.0	0.4	1.0	0.2	0.6	0.3	0.8	0.2	0.6	0.3	0.5	0.2
16:0	22.6	3.6	21.2	2.0	21.3	1.2	20.7	0.4	19.0	2.3	19.4	1.9	19.0	1.1
16:1n-7	5.8	1.9	5.5	2.3	7.4	1.4	5.7	2.9	5.7	2.1	4.6	1.7	5.1	0.6
17:1	0.9	0.2	1.0	0.2	0.8	0.3	0.5	0.3	0.8	0.3	0.6	0.4	0.5	0.1
18:0	6.6	1.0	8.0	2.7	6.7	1.7	7.3	3.7	8.2	2.9	7.7	2.9	6.1	1.0
18:1n-9	13.9	2.8	11.4	4.3	13.3	3.0	9.1	2.6	11.5	5.1	20.9	9.7	26.7	7.9
18:1n-7	7.6	0.1	7.0	0.9	6.4	0.8	6.1	1.2	6.9	0.7	6.2	1.1	6.0	1.4
18:2n-6	0.9	0.1	0.8	0.3	1.3	1.5	2.0	0.8	1.8	1.4	5.1	3.7	7.3	3.2
18:3n-3	0.6	0.1	0.4	0.2	0.5	0.3	0.5	0.3	0.6	0.4	0.9	0.6	1.3	0.6
2001	0.2	0.4	0.0	0.0	1.8	1.0	0.0	0.0	0.0	0.0	1.0	0.7	0.0	0.0
10,12 18:2 cLA	0.8	0.4	1.7	1.3	0.2	0.5	0.6	0.5	2.4	1.1	0.0	0.1	1.0	0.8
20:1n-9	0.1	0.1	0.0	0.0	1.8	0.6	0.0	0.0	0.1	0.2	1.9	0.5	0.0	0.0
20:2n-9	2.0	0.3	1.8	0.4	0.1	0.0	1.3	0.3	1.8	0.2	0.1	0.0	2.3	0.6

20:2n-6	0.2	0.1	0.1	0.1	0.8	0.3	0.0	0.0	0.1	0.1	1.0	0.3	0.0	0.0
20:3n-9	0.8	0.3	0.8	0.3	0.0	0.0	0.6	0.2	1.1	0.3	0.0	0.0	1.1	0.1
20:4n-6	2.9	0.8	5.9	8.5	2.3	0.3	6.1	7.9	4.1	3.4	5.7	9.2	1.3	0.6
20:4 n-3	0.2	0.0	0.4	0.2	0.8	0.7	1.2	0.7	0.6	0.3	0.3	0.1	0.3	0.1
20:5n-3	2.1	0.9	1.2	0.5	3.4	3.3	5.1	2.0	1.3	1.0	0.6	0.1	0.5	0.1
22:4n-6+22:3n-3	4.9	0.9	5.6	1.3	4.9	2.2	3.3	0.6	6.4	4.1	4.7	1.8	4.0	2.9
22:4n-3	1.0	0.3	1.3	0.4	0.1	0.0	1.4	0.6	1.9	1.4	0.1	0.1	0.9	0.6
22:5n-3	6.2	0.9	6.2	2.0	6.4	0.7	6.1	2.6	6.3	0.6	4.4	2.3	4.1	2.1
22:6n-3	13.0	2.8	11.8	0.9	11.7	1.8	13.5	2.1	12.0	3.8	8.8	3.0	6.1	2.6
∑ SFA	32.5	2.6	32.6	2.9	31.9	1.0	32.5	3.1	30.2	1.4	29.0	4.0	27.2	2.0
∑ PUFA	36.0	6.5	37.8	5.6	33.7	3.8	42.6	4.3	39.1	4.9	33.1	7.3	30.7	4.2
∑ MUFA	31.6	4.4	29.6	7.8	34.4	3.9	24.9	7.2	30.6	5.7	37.9	10.8	42.1	6.0
∑ n-3	23.5	4.6	21.9	2.5	23.2	5.0	28.3	3.1	23.1	4.3	15.3	4.1	13.8	4.6
∑ n-6	4.5	0.8	7.4	8.1	5.2	1.0	9.0	6.9	6.7	2.8	12.6	7.5	9.3	2.7
∑ n-9	18.5	2.2	15.7	4.8	16.8	2.9	12.0	3.2	15.9	5.0	24.3	10.3	31.8	7.8
Total lipid (%)	35.9	2.6	39.9	20.0	33.5	11.7	29.6	18.2	31.3	19.3	23.5	15.5	36.0	9.5

Table 5.3 Mean FFA composition (mol% of total FFA, ± standard deviation) of Port Jackson shark H. portusjacksoni blood serum sampled prior to feeding any experimental diet (control), and after feeding at 6, 12 and 18 weeks on poultry oil (PO) or fish oil (FO) pellet diet. Minor FAs (≤1%) are not shown.

	Control		FO				PO					
	Week 0 (n = 3)		Week 6 (n = 6)		Week 12 (n = 6)		Week 18 (n = 7)		Week 6 (n = 6)		Week 12 (n = 5)		Week 18 (n = 10)	
	Mean	±	Mean	±	Mean	±	Mean	±	Mean	±	Mean	±	Mean	±
9:0	0.7	0.1	1.4	1.3	0.0	0.0	0.0	0.0	1.2	0.4	0.0	0.0	0.0	0.0
11:0	1.6	0.2	1.8	0.8	2.2	0.8	2.6	1.9	1.5	0.8	3.6	2.0	3.3	1.6
12:0	0.4	0.1	0.5	0.4	0.1	0.2	0.6	0.2	0.5	0.2	0.1	0.3	1.2	1.6
14:0	1.4	0.3	1.2	0.3	1.8	1.0	2.9	1.2	0.9	0.2	0.1	0.2	0.6	0.7
15:0	1.1	0.5	0.2	0.4	0.0	0.0	0.0	0.0	0.4	0.3	0.0	0.0	0.1	0.3
16:0	32.7	3.5	31.1	6.0	24.0	11.2	23.4	7.1	28.0	6.3	24.3	3.2	27.2	5.3
16:1n-7	3.8	2.0	1.9	1.8	4.9	2.1	2.9	1.7	2.2	1.9	2.4	1.5	1.9	1.5
18:0	16.3	1.4	17.3	5.8	9.5	3.3	11.7	6.5	15.0	5.7	12.7	2.9	15.6	4.3
18:1n-9	5.4	3.9	3.0	2.2	4.5	2.6	3.1	0.6	8.1	6.5	8.4	5.4	10.8	4.0
18:1n-7	3.4	0.5	2.1	1.6	4.1	1.5	3.2	1.2	3.2	1.4	2.7	1.5	2.1	1.9
18:2n-6	0.7	0.3	1.0	0.8	1.2	0.7	1.0	0.6	2.5	2.0	4.3	3.1	5.2	2.5
19:1	6.0	2.6	9.4	4.4	9.8	2.0	11.2	5.9	7.2	4.4	20.1	9.0	16.5	5.7
20:1n-9	0.0	0.0	0.3	0.6	0.7	1.2	1.4	1.3	0.0	0.0	0.0	0.0	0.0	0.0
20:4n-6	8.2	4.5	7.1	1.9	2.4	1.4	1.3	1.5	6.0	3.8	4.0	4.8	1.2	2.1
20:4 n-3	0.0	0.0	0.3	0.4	0.2	0.5	1.0	0.7	0.2	0.3	0.0	0.0	0.0	0.0
20:5n-3	2.1	0.4	3.1	3.2	15.9	8.5	13.8	8.8	1.5	1.1	1.5	1.4	1.3	1.5
22:4n-6+22:3n-3	1.9	1.1	1.9	1.0	0.0	0.0	0.2	0.4	3.7	3.9	1.2	1.3	1.0	1.3
22:4n-3	0.6	0.5	0.5	0.5	0.0	0.0	0.1	0.2	1.3	1.3	0.0	0.0	0.1	0.3
22:5n-3	3.2	1.5	4.1	3.1	5.0	2.6	5.5	3.6	4.4	1.3	4.5	2.9	3.0	2.6
22:6n-3	7.8	3.5	9.5	7.1	12.2	6.4	13.0	5.1	7.7	2.9	9.4	5.7	7.3	4.8

∑ SFA	54.8	5.9	54.4	14.0	37.8	13.1	41.3	16.4	48.6	11.9	40.8	7.6	48.1	11.8
∑ PUFA	25.7	10.0	28.2	13.7	37.4	18.5	36.6	18.7	29.0	11.1	25.5	8.0	20.2	11.8
∑ MUFA	19.4	4.5	17.4	3.7	24.8	5.9	22.1	2.5	22.4	6.6	33.8	2.4	31.6	6.0
∑ n-3	13.7	5.7	17.6	11.9	33.4	16.6	33.7	17.0	15.7	4.8	15.8	9.2	12.6	8.9
∑ n-6	8.9	4.3	8.1	2.1	3.7	1.9	2.6	1.9	8.6	3.0	8.4	3.2	6.6	3.7
∑ n-9	7.1	4.4	4.3	3.4	5.6	2.4	4.8	0.9	10.0	7.0	8.6	5.7	11.1	4.2

Table 5.4 Mean FA composition (mol% of total FA, ± standard deviation) of Port Jackson shark *H. portusjacksoni* muscle tissue sampled prior to feeding any experimental diet (control), and after feeding at 6, 12 and 18 weeks on poultry oil (PO) or fish oil (FO) pellet diet. Minor FAs (≤1%) not shown.

	Control		FO						PO					
	Week 0 (n = 4)		Week 6 (n = 6)		Week 12 (n = 6)		Week 18 (n = 7)		Week 6 (n = 6)		Week 12 (n = 6)		Week 18 (n = 10)	
	Mean	±	Mean	±	Mean	±	Mean	±	Mean	±	Mean	±	Mean	±
dma 16:0	4.8	0.5	4.2	0.5	4.4	0.8	4.4	0.9	4.6	0.3	4.2	0.6	4.6	0.6
16:0	21.9	1.4	21.2	0.9	22.6	1.4	22.4	1.3	21.4	0.6	21.9	0.7	21.8	1.3
16:1n-7	4.5	1.1	4.0	0.5	4.1	1.1	3.3	1.1	4.0	1.2	3.0	0.8	2.9	0.6
17:1	0.8	0.5	1.2	0.4	0.7	0.1	0.6	0.2	0.7	0.1	0.7	0.1	0.7	0.1
18:0	8.6	0.4	8.1	0.9	9.1	1.1	9.7	2.5	8.2	0.9	10.1	2.1	9.6	1.7
18:1n-9	13.0	0.7	11.7	0.7	10.1	1.5	9.9	1.3	10.9	1.6	11.1	2.7	11.5	1.3
18:1n-7	6.9	1.5	6.6	1.0	5.7	1.3	5.2	1.1	6.4	0.9	5.4	0.9	5.1	0.8
18:2n-6	0.7	0.1	0.8	0.2	0.7	0.1	0.9	0.1	1.1	0.2	1.1	0.4	1.9	0.6
20:4n-6	10.7	2.6	11.9	1.1	14.8	2.6	13.5	3.2	12.7	1.9	14.2	4.0	14.6	3.0
20:5n-3	2.7	0.6	3.9	0.4	4.0	0.5	4.3	0.7	4.2	0.9	3.8	0.8	2.9	0.7
22:4n-6+22:3n-3	3.0	0.4	3.2	0.5	2.7	0.2	2.3	0.2	2.8	0.5	3.0	0.5	2.5	0.4
22:5n-3	4.2	0.5	3.8	0.7	3.0	0.7	3.6	1.1	3.6	0.6	3.5	0.7	3.2	0.4
22:6n-3	12.0	1.8	12.8	0.8	13.3	1.0	14.0	1.6	13.3	1.5	12.8	1.7	13.4	1.3
∑SFA	36.3	1.5	34.6	1.5	37.2	1.5	37.7	0.8	35.1	1.4	37.5	1.9	36.8	1.2
∑PUFA	35.7	4.2	39.1	0.9	39.9	2.6	41.2	2.7	40.3	2.2	40.0	2.5	41.0	1.9
∑MUFA	28.0	3.2	26.2	2.0	22.9	3.8	21.0	3.4	24.6	3.5	22.5	4.3	22.2	2.5
∑n-3	19.8	2.1	21.9	1.1	20.8	0.7	23.4	0.7	22.5	1.3	20.6	2.7	20.9	1.9
∑n-6	12.0	2.5	13.2	1.1	15.7	2.7	14.9	3.1	14.3	1.8	15.7	3.8	16.8	2.7
∑n-9	15.0	1.0	13.8	0.8	11.8	1.8	11.3	1.4	12.6	1.8	12.9	3.0	13.1	1.6
∑Total lipid (%)	0.7	0.1	0.9	0.2	1.0	0.1	0.8	0.2	0.9	0.0	1.0	0.2	0.9	0.1

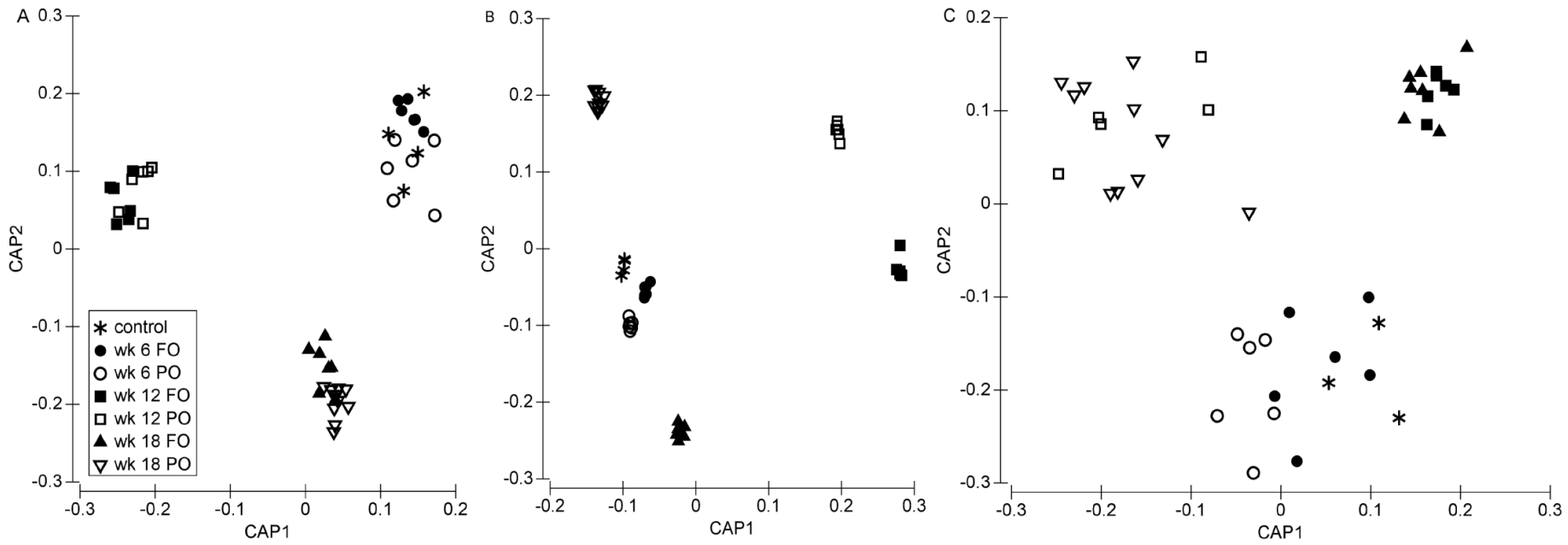

Figure 5.1 Canonical analysis of principal coordinates (CAP) plots from shark **(**A**)** muscle fatty acid profiles, **(**B**)** liver fatty acid profiles, and (C) blood free fatty acid profiles of *Heterodontus portusjacksoni* compared by diet and week sampled, as well as unfed control sharks.

5.4.6 Major drivers of differences between sharks: non-dietary drivers

High levels of variation in 20:4n-6 were seen, despite low proportions in both diets (Table 5.1). 20:4n-6 was a major driver of the dissimilarity between FO-and PO-fed sharks in the liver at all weeks sampled, in the blood serum at week 12 and in the muscle at weeks 12 and 18 (Table 5.7). The liver FA profiles showed the least change over time, despite 20:4n-6 driving dissimilarity between FO and PO-fed sharks at all weeks sampled, high levels of variation were observed and there was only a 4.8% difference by week 18 (Table 5.2). Levels of 20:4n-6 decreased over time in both FO- and PO-fed shark blood serum. Despite being a driver of dissimilarity between FO- and PO-fed sharks, levels of 20:4n-6 in the blood serum differed the most at week 12 (1.6%), but showed similar levels in FO- and PO-fed sharks by week 18 (Table 5.3). There was a slight increase in 20:4n-6 over time in the muscle.However, high levels of variation between individuals were observed and the difference was only 1% by week 18, with a peak of 6.3%at week 6 (Table 5.4).

Despite high levels of variation amongst individuals, 22:5n-3 was only identified as a major driver of the dissimilarity between the blood serum FA profiles of PO- and FO-fed sharks at week 18 and in the muscle at week 12 (Table 5.7). Levels of 22:5n-3 increased over time in FO-fed shark blood serum, peaking at a 2.6% difference by week 18 (Table 5.3). In the liver, FO-fed sharks also had higher levels of 22:5n-3 than PO-fed sharks, peaking at 7.4% by week 18 (Table 5.2). The muscle showed a different pattern, with higher levels of 22:5n-3 in FO-fed sharks at week 6, followed by similar levels to PO-fed sharks at weeks 12 and 18 (Table 5.4). Higher levels of 22:5n-3 in FO-fed shark blood serum were consistent with diet as FO-pellets contained 1% more 22:5n-3 than PO-pellets (Table 5.1).

Table 5.5 Two-way non-parametric multivariate analysis of variance (PERMANOVA) results of fatty acid profile differences between the liver, muscle and blood (FFA) of experimentally fed *H. Portusjacksoni* fed fish oil and poultry oil (diet) nested by tank sampled crossed with week sampled (week).

		df	MS	F	P
Liver	Diet	1	19.2	6.2	0.035*
	Week	2	42.5	9.8	<0.001*
	Tank (diet)	3	1.9	0.4	0.988
	Diet x	2	13.3	3.1	0.030*
	Tank (diet)	4	4.3	0.9	0.641
Muscle	Diet	1	2.1	2.0	0.073
	Week	2	9.8	8.0	<0.001*
	Tank (diet)	3	1.0	1.1	0.332
	Diet x	2	1.3	1.1	0.405
	Tank (diet)	4	1.2	1.3	0.144
Blood	Diet	1	73.9	6.9	0.332
	Week	2	42.8	3.7	0.010*
	Tank (diet)	2	10.6	0.9	0.495
	Diet x	2	15.9	1.4	0.270
	Tank (diet)	4	11.6	1.0	0.447

Table 5.6 Pairwise statistical tests of dietary differences in liver and muscle FA, and blood FFA profiles between control *H. Portusjacksoni* and sharks fed fish oil (FO) pellets or poultry oil (PO) pellets by two-way factorial non-parametric multivariate analysis of variance (PERMANOVA).

	Liver		Muscle		Blood serum	
Groups	t	*P*	t	*P*	t	*P*
wk 6 FO, wk 6 PO	0.8	0.626	1.3	0.193	1.0	0.371
wk 12 PO, wk 12 FO	1.7	0.009*	1.0	0.355	1.8	0.013*
wk 18 FO, wk 18 PO	3.5	<0.001*	1.6	0.034*	2.3	0.007*
wk 12 FO, wk 6 FO	2.6	0.002*	2.7	0.002*	2.0	0.011*
wk 18 FO, wk 12 FO	2.5	0.002*	2.0	0.008*	0.8	0.456
wk 18 FO, wk 6 FO	1.5	0.137	2.1	<0.001*	1.8	0.046*
wk 12 PO, wk 6 PO	2.6	0.003*	2.7	0.003*	1.6	0.041*
wk 18 PO, wk 12 PO	2.9	<0.001*	2.2	<0.001*	1.0	0.397
wk 18 PO, wk 6 PO	3.1	<0.001*	2.9	<0.001*	2.2	0.003*
control, wk 6 FO	1.0	0.517	1.3	0.090	0.9	0.552
wk 12 FO, control	2.5	0.005*	2.6	0.004*	1.8	0.051
wk 18 FO, control	1.5	0.119	2.1	0.003*	1.7	0.070
control, wk 6 PO	1.4	0.135	2.0	0.014*	0.9	0.520
wk 12 PO, control	2.2	0.004*	2.2	0.0057*	1.6	0.039*
wk 18 PO, control	3.0	0.002*	2.5	<0.001*	2.1	0.007*

Table 5.7 Major drivers (>5%) of the differences between fish oil (FO) and poultry oil- (PO) fed sharks identified by SIMPER analysis. The drivers of difference between FO- and PO-pellets are identified in bold. The weeks analysed are those which were found to be significantly different using PERMANOVA. * indicates less than 5% in diet, where dietary FA was <1% drivers are not shown.

		Major driver of difference	
Tissue	Weeks	Fish Oil	Poultry Oil
Liver	6, 12, 18	20:4n-6*	**18:1n-9**
	12, 18	**20:5n-3**	**18:2n-6**
	18	**22:6n-3**	
Blood	6, 12, 18	**22:6n-3**	**18:1n-9**
	12, 18	**20:5n-3,** 14:0, 22:5n-3*	**18:2n-6**
	6	16:1n-7	
	12	20:4n-6*	
Muscle	12, 18	20:4n-6*	
	12	16:1n-7	**18:1n-9**
	18	**20:5n-3**	**18:2n-6**

5.5 Discussion

5.5.1 Temporal changes in blood serum free fatty acid profiles in relation to diet

Dietary FA were incorporated into the blood serum of fed sharks, with significant differences seen within 12 weeks and high levels of dissimilarity between sharks fed different diets relative to the other tissues sampled. This suggests that blood serum FFA is a useful indicator of acute dietary changes in Port Jackson sharks. Rapid incorporation of dietary FA has been previously demonstrated in other animals such as birds, where previously dominant dietary FA were extinguished in the blood serum rapidly following a dietary change (Käkelä et al. 2009). The serum FFA profiles of the southern stingray *Dasyatis americana* were successfully used to discriminate tourist fed stingrays from unfed individuals (Semeniuk et al. 2007). Furthermore, blood plasma FA have also corresponded previously with prey FA in the Greenland shark, *Somniosus microcephalus* (McMeans et al. 2012).

Although significant FA oxidation does not occur outside of the liver in sharks, the use of blood serum FFA to distinguish between diets is justified as the lipids stored in the liver must still be transported to other tissues to carry out a variety of metabolic functions (Ballantyne et al. 1993). Investigations of the FA transport mechanisms from the liver to the blood serum shows that lipoproteins, which bind to the FFA, were absent in many cases (Metcalf and Gemmell 2005). The detection of dietary patterns in the blood serum FFA in this study shows that FFA are being transported in the blood. This is despite previous suggestions that chondrichthyans do not need a FFA transport because of their low levels of extrahepatic *β*-oxidation (Metcalf and Gemmell 2005).

5.5.2 Stability of the liver and muscle fatty acid profiles

Similar to the blood serum FFA, the liver FA profiles of FO- and PO-fed sharks showed significant differences at week 12 and 18. This is supported by previous controlled experiments, which showed significant differences in the FA profiles of *H. portusjacksoni* fed different diets and sampled after 10 weeks (Beckmann et al. 2013a). This result is reflective of the function of the liver as the main site of lipid storage (Bone and Roberts 1969; Hallgren and Larsson 1962; Malins 1968; Medzihradsky et al. 1992). Previous studies using wild specimens of *S. microcephalus* have shown that shark liver has high levels of intraspecific variation and large differences in prey and predator FA patterns because of high levels of FA modification (McMeans et al. 2013). Similar variation between individual shark FA concentrations in the liver was also observed in this study. However, some specific FA showed strong correlations with diet and low levels of variation between individuals. The current study also showed that proportions of MUFA increased over time in correlation with high levels in the PO diet. This is consistent with a previous study on deepwater chondrichthyans, which showed that the liver is biased towards representing MUFA-rich prey species (Pethybridge et al. 2013).

Unlike the liver, significant differences between the muscle FA profiles of sharks fed different diets were not observed at any interval. The lack of significant

differences between the total FA profile of sharks fed differing diets may be a result of lower overall levels of fat (<1%) found in the muscle tissue. In lean species of fish (<2% fillet lipid), there is very little storage of fat in the muscle and the lipids are dominated by structural, cell-membrane phospholipids (Jobling 2013) The differences seen in muscle FA profiles were driven by PUFA such as 20:4n-6, 22:6n-3, 18:2n-6, and 20:5n-3. This supports the idea that PUFA, which characterise muscle tissue, are preferentially diverted to the muscle to meet metabolic requirements (Pethybridge et al. 2010). These essential PUFA are required for normal growth and development of cells and tissues (Sargent 1995). In fish, these FA are also normally used as a source of energy through β-oxidation, however, sharks do not have the necessary enzymes to transport FA into the mitochondria for catabolism in tissues (Watson and Dickson 2001). The diet induced alterations of FA profiles in the present study suggest that the physiological function and selective incorporation of PUFA makes them suitable indicators of diet in the muscle.

Previously, the muscle and liver triacylglycerol (TAG) component of the FA profile have been used to demonstrate the long-term feeding history of the sleeper shark *Somniosus pacificus* (Schaufler et al. 2005). Unusually, *S. pacificus* had high proportions of MUFA in the muscle and liver, likely as a result of a long-term diet of cetacean blubber (Schaufler et al. 2005). This suggests that if a dietary pattern is well established, it is possible for the muscle to become dominated by MUFA rather than PUFA as previously seen. Higher proportions of MUFA were observed by week 18 in the liver and this was in line with high proportions of this FA in the PO diet. Proportions of PUFA always, however, dominated the muscle FA profile of fed sharks.

5.5.3 Fatty acids as indicators of diet in the liver, blood serum, and muscle

This study suggests that 20:5n-3 and 22:6n-3 are dietary derived. After 18 weeks of feeding, consistent patterns with diet were seen in 20:5n-3 proportions in the liver and blood serum FA profiles and 22:6n-3 in the liver FA profiles. Marine fish inhabit environments rich in highly unsaturated fatty acids, particularly 22:6n-3.

There is, therefore, no evolutionary pressure for them to retain the ability to produce these FA endogenously (Tocher 2010). 20:5n-3 and 22:6n-3 are considered essential FA as carnivores and many marine fish only have a limited ability to convert 18:3n-3 to 22:6n-3 through Δ6 and Δ5 desaturation (Tocher 2010).

Despite their potential for modification, dietary proportions of 14:0, 18:1n-9, 18:2n-6 and 20:5n-3 were reflected in the blood serum and liver by week 18. 20:5n-3, 22:6n-3, 18:1n-9, and 18:2n-6 have previously been identified as useful indicators of diet in the liver and muscle of deepwater chondrichthyans (Pethybridge et al. 2010) and in the liver, muscle, and blood serum of Greenland sharks *S. microcephalus* (*6*). 22:6n-3 has also previously been used as biomarker of dinoflagellates (McMeans et al. 2012) and 20:5n-3 as a biomarker of diatoms (Graeve et al. 1994; Viso and Marty 1993). However, levels of 22:6n-3 fluctuated in the muscle and blood serum of PO-fed sharks fed a 22:6n-3 deficient diet and this reduced the overall difference observed between sharks fed different diets. Similarly, levels of 20:5n-3 in the muscle of fed sharks fluctuated likely as a reflection of the low level of dietary input and mediation from stores in the lipid. Levels of 22:6n-3 in the liver of PO-fed sharks declined over time. However, as they were not exhausted, mediation from stores cannot be ruled out. By week 18, liver 20:5n-3 had decreased to only 0.5% in PO-fed sharks. This pattern correlated with reduced 20:5n-3 in the muscle of PO-fed sharks (2.6% by week 18) and is supported by low levels of 20:5n-3 in the blood of PO-fed sharks at all weeks (1.3–1.5%) compared to 22:6n-3 blood serum levels (3–4.4%). 20:5n-3 appears to be a more accurate indicator of diet in this study and relating 22:6n-3 to diet using the muscle and blood serum should be done with caution due to the fluctuations observed.

The difference in 18:1n-9, which accounts for approximately 9 to 16% of total FA in fish oils (Young 1986), was reflected in the blood serum and liver FA profiles, while a high degree of variability was observed in the muscle. Fluctuations in 18:1n-9 may be a result of its conversion to 20:3n-9. The accumulation of 18:1n-9 in the liver of PO-fed sharks in response to diet suggests that 18:1n-9 was not used as an oxidative substrate and that patterns are related to

direct differences in consumption of 18:1n-9. Levels of 20:2n-9 and 20:3n-9, the products of 18:1n-9 elongation and desaturation, decreased to trace levels in the liver of FO- and PO-fed sharks at week 12, followed by an increase back to the week 6 levels at week 18. Increased 18:1n-9 and subsequently 18:2n-9, 20:2n-9, and 20:3n-9 are typically indicators of an essential fatty acid deficiency (Sargent et al 1993; Tocher 2003). Low levels of 18:2n-9 accumulated in FO-fed sharks up until week 12 and as this cannot be explained by direct dietary input it may actually be a result of deficiencies in both 18:2n-6 and 18:3n-3. This finding is relevant to dietary studies as 18:1n-9 has previously been used as a biomarker of brown algae (Alfaro et al. 2006), bacteria, and omnivory (Stevens et al. 2004a,b). While these FA may be useful in controlled dietary studies, using them to detect unknown diets is complicated as the sources of 18:2n-6 and 18:3n-3 are not clear. 18:2n-6 and 18:3n-3 are considered essential FA and sharks cannot form 18:2n-6 and 18:3n-3, as the enzymes Δ12 and Δ15 desaturase necessary to form 18:2n-6 and 18:3n-3 from 18:1n-9 are generally only found in plants (Tocher 2003). 18:2n-6 proportions between PO- and FO-pellets were also reflected in the FA profile of liver, muscle, and blood serum by week 18. As long as 18:2n-6 and 18:3n-3 dietary intakes are adequate, elongation and desaturation of 18:1n-9 to 20:3n-9 are unlikely to occur (Curtis-Prior 2004), and 18:1n-9 and 18:2n-6 in liver and blood serum and 18:2n-6 in the muscle should be considered suitable indicators of diet.

18:1n-9 has previously been suggested as an indicator of diet for sharks fed prawns in a controlled setting (Beckmann et al. 2013a) and grazers consuming brown algae (Alfaro et al. 2006), while 18:2n-6 has been used as a biomarker of fresh plant material, indicating secondary predation on herbivorous species (Dalsgaard et al. 2003; Wai et al. 2011). Similarly, both 18:1n-9 and 18:2n-6 have been shown to accumulate in Greenland shark , *S. microcephalus* tissues in proportions similar to those observed in the diet based on halibut (McMeans et al. 2012).

5.5.4 Non-dietary drivers of the differences between fed sharks

22:5n-3 was a major driver of the difference between FO- and PO-fed sharks in the blood serum, and 20:4n-6 in the liver, blood serum, and muscle FA profiles. These FA were not drivers of the differences between diets, indicating that the differing patterns observed in the FO- and PO-fed sharks are indirectly related to diet through the metabolism of dietary FA. 22:5n-3, and 20:4n-6 have been reported as dietary indicators in liver and muscle tissue of deepwater chondrichthyans (Pethybridge et al. 2011a), while 22:5n-3 has been reported as a dietary indicator in the liver and muscle tissue, and blood serum of Greenland sharks *S. microcephalus* (McMeans et al. 2012).

20:4n-6 was present in low proportions in both diets and was seen to fluctuate in the liver, muscle and blood serum FA profiles of sharks fed both diets. Decreases in 20:4n-6 may be reflective of the interconversion of the FA to 18:2n-6. This suggestion is supported by increasing levels of 18:2n-6, particularly in PO-fed sharks, which were fed higher dietary proportions of 18:2n-6. 20:4n-6 becomes an essential FA and is required in the diet when a deficiency of 18:2n-6 occurs, such as in FO-fed sharks. This suggests that 20:4n-6 is not a suitable indicator of diet as it can be affected by fluctuations in 18:2n-6.

5.6 Conclusion

Our study provides important baseline information required to interpret the incorporation of dietary FA in Port Jackson sharks and potentially other chondrichthyans. We have demonstrated that the liver or blood serum FA profiles of sharks fed different diets were distinguishable within 12 weeks of feeding and that the muscle FA profiles were not distinguishable within 18 weeks. Dietary FAs fluctuated in the muscle indicating that complete turnover in this tissue occurs at a slower rate than the liver, and that the liver tissue and blood serum are in fact more indicative of the incorporation of dietary FA. This is the first controlled study to report blood serum FFA in relation to diet in a chondrichthyan species The most consistent dietary indicators were 14:0, 18:2n-6, 20:5n-3 and 18:1n-9 which were

consistently reflected in the blood serum and liver, and 22:6n-3 which was reflected in the liver.

While shifts in wild diet are likely to be less extreme than those simulated in this captive feeding trial, baseline data is required to interpret the differences observed in the fatty acid profiles of wild sharks. Although there is a plethora of information available about the fatty acid profiles of teleosts and other vertebrates (Sargent et al. 1987; Tocher 2003), the physiological and metabolic differences between species can limit the application of this information across taxa. Recent studies suggest chondrichthyans exhibit complex feeding ecology which may not be detected without the use of complimentary techniques including fatty acid profile analysis (Belicka et al. 2012; Couturier et al. 2013; McMeans et al 2012, 2013; Pethybridge et al. 2011a,b; Schaufler et al 2005; Semeniuk et al 2007; Wai et al. 2011). The knowledge of which fatty acids are suitable indicators of dietary intake and the timescales over which fatty acids are integrated into the diet in the different tissues is fundamental to the use of fatty acids to determine dietary preferences in shark.

Where less invasive sampling methods are desirable, the muscle and blood serum fatty acids have shown potential to indicate long-term dietary patterns. Caution should be exercised when using the muscle tissue in particular because of the low levels of fat and the physiological role the muscle plays in structural, cell-membrane phospholipids (Jobling 2003). The blood serum was representative of mobilised fatty acids from the liver and may provide a more suitable method for dietary analysis than the muscle.

Future studies should examine the differences in fatty acid profiles of shark species at different spatial scales using a combination of stomach content and fatty acid analyses of multiple tissues to investigate how fatty acid profiles vary between regions and among demographic groups.

5.7 Supplementary material

Supplementary Table 5.1 Ingredient formulation and proximate composition of two experimental diets: fish oil (FO), poultry oil (PO), modified from Bowyer et al. (2012).

	Diets	
	FO	PO
Ingredients (g kg^{-1})		
Herring meal[1]	500	500
Wheat gluten meal	95.3	95.3
Fish oil[1]	203.6	
Poultry oil[1]		203.6
Tapioca starch	96.1	96.1
Mill run	97	97
Choline choloride	3	3
Vitamin/mineral premix[2]	2	2
Vitamin C3	3	3
Total	1000	1000
Analysed composition (% DM)		
Moisture	4.2	3.7
Crude protein	42.4	42.8
Crude fat	24.2	24
Ash	9.2	9.5
Gross energy (MJ kg^{-1})	23.1	23.4

Chapter 6: Stomach content and fatty acid biomarkers as indicators of spatial variations in the diet of Port Jackson shark (*Heterodontus portusjacksoni*) between southern and eastern Australia

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6.1 Abstract

The diet of wild Port Jackson sharks (*Heterodontus portusjacksoni*) was investigated by comparing the fatty acid profiles of liver and muscle tissue with the stomach contents from three locations obtained between February and April 2013. According to cumulative prey curves, stomach content based on lowest possible taxonomic levels did not represent diet as accurately as when characterised using a broad taxonomic classification. The diet of Port Jackson sharks was significantly different between Spencer Gulf and New South Wales, and Gulf St Vincent and New South Wales, but not between Gulf St Vincent and Spencer Gulf. The fatty acid profiles of shark tissues were, however, significantly different between all three locations. The liver fatty acid profiles were the best predictor of sample location at 92.6% correct allocation, with the least allocation success obtained from stomach content analysis with broad taxonomic classification at 55.8%. Shark stomachs from Spencer Gulf were characterised by high levels of nematodes and arthropods, while stomachs from Gulf St Vincent contained high levels of annelids and molluscs. Nematodes were generally found unattached to the stomach wall, indicating that they may have been ingested with prey (Marcogliese, 2002). Sharks from New South Wales had high levels of teleosts compared to the other two locations, which was supported by the accumulation of docosahexaenoic acid (DHA, 22:6n-3), a biomarker of flagellate predation as a result of secondary or tertiary consumption, in the liver tissue. Fatty acid biomarkers of diatoms and herbivory, such as 16:1n-7, revealed that molluscs were underrepresented in stomach contents, likely due to the rapid digestion of soft tissues and crushing of shells making them unrecognisable. This study demonstrated that fatty acid profile analysis of liver and muscle tissue can reduce some of the biases of stomach content analysis, and identified spatial differences that were undetected by stomach content analysis.

Key Words: Lipids, chondrichthyan, Gulf St Vincent, Spencer Gulf, New South, Wales, DHA, docosahexaenoic acid

6.2 Introduction

The study of chondrichthyan feeding biology is required to understand trophic interactions and the effect of energy flow through food webs (Braccini & Perez 2005; Myers et al. 2007). Many shark species are important predators and can regulate the lower trophic levels of the ecosystem they live in (Bethea et al. 2004; Ferretti et al. 2010; Worm et al. 2013). Knowledge of the diet of sharks that interact with commercial fisheries is crucial to understand the effects that changes in population size, due to overexploitation, can have on the ecosystem (Cortés 1999; Wetherbee & Cortés 2004).

Stomach content analysis has been widely used to study the feeding habits of sharks (Hyslop 1980; Cortés 1997). It is, however, biased towards recently ingested prey items, only provides a snapshot of a species diet (Pinnegar & Polunin 1999, Pinnegar et al. 2001; MacNeil et al. 2005), and is generally lethal. In addition, low sample sizes, empty stomachs, and variable digestibility of prey items can bias dietary assessments based on stomach contents alone (Simpfendorfer 1998). Complementary biochemical methods such as fatty acid (fatty acid) analysis can provide additional insight into the diet of a species, trophic interactions, and ecological niches (Iverson et al. 1997). Studies examining the fatty acid composition of chondrichthyans are becoming increasingly common (Pethybridge et al. 2011; Wai et al. 2011; McMeans et al. 2012). Although the use of tissue and blood serum fatty acid profiles to determine diet has been validated using controlled feeding experiments in various species (Casper et al. 2006; Iverson et al. 2007; Fluckiger et al. 2008; Käkelä et al. 2009; Wang et al. 2010), it has only recently been investigated in sharks. Fatty acid analysis requires an understanding of how ingested fatty acids are metabolised and deposited in various tissues of the consumer. The extent to which specific fatty acids can be created or modified by sharks is important to consider when distinguishing between dietary and non-dietary fatty acid sources. Controlled feeding experiments with Port Jackson sharks, *Heterodontus portusjacksoni*, identified the dietary fatty acid and biomarkers in the blood serum, liver, and muscle tissue associated with diets fed to the sharks (Beckmann et al. 2013a, b, 2014a, b.). Used in combination with stomach content analysis, dietary tracer fatty acids, such as eicosapentaenoic acid

(EPA, 20:5n-3) and Docosahexaenoic acid (DHA, 22:6n-3), which are not biosynthesised by most marine consumers (Parrish 2009), may be used to infer diet. Furthermore, diatom-based food webs can be identified through EPA, 16:1n-7 and high levels of polyunsaturated fatty acids, whereas flagellate consumption is characterised by high concentrations of DHA and 18 carbon polyunsaturated fatty acids such as 18:4n-3 (Thompson et al. 1992; Viso and Marty, 1993; Graeve et al. 1994, 2005; Stevens et al. 2004b). Comparison of fatty acid profiles between tissues also showed that liver tissue and blood serum are more accurate indicators of dietary fatty acid profiles than muscle tissue because of selective deposition of polyunsaturated fatty acids (Beckmann et al. 2014a). Preferential incorporation of certain fatty acids has shown that polar phospholipids are less responsive to dietary change than neutral, non-polar lipids (Jobling et al. 2003). High proportions of polar phospholipids have been identified in the muscle, while the liver contains higher proportions of neutral non-polar lipid (Pethybridge et al. 2010). Separation of the polar and neutral lipid may reveal that the muscle actually reflects the storage of fatty acids as seen in the liver (Nanton et al. 2001).

In the present study, we used *H. portusjacksoni* to compare the information obtained from stomach contents to fatty acid analysis. *Heterodontus portusjacksoni* is an abundant demersal endemic species to Australian waters (Last and Stevens 2009; Jones et al. 2010; McAuley et al. 2003). Port Jackson sharks are also highly resistant to capture stress as evidenced by minimal disruptions to stress parameters in their blood and lack of mortality after exposure to gillnet and longline soak times of up to 360 minutes (Frick et al. 2010). The dietary compositions of Port Jackson sharks from south-western Australia (Sommerville et al. 2011) and eastern populations sampled in New South Wales (Powter et al. 2010) have been shown to vary, with higher reliance on teleosts in the south-western populations (Sommerville et al. 2011). Variations in diet between locations are likely to be related to differing patterns in prey availability and environmental differences between locations. This suggests that *H. portusjacksoni* are either responding to differences in prey availability or that their prey selectivity differ between locations (Sommerville et al. 2011). The diet of the eastern population varies by ontogenetic stage (Powter et al. 2010), which is typical of other elasmobranch species (Ebert 2002; Marshall et al. 2007).

Variations in diet have been linked to changing dentition and mouth morphology and may also be explained by changing movement patterns as sharks mature (McLaughlin & O'Gower 1971; Powter et al. 2010).

In the present study, we investigated whether the spatial variation seen in *H. portusjacksoni* also occurs between spatially close locations. In South Australia, sharks were collected from Spencer Gulf and Gulf St Vincent, two large semi-enclosed hypersaline inverse estuaries (Dixon et al. 2011). These ecosystems are supported by mangroves in Barker inslet, Gulf St Vincent and seagrass meadows, intertidal mudflats and temperate mangrove forests in Spencer Gulf provide critical nursery habitats for a range of fish species (Edwards et al. 2001). Large-scale variations were investigated through comparisons to sharks from the central coast of New South Wales. The waters of the central coast of New South Wales are influenced by the East Australian current which is most prevalent between September and March (Gray and Mikiewicz 2000). Compared to the locations in Southern Australia, the New South Wales coast also has considerable freshwater input through estuaries such as the Hawkesbury River, which is known to flood and cause changes in zoobenthos abundance (Jones 1990). The diet of *H. portusjacksoni* was analysed from these three different locations using a combination of stomach content and fatty acid analyses of muscle and liver tissue to investigate how fatty acid profiles vary between locations and examine the relationship between fatty acid profiles and stomach contents. Our hypothesis was that fatty acid analysis would more accurately describe diet compared to stomach content analysis in Port Jackson sharks. Specifically, fatty acid profiles will reveal diet differences between geographically close locations that stomach content analyses will not detect.

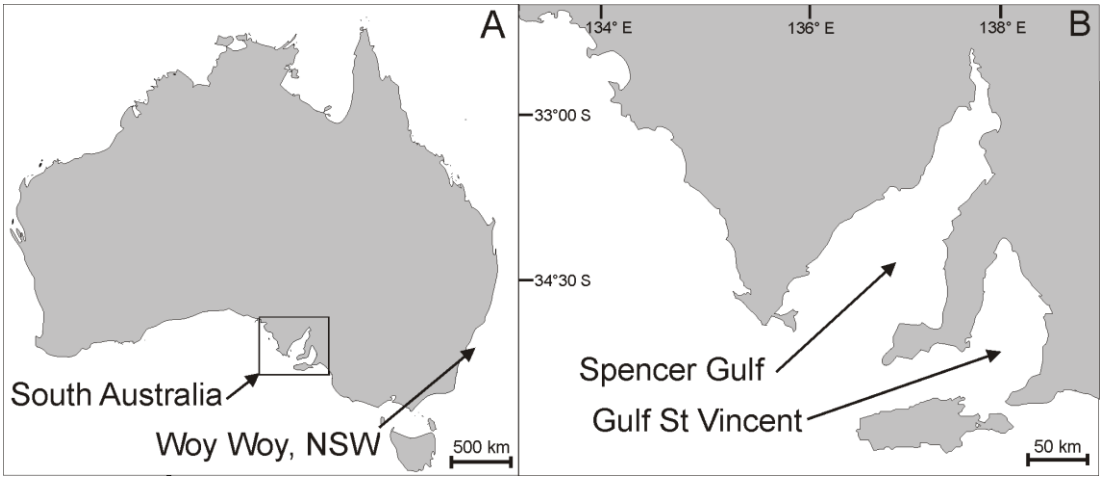


Figure 6.1 *Heterodontus portusjacksoni* were obtained from (A) Woy Woy, New South Wales and inset the South Australian samples were obtained from (B) Gulf St Vincent and Spencer Gulf.

6.3 Materials and Methods

Port Jackson sharks were collected during fishery-independent surveys of the South Australian Prawn Trawling Fishery in Gulf St Vincent (n = 25, 25th of April 2012) and Spencer Gulf (n = 33, 21–23rd of February 2012) in South Australia, and were provided by a commercial trawl fisher operating from Woy Woy, in the New South Wales Ocean Trawl Fishery (n = 23, 20–22nd of March 2012) (Figure 6.1). New South Wales and South Australian samples were separated by approximately 1,200–1,400 km. Sampling depth was 10–100 and 10–60 m for the New South Wales and South Australian sites, respectively. Trawling was undertaken at night using standard demersal otter-trawl gear.

The gender of all sharks was recorded based on the presence or absence of claspers. Sharks were also measured (total length, TL, ±1 mm), and weighed (±0.1 g) upon capture. Shark maturity was assessed according to Powter et al (2010). Eighty-one sharks were collected across the three locations, 33 from Spencer Gulf, 25 from Gulf St Vincent, and 23 from New South Wales. More male sharks were collected in all locations with 18 in Spencer Gulf, 15 in New South Wales, and 13 in Gulf St Vincent. In total, 69 sharks were immature (24 from Gulf St Vincent, 23 from Spencer Gulf and 22 from New South Wales), 11 were subadults (9 from Spencer Gulf, 1 from New South Wales, and 1 from Gulf St Vincent) and 1 was

mature (Spencer Gulf).

Sharks were sacrificed on capture by spinal section and pithing, snap frozen at -40 ºC on board, and stored at -20 ºC until dissection. Sharks were removed from the freezer and dissected prior to being completely thawed to decrease the chance of fatty acid oxidation. Five to ten grams of muscle and liver tissue were collected and frozen at -20ºC until analysed. Samples were analysed by the FOODplus Fatty Acid Lab (Urrbrae, South Australia, Australia). Lipids were extracted from shark tissue samples using a chloroform/methanol (2:1) method as described by (Bligh & Dyer, 1959). The percent lipid was calculated on a wet weight basis and the lipid was extracted in chloroform. Fatty acid methyl esters were produced by heating the extracted lipids in 1% H_2SO_4 in methanol for 3 hrs in a 70^{o}C water bath. After cooling, 750 µl distilled water was added along with 2.0 ml of *n*-heptane and the solution was centrifuged allowing the phases to separate. The extracted fatty acid methyl esters were separated and quantified using a gas chromatograph (Palo Alto, CA, USA) to determine fatty acid composition. Samples were run on a gas chromatograph with a flame ionization detector and an external standard was used with approximately 46 different fatty acid methyl ester types. Additional fatty acids were identified by the relative locations of other peaks in human blood. Fatty acid values are presented both as concentrations (mg g^{-1}) and proportions as a percent of the total fatty acid present.

During dissection, stomachs were removed to analyse and identify dietary content. For each stomach, prey items were identified to the lowest possible taxon (LPT) and weighed using an electronic balance (±0.01 g). Due to the disarticulated and crushed nature of the majority of prey items, prey was also categorised into broad taxa at phyla level or higher (algae, nematoda, nemertea, arthropoda, annelida, mollusca, echinodermata, chordata).

Unidentifiable items were excluded from analysis of stomach contents due to potential bias created by difficulty in obtaining reliable counts of unidentifiable items (Schafer et al. 2002). Stomach contents were described using percentage frequency of occurrence (%FO) and percentage by weight (%W) (Cortés 1997; Pinkas et al. 1971). The disarticulated and crushed nature of the majority of prey items meant that the number of prey items (%N) could not be reliably determined,

and hence that the Index of Relative Importance (%IRI) (Pinkas et al. 1971) could not be calculated. "As the accuracy and utility of different estimators may depend on the data in question, cumulative numbers of observed taxa across samples (hereafter referred to as 'Sobs') and non-parametric estimators of total prey taxon richness (Chao1 and 2, Jacknife1 and 2, and bootstrap) were calculated to determine whether sample size was sufficient to accurately describe the diet of *H. portusjacksoni* (Colwell & Coddington 1994, Tirasin & Jørgensen 1999, Bartels & Nelson 2007, Haddon 2010).

Total lipid (%) in the liver and muscle, and total length (mm) were tested for normality using the Shapiro-Wilk test and homogeneity of variance using a Levene's test. Data that did not show normality and homogeneous variances were log-transformed. A one-way analysis of variance (ANOVA) was employed to test differences amongst locations for each the liver and muscle tissue, and total length. When significant differences were detected by the one-way ANOVA, the non-parametric Games-Howell post-hoc test was used to determine which locations differed, because this test is more powerful and specifically designed for lack of homogeneity of variances (Field 2009).

Liver and muscle fatty acid composition (%) was square-root transformed and Euclidian distance matrices were calculated and stomach content (%W) was square root transformed and Bray-Curtis similarity matrices were calculated using Primer Version 6.1.13 (http://www.primer-e.com). Within- and between-assemblage overlap matrices of stomach content and fatty acid composition were compared with Mantel-type and permutation tests (999 permutations using the RELATE routine in Primer (Clarke & Warwick 2001). Correlations (ρ) between matrices in the RELATE routine were calculated using the Spearman rank correlation method. The null hypothesis tested was that there was no linkage between fatty acid profiles and stomach content and no linkage between liver and muscle fatty acid profiles. The null hypothesis was rejected where $P \leq 1\%$, with ρ values close to 1 indicating a strong match. Where significant correlations were found SIMPER was used to identify typifying and distinguishing prey items or fatty acids (Clarke 1993). A covariate PERMANOVA with sequential (Type I) sums of squares was used to analyse fatty acid and stomach content assemblage

data PERMANOVA+ version 1.0.3 (Anderson 2001). Fat content (% lipid) of liver and muscle samples and total length were included in the analysis as covariates, with location and sex being fixed factors. Factors were removed from the PERMANOVA model where components of variance (CV) were negative, negligible, or zero (Anderson, 2001; Fletcher and Underwood, 2002). Additional pair-wise tests were then performed using the square root of the pseudo-F statistic (t-test). Constrained Canonical Analysis of Principal coordinates (CAP) was used to provide a graphical representation of the differences between sharks from separate locations in two dimensions. Standard error of the mean was used where data is expressed as mean values.

6.4 Results

6.4.1 Shark length and total lipid

Using log transformed data, total length and proportions of lipid in the liver and muscle were normally distributed (Shapiro-Wilk test, $P > 0.05$) and had homogeneous variances (Levene's test, $P > 0.05$). The overall mean TL of sharks was 398 ± 118 mm, but TL was significantly different between regions (ANOVA, $F_2 = 9.3$, $P < 0.01$).The TL of sharks from Spencer Gulf was 469 ± 22 mm and was significantly longer than those from New South Wales at 352 ± 15 mm, (Games-Howell test $P = 0.002$) and Gulf St Vincent at 346 ± 18 mm (Games-Howell test $P = 0.002$). The overall mean proportion of liver lipid was 19.56 ± 11.65%, but liver lipid content was also significantly different between regions (ANOVA $F_2 = 4.8$, $P = 0.011$). Sharks from Spencer Gulf had significantly greater total lipid in the liver (23.82 ± 2.02%) than those from New South Wales (14.02 ± 1.93%, Games-Howell test $P = 0.009$). However, sharks from Gulf St Vincent (19.03 ± 2.37%) were not significantly different to sharks from Spencer Gulf (Games-Howell test $P = 0.164$) or New South Wales (Games-Howell test $P = 0.476$). The mean total lipid content in the muscle was 0.91 ± 0.14% and was not significantly different between regions (ANOVA $F_2 = 0.8$, $P = 0.460$).

6.4.2 Relationship between tissues, fatty acids and prey items

Fatty acid composition was positively correlated with prey items identified in stomach content at LPT (liver, $\rho = 0.281$, $P = 0.001$, muscle, $\rho = 0.257$, $P = 0.001$) and at a broad taxonomic level (liver $\rho = 0.131$, $P = 0.007$, muscle $\rho = 0.148$, $P = 0.002$). Liver fatty acid composition was positively correlated with muscle fatty acid composition ($\rho = 0.49$, $P = 0.001$).

6.4.3 Spatial variability in fatty acid profiles

Overall, 51 fatty acid were identified in the liver and 44 in the muscle of the *H. portusjacksoni* sampled. Only those which were present at average levels ≥ 1% are presented to reduce table sizes (Table 6.1). The total sum fatty acids in the liver were highest in sharks from Spencer Gulf and this was positively correlated with higher total lipid.

The PERMANOVA test showed that fatty acid composition was significant different between locations, with the covariates total length and lipid % also affecting fatty acid composition, justifying their inclusion within the analysis (Table 6.2). Sex was not significantly different in either analysis and was removed due to low or negative co-variance (liver pseudo-$F_1 = 0.747$, $P = 0.608$, CV = -0.11, muscle pseudo-$F_1 = 1.44$, $P = 0.170$, CV = 0.11).

In both tissues, pair-wise tests indicated differences between Spencer Gulf and New South Wales (liver: $t = 4.08$, $P < 0.001$, muscle: $t = 5.31$, $P < 0.001$), between New South Wales and Gulf St Vincent (liver: $t = 2.75$, $P < 0.001$, muscle: $t = 3.12$, $P < 0.001$), and between Spencer Gulf and Gulf St Vincent (liver $t = 3.02$, $P < 0.001$, muscle: $t = 3.12$, $P < 0.001$). CAP analysis showed high levels of allocation success of liver and muscle fatty acid profiles by location at 92.6% and 88.9%, respectively (Figure 6.2). Using liver fatty acid profiles, sharks from New South Wales were correctly allocated 95.7% of the time followed by Gulf St Vincent and Spencer Gulf with 92.0% and 90.9% correct allocations, respectively

(Figure 6.2a). Using muscle fatty acid profiles, sharks from New South Wales were correctly allocated 95.6% of the time, followed by Spencer Gulf and Gulf St Vincent with 87.9% and 84.0% correct allocations, respectively (Figure 6.2b).

SIMPER analysis indicated that fatty acid profiles of sharks from Spencer Gulf and New South Wales were the most dissimilar, followed by sharks from New South Wales and Gulf St Vincent, while sharks from Gulf St Vincent and Spencer Gulf were the most similar (Table 6.3). Dissimilarity between locations was consistently driven by ARA in the liver and muscle of sharks from Spencer Gulf (Table 6.3), which contained higher proportions and concentrations than New South Wales and Gulf St Vincent (Table 6.1 Figure 6.3). In the liver tissues, 18:1n-9 also drove dissimilarity between locations (Table 6.3), with higher proportions observed in Spencer Gulf compared to Gulf St Vincent and New South Wales (Figure 6.3). Sharks from New South Wales displayed consistently higher proportions and concentrations of 22:1n-11, 16:1n-7 and EPA in their muscle than sharks from Gulf St Vincent or Spencer Gulf (Table 6.2, Figure 6.3) and these fatty acid were identified as major drivers of dissimilarity (Table 6.3). Sharks from New South Wales also displayed higher proportions of DHA, a major driver of dissimilarity (Table 6.3), in the liver than sharks from Spencer Gulf or New South Wales, but this was not reflected in the concentration values (Table 6.1). DHA was a major driver of dissimilarity (Table 6.3) as sharks from Gulf St Vincent contained higher levels of DHA in the muscle than sharks from New South Wales and higher levels of DHA in the liver of sharks from Gulf St Vincent compared to sharks from Spencer Gulf (Table 6.1, Figure 6.3).

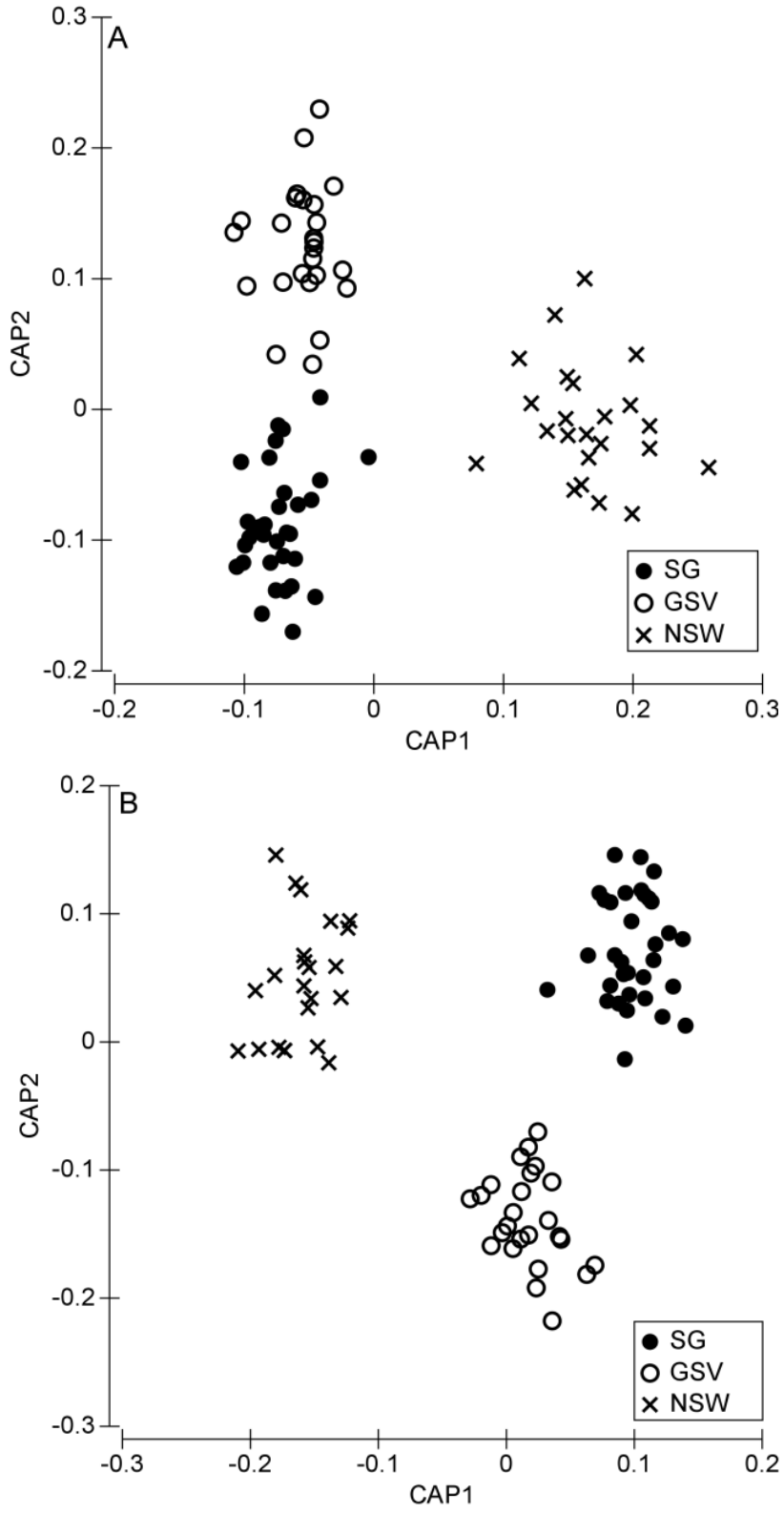


Figure 6.2 Canonical analysis of principal coordinates (CAP) plots from shark (A) liver, and (B) muscle fatty acid profiles compared by location sampled.

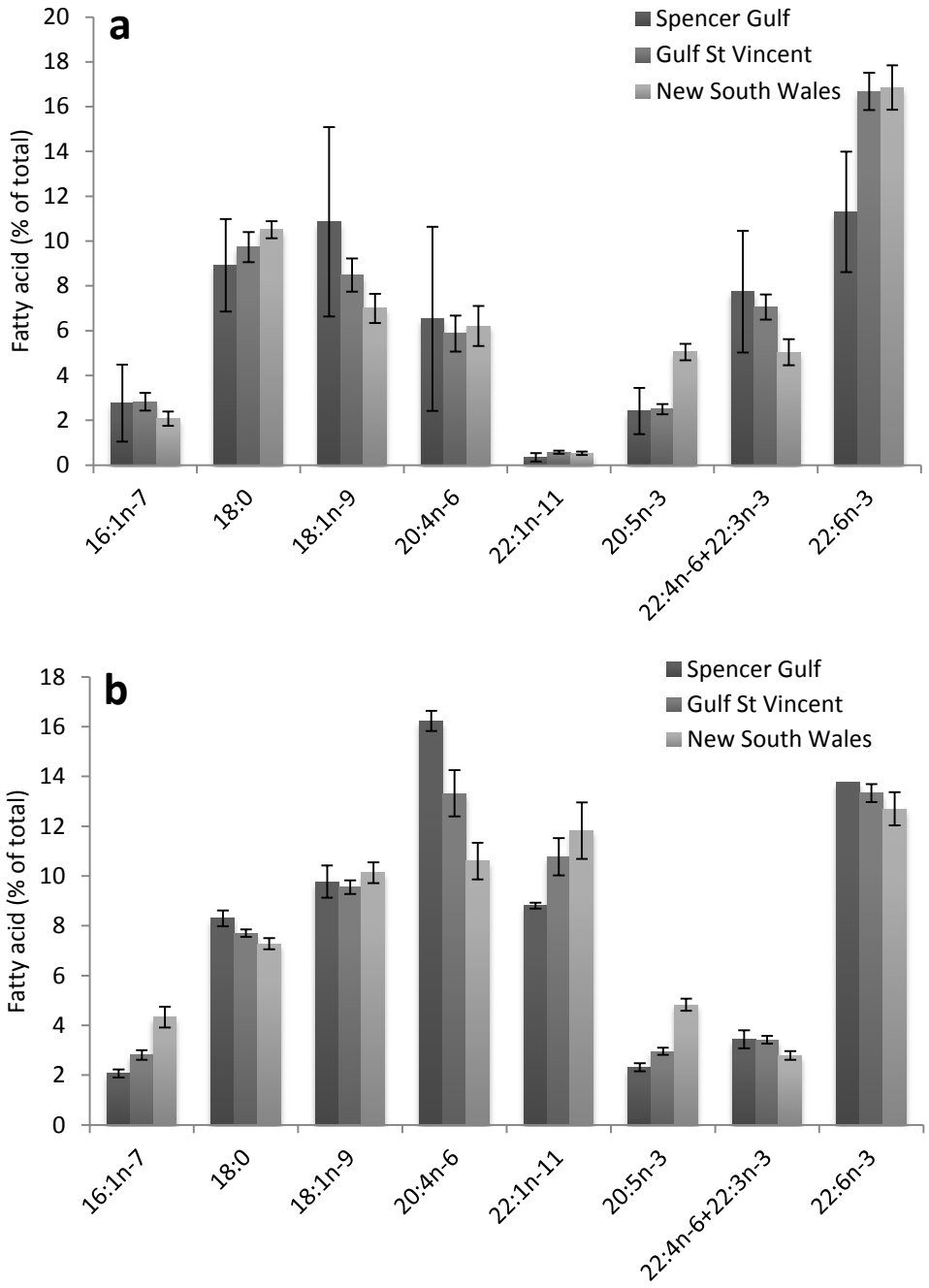


Figure 6.3 Fatty acid composition of *Heterodontus portusjacksoni* (a) liver and (b) muscle with sharks grouped by location sampled (Spencer Gulf Spencer Gulf, New South Wales New South Wales and Gulf St Vincent Gulf St Vincent). Values are presented as a percent of the total fatty acids present (± standard error).

Table 6.1 Fatty acid composition of *Heterodontus portusjacksoni* liver with sharks grouped by location sampled (Spencer Gulf, New South Wales, and Gulf St Vincent). Values are presented as $mg.g^{-1}$ (± standard error).

	Spencer Gulf						New South Wales						Gulf St Vincent					
	Liver			Muscle			Liver			Muscle			Liver			Muscle		
% lipid	23.82	±	2.02	0.92	±	0.02	14.00	±	1.93	0.92	±	0.03	19.03	±	2.37	0.88	±	0.03
14:0	1.42	±	0.17	0.01	±	0.00	0.90	±	0.42	0.01	±	0.00	1.22	±	0.24	0.01	±	0.00
dma 16:0	0.18	±	0.01	0.08	±	0.00	0.20	±	0.01	0.07	±	0.00	0.16	±	0.01	0.07	±	0.00
16:0	22.68	±	2.55	0.63	±	0.02	13.00	±	2.53	0.62	±	0.02	18.57	±	2.92	0.63	±	0.02
16:1n-7	4.38	±	0.80	0.07	±	0.01	2.10	±	0.89	0.15	±	0.01	3.80	±	0.83	0.10	±	0.01
17:1	1.11	±	0.14	0.02	±	0.00	0.50	±	0.06	0.02	±	0.00	0.68	±	0.12	0.02	±	0.00
18:0	10.05	±	0.74	0.29	±	0.01	6.50	±	0.78	0.25	±	0.01	7.89	±	0.72	0.27	±	0.01
18:1n-9	15.92	±	2.30	0.35	±	0.02	5.80	±	1.75	0.35	±	0.02	10.55	±	2.09	0.34	±	0.02
18:1n-7	8.71	±	0.86	0.19	±	0.01	4.40	±	0.75	0.20	±	0.01	7.18	±	0.99	0.21	±	0.01
18:2n-6	1.57	±	0.15	0.03	±	0.00	1.00	±	0.21	0.03	±	0.00	1.29	±	0.18	0.03	±	0.00
20:1n-11	2.54	±	0.38	0.00	±	0.00	0.40	±	0.08	0.00	±	0.00	1.38	±	0.21	0.00	±	0.00
20:1n-9	4.12	±	0.49	0.02	±	0.00	1.00	±	0.16	0.02	±	0.00	1.83	±	0.29	0.02	±	0.00
20:2n-6	1.50	±	0.15	0.01	±	0.00	0.60	±	0.09	0.01	±	0.00	1.05	±	0.13	0.01	±	0.00
20:4n-6	6.19	±	0.36	0.56	±	0.02	2.70	±	0.17	0.35	±	0.02	3.79	±	0.27	0.46	±	0.03
22:1n-11	0.45	±	0.06	0.31	±	0.02	0.60	±	0.26	0.44	±	0.06	0.73	±	0.16	0.39	±	0.03
20:5n-3	2.80	±	0.31	0.08	±	0.00	3.60	±	0.90	0.17	±	0.01	2.38	±	0.35	0.11	±	0.01
22:4n-6+22:3n-3	8.62	±	0.68	0.12	±	0.00	3.10	±	0.52	0.09	±	0.00	6.36	±	0.78	0.12	±	0.01
22:5n-6	1.63	±	0.17	0.02	±	0.00	0.70	±	0.10	0.01	±	0.00	1.37	±	0.15	0.02	±	0.00
22:5n-3	8.51	±	0.78	0.12	±	0.01	6.30	±	0.99	0.15	±	0.01	7.39	±	1.04	0.14	±	0.01

22:6n-3	14.51	±	1.66	0.48	±	0.02	12.00	±	2.47	0.45	±	0.03	17.05	±	2.76	0.47	±	0.02
∑ SFA	36.32	±	3.55	1.05	±	0.03	21.00	±	3.82	0.99	±	0.04	29.29	±	3.91	1.01	±	0.03
∑MUFA	40.33	±	4.82	1.00	±	0.04	16.00	±	4.02	1.21	±	0.09	28.18	±	4.67	1.12	±	0.07
∑PUFA	48.43	±	4.12	1.45	±	0.04	32.00	±	5.35	1.30	±	0.06	43.46	±	5.38	1.39	±	0.05
∑ FA	125.09	±	12.25	3.49	±	0.10	69.00	±	12.89	3.49	±	0.15	100.93	±	13.84	3.52	±	0.12

DMA, dimethyl acetal; ARA, arachidonic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid, DHA, docosahexaenoic acid; SFA saturated fatty acid, MUFA monounsaturated fatty acid, PUFA polyunsaturated fatty acid, FA fatty acid

Table 6.2 Type I SS PERMANOVA of fatty acid assemblage data from *Heterodontus portusjacksoni* liver and muscle tested across locations (Spencer Gulf, New South Wales, and Gulf St Vincent), and accounting for total length and lipid percentage as covariates.

Source	df	SS	MS	Pseudo-F	P(perm)	CV
Liver						
Total length	1	69.6	69.6	34	< 0.001*	0.91
Lipid %	1	24.5	24.5	12	< 0.001*	0.71
Location	2	45.2	22.6	11	< 0.001*	0.96
Total length x lipid %	1	10.6	10.6	5.2	< 0.001*	0.29
Total length x location	2	8.1	4.1	2.0	0.018*	0.38
Lipid % x location	2	6.6	3.3	1.6	0.068	0.34
Total length x lipid % x location	2	7.9	4	1.9	0.015	0.36
Res	69	141.3	2			1.43
Total	80	313.9				
Muscle						
Total length	1	7.5	7.5	6.9	< 0.001*	0.28
Lipid %	1	8.2	8.2	7.5	< 0.001*	0.40
Location	2	39	19.5	17.9	< 0.001*	0.91
Total length x lipid %	1	6.8	6.8	6.2	0.005*	0.24
Total length x location	2	4.8	2.4	2.2	0.026*	0.31
Lipid % x location	2	2.2	1.1	1.0	0.397	0.05
Total length x lipid % x location	2	5.3	2.6	2.4	0.01*	0.32
Res	69	75.2	1.1			1.04
Total	80	149				

Table 6.3 Major contributors to the average squared Euclidean distance calculated using fatty acid (%) in the similarity percentage analysis between Spencer Gulf, New South Wales, and Gulf St Vincent. Oleic acid (18:1n-9, 18:1n-9), arachidonic acid (ARA, 20:4n-6), docosahexaenoic acid (DHA, 22:6n-3), palmitoleic acid (16:1n-7, 16:1n-7). *Values are higher in proportions but this is not reflected in concentrations.

		Spencer Gulf & New South Wales		New South Wales & Gulf St Vincent		Gulf St Vincent & Spencer Gulf	
		Liver	Muscle	Liver	Muscle	Liver	Muscle
Average squared distance		9.56%	4.97%	8.00%	4.21%	7.57%	3.20%
Drivers of dissimilarity		18:1n-9, ARA	ARA, DHA			18:1n-9, ARA, 20:1n-11, 16:1n-7	ARA
	Higher proportions in Spencer Gulf	DHA*	22:1n-11, 16:1n-7, EPA	ARA*, DHA*, EPA	22:1n-11, 16:1n-7, EPA		
	Higher proportions in New South Wales			18:1n-9, 16:1n-7, 22:4n-6 + 22:3n-3	ARA, DHA	DHA 18:0*, 22:4n-6 + 22:3n-3	22:1n-11, 16:1n-7,
	Higher proportions in Gulf St Vincent						
Contribution of drivers to average squared difference		34.57%	70.49%	59.10%	71.12%	66.69%	55.78%

6.4.4 Stomach content analysis

In total, 78 stomachs were dissected, 88.5% of which contained prey items and 66.6% of these stomachs contained identifiable prey items (Table 6.4, 6.5).The cumulative prey curves reached an asymptote when combining all locations and grouping prey items into broad taxonomic groups (Figure 6.4a), indicating that sufficient samples were collected to adequately characterise the diet of this species. However, the prey curves did not completely reach an asymptote when combining all locations with stomach content classified as the lowest possible taxonomic level (Figure 6.4b), or when separated by locations at a broad taxonomic level (Figure 6.4 c, d, e), suggesting that the sample size may not be sufficient to accurately characterise the diet of *H. portusjacksoni* within individual locations.

Table 6.4 Summary table of Port Jackson sharks *Heterodontus portusjacksoni* sampled and numbers of prey items.

Parameter	Gulf St Vincent	New South Wales	Spencer Gulf	Total
Stomachs dissected	25	22	31	78
Stomachs with prey item	20	21	28	69
Stomachs with identifiable prey item	11	19	22	52
Empty stomachs (%)	20	4.5	9.7	11.5

Number dissected = the numbers of Port Jackson sharks dissected; Stomachs with prey item = the numbers of stomachs with at least one prey item; Stomachs with identifiable prey item = the numbers of stomachs where prey could be identified to lowest taxonomic group; Prey items = the numbers of prey items found in stomachs; Empty stomachs = the proportion of stomachs that were empty or only contained mucous.

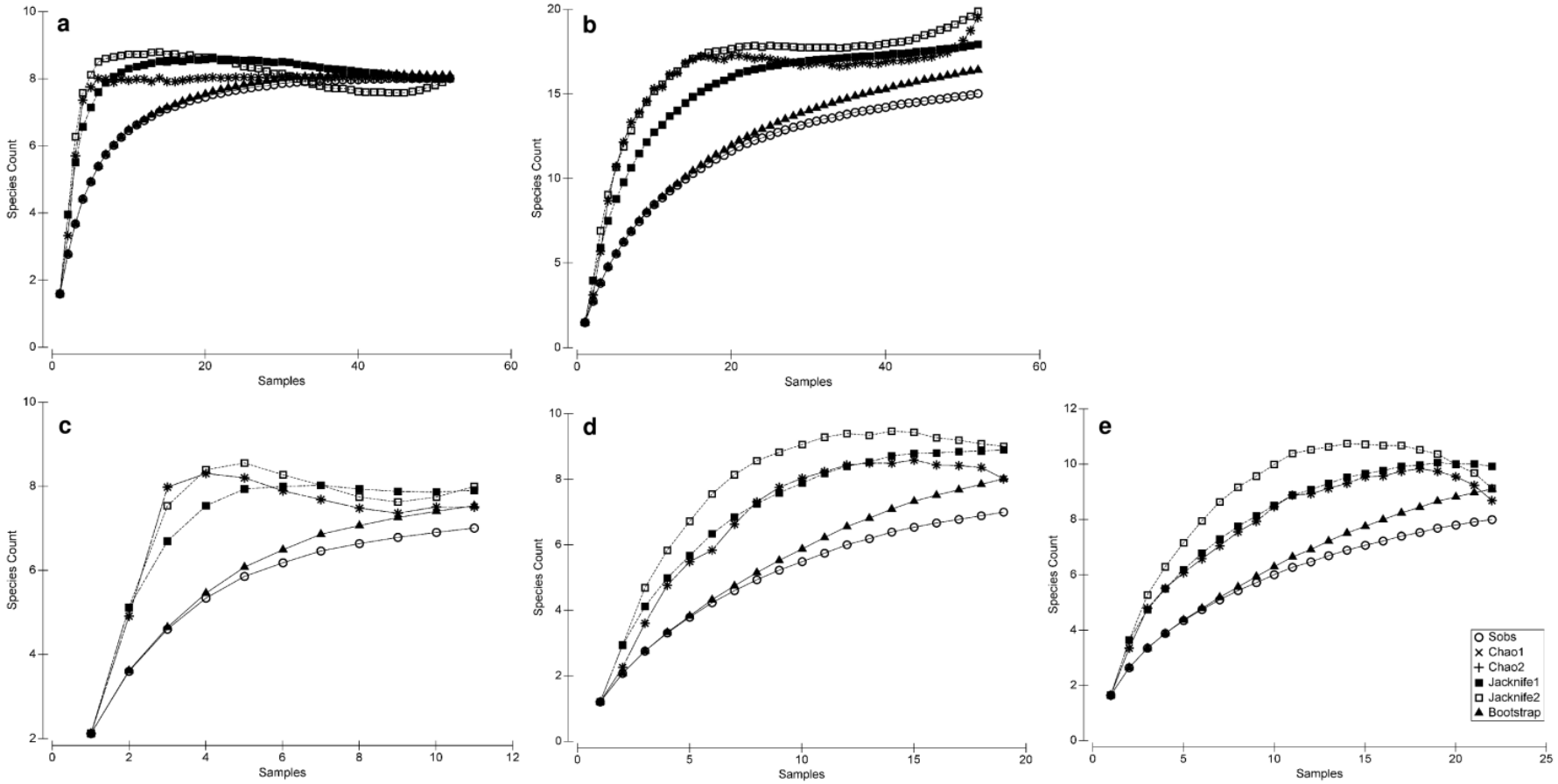


Figure 6.4 Randomized cumulative prey curve of Port Jackson sharks *Heterodontus portusjacksoni* (a) at a broad taxonomic level, (b) at the lowest possible taxonomic level (LPT), and at a broad taxonomic level in (c) Gulf St Vincent, (d) New South Wales and (e) Spencer Gulf.

Table 6.5 Frequency of occurrence (%FO) and gravimetric contribution (%W) of dietary items and dietary categories in stomach contents to the diet of *Heterodontus portusjacksoni* overall and by location sampled.

	%W				%FO			
	Combined	Spencer Gulf	Gulf St Vincent	New South Wales	Combined	Spencer Gulf	Gulf St Vincent	New South Wales
Algae	2.51	1.11	0.32	5.26	3.85	2.94	4.76	4.35
Nematoda	17.22	31.30	21.37	0.00	24.36	44.12	19.05	0.00
Nemertea	19.42	0.00	7.86	46.56	16.67	0.00	14.29	43.48
Arthropoda	21.82	36.77	3.33	16.78	17.95	26.47	4.76	17.39
Malacostraca	9.78	22.62	3.33	0.00	7.69	14.71	4.76	0.00
Crustacea	2.51	5.34	0.00	0.99	3.85	5.88	0.00	4.35
Decapoda	9.52	8.80	0.00	15.79	6.41	5.88	0.00	13.04
Annelida	17.01	12.23	36.91	10.53	15.38	11.76	28.57	8.70
Sipuncula	10.99	12.23	18.61	5.26	10.26	11.76	14.29	4.35
Polychaeta	6.03	0.00	18.30	5.26	5.13	0.00	14.29	4.35
Mollusca	8.33	9.88	19.29	0.35	10.26	5.88	19.05	8.70
Bivalvia/Gastropoda	0.35	0.00	0.96	0.35	3.85	0.00	4.76	8.70
Cephalopoda	5.12	5.00	14.20	0.00	3.85	2.94	9.52	0.00
Octopoda	0.91	0.00	4.14	0.00	1.28	0.00	4.76	0.00
Teuthida	1.95	4.88	0.00	0.00	1.28	2.94	0.00	0.00
Echinodermata	3.28	3.71	0.00	4.73	3.85	5.88	0.00	4.35
Asteroidea	1.48	3.71	0.00	0.00	2.56	5.88	0.00	0.00
Ophiuroidea	1.80	0.00	0.00	4.73	1.28	0.00	0.00	4.35
Teleostei	10.40	5.00	10.91	15.79	7.69	2.94	9.52	13.04

6.4.5 Spatial variability in stomach contents

At a broad taxonomic level, the PERMANOVA indicated that stomach contents (%W) were significantly different between locations (Table 6). No significant differences by sex were seen at either a broad taxonomic level or a LPT and sex was removed from further analysis (broad pseudo-F_1 = 1.16, P = 0.308, CV = -0.26, LPT pseudo-F_1 = 0.93, P = 0.496, CV = -3.22). At a broad taxonomic level, pair-wise tests indicated significant differences in sharks from Gulf St Vincent and New South Wales (t = 1.8, P = 0.041), and between New South Wales and Spencer Gulf (t = 2.0, P = 0.002). No significant differences, however, occurred between Gulf St Vincent and Spencer Gulf (t = 1.4, P = 0.114).

When diet was classified at a broad taxonomic level, location was successfully allocated 55.78% (Figure 6.5a) of the time and 67.3% for diet classified at LPT (Figure 6.5b). Sharks from New South Wales showed the highest level of allocation success by location (broad: 73.68%, LPT: 84.21%), followed by sharks from Spencer Gulf (Broad: 59.09%, LPT: 68.18%), and Gulf St Vincent (Broad: 18.18%, LPT: 36.36%). Stomach content was not significantly different at a broad taxonomic level, however, pair-wise tests indicated that stomach contents were significantly different at LPT between Spencer Gulf and New South Wales (t= 2.29, P < 0.001), and Gulf St Vincent and New South Wales (t = 1.60, P = 0.020), but not between Gulf St Vincent and Spencer Gulf (t = 1.12, P = 0.261).

Sharks from New South Wales and Spencer Gulf were the most dissimilar and this was driven by nemerteans, nematodes, prawns, crabs, fish, and sipunculans. The diet of sharks from Spencer Gulf was characterised by higher occurrence and contribution by weight of nematodes, sipunculans, crabs and other crustaceans (Table 6.5), while sharks from New South Wales had high levels of nemerteans, prawns and teleosts (Table 6.5).

Stomach contents from Gulf St Vincent sharks did not significantly differ from those caught off New South Wales and Spencer Gulf. Sharks from Gulf St Vincent were characterised by a more frequent occurrence and greater weight of nematodes, sipunculans, polychaetes, and cephalopods (Table 6.5) compared to sharks from New South Wales, which consumed more nemerteans, prawns and

fish (Table 6.5). Sharks from Gulf St Vincent were characterised by more frequent occurrence and a high contribution by weight of nemerteans, sipunculans, polychaetes, and cephalopods (Table 6.5), compared to sharks from Spencer Gulf which consumed more nematodes and crabs (Table 6.5).

Table 6.6 Type I SS PERMANOVA of stomach content assemblage data from *Heterodontus portusjacksoni* liver and muscle tested across locations (Spencer Gulf, New South Wales, and Gulf St Vincent), and accounting for total length as covariate.

Source	df	SS	MS	Pseudo-F	P(perm)	CV
LPT						
Total length	1	26863	26863	7.4	< 0.001*	21.13
Location	2	10918	5459	1.5	0.101	11.93
Total length x location	2	8731	4366	1.2	0.284	8.40
Res	46	168050	3653			60.44
Total	51	214570				
Broad						
Total length	1	19429	19429	7.0	0.002*	17.89
Location	2	15090	7545	2.7	0.010*	19.37
Total length x location	2	9891	4946	1.8	0.100	14.64
Res	46	128070	2784			52.77
Total	51	172480				

6.5 Discussion

The New South Wales coastal location and the two semi-enclosed estuarine environments sampled in South Australia are under the influence of vastly different oceanic processes. The gulfs in South Australia are shallow with mean depths of about 20 m and both experience high rates of evaporation with very little freshwater inflow into either gulf (Bryars and Havenhand, 2006; Nunes and Lennon, 1986). This is compared to much larger depth ranges in the central coast of New South Wales, where the continental shelf is relatively narrow and depths increase from the shoreline to 200 m in about 25 km (Middleton et al. 1996). In the gulfs, limited nutrient enrichment results in low levels of primary and

secondary production (Dimmlich et al. 2004). This is in stark contrast to the coastline of New South Wales which is periodically influenced by the East Australian Current driving nutrient rich waters towards the coast (Rendell and Pritchard, 1996). In addition to sporadic upwelling, a large number of fresh water outflows exist along the New South Wales coastline contributing to the nutrient load (Suthers et al. 2011). Phytoplankton blooms such as those observed on the south-east Australian continental shelf are likely to lead to increases in fatty acids that dominate in marine algae such as EPA and DHA (Sargent et al. 2002). In addition, estuarine plumes as seen along the New South Wales coastline are likely to result in increased terrestrial organic matter indicated by the higher proportion of long chain saturated fatty acids in sharks from New South Wales (Wai et al. 2011).

Although sharks from Spencer Gulf had higher concentrations of DHA than sharks from other regions, the relative proportions of DHA were the lowest. The high absolute amounts of DHA in Spencer Gulf sharks are likely a result of the higher total lipid levels in these sharks compared to those from New South Wales and Gulf St Vincent, rather than due to dietary differences. This higher proportion of DHA in sharks from New South Wales and Gulf St Vincent was supported by the high levels of fishes found in the stomachs from New South Wales and Gulf St Vincent, and with fishes being a major driver of the difference between regions. This reinforces that fatty acid proportions are more reflective of diet than absolute concentration values. Increased proportions of dinoflagellate markers such as DHA may be indicative of increased secondary or tertiary predation on dinoflagellates (Viso & Marty, 1993). Dinoflagellates are pelagic microalgae that make up the diet of filter feeders such as bivalves, which are then predated upon by scavengers such as snails and crabs or predators such as fish and prawns (Alfaro et al. 2006). Furthermore, sharks from New South Wales were expected to have higher DHA levels due to higher levels of productivity and river input in this location. Sharks from Gulf St Vincent were more similar to those from New South Wales than from Spencer Gulf and this is likely due to the size ranges sampled. Smaller sharks, such as those sampled in New South Wales, were characterised by higher proportions of DHA. Higher levels of DHA would be, however, expected in larger sharks, as ontogenetic shifts have previously been

demonstrated in *H. portusjacksoni* (McLaughlin & O'Gower 1971; Powter et al. 2010) and DHA indicates increasing predation at higher trophic levels such as fish (Viso & Marty, 1993). High proportions of DHA in smaller sharks may be the result of growth and development (Bell et al. 1995; Horrocks & Yeo 1999). Docosahexaenoic acid has also been shown to be more reflective of maternal egg yolk composition than of diet in neonate sharks (Beckmann et al. 2014b). These findings suggest that while DHA may be suitable indicators of diet in larger sharks, caution needs to be taken when interpreting immature specimens. The preferential retention of polyunsaturated fatty acids in immature individuals has been previously observed and may be linked to the demands of growth and development (Beckmann et al. 2014b).

Biomarkers of diatom consumption, such as EPA and 16:1n-7, are typically observed in coastal herbivorous species and indicate assimilation of benthic microalgae and plant materials associated with ingesting fine sediments (Wai et al. 2011, Graeve et al 1994, Viso & Marty 1993). Higher proportions and concentrations of EPA were seen in the liver and muscle of sharks from New South Wales compared to sharks from Spencer Gulf and Gulf St Vincent. Higher proportions and concentrations of 16:1n-7 were also observed in the muscle of sharks from New South Wales compared to the other locations. Similar to DHA, EPA is an essential fatty acid, which is dietary derived and is needed to satisfy the essential fatty acid requirements of marine fish (Sargent et al. 2002). Accumulation of 16:1n-7 may not, however, be solely derived from the diet and could be a result of elongation of 16:0 and may be continually elongated to 18:1n-7 (Tocher 2003.). Proportions of 16:0, the precursor to 16:1n-7 were similar in tissues analysed across all locations, as were proportions of 18:1n-7. This may suggest that the levels of non-dietary input were similar across all locations and elevated proportions of 16:1n-7 in the muscle and of sharks from New South Wales are reflective of diet. In the liver, 16:1n-7 was similar across all locations, and the use of 16:1n-7 as a biomarker is not as reliable due to its role in fatty acid synthesis and elongation (Bone & Roberts 1969; Hallgren & Larsson 1962; Malins 1968; Medzihradsky et al. 1992). Enhanced levels of diatom fatty acid have previously been observed in juvenile sharks (Wai et al. 2011). This has been explained by the consumption of polychaetes, which can biosynthesise and can

contain high levels of EPA (Wai et al. 2011). Analysis of the stomach content analysis is not supportive of this finding as higher levels of annelids were seen in sharks from Gulf St Vincent and Spencer Gulf than sharks from New South Wales. Sharks from New South Wales, however, consumed more nemerteans than in the other two locations, and most nemerteans are known to prey on polychaetes (McDermott & Roe 1985).

Higher proportions and concentrations of ARA were observed in the muscle of sharks from Spencer Gulf and Gulf St Vincent compared to New South Wales. ARA is only considered purely dietary derived when there is a deficiency in 18:2n-6 or if there is an inability to convert 18:1n-6 to ARA (Tocher 2003). 18:3n-3, the metabolic precursor of ARA and 18:2n-6, may be used as an indicator of this process (Sargent et al. 2002). In the present study, similar proportions of 18:3n-3 were seen in the liver and muscle of sharks from all locations and this suggests that non-dietary input had a negligible effect on the proportions of ARA. Higher proportions of ARA in the muscle of sharks may indicate increased levels of prey that feed on macroalgae, such as molluscs (Sargent & Whittle 1981; Wai et al. 2011). In particular, ARA has previously been used to distinguish omnivorous and carnivorous teleosts, and high levels of ARA have been reported in *H. portusjacksoni* as a result of feeding on macroalgae via predation on sea urchins and snails (Dalsgaard et al. 2003). Sharks from Gulf St Vincent had the highest levels of mollusc prey items, with high levels of cephalopods and octopus, however, Spencer Gulf had high levels of squid. Levels of bivalves/gastropods were low across all locations, occurring most frequently in New South Wales. This may indicate that molluscs are underrepresented in the stomach contents and higher levels of consumption may occur in sharks from Spencer Gulf than those from Gulf St Vincent and New South Wales. A high proportion of stomachs contained mucous, which might have been partially digested molluscan prey. Mucous was, however, excluded from the analysis as it was difficult to determine its origin.

Sharks from Spencer Gulf had higher levels of total lipid in the liver and larger total length than those from New South Wales. While lipid classes were not separated in this study, levels of triacylglyceride, which is used in energy storage,

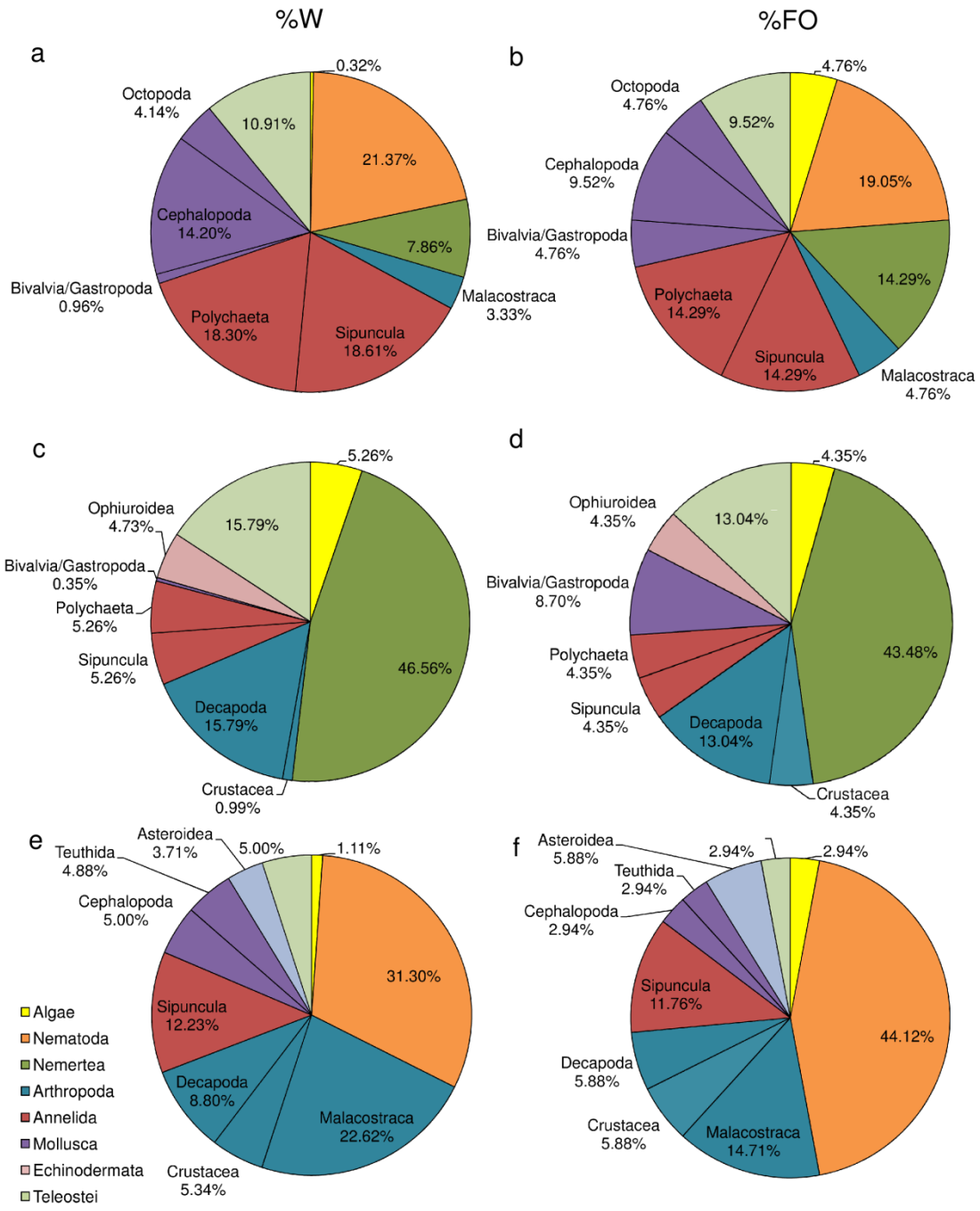
have been shown to increase with shark size due to accumulation and this may explain the high total lipid content from Spencer Gulf sharks (Phleger 1998, Pethybridge et al. 2010). Levels of 18:1n-9, a biomarker of omnivorous feeding on flagellates (Thompson et al. 1992, Viso and Marty, 1993) and the ratio of 18:1n:9/18:1n-7 is a biomarker of bacteria and omnivory (Stevens et al. 2004a,b), also increased with shark size. High levels of 18:1n-9 represent diatom consumption and have been associated with herbivory (Falk-Petersen et al. 2000). The presence of biomarkers of feeding low trophic levels, such as 16:1n-7, and biomarkers of feeding at high trophic levels, such as 18:1n-9, may suggest that *H. portusjacksoni* are scavengers. This is supported by Powter et al. (2010) who found that subadults fed on both demersal benthic prey and to a lesser extent of benthic infauna and epifauna compared to juveniles, which fed largely on the latter two groups. Previous controlled dietary studies have shown that 18:1n-9 accumulates in the liver of sharks fed high dietary concentrations (Beckmann et al. 2014a).

At a broad level of taxonomic classification, no differences in stomach content were identified between Spencer Gulf and Gulf St Vincent, however, using the fatty acid profiles of liver and muscle tissues, spatial differences were apparent. No differences were observed between locations in stomach content based on LPT and cumulative prey curves showed that stomach content based on LPT did not represent diet as accurately as when characterised using a broad taxonomic classification. Using stomach content alone at a broad taxonomic level, sharks were only allocated to their correct location 55.8% of the time, compared to 92.6% using liver fatty acids and 88.9% using muscle fatty acids. Spatial variability in the diet *of H. portusjacksoni* is not unusual and has previously been observed among eastern and south-western populations (Powter et al. 2010; Sommerville et al. 2011). The inability of stomach content analysis to distinguish dietary differences between locations might be due to the small sample size that may not have been sufficient to accurately characterise the diet of each location. Furthermore, Port Jackson sharks have grinding plates, which result in disarticulated and mostly unidentifiable stomach contents, hence limiting taxonomic resolution. A larger sample size may have resulted in identifiable differences between locations. Liver and muscle tissue fatty acid profiles may,

therefore, be a more suitable method of dietary differentiation analysis when a large number of animals cannot be collected for stomach content analysis.

6.6 Conclusion

The present study used fatty acids and stomach content analysis to investigate differences between locations in the diet of Port Jackson sharks. The dominant prey items observed in stomach contents were reflected in the fatty acid profiles of liver and muscle tissues. Although the diet of Port Jackson sharks did not differ between Spencer Gulf and Gulf St Vincent when grouped by broad taxonomic levels, the fatty acid profiles were significantly different between all locations. The liver fatty acid profiles showed the most accurate classification of sharks by diet, followed by muscle fatty acids and then stomach content (broad classification). This study demonstrated that fatty acid profile analysis of liver and muscle tissue can be complementary to stomach content analysis, and identified spatial differences that were undetected by stomach content analysis. Where high levels of taxonomic classification cannot be achieved, fatty acid analysis is an advantageous complimentary approach to stomach content analysis.



Supplementary Figure 6.1Stomach contents presented as a percentage of total weight (W%) and frequency occurrence (FO%) from Port Jackson sharks (*Heterodontus portusjacksoni)* sampled in Gulf St Vincent (a, b) Spencer Gulf (c, d) and New South Wales (e, f) grouped broadly with lowest possible taxonomic level values inset.

Chapter 7: General Discussion

7.1 Overview

Fatty acids have historically been used as qualitative markers to determine dietary relationships in the marine environment and more recently their use has been extended to identifying the key processes which impact ecosystem dynamics. One of the key issues of using fatty acids to infer diet is the relatively poor understanding of fatty acids incorporation rate in different tissues as this can vary between species. The objective of this thesis was to determine whether fatty acids reflected dietary change in Port Jackson sharks and this was investigated through controlled feeding experiments. While studies of chondrichthyan feeding ecology have increasingly used fatty acid profiles to investigate diet, feeding experiments in a controlled environment had never been performed to validate how the fatty acid profiles of chondrichthyans reflect diet.

Fatty acid profiles in Port Jackson sharks were shown in this thesis to reflect dietary patterns. The liver showed differences in fatty acid composition after 10 weeks of feeding while muscle took up to 18 weeks to show differences. Similarly, the blood serum free fatty acids showed dietary change after 12 weeks of feeding. Specific fatty acids, such as, DHA were shown to increase in the muscle following dietary change, however, the muscle fatty acids quickly reverted back to their characteristic profile.

The key findings of this study included:

- Confirmation of liver and muscle fatty acid profiles as indicators of recent dietary change, through comparison of control and experimentally fed sharks, and confirmation of liver fatty acid profiles as an indicator of dietary patterns after 10 weeks of feeding (Chapter 2);
- Tracking of fatty acid profiles over time in the muscle and liver, which identified potential fatty acid biomarkers, and metabolic pathways that influence fatty acid profiles during recent dietary change (Chapter 3);
- Demonstration of the maternal influence on liver fatty acid profiles in young-of-the-year (YOY) sharks and low levels of dietary influence on the muscle fatty acid profiles of hatched sharks (Chapter 4);

- Evidence of fluctuation in the levels of muscle fatty acids during 18 weeks of feeding, indicating that this tissue is more resistant to dietary change and takes longer to turn over than the liver and blood serum, which reflected dietary patterns within 12 weeks of feeding compared to 18 weeks in the muscle (Chapter 5); and
- Identification of high diet dissimilarity across large spatial scales, better diet discrimination based on fatty acid profiles compared to stomach content analysis, and the ability of fatty acid profiles to detect spatial and demographic differences that were undetected by stomach content analysis (Chapter 6).

7.2 Tissue-specific differences in fatty acid deposition

Controlled feeding experiments from 10 to 26 weeks in duration resulted in the collection of fatty acid profiles of 84 experimentally fed sharks, 10 shark egg yolk, and 85 wild shark fatty acid profiles. These experiments provided valuable baseline information on the effect of diet on fatty acid profiles in the liver, muscle, and blood serum. The benefits of fatty acid profile analysis as a complementary method include: the potential for less invasive sampling using the muscle and blood, higher levels of resolution than stomach content analysis when only small sample sizes are available, and the provision of detailed information on consumer diets with higher levels of complexity than are possible through other methods of analysis such as stomach contents and stable isotopes.

The high degree of affinity between the fatty acid profiles of shark liver and the diet fed to sharks was a key finding of the study. The liver fatty acid profiles of wild sharks and experimentally fed sharks showed significant differences after a recent dietary change, within three weeks. Significant differences were also observed between the liver fatty acid profiles of experimentally fed sharks, which had consumed different diets for a longer period of time, 10–18 weeks. Dietary proportions of vaccenic acid (VA, 18:1n-7), docosapentaenoic acid (DPA, 22:5n-3), arachidic acid (20:0), oleic acid (OLA, 18:1n-9), palmitic acid (PAM, 16:0), and docosahexaenoic acid (DHA, 22:6n-3)

were reflected in the liver of sharks fed squid and prawns. When sharks were fed artificial pellets treated with fish or poultry oil the dietary fatty acids which drove dissimilarity between feeding groups were linoleic acid 14:0, (LNA, 18:2n-6), eicopentaenoic acid (EPA, 20:5n-3), DHA, and OLA. In contrast to these findings, liver fatty acids have previously been predicted to show the most modification and highest difference to that of prey consumed and this was previously supported by high variability in liver FA profiles among sharks (McMeans et al. 2012). Despite the potential for fatty acid modification in the liver, monitoring sharks over time revealed the fatty acids that were the most reliable and stable indicators.

The energy stored in the liver is transported to other tissues such as the muscle, via the blood, to fuel a variety of metabolic functions (Ballantyne et al. 1993). Muscle fatty acid profiles could be used to identify a diet switch (chapter 3), but could not distinguish sharks fed different diets for 10 weeks (chapters 2). An extended experiment, however, revealed that muscle fatty acid profiles could differentiate sharks feeding on different diets following 18 weeks of controlled feeding (chapter 5). Muscle fatty acid profiles were also useful to distinguish wild sharks from different locations (chapter 6), demonstrating that dietary fatty acids are in fact incorporated into the muscle. Rapid mediation of fatty acids to the muscle (as seen in chapter 3) is supported by Pethybridge et al. (2011) who suggests that the polyunsaturated fatty acids, which characterise muscle tissue, are preferentially diverted to the muscle to meet physiological requirements. Muscle fatty acid profiles showed a high amount of variability and the longest turnover time, as evidenced by significant differences in FO- and PO-fed shark muscle within 12 weeks of feeding, while significant differences were seen in the blood serum and liver FA profiles within 12 weeks of feeding. After 18 weeks of feeding, significant differences in the muscle FA profiles of FO- and PO-fed sharks were driven by proportions of LNA and EPA. The other fatty acids that drove dissimilarity between diets, such as DHA showed high degrees of variation between individual sharks, resulting in less difference between the fatty acid profiles of sharks fed the different diets. Furthermore, fatty acids which were present in relatively low proportions in the diet such as 20:4n-6 and 22:5n-3 also drove dissimilarity between feeding groups. This may suggest that fluctuations in fatty acid levels may not be as a result of diet but a result of physiological

processes and/or the metabolism of dietary fatty acids. Previously, high levels of variability have been observed in the muscle of wild sharks and this was suggested to be a result of dietary differences among individuals (McMeans et al. 2012). Here, high levels of variability in muscle fatty acid profiles occurred among individuals which were feeding on the same diet, suggesting that the muscle is a dynamic tissue and natural variability occurs independent of diet. In addition to dietary input, levels of fatty acids in the muscle could be influenced by their involvement in the maintenance of the structures and functions of phospholipid cell membranes and their role as precursors to the paracine hormones known as eicosanoids (Sargent et al. 1999). While sampling of the muscle is advantageous as the method is not lethal, the timescales of integration make interpretations surrounding this tissue difficult.

In chondrichthyans, the profiles of blood serum FA have not previously been reported in a controlled setting and are an important component to take into consideration due to the role of blood in FA transport to tissues for oxidation (Henderson and Tocher 1987). Controlled feeding experiments demonstrated that dietary inputs of 14:0. EPA, OLA, and LNA were reflected in the blood serum of sharks. Similar to the liver, blood serum fatty acid profiles of sharks fed different diets were distinguishable within 12 weeks of feeding. This is supported by a previous study which suggested the blood serum fatty acids represent mobilised fatty acids from the liver (Semeniuk et al. 2007). In contrast to this, blood serum fatty acids have been reported as more similar to muscle than to liver fatty acids (McMeans et al. 2012). These differences between species are likely a result of the varying levels of reliance fatty acid transport in the blood from fat depots such as the liver to various tissues including the muscle. Blood serum fatty acids have, however, been demonstrated as a promising non-lethal method for examining long-term dietary patterns, such as anthropogenically induced diet shifts (Semeniuk et al. 2007). Unlike the collection of body tissue fatty acids, the collection of blood serum requires some technical knowledge to extract the blood from the caudal vein. This may restrict the number of samples which can be obtained by researchers. Furthermore, in a controlled situation, the amount of time since the animal was last fed can be controlled. This is likely to be more variable

in wild animals and may result in further fluctuations as fatty acids are transported around the body.

7.3 Maternal inputs

The fatty acid profile of any organism, including sharks, is dependent on its original fatty acid composition and the cumulative intake of dietary fatty acids, but is also affected by various metabolic processes associated with growth and maturation (Robin et al. 2003). Consequently, the interpretation of fatty acid profiles can be hampered by these factors and controlled experiments are required to investigate which fatty acids are preferentially retained through the various metabolic processes. The liver and muscle fatty acid profiles of young-of-the-year (YOY) sharks were compared to egg yolks. Maternal input was high in the liver, which was more similar to egg yolks than to diet, while some indications of dietary input were detected in the muscle, which was more similar to diet than to egg yolks. Specifically, arachidonic acid (ARA, 20:4n-6) was preferentially retained likely as a result of eicosanoid production during growth, while dietary DPA was not reflected in shark tissues and is likely catabolised for energy.

Docosahexaenoic acid (DHA, 22:6n-3) was shown to accumulate in the liver, to higher levels than seen in egg yolks alone, suggested some influence of the diet on the fatty acids profiles of young sharks. This is likely due to the role of DHA in developing visual and neural tissues, which account for a relatively great proportion of total body mass in larval stages (Sargent et al. 2002). High proportions of saturated fatty acids were retained in the muscle at the expense of dietary polyunsaturated fatty acids, making the muscle an unsuitable indicator of diet in these sharks. These differences in the fatty acid profiles reflect the influence of maternal fatty acid input on YOY sharks. Combine this with rapid growth and development and the fatty acid profiles of YOY sharks are very difficult to relate to diet and are more likely reflective of maternal inputs and indicative of limited foraging ability during this time. High levels of maternal investment have also been observed in deepwater chondrichthyans (Pethybridge et al. 2011b) and YOY bull sharks (Belicka et al. 2012). This suggests that the

composition of neonatal animals needs to be interpreted cautiously as after 185 days of controlled feeding, sharks tissues were still highly reflective of maternal fatty acid composition and not diet.

7.4 Ecological applications

Fatty acid analysis is a powerful tool increasingly used in studies of trophic ecology in marine ecosystems. This biochemical technique has become useful in deciphering spatial and temporal variability in diets, identifying predation on key species, and in some cases providing quantitative dietary information. Such quantitative information about dietary composition is extremely valuable, but requires controlled feeding experiments to determine the underlying factors affecting fatty acid metabolism.

Prior to this study, significant gaps existed in the basic information required to assess chondrichthyan feeding ecology using fatty acid profiles. Using the knowledge gained from controlled feeding studies the fatty acid profiles of liver and muscle tissue were compared to stomach contents of sharks collected from three locations. Large-scale (1,200–1,400 km) spatial variations were apparent in fatty acid profiles and the same level of difference was not observed utilising the stomach content. Fatty acid profiles which showed the highest allocation success to region (CAP analysis) at 92.6% using liver fatty acids profiles and 88.9% using muscle fatty acid profiles, compared to only 55.8% success achieved using stomach contents alone. Large-scale spatial variations were supported by previous reports of stomach content analysis that showed differences among eastern and south-western populations (Powter et al. 2010; Sommerville et al. 2011). The inability of stomach content analysis to distinguish dietary differences between regions might be due to the small sample size that may not have been sufficient to accurately characterise the diet of each region. A larger sample size may have resulted in identifiable differences between the two regions. Liver and muscle tissue fatty acid profiles may, therefore, be a more suitable method of dietary differentiation analysis when a large number of animals cannot be collected for stomach content analysis.

The spatial trends in fatty acid profiles suggests that *H. portusjacksoni* are either responding to differences in prey availability or that their prey selectivity differ between locations (Sommerville et al. 2011). Sharks from NSW had high levels of teleosts compared to the other two regions, which was supported by the accumulation of DHA, a biomarker of dinoflagellate-based food webs, in the liver tissue. Higher levels of fish consumption in NSW and GSV than in SG was also indicated by higher levels of 22:1n-11, which is a biomarker of marine copepods transferred to planktivores through the food web (Kirsch et al. 2000; Sargent et al. 1987). Fatty acid biomarkers of herbivory such as ARA were higher in sharks from SG and GSV compared to NSW. This may indicate increased levels of predation on molluscs, which feed on macroalgae (Sargent and Whittle 1981; Wai et al. 2011), however, molluscs were underrepresented in stomach contents likely due to the rapid digestion of soft-bodied tissues. This study demonstrated that using stomach contents alone does not provide a complete picture of diet due to the high number of empty stomachs and disarticulated and often unidentifiable nature of the prey items that were recovered. Recent studies suggest chondrichthyans exhibit complex feeding ecology which may not be detected without the use of complimentary techniques including fatty acid profile analysis (Belicka et al. 2012; Couturier et al. 2013; McMeans et al. 2012, 2103; Pethybridge et al. 2011a, b; Schaufler et al. 2005; Semeniuk et al. 2007; Wai et al. 2011).

Demographic differences were also detected by fatty acid profile analysis but not through stomach content analysis. Sub-adult sharks and sharks from SG had an elevated 18:1n-9/18:1n-7 ratio in liver tissue suggesting increasing levels of carnivory (El-Sabaawi et al. 2009; Stevens et al. 2004a, b). This suggests an ontogenetic shift from immature sharks to maturing sub-adults. Variations in diet have been linked to changing dentition and mouth morphology and may also be explained by changing movement patterns as shark's mature (McLaughlin & O'Gower 1971; Powter et al. 2010). These findings highlight the importance of a wide variety of size classes in dietary studies.

This study demonstrated that FA profile analysis of liver and muscle tissue can reduce some of the biases in stomach content analysis, and identified spatial

and demographic differences that were undetected by stomach content analysis. While it is difficult to determine diet to species level in generalist feeders with high levels of unidentifiable prey items, trophic level differences and differences at a broad taxonomic level were apparent.

7.5 Future directions

The interpretation of fatty acid profiles relies upon an understanding of the ecological, physiological, and biochemical mechanisms that affect them. While the results of this study indicate that fatty acid profiles are a valuable tool for studying dietary patterns in chondrichthyans, further experimentation is still required. Firstly, differences in locomotory mode and phylogeny may contribute to differences in lipid classes and fatty acid profiles among shark species (Pethybridge et al. 2010). For example, fast-swimming pelagic sharks require different levels of energy to be supplied to their muscle than the less-active benthic species. There are also some suggestions that large free-swimming sharks use ketone bodies rather than fatty acids to provide energy for aerobic tissues (Watson and Dickson 2001). While large, free-swimming sharks are difficult to maintain in captivity, a range of species from different habitats, with different activity levels, and life histories should be examined and compared.

Controlled experiments in teleost fish have also demonstrated the effects of environmental parameters on fatty acid profiles. Temperature has been shown to interact with diet to affect fatty acid composition in fish (Person-Le Ruyet et al. 2004). It could, therefore, be difficult to relate fatty acid composition to diet without a knowledge of potential migrations or residency periods driven by temperature. Specifically, high amounts of PUFA have been linked with low temperatures and the resulting adaptation and maintenance of cellular membranes (Bell et al. 1986; Cowey and Sargent 1977; Dalsgaard et al. 2003; Farkas et al. 1994). Studies have also suggested that temperature affects levels of food intake, metabolic rates, and the digestibility and nutrititive value of lipid sources (Bureau et al. 2002). Further feeding experiments are required to determine the effects of environmental variables on chondrichthyan fatty acid profiles.

More detailed information should also be obtained surrounding the turnover of specific fatty acids. The influence of differential deposition, modification, utilisation, and *de novo* synthesis of individual fatty acids can affect the overall fatty acid composition of fat stores. The relative importance of these processes varies with the fatty acid composition and fat content of the diet. In fish and seals, radiolabelled carbons have been used to describe linear changes in fatty acids over time and demonstrate the uptake and clearance of specific FAs within cellular structures (Bell et al. 2002; Budge et al. 2004; Cooper et al. 2006). Radiolabelling can be used to directly investigate the modification and deposition of specific fatty acids, providing insight into the underlying biochemical processes and can be used to understand the effects of variation in diet on these factors (Budge et al. 2004). This information can then be applied to quantitative models, which require calibration coefficients to account for consumer metabolism, and provide estimates of the proportions of prey species in the diets of individual predators using fatty acid profiles (Iverson et al. 2004). Quantifying the relative abundance of the specific prey items using these models is desirable to detect the effect of changes in potential prey or on consumers which may be relevant in management and conservation particularly of vulnerable species. As calibration coefficients are not currently available for any chondrichthyan species, such methods cannot yet be used in chondrichthyan ecology. Even with calibration coefficients, quantitative methods will require further development as calibration coefficients may differ by family, species, and prey type (Rosen and Tollit 2012). This should not discourage researchers from using fatty acid profiles in dietary studies, but should re-enforce the need to use multiple complimentary methods such as stomach contents and stable isotopes.

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