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Lipid Metabolites as Energy Stores in Four Stingray Species

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LIPID METABOLITES AS ENERGY STORES IN FOUR STINGRAY SPECIES

by

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(Under the Direction of Christine N. Bedore)

ABSTRACT

Assessing macronutrient transfer is important for estimating ecosystem health and structure. This nutrient transfer is facilitated through trophic position interactions and the consumption of biomass. Lipids are macronutrients that can be used to assess energy flow. Triglyceride (TAG) and free fatty acids (FFA) are important lipids that are obtained from diet and integrate into tissues. They are representative of energy stores and potential energy available for metabolic processes. In marine ecosystems, stingrays occupy the mesopredator niche, facilitating nutrient transfer from lower to higher trophic positions. Stingrays consume a variety of prey items ranging in lipid content, but how lipid metabolites compare between batoid tissues and across species is poorly understood. This study aims to determine tissue-specific and species-specific differences in TAG and FFA in liver, plasma, and muscle tissues of four stingray species. Liver, muscle, and plasma samples were collected from butterfly rays (*Gymnura lessae*), Atlantic stingrays (*Hypanus sabinus*), bluntnose stingrays (*Hypanus say*), and southern bullnose rays (*Myliobatis freminvillii*) from the Northwest Atlantic. Tissue concentrations of TAG and FFA were quantified using colorimetric assays and analyzed using a linear mixed-effects model. Overall, liver had higher TAG and FFA concentrations than plasma and muscle. However, bullnose ray and Atlantic stingray muscle TAG and FFA were not significantly different from liver. Butterfly rays had significantly greater liver TAG than Atlantic and bluntnose stingrays. Bullnose rays had significantly greater muscle TAG and FFA than all three species. The butterfly

rays' liver TAG content may be attributed to their diet since they primarily consume teleosts. Bullnose rays' muscle TAG and FFA are unusual and whether muscle has the capacity for lipid oxidation or is an alternative lipid storage tissue should be further researched. Results from this study can be used as to further understand energy flow through trophic positions.

INDEX WORDS: Stingray, Mesopredator, Triglycerides, Free fatty acids, Liver, Plasma, Muscle, Trophic position

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by

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by

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DEDICATION

To the southern bullnose, butterfly, Atlantic, and bluntnose stingrays

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CHAPTER 1

INTRODUCTION

Understanding nutrient transfer through ecosystems is a useful indicator for assessing energy flow and ultimately ecosystem function, structure, and health (Parrish, 2013; Lu *et al*., 2015). Nutrient transfer through trophic positions is facilitated when organisms from one trophic position consume organisms from another and obtain energy that was stored as biomass. These food-web interactions form complex links between producers and consumers (Doughty *et al*., 2016; McDonald-Madden *et al.*, 2016). Nutrient uptake and transfer are complex processes involving many factors not limited to trophic position, trophic position interactions, and energy metabolism (Rombouts *et al.*, 2013; Welti *et al*., 2017; Degerman *et al*., 2018; Williams *et al.*, 2018). The efficiency of this transfer determines how much energy in the form of biomass moves to higher trophic positions (Lefébure *et al.*, 2013). Previous research has focused on how apex predators regulate lower trophic positions (Ripple *et al.*, 2014; Myers *et al*., 2017; Feit *et al*., 2019) and the effect of how primary producers input energy into an ecosystem (Iverson, 1990). However, fewer studies have focused on mid-trophic position predators or mesopredators which are integral for nutrient transfer in many ecosystems.

Mesopredators are small to mid-sized predators that are important to ecosystems because they facilitate structure, dynamics, and energy flow (Tambling *et al*., 2018). Mesopredators consume prey species in lower trophic positions and are also eaten by apex predators (Ritchie and Johnson, 2009). They regulate smaller prey species populations that are not consumed by apex predators (Nishijima *et al.*, 2014). In this way, mesopredators act as fulcrums between upper and lower trophic positions making them species of interest when investigating nutrient transfer through ecosystems. To assess nutrient transfer from the mesopredator trophic position,

there needs to be information about how these species metabolize and store energy. Lipids are energy dense and long-term energy sources involved in many biological processes making them the ideal macronutrient to investigate energy metabolism in context of energy flow through ecosystems.

Lipids are macronutrients and major sources of metabolic fuel that many taxa need to survive. Lipids can provide up to and exceeding two times more energy per gram than carbohydrates or proteins (Parrish, 2013; Parzanini *et al.*, 2018). Many vertebrate taxa rely on carbohydrates (sugars and starches) as the main and immediate fuel source for reasons including the speed at which adenosine triphosphate (ATP) is produced from glycolysis and cellular respiration, their solubility in water, and their generally quick availability for physiological work, such as exercise (Weber, 2010). In comparison with lipids, stored carbohydrates have lower energy density and are short term energy stores while lipids are long term energy stores (Weber, 2010). Carbohydrate stores (glycogen) are depleted during intense exercise or stress (Vijayan and Moon, 2011). When animals lack carbohydrates in their diet or enter a fasting state, they will start metabolizing proteins and fatty acids for energy (Puchalska and Crawford, 2017). Fatty acids can be metabolized extrahepatically in tissues like muscle in some species but can also be metabolized into ketone bodies for fuel (Puchalska and Crawford, 2017).

Triglycerides or triacylglycerols (TAG) are composed of three fatty acid chains esterified to a glycerol molecule and function as major energy storage units (Budge *et al*., 2006). They are acquired either through diet or synthesized in the liver. Triglycerides are transported in plasma and can be stored in adipose, liver, and muscle tissues (Zammit and Newsholme, 1979; Tocher, 2003). Many taxa use TAG as energy stores to fuel migrations and other energetically costly functions such as ontogenetic processes, reproduction, gametogenesis, and vitellogenesis

(Garcia-Garrido *et al.*, 1990; Norton *et al*., 2001; Pethybridge *et al*., 2014). Triglycerides are also metabolized during times of food deprivation (Alkanani *et al*, 2005). When TAG are mobilized for physiological energy they are metabolized into free fatty acids (FFA) via lipid oxidation (McClelland, 2004).

Free fatty acids are saturated and unsaturated fatty acid chains not esterified to glycerol and are either oxidized into ketone bodies for ATP production or are synthesized to make TAG via lipogenesis (Larsson and Fänge, 1977; McClelland, 2004; Gallagher, 2017). Free fatty acids are acquired through diet but are also synthesized *de novo* from carbohydrates or proteins (Budge *et al*., 2006). Many taxa use FFA in muscle for routine movements and activities, such as sustained swimming and recovery from exercise (John *et al.*, 1988; Tocher, 2003; Li *et al.*, 2015), as well as an energy source during times of limited prey availability (Cherel *et al.*, 1992; Simpkins *et al.*, 2003). Levels of high plasma FFA may indicate depleted energy stores and increased energetic demand (Alkanani *et al.*, 2005).

Lipid metabolism in mammals

The liver is an important organ concerning lipid metabolism because it regulates lipid homeostasis via storage, beta-oxidation, and lipogenesis (Figure 1). The liver is a lipid depot that mainly stores fatty acids as TAG from dietary and endogenous fatty acids (Alves-Bezerra and Cohen, 2019). Beta-oxidation also occurs in the liver and is the breakdown of TAG into FFA (via lipolysis) and acetyl-CoA which then undergo more reactions to form ketone bodies. The liver is also capable of creating fatty acids *de novo* using lipogenesis (Harlan *et al.*, 1963; Alvarez *et al*., 2000). Lipogenesis is the conversion of carbohydrates, such as glucose, or proteins into fatty acids. These intrinsic functions help regulate TAG and FFA stores intra and extrahepatically. The liver regulates TAG and FFA concentrations depending on whether an

animal is feeding or fasting (Simpkins *et al.*, 2003; Wang *et al*., 2018). When feeding, FFA are converted to TAG for energy storage and this process reverses during the fasting state (Cherel *et al.*, 1992). Other processes that directly affect liver TAG and FFA concentrations are growth, reproductive status, movement ecology, hibernation, and extrinsic factors such as temperature (Shen and Gao, 2005; Gallagher *et al.*, 2014).

Plasma is another important tissue to consider when discussing lipid metabolism as it is the main transport tissue. Plasma distributes TAG, FFA, and other lipids using carrier proteins, such as albumin and low-density lipoproteins, to the liver or adipose tissue after digestion and absorption. Once TAG have undergone beta-oxidation to form free fatty acids, they are transported in plasma to tissues like muscle for energy (Figure 1). Consequently, the plasma TAG lipolysis can also occur using clearing factor lipase, which removes the TAG from the bloodstream and into extrahepatic tissues as FFA (Robinson,1973). Plasma concentrations of TAG and fatty acids fluctuate based on the timing and frequency of feeding events and the lipid content of prey items (Wood *et al.,* 2010). Plasma fatty acids and TAG increase post-feeding events, but plasma TAG and FFA also increase during times of low food availability (Wood *et al*., 2010; Jenkins *et al*., 2019)

Skeletal muscle is one of the largest organs and expends energy to power movement and maintain stability (Davison and Goldspick, 1984; Holloway *et al.*, 2010). Red muscle is a slow-twitch, aerobic tissue used for sustained movements such as routine swimming. In contrast, white muscle is made of fast-twitch, anaerobic fibers used for sudden burst activity like burst swimming to avoid predators or catch prey. White muscle relies on stored carbohydrates like glycogen and fats, such as triglycerides, for recovery. Muscles mobilize fatty acids based on metabolic requirement as an alternative fuel source to carbohydrates and proteins. Muscle tissue

obtains TAG and FFA from liver through plasma transport (Holloway *et al.*, 2010). In some instances, muscle also functions as a lipid storage tissue when the rate of fatty acid uptake surpasses the rate of beta-oxidation (Figure 1). Muscle can also store TAG in the form of droplets as smaller energy depots (Shen and Gao, 2005; Görgün and Akpinar, 2007). Some taxa store fatty acids in their muscle as an immediate energy source (Sheridan, 1994; Zhol *et al*., 1995).

Lipid metabolism in elasmobranchs

Elasmobranchs (sharks, skates, and rays) are a subclass of Chondrichthyes, ancient cartilaginous fishes that appeared 420 million years ago (Grogan and Lund, 2004). They developed an atypical lipid metabolism strategy that differs greatly from other vertebrate taxa including teleosts. Elasmobranchs store a range of lipid classes in their livers as opposed to adipose tissue to sustain energy stores and maintain buoyancy (Sargent *et al.,* 1971; Zammit and Newsholme, 1979; Phleger, 1998; Davidson *et al*., 2014) (Figure 2). In addition, elasmobranchs lack albumin, a common lipid transport protein, and instead transport their lipids in plasma using low density lipoproteins and converting fatty acids to ketone bodies, which are water-soluble (Lauter *et al.*, 1968; Metcalf and Gemmell, 2005) (Figure 2). Additionally, elasmobranchs have a limited capacity for beta-oxidation in both red and white muscle (Speers-Roesch and Treberg, 2006). Instead, elasmobranchs use ketone bodies in red muscle for aerobic activity and 3-hydroxyacyl CoA dehydrogenase (an enzyme involved in ketosis) has also been found in white muscle (Zammit and Newsholme, 1979; Watson and Dickson, 2001; Speers-Roesch *et al*., 2006).

Given that elasmobranchs store lipids primarily in their livers, transport them in plasma, and mobilize lipid derivatives in muscle, research has focused on these tissue types (including the presence and concentrations of TAG and FFA) for insights into how lipid content relates to

elasmobranch ecology. In great white sharks (*Carcharodon carcharias*), TAG concentrations were observed to be greater than 93% of the total lipid content in the liver, which reflects this species' high fat diet to fuel long migrations (Pethybridge *et al.*, 2014). Other studies have focused on plasma TAG and FFA since species transport lipid metabolites in their plasma on a requirement basis. Shark plasma TAG and FFA concentrations in sharks are influenced by diet, ontogeny, sex, seasonality, and activity levels (Beckmann *et al*., 2014; Valls *et al*., 2016; Gallagher *et al*., 2017). In muscle, TAG concentrations are low, however, fatty acids show changes in diet over time (Beckmann *et al*., 2014; Pethybridge *et al*., 2014).

Previous research on TAG and FFA in elasmobranchs has focused primarily on sharks with few studies assessing lipid metabolism in stingrays. Batoids (rays and skates) are a diverse group of dorsally-ventrally compressed cartilaginous fishes with elongated pectoral fins that are fused to their heads. They interact heavily with the benthos (with a few exceptions) when foraging for invertebrates or small teleost fishes and rely primarily on undulation and oscillation swimming modes (Fish and Hoffman, 2015). Stingrays fulfill the mesopredators niche in many marine ecosystems acting as fulcrums between higher and lower trophic positions, thus facilitating energy flow (Stevens *et al.*, 2000; Bornatowski *et al.*, 2014; Navia *et al.*, 2016). Despite the presence of several stingray species in several marine ecosystems, their energetic requirements and constraints are not well-understood due to the majority of studies focusing on the large, apex predator shark species.

Study species

This study aims to investigate the use of lipid metabolites as energy stores in stingrays. Four different species were chosen to encompass a range of diets and movement ecologies seen in this group of elasmobranchs. All inhabit the same coastal region of the Northwestern Atlantic,

but each occupy a unique niche. Butterfly rays (*Gymnura lessae*) are a demersal species that mainly consume teleosts and have intermittent feeding/digestion (Yokota and Carvalho, 2017). Atlantic stingrays (*Hypanus sabinus*) are a sedentary, benthic species that feed on invertebrates (polychaetes, clams, shrimp, tube anemones, serpent stars, and small crustaceans) and some teleosts (Robins and Ray, 1986). Bluntnose stingrays (*Hypanus say*) are also coastal, benthic, and sedentary with diets consisting of shrimp and teleosts (Compagno, 1999). Bullnose rays (*Myliobatis freminvillii*) are benthopelagic coastal species that migrate and consume bivalves and gastropods (Szczepanski and Bengston, 2014). Since teleosts (generally higher in lipid content than marine invertebrates) form a large portion of butterfly rays' diet and Atlantic stingrays, bluntnose stingrays, and bullnose rays mainly consume invertebrates, differences in lipid content may contribute to increased lipid concentrations in tissue (Table 2) (Wilder *et al*., 2019; Diaz Gomez *et al.*, 2020).

Research objectives, hypotheses, and predictions

To further understand lipid metabolism in batoid fishes, my thesis aims to quantify triglyceride and free fatty acid concentrations in the liver, plasma, and muscle tissues of four stingray species: butterfly rays, Atlantic stingrays, bluntnose stingrays, and southern bullnose rays.

Hypothesis I: Triglyceride and FFA concentrations will vary between tissue types within a species based on tissue function.

Predictions:

1. Liver will have the highest TAG and FFA concentrations amongst all three tissue types because it is the main lipid storage tissue in elasmobranchs.

2. Plasma TAG and FFA concentrations will be low since elasmobranchs utilize ketone bodies instead of FFA for fuel.
3. Muscle TAG and FFA will be lower than liver, but higher than plasma since it is a metabolically active tissue.

Hypothesis II: TAG and FFA concentrations of each tissue type will vary between species.

Predictions:

1. Triglyceride and FFA concentrations will vary between species based on the different lipid content in diets.
2. Butterfly rays will have the highest TAG and FFA content due to their high-lipid content diet of teleosts.
3. Bullnose ray, Atlantic stingray, and bluntnose stingray will have similar TAG and FFA because they mainly consume invertebrates.

Table 1: Diet and swimming ecology of four stingray species: Butterfly ray, Atlantic stingray, bluntnose stingray, and southern bullnose ray.

Family	Species	Species Outline (Dorsal View)	Diet	Swimming Ecology
Gymnuridae	Butterfly ray (*Gymnura lessae*)		Teleosts	Demersal
Dasyatidae	Atlantic stingray (*Hypanus sabinus*)		Bivalves, crustaceans, isopods, and polychaetes	Benthic
Dasyatidae	Bluntnose stingray (*Hypanus say*)		Bivalves, crustaceans, polychaetes, and teleosts	Benthic
Myliobatidae	Southern bullnose ray (*Myliobatis freminvillii*)		Gastropods, crustaceans, and bivalves	Benthopelagic

Table 2: Foraging species lipid content (% lipid by wet weight).

Location	Group	Species	Total Lipid Content % (wet mass)
Northwest Atlantic (Nova Scotia)*	Teleosts	Atlantic cod	2.6
		Sand lance	2.9
		American plaice	3
		Arctic cod	3.7
		Daubed shanny	5
		Redfish	6
		Greenland halibut	7.5
		Capelin	13.7
		Atlantic herring	13.7
	Crustaceans	Northern shrimp	3.6
	Cephalopods	Squid (*Illex*)	6.6
		Squid (*Gonatus*)	10.9
Various**	Bivalves	Clams	0.5–5.0
		Scallops	0.6–2.8
		Mussels	1.0–3.0
		Oysters	3.3–6.7
	Gastropods	Cockles	1.6–1.9
Prince William's Sound, Alaska***	Teleosts	Righteye flounders	0.8–1.9
		Codfishes	0.8–5.1
		Sculpins	1.3–1.5
		Greenlings	1.3–4.4
		Salmonids	1.4-3.8
		Sand lances	1.5–5.2
		Sablefishes	2.6
		Rockfishes	3
		Herrings	3.5–14.2
		Smelts	1.4–19.0
	Crustaceans	Shrimp	0.9–1.7
	Cephalopods	Octopus	1.1–1.6
		Squid	1.6–8.0

*(Lawson *et al*., 1998), **(Tan *et al.*, 2020),***(Iverson *et al*., 2002)

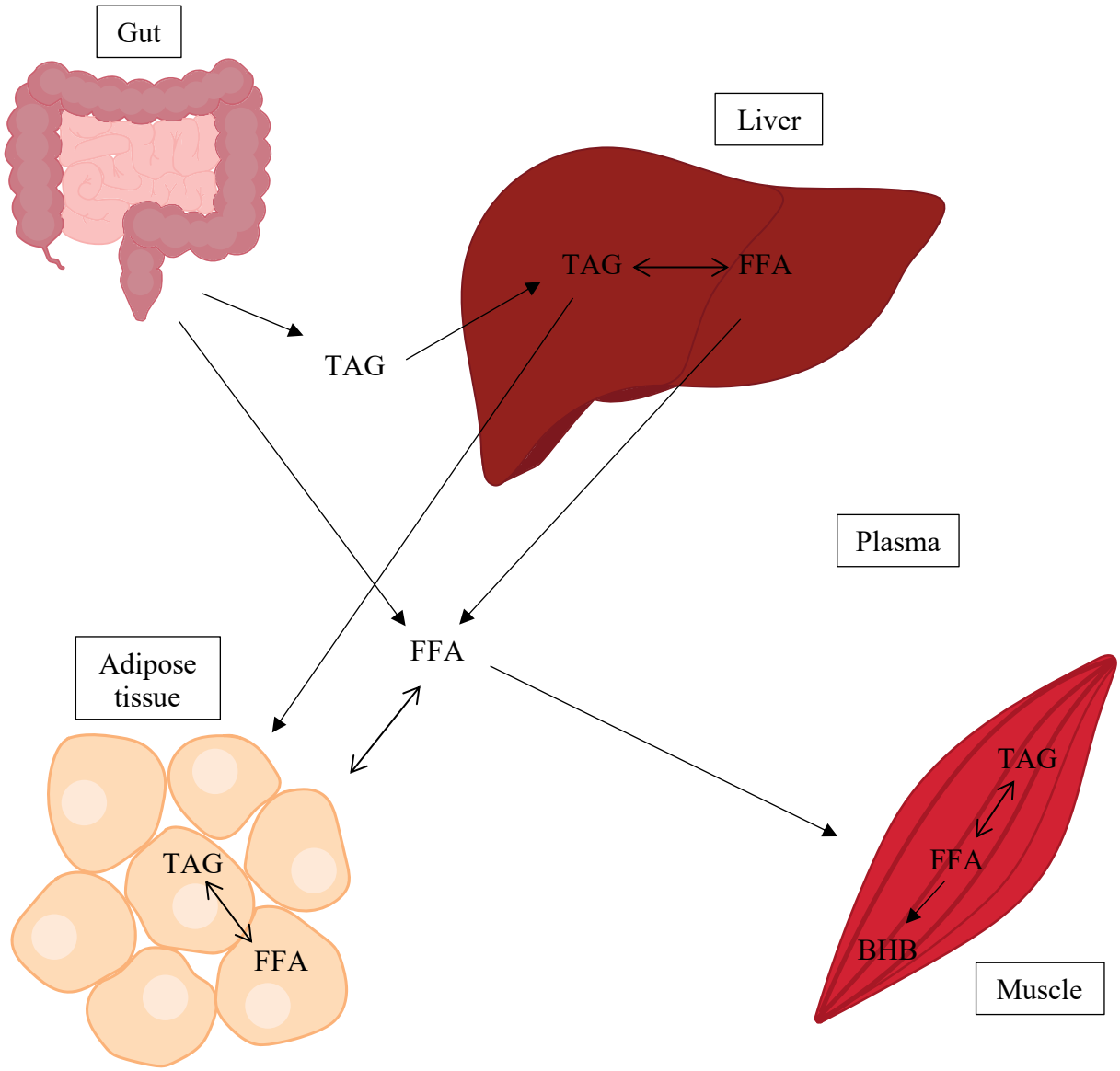

Figure 1: Diagram of mammalian triglyceride (TAG), free fatty acid (FFA), and beta-hydroxybutyrate (BHB) metabolic pathway in gut, liver, adipose tissue, and muscle. The arrows represent transportation between tissue types and the double-facing arrows represent lipogenesis and beta-oxidation.

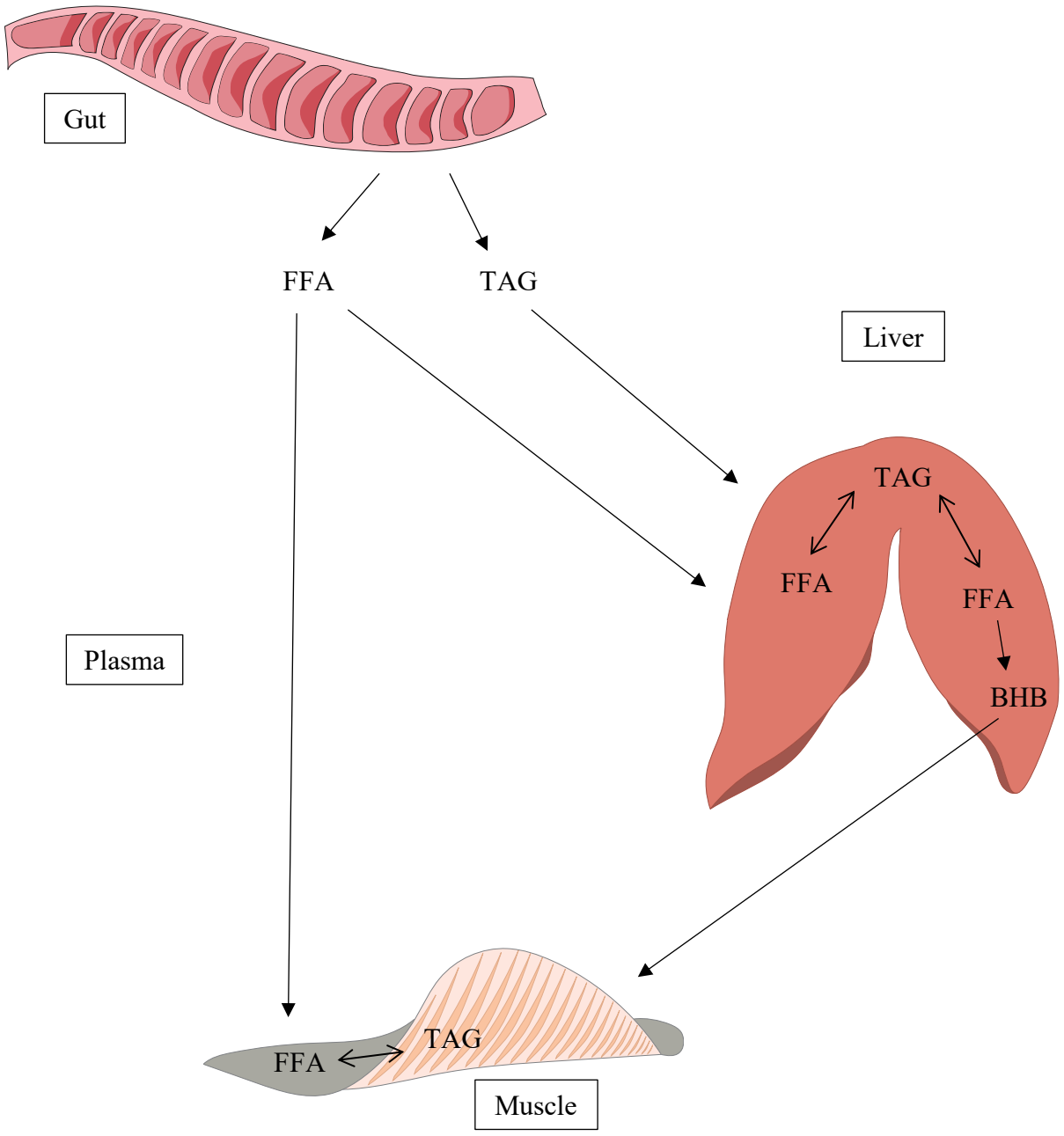

Figure 2: Diagram of elasmobranch triglyceride (TAG) free fatty acid (FFA), and beta hydroxybutyrate (BHB) metabolic pathway in gut, liver, adipose tissue, and muscle. The arrows represent transportation between tissue types and the double-facing arrows represent lipogenesis and beta-oxidation.

CHAPTER 2

METHODS

Study species and tissue collection

Butterfly rays (n =13), Atlantic stingrays (n = 9), and bluntnose stingrays (n = 11) caught as bycatch on commercial shrimp trawls off the coast of Tybee Island, Georgia, June-August of 2019. Bullnose were sampled on the commercial fishing trawls in 2019 (n=2) and with the South Carolina Department of Natural Resources research trawls during April 2021 (n=5). Butterfly ray body mass ranged from 153–505g, Atlantic stingray 66–313g, bluntnose stingray 130–915g, and bullnose ray 425-7257g. Approximately 1ml of whole blood was taken via pectoral fin puncture or cardiac puncture, placed into 2ml heparinized microcentrifuge tubes, and then stored on ice. Whole blood was centrifuged at 3000rpm for 5 minutes to separate plasma from packed red blood cells. Plasma was removed and stored at -80˚C until analyzed. Incidental mortalities were retained and stored at -20 for 3-6 months, then liver and muscle were sampled and stored at -80˚C until homogenized for analysis.

Tissue preparation

Liver and muscle tissues (0.1g of each tissue type) were homogenized prior to colorimetric assay analysis in a 2:5:2 Triton X 100: isopropanol: diH_2O solution (1:10, sample: solution) and a Fisher Scientific PowerGen 125 homogenizer followed by centrifugation at 14,000rpm for 5 minutes at 4˚C. The supernatant was kept and frozen at -80˚C for analysis. All samples were analyzed within three months of acquiring from the trawls. Due to the variation in liver TAG and FFA concentrations among species, *Gymnura lessae* samples were diluted 1:15 (supernatant: diH_2O) for TAG and 1:30 (supernatant: kit assay buffer) for FFA while *Hypanus sabinus* and *Hypanus say* were diluted 1:3 for TAG and 1:20 for FFA respectively. Plasma was

diluted 1:5 (sample: diH2O) for TAG, but no dilution was used for FFA. Muscle samples were not diluted for either assay.

TAG and FFA quantification

Liver, plasma, and muscle triglycerides and free fatty acid concentrations (mmol L^{-1}) were quantified in triplicate using EnzyChrom™ Triglyceride Assay Kit, BioAssay Systems; Haywood, CA, USA, and EnzyChrom™ Free Fatty Acid Assay Kit, BioAssay Systems; Haywood, CA, USA (Moorhead *et al*., 2020; Gallagher *et al.*, 2017; Valls *et al.*, 2016). Absorbance was measured at 570nm using a Molecular Devices SpectraMax® M3 Microplate Reader. Concentrations of triglycerides and free fatty acids were calculated using the corresponding standard curves.

Statistical analysis

Both hypotheses were tested using one linear mixed-effects model with tissue type and species as the fixed effects. The assumptions of a linear mixed-effects model are normality of residuals, homoscedasticity, independence of observations, and a linear relationship between the independent and dependent variables. Triglyceride and FFA values were $\log_{10}$ transformed for the linear mixed-effects model to fit assumptions. Comparisons of TAG and FFA mean effect sizes of each tissue type between species and within a species were calculated using mean effect size overlap within 95% confidence intervals. All statistical analyses were conducted using R version 4.1.0 (2021-05-18) software.

CHAPTER 3

RESULTS

Triglyceride concentrations were quantified in liver, plasma, and muscle of all four species; however, FFA concentrations were only quantified in liver and muscle of the four species due to the majority of plasma samples being below assay detection limit within each species. Any sample below detection limit was removed from the sample size.

Comparison of triglyceride and free fatty acids between tissue types within species

Within individual species, TAG differed significantly between tissue types (Figure 3). Liver TAG mean effect size was significantly higher than plasma and muscle in butterfly and bluntnose stingrays based on 95% confidence intervals. Both species plasma and muscle TAG were not significantly different. Atlantic stingray liver TAG mean effect size was significantly higher than plasma, but liver TAG mean effect size did not differ significantly from muscle. Bullnose ray liver and muscle TAG mean effect sizes were significantly higher than plasma. No significant difference was observed for liver and muscle (Table 3, Figure 4).

Free fatty acid concentrations differed significantly between liver and muscle (Figure 5). Liver FFA mean effect size was significantly greater than muscle FFA in butterfly and bluntnose stingrays. Atlantic stingray liver and muscle FFA mean effect sizes were not significantly different. Bullnose ray liver and muscle FFA mean effect size overlapped in both tissue 95% confidence intervals and fell within the upper range of liver 95% confidence intervals (Table 3, Figure 6).

Comparison of triglyceride and free fatty acids within tissues between species

Species-specific differences in TAG concentrations were seen in liver and muscle (Figure 7). Mean liver TAG concentrations ranged from 22.29 ± 18.69 – 85.90 ± 52.90 mmol L^{-1} across

species (Table 2). Butterfly ray liver TAG mean effect size was significantly greater than Atlantic and bluntnose stingrays, however, butterfly ray liver mean effect fits within the bluntnose stingrays towards the upper limit of the 95% confidence interval (Table 3, Figures 7 and 8). Plasma TAG concentrations ranged from 0.65 ± 0.28 – 1.76 ± 0.52 mmol L^{-1} across species (Table 2). Plasma TAG mean effect sizes fit within each other's 95% confidence intervals indicating no significant differences between species (Figure 7, Figure 8). Muscle TAG concentrations ranged from 1.67 ± 0.49 – 68.51 ± 22.69 mmol L^{-1} across species (Table 2). Bullnose ray muscle TAG was significantly higher than the other three species (Figures 7 and 8).

Species-specific differences in FFA were only observed in liver and muscle (Figure 9). Liver FFA concentrations ranged from 64.89 ± 89.24 –160.4 ± 90.50 mmol L^{-1} across species (Table 2). There was no significant difference in liver FFA between species (Table 3, Figure 10). Muscle FFA concentrations ranged from 1.99 ± 1.49 – 31.77 ± 11.31 mmol L^{-1} across species (Table 2). Bullnose ray muscle FFA mean effect size was significantly greater than the other three species. Butterfly ray muscle FFA mean effect size was significantly greater than Atlantic stingray. However, the Atlantic stingray upper 95% confidence interval overlap with butterfly ray lower 95% confidence interval (Figure 10).

Table 3: Triglyceride (TAG) and free fatty acid (FFA) concentrations (mmol L^{-1}) in liver, plasma, and muscle of butterfly ray, Atlantic stingray, bluntnose ray, and bullnose ray. Plasma FFA concentrations are preliminary and based on a sample size of n=3. Bullnose ray plasma FFA concentration was below the assay detection limit (BDL). Data are represented as mean ± standard deviation.

	TAG (mmol L^{-1})			FFA (mmol L^{-1})		
Species	Liver	Plasma	Muscle	Liver	Plasma	Muscle
Butterfly ray (*Gymnura lessae*)	85.90 ± 52.90	1.76 ± 0.52	1.97 ± 1.28	160.4 ± 90.50	0.08 ± 0.02	2.89 ± 1.66
Atlantic stingray (*Hypanus sabinus*)	22.29 ± 18.69	0.75 ± 0.40	1.67 ± 0.49	67.04 ± 64.48	0.15 ± 0.03	1.99 ± 1.49
Bluntnose stingray (*Hypanus say*)	28.21 ± 18.46	0.94 ± 0.86	3.04 ± 3.14	97.59 ± 77.41	0.16 ± 0.09	3.10 ± 2.63
Southern bullnose ray (*Myliobatis freminvillii*)	60.19 ± 66.09	0.65 ± 0.28	68.51 ± 22.69	64.89 ± 89.24	BDL	31.77 ± 11.31

Table 4: Linear mixed effects model log_{10} mean effect size (MES) and 95% confidence intervals (CI) of triglyceride (TAG) and free fatty acid (FFA) for between species and within tissue type comparisons.

			Triglyceride			Free Fatty Acid		
Species	**Tissue**	**N**	**Log10 Mean**	**Lower 95% CI**	**Upper 95% CI**	**Log10 Mean**	**Lower 95% CI**	**Upper 95% CI**
Butterfly ray	Liver	10	1.80	1.58	2.03	1.47	0.55	2.38
(*Gymnura lessae*)	Plasma	13	0.23	-0.30	0.75	-3.33	-5.46	-1.20
	Muscle	10	0.18	-0.37	0.72	-0.42	-2.63	1.78
Atlantic stingray	Liver	8	1.20	0.63	1.76	1.41	-0.88	3.69
(*Hypanus sabinus*)	Plasma	9	0.42	-0.15	0.99	-2.89	-5.20	-0.57
	Muscle	8	0.81	0.23	1.39	-0.36	-2.72	1.99
Bluntnose stingray	Liver	9	1.36	0.81	1.92	1.23	-1.02	3.47
(*Hypanus say*)	Plasma	11	0.32	-0.23	0.86	-2.89	-5.11	-0.68
	Muscle	9	0.60	0.04	1.16	0.09	-2.18	2.37
Bullnose ray	Liver	7	1.61	1.03	2.19	1.47	-0.87	3.81
(*Myliobatis freminvillii*)	Plasma	7	-0.04	-0.64	0.57	-3.67	-6.13	-1.21
	Muscle	7	2.01	1.40	2.61	1.47	-0.99	3.93
Butterfly ray	Liver	10	1.80	1.58	2.03	1.47	0.55	2.38
Atlantic stingray		8	1.20	0.63	1.76	1.41	-0.88	3.69
Bluntnose stingray		9	1.36	0.81	1.92	1.23	-1.02	3.47
Bullnose ray		7	1.61	1.03	2.19	1.47	-0.87	3.81
Butterfly ray	Plasma	13	0.23	-0.30	0.75	-3.33	-5.46	-1.20
Atlantic stingray		9	0.42	-0.15	0.99	-2.89	-5.20	-0.57
Bluntnose stingray		11	0.32	-0.23	0.86	-2.89	-5.11	-0.68
Bullnose ray		7	-0.04	-0.64	0.57	-3.67	-6.13	-1.21
Butterfly ray	Muscle	10	0.18	-0.37	0.72	-0.42	-2.63	1.78
Atlantic stingray		8	0.81	0.23	1.39	-0.36	-2.72	1.99
Bluntnose stingray		9	0.60	0.04	1.16	0.09	-2.18	2.37
Bullnose ray		7	2.01	1.40	2.61	1.47	-0.99	3.93

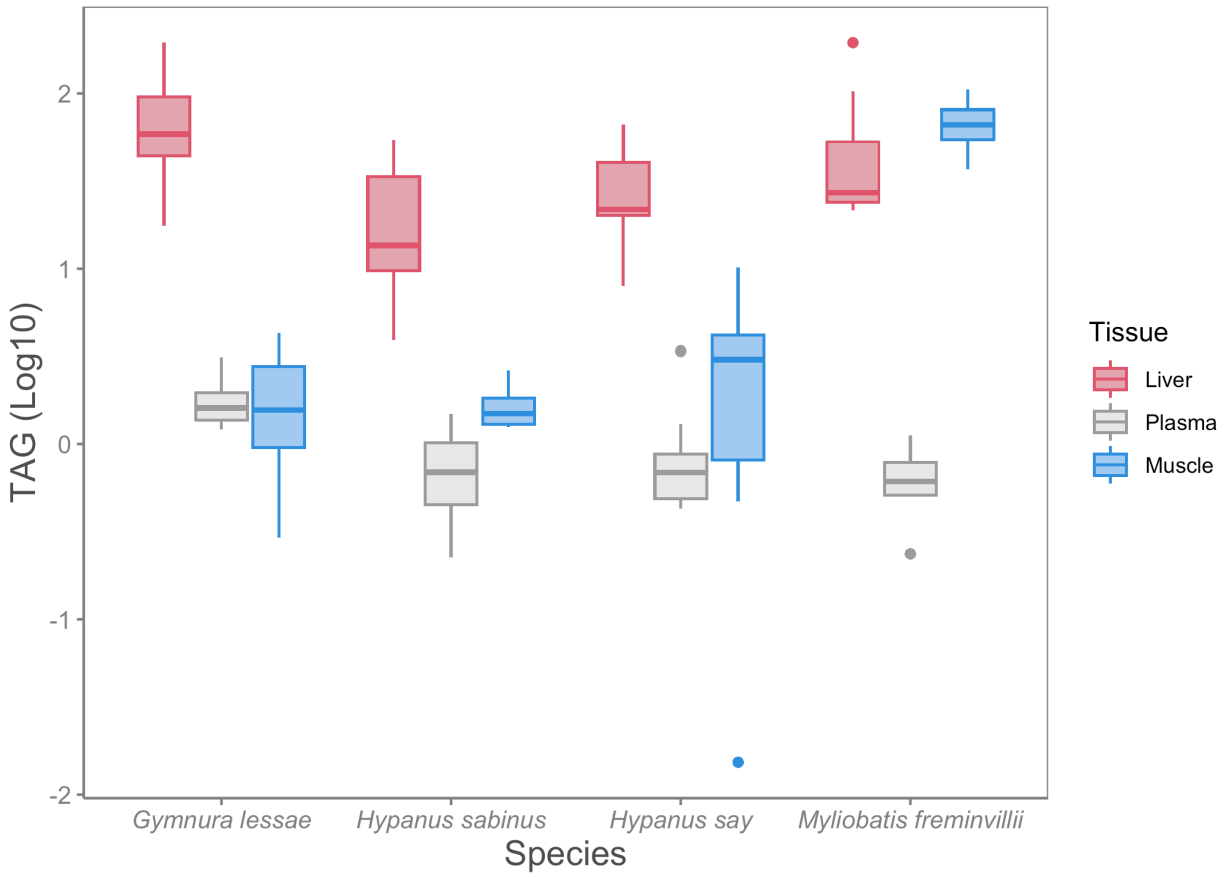

Figure 3: Tissue $\log_{10}$ triglyceride (TAG) (mM) comparison between liver, plasma, and muscle of four species of stingray: butterfly ray (*Gymnura lessae*) (liver n=10, plasma n=13, muscle n=10), Atlantic stingray (*Hypanus sabinus*) (liver n=8, plasma n=9, muscle n=8), bluntnose stingray (*Hypanus say*) (liver n=9, plasma n=11, muscle n=9), and southern bullnose ray (*Myliobatis freminvillii*) (n=7). Data are displayed as median (bolded bar), interquartile range (box), and maximum and minimum (vertical lines). Extreme values are denoted by points outside of the minimum and maximum range.

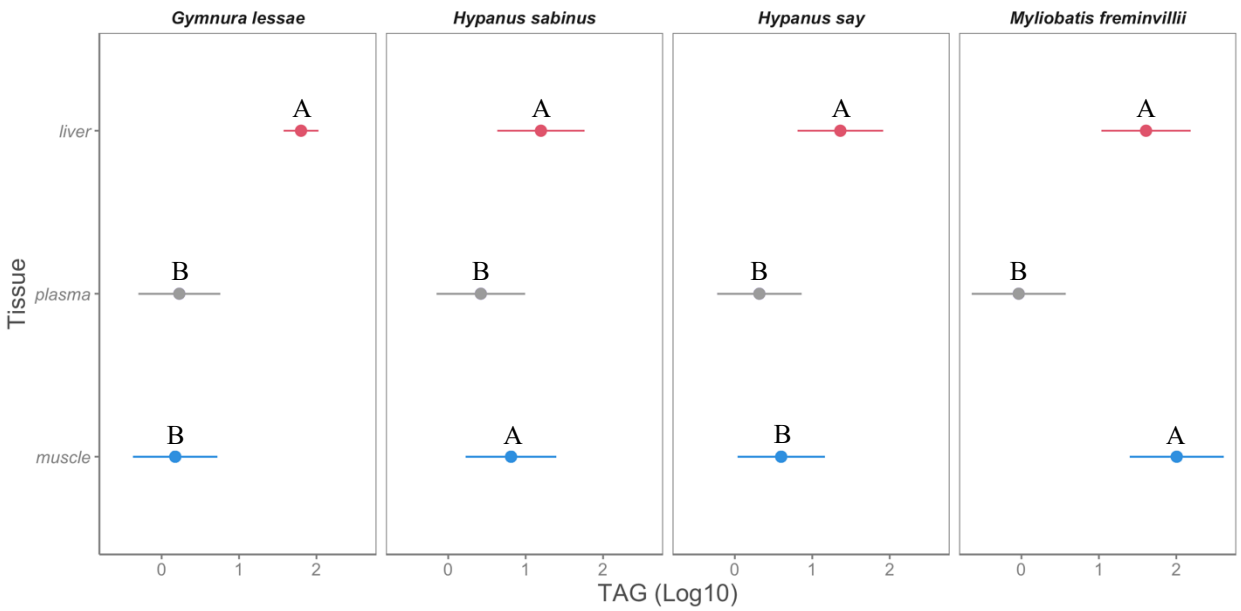

Figure 4: Coefficient plot showing linear mixed-effects model $\log_{10}$ triglyceride (TAG) mean effect size (points) and corresponding 95% confidence intervals (horizonal lines) between liver, plasma, and muscle of four stingray species: butterfly ray (*Gymnura lessae*) (liver n=10, plasma n=13, muscle n=10), Atlantic stingray (*Hypanus sabinus*) (liver n=8, plasma n=9, muscle n=8), bluntnose stingray (*Hypanus say*) (liver n=9, plasma n=11, muscle n=9), and southern bullnose ray (*Myliobatis freminvillii*) (n=7). Pairwise comparisons are denoted by connecting letters report.

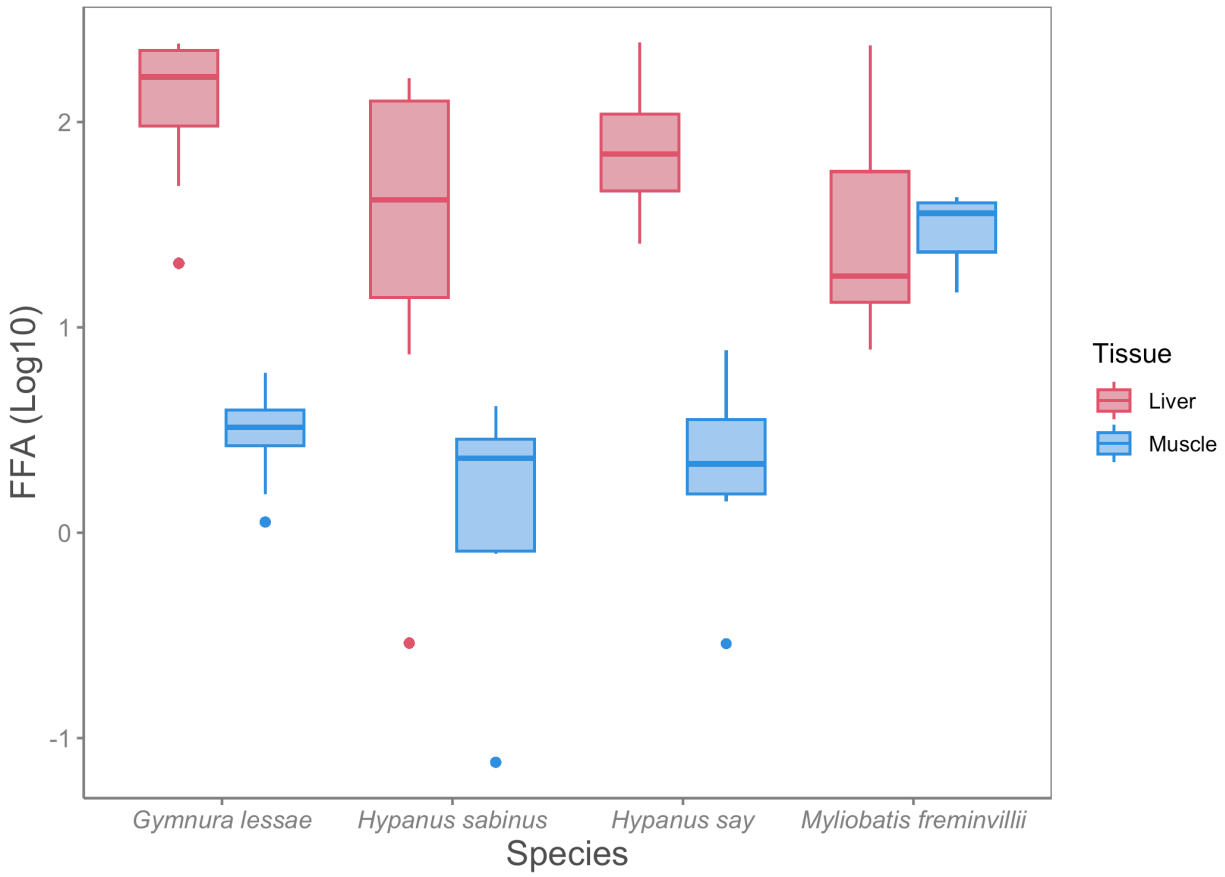

Figure 5: Tissue $\log_{10}$ free fatty acid (FFA) (mM) comparison between liver and muscle of four species of stingray: butterfly ray (*Gymnura lessae*) (n=10), Atlantic stingray (*Hypanus sabinus*) (n=8), bluntnose stingray (*Hypanus say*) (n=9), and southern bullnose ray (*Myliobatis freminvillii*) (n=7). Plasma was not included in this analysis since most of the samples were below detection limit. Data are displayed as median (bolded bar), interquartile range (box), and maximum and minimum (vertical lines). Extreme values are denoted by points outside of the minimum and maximum range. Plasma FFA were below detection.

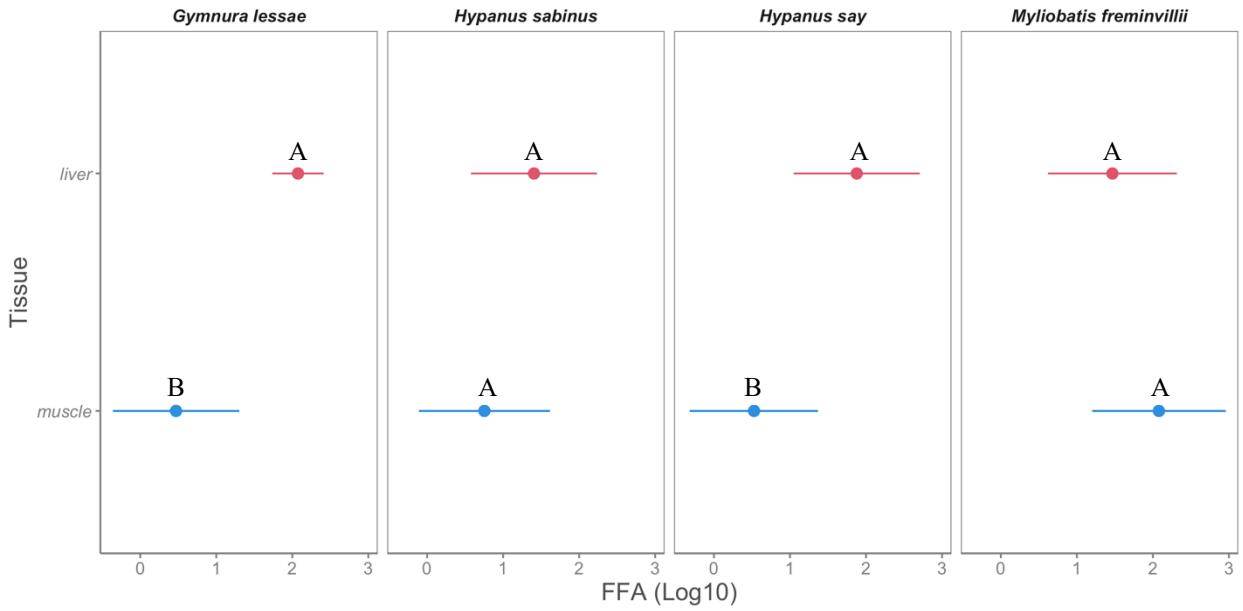

Figure 6: Coefficient plot showing linear mixed-effects model $\log_{10}$ free fatty acid (FFA) mean effect size (points) and corresponding 95% confidence intervals (horizonal lines) between liver and muscle of four stingray species: butterfly ray (*Gymnura lessae*) (n=10), Atlantic stingray (*Hypanus sabinus*) (n=8), bluntnose stingray (*Hypanus say*) (n=9), and southern bullnose ray (*Myliobatis freminvillii*) (n=7). Plasma FFA were below detection. Pairwise comparisons are denoted by connecting letters report.

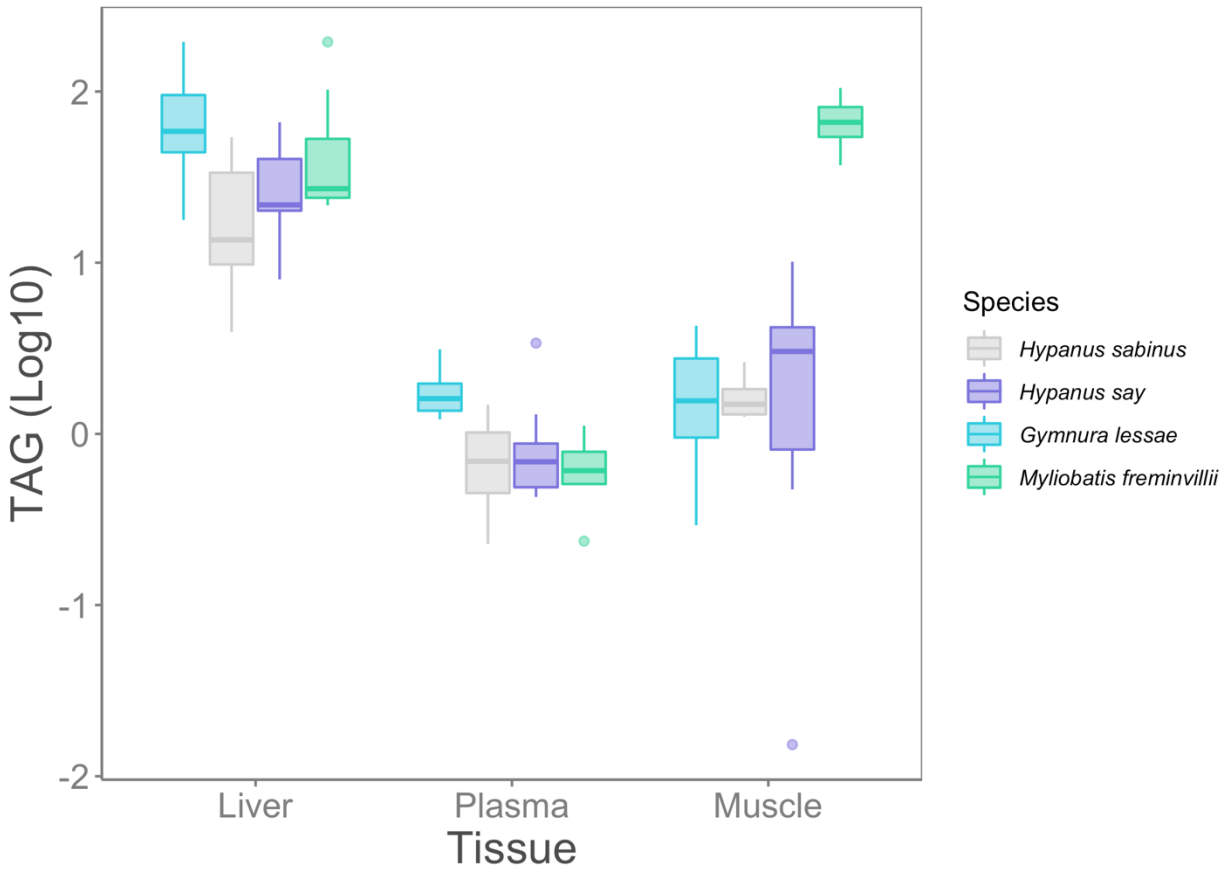

Figure 7: Comparison of $\log_{10}$ triglyceride (TAG) (mM) in liver, plasma, and muscle between four stingray species: butterfly ray (*Gymnura lessae*) (liver n=10, plasma n=13, muscle n=10), Atlantic stingray (*Hypanus sabinus*) (liver n=8, plasma n=9, muscle n=8), bluntnose stingray (*Hypanus say*) (liver n=9, plasma n=11, muscle n=9), and southern bullnose ray (*Myliobatis freminvillii*) (n=7). Data are displayed as median (bolded bar), interquartile range (box), and maximum and minimum (vertical lines). Extreme values are denoted by points outside of the minimum and maximum range.

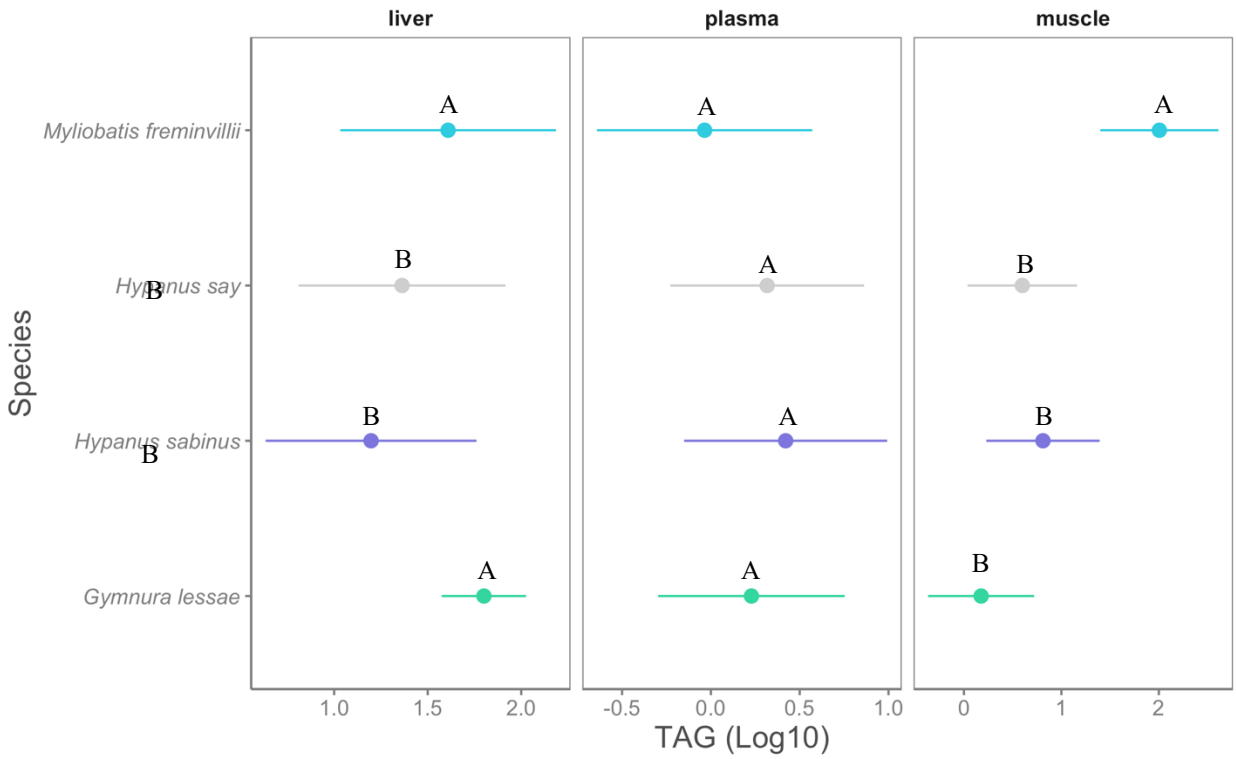

Figure 8: Coefficient plot showing linear mixed-effects model $\log_{10}$ triglyceride (TAG) mean effect size (points) and corresponding 95% confidence intervals (horizonal lines) within liver, plasma, and muscle compared across the four stingray species: butterfly ray (*Gymnura lessae*) (liver n=10, plasma n=13, muscle n=10), Atlantic stingray (*Hypanus sabinus*) (liver n=8, plasma n=9, muscle n=8), bluntnose stingray (*Hypanus say*) (liver n=9, plasma n=11, muscle n=9), and southern bullnose ray (*Myliobatis freminvillii*) (n=7). Pairwise comparisons are denoted by connecting letters report.

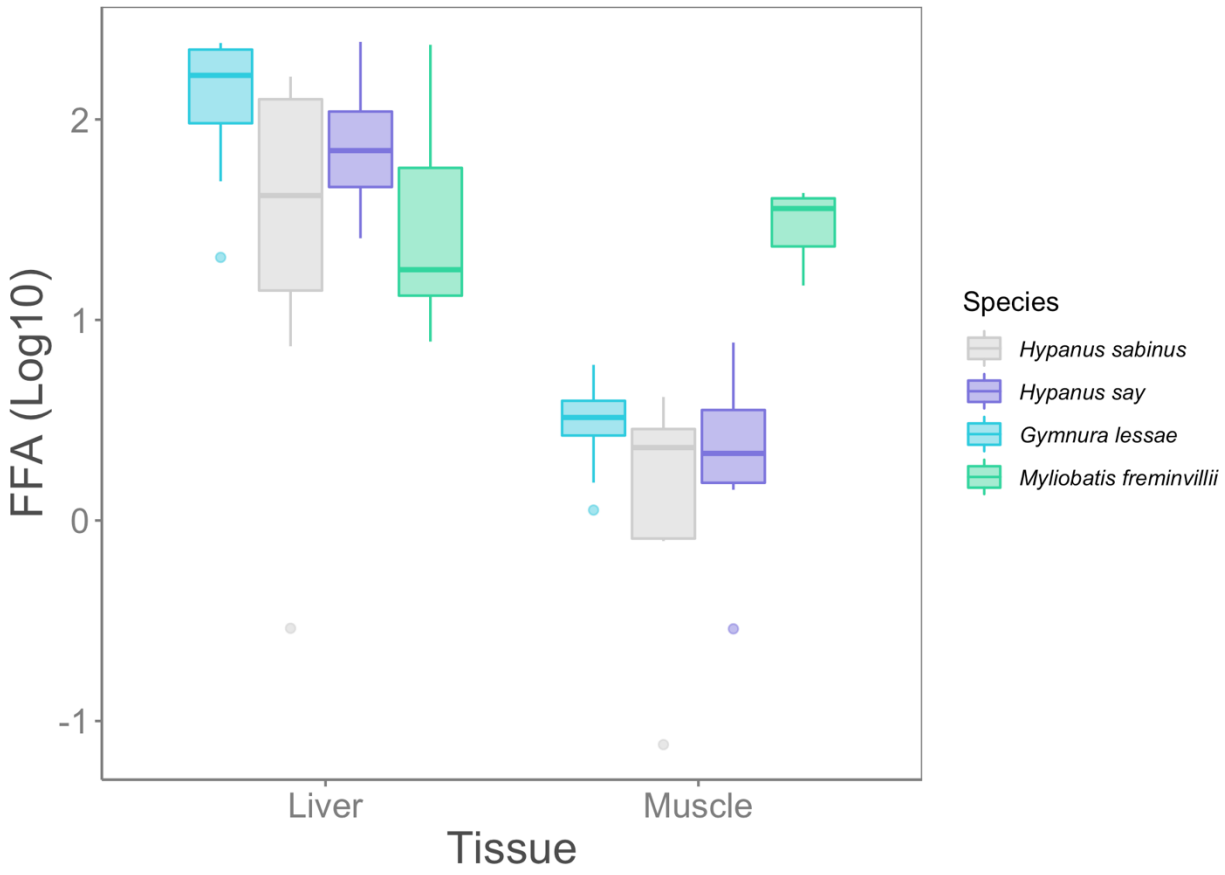

Figure 9: Comparison of $\log_{10}$ free fatty acid (mM) in liver, plasma, and muscle between four stingray species: butterfly ray (*Gymnura lessae*) (n=10), Atlantic stingray (*Hypanus sabinus*) (n=8), bluntnose stingray (*Hypanus say*) (n=9), and southern bullnose ray (*Myliobatis freminvillii*) (n=7). Data are displayed as median (bolded bar), interquartile range (box), and maximum and minimum (vertical lines). Extreme values are denoted by points outside of the minimum and maximum range. Plasma FFA were below detection.

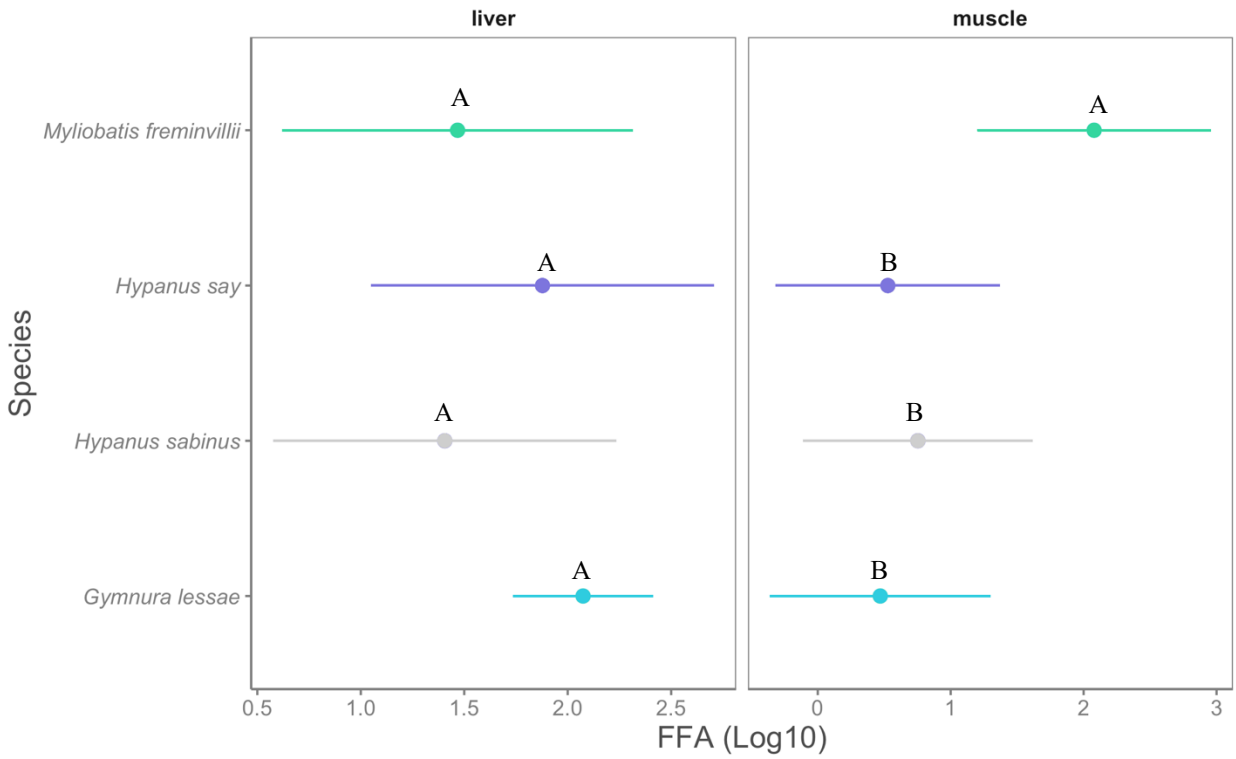

Figure 10: Coefficient plot showing linear mixed-effects model $\log_{10}$ free fatty acid mean effect size (points) and corresponding 95% confidence intervals (horizonal lines) within liver and muscle compared across the four stingray species: butterfly ray (*Gymnura lessae*) (n=10), Atlantic stingray (*Hypanus sabinus*) (n=8), bluntnose stingray (*Hypanus say*) (n=9), and southern bullnose ray (*Myliobatis freminvillii*) (n=7). Plasma FFA were below detection. Pairwise comparisons are denoted by connecting letters report.

CHAPTER 4

DISCUSSION

Between tissue type comparison of triglyceride and free fatty acids within species

Elasmobranch exhibit atypical lipid metabolism by storing lipids in their livers, lacking the transport protein, albumin, and preferentially using ketone bodies for routine fuel in muscle. It has been established that elasmobranchs lack adipose tissue and instead use their livers as the main lipid depot (Ballantyne, 1997). Previous research on liver TAG content in Bleeker's whipray (*Pateobatis bleekeri*) showed TAG forming 92.7% of liver neutral lipids by wet weight (Pal *et al.*, 1998). Similarly, cownose rays (*Rhinoptera bonasus*), spotted eagle rays (*Aetobatus narinari*), and southern stingrays (*Dasyatis americana*) TAG formed 68.9%, 85.9%, and 81.6% of total liver lipid class respectively (Navarro-García *et al*., 2009). Free fatty acids only formed 8% of the 54% total lipid by wet weight in liver of Bleeker's whipray (Néchet *et al*., 2007). In comparison, elasmobranch plasma TAG and FFA concentrations are lower. Plasma TAG and FFA concentrations have been reported in four mobile shark species with TAG ranging from 0.30–1.66 mmol L^{-1} and FFA ranging from 0.13–0.80 mmol L^{-1} (Gallagher *et al*., 2017). As part of their atypical lipid metabolism, elasmobranchs have limited capacity for lipid oxidation in muscle (Anderson, 1990). However, studies have reported muscle TAG/FFA percentages and concentrations in great white sharks (FFA: 1.4% lipid faction), kitefin sharks (*Dalatias licha*) (TAG: 18.5% total lipid by wet weight), and Port Jackson sharks (*Heterodontus portusjacksoni*) (Hayashi and Takagi, 1981; Pethybridge *et al*., 2014; Meyer *et al*., 2019).

When comparing liver and plasma, all study species liver TAG were approximately 30 to 90 times greater than plasma TAG, which was expected since liver is the lipid storage tissue and

plasma transports TAG on a requirement basis (Table 3, Figures 3 and 4). Plasma FFA were below detection limit for most individuals in all four species. Low concentrations of plasma FFA have been reported in some elasmobranch species (193–399 nmol ml^{-1}); therefore, the species in this study may also exhibit low plasma FFA concentrations that are not within detectable limits of the assay used in this study (Ballantyne, 1993; Speers-Roesch *et al*., 2006; Speers-Roesch and Treberg, 2010).

When comparing liver and muscle, liver TAG concentrations that were 9 and 40 times significantly greater than muscle TAG and FFA concentrations that were 30 and 55 times greater than muscle FFA in two study species (Table 3, Figures 3–6). Low levels of muscle TAG and FFA were expected since elasmobranchs use ketone bodies for fuel in both red and white muscle. However, Atlantic stingray and bullnose ray liver TAG and FFA concentrations were similar to muscle TAG and FFA (Figures 3–6). These findings are surprising since elasmobranchs have a limited capacity for beta-oxidation in muscle and further research is needed. Since the muscle type used in this study was white muscle, lipid content in red muscle should also be investigated due to its aerobic activity. Finally, due to the unusually high concentrations observed in these species, it is possible that muscle to be an alternative lipid storage tissue. Salmonids store lipids in adipose tissue, subcutaneous tissue, and intramuscularly (Zhol *et al.*, 1995).

Between species comparison of triglyceride and free fatty acids in liver, plasma, and muscle

Overall, liver TAG concentrations were not significantly different between the four study species except for butterfly rays having three to four times greater liver TAG than Atlantic and bluntnose stingrays, and two times greater FFA than Atlantic stingrays and bullnose rays (Table 3, Figures 5–8). Diamond stingrays (*Dasyatis brevis*) and California butterfly rays (*Gymnura marmorata*) have higher liver TAG concentrations (577–758mg g^{-1} or approximately 651.4–855.7 mmol L^{-1}) than those reported in this study (Navarro-García *et al.*, 2004). Similar liver TAG concentrations (19.7mg g^{-1} or approximately 22.2 mmol L^{-1}) were observed in small-spotted cat sharks (*Scyliorhinus canicula*) (Ruiz-Jabaro *et al*., 2019). The conversion from mg g^{-1} to mmol L^{-1} were done using the TAG conversion factor 0.01129 and are likely under-approximations (Haney *et al*., 2007). Butterfly ray's liver TAG and FFA content may be due to greater lipid content in teleosts than the invertebrates Atlantic and bluntnose stingrays consume (Jargowsky *et al*., 2019). Atlantic and bluntnose stingrays' similar liver TAG and FFA concentrations may be attributed to their similar lower lipid content diets as they are both benthic species that consume invertebrates (Rosenberger, 2001; Schaefer and Summers, 2005). Bullnose ray's similar liver TAG and FFA concentrations to Atlantic and bluntnose stingrays may be due to diet.

Plasma TAG concentrations present in this study were similar to concentrations observed in tiger sharks (*Galeocerdo cuvier*) 0.30 mmol L^{-1}, bull sharks (*Carcharhinus leucas*) 0.83 mmol L^{-1}, blacktip sharks (*Carcharhinus limbatus*) 1.59 mmol L^{-1}, and nurse sharks (*Ginglymostoma cirratum*) 1.66 mmol L^{-1} (Gallagher *et al*., 2017). The presence and concentrations of plasma TAG are influenced by recent feeding events, digestion, and absorption (Borges *et al.*, 2013). Considering that the feeding, digesting, and absorbing statuses of the stingrays in this study are

unknown, the observed plasma TAG concentrations may have been influenced by these processes. Additionally, like TAG, the presence and concentration of plasma FFA is influenced by feeding events (Larsson and Fänge, 1977). If plasma FFA is most readily detectable post-feeding and digestion, then it is possible that the majority of individuals were in the post-absorptive state and had not recently fed. Future studies should analyze stomach contents (not only assessing prey items but also the digestive status of prey) as well as analyze plasma TAG and FFA concentrations pre- and post-feeding events. During feeding trials, Senegalese sole (*Solea senegalensis*) displayed an increase in plasma TAG post feeding event, and the TAG concentrations were also affected by percentage of lipid content in diet (Borges *et al.*, 2013).

Between species comparison of muscle TAG and FFA showed bullnose rays had 35–40 times greater TAG and 10–16 times greater FFA concentrations than the other three species (Figures 7–10). Muscle TAG and FFA have been observed in low concentrations in small-spotted catsharks (Garcia-Garrido, Muñoz-Chapuli, and Andres *et al*., 1990) and Port Jackson shark (Meyer *et al*., 2021). Meyer *et al*. 2021 measured muscle TAG in Port Jackson shark post long-term exhaustive exercise and found that muscle TAG decreased after 33 days of daily 3-minute exhaustive exercise. Bullnose rays' mobile foraging strategy may contribute to the unusually high TAG concentrations present in muscle as they repeatedly oscillate their pectoral fins during foraging to clear sediment and expose prey (Szczepanski and Bengston *et al*., 2014). Utilizing intramuscular TAG for exercise would reduce the immediate need for extra-muscular TAG stores (i.e., in the liver). Species, such as rainbow trout (*Oncorhynchus mykiss*), use white muscle intramuscular TAG to recover from exhaustive exercise (Richards *et al.*, 2002). Future studies should focus on determining of enzymes involved in beta-oxidation, such as carnitine palmitoyl transferase and 3-hydroxy-o-acyl-CoA dehydrogenase are present in white muscle as

previous research has indicated a severely limited capacity for lipid oxidation in red and white muscle of other elasmobranch species (Anderson, 1990; Watson and Dickson, 2001; Speers-Roesch *et al*., 2006). In addition, further investigation into the presence of ketone bodies like beta-hydroxybutyrate is needed to establish if muscle relies on beta-hydroxybutyrate as a main fuel source in muscle of all four species.

Triglyceride and FFA concentrations reported in this study may be lower than actual concentrations in all three tissue types due to the high ambient air temperatures (34-37˚C) on the commercial fishing vessels and the delay in retrieving expired stingrays to store on ice for transport. Additionally, whole blood was taken from these expired animals, placed on ice, and then centrifuged 3-6 hours later. The air temperature and prolonged time on deck may have expedited enzymatic activity in these tissues leading to increased oxidation. Previous research has indicated that lipid stability in muscle tissue decreases after being exposed to temperatures ~20˚C for 24 hours; however, subdermal tissue lipids remained the same when exposed to the same conditions (Meyer *et al.*, 2017). This may explain the below detectable TAG and FFA concentrations in plasma and some muscle samples. Future studies should mitigate the amount of time expired animals are exposed to high ambient air temperatures that increase rates of oxidation.

Energy transfer between prey and predator species

The range of dietary items between species may strongly contribute to the differences in TAG and FFA concentrations since prey species' lipid profiles heavily influences lipid profiles of predators (McMeans *et al.*, 2012; Beckmann *et al*., 2014; Meyer *et al*., 2019). Additionally, the majority of TAG and FFA are obtained through diet with little to no modification during digestion and absorption (Iverson *et al*., 2002). Atlantic stingrays are generalist feeders that

consume a range of prey items including polychaetes, small crustaceans, such as crabs and shrimp, tube anemones, clams, serpent stars, and some teleosts (Robins and Ray, 1986). Likewise, bluntnose stingrays mainly consume invertebrates (clams, shrimp, and worms), but also feed on teleosts more frequently than Atlantic stingrays (Compagno, 1999). Butterfly rays primarily feed on teleosts, which have a higher lipid content and, therefore, a higher energy density than most marine invertebrates (Iverson, Frost, and Lang, 2002; Spitz *et al*., 2010; Jargowsky *et al.*, 2019). Furthermore, butterfly rays are ambush predators that exhibit intermittent feeding, which can be supported by a lipid-dense diet of teleosts (Jargowsky *et al*., 2019). These differences in the lipid content of prey items may drive the species variation in liver, plasma, and muscle TAG and FFA concentrations.

Butterfly, Atlantic, bluntnose, and bullnose rays are abundant mesopredators in coastal marine ecosystems and regulate these ecosystems via top-down interactions; however, they are also important energy sources for apex predators. Many species of hammerhead sharks (*Sphyrna sp.*) and dolphins fulfill the apex predator niche and have been documented to consume stingrays to varying degrees with sharks and stingrays making up a significant portion of hammerhead shark diets (Cliff, 1995). The species in this study represent a range of both lipid content and protein content based on pectoral fin muscle mass with Atlantic and bluntnose stingrays having less pectoral fin muscle than butterfly and bullnose rays. Butterfly rays may prove to be a more energy-dense, higher quality prey item than the other batoid species that form apex predators' diets because of their overall higher TAG and FFA concentrations in liver. However, bullnose rays have higher muscle TAG and FFA, and, therefore, should still be considered viable sources of energy due to the protein content coupled with TAG and FFA present in pectoral fin muscle.

Conclusions

In conclusion, TAG was present in all tissue types while FFA was detectable in liver and muscle. Butterfly rays had significantly greater liver TAG than Atlantic and bluntnose stingrays. The high concentration of liver TAG and FFA present in may be attributed to diet since butterfly rays consume more teleosts (more lipid-dense prey item) than the other three species. Bullnose rays had significantly higher muscle TAG and FFA, which may be explained by their movement ecology and foraging strategy, but also may be an alternative lipid storage tissue. Future research should investigate the presence of beta-oxidation proteins in bullnose ray red and white muscle, and assess liver, plasma, and muscle lipid content in other batoid species.

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