Diet analysis of two data deficient stingray species, the southern stingray (*Hypanus americanus*) and the Caribbean whiptail ray (*Styracura schmardae*), with methodological insights into the use of stomach content analysis and stable isotope analysis

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Abstract

The study of animal diet has long been a fundamental area of biological sciences and has developed significantly over the centuries. An understanding of animal diet goes beyond species-specific biology providing insights into interspecific interactions and whole ecosystem functions essential for ecosystem-based management. Anthropogenic activities have caused major declines in marine vertebrate populations many of which are top predators. It is therefore vital to establish an ecological understanding of mesopredators in the oceans who may either mediate or exacerbate the cascading impacts of such declines. Elasmobranch batoids (otherwise referred to as rays) are a diverse, yet highly vulnerable group of mesopredators many of which are considered data deficient and lack comprehensive dietary assessments. Studies that do exist use stomach content analysis (SCA) or stable isotope analysis (SIA) independently of one another. In contrast, the present thesis integrates these two methods. Chapter one aimed to utilize both SCA and SIA techniques to provide the first integrative dietary assessment of the southern stingray (*Hypanus americanus*), and the first ever quantitative dietary study of the Caribbean whiptail ray (*Styracura schmardae*), two sympatric and data deficient species. Our results suggested that the diets of both species were similar in structure and composition, though Caribbean whiptail ray diet was dominated by arthropod and annelid prey, while southern stingray diet was dominated by molluscs. The broad variety of taxa identified in the stomachs of both species indicates opportunistic feeding, likely as mesopredators at a trophic level similar to other ray species in their respective families. Our integration of SCA and SIA highlights the advantages of combining the two methods, for example, the higher representation of soft-bodied prey in stable isotope mixed models (SIMM) compared to those of SCA. The focus of Chapter two was the isotopic variances between three metabolically different tissues (blood, white muscle and barb) from both the southern stingray and the Caribbean whiptail ray, highlighting how the use of multiple tissues in diet assessments may give better insight into the temporal variability of diet. This was the first quantitative comparison of SCA and SIA across tissue types in rays, the results of which suggest that method agreement is influenced by tissue type incorporated in SIMM. A limitation of our inferences, however, is the lack of data available on isotopic turnover rates in ray and elasmobranch tissues, thus we

recognise the need for further literature and diet manipulation experiments. Nonetheless, the results of the present thesis provide a novel insight into the diets of these two data deficient stingray species and highlight potential avenues for future research which would improve our understanding of these ecologically significant and vulnerable animals.

Table of Contents

List of Tables and Figures

Tables

General Introduction

Table 1 - Synthesis of trophic studies of rays employing either stomach content analysis (SCA), stable isotope analysis (SIA), or an integration of the two (SCA/SIA) since the year 2000. Genus represented by three or more species within an individual study are signified with *; while higher taxonomies where species were not assessed independently or were unspecified, are represented by **.

Chapter One

Table 1-. Dietary compositions of the Caribbean whiptail rays (*Styracura schmardae*) and southern stingrays (*Hypanus americanus*) as seen via gastric lavage stomach content analysis. Identified prey items are listed below the appropriate phylum and noted with the lowest taxonomic level to which they were identified; phylum (p), sub-phylum (s-p), infraclass (ic), order (o), infraorder (io), family (f) and genus (g). Prey proportions are represented as percentage numerical composition (N_c %), percentage weight composition (W_c %), percentage frequency of occurrence (F_o %), and overall percentage index of relative importance (IRI %).

Chapter Two

Table 1 - P-values of post-hoc analyses for $\delta^{15}N$ and $\delta^{13}C$ isotope distributions between tissues using Dunn's nonparametric test for Caribbean whiptail ray (*S. schmardae*) tissues and Tukey HSD parametric tests for southern stingray (*H. americanus*) tissues. Highlighted cells represent values of significance.

Table 2 - P-values and test statistics for post-hoc analyses of repeated measures tests of $\delta^{15}N$ and $\delta^{13}C$ isotopic differences between tissues. Non-parametric Friedman's test with post-hoc analysis for Caribbean whiptail ray (*S. schmardae*)

and parametric ANOVA with repeated measures post-hoc analysis for southern stingray (*H. americanus*). Highlighted cell represent values of significance.

Table 3 - Contribution of Arthropoda, Annelida, Sipuncula and Chordata among the stomach contents of Caribbean whiptail ray (top) and southern stingray (bottom) sampled for blood, white muscle, and barb tissues. Dietary contribution is described by numeric contribution (N_c %) gravimetric contribution (W_c %), frequency of occurrence (F_o %) and a resulting index of relative importance (IRI %).

Table 4 - Contribution of Arthropoda, Annelida, Sipuncula, and Chordata as estimated by Stable Isotope Mixed Models in R (SIMMR) for Caribbean whiptail ray (*S. schmardae*) and southern stingray (*H. americanus*) sampled for blood, white muscle, and barb. Proportional values are means ± standard deviations of 1000 iterations per prey source.

Supplementary Material

Table S1 - Sample size (n), and minimum (Min); mean (± s.d.); and maximum (Max) isotopic values of $\delta^{13}C$ and $\delta^{15}N$ for prey phyla used in stable isotope analysis.

Figures

Chapter One

Figure 1 - Map showing the capture locations of Caribbean whiptail rays (blue points) and southern stingrays (red points) at sites surrounding the Exuma Cays, and Eleuthera Island.

Figure 2 - Cumulative prey curves for the Caribbean whiptail ray (*Styracura schmardae)* (a) and the southern stingray (*Hypanus americanus)* (b) after 1000 randomisations of sampled stomachs. Bars represent the standard deviation around each sample.

Figure 3 - Bivariate plot of values for Caribbean whiptail rays (a), southern stingray (b) and the four main prey groups; Arthropoda (blue), Annelida (red),

Sipuncula (green), Chordata (yellow) and Mollusca (pink). Crossed points represent isotopic ratios of individual stingray for both species, whilst prey groups are presented as combined mean (± s.d.).

Figure 4 - Box plots of stable isotope mixing model (SIMM) dietary contribution estimates for Arthropoda (blue), Annelida (red), Sipuncula (green), Chordata (yellow) and Mollusca (pink) in Caribbean whiptail rays (a) and southern stingrays (b). Central boxes present 2.5 – 97.5% confidence intervals, with mid-lines showing the median.

Chapter Two

Figure 1 - Distribution of δ^{15}N (a & b) and δ^{13}C (c & d) isotope ratios for Caribbean whiptail ray (left) and southern stingray (right) blood (white), white muscle (light grey), and barb (dark grey) tissues. Boxes represent 25 – 75 % interquartile ranges with notched means. Absence of overlap in notches between tissues is indicative of significant differences between tissues.

Figure 2 - Dietary contribution (%) of Arthropoda, Annelida, Sipuncula, and Chordata as estimated by stable isotope analysis (SIA) and stomach content analysis (SCA) for southern stingrays sampled for blood (a & b), white muscle (c & d) and barb (e & f). Boxes represent 25 – 75 % interquartile ranges of estimates with notches and mid-lines presenting mean values.

Figure 3 - Dietary contribution (%) of Arthropoda, Annelida, Sipuncula, and Chordata as estimated by stable isotope analysis (SIA) and stomach content analysis (SCA) for Caribbean whiptail rays sampled for blood (a & b), white muscle (c & d) and barb (e & f). Boxes represent 25 – 75 % interquartile ranges of estimates with notches and mid-lines presenting mean values.

Figure 4 - Pairwise comparisons of diet contribution estimates from stomach content analysis and stable isotope analysis of Caribbean whiptail rays (*Ss*; light grey) and southern stingrays (*Ha*; dark grey) sampled for blood, white muscle and barb tissues. Czekanowski's similarity index ranges from 0 to 1, with a result of 1 representing complete overlap between the methods, while 0 denotes no overlap.

Author's declaration

I conducted all aspects of this research under the regulations and ethical considerations of both the University of Exeter, and Cape Eleuthera Institute, with the aid of Molly Meadows and under the guidance of Dr Lucy Hawkes, Dr Owen O'Shea, Dr Jason Newton, and Dr Matthew Witt.

Between August and December 2016 I undertook, or supervised, all ray captures and sample collection procedures alongside Molly Meadows and under the tutorship of Dr Owen O'Shea. Dr Lucy Hawkes supported and advice from overseas during this field period. All sample processing was done by myself and Molly Meadows under the guidance of Dr Lucy Hawkes at the University of Exeter Cornwall Campus, and were further processed through mass-spectrometry at NERC's Life Science Mass-Spectrometry Facility (LSMSF) after a successful grant application written by myself, Molly Meadows, Dr Lucy Hawkes, and Dr Matthew Witt. Dr Jason Newton supervised and guided in all aspects of mass-spectrometry, and stable isotope analysis throughout processing and thesis writing.

I conducted all literature searches and collation, as well as manuscript/thesis structuring, writing, and formatting. Dr Lucy Hawkes and Dr Owen O'Shea contributed significantly to these steps, advising and guiding throughout.

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General Introduction

Phylogeny of rays

Sharks, skates, and rays, collectively categorised as Elasmobranchii, are a diverse subclass of Chondrichthyes consisting of 1,200 cartilaginous fish species, the most abundant of which are the rays (Last & Stevens, 2009). Rays collectively consist of electric rays, sawfishes, guitarfishes, skates, and stingrays, and are organised under the superorder Batoidea (McEachran & Aschliman, 2004). It is thought that there are approximately 600 species of extant ray, though uncertainties in inter- and intra-specific taxonomy (Griffiths et al., 2010; Naylor et al., 2012; Carvalho et al., 2016) make this likely an underestimation. Batoidea as a superorder has been subject to phylogenetic investigation with some studies suggesting that sharks and rays may be distinct monophyletic groups (McEachran & Aschliman, 2004), whilst others support the 'Hypnosqualea' hypothesis which places batoids as derived sharks, closely related to sawsharks and angelsharks (Shirai, 1992; Carvalho, 1996).

Life history and ecological role

Rays generally share a long life and slow growth strategy to those of many elasmobranch groups, though ray life histories vary along a continuum from slow to fast (Frisk, 2010), often governed by environmental factors such as depth (Cailliet et al., 2001; Simpfendorfer & Kyne, 2009). Rays occupy all major marine ecosystems from the Arctic to the tropics and coastal waters to the deep sea (Last & Stevens, 2009), with some families such as Potamotrygonidae having evolved physiological adaptations to freshwater and low salinity environments (Lovejoy, 1996; Frisk, 2010). Though typically associated with dorsoventrally compressed and circular bodies, there is great diversity in body plan (e.g. the large winged mobulid rays), as well as a variety of specialisations in jaw morphology (e.g. the elongated rostrums of sawfish) (Dean et al., 2007; Aschliman et al., 2012). The typical body plan of skates, stingrays, and electric rays is that of a flattened profile well suited for demersal life in areas of tidal flow (Gilliam, & Sullivan, 1993; Matern et al., 2000). Consequently, the majority of ray species inhabit the soft-sediments of coastal and continental shelf environments, such as mangroves, sand bars,

and coral reefs (Frisk, 2010). Among these environments, many rays have become cryptobenthic submerging themselves among soft-sediments for rest, predator avoidance, and whilst feeding upon infauna by jetting water and beating pectoral fins to expose buried prey (Heithaus, 2004; O'Shea et al., 2012). The resulting disturbance of sediment from all these activities is known as 'bioturbation' (Meysman et al., 2006) and has been shown to have significant biological (Meysman et al., 2006; O'Shea et al., 2012) and physical impacts on benthic sediments (Grant, 1983). Rays largely occupy intermediate trophic positions as 'mesopredators' facilitating vital linkages between apex predators and primary consumers (Yick et al., 2012). Community networks that include mesopredators are generally more complex and can be more flexible, potentially mediating the detrimental impacts of extrinsic pressures (Eriksson et al., 2011) such as fisheries, shipping traffic, tourism, and climate change (Halpern et al., 2007; Crain et al., 2008). However, it may be argued that reduced populations of apex predators, via anthropogenic persecution or overexploitation, may release mesopredator populations from top-down control causing a cascade effect throughout a trophic network (Prugh et al., 2009). This is known as 'mesopredator release', and has been suggested to have occurred among mesopredatory elasmobranchs experiencing increasing populations following declines in those of large shark predators (Shepherd & Myers, 2005). The release of populations of cownose rays (*Rhinoptera bonasus*) within their U.S. Atlantic range, hint at the potential consequences of mesopredator release after their predation of benthic bivalves was linked to the collapse of a scallop fishery in the USA (Myers et al., 2007), though the link has since been suggested to not be well-supported (Grubbs et al., 2016).

Threats and data deficiency

Despite their ecological importance, rays are among the most vulnerable marine vertebrates in the world (Dulvy et al., 2014). Rays are commonly caught as by-catch among trawl and longline fisheries (Domingo et al., 2005; Tamini et al., 2006; Dulvy et al., 2008; Piovano et al., 2010; Clarke et al., 2016). There are, however, targeted fisheries of rays the largest of which is in Southeast Asia (White et al., 2006; Couturier et al., 2012), though there are significant artisanal elasmobranch fisheries in Central America whose ray landings can be similar in

quantity to those of sharks (Smith et al., 2009), while others exhibit seasonal dominance of ray catch (Bizzarro et al., 2009). Responses to exploitation vary in accordance to life history strategies, with species of faster growth to maturity and shorter life spans, exhibiting increased rebound potentials (Frisk, 2010). Deep sea rays have been observed to have lower intrinsic rebound potentials to those of shallower species (Simpfendorfer & Kyne, 2009), though rays that live in coastal environments are exposed to additional anthropogenic pressures such as pollution (Rainer Froese & Garilao, 1997), habitat degradation (Worm et al., 2006), and tourism (Maljković & Côté, 2011). Numerous species of ray are classified as data deficient (DD) by the International Union for Conservation of Nature Red List of Threatened Species (IUCN) due to a lack of taxonomic, distribution, and demographic data. Dulvy et al (2014) assessed the conservation status of 1,041 Chondrichthyen species, finding that of the 487 classified as DD, over half (256 species) were rays. Tools such as the IUCN Red List are important when allocating conservation resource and efforts effectively (Morais et al., 2013). However, truly threatened species classified as DD may be overlooked (Bland et al., 2015).

Importance of trophic studies and application to rays

Trophic studies provide researchers with valuable insights into inter- and intra-specific interactions, facilitating an understanding of species-specific ecological role, and whole ecosystem functions, essential for ecosystem-based management (Navia et al., 2010; Bornatowski et al., 2014; Espinoza et al., 2015). Furthermore, a detailed understanding of the ecology of mesopredators, such as rays, is required to manage threats effectively (Heithaus et al., 2008). Given the ecological role of rays as mesopredators and the insufficiencies of data that inhibit their conservation, rays present ideal candidates for trophic studies.

A species' diet may shift in accordance with spatial (Ajemian & Powers, 2012; Espinoza et al., 2015) or temporal influences (McMeans et al., 2015), or as a response to environmental change (Tunney et al., 2014), making a comprehensive understanding of species diet challenging to achieve (Nielsen et al., 2018). Numerous methodologies ranging from visual observation of gut, stomach, or scat contents (Hyslop, 1980; Cortés, 1997; Klare et al., 2011), DNA

barcoding of prey (Valentini et al., 2009), and analysis of stable isotopes (Inger & Bearhop, 2008; Rundel et al., 2012) and fatty acids (Iverson et al., 2004) have been used on a wide variety of terrestrial and marine vertebrates. However, each method has differing benefits and limitations (Traugott et al., 2013). To date, the majority of studies of ray species have used stomach content analysis and stable isotope analysis (Table 1).

Stomach content analysis (SCA) is the most prevalent method used in dietary studies of rays (Bigelow & Schroeder, 1953; Snelson & Williams, 1981; VanBlaricom, 1982; Gilliam & Sullivan, 1993). Stomach content analysis assesses the contribution of identified items using descriptive indices (e.g. numeric contribution, gravimetric contribution, and frequency of occurrence), which can be integrated into composite measures such as the index of relative importance (IRI) (Pinkas et al., 1970; Hart et al., 2002) or the Prey-Specific Index of Relative Importance (PSIRI) (Brown et al., 2012; Espinoza et al., 2015). The addition of abundance surveys of the various prey alongside SCA allows researchers to investigate the feeding strategies - the extent to which prey may be disproportionately targeted in the environment (O'Shea et al., 2017). However, SCA can underestimate the presence of soft-bodied prey, which are more rapidly digested than animals with hard exoskeletons or other conspicuous body parts (Hyslop, 1980; Wetherbee, Cortés, & Bizzarro, 2004). Additionally, SCA only represents recent diet, requiring repeated sampling over time to gain insight on temporal variance (Inger & Bearhop, 2008; Tierney, 2009). There are also ethical considerations - the use of lethal sampling to collect post-mortem stomach content is probably rarely acceptable today, though by-catch fisheries can be utilised for this purpose (Heupel & Simpfendorfer, 2010). Non-lethal methods of SCA, however, are present and have been successfully employed in elasmobranch studies, for example, gastric lavage (Vaudo & Heithaus, 2011; Elston et al., 2015 & 2017; O'Shea et al., 2017).

Studying the diet of elasmobranchs via SCA has been used for at least a hundred years (Coles, 1919). In comparison, stable isotope analysis (SIA) has only been used for the study of elasmobranch diet for the past two decades (Estrada et al., 2003; Domi et al., 2005; Galván-Magaña et al., 2012; Hussey et al., 2012;

Espinoza et al., 2015) with the exception of Rau et al. (1983). Elements naturally occur in multiple forms known as isotopes which differ in mass based on the number of neutrons they contain (e.g. Carbon: C^{12}/C^{13}, and Nitrogen: N^{14}/N^{15}) (Inger & Bearhop, 2008). Stable isotope analysis measures the differences in the relative abundance of stable isotopes - isotopes that do not decay over time unlike radiogenic isotopes - which are assimilated predictably into the organic tissues of a consumer, and can reflect trophic (Nitrogen), or locational (Carbon, Sulphur, Hydrogen and Oxygen; Inger & Bearhop, 2008) information. The use of SIA in elasmobranch studies in recent decades has thus yielded valuable insights into trophic niche widths (Vaudo & Heithaus, 2011), trophic positions (Jacobsen & Bennett, 2013), and habitat utilisation (Lesage et al., 2010) of numerous species. Furthermore, Bayesian approaches to stable isotope mixing models (SIMM) (e.g. Stable Isotope Analysis in R (SIAR; Inger et al., 2013) and Stable Isotope Mixing Models in R (SIMMR; Parnell & Inger, 2016)) allow for estimates of food source contributions in a consumer diet, while incorporating uncertainty and fractionation factors for more robust models (Inger & Bearhop, 2008; Phillips et al., 2014; Weidner et al., 2017). A major benefit of SIA is its potential to provide temporal insights into diet. The rate of isotope assimilation can be attributed to the metabolic activity of a tissue (Kim et al., 2012; Logan & Lutcavage, 2010; Malpica-Cruz et al., 2012). Experimental diet manipulation studies in laboratory conditions of a multitude of taxa (Vander Zanden et al., 2015) have shown that the greater the metabolic activity of a tissue the faster the isotopic turnover, and thus a more recent representation of diet (Boecklen et al., 2011; Kim et al., 2012; Logan & Lutcavage, 2010; MacNeil et al., 2006). Experimental studies are lacking for ray species with only one exception (MacNeil et al., 2006) which measured the uptake and elimination of N^{15} across metabolically different tissues (liver, blood, cartilage and muscle) of freshwater stingrays, with resulting trends similar to those of other taxa. Where validated, the use of differing tissues can reveal insights into temporal variations in diet (Barría et al, 2015; Tilley et al., 2013; Weidner et al., 2017). However, there are several considerations to be made when using SIA, such as trophic discrimination factors (TDF) - the offset between isotope ratios from prey to consumer due to the excretion of light isotopic forms (e.g. C^{12} & N^{13}) (Nielsen et al., 2018). Trophic discrimination factors have been proposed for stingrays lacking in validation experiments (Tilley et al., 2013) based on experimental and modelling studies (Hussey et al., 2010; Kim et al., 2012),

though temperature, growth rates, and resource availability may influence TDF values, making it unclear whether laboratory derived estimations can be used for wild rays in natural conditions (Martínez del Rio, Wolf, Carleton, & Gannes, 2009). Furthermore, putative diet reconstructions using SIMM require informed priors on the most relevant food sources, often acquired through SCA (Nielsen et al., 2018). However, strong biases associated with such priors, especially if based on one method, can influence SIMM so that they merely reflect the biased results (Moore & Semmens, 2008). Finally, SIA cannot easily differentiate between isotopically similar prey sources without the addition of alternative markers (e.g. Sulphur: $\delta^{34}S$; Hydrogen: $\delta^{2}H$; and Oxygen: $\delta^{18}O$), though knowledge gaps of their utility complicates interpretations (Nielsen et al., 2018).

Dietary assessments on a range of taxa are increasingly employing SCA and SIA alongside one another as a means of better describing trophic ecology, complex food webs and dietary composition (Chiaradia et al., 2014; Nielsen et al., 2018). The contrasting features of these analyses – high resolution, but short temporal scale of SCA data and vice versa for SIA – are complementary when combined. For example, in a study integrating SCA and SIA in the diet assessment of Gentoo and Chinstrap penguins, it was observed that SIA avoided the temporal biases of SCA providing less variable and more accurate insights into inter-annual diet, while SCA allowed for more fine scale estimation of taxonomic composition (Polito et al., 2011). The study concluded that the simultaneous use of SCA alongside SIA (in particular SIMM) can produce more refined estimates of diet and better informed models for interpretation. Several studies on rays have also used SCA alongside SIA, (Dale et al., 2011; Vaudo & Heithaus, 2011; Galván-Magaña et al., 2012; Barría et al., 2015; Espinoza et al., 2015; Oñate-González et al., 2017; Weidner et al., 2017), however, most such studies avoid direct integration of these methods, but utilise them to address broad aspects of trophic ecology. Dale et al. (2011), for example, primarily employs SCA to investigate the dietary composition of brown stingray (*Bathytoshia lata*), while using SIA to identify ontogenetic habitat shifts and trophic position. Similarly, Espinoza et al. (2015) also reconstructed diet via SCA, but used SIA to infer trophic niche widths and broad changes in diet across seasons. In comparison, integrative approaches for accurate assessments of dietary composition, as seen

in Polito et al. (2011), have been few and far between among studies of rays (Barría et al., 2015; Valenzuela-Quiñonez et al., 2017; Weidner et al., 2017). Among these, however, have been insights into how direct integration of SCA and SIA in the reconstruction of diet can validate one another (Barría et al., 2015; Valenzuela-Quiñonez et al., 2017), or highlight novel dietary data that would not be evident had the analyses not been integrated (Weidner et al., 2017). It is, therefore, evident that dietary analysis can benefit from combining SCA and SIA, particularly in direct integration of one another, and should be increasingly implemented within studies of mesopredatory rays to ensure accurate interpretation of data.

Thesis content

Given the trophic significance of rays, comprehensive assessments of their diet are a legitimate line of enquiry with the potential to facilitate conservation frameworks based around the life histories of these vulnerable species, and the ecological networks they inhabit. In **Chapter One** I quantitatively assess, for the first time, the diets of two sympatric and DD species of stingray found in the shallow shelf waters of The Grand Bahama Bank; the southern stingray (*Hypanus americanus*), and the Caribbean whiptail ray (*Styracura schmardae*). Using SCA and SIA I reconstruct the putative diets of both species gaining insights into the prey taxa consumed, the quantitative contribution of major prey types to diet, and make inferences on their trophic ecology. **Chapter Two** investigates the use of multiple tissues in the analysis of ray diet, providing novel insights into method agreement between SCA and SIA. Using three metabolically different tissues (blood, white muscle and barb), the chapter additionally provides insights into possible temporal dietary shifts and highlights deficiencies prevalent in our understanding of elasmobranch isotopic dynamics.

Table 1: Synthesis of trophic studies of rays employing either stomach content analysis (SCA), stable isotope analysis (SIA), or an integration of the two (SCA/SIA) since the year 2000. Genus represented by three or more species within an individual study are signified with *; while higher taxonomies where species were not assessed independently or were unspecified, are represented by **.

Authors	Year	Study species	SCA	SIA	SCA/SIA
Valenzuela-Quioñez et al.	2017	*Pseudobatos productus*			x
Oñate-González et al.	2017	*Urotrygon chilensis*			x
Weidner et al.	2017	*Pteroplaytrygon violacea*			x
Elston et al.	2017	*Urogymnus asperrimus*	x		
Rohner at al.	2017	*Manta birostris, Mobula**	x		
O'Shea et al.	2017	*Urobatis jamaicensis*	x		
Ponte et al.	2016	*Dasyatis pastinaca, Myliobatis aquil*	x		
Burgess et al.	2016	*Manta birostris*		x	
Barria et al.	2015	*Gymnura altavela, Myliobatis aquil, Mobula mobular, Dipturus oxyrinchus, Leucoraja naevus, Raja spp.*, Torpedo spp.**			x
Espinoza et al.	2015	*Raja velezi, Zapteryx xyster, Torpedo peruana*			x
Costa et al.	2015	*Hypanus marianae*	x		
Pardo et al.	2015	*Neotrygon kuhlii, Hemitrygon fluviorum, Maculabatis toshi*	x		
Sczczepanski & Bengtson	2014	*Myliobatis freminvillii*	x		

			Col1	Col2	Col3
Jacobsen and Bennett	2013	Myliobatoidei *, Torpedinoidei*	x		
O'Shea et al.	2013	*Pastinachus ater, Taeniura lymma, Neotrygon kuhlii, Urogymnus asperrimus, Himantura uarnak*	x		
Tilley et al.	2013	*Hypanus americanus*		x	
Rosa-Meza et al.	2013	*Pseudobatos glaucostigmus*	x		
Espinoza et al.	2013	*Zapteryx xyster*	x		
Navarro et al.	2013	*Raja asterias*	x		
Heithaus et al.	2013	*Rhynchobatus laevis*		x	
Blanco-Parra et al.	2012	*Zapteryx exasperata*			x
Navarro-González et al.	2012	*Rhinoptera steindachneri, Pseudobatos glaucostigmus, Hypanus dipterurus, Urotrygon spp.*	x		
Jacobsen and Bennett	2012	*Neotrygon spp.*	x		
Espinoza et al.	2012	*Raja velezi*	x		
López-García et al.	2012	*Hypanus longus*	x		
Vaudo and Heithaus	2011	*Glaucostegus typus, Neotrygon spp.*, Himantura spp.*, Rhynchobatus laevis, Pastinachus atrus, Aetobatus ocellatus*			x
Dale et al.	2011	*Dasyatis lata*			x
Jacobsen and Bennett	2011	*Maculabatis astra*	x		

Borrell et al.	2011	*Pristis pectinata, Pateobatis uarnacoides, Aetomylaeus maculatus, Mobula diabolus, Rhina ancylostoma, Glaucostegus granulatus, and Rhynchobatus djiddensis.*		x
Flores-Ortega et al.	2011	*Urobatis halleri, Urotrygon munda, U. rogersi*	x	
Saglam et al.	2010	*Dasyatis pastinaca*	x	
Schluessel et al.	2010	*Aetobatus narinari*	x	
Sampson et al.	2010	*Mobula thurstoni, M. japonica*		x
Bornatowski et al.	2010	*Pseudobatos percellens*	x	
Shibuya et al.	2009	*Potamotrygon spp.*, Paratrygon aiereba*	x	
Charvet-Almeida	2008	*Fontitrygon colarensis*	x	
Marshall et al.	2008	*Trygonoptera testacea, Urolophus kapalensis*	x	
Collins et al.	2007	*Rhinoptera bonasus*	x	
Silva and Uieda	2007	*Potamotrygon falkneri, P. motoro*	x	
Ebert and Bizzarro	2007	Rajiformes**	x	
Bizzarro et al.	2007	*Beringraja binoculata, Raja inornata, R. rhina, Bathyraja kincaidii*	x	
San Martin et al.	2007	*Psammobatis bergi*	x	
Mabragaña and Giberto	2007	*Psammobatis normani, P. rudis*	x	
Ebert and Cowley	2003	*Dasyatis chrysonota*	x	

Chapter One: Dietary assessment of the Caribbean whiptail stingray (*Styracura schmardae*) and the southern stingray (*Hypanus americanus*), using stomach content and stable isotope analysis

Ethan E. Wrigglesworth,[1] Molly H. Meadows,[1] Jason Newton,[2] Owen R. O'Shea,[3,4,*] Matthew J. Witt,[1,5] and Lucy A. Hawkes[1,5]

[1]University of Exeter, Centre for Ecology and Conservation, College of Life and Environmental Sciences, Penryn Campus, Cornwall TR10 9FE, UK; [2]NERC Life Sciences Mass Spectrometry Facility, SUERC, Rankine Avenue, East Kilbride, Glasgow G75 OQF, UK; [3]Shark Research and Conservation Program, The Cape Eleuthera Institute, Eleuthera, Bahamas; [4]The Centre for Ocean Research and Education, Po Box 255-16, Gregory Town, Eleuthera, The Bahamas; [5]University of Exeter, College of Life and Environmental Sciences, Environmental Biology Group, Hatherly Laboratories, Exeter, EX4 4PS, UK

* Corresponding author: owen@coresciences.org

Abstract

Many ray species are considered data deficient (DD), which hinders effective conservation management in the face of extrinsic pressures. Rays are also thought to be mesopredators, however, comprehensive diet assessments are lacking for most species. In the present study, stomach content analysis (SCA) and stable isotope analysis (SIA) were used to assess the dietary composition and trophic level of two DD and sympatric species of stingray in The Bahamas; the southern stingray (*Hypanus americanus*), and the Caribbean whiptail ray (*Styracura schmardae*). Stomach contents were sampled from 68 stingrays between August 2016 and February 2017, and muscle tissues were collected from 198 stingrays (Caribbean whiptail ray: n = 96, southern stingray: n = 102) between January 2015 and June 2017 for SIA. Both species shared similar diets dominated by arthropod and annelid prey, with minor discrepancies in the composition of three other prey taxa. Caribbean whiptail ray and southern stingray trophic levels were also similar, indicating mesopredatory roles for both species. Our results suggest both species have broad opportunistic diets rather than preferential targeting of specific taxa. This study presents the first integrative use of SCA and SIA for southern stingray, and the first ever dietary assessment for Caribbean whiptail ray. These data should be of utility for future ecological assessments of these two DD ray species, and further supports the integration of dietary techniques to further trophic understanding.

Introduction

Chondrichthyan fishes (sharks, rays and chimera) are among the most vulnerable vertebrates in the world and are highly threatened by anthropogenic activities such as pollution, habitat degradation and overexploitation (Stevens, 2000; Dulvy et al., 2008). Rays (guitarfish, sawfish, skates, electric rays and stingray) are poorly studied in contrast to sharks (Dean, et al., 2007) resulting in data deficiency for many ray species, contributing to over half of all data deficient (DD) chondrichthyans (Dulvy et al., 2014). Data deficiency impairs the evaluation of a species risk of extinction, limiting conservation (Morais et al., 2013) and, when coupled with the conservative life history (Frisk, 2010) and low rebound potentials (Cortés, 2000; Simpfendorfer & Kyne, 2009) of many ray species, the status of DD for a truly vulnerable ray may be detrimental. However, an understanding of trophic ecology and ecosystem dynamics may facilitate conservation frameworks (Yick et al., 2011), and has been identified as an area of priority for elasmobranch conservation (Simpfendorfer et al., 2011).

Numerous ray species are considered mesopredators, subject to predation from top predators while feeding on primary consumers (Yick et al., 2012). They, therefore, provide complexity to trophic networks and potentially mediate the impacts of extrinsic pressures such as apex predator loss (Myers et al., 2007; Eriksson et al., 2011; Yick et al., 2012). Comprehensive studies of ray trophic ecology are lacking with the majority employing traditional methods such as stomach content analysis. Stomach content analysis (SCA), via either post-mortem or gastric lavage, provides insight into recently consumed prey (Cortés, 1997; Nielsen et al., 2018). However, SCA has been associated with numerous biases from misrepresentation of soft-bodied prey, to a limited temporal view of the diet (Hyslop, 1980; Tierney, 2009). With the addition of stable isotope analysis (SIA) such biases can be ameliorated from dietary studies. Stable isotope analysis measures the ratios of light and heavy forms of stable isotopes (i.e. Carbon; C^{12}/C^{13}, and Nitrogen; N^{14}/N^{15}) that are assimilated from the environment and diet into the tissues of an organism (Inger & Bearhop, 2008). The rate of isotopic assimilation, therefore, results in SIA offering a temporal insight into the diet of a consumer in accordance to the metabolic rate of the tissue sampled

(Dalerum & Angerbjörn, 2005; Inger & Bearhop, 2008). Both SCA and SIA complement one another – SCA providing a high resolution of prey identification and SIA offering a broad temporal view of diet (Dale et al., 2011; Espinoza et al., 2015) – but when applied in integration, i.e. SCA identifying key priors for stable isotope mixed models (SIMM), the combination can prove a powerful tool for accurate dietary analysis (Polito et al., 2011). Despite this, only a few dietary studies of rays have implemented an integrative approach to their analyses (Barría et al., 2015; Valenzuela-Quiñonez et al., 2017; Weidner et al., 2017). Weidner et al. (2017) presents an case where SIMM of pelagic stingray (*Pteroplaytrygon violacea*), suggested a crustacean dominated diet as opposed to the cephalopod dominated diet observed via SCA and within previous studies (Ribeiro-Prado & Amorim, 2008). The study stands as an example of how the integration of analyses can identify dietary traits which would otherwise remain unknown. This may particularly be the case for species whose diets are broad, consisting of hard and soft bodied prey which are often misrepresented when assessed using singular approaches.

In The Bahamas, two species of stingrays, the southern stingray (*Hypanus americanus*) (Hildebrand & Schroeder, 1928; Last et al., 2016) and the Caribbean whiptail ray (*Styracura schmardae*) (Werner, 1904; Carvalho et al., 2016) inhabit mangrove creeks, sand flats and coral reefs in sympatry. Unlike other species of stingray inhabiting The Bahamas, both the southern stingray and the Caribbean whiptail are of DD status (Charvet-Almeida & de Almeida, 2006; Grubbs et al., 2016), with the latter having only recently been recognised as a resident of The Bahamas (O'Shea et al., 2017). Southern stingrays have been subject to research efforts as a product of their integration in the ecotourism industry (Shackley, 1998; Semeniuk & Rothley, 2008; Semeniuk et al., 2009; Corcoran et al., 2013; Bird et al., 2018), yet, few studies have addressed critical aspects of their ecology. There have been previous dietary assessments of southern stingrays (Bigelow & Schroeder, 1953; Gilliam & Sullivan, 1993; Tilley et al., 2013), though none have directly integrated SCA and SIA within a single study. The Caribbean whiptail ray, on the other hand, is completely lacking data for almost all aspects of its biology, and to date, its diet has not been studied. Such deficiencies in data make effective conservation of both species challenging.

In the present study, I aim to reconstruct the diet of the southern stingray and the Caribbean whiptail ray, integrating for the first time SCA and SIA, while providing the first ever dietary assessment for the Caribbean whiptail ray in The Bahamas. I additionally provide the first use of gastric lavage in large batoids in the Caribbean, with insights into its application.

Methods

Study site

Stingrays were captured among nearshore ecosystems of three locations: 1) the Schooner cays (24.900823 °N, - 76.370323 °W) a group of limestone islands just north of Powell Point Eleuthera, surrounded by shallow sand flats of the Great Bahama Bank, approximately one meter in depth; 2) Powell Point, Southern Eleuthera (24.831748 °N, - 76.334907 °W) a Cape lined with shallow mangrove creek inlets, sand flats, and patch reefs to the north, and the deep oceanic waters of the Exuma sound to the south; and 3) the Exuma Cays (24. 717711 °N, - 76.822376 °W) a chain of small islands along the Great Bahama Bank, adjacent to the Exuma sound to the east (Figure 1). Capture sites consisted of shallow subtidal areas of sandy substrate and had been selected for stingray capture due to prior identification as areas of abundance for either or both stingray species.

Ray capture

Stingrays were observed from on board a slow-moving, shallow draft boat, and upon identification were captured via spot seining methods (O'Shea et al., 2017) as follows. Stingrays were herded into a 10-metre seine net and transferred into a large dip net for sample collection. Their venomous tail barbs were wrapped and secured flush to the tail using a cotton bandage. Any pre-existing identification tags were noted, and disc width (the distance between the two widest points of the stingray's pectoral fins) was measured using a flexible measuring tape. All new captures were tagged in the dorsal surface of the left pectoral fin with a new spaghetti tag bearing a unique identification number.

Sampling

Prior to sampling, stingrays were placed into a state of tonic immobility via dorso-ventral recumbence, resulting in a natural state of paralysis which is thought to reduce stress during sampling procedures (Henningsen, 1994). White muscle tissue was sampled from pelvic fins using a clamp and surgical scissors to extract a small segment of tissue, which was frozen for later analysis.

Gastric lavage

Gastric lavage was performed following methods outlined by O'Shea et al., (2017). A sterile silicone tube (~ 10 mm diameter) was inserted through the buccal cavity into the stomach using an external marker on the ventral surface of the animal to visualise the appropriate distance. Sixty millilitres of ambient seawater was then introduced to the tube via a syringe, and resulting regurgitated stomach content was captured in a catchment tray lined with meshed netting (1 mm). Stomachs were considered to be 'empty' after three lavage attempts (i.e. a maximum of 180 ml of stomach flushing). All collected content was stored on ice for later identification and analysis. Putative prey samples were collected using small aquarium nets from sites where stingrays were captured. Any prey items from gastric lavage samples that had been minimally digested were also collected for stable isotope analysis.

Processing and analysis

Gastric lavage samples

Total wet mass of stomach contents was recorded to the nearest 0.1 g. Any highly digested and/or unidentifiable items were weighed, inspected, and discarded if further identification was not possible. The remaining prey items were identified to the lowest taxonomic resolution and grouped by phylum (Arthropoda, Annelida, Sipuncula, Mollusca and Chordata). For each identified prey taxa and collective prey phylum, the number of prey items were counted and weighed to the nearest 0.1 g. Four metrics were used to describe identified prey; (i) numerical contribution (N_c %) calculated as the total count of a given prey taxa / total count of all prey taxa x 100 (Hyslop, 1980); (ii) gravimetric contribution (W_c %) calculated as the total mass (g) of a given prey taxa / total mass of all prey taxa x 100 (Hyslop, 1980); and (iii) the frequency of occurrence (F_o %) calculated as the total count of stomachs containing a given prey taxa / total count of stomachs with identifiable content x 100 (Vaudo & Heithaus, 2011). Finally, (iv) N_c %, W_c % and F_o % were used to calculate an Index of Relative Importance (IRI) (Pinkas et al., 1970):

$$IRI = (N_c\% +\ W_c\%)\ \times\ F_o\%$$

The resulting IRI for each prey taxa (IRI_i) was then calculated as a percentage (IRI%) of the sum total of all prey taxa IRI (IRI_t) using the following equation (Cortés, 1997);

$$IRI\% = \left(IRI_i \div \sum IRI_t \right) \times 100$$

In order to estimate whether the number of stingrays sampled was sufficient enough to have described diet compositions of both stingray species, the mean cumulative number of prey taxa found within lavage samples was plotted against the number of stingrays sampled. The number of stingrays sampled was considered to be sufficient when the curve reached an asymptote (Ferry et al., 1997).

Stable isotope analysis and Bayesian mixed models

Samples were oven dried at 70°C for 24 hours and homogenised using a pestle and mortar. The presence of lipids within the body tissue of an organism can cause bias in the analysis of carbon isotopes (Post et al., 2007). However, in the present study carbon-to-nitrogen ratios (C:N) were less than 3.5 % (Caribbean whiptail ray; mean 3.06 ‰ ± 0.1 ‰ s.d., and southern stingray; 3.07 ‰ ± 0.1 ‰) and thus lipid extraction was deemed unnecessary. Additionally, the unique retention of urea in elasmobranch tissues due to osmoregulatory processes may cause bias in the analysis of nitrogen isotopes (Kim & Koch, 2012; Carlisle et al., 2016; Li, Zhang et al., 2016), and thus should be extracted before analysis, although see (Logan & Lutcavage, 2010). However, Shipley et al (2017) found no significant changes in $\delta^{15}N$ within tissues of both Caribbean whiptail rays and southern stingrays before and after urea extraction, and thus I did not remove urea before analysis.

A sub-sample of the dried, homogenised material (0.700 mg ± 0.050 mg) was enclosed into a 5 x 3.5 mm tin capsule (Elemental Microanalysis) and combusted in an Elementar Pyrocube purge-and-trap elemental analyser run in Nitrogen, Carbon, and Sulphur (NCS) mode interfaced with an Elementar VisION isotope ratio mass spectrometer. Two international standards (United States Geological Survey L-glutamic acid (USGS40) and IAEA-S-1, S-2 and S-3 Silver Sulphides) and three internal standards (methane sulphonamide/gelatine (MSAG2),

methionine/gelatine (M2) and sulphanilamide/gelatine (SAAG2)) were run alongside the stingray samples for calibration and cross checking against other SIA centres to ensure the data is globally matched (Newton, 2001). Stable isotope ratios of $^{12}C/^{13}C$ and $^{14}N/^{15}N$ are expressed with delta notation (δ) as parts per mil (‰) (McKinney et al., 1950), with resulting values representing the relative difference between isotopic ratios of the sample compared to a standard (Slater et al., 2001).

The R package Stable Isotope Mixing Models in R (SIMMR, (Parnell & Inger, 2016)) was used to model the proportional contribution of prey phyla identified through stomach content analysis to the diets of both Caribbean whiptail rays and southern stingrays. Prey phyla were treated separately throughout analysis as there was a lack of rationale for combining any of the groups (Phillips et al., 2014). Diet-tissue discrimination factors (DTDF's) of + 2.7 ‰ $\delta^{15}N$ and + 0.9 ‰ $\delta^{13}C$ were applied to prey stable isotope ratios as recommended by Tilley et al (2013) for stingray species lacking in validation studies. Sensitivity of SIMMR dietary proportion estimates to DTDF's was tested with model re-runs adjusting $\delta^{13}C$ by 0.5 ‰ and 1 ‰, and adjusting $\delta^{15}N$ by 0.5 ‰ and 1 ‰. Caribbean whiptail and southern stingray $\delta^{13}C$ and $\delta^{15}N$ ratios from muscle tissues were plotted in two-dimensional isotopic space, with DTDF adjusted isotopic ranges of prey phyla, and proportional contribution estimates (n = 1000 iterations per prey phyla) from SIMMR output models were constructed.

Trophic level

Trophic levels (TL) of putative prey phyla were obtained from several published studies. Arthropoda, Annelida, Sipuncula and Mollusca TL values were taken from Cortés (1999) following calculations based on several published sources (Hobson & Welch, 1992; Pauly & Christensen, 1995; Pauly et al., 1998) during a study of global shark TL values. For Chordata (teleost fish) a mean TL value was obtained from FishBase (Froese & Pauly, 2000), as used in previous TL calculations for the southern stingray (Tilley et al., 2013). These prey data were used to calculate the TL's for the Caribbean whiptail ray and the southern stingray using the following equation (Cortés, 1999):

$$TL_K = 1 + \left(\sum_{i=1}^{n} P_i \times TL_i \right)$$

where TL_K is the trophic level of a consumer, P_i is the proportional contribution of the ith prey source to consumer diet, n is the total count of prey sources observed within the consumers' diet, and TL_i is the trophic level of the ith prey source. This was repeated for both species of stingray using the proportion estimates of SCA and SIMM.

$$TL_K = 1 + \left(\sum_{i=1}^{n} P_i \times TL_i \right)$$

Results

A total of 74 stingrays (Caribbean whiptail ray n = 31, southern stingray n = 43) were captured between August 2016 and February 2017, of which 68 (Caribbean whiptail ray n = 31, southern stingray n = 37) underwent gastric lavage. Between January 2015 and June 2017, white muscle tissues were extracted opportunistically for SIA from a total of 198 stingrays (Caribbean whiptail ray n = 96, southern stingray n = 102), 38 of which were among those sampled for stomach content (Caribbean whiptail ray n = 18, southern stingray n = 20). Additionally, a total of 100 tissue samples were collected from putative prey (Arthropoda n = 64, Annelida n = 14, Sipuncula n = 10 and Chordata n = 12). Caribbean whiptail rays ranged from 228 mm to 1,472 mm disc width (mean 629 mm ± 283 s.d.), whilst southern stingray ranged from 342 mm to 1,102 mm disc width (674 mm ± 175).

Gastric lavage

Gastric lavage was performed on a total of 68 stingrays of which 47 had prey among their stomachs (Caribbean whiptail ray n = 19*,* southern stingray n = 28). A total of 183 prey items were identified (Caribbean whiptail ray n = 109*,* southern stingray n = 74) belonging to five phyla (Table 1; Arthropoda, Annelida, Sipuncula, Chordata, and Mollusca). In 54 cases it was not possible to assign prey items to lower than a sub-phylum level. Cumulative prey curves for each species reached asymptotes after approximately 20 (Caribbean whiptail ray) and 30 (southern stingray) individuals had been sampled (Figure 2) suggesting that a sufficient number of stingrays had been sampled to represent the diet of both species*.*

Caribbean whiptail ray – Stomach contents were categorised into four phyla (Arthropoda, Annelida, Sipuncula, and Chordata; Table 1). Arthropoda (n = 56 prey items) had the highest proportional contribution to diet of all four prey phyla found (70.41 IRI %)*,* having been present in all 19 Caribbean whiptail rays with a mean cumulative mass of 1.0 g. Arthropoda comprised of seven prey groups of varying taxonomic levels, which contributed to diet from highest to lowest as follows: *Alpheus sp.*, Decapoda, Palaemonidae, Brachyura, Stomatopoda,

Crustacea, and Penaeidae. Annelida (n = 44 prey items) had the next highest contribution to diet of all prey phyla (27.02 IRI %), being present in 13 of the 19 rays sampled with a mean cumulative mass of 0.4 g. The family Arenicolidae (n = 12 prey items) was the only taxa identified beyond the phylum of Annelida but contributed relatively little to diet (2.02 IRI %). Collectively the remaining 32 annelid prey items contributed the most towards diet of all individual prey taxa (51.22 IRI %). Sipuncula (n = 8 prey items), had the third highest dietary proportion of all phyla (2.53 IRI %) being present in four of the 19 rays sampled, with a mean cumulative mass of 0.8 g. Lastly, the phylum Chordata, which consisted of teleost fish within the stomach of one Caribbean whiptail, accounted for the lowest dietary contribution (0.03 IRI %).

Southern stingray – Five phyla (Arthropoda, Annelida, Sipuncula, Chordata and Mollusca; Table 1) were identified in the stomachs of southern stingrays**.** Arthropoda (n = 50 prey items) contributed the most to diet (89.43 IRI %) being present in all 28 rays sampled, accounting for a mean cumulative mass of 0.75 g. The phylum comprised of 10 taxa, which ordered from highest to lowest contribution to southern stingray diet were: Crustacea, Penaeidae, Brachyura, Stomatopoda, Palaemonidae, *Metapenaeopsis sp.*, Portunidae, *Alpheus sp.*, Decapoda, and Diogenidae. Two families (Portunidae and Diogenidae) and one genus of velvet shrimp (*Metapenaeopsis sp.*) present among southern stingray stomachs were not observed in those of Caribbean whiptail rays. Chordata (n = 10 prey items) contributed the second highest proportion to diet of the five prey phylum (5.98 IRI %), with teleost fish identified in nine of the 28 rays sampled, with a mean cumulative mass of 0.3 g. Annelida (n = 8 prey items) had the third highest contribution (2.98 IRI %), being present in six of the 28 rays, with a mean cumulative mass of 0.3 g. Sipuncula (n = 4 prey items) contributed little to diet (1.28 IRI %) being present in only three of the 28 rays sampled, with a mean cumulative mass of 1.8 g. Mollusca (n = 2 prey items) was only observed in two of the rays sampled, with a mean cumulative mass of only 0.8 g, and had the lowest contribution to diet (0.33 IRI %).

Stable isotope analysis

A total of 208 white muscle tissue samples were processed for SIA (Caribbean whiptail ray n = 102, southern stingray n = 106). $\delta^{13}C$ values for Caribbean whiptail white muscle ranged from -13.32 ‰ to -5.65 ‰ (Figure 2; mean -9.54 ‰ ± 1.64 ‰ s.d.), and $\delta^{15}N$ values ranged from 2.07 ‰ to 7.96 ‰ (4.81 ‰ ± 1.19 ‰). $\delta^{13}C$ ratios for southern stingray white muscle ranged from -12.68 ‰ to -6.51 ‰ (mean -8.95 ‰ ± 1.19 ‰ s.d.), and $\delta^{15}N$ ratios ranged from 3.13 ‰ to 8.95 ‰ (6.67 ‰ ± 1.11 ‰). Mean values and standard deviations of $\delta^{13}C$ and $\delta^{15}N$ ratios for putative prey are shown in Figure 3 (see also Table S1).

For the Caribbean whiptail ray, SIMMR estimates indicated that the largest dietary contribution was likely from Arthropoda (Figure 4; mean contribution 48 % ± 6 % s.d.), followed by Annelida (45 % ± 4 %), Sipuncula (5 % ± 3 %), and Chordata (1 % ± 1 %).

SIMMR for southern stingray (Figure 4) estimated that Mollusca were the largest contributors to diet (47 % ± 6 %), followed by Annelida (19 % ± 6 %), Arthropoda (16 % ± 5 %), Chordata (16 % ± 3 %) and Sipuncula (3 % ± 2 %).

Trophic level

Trophic levels for prey sources were 2.52 for Arthropoda, 2.5 for Annelida and Sipuncula, 2.1 for Mollusca, and 2.8 for Chordata. Caribbean whiptail ray had a trophic level of 3.48 when calculated using both SCA and SIA prey contribution estimates. Southern stingray had a trophic level of 3.51 from SCA, and 3.39 with SIA.

Discussion

Our results present the first quantitative insights into the dietary ecology of the Caribbean whiptail ray within The Bahamas and represent the first direct integration of methods in such a study for the southern stingray. For both species, SCA suggested diet primarily consisted of arthropods with varying contributions of Annelid worms, Sipuncula, Mollusca, and Chordata between the two species. Stomach contents of Caribbean whiptail rays primarily consisted of shrimp, though the wide range of prey identified in the stomachs suggested that there was unlikely to be a strong dietary preference. Prey taxa identified in the stomach content of southern stingrays in the present study aligns with previous works in The Bahamas and surrounding areas (Bigelow & Schroeder, 1953; Randall, 1967; Gilliam & Sullivan, 1993). Southern stingrays exhibited a narrower dietary range than Caribbean whiptail ray, indicating a dominance of arthropod prey, as seen by Gilliam and Sullivan (1993), with little contribution from other prey phyla. However, for both stingray species, dietary contributions from SIA differed from those of SCA with results indicating higher contributions of soft-bodied prey such as Annelida, and even a dominance of Mollusca in the diet of southern stingray. Similar differences in the contribution of annelids and molluscs between methods (SCA and SIA) have been observed in previous studies of southern stingray (Tilley et al., 2013), where it was suggested that SCA underrepresented rapidly digested soft-bodied prey (Hyslop, 1980). These results underscore the importance of integrating dietary assessment methods for more representative interpretations.

The varieties of prey consumed by both Caribbean whiptail rays and southern stingrays may suggest opportunistic foraging rather than a dietary specialism. This has been previously suggested for southern stingrays (Bigelow & Schroeder, 1953; Gilliam & Sullivan, 1993; Tilley et al., 2013), and other stingray taxa (Collins et al., 2007; Silva & Uieda, 2007; Ponte et al., 2016). Opportunistic foraging targets prey in proportion to their availability in the habitat, with little selectivity of prey type or energetic quality (Davis & Smith, 2001). Abundance surveys of infaunal biomass around the study sites could help provide insight into this for southern stingrays and Caribbean whiptail rays. We, therefore, recommend the

use of diversity analyses (Shannon, 1948) of benthic communities, alongside foraging strategy analyses (Costello, 1990) for future diet assessments of stingray, as done by O'Shea et al (2017).

Where ecologically similar species co-inhabit an environment, a division in resources is expected in order to avoid competitive exclusion (Langeland et al, 1991; Papastamatiou et al., 2006). Previous work using SCA has suggested that partitioning may occur in coastal communities of sympatric stingray species occupying fringe reefs and seagrass flats of Australia (O'Shea et al., 2013; Pardo et al., 2015). In the present study, there is little evidence of trophic partitioning between southern stingrays and Caribbean whiptail rays, besides the contribution of Mollusca in the diet of southern stingrays. However, the absence of molluscs in the stomach contents of Caribbean whiptail rays, and its consequential exclusion from SIA, may be influenced by biases of SCA, and is insufficient for the determination of trophic partitioning. It remains to be investigated whether they mitigate competition by other means such as temporal or spatial segregation as seen in sympatric stingrays of Ningaloo Reef (O'Shea et al., 2013) and future studies could seek to investigate this using bio-logging approaches (Hussey et al., 2015)

The present study suggests that Caribbean whiptail rays and southern stingrays forage at similar mesopredatory trophic levels to previous studies (Cortés, 1999; Tilley et al., 2013) though at the lower range for Myliobatoidei, particularly the families Dasyatidae and Potamotrygonidae (mean trophic levels; 3.62, 3.62 and 3.71, respectively) (Jacobsen & Bennett, 2013). However, it is possible that trophic levels fluctuate between individuals within these species due to potential ontogenetic or size related dietary shifts, as was seen in the Brown stingray (*Bathytoshia lata*) (Dale et al., 2011), and remains to be investigated.

This study further supports the use of gastric lavage as an effective method for non-lethal stomach sampling of stingrays in the field. Stomach contents were successfully excised on 69 % of attempts which is a lower success rate than previously reported (94.5 % (Elston et al., 2015), 77.8 % (Ajemian et al., 2012), and 74 % (Vaudo & Heithaus, 2011)). It is possible that the use of a bilge pump

(Vaudo & Heithaus, 2011; Elston et al., 2015), or a continuous flow of salt water into the stomach cavity, may prove to be more successful at expelling stomach contents. However, if Caribbean whiptail ray and southern stingray are nocturnal foragers, as has been observed in multiple myliobatiformes (Corcoran et al., 2013; Tilley et al., 2013), and river rays (Garrone Neto & Uieda, 2012), sampling of these species during the day may be less likely to identify prey within stomach content than overnight or early morning sampling. Studies of the diel movements of both species would be helpful in ascertaining this information.

There are, however, a few limitations to the study that must be considered when interpreting the data. Firstly, though the vast majority of samples were collected within a small geographic area (namely from Powell Point and the Schooner cays, see Figure 1), several Caribbean whiptail and southern stingray samples were collected along the length of the Exuma cays, but were not treated independently in analysis due to small sample sizes per site. There may well be differences in δ^{13}C and/ or δ^{15}N signatures between sample locations, though without sufficient samples, or application of iso-scaping models such as those conducted around the UK (Glew et al., 2018), we cannot accurately predict in what way such differences may have influenced our data. Secondly, the use of IRI % as a generalised index of diet has been criticized as not being as un-biased as once thought largely due to its emphasis on FO % which, algebraically, may be misrepresentative (Brown et al., 2012). Brown et al. (2012) have proposed the use of the Prey-Specific Index of Relative Importance (PSIRI %) – an index which incorporates prey-specific measures in the calculation of N % (becoming PN %) and W % (becoming PW %) indices – as a solution to the weaknesses of IRI %. Lastly, the study employs DTDF values of + 2.7 ‰ δ^{15}N and + 0.9 ‰ δ^{13}C due to a lack of previous validation studies (Tilley et al., 2013), however, there are sizeable caveats associated with this. Stable isotope mixed model results have been shown to vary significantly according to the DTDF values applied (Bond & Diamond, 2011; Hussey et al., 2010), which in turn can vary due to a multitude of environmental and physical factors such as taxa, prey diet, spatio-temporal factors, metabolic rate and more (Phillips et al., 2014). Without validated controlled feeding experiments of ray species, estimates of appropriate DTDF values used in studies such as the present must be interpreted with great caution

as their inaccurate use can lead to misrepresentation of true diet (Hussey et al., 2010).

Conclusions

Mesopredatory rays likely have key ecosystem roles (Polis & Strong, 1996), but are largely considered DD hindering the establishment of robust conservation (Simpfendorfer et al., 2011; Morais et al., 2013). Our study presents the first integrated reconstruction of diet for the southern stingray, and the first ever dietary assessment for the Caribbean whiptail ray in The Bahamas. Our results show that the dietary composition of both species are similar, though Caribbean whiptail diet is dominated by arthropods and annelids, while molluscs dominate the diet of southern stingrays. Additionally, both species occupy similar trophic positions, indicative of mesopredatory roles (Cortés, 1999). The integration of SCA and SIA negated biases that otherwise may have influenced results (Hyslop, 1980), thus this data supports integrative approaches to diet assessments for future studies. Our findings have advanced the knowledge of the dietary ecology of two DD stingray species, however, further study of their habitat use, diel movements, foraging strategy, and ontogenetic shifts are required to contribute towards an improved biological understanding for these two species.

Table 1: Dietary compositions of the Caribbean whiptail rays (*Styracura schmardae*) and southern stingrays (*Hypanus americanus*) as seen via gastric lavage stomach content analysis. Identified prey items are listed below the appropriate phylum and noted with the lowest taxonomic level to which they were identified; phylum (p), sub-phylum (s-p), infraclass (ic), order (o), infraorder (io), family (f) and genus (g). Prey proportions are represented as percentage numerical composition (N_c %), percentage weight composition (W_c %), percentage frequency of occurrence (F_o %), and overall percentage index of relative importance (IRI %).

	Styracura schmardae				*Hypanus americanus*			
	N_c%	*W_c%*	*F_o%*	*IRI%*	*N_c%*	*W_c%*	*F_o%*	*IRI%*
Arthropoda	**51.38**	**58.63**	**100**	**70.41**	**67.57**	**62.46**	**100**	**89.43**
Crustacea [(s-p)]	1.83	2.30	10.53	0.69	17.57	16.56	35.71	32.28
Stomatopoda [(o)]	2.75	1.92	10.53	0.78	6.76	1.91	14.29	3.28
Decapoda [(o)]	18.35	13.09	26.32	13.06	1.35	3.32	3.57	0.44
Brachyura [(io)]	3.67	1.65	10.53	0.88	10.81	5.46	17.86	7.69
Portunidae [(f)]	0	0	0	0	5.41	2.60	14.29	2.02
Diogenidae [(f)]	0	0	0	0	1.35	0.33	3.57	0.16
Palaemonidae [(f)]	6.42	16.13	15.79	5.62	5.41	3.07	10.71	2.40
Penaeidae [(f)]	0.92	4.23	5.26	0.43	9.46	16.00	17.86	12.04
Alpheus sp. [(g)]	17.43	19.30	31.58	18.31	5.41	5.50	3.57	1.03
Metapenaeopsis [(g)]	0	0	0	0	4.05	7.72	7.14	2.23
Annelida	**40.37**	**27.83**	**68.42**	**27.02**	**10.81**	**4.37**	**21.43**	**2.98**
Annelida [(p)]	29.36	26.68	57.89	51.22	10.81	4.37	21.43	8.62

Arenicolidae [f]	11.01	1.15	10.53	2.02	0	0	0	0
Mollusca	**0**	**0**	**0**	**0**	**2.10**	**5.91**	**7.14**	**0.33**
Mollusca [p]	0	0	0	0	2.70	5.91	7.14	1.63
Sipuncula	**7.34**	**13.41**	**21.05**	**2.53**	**5.41**	**19.51**	**10.71**	**1.28**
Sipuncula [p]	7.34	13.41	21.05	6.90	5.41	19.51	10.71	7.07
Chordata	**0.92**	**0.13**	**5.26**	**0.03**	**13.51**	**7.75**	**32.14**	**5.98**
Teleostei [ic]	0.92	0.13	5.26	0.09	13.51	7.75	32.14	18.10

Figure 1: Map showing the capture locations of Caribbean whiptail rays (blue points) and southern stingrays (red points) at sites surrounding the Exuma Cays, and Eleuthera Island.

Figure 2: Cumulative prey curves for the Caribbean whiptail ray (*Styracura schmardae)* (a) and the southern stingray (*Hypanus americanus)* (b) after 1000 randomisations of sampled stomachs. Bars represent the standard deviation around each sample.

Figure 3: Bivariate plot of values for Caribbean whiptail rays (a), southern stingray (b) and the four main prey groups; Arthropoda (blue), Annelida (red), Sipuncula (green), Chordata (yellow) and Mollusca (pink). Crossed points represent isotopic ratios of individual stingray for both species, whilst prey groups are presented as combined mean (± s.d.).

Figure 4: Box plots of stable isotope mixing model (SIMM) dietary contribution estimates for Arthropoda (blue), Annelida (red), Sipuncula (green), Chordata (yellow) and Mollusca (pink) in Caribbean whiptail rays (a) and southern stingrays (b). Central boxes present 2.5 – 97.5% confidence intervals, with mid-lines showing the median.

Chapter Two: Isotopic variance of metabolically different body tissues, with applications to method comparison and dietary assessments of ray species.

Ethan E. Wrigglesworth,[1] Molly H. Meadows,[1] Jason Newton,[2] Owen R. O'Shea,[3,4,*] Matthew J. Witt,[1,5] and Lucy A. Hawkes[1,5]

[1]University of Exeter, Centre for Ecology and Conservation, College of Life and Environmental Sciences, Penryn Campus, Cornwall TR10 9FE, UK; [2]NERC Life Sciences Mass Spectrometry Facility, SUERC, Rankine Avenue, East Kilbride, Glasgow G75 OQF, UK; [3]Shark Research and Conservation Program, The Cape Eleuthera Institute, Eleuthera, Bahamas; [4]The Centre for Ocean Research and Education, Po Box 255-16, Gregory Town, Eleuthera, The Bahamas; [5]University of Exeter, College of Life and Environmental Sciences, Environmental Biology Group, Hatherly Laboratories, Exeter, EX4 4PS, UK

Abstract

The study of animal diet has evolved over time and now presents ecologists with numerous techniques from which to choose. Stomach content analysis (SCA) and stable isotope analysis (SIA) have been used in the study of marine rays, but often in isolation. The present study investigates how the use of multiple, metabolically differing tissues (blood, white muscle, and barb) can provide differing temporal insights into the trophic ecology of two data deficient (DD) stingray species, the Caribbean whiptail ray (*Styracura schmardae*) and the southern stingray (*Hypanus americanus*). Between January 2015 and July 2017, 204 stingrays (Caribbean whiptail ray; n = 94, southern stingray; n = 110) were sampled for stable isotope ratios of Carbon ($\delta^{13}C$) and Nitrogen ($\delta^{15}N$) in blood, white muscle, and barb. There were significant differences in $\delta^{13}C$ and $\delta^{15}N$ ratios between all tissues except for southern stingray blood and white muscle. In addition, 38 stingrays (Caribbean whiptail ray: n = 18, southern stingray: n = 20) also underwent gastric lavage between August and December 2016. Dietary contribution estimates from both methods were quantitatively assessed for agreement, the results of which varied according to tissue type used in SIA. There was little fluctuation in method agreement among Caribbean whiptail ray tissues, though an increase in agreement with more metabolically active tissues was observed among southern stingray, indicating possible temporal variance in diet. This study provides the first quantitative comparison of SCA and SIA among rays, and highlights how multi-tissue approaches to SIA may better validate results of temporally limited methods, and presents the potential for multi-tissue approaches to analysis in future dietary assessments.

Introduction

The study of diet is fundamental in biology (Nielsen et al., 2018), and the ecological insights it can provide (i.e. predator-prey interactions, trophic position, resource/niche overlap and/or specialisation, etc.) have proven to be valuable in piecing together whole ecosystem functions among marine environments (Estes et al., 2011), facilitating conservation efforts, and may help predict responses to anthropogenic exploitation (Myers et al., 2007) or climate change (Edwards & Richardson, 2004). Ecologists are presented with an arsenal of techniques for diet and trophic assessments (i.e. visual analysis, bulk or compound stable isotope analysis, and DNA approaches) (Nielsen et al., 2018). Though all methods share the aim of describing species diet and ecological function, due to differing advantages and limitations, consideration of method selection must be taken (Traugott et al., 2013; Matley et al., 2018).

Two methods in particular are prevalent among dietary studies; stomach content analysis (Hyslop, 1980; Cortés, 1997) and stable isotope analysis (Inger & Bearhop, 2008; Logan & Lutcavage, 2010; Boecklen et al., 2011). Stomach content analysis (SCA) assesses dietary composition via visual identification of prey in stomach contents, and describes diet using indices (e.g. numeric/gravimetric contribution, and frequency of occurrence) and composite measures (e.g. Index of relative importance (IRI)) (Pinkas et al., 1970; Hyslop, 1980). Stable isotope analysis (SIA), on the other hand, measures the difference in the relative abundance of light and heavy stable isotopes (e.g. Carbon; C^{12}/C^{13}, and Nitrogen; N^{14}/N^{15}) which are assimilated into organic tissues primarily from diet (Inger & Bearhop, 2008). Stable isotope ratios enrich with trophic level (Nitrogen), and can be indicators of geographic location (Carbon, Hydrogen, Oxygen), therefore, they provide insights into the trophic ecology and habitat use of mobile or elusive species (Post, 2002; Inger & Bearhop, 2008; Hussey et al., 2011). Integrating SCA and SIA can reduce uncertainly in results, or highlight aspects of trophic ecology that may otherwise have remained undetected (Nielsen et al., 2018). The misrepresentation of inconspicuous prey items and 'snapshot' view of diet that limits SCA (Hyslop, 1980; Tierney, 2009), is complemented by the representation of assimilated diet and the temporal scales

that SIA can provide (Inger & Bearhop, 2008; Nielsen et al., 2018), while, without sufficient priors from methods such as SCA, SIA cannot easily differentiate between isotopically similar dietary items and is unable to identify prey sources to a suitable resolution (Nielsen et al., 2018).

A primary benefit of SIA is the previously mentioned temporal scales of diet that it can provide. This is based on the principle that metabolically different tissues represent varying assimilation rates of isotopic signatures, thus differing temporal insights (Bearhop et al., 2002; Dalerum & Angerbjörn, 2005; Malpica-Cruz et al., 2012). Controlled feeding experiments of terrestrial and marine vertebrates have suggested a general trend of more metabolically active tissues (e.g. blood) exhibiting faster turnover rates of assimilation, resulting in a more recent representation of diet (Tieszen et al., 1983; MacNeil et al., 2006). Under these assumptions tissue choice may greatly influence the interpretations of SIA in dietary studies, and approaches utilising multiple metabolically different tissues could be exploited to (1) validate recent diet trends observed via SCA, and (2) observe changes in diet over several temporal scales (MacNeil et al., 2005).

Batoids (skates and rays; hereafter referred to as rays) are cartilaginous elasmobranchs whose trophic ecology has largely been studied using SCA and SIA. In the vast majority of these studies, white muscle tissues are sampled for SIA, which, in the only experimental investigation of isotope assimilation rates among ray tissues, was estimated to have a turnover half-life of 98 days (MacNeil et al 2006). With such a broad period of temporal insight, SIA of white muscle may not reflect the results of SCA, but may better indicate temporal variability in diet, potentially explaining discrepancies in the independent outputs of these analyses. Among elasmobranchs, few studies have utilised multiple tissues in SIA (MacNeil et al., 2005; Matich & Heithaus, 2014), though data supports the use of such analysis as a means of providing greater trophic resolution over single tissue approaches (MacNeil et al., 2005). To our knowledge, no studies have utilised varying tissues in the quantitative assessment of dietary composition among ray species.

In the present study, SIA of Carbon and Nitrogen obtained from blood, white muscle, and barb samples of two data deficient (DD) stingray species, the southern stingray (*Hypanus americanus*) and the Caribbean whiptail ray (*Styracura schmardae*), are used alongside SCA to investigate potential temporal changes in diet, and provide inter-analyses comparisons of dietary outputs. In doing so, this study provides the first quantitative comparison of SCA and SIA among ray species, as well as insights into multi-tissue applications to dietary assessments.

Methods

Study site

Stingrays were captured in the coastline waters of three islands of the Bahamian archipelago; 1) the Schooner cays (24.5404.2 ° N; 76.2212.1 ° W); 2) Powell point, Southern Eleuthera (24.4932.4 ° N; 76.1935.2 ° W); and 3) the Exuma Cays (24.4857.0 ° N; 76.4926.9 ° W) (see Chapter One, Figure. 1). All three locations share the shallow waters of the Grand Bahamas Bank, though, while the Schooner cays are entirely surrounded by shallow waters, the southern and eastern coastlines of Eleuthera and the Exuma cays, respectively, neighbour the deep Exuma Sound. Stingrays were captured in shallow, sandy areas including mangrove creek systems and sandbars, which had been identified as areas of stingray abundance for Caribbean whiptail rays and/or southern stingrays.

Ray capture

Visual surveys from slow-moving shallow draft boats were conducted to locate stingrays prior to capture using spot seining methods as described by O'Shea et al (2017). The boat was used to drive stingrays into shallow waters before being herded by foot into a 10-meter seine net and transferred into a large dip net for data collection. The venomous tail barbs were wrapped with a cotton bandage and secured flush to the tail surface. Pre-existing identification tags were noted, and disc width (the distance between the two widest points of the stingray's pectoral fins) was measured using a flexible measuring tape. All new captures were tagged in the dorsal surface of the left pectoral fin with a new spaghetti tag bearing a unique identification number.

Sample collection

Tissue isotope collection and processing

Three tissue types were collected from rays. First, white muscle samples (~1g) were taken from the pelvic fin of the stingray using a clamp and scissors and were stored on ice in Eppendorf tubes for later processing and analysis. Second, blood was extracted using 18 – 22 G needles and 1 ml syringes from the caudal vein, accessed via the ventral surface of the stingray at the base of the tail. Blood was

then transferred from syringe to a heparinised vacutainer and later centrifuged to separate red blood cells from blood plasma; the red blood cells were retained for further use in the present study. Finally, barb (the vasodentin forming the stinging organ of the tail) was sampled from the tip (~1g) using a clamp and scissors and was stored on ice for later processing and analysis.

Samples of putative prey were collected from stingray capture sites using small aquarium nets. These were also stored on ice for later processing and analysis.

All tissue samples, including those of putative prey, were oven dried at 70°C for 24 hours. Due to carbon-to-nitrogen ratios (C:N) below 3.5 % (Caribbean whiptail ray; mean 3.06 ‰ ± 0.1 ‰ s.d., and southern stingray; 3.07 ‰ ± 0.1 ‰), lipid extraction was not conducted (Post et al., 2007). Urea was also not extracted from the stingray tissues as Shipley et al (2017) suggested that extraction of urea had no effect on $\delta^{15}N$ values in either Caribbean whiptail rays or southern stingrays. Dried samples were homogenised using a pestle and mortar, and a sub-set of the resulting material (0.700 mg ± 0.050 mg) was enclosed into a 5 × 3.5 mm tin capsule (Elemental Microanalysis). Sample combustion was done in an Elementar Pyrocube purge-and-trap elemental analyser run in Nitrogen, Carbon and Sulphur (NCS) mode interfaced with an Elementar VisION isotope ratio mass spectrometer. Two international standards (United States Geological Survey L-glutamic acid (USGS40) and IAEA-S-1, S-2 and S-3 Silver Sulphides) and three internal standards (methane sulphonamide/gelatine (MSAG2), methionine/gelatine (M2) and sulphanilamide/gelatine (SAAG2)) were run alongside the stingray samples for calibration and cross checking against other SIA centres to ensure globally matching (Newton, 2001). Stable isotope ratios of $^{12}C/^{13}C$ and $^{14}N/^{15}N$ are expressed with delta notation (δ) as parts per mil (‰) (McKinney et al., 1950), with resulting values representing the relative difference between stable isotopes of Carbon, Nitrogen and Sulphur in the sample compared to a standard form (Slater et al., 2001).

Stomach content sampling

For all individuals sampled for stomach content, a state of tonic immobility was induced prior to sampling via dorso-ventral encumbrance, resulting in a natural state of paralysis that is thought to reduce stress during sampling procedures (Henningsen, 1994). Using methods outlined by O'Shea et al (2017), gastric lavage was used to extract stomach contents via non-lethal means. The stomach was visualised on the ventral surface of the stingray, and a sterile silicone tube (~ 10 mm diameter) was inserted through the buccal cavity into the stomach. A 60 ml syringe was then used to pump ambient seawater down the tube, inducing regurgitation. Expelled stomach content was captured on meshed netting (1 mm) that lined a catchment tray. This was repeated a maximum of three times (i.e. a maximum of 180 ml of stomach flushing) by which point absence of content was considered an indication of an 'empty' stomach. All collected content was stored in-situ on ice for later identification and analysis.

Analysis

Tissue isotope variance

$\delta^{13}C$ and $\delta^{15}N$ isotope ratios of blood, white muscle, and barb tissues for both stingray species were tested for normality with Shapiro-wilks tests, and ANOVA or Kruskal-Wallis tests were used as appropriate to test for significant differences in isotope ratios between the tissue types. Post-hoc tests were used to indicate the direction of significant results between tissues. Isotope differences between tissues were also tested for significance within individuals using a one-way ANOVA with repeated measures or Friedman's repeated measures tests in relation to the aforementioned normality tests, with relevant post-hoc identification of trend direction.

Stomach content analysis

Total wet mass of stomach contents was recorded to the nearest 0.1 g. Any highly digested and/or unidentifiable items were weighed, inspected, and discarded if further identification was not possible. The remaining prey items were identified to the lowest taxonomic level possible and categorised by respective phylum. For every stomach sample, prey items of each present phyla were counted and weighed to the nearest 0.1 g. These values were then collated into three groups

depending on whether the corresponding individual stingray had been sampled for blood, white muscle, or barb body tissues. For each category, four metrics were used to describe dietary contributions of prey phyla: (i) numerical contribution (N_C %) calculated as total count of a given prey phyla / total count of all prey phyla x 100 (Hyslop, 1980); (ii) gravimetric contribution (W_C %) calculated as total mass (g) of a given prey phyla / total mass of all prey phyla x 100 (Hyslop, 1980); (iii) the frequency of occurrence (F_O %) calculated as total count of stomachs containing a given prey phyla / total count of stomachs with identifiable content x 100 (Vaudo & Heithaus, 2011); and lastly (iv) the index of relative importance (IRI) calculated using the following equation (Pinkas et al.c 1970):

$$IRI = (N_c\% + W_c\%) \times F_o\%$$

The resulting IRI for each prey taxa (IRI_i) was then calculated as a percentage (IRI %) of the sum total of all prey taxa IRI (IRI_t) using the following equation (Cortés, 1997):

$$IRI\% = \left(IRI_i \div \sum IRI_t\right) \times 100$$

Stable isotope analysis and mixing models

Using isotope values of blood, white muscle, and/or barb tissues from individual stingrays of both species sampled for SCA, stable isotope mixing models (SIMM) were used to model the dietary contributions of prey phyla using the package Stable Isotope Mixing Models in R (SIMMR) (Parnell & Inger, 2016). Prey phyla were removed from SIMM if they were not present in the collected stomach contents. Each phylum was treated independently throughout analysis as there was a lack of rationale for combining any of the groups (Phillips et al., 2014). Diet-tissue discrimination factors (DTDF's) of + 2.7 ‰ ± 1 ‰ $\delta^{15}N$ and + 0.9 ‰ ± 1 ‰ $\delta^{13}C$ were applied to prey stable isotope ratios as recommended by Tilley et al (2013) for stingray species lacking validation experiments.

Method agreement

The Czekanowski index of similarity (Feinsinger et al., 1981) was used to measure the agreement between SCA and SIA for Caribbean whiptail rays and

southern stingrays sampled for blood, white muscle, and/or barb tissues. Czekanowski index (CI) is calculated as follows:

$$CI = 1 - \left(0.5 \times \left(\sum_{i=1}^{n} Px_i - Py_i \right) \right)$$

where Px_i (estimate of method x) and Py_i (estimate of method y) are the diet contribution estimates of the ith prey phylum, and n is the total number of prey phyla identified. The CI ranges from 0 to 1, with 0 signifying no agreement in estimates, and 1 signifying total agreement.

Results

Sampling

Between January 2015 and June 2017 a total of 204 stingrays (Caribbean whiptail ray n = 94, southern stingray n = 110) were sampled for body tissues from which a total of 154 samples were acquired from Caribbean whiptail rays (blood n = 30, white muscle n = 94, and barb n = 30), and 157 samples were acquired from southern stingrays (blood n = 26, white muscle n = 101, and barb n = 30). Thirty-eight of these stingrays (Caribbean whiptail ray n = 18, southern stingray n = 28) were captured between August and December 2016 and were additionally sampled for stomach content via gastric lavage.

Tissue isotope variance

Caribbean whiptail ray blood samples had a $\delta^{13}C$ range of -14.71 ‰ to -9.67 ‰ (mean -11.96 ‰ ± 1.30 ‰ s.d.) while $\delta^{15}N$ ranged from 2.94 ‰ to 7.82 ‰ (5.09 ‰ ± 0.85 ‰). White muscle $\delta^{13}C$ ranged from -13.32 ‰ to -5.65 ‰ (-9.46 ‰ ± 1.62 ‰) with a $\delta^{15}N$ range of 2.07 to 7.96 (4.81 ‰ ± 1.19 ‰). Lastly, barb $\delta^{13}C$ ranged from -13.03 to -6.81 (-8.93 ± 1.51 ‰), and $\delta^{15}N$ ranged from 2.78 ‰ to 5.07 ‰ (4.08 ‰ ± 0.61 ‰).

Southern stingray blood samples had a $\delta^{13}C$ range of -13.67 ‰ to -8.40 ‰ (-11.41 ‰ ± 1.46 ‰), and a $\delta^{15}N$ range of 4.68 ‰ to 8.27 ‰ (6.59 ‰ ± 1.02 ‰). White muscle $\delta^{13}C$ ranged from -12.68 ‰ to -6.51 ‰ (-8.95 ‰ ± 1.19 ‰), while $\delta^{15}N$ had a range of 3.13 ‰ to 8.95 ‰ (6.69 ‰ ± 1.11 ‰). Lastly, barb $\delta^{13}C$ had a range of -12.33 ‰ to -6.23 ‰ (-8.22 ‰ ± 1.36 ‰) with a $\delta^{15}N$ range of 3.75 ‰ to 7.16 ‰ (5.56 ‰ ± 0.91 ‰).

$\delta^{15}N$ between tissues

There were significant differences in $\delta^{15}N$ between tissues across both species (Figure 1a & b: Caribbean whiptail ray; Kruskal-Wallis test, $X_2 = 20.7$, df = 2, $p < 0.001$, southern stingray; ANOVA, $F_{2,154} = 13.4$, $p < 0.001$). In Caribbean whiptail

rays, blood had the highest δ^{15}N (mean 5.16 ‰ ± 0.851 ‰ s.d.), followed by white muscle (4.62 ‰ ± 1.20 ‰), then barb (4.22 ‰ ± 0.615 ‰). All tissues were significantly different from one another (Dunn's test, Table 1). In southern stingrays, there was no significant difference between blood (6.59 ‰ ± 1.02 ‰) and white muscle (6.70 ‰ ± 1.12 ‰, Tukey HSD, p = 0.888), however, barb (5.56 ‰ ± 0.917) had significantly lower δ^{15}N distribution than both white muscle, and blood (Tukey HSD, Table 1). The δ^{15}N distribution for Caribbean whiptail rays and southern stingrays was significantly different between tissues across individuals (Friedman's test, X_2 = 38, df = 2, p < 0.001, and ANOVA with repeated measures, $F_{2,34}$ = 87.2, p < 0.001, respectively), though no significance was found between southern stingray blood and white muscle (ANOVA with repeated measures, $F_{2,34}$ = 87.2, p = 1, Table 2). Isotope variance was equal across tissues for δ^{15}N in southern stingray (Bartlett's test, K_2 =1.73, df = 2, p = 0.422), however the variance was not equal for Caribbean whiptail rays (Fligner-Killeen; X_2 = 11.1, df = 2, p = 0.00395).

δ^{13}C between tissues

There were significant differences in δ^{13}C between tissues across both species (Figure 2 c & d: Caribbean whiptail ray; Kruskal-Wallis test, X_2 = 50.2, df = 2, p < 0.001, southern stingray; ANOVA, $F_{2,154}$ = 49.9, p < 0.001). Both species had the same trend in δ^{13}C among tissues with barb representing the highest δ^{13}C distribution (Caribbean whiptail ray mean -8.37 ‰ ± 1.51 ‰ s.d., southern stingray -8.22 ‰ ± 1.37 ‰), followed by white muscle (Caribbean whiptail ray -9.38 ‰ ± 1.62 ‰, southern stingray -8.95 ‰ ± 1.20 ‰), and then blood (Caribbean whiptail ray -11.8 ‰ ± 1.28 ‰, southern stingray -11.4‰ ± 1.46 ‰). All tissues in both species showed significant differences in δ^{13}C distribution from one another (Table 1). There were also significant differences between tissues in both species across individuals (Caribbean whiptail ray; Friedman's test, X_2 = 47.2, df = 2, p < 0.001, southern stingray; ANOVA with repeated measures, $F_{2,52}$ = 147.8, p < 0.001, Table 2), though there was no significant difference between white muscle and barb in southern stingrays (ANOVA with repeated measures post-hoc; Z = -1.93, p = 0.16). Isotope variance of δ^{13}C was equal across tissues for both species (Caribbean whiptail ray; Fligner-Killeen; X_2 = 3.26, df = 2, p = 0.196; southern stingray; Bartlett's test, K_2 = 2.06, df = 2, p = 0.358).

Caribbean whiptail ray SCA

Stomach contents of Caribbean whiptail rays consisted of four prey phyla: Arthropoda, Annelida, Sipuncula, and Chordata. Among the stomachs of rays sampled for blood, phyla from highest to lowest dietary contributions were: Arthropoda, followed by Annelida, Sipuncula, and Chordata. The same trend was observed in the SCA of Caribbean whiptail rays sampled for white muscle, and for those sampled for barb (Figure 2b, d, & f; Table 3).

Southern stingray SCA

Stomach contents of southern stingrays consisted of four prey phyla: Arthropoda, Annelida, Sipuncula, and Chordata; though among the stomach content of southern stingrays sampled for blood, only Arthropoda and Chordata were identified. Among the stomachs of southern stingrays sampled for blood, Arthropoda had the highest dietary contribution followed by Chordata. Within the stomachs of southern stingrays sampled for white muscle and barb, phyla from highest to lowest dietary contribution were: Arthropoda, followed by Chordata, Sipuncula, and Annelida (Figure 3b, d, & f; Table 3).

Stable isotope analysis and mixing models

$\delta^{13}C$ isotopes of Caribbean whiptail ray blood ranged from -14.71 ‰ to -10.24 ‰ (mean -11.79 ‰ ± 1.19 ‰ s.d., n = 16), while $\delta^{15}N$ isotopes ranged from 2.94 ‰ to 6.51 ‰ (5.01 ‰ ± 0.75 ‰). White muscle $\delta^{13}C$ ranged from -12.63 ‰ to -8.06 ‰ (-9.84 ‰ ± 1.37 ‰, n = 17), while $\delta^{15}N$ ranged from 3.24 ‰ to 5.06 ‰ (4.37 ‰ ± 0.43 ‰). Barb tissue $\delta^{13}C$ ranged from -13.03 ‰ to -6.81 ‰ (-9.04 ‰ ± 1.77 ‰, n = 17), while $\delta^{15}N$ ranged from 2.78 ‰ to 5.07 ‰ (4.11 ‰ ± 0.67 ‰).

$\delta^{13}C$ isotopes of southern stingray blood ranged from -13.49 ‰ to -8.04 ‰ (-11.04 ‰ ± 1.47 ‰, n = 11), while $\delta^{15}N$ ranged from 5.30 ‰ to 8.27 ‰ (6.43 ‰ ± 0.97 ‰). White muscle tissue $\delta^{13}C$ ranged from -10.53 ‰ to -6.89 ‰ (-8.67 ‰ ± 0.83 ‰, n =17), while $\delta^{15}N$ ranged from 4.70 ‰ to 7.92 ‰ (6.61 ‰ ± 0.97 ‰). Lastly,

barb tissue $\delta^{13}C$ ranged from -11.48 ‰ to -6.23 ‰ (-8.03 ‰ ± 1.16 ‰, n = 16), while $\delta^{15}N$ ranged from 4.79 ‰ to 7.11 ‰ (5.73 ‰ ± 0.69 ‰). $\delta^{13}C$ and $\delta^{15}N$ isotope ranges and means (± s.d.) of Arthropoda, Annelida, Sipuncula, and Chordata putative prey samples can be seen in supplementary material (Table S1).

Stable isotope mixing models of Caribbean whiptail ray blood estimated the order of phyla, from highest to lowest dietary contributions, as Arthropoda, followed by Sipuncula, Annelida, and Chordata. For white muscle, the estimated order was Arthropoda, followed by Annelida, Sipuncula, and Chordata; while for barb, the estimated order was Annelida, followed by Arthropoda, Sipuncula, and Chordata. (Figure 2a, c, & e; Table 4).

Stable isotope mixing models of southern stingray blood estimated Arthropoda as the highest contributor of diet, followed by Chordata. Estimates from highest to lowest diet contribution using white muscle were Annelida, followed by Arthropoda, Chordata, and Sipuncula, and for barb, Annelida, followed by Arthropoda, Sipuncula, and Chordata (Figure 3a, c, & e; Table 4).

Method agreement

Overall, there was a moderate to good agreement between dietary estimates from SIA and SCA for Caribbean whiptail rays (Figure 4). Of the three tissues sampled, dietary estimates from SIA of blood had the least agreement with SCA (Czekanowski index (CI) = 0.69), followed by barb (CI = 0.77), then white muscle (CI = 0.82). Among southern stingray, however, agreement between methods varied considerably from very good to poor depending on the tissues used in SIMM (Figure 4). Agreement was highest between SIA and SCA for southern stingray blood (CI = 0.94), followed by white muscle (CI = 0.48), and barb (CI = 0.33).

Discussion

Our results show that among both stingray species there is significant variance in isotopic values between all tissues, other than blood and muscle $\delta^{15}N$ in southern stingrays. The metabolic activity of a tissue is thought to drive this variance and the resulting turnover rate of assimilated isotopes can be used to provide insight into differing temporal scales of diet (Tieszen et al., 1983; Logan et al., 2006; MacNeil et al., 2006; Logan & Lutcavage, 2010). Based on this assumption, our results may be indicative of temporal variance in diet, particularly among Caribbean whiptail rays whose tissues showed the most isotopic variability. MacNeil et al. (2005) present a similar study using SIA of liver, white muscle and cartilage of several shark species from the West-Atlantic. Based on the variability of isotopes among these tissues they determined that shortfin mako sharks (*Isurus oxyrinchus*) showed a diet of seasonal variability while consistency in isotopes across tissues in blue (*Prionace glauca*) and common thresher sharks (*Alopias vulpinus*) supported previous theories of year-round generalist diets. Though further study would be required to validate our results, our data thus far suggests that southern stingrays may have a relatively consistent diet over time compared to Caribbean whiptail rays. For both species more metabolically active tissues, blood and/ or white muscle, were more enriched in $\delta^{15}N$ suggesting more recent consumption of prey of a higher trophic level. However, accurate estimates for the timescale of potential dietary shifts cannot be made without validated controlled experiments. There is of only one study to date for rays (MacNeil et al., 2006) which estimated turnover half-lives of 61 days, 98 days and 134 days for blood, white muscle and cartilage from freshwater ocellate river stingrays (*Potamotrygon motoro*), respectively. Changes in prey contributions to the diet across tissue types, as shown via SIMM, do however support a recent diet of a higher trophic level. Stable isotope mixing models showed decreases in the contribution of Annelida and increases in Arthropoda and Sipuncula to the diets of both species from metabolically low to high body tissues (barb, white muscle and blood, respectively).

The trends observed among method agreements somewhat contradict our previous interpretations from isotopic variance in tissues. Stomach content

analysis samples recently ingested dietary components (Hyslop, 1980), therefore, one would expect to see an increase in agreement between SCA and SIA of more metabolically active tissues due to a mutal reflection of recent diet. For example, in MacNeil et al. (2005) SIA of the highly metabolic liver tissue of shortfin mako sharks corresponded well with the previously observed diet shift to inshore populations of bluefish (*Pomatomus saltatrix*) as determined by SCA (Stillwell & Kohler, 1982). In the present study, agreement of SCA and SIA dietary contribution estimates varied in accordance with tissue type, but more so for southern stingray ranging from very good to poor with a trend of increasing agreement from barb, white muscle, and blood. Poor agreement between less metabolically active tissues, such as white muscle and barb, in southern stingrays suggests a more variable diet over time, compared to the relatively consistent concordance seen between SCA and SIA across tissues of Caribbean whiptail rays.

While there maybe contradictions in the temporal interpretations of diet between the analysis of isotopic variance and method agreement, there are important considerations to be made which may be influencing our results. Firstly, as SIA relies heavily on the incorporation of priors for representative estimations, the influence of biases that accompany prior methods such as SCA must be considered with caution (Moore & Semmens, 2008; Nielsen et al., 2018). For example, the agreement between SCA and blood SIA of southern stingrays could be due to the identification of only two prey phyla among stomach content of individuals sampled for blood, resulting in the incorporation of only two food source priors in SIMM. As food source contributions in SIMM must sum to 100% (Phillips et al., 2014) both phyla (Arthropoda and Chordata) resulted in high dietary contribution estimates similar to those of SCA, however, soft-bodied prey such as Annelida and Sipuncula, may have initially been underrepresented by the bias SCA (Cortés, 1997; Hyslop, 1980). In addition to this, multi-tissue SIA would likely profit from priors sampled across relevant timeframes if they are to better represent true shifts in diet (MacNeil et al., 2005). Lastly, isotopic variance between tissues may have several influences beyond metabolic rate. For example, the variation of Carbon and Nitrogen rich/poor nutrient components (lipids, proteins, and carbohydrates) among tissues, can alter the assimilation

and turnover rate of $\delta^{13}C$ and $\delta^{15}N$ through a process called isotopic routing (Schwarcz, 1991; Gannes et al., 1997; Bearhop et al., 2002). Without sufficient data of such influences on ray tissues, temporal inferences of diet remain uncertain.

Conclusions

The vast majority of dietary assessments of rays using SIA utilise white muscle tissue alone (Dale et al., 2011; Barría et al., 2015; Burgess et al., 2016; Weidner et al., 2017), however, exploiting isotopic differences across metabolically different tissues via multi-tissue SIA can provide broader temporal insight (MacNeil et al., 2005). The metabolic activity of a tissue is a primary driver of isotopic turnover rate, with more metabolically active tissues representing recent dietary assimilation (Logan et al., 2006; Logan & Lutcavage, 2010; MacNeil et al., 2006; Malpica-Cruz et al., 2012). The present study identifies how tissue type influences the agreement of SCA and SIA dietary estimates, indicating potential temporal shifts in diet among two DD stingrays. Method agreement among southern stingrays varied greatly suggesting a more temporally variable diet to that of Caribbean whiptail rays. The close agreement of SCA and SIA of southern stingray blood supports the use of highly metabolic tissues as a validator of recent diet, though caution of prior biases must be taken (Moore & Semmens, 2008). Using multiple tissues in SIA also suggested changes in diet over time, though repeated sampling of stomach content, or other indicators of recent consumption (e.g. gut/scat DNA analysis: Traugott et al., 2013; Nielsen et al., 2018), would be required for validation. The lack of experimentally derived insights of turnover rates among rays and elasmobranchs are major deficiencies that must be addressed for better interpretation of isotope studies (Fisk et al., 2008; Martínez del Rio et al., 2009). The duration required for studies to observe total isotopic turnover has resulted in many making estimates based on rates of incomplete turnover (Logan & Lutcavage, 2010; MacNeil et al., 2006). Such estimations are considered unreliable (Martínez del Rio et a., 2009), with true total turnover being at a far slower rate than previously estimated (Kim et al., 2012). Captive rays, such as southern stingrays that are common species in aquariums, could prove to be great tools for future tissue-isotope turnover studies.

Table 1: P-values of post-hoc analyses for δ^{15}N and δ^{13}C isotope distributions between tissues using Dunn's nonparametric test for Caribbean whiptail ray (*S. schmardae*) tissues and Tukey HSD parametric tests for southern stingray (*H. americanus*) tissues. Highlighted cells represent values of significance.

	δ^{15}N		δ^{13}C	
	S. schmardae Dunn's test	*H. americanus* Tukey HSD	*S. schmardae* Dunn's test	*H. americanus* Tukey HSD
Blood vs WM	0.010	0.888	< 0.001	< 0.001
Blood vs Barb	< 0.001	0.001	< 0.001	< 0.001
WM vs Barb	< 0.001	< 0.001	0.017	0.036

Table 2: P-values and test statistics for post-hoc analyses of repeated measures tests of δ^{15}N and δ^{13}C isotopic differences between tissues. Non-parametric Friedman's test with post-hoc analysis for Caribbean whiptail ray (*S. schmardae*) and parametric ANOVA with repeated measures post-hoc analysis for southern stingray (*H. americanus*). Highlighted cell represent values of significance.

	δ^{15}N			δ^{13}C		
	S. schmardae Friedman's post-hoc		*H. americanus* ANOVA RM	*S. schmardae* Friedman's post-hoc		*H. americanus* ANOVA RM
	Z	P-value	P-value	Z	P-value	P-value
Blood vs WM	0.368	< 0.001	1	10.5	< 0.001	< 0.001
Blood vs Barb	11.3	< 0.001	< 0.001	-12.4	< 0.001	< 0.001
WM vs Barb	11.6	< 0.001	< 0.001	-1.93	< 0.001	0.16

Table 3: Contribution of Arthropoda, Annelida, Sipuncula and Chordata among the stomach contents of Caribbean whiptail ray (top) and southern stingray (bottom) sampled for blood, white muscle, and barb tissues. Contribution is described by numeric contribution (N_c %) gravimetric contribution (W_c %), frequency of occurrence (F_o %) and a resulting index of relative importance (IRI %).

	Blood				White Muscle				Barb			
	N_c %	W_c %	F_o %	IRI %	N_c %	W_c %	F_o %	IRI %	N_c %	W_c %	F_o %	IRI %
Arthropoda	53.1	68.1	56.3	61.6	43.9	52.8	56.3	49.7	52.0	66.3	56.3	59.8
Annelida	38.8	17.1	68.8	34.7	46.3	25.2	68.8	45.0	40.0	19.2	68.8	36.6
Sipuncula	7.1	14.7	18.8	3.7	8.5	21.7	18.8	5.2	7.0	14.3	18.8	3.6
Chordata	1.0	0.1	6.3	0.1	1.2	0.2	6.3	0.1	1.0	0.1	6.3	0.1
Arthropoda	86.4	80.1	90.9	94.3	68.3	76.9	88.2	89.7	74.3	60.2	93.8	91.0
Annelida	-	-	-	-	7.3	0.3	11.8	0.6	2.6	0.01	6.3	0.1
Sipuncula	-	-	-	-	4.9	10.9	5.9	0.7	7.7	31.4	12.5	3.5
Chordata	13.6	19.9	27.3	5.7	19.5	11.8	41.2	9.0	15.4	8.3	31.3	5.3

Table 4: Contribution of Arthropoda, Annelida, Sipuncula, and Chordata as estimated by Stable Isotope Mixed Models in R (SIMMR) for Caribbean whiptail ray (*S. schmardae*) and southern stingray (*H. americanus*) sampled for blood, white muscle, and barb. Proportional values are means ± standard deviations of 1000 iterations per prey source.

	S. schmardae			*H. americanus*		
	Blood	White Muscle	Barb	Blood	White Muscle	Barb
Arthropoda	0.55 ± 0.16	0.55 ± 0.17	0.37 ± 0.17	0.88 ± 0.06	0.38 ± 0.12	0.26 ± 0.91
Annelida	0.11 ± 0.08	0.28 ± 0.13	0.47 ± 0.16	-	0.42 ± 0.11	0.62 ± 0.00
Sipuncula	0.31 ± 0.12	0.11 ± 0.08	0.09 ± 0.07	-	0.07 ± 0.05	0.08 ± 0.03
Chordata	0.04 ± 0.03	0.06 ± 0.05	0.06 ± 0.05	0.11 ± 0.06	0.12 ± 0.06	0.04 ± 0.05
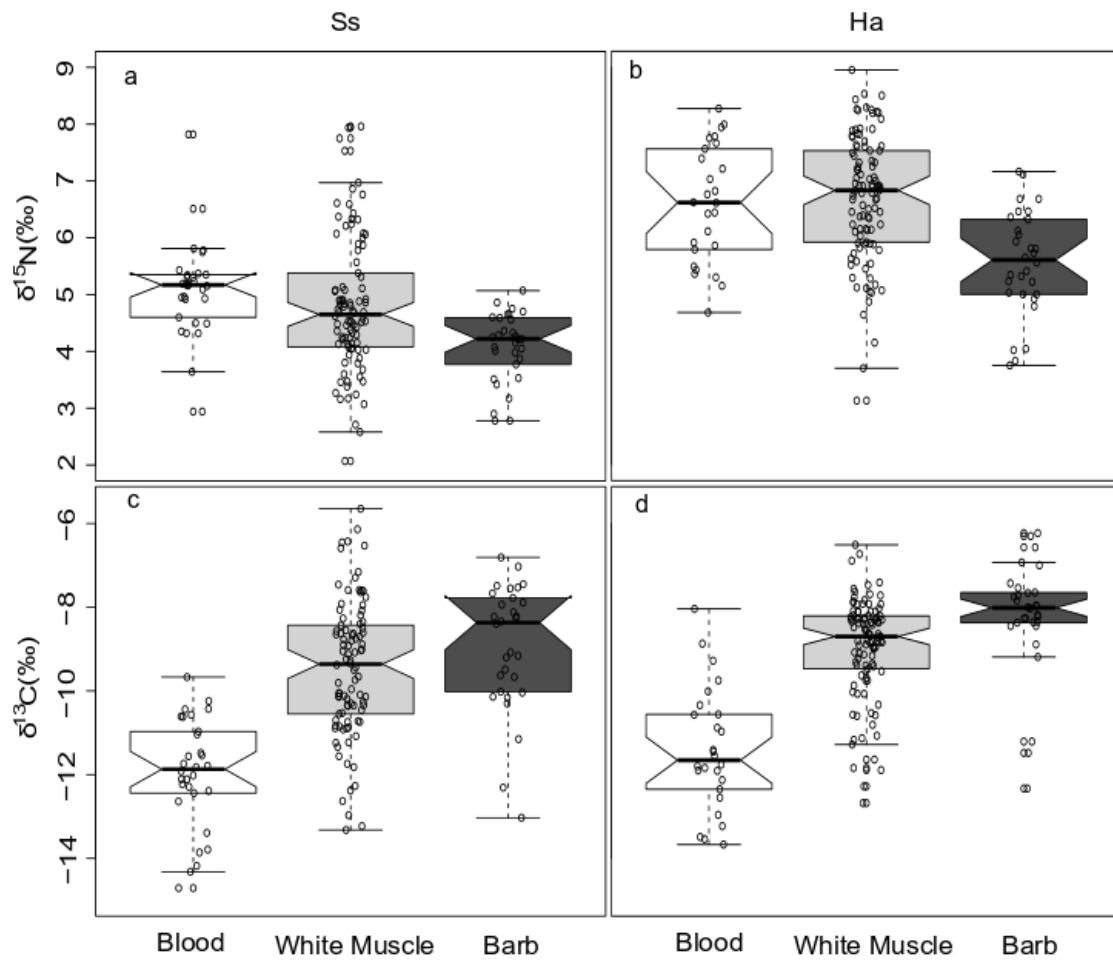
Figure 1: Distribution of $\delta^{15}N$ (a & b) and $\delta^{13}C$ (c & d) isotope ratios for Caribbean whiptail ray (left) and southern stingray (right) blood (white), white muscle (light grey), and barb (dark grey) tissues. Boxes represent 25 – 75 % interquartile ranges with notched means. Absence of overlap in notches between tissues is indicative of significant differences between tissues.

Figure 2: Dietary contribution (%) of Arthropoda, Annelida, Sipuncula, and Chordata as estimated by stable isotope analysis (SIA) and stomach content analysis (SCA) for southern stingrays sampled for blood (a & b), white muscle (c & d) and barb (e & f). Boxes represent 25 – 75 % interquartile ranges of estimates with notches and mid-lines presenting mean values.

Figure 3: Dietary contribution (%) of Arthropoda, Annelida, Sipuncula, and Chordata as estimated by stable isotope analysis (SIA) and stomach content analysis (SCA) for Caribbean whiptail rays sampled for blood (a & b), white muscle (c & d) and barb (e & f). Boxes represent 25 – 75 % interquartile ranges of estimates with notches and mid-lines presenting mean values.

Figure 4: Pairwise comparisons of diet contribution estimates from stomach content analysis and stable isotope analysis of Caribbean whiptail rays (*Ss*; light grey) and southern stingrays (*Ha*; dark grey) sampled for blood, white muscle and barb tissues. Czekanowski's similarity index ranges from 0 to 1, with a result of 1 representing complete overlap between the methods, while 0 denotes no overlap.

General discussion

The study of animal diet is a useful tool to understand species contributions to whole ecosystem ecological functions (Navia et al., 2010). Many ray species are known to be mesopredators but are otherwise poorly studied, lacking sufficient data to facilitate the conservation management required to mitigate extrinsic pressures (Shiffman et al., 2012; Simpfendorfer et al., 2011)

In **Chapter One,** I integrated stomach content analysis (SCA) and stable isotope analysis (SIA) to gain insight into the ecology of two data deficient (DD) stingrays providing the first integrative use of these methods in dietary assessments of the southern stingray (*Hypanus americanus*), and the first ever trophic assessment of the Caribbean whiptail ray (*Styracura schmardae*). Prey identified through SCA aligned with previous findings of southern stingray in The Bahamas and surrounding areas (Bigelow & Schroeder, 1953; Randall, 1967; Gilliam & Sullivan, 1993) and was similar to those of other benthic ray species (Espinoza et al., 2013; Jacobsen & Bennett, 2013; O'Shea et al., 2013; O'Shea et al., 2017). Despite sympatry, dietary compositions of both species were similar composing of four phyla (Arthropoda, Annelida, Sipuncula, and Chordata), though southern stingray also consumed Mollusca. Arthropoda and Annelida dominated the diet of Caribbean whiptail rays and contributed notably to the diet of southern stingrays, but Mollusca dominated SIA estimates of southern stingrays. The absence of molluscs in Caribbean whiptail stomach content, and its subsequent exclusion from corresponding SIA, may be influenced by misrepresentative biases of SCA and may not be evident of trophic resource partitioning. The breadth of prey taxa identified among the stomach contents of both species was indicative of opportunistic feeding (Davis & Smith, 2001). Stomach content analysis underestimated the contribution of soft-bodied prey, such as Annelida, in dietary estimates of both species compared to SIA. Such discrepancies support the suggestions of Tilley et al. (2013) when comparing their own SIA dietary estimates of southern stingray to previous independent uses of SCA (Bigelow & Schroeder, 1953; Gilliam, & Sullivan, 1993). Furthermore, the study supported the integration of SCA and SIA as a way of better representing true diet (Dalerum & Angerbjörn, 2005; Espinoza et al., 2015).

Chapter Two further investigated diet with a focus on multi-tissue variance and temporal insights of SIA. Metabolically different tissues have been observed to have differing rates of isotopic turnover (MacNeil et al., 2006; Malpica-Cruz et al., 2012) resulting in differing temporal insights into trophic ecology when used in SIA (Inger & Bearhop, 2008). Thus far few studies have incorporated the use of multiple metabolically different tissues into dietary assessments (MacNeil et al., 2005). Our results suggest that δ^{13}C and δ^{15}N differed between blood, white muscle and barb tissues in both species of stingray which could be attributed to the respective metabolic activities of the different tissues. Following this, the most metabolically active tissues, reflecting the most recent prey consumption, can be used to validate methods such as SCA. The potential application of multiple tissues in SIA can thus be used to investigate temporal shifts in diet, though with the caveat of appropriate priors.

This thesis has furthered our understanding of trophic ecology for the DD southern stingray and Caribbean whiptail ray, while outlining possible applications of dietary assessment techniques in isolation and integration, which could prove valuable in future studies of rays. However, there is still a lack of understanding of ray spatial and diel patterns, as well as the isotopic turnover rates and influences of ray tissues. The use of multi-tissue approaches to isotopic dietary assessment would greatly attribute to ecological understanding of these significant and vulnerable animals, but the aforementioned deficiencies must be addressed if we are to better ecological inferences.

Supplementary Material

Table S1: Sample size (n), and minimum (Min); mean (± s.d.); and maximum (Max) isotopic values of $\delta^{13}C$ and $\delta^{15}N$ for prey phyla used in stable isotope analysis.

		$\delta^{13}C$				$\delta^{15}N$	
Prey	n	Min	Mean	Max	Min	Mean	Max
Arthropoda	64	- 15.56	-11.73 ± 1.59	- 8.52	1.39	3.81 ± 1.23	7.51
Annelida	15	- 15.03	-10.14 ± 3.91	- 2.84	2.57	3.47 ± 0.63	5.21
Sipuncula	8	- 22.51	-13.76 ± 3.97	- 8.79	2.71	3.35 ± 0.49	4.28
Chordata	12	- 14.23	-10.43 ± 2.93	- 3.73	4.53	7.35 ± 1.82	10.68
Mollusca	6	- 14.49	-10.79 ± 3.03	- 7.24	1.28	3.21 ± 1.71	5.46

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