Examining spatial and trophic ecology of Bahamian stingrays, *Styracura schmardae* and *Hypanus americanus*, using stable isotope analysis

Submitted by Molly Hebe Meadows to the University of Exeter as a thesis for the degree of Master of Science by Research in Biological Sciences, September 2018.

I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

Signature: ……..

Abstract

In this thesis I use stable isotope analysis to investigate the spatial and dietary ecology of two species of tropical stingray, the southern stingray (*Hypanus americanus*) and the Caribbean whiptail ray (*Styracura schmardae*) from Eleuthera island, The Bahamas. In **Chapter 1,** I directly compare stable isotopes of carbon, nitrogen and sulphur between the two species (*S. schmardae*, n = 96 ; *H. americanus*, n = 102) to investigate if and how these sympatric stingrays exhibit resource partitioning. I show that mangrove creek systems may be important habitat for *S. schmardae*, mitigating competition with *H. americanus*, and that trophic resource partitioning may also be occurring, with *H. americanus* feeding at a higher trophic level than *S. schmardae.* In **Chapter 2**, I explore the use of stable isotope analysis in detecting ontogenetic shifts in *H. americanus* (n = 110) and *S. schmardae* (n = 94). Here, I use breakpoint analysis to pinpoint shifts in mean $\delta^{15}N$ and $\delta^{13}C$ as body size increases, on three metabolically distinct tissues, which therefore give insights into different time periods: whole blood, white muscle and cartilage (barb). There were four breakpoints in white muscle samples, two in blood and in cartilage only one. We recommend that future research determining ontogenetic shifts via stable isotopes utilise this range of tissues. Breakpoints in $\delta^{13}C$ were observed in both species, indicating ontogenetic habitat shifts occurring at juvenile sizes. A second shift was detected at larger body sizes in both $\delta^{15}N$ and $\delta^{13}C$ for *S. schmardae,* we suggest this second ontogenetic niche shift indicates a return to mangroves and concurrent increase in higher trophic level prey by adults. The findings presented in this thesis are novel for both species, emphasising the significance of mangroves habitats as well as providing the first ever assessment of resource use by the poorly studied Caribbean whiptail ray. Findings could be used to build

conservation frameworks to protect southern stingrays, Caribbean whiptail rays, and the mangroves that appear to be intrinsic to their ecology.

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Author's Declaration

This work was carried out in conjunction with the Cape Eleuthera Institute Stingray Research programme and was also supported by a grant from the Natural Environment Research Council (NERC) for isotope analysis at the LSMSF East Kilbride (EK287-10/17). I coordinated, supervised and participated in fieldwork collecting stingray biopsy samples between August and December 2016. Following this, alongside Ethan Wrigglesworth, I carried out all sample processing at the University of Exeter. I co-wrote the grant for the LSMSF facility use with Ethan Wrigglesworth and Lucy Hawkes. Stable isotope analysis took place at the East Kilbride Life Sciences Mass Spectrometry Facility, led by Dr. Jason Newton. I carried out all subsequent data analysis, manuscript writing, thesis compilation and formatting. Dr. Lucy Hawkes and Dr. Owen O'Shea provided guidance and comments throughout the project. Dr. Matthew Witt and Dr. Richard Inger supplied guidance for statistical analysis. Dr. Jason Newton provided additional comments and guidance for Chapter 1. The project was designed based upon work by Dr. Owen O'Shea at the Cape Eleuthera Institute and was used to help inform the methodology and supply additional biopsy samples from additional stingray sampling between 2015 and 2017.

Acknowledgements

There are a number of people I would like to thank for supporting me throughout the course of my Master's degree. Firstly, I would like to thank my supervisors, Dr Lucy Hawkes, Dr Owen O'Shea and Dr Matthew Witt, for their endless advice and guidance. A special thank you to Lucy for all of the edits you have supplied to me (working throughout weekends!), from your guidance I can really see the growth in myself as a scientist and thank you for taking a leap of faith in me from the outset. Thank you to Owen for your constant encouragement during both fieldwork and the write up stage, apologies for spontaneously crying every now and again. I would like to thank the Cape Eleuthera Institute (CEI) Shark and Ray interns, Morgan Eason, Celine Terfloth, Vicky Herbert, Casey Sullivan, Catherine Brown and Chris Brown, as well as all of the Ray Team Fall 2016 (Jake, Neila, Garrett, Ellie, Claire, Cianan and Ethan) for your unwavering endurance during data collection (All Ray, All Day!). Thanks to all the CEI community for being an example of sustainable living which we should all seek to emulate, especially to Brad, Maggie, Mark, Ron, Karen, Georgie and Mackie for the all the good times spent both at work and play. Thank you to Jason Newton and Richard Inger for teaching me all about the magical world of stable isotopes, and thank you to LSMSF and NERC for supplying the lab facilities for said, stable isotope analysis. I would like to thank Josh, Ju and Tom for the dungeon and dragons based respite during the hard times. A very special thank you to my family for always, always being on my side, especially for your patience and support during the toughest of times, you are all amazing! Shout out to Tiffy and Grandad for housing, feeding and cleaning up after me for approximately 8 months. Finally, thank you to Ethan Wrigglesworth for being my stalwart partner in this adventure, it was not the first, nor will it be the last.

General Introduction

An introduction to batoids

Elasmobranchs are one of the most ancient groups in the animal kingdom, characterised by cartilaginous skeletons as well as delayed maturity, low fecundity and long lives. These features are ultimately less compatible with exploitation and increasing habitat change which is conducive to 74 of the total 465 species being of conservation concern (Dulvy *et al.*, 2008; Lucifora *et al.*, 2011; Dulvy *et al.*, 2014). Batoid elasmobranchs, otherwise known as skates and rays, have been subject to far less research effort and perhaps consequently the knowledge base behind them is much smaller than in sharks. There are over 600 batoid species globally, occupying ecological niches across all major aquatic bio-realms (Kriwet *et al.*, 2009), however almost 50% of skates and rays are classified as data deficient by the International Union for the Conservation of Nature (IUCN) Red List (Dulvy *et al.*, 2014), hindering conservation efforts (Bland *et al.*, 2015). Myliobatoidei contain the two groups commonly known as stingrays and eagle rays. Henceforth, the term 'stingray' will refer to demersal members of myliobatoidei, excluding eagle rays. Stingrays (n=220 species) usually have venomous, barbed tails and are largely found in subtropical coastal waters (Lovejoy, 1996; Aschliman *et al.*, 2012).

Diet of stingrays

Almost all (98% of species) batoids are demersal feeders, with the notable exception of pelagic species such as oceanic manta rays (Camhi *et al.*, 2009). Demersal stingrays hunt buried, benthic prey and a body of research has addressed diet preferences in several stingray species (Table 1). Most research to date has concluded that stingrays are carnivorous and the primary sources of

food are crustaceans, annelids, molluscs and bivalves (Collins *et al.*, 2007; Table 1). Stingrays are extremely well adapted to preying on benthic invertebrates, with ventral mouths, electrosensory capabilities for detecting bioelectric cues from prey up to 25 cm deep in benthos (Haine *et al.*, 2001), and having grinding plate dentition (Summers *et al.*, 1998). These adaptations are specialized towards hunting benthic prey, however stingrays will occasionally feed on more unusual prey items, including lancelets, octopus, and even other stingrays (Stokes and Holland, 1992; Gilliam and Sullivan, 1993; Branco-Nunes *et al.*, 2016; Dean *et al.*, 2017). Southern stingrays (*Hypanus americanus)* in San Salvador, for example, have been observed opportunistically preying upon sea urchins (Grun, 2016; Elston *et al.*, 2017). Stomach content analysis has previously suggested that stingrays feed on at least seven different taxa (Table 1; Pardo *et al.*, 2015), although they have also been categorised as dietary specialists (Collins *et al.*, 2007; Ajemian and Powers, 2012).

Stingrays tend to be mesopredators - organisms that occupy the middle range of the trophic system, providing prey for some species while being prey for others. Jacobsen and Bennett (2013) compared the diet and trophic level (T_L) estimates of 75 batoid species. Although the diet of these batoids were similar (varying only by 3.92% for crustacean prey and 3.43% for teleost prey), they classified stingrays across both secondary and tertiary consumer levels, ranging from 3.10 T_L to 4.24 T_L. This may reflect the trophic distribution of prey within the study ecosystems (McCann, 2000; Cardinale *et al.*, 2006) but also may suggest that stingrays can act as ecosystem stabilisers with the ability to absorb trophic perturbations (Tilley *et al.*, 2013a). The strength of omnivory by generalist species in Caribbean marine food webs reduces the likelihood of trophic cascades by removal of apex predators (Bascompte *et al.*, 2005).

Methods used to study stingray diet

Studying the diet of wild rays has been predominately approached by using stomach content analysis. Stomach contents can be obtained in one of two ways, either by excising the stomach and its contents postmortem, or non-lethally using gastric lavage - flushing and collecting the stomach contents from live individuals. Stomach content analysis usually reports diet via the index of relative importance (IRI) proposed by Pinkas (*et al.*, 1971). Both methods of extracting stomach contents are technically simple, but have important ethical implications (Barnett *et al.*, 2010; Heupel and Simpfendorfer, 2010). Stomach content analysis can reveal recently consumed prey items, but for easily digestible soft-bodied prey it may not give insight into longer-term diet (Hyslop, 1980). For example, Santic *et al.* (2011) and Ponte (2016) found decapod prey to be the most statistically important dietary item for the common stingray (*Dasyatis pastinaca*), whereas Saglam *et al.* (2010) found that shrimp was the main dietary component.

More recently stable isotope analysis (SIA) has been adopted for use in dietary studies, as a less invasive and potentially more comprehensive way to estimate diet in wild animals (Hussey *et al.*, 2012). The elements used in SIA and which are of ecological relevance contain at least two forms of stable isotope, each form consists of a different number of neutrons and therefore have differing masses. These slight discrepancies in mass create different reaction kinetics and bond energies within biological processes which in turn produces more significant disparity between the isotopic composition of prey and that of the body tissues of the consumer, a process known as isotopic fractionation (Ramos and González-Solís, 2012). The proportion of various elemental isotopes which have been assimilated into body tissues via digestion can be used to represent certain

ecological gradients. The ratio of nitrogen isotopes is used to make assumptions regarding a species' trophic ecology when compared to the baseline of a trophic system (Inger and Bearhop, 2008). Stable isotopes can reflect diet because the lighter nitrogen isotope (^{14}N) is preferentially absorbed and subsequently excreted within metabolic products with each trophic step, therefore leading to kinetic fractionation where a greater residue of the heavy nitrogen isotope remains for tissue synthesis with increasing trophic level. Stable isotopes can be used to predict other ecological patterns; carbon and sulphur isotopes are used as tracer elements due low isotopic fractionation between trophic levels. Carbon isotopes can indicate the origin of their prey; comparisons between tracer isotopes from a certain environment and carbon within an organism's body tissues can be used to infer habitat use (Fry, 1983; Wolf *et al.*, 2009; Trueman *et al*., 2012). Sulphur isotopes can predict a species' habitat preference within anaerobic environments such as mangroves systems and salt marshes, the ratio of ^{34}S within their tissues reflects a sulphide-rich versus sulphate-rich environment. Stable isotope analysis only requires a small amount of biological tissue (<1 g), and therefore can usually be taken from the animal as a non-lethal and far less invasive method than gastric lavage. In addition, different body tissues have different metabolic rates – from highly metabolically active blood to cartilage, which is significantly less metabolically active following synthesis (Hussey *et al.*, 2012). Because isotopes are assimilated into tissues during synthesis, the various tissues will reflect different time periods, thus differing temporal scales of resource use can be studied by sampling across tissues. Although SIA has many benefits which are useful in ecological studies of marine species, there are a number of limitations which must be considered. Isotopic tracer elements such as carbon and sulphur rely heavily on past literature for

comparison with environmental tracer values. Another important consideration is that isotopic fractionation can be influenced by inconsistent biological processes such as isotopic routing, which may influence the isotopic signature measured in different species/tissues in unpredictable ways (Boecklen *et al.*, 2011). If these limitations are considered and appropriate measures are taken to mitigate against them then SIA could be an effective way of analysing resource use patterns within ecosystems to answer critical ecological concerns regarding elasmobranch species (Shiffman *et al*., 2012).

Habitat preference in stingrays

Batoids occupy almost all marine habitats across the globe, from the open ocean to sub-tropical nearshore waters, and freshwater species of batoids are also found, for example in rivers of South America (Compagno, 1990; Last *et al*., 2016). Stingrays are most commonly found in reef, mangrove or sand bar/beach habitats, and make directed movements between these key habitats (Davy *et al*., 2015) where a sufficient abundance of preferred prey may be located (Costa *et al*., 2015). Spatial feeding zones are important to stingrays (O'Shea *et al*., 2013), and most species undertake solitary foraging activities (Semeniuk and Rothley, 2008). Adult southern stingrays are proposed to have a home range, potentially using reefs as landmarks (Tilley *et al*., 2013b). They require shallow nearshore environments to breed and give birth (Jirik and Lowe, 2012). Stingrays are ovoviviparous, which distinguishes them from skates, which lay egg cases (Wourms and Demski, 1993). Live born young require shelter from birth onwards (Leis and McCormick, 2002); juvenile stingrays are faced with trade-offs between slow growth and shelter in mangrove creek systems versus predation (Ajemian and Powers, 2012; Dale *et al*., 2014; Ajemian and Powers, 2016). Among

elasmobranch taxa, juvenile shifts in habitat use have been linked to increased body size (Heupel *et al*., 2007). For example, southern stingrays (*Hypanus americanus*) apparently leave nursery areas only when they reach a size where predation poses less risk (Aguiar *et al*., 2009). This may be common amongst many species of stingrays, including neotropical stingrays in freshwater river habitats (Garrone Neto and Uieda, 2012). There are also examples of stingrays segregating according to sex (Costa *et al*., 2015; Jirik and Lowe 2012). In 2006, Wallman and Bennett found that female Atlantic stingrays (*Hypanus sabinus)* preferred areas of warm, shallow open water, whereas males showed no preference, this was proposed to be because higher temperatures offer sex specific reproductive benefits.

Stingrays in The Bahamas

The Caribbean whiptail stingray (*Styracura schmardae*) has only recently been officially recorded as resident in The Bahamas (O'Shea *et al.*, 2017). In more recent years, studies concerning parasites of *S. schmardae*, and studies of their phylogeny have been published (See Table 2 for a comprehensive list of all publications which refer to *S. schmardae*). The species has undergone multiple taxonomic revisions since it was named as *Trygon schmardae* in 1904. It has recently been placed as a member of the Potamotrygonidae family, in a new genus containing *S. schmardae* and its Pacific counterpart *Styracura pacifica* (Carvalho *et al.*, 2016; see supplementary materials for phylogenetic tree). The southern stingray (*Hypanus americanus*) is also found in The Bahamas, this more common species is more comprehensively described than the Caribbean whiptail ray, but both the southern stingray and the Caribbean whiptail ray are listed as data-deficient by the IUCN. Studies of *H. americanus* have been quite varied,

ranging from mutualistic interactions with cleaner fish to mating behaviour of captive individuals (See Table 3 regarding all publications regarding *H. americanus* as study species).

This research

Using multivariate analyses of nitrogen, carbon and sulphur isotopes, I explore ecological patterns of wild stingrays, *H. americanus* and *S. schmardae*. Throughout this thesis I examine resource distribution by these species both inter- and intra-specifically. This research includes the first comprehensive study of ecological resource use by *S. schmardae* and offers new insights of the trophic and spatial ecology of *H. americanus* in The Bahamas.

Table 1: Most recent diet descriptions from stomach content analysis of marine demersal myliobatoid species showing percentage index of relative importance (standardised diet measurement: Pinkas *et al.,* 1971) for each prey group, species and reference.

Species	Reference	Decapoda - other	Decapoda - shrimp	Molluscs	Sipuncula	Teleostei	Nemertea	Polychaeta	Echinodermata	Enteropneusta
Chilean round ray (*Urotrygon chilensis*)	Onate-Gonsalez *et al.*,, 2017.	16.2	58.3	0.1		2		23.3		
Munda round ray (*Urotrygon munda*)	Flores-Ortega, 2011.	19.2	78.5			2.4				
Roger's round ray (*Urotrygon rogersi*)	Flores-Ortega, 2011.	5.3	90			2		2.6		
Round stingray (*Urobatis halleri*)	Flores-Ortega, 2011.	6.7	92.7			0.5		0.1		
Crossback stingaree (*Urolophus cruciatus)*	Yick *et al.*,, 2011.	1.9	84.8	0.6	2.2		0.3	30.3		
Porcupine ray (*Urogymnus asperrimus*)	Elston *et al.*,, 2017.	7.6	9.4		3		1.8	78.1		
Brown whipray (*Himantura toshi)*	Pardo *et al.*,, 2015.	15.9	84.2			0.1				
Black-spotted whipray *(Himantura astra)*	Jacobsen & Bennett, 2011.	12.3	86.9	0.1		0.2		0.9	0.1	
Southern stingray (*Hypanus americanus)*	Gilliam & Sullivan, 1993.	77.8	19.1	3.4		9.2		0.2		
Common stingray *(Dasyatis pastinaca)*	Ponte *et al.*,, 2016.	87.5	15.7			0.5		18.2		
Estuary stingray *(Dasyatis fluviorum)*	Pardo *et al.*,, 2015.	52.8	12.5			1.9		32.9		
Brazilian large-eyed stingray *(Dasyatis marianae)*	Costa *et al.*,, 2015.	39.5	43.9		3.8		0.2	12.2	0.4	
Blue stingray *(Dasyatis chrysonota)*	Ebert & Cowley, 2003.	1.5	24.3	1.3		1.4	2.3	59.3		9.3
Groovebelly stingray *(Dasyatis hypostigma)*	Ruocco & Lucifora, 2017.	3.5	89.5	1.1				7		0.2
Bluespotted maskray (*Neotrygon kuhlii)*	Pardo *et al.*,, 2015.	0.6	4.7	3.9		0.8		90		
Peppered maskray (*Neotrygon picta*)	Jacobsen & Bennett, 2012.	0.2	82.5	0.1		0.6		15	0.1	0.4
Plain maskray *(Neotrygon annotata)*	Jacobsen & Bennett, 2012.	0.1	70.3	0.2	0.2	4.0		25.5	0.1	

Table 2: Comprehensive list of all publications (Pre 2018) which refer to the Caribbean whiptail ray (*Styracura schmardae*) sorted into subject categories.

Phylogeny	**Parasite**	**Descriptive/Range**	**Stable Isotope Methodology**
De Carvalho *et al.*, 2016	Trevisan and Marques, 2017	O'Shea *et al.*, 2017	Shipley *et al.*, 2017
Last *et al.*, 2016	Marques *et al.*, 2001	Bohlke, ~1961	
Bertozzi *et al.*, 2016	Marques *et al.*, 1997		
Rosenberger, 2001	Marques *et al.*, 1996		
Lovejoy, 1996	Brooks, 1977		

Table 3: Comprehensive list of all publications (Pre 2018) which use the southern stingray (*Hypanus americanus)* sorted into subject categories.

Reproduction	**Electrosense**	**Morphology**	**Biochemistry**	**Diet**	**Habitat use**	**Population**	**Interaction with other species**	**Tourism effects**	**Fisheries Bycatch**	**Husbandry**
Ramírez-Mosqueda *et al.*, 2012	O'Connell, 2011	Mendoza-Carranza *et al.*, 2016	Shipley *et al.*, 2017	Tilley *et al.*, 2013a	Aguiar *et al.*, 2009	Branco-Nunes *et al.*, 2016	Kajiura *et al.*, 2009	Corcoran *et al.*, 2013	Briones *et al.*, 2017	Henningsen, 2010
Chapman *et al.*, 2003	O'Connell, 2010	Wakida-Kusunoki, 2015	Phillips *et al.*, 2016	Gilliam and Sullivan, 1993		Tagliafico *et al.*, 2013	Souza *et al.*, 2007	Semeniuk and Rothley, 2008		Henningsen, 1994
Henningsen, 2000		Schwartz and Safrit, 1977	Grant *et al.*, 2013	Stokes and Holland, 1992		Tilley & Strindberg, 2013	Snelson *et al.*, 1990	Semeniuk *et al.*, 2007		
Hamlett *et al.*, 1996a			Cain *et al.*, 2004			Tilley *et al.*, 2013b		Corcoran, 2006		
Hamlett *et al.*, 1996b			Nunez and Trant, 1997			Carvalho *et al.*, 2010		Shackley, 1998		
Brockman, 1975						Pikitch *et al.*, 2005				

Chapter 1: Resource partitioning in two species of sympatric stingray from The Bahamas

Key words: *Stable isotope analysis, Resource partitioning, Elasmobranch, Stingray, Mangrove*

Abstract

Tropical coastal environments including mangrove creek systems are threatened by anthropogenic disturbances. Stingrays fill important roles that support and maintain these ecosystems. In The Bahamas, two data-deficient demersal stingrays coexist within these habitats, but whether they exhibit resource partitioning in order to avoid competition had yet to be investigated. Analysis of stable isotopes was carried out on white muscle samples of 96 Caribbean whiptail rays (*Styracura schmardae*) and 102 Southern stingrays (*Hypanus americanus*), carbon, nitrogen and sulphur isotopic compositions were measured for each species and used to compare and distinguish ecological factors. Nitrogen isotopes suggested that *H. americanus* feeds at a higher trophic level than *S. schmardae,* potentially implying dietary resource partitioning; however, competition is still present due to an overlap of diets by 35.6% (total ellipse area). Positive correlation between sulphur and nitrogen distribution suggest trophic differences between species are due to differences in habitat use of sulphide-rich environments, specifically mangroves. The combination of carbon and sulphur isotopes suggests that mangrove creek systems are a vital habitat for both species but especially for the poorly-studied Caribbean whiptail ray. Spatial resource partitioning could be occurring in the Bahamas between these two species. However, it is entirely possible that the coexistence of these

sympatric stingray species could be due to pre-existing ecological tendencies of *S. schmardae* in relation to mangrove habitat use. *S. schmardae* may not be restricted to mangrove systems due to resource partitioning, the mitigation of competition could be a secondary effect of their innate habitat use.

Introduction

Coastal environments in the tropics contain a multitude of interconnecting habitats, including coral reefs, lagoons and mangrove creek systems, and are among some of the most productive ecosystems in the world (Moberg and Folke, 1999; Nagelkerken, 2009). Such ecosystems occupy areas which are valuable to tourism, and consequently face increased anthropogenic impacts (Ellison and Farnsworth, 1996; Davenport and Davenport, 2006; Lotze, 2006). For example, The Bahamas is a developing nation that relies significantly on economic input from tourism, yet being an island nation lacks effective means in regulating access to these vulnerable marine environments (Orams, 2002). Mangrove habitats especially are usually offered less protection than the more popular yet fragile coral reefs (Rönnbäck, 1999; Lugo, 2002). For the majority of coastal communities, mangroves offer essential nursery habitat to economically important reef and fishery species (Mumby *et al.*, 2004; Barbier *et al.*, 2011). Despite all these benefits, mangroves are often undervalued and within The Bahamas they are afforded no protective legislation or status (O'Shea *et al.*, 2017). The functional diversity of a community contributes to the performance and overall health of the ecosystem they occupy; they modify and enhance productivity through ecological behaviours and interactions (Cadotte *et al.*, 2011).

Stingrays belong to the superorder Batoidea, comprising around 650 extant species globally, occupying every major aquatic bioregion on the planet including Caribbean mangrove creek habitats (Kriwet *et al.*, 2009). Almost 50% of this group are classified as data deficient by the International Union for the Conservation of Nature's (IUCN) Red List (Dulvy *et al.*, 2014) limiting informed design of effective conservation legislation (Bland *et al.*, 2015). 'Stingray' is a general term and is used to describe a number of species of batoid rays, the

phrase does not denote any fixed order or family; in the present study we distinguish stingrays as demersal members of the suborder Myliobatoidei. Stingrays are thought to provide services critical to ecosystem function, this has largely been centred around their feeding and predator avoidance behaviours. They act as mesopredators, occupying intermediate trophic positions that impact both their predators and prey (Vaudo and Heithaus, 2011). They are also bioturbators (Cadée, 2001; O'Shea *et al.*, 2012), sifting through sediment for prey and to avoid predators (Aller, 1994), which oxygenates the sediment (increasing the population of the microbial denitrifying bacteria (Gilbert *et al.*, 1995)) and changes the topography of the seabed (VanBlaricom 1982, Cross and Curran 2000, Zajac, 2004). As potential 'ecosystem engineers' in these back-reef ecosystems they provide essential services to the habitats they occupy (Meysman *et al.*, 2006).

Another consideration for animal influences on an environment are the ways in which they interact with other species sharing the environment. When multiple species, which favour the same ecological niche, also inhabit the same ecosystem there will be increased pressure on resource availability, therefore resource partitioning on one or more ecological gradients usually occurs to reduce pressure and avoid competitive exclusion (Schoener, 1974; Kappes *et al.*, 2011). Partitioning of dietary resources may be most likely to occur between stingray species due to their ability to eat a variety of benthic food sources through specialist feeding morphology and behaviours (Haine *et al.*, 2001; Vaudo and Heithaus, 2011; Tilley *et al.*, 2013a; Varghese *et al.*, 2014). Resource partitioning does not always occur in diet; spatial partitioning is another ecological gradient that can be utilised by sympatric stingrays (O'Shea *et al.*, 2012; Bangley and Rulifson, 2017), which are defined as multiple organisms occupying the

same space. However, within this space there may be differences in microhabitat use, as a way of mitigating competition (O'Shea *et al.* 2013). Another ecological axis which could be used as a platform to distribute resources is time. Temporal partitioning between batoids is more rarely reported in past studies as most stingrays share similar diel cycles and evidence is harder to verify (Cartamil *et al.*, 2003; Vaudo and Heithaus, 2012).

In the present study we investigated two sympatric species of stingrays occupying coastal habitats around The Bahamas: (i) The southern stingray (*Hypanus americanus*) belongs to the family Dasyatidae, and is a species widely distributed from the east coast of USA to south east Brazil; (ii) The Caribbean whiptail stingray (*Styracura schmardae*), recently reclassified into the Potamotrygonidae family (Carvalho *et al.*, 2016), has only recently been officially recorded as a resident of The Bahamas (O'Shea *et al.*, 2017). The southern stingray generally occupies areas with greater accessibility, therefore they are better studied than the Caribbean whiptail ray, but both species are listed as data deficient by the IUCN (www.iucnredlist.org).

Studying the trophic ecology of wild stingrays has been predominantly approached in past literature using stomach content analysis (Cortés, 1997). Extraction of stomach contents is technically straightforward but the methodology has important ethical implications (Barnett *et al.*, 2010; Heupel and Simpfendorfer, 2010). While stomach content analysis can reveal recently consumed prey items, it does not give insight into longer-term diet and therefore may not be representative of true diet (Hyslop, 1980). More recently, stable isotope analysis (SIA) has been adopted for use in resource studies and involves measuring the distribution of assimilated elemental isotopes within a tissue of an organism. Stable isotope analysis enables examination of multiple ecological

tendencies (e.g trophic level, geographic location) of a species from the same set of data (Cherel *et al.*, 2008), and this multivariate approach gives a more comprehensive representation of an organism's overall niche (Bearhop *et al.*, 2004; Newsome *et al.*, 2007). Stable isotope analysis is proposed to be an effective way of analysing resource use patterns within entire ecosystems to answer critical ecological and conservation questions regarding elasmobranch species (Shiffman *et al.*, 2012; Hussey *et al.*, 2012; Bird *et al.*, 2018).

The present study aims to use SIA to examine the trophic and spatial resource use of *H. americanus* and *S. schmardae*. Subsequently, this information will be used to infer if and how these sympatric species partition resources. We predict that dietary partitioning will occur between *S. schmardae* and *H. americanus* and we posit that *H. americanus* will feed at a higher trophic level than *S. schmardae*. We also predict that mangrove habitats will be important foraging grounds for both species.

Methods

Study location

The island of Eleuthera and the Exuma Cays lie in the northern half of The Bahamas archipelago in the Western North Atlantic (Figure 1), where mangrove creek, coral reef and sand flats across southern Eleuthera and northern Exuma Cays provide an abundance of suitable habitats for tropical stingrays (Garrone Neto and Uieda, 2012, Aguiar *et al.*, 2009). The capture locations of stingrays were categorised into mangrove habitats – locations within 200 m of a mangrove creek system and sandbar/beach habitats – locations which were offshore or more than 200 m from a mangrove creek system (See supplementary materials). Stingrays were captured across 23 sites around Cape Eleuthera and the Exuma Cays between January 2015 and June 2017 using spot seining (Figure 1). The

process involved locating a stingray in shallow clear water from a shallow hulled boat. The research team would then work to encircle and herd the stingray on foot in the water (< 1 m depth) with a 10 m seine net, once within the seine net, a large (1 m diameter) dip net was used to capture the stingray (See supplementary materials). The stingray was restrained using puncture proof gloves and the venomous barb secured with cloth and Velcro straps.

Tissue sampling and sample processing

Morphometric measurements including disc width were first taken using a flexible tape measure. Following this samples of white muscle were taken (~1 cm^2) from the left pelvic fin using sterilised scissors. The samples were kept on ice and frozen during transportation from the field and temporarily stored in the lab. The samples were oven dried at 70℃ for 24 hours for transportation to the UK and subsequently freeze-dried at the University of Exeter. In preparation for SIA, samples were ground to a fine powder using a pestle and mortar and weighed into tin cups to 0.70 mg ± 0.05 mg (for $\delta^{15}N$ and $\delta^{13}C$ analysis, see below). In a subset of 100 muscle samples, the powdered tissue was weighed into tin cups to 2.00 mg ± 0.05 mg for $\delta^{34}S$ analysis. Extraction of urea was considered due to the potential for confounding isotopic effects by urea excretion in elasmobranch muscle tissues. However, Shipley *et al.* (2017) concluded that neither lipid nor urea affected stingray stable isotopes so chemical extraction ultimately did not occur. Stable isotope ratios in muscle tissue were measured using continuous flow isotope ratio mass spectrometry, using an Elementar Pyrocube purge-and-trap elemental analyzer (EA) interfaced with an Isoprime VisION stable isotope ratio mass spectrometer (IRMS; Fourel *et al.*, 2014). Briefly, IRMS measures the ratio of stable isotopes of nitrogen, carbon and

sulphur relative successively in the same sample. Isotope ratios are expressed as $\delta^{15}N$, $\delta^{13}C$ and $\delta^{34}S$ against international references (AIR, V-PDB and V-CDT respectively) where the international references is defined as 0‰ in each case (Brand *et al.*, 2014). Each isotope ratio is indicative of ecological characters such as foraging and geographic positions (Inger and Bearhop, 2008). Lighter nitrogen isotopes (^{14}N) are excreted more with each step in a food web, leaving a residue of assimilated ^{15}N within body tissues and thus the proportion of ^{15}N can indicate relative trophic level (Michener and Kaufman, 2007). Carbon isotopes reflect the sources of carbon used for primary production (e.g. C3 vs. C4 plants) and therefore $\delta^{13}C$ can broadly indicate foraging location (Marshall *et al.*, 2007). Sulphur is useful for its functionality regarding whole food webs, there is minimal fractionation of sulphur isotopes with increasing trophic level; data using $\delta^{34}S$ offers new insights for community interactions (McCutchan *et al.*, 2003; Layman *et al.*, 2012). The degree of $\delta^{34}S$ within an organism's tissue is useful to distinguish between feeding habitats with a high concentration of sulphates (e.g. in the open ocean marine sulphates are a uniform + 21‰) and those with a high concentration of sulphides (e.g. estuarine environments; Rees *et al.*, 1978). The measurement of traceable elements such as carbon and sulphur is particularly helpful in this study as the habitats which stingrays are observed occupying have considerable isotopic variability, making environmental tracer values distinct. Organisms which predominantly inhabit mangroves particularly are characterised by relatively low $\delta^{34}S$ and $\delta^{13}C$ values. ^{34}S values are low within mangroves due to the widespread anoxia in sediments leading to the incorporation of methane within primary producers. Thus, sulphur is released during oxidation of sulphides at the sediment/water interface, leaving behind a depletion of ^{34}S in plant tissues, the low $\delta^{34}S$ signature is subsequently reflected in any consumers within the

ecosystem. Organisms inhabiting mangroves also have relatively low $\delta^{13}C$ values because mangroves are categorised as C3 plants. During carbon fixation within C3 plants the heavier carbon isotope (^{13}C) is preferentially removed leaving an enrichment of carbon-12 in their tissues (Peterson, 1999; Layman, 2007). As an organism migrates offshore their $\delta^{34}S$ and $\delta^{13}C$ values will be expected to increase due to the influence of sulphates with a high proportion of ^{34}S and diminishing occurrence of low $\delta^{13}C$ C3 plants (Hill *et al.*, 2006).

Statistics

SIA data were tested for normality, and, failing assumptions of normality, differences between species were tested using Wilcoxon rank sum tests. Other potential influencing factors that may have influenced isotopic signatures of body tissues included disc width, sex, island of capture and season of capture and were incorporated in analyses using a Generalised Linear Mixed Model as fixed effects. All plotting and statistics were carried out using R software (Version 3.4.2). In order to test for dietary and habitat overlap between the two species, Bayesian ellipses of isotopic space were generated for both stingray species using the R package 'Stable Isotope Bayesian Ellipses in R' (SIBER; Jackson *et al.*, 2011). Bayesian ellipses describe isotopic niche space and have been used to denote dietary overlap in species and communities (Layman *et al.*, 2012; Jackson *et al.*, 2012; Hill *et al.*, 2015). Standard Ellipse Area (SEA) is calculated from the Bayesian ellipses, where SEA quantifies the isotopic niche space as the area bounded by a standard Bayesian ellipse for 95% of isotopic values. The standard ellipse area can be used to estimate the extent of overlap between two sets of isotope data which can be used as an indirect proxy for niche overlap between species (Guzzo *et al.*, 2013). The ecological gradient that can be

represented depends on the specific isotopes combined in the bivariate analysis. Nitrogen and carbon bivariate plots are classically used in scientific literature, the combination of both these isotopes gives a more realistic depiction of feeding ecology, including both trophic effects and source of prey (Thornton and McManus, 1994). The comparison of carbon and sulphur isotopic ratios within a plot is most likely to represent overall habitat niche, as both carbon and sulphur isotopes infer aspects of the origin of elemental tracers. The sulphur and nitrogen plot is not typically used in past literature as it does not represent a certain ecological gradient any better than the other bivariate combinations. However, there is possible utility in its representation of information specifically regarding foraging within estuarine environments.

Ethics

All work (including stingray capture and tissue sampling) was undertaken under permits from the Bahamas Fisheries Department, and complied with the University of Exeter Research Ethics framework and ethical policy, and was approved by the College of Life and Environmental Sciences (2016/1546(rev2), 2016/1543(rev2)).

Results

Catch data

In total, 96 *Styracura schmardae* and 102 *Hypanus americanus* were captured. *Styracura schmardae* ranged from 228 to 1,472 mm disc width and 47 were female and 48 were male (All associated metadata except species was lost for 1 *S. schmardae* individual – it has been removed from all applicable data analysis). Almost 19 % (18.8) of individuals were captured around the Exuma Cays and 81.2% were captured around the coast of Eleuthera. Ninety-nine

individuals were captured in habitats characterised as ‘mangrove’ systems and 97 individuals were captured in habitats characterised as ‘sandbar/beach’ locations. Of *S. schmardae*, 61 individuals were captured during the wet season (categorised between August and January) whereas 34 individuals were captured during the dry season (February to July). *Hypanus americanus* ranged from 342 to 1,102 mm disc width and 84 were female and 18 were male. Seventeen percent of individuals were captured in the Exuma Cays and 83% were captured around the coast of Eleuthera. Of *H. americanus*, 66 individuals were captured during the wet season, whereas 36 individuals were captured during the dry season.

Inter-species differences

$\delta^{15}N$ values in white muscle tissues of *H. americanus* (6.77‰ ± 1.07 s.d.) were significantly higher than in *S. schmardae* (4.82‰ ± 1.06 s.d.; Wilcoxon rank sum; $W_{96,102} = 8493$, $p < 0.001$), indicating a higher trophic position. Homogeneity of variance tests for $\delta^{15}N$ values found the variances to be homogeneous for both species (Fligner-Killeen; $X^2 = 0.0928$, df = 1, $p = 0.760$), suggesting that both species have a similar dietary breadth.

$\delta^{13}C$ values in white muscle tissues of *H. americanus* (-8.76‰ ± 1.05 s.d.) were also significantly higher than in *S. schmardae* (-9.31‰ ± 1.59 s.d.) (Wilcoxon rank sum; $W_{96,102} = 6051.5$, $p < 0.01$). A greater prevalence of low delta values in a sample indicates a source of carbon from mangrove (C3 plant) creek systems (Lin *et al*., 1991; Layman, 2007). Homogeneity of variance tests for $\delta^{13}C$ values, however, showed that the variance was significantly greater in *S. schmardae* (1.59 s.d.) than in *H. americanus* (1.05 s.d., Fligner-Killeen; $X^2 = 13.6$, df = 1, $p < 0.001$), implying that *S. schmardae* feed on prey from a wider range

of origins. The isotopic niche space was larger in *S. schmardae* (5.82 ‰^{2}) than in *H. americanus*, (3.43 ‰^{2}) although 35.6% of the total ellipse area overlapped between the two species (Figure 2a).

Isotopic values of $\delta^{34}S$ in white muscle tissues of *H. americanus* (9.20‰ ± 3.82 s.d.) were significantly higher than in *S. schmardae* (3.50‰ ± 4.69 s.d.; Wilcoxon rank sum; W = 2068, p < 0.001), but the variances were homogeneous (Fligner-Killeen; X^2 = 1.89, df = 1, p = 0.169). The difference in $\delta^{34}S$ distribution between species is not large enough to denote completely separate ecologies, however, at 5.7‰ the difference between the averages of $\delta^{34}S$ distribution is far greater than in the carbon and nitrogen isotopic ratios. Isotopic niche space for $\delta^{34}S$ and $\delta^{15}N$ was slightly larger in *S. schmardae* (11.8‰^{2}) than in *H. americanus* (9.10‰^{2}) and 41.1% of the total ellipse area overlapped (Figure 2b). The isotopic niche space represented by $\delta^{34}S$ and $\delta^{13}C$ was largest out of all isotopic combinations for both species, however, the niche space in *S. schmardae* (21.8‰^{2}) was almost double that of *H. americanus* (12.3‰^{2}), and the extent of overlap of total ellipse area was 61.5%.

Other factors affecting dietary and habitat use

Neither sex nor season of capture influenced isotopic ratios of ^{15}N, ^{13}C or ^{34}S in either species (GLMM: P > 0.05, see supplementary materials). Instead, $\delta^{15}N$ values were significantly predicted only by disc width (GLMM: X_1 = 7.44, P < 0.01; Figure 3), and had a significant interaction effect between species ($F_{1,193}$ = 14.4, P < 0.01), with a significant positive effect (although with a small effect size) in *S. schmardae* but not on *H. americanus* (Figure 3: Linear regression: R_2 = 0.179 t_{93} = 4.50, p < 0.001; $\delta^{15}N$ = 0.00182 disc width (mm) + 3.69, see

supplementary materials for carbon and sulphur). Disc width did not predict $\delta^{34}S$ (GLMM: $X_1 = 1.57$, $P > 0.05$) or $\delta^{13}C$ (GLMM: $X_1 = 2.72$, $P > 0.05$).

Island of capture was found to be a significant predictor for $\delta^{13}C$ in both species (GLMM: $X_1 = 6.21$, $P = 0.0127$) with higher $\delta^{13}C$ values on Exuma compared with Eleuthera (Wilcoxon rank sum; $W_{38,161} = 2183$, $p = 0.0130$; Figure 4), but neither $\delta^{15}N$ (GLMM: $X_1 = 2.94$, $P > 0.05$) nor $\delta^{34}S$ values (GLMM: $X_1 = 1.69$, $P > 0.05$) varied by island of capture (see supplementary materials). The difference in $\delta^{13}C$ values between stingrays sampled on Eleuthera and the Exuma Cays, although significant, was only an average of ~0.25‰. This difference in $\delta^{13}C$ distribution is biologically negligible and likely does not denote distinct habitat use between the populations.

Discussion

Implications for diet distribution

The results of the present study suggest that while *S. schmardae* feeds at a higher trophic level than *H. americanus*, there is perhaps considerable dietary overlap (possibly 35.6%) between the two species. However, it should be noted that this overlap is not an absolute representation of diet and is only suggested by geometric indices of carbon and nitrogen isotopes of consumer muscle tissues. *Hypanus americanus* appears more likely to have a diet consisting of higher trophic level prey such as teleost fish, whereas *S. schmardae* is appears to have a diet more reliant on lower trophic level prey. Previous work has shown that, *H. americanus* maintains a trophic level of approximately 3.5, placing it firmly in mesopredator category (Cortés, 1999; Tilley *et al.*, 2013a). Gilliam and Sullivan (1993) described the diet of *H. americanus* on the island of Bimini (Bahamas), but it is unknown whether the diet of this population of southern stingrays would include perturbations from possible resource partitioning by a sympatric

Caribbean whiptail ray. Although not officially a resident as far north as Bimini, there was a single *S. schmardae* noted by O'Shea *et al.* (2017). The present study is the first to scrutinise the dietary ecology of *S. schmardae* due to its somewhat elusive and taxonomically cryptic nature (Carvalho *et al.*, 2016).

The sulphur/nitrogen bivariate plot demonstrates a positive trend of increased $\delta^{34}S$ values with increased $\delta^{15}N$ values. This correlation has been reported in the past and is attributed to protein within samples affecting sulphur in a similar way to nitrogen (McCutchan *et al.*, 2003; Florin *et al.*, 2011). This could represent a biological trend of prey from sulphide rich environments generally being at a lower trophic level than prey items from sulphate rich environments (de la Morinière *et al.*, 2003). The noteworthy conclusion from this bivariate analysis is that the difference in diet between these two species may be intrinsically linked to the difference in sulphur. Mangrove feeding behaviours may be directly contributing to the difference in trophic levels between *H. americanus* and *S. schmardae*.

The results of the present study indicate larger *S. schmardae* feed at a higher trophic level. This could potentially be due to an ontogenetic shift of juveniles moving from inshore nursery habitats with a prevalence of lower trophic level prey, to habitats that support higher trophic prey (de la Morinière *et al.*, 2003; Grubbs, 2010,). There was no apparent relationship between trophic level and size in *H. americanus*.

The present study found that the dietary isotopic niche width, using carbon and nitrogen isotopic ratios in conjunction, is much larger in *S. schmardae* than in *H. americanus*. Increased variation in carbon isotopic ratios implies that *S. schmardae* prey on a large range of different food sources from different origins. *Styracura schmardae* may utilise more generalist tendencies than *H. americanus*,

the adoption of a generalist diet could be a technique of dietary resource partitioning. This strategy allows *S. schmardae* to opportunistically feed on a greater diversity of prey, thus avoiding competition with the more specialist *H. americanus* (Bearhop *et al.*, 2004; Kinney *et al.*, 2011). The upper limit of isotopic carbon distribution is only 0.01‰ apart for each species; it is possible that a biological constraint is restricting both species to the maximum $\delta^{13}C$ value of ~-10.40‰.

Dietary niche shift has been measured in the past by comparing the diet of sympatric populations of a species with allopatric populations of the same species (Langeland *et al.*, 1991). Asymmetric competition between two species in sympatry could cause only one of the species to change diet. The 'dominant' species (the species which maintains a similar diet to that in an allopatric population (Schutz and Northcote, 1972; Hindar *et al.*, 1988; Klawinski *et al.*, 1994)) may be *H. americanus* in the present study site, supported by the greater extent of variation in $\delta^{13}C$ distribution for *S. schmardae* indicating a larger more generalist niche (Kinney *et al.*, 2011).

The degree of resource partitioning (estimated using SEA) between stingrays is an advancing statistical technique, but should be used with caution (Swanson *et al.* 2015). Knickle and Rose (2014) concluded that the degree of overlap between sympatric *gadoid* species (43.3%) was not large enough to indicate significant competition for dietary resources, but Schoener (1968) proposed that an overlap greater than 60% was sufficiently high enough to warrant significant competition between species. Using this metric, competition is not significant between *H. americanus* and *S. schmardae* and therefore dietary resource partitioning may be occurring instead. However, there is still a moderate amount of overlap between the dietary resources between these species,

especially in comparison to past studies where resource partitioning is concluded with distinct trophic niches (Tilley *et al.*, 2013a; Ryan *et al.*, 2013; Albo-Puigserver *et al.*, 2015). Although our data shows a statistically significant difference in $\delta^{15}N$ distribution, denoting disparate dietary resources between *S. schmardae* and *H. americanus*, the extent of overlap between the sympatric species is too great to suggest absolute trophic resource partitioning.

Implications for habitat distribution

Tracer values for inshore and offshore environments were collected by Fry (1983). Shrimp which utilised inshore flats assimilated a $\delta^{13}C$ distribution in the range of -11 to -14‰, however, when they migrated to offshore zones their tissues showed carbon isotopic ratio values closely clustered at -15‰. As both species had average $\delta^{13}C$ values in the range of Fry's inshore tracer range (with a minimal difference of 0.55‰), they both likely occupy inshore habitats. Additionally, there are environmental carbon tracer values specifically for seagrass from mangrove areas (values of -12.8‰) and for seagrass away from mangrove areas (values of -8.3‰; Lin *et al.*, 1991). The range of $\delta^{13}C$ distribution for *S. schmardae* (-10.42 to -13.60‰ with trophic enrichment correction (Tilley *et al.* 2013a)) in the present study more closely matches that of seagrass from mangrove habitat than *H. americanus* (-10.41 to -12.51‰ with trophic enrichment correction) (Lin *et al.*, 1991). Thus, it is worth noting, the lower average $\delta^{13}C$ distribution of stingrays sampled around Eleuthera island could possibly be attributed to the Schooner Quays, a significant offshore sand bar habitat where a high portion of stingrays were sampled.

$\delta^{34}S$ distribution within both stingray species tissue samples indicates prey from an environment with greater prevalence of ^{34}S-depleted sulphides such as

mangroves (Fry *et al.*, 1982; Currin *et al.*, 1995). Yet again, *S. schmardae* isotopic signatures are shifted more towards sulphide rich mangrove habitats than *H. americanus*. The results of sulphur isotopic ratios showed the largest distinction between the ecologies of the two species of all the isotopes measured. Enrichment by fractionation between trophic levels is minimal with sulphur isotopes, so an organism's isotopic signature is not confounded by trophic effects (Peterson *et al.*, 1986; McCutchan *et al.*, 2003). The variation in $\delta^{34}S$ distribution indicates that the degree of individual variation has a finite point, and this point is the same for both species. Sulphur isotopic distribution in sampled stingrays had a much larger variation than the distribution of carbon and nitrogen. Large variation in isotopic distribution is a common occurrence in sulphur isotopic studies due to the high number of sulphuric pathways available in a coastal ecosystem (Mekhtiyeva *et al.*, 1976; Peterson *et al.*, 1985; Layman, 2007).

Unlike other isotopic ratios, sulphur does not have specific tracer values to label mangroves habitats, this is due to the 'open' nature of mangrove systems with high connectivity to other systems such as seagrass and reefs (Layman, 2007). The $\delta^{34}S$ values indicate a more benthic or pelagic ecology of an organism (Fry *et al.*, 1982). In conjunction with carbon, data confirms that benthic food sources are prevalent in the diets of both species. Due to higher levels of sulphides as well as carbon isotopic signatures which more closely match seagrass from near mangroves, we can deduce that although both species likely utilise mangrove habitats, *S. schmardae* relies on these habitats much more. As with trophic partitioning, asymmetrical niche shift can occur between sympatric species for spatial partitioning. *S. schmardae* appears to be the outcompeted species due to larger C/S isotopic niche space and greater range of $\delta^{13}C$ distribution, they are more able to deal with deviations in their habitat use than *H.*

americanus. Although knowledge of habitat use of these specific species is limited in scientific literature, inferences can be made from studies with similar species. Pikitch *et al.* (2005) found that out of twelve elasmobranch species studied *H. americanus* was one of four which was found in both deep and shallow lagoon habitats across all four study years, they also found a large presence of *H. americanus* in mangrove-fringed cayes. Southern stingrays seem to utilise a variety of nearshore habitats, with adult southern stingrays proposed to have a home range whilst using reefs as important features in the spatial network (Tilley, 2013b). Again the lack of literature on *S. schmardae* makes these comparisons incomplete; their ecology is superficially similar to that of the southern stingray, however their closest relatives are freshwater river rays. In the present study we have provided evidence that mangroves are important ecosystems to *S. schmardae*. There is a possibility that their affinity for mangrove habitat use is evolutionary, freshwater river rays inhabit structurally similar ecosystems to Bahamian mangrove creek systems (Garrone Neto and Uieda, 2012). Evolutionary inheritance may offer benefits such as a greater tolerance of salinity perturbations and foraging in anaerobic sediments (Lugo and Snedaker, 1974; Carvalho *et al.*, 2016). The segregation of habitat use between species, specifically regarding utilisation of mangrove systems, could be a form of habitat resource partitioning; *S. schmardae*'s preference of mangrove habitats mitigates competition between these two sympatric stingray species.

Stingrays exhibit ontogenetic habitat segregation, with juvenile stingrays utilising mangroves as nurseries (Leis and McCormick, 2002; Heupel *et al.*, 2007; Aguiar *et al.*, 2009; Jirik and Lowe, 2012). Although there was a positive relationship between size and trophic level of prey in *S. schmardae*, we had no evidence of an ontogenetic habitat shift occurring in either species. It is

interesting that while nitrogen data were suggestive of an ontogenetic dietary niche shift in *S. schmardae*, there was no evidence using carbon or sulphur isotopic ratios. It is possible that mature *S. schmardae* are still utilising mangroves habitats to the same extent as juveniles but are feeding on a source of prey higher within the mangrove trophic system. This may be indicative of ontogenetic niche expansion rather than a discrete niche shift; *S. schmardae* increases the trophic range of prey items it consumes whilst occupying the same habitat, this may be further supported by the large range in $\delta^{13}C$ distribution for *S. schmardae* (Werner and Gilliam, 1984; Hammerschlag-Peyer *et al.*, 2011). The difference in ecology between *S. schmardae* and other species might again be attributed to evolutionary relics of the family Potamotrygonidae. Small scale spatial segregation and dietary ontogenetic shifts can occur for these freshwater river rays but discrete habitat transition is not possible as it is in marine habitats (Garrone Neto and Uieda, 2012), *S. schmardae* may be utilising this ecological technique as a relic of ancestry with secondary benefits of competition avoidance.

Conclusion

Our study offers evidence for both habitat and dietary partitioning between these two sympatric stingray species. However, correlation between sulphur and nitrogen isotopic ratios indicate trophic differences between species are directly linked to disparity in mangrove habitat use. Asymmetric niche shift may be occurring with *H. americanus* fulfilling the role of dominant species and *S. schmardae* as the submissive (outcompeted) species in sympatry. Although mangrove habitats are clearly important to both stingray species analysed in the present study, they appear to be fundamental in the ecology of the poorly studied Caribbean whiptail ray. This finding should be added to conservation frameworks

for the protection of mangroves in The Bahamas. Resource partitioning should also be considered when deciding conservation frameworks as pressures from competing sympatric species may cause shifts from a species' normal ecological preferences. This study was limited by stingray capture methodology, sampled stingrays were only captured in shallow water (< 1 m), this bias could potentially lead to the exclusion of an entire population of stingrays utilising deeper environments. Further studies should use electronic tagging methods that record depth movements to test whether one or both species could potentially be using deep environments for further resource partitioning. Further research is urgently needed about all aspects of ecology of the Caribbean whiptail ray. Ontogenetic use of mangroves by stingrays should be further investigated, in particular further research on the use of typical nursery habitats by adult *S. schmardae* that has been proposed in this study.

Figures and Tables

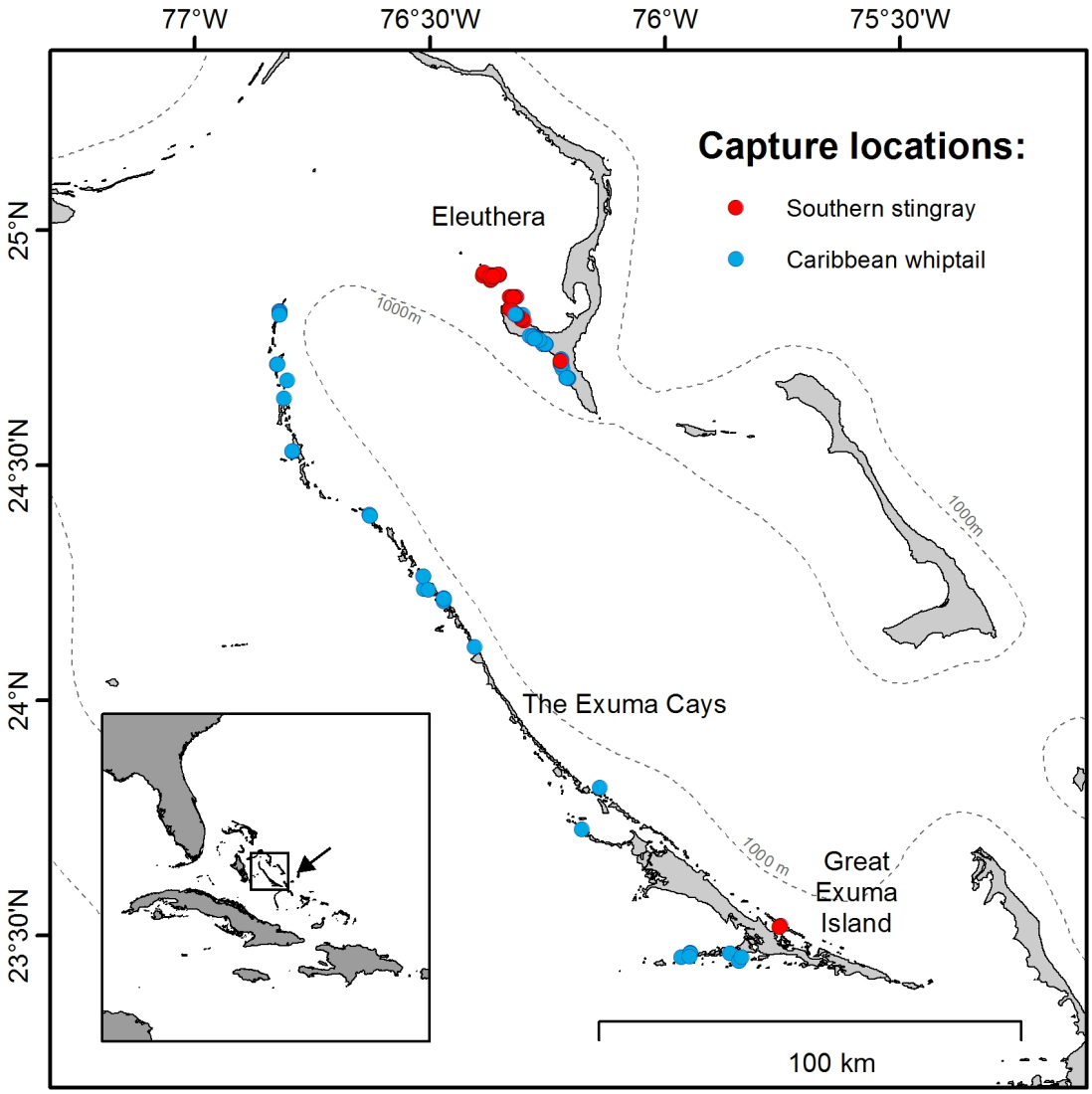

Figure 1: Map showing the location of 23 sites where stingray biopsy samples were collected, across the Northern Exuma Cays and Southern Eleuthera within the Bahamas archipelago. Sampling locations of *S. schmardae* are displayed by blue circles and *H. americanus* by red.

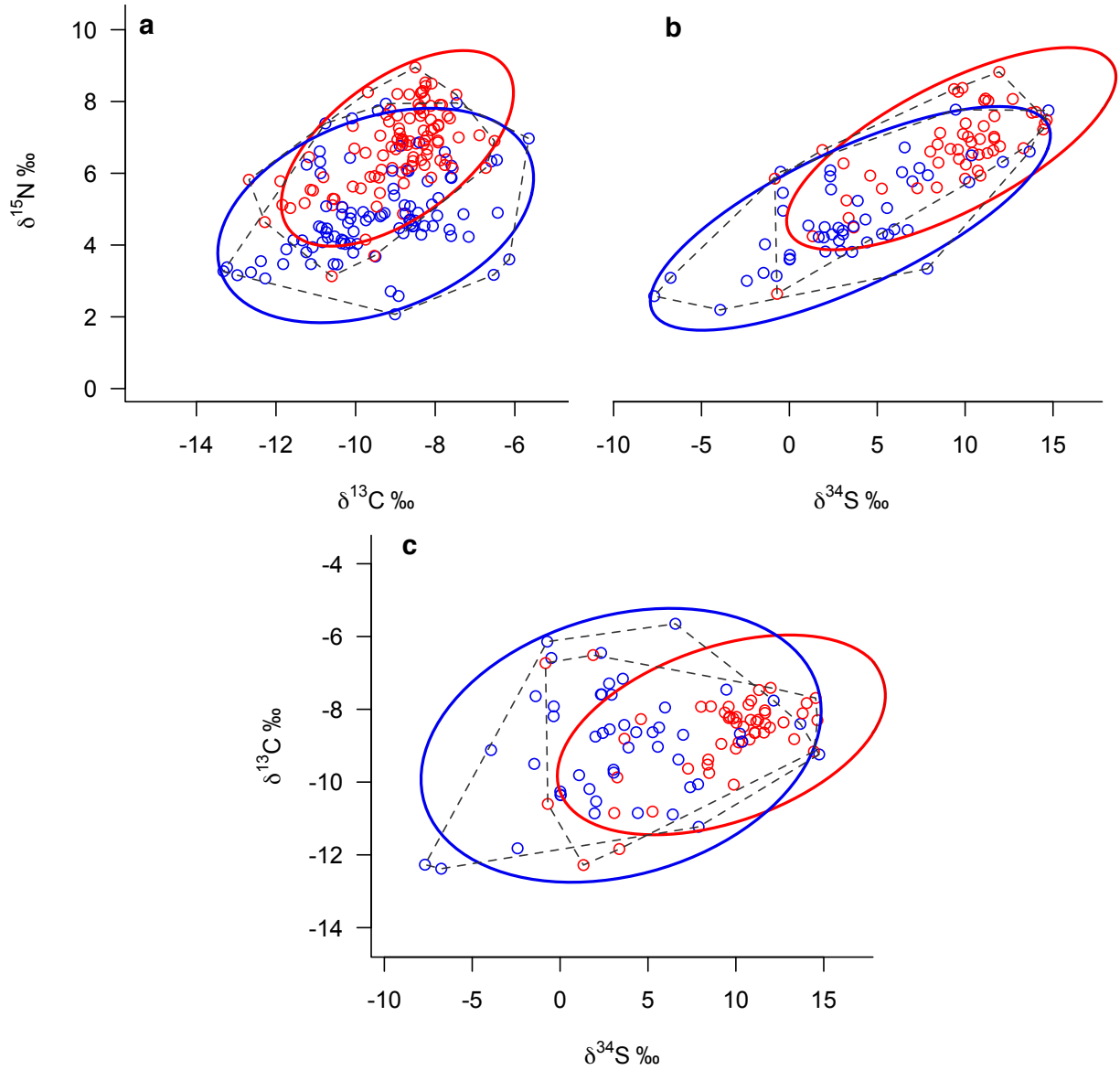

Figure 2: Standard Bayesian Ellipses illustrating the isotopic niches from white muscle samples of *H. americanus* (red) and *S. schmardae* (blue) for (a) carbon and nitrogen isotope ratios; (b) nitrogen and sulphur isotope ratios; and (c) carbon and sulphur isotope ratios. Solid lines enclose standard ellipse areas (*SEA*) for each species which could be used to represent the total niche area occupied by each species. Dashed lines represent convex hulls which encompass all data points for each species.

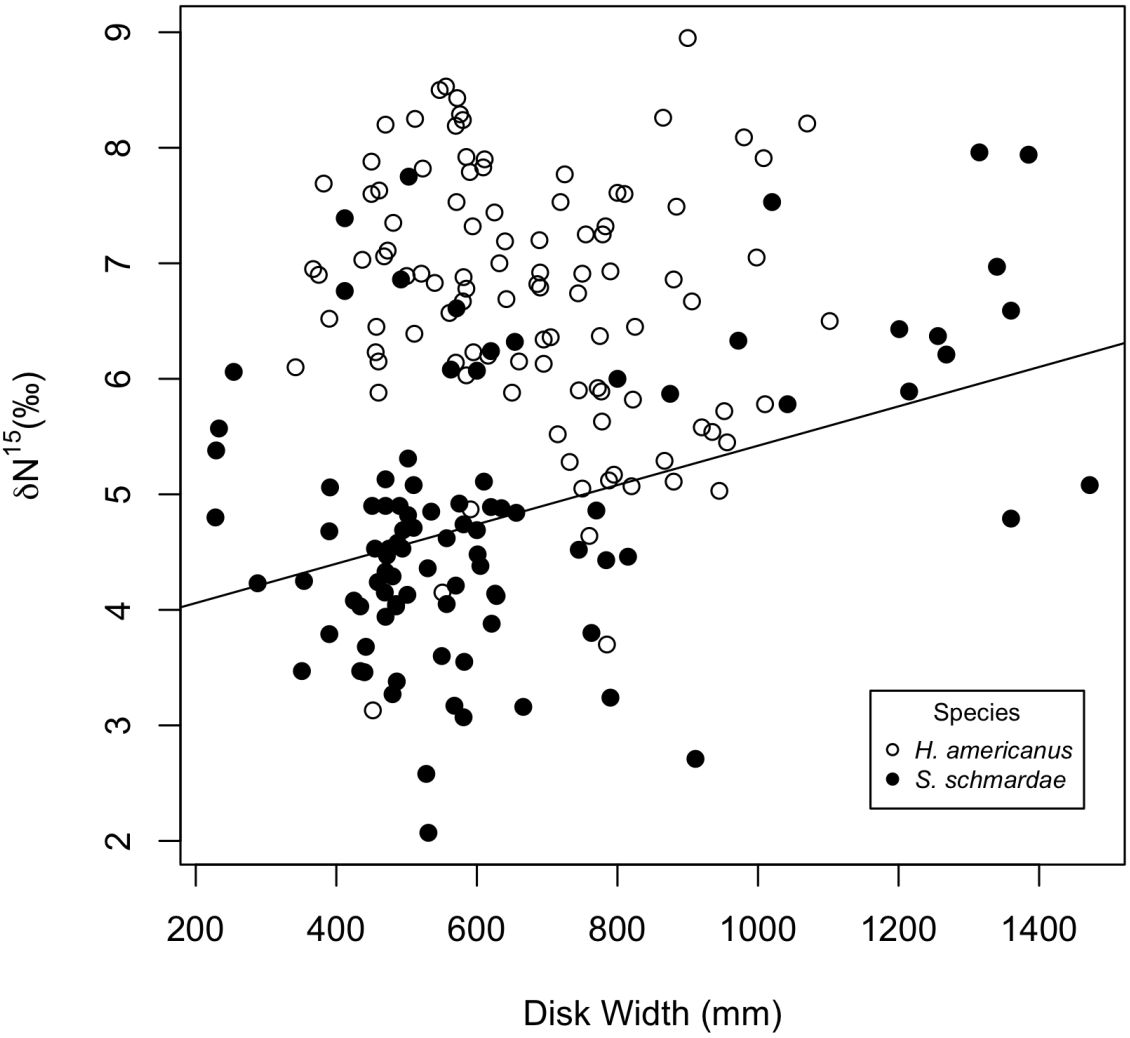

Figure 3: The relationship between individual stingray disc width (mm) and isotopic values of $\delta^{15}N$ (‰) in white muscle of all individual stingrays for species *H. americanus* (empty circles) and *S. schmardae* (solid black circles). A significant least squared linear regression line for *S. schmardae* is displayed on the graph.

Chapter 2: Detecting ontogenetic shift using stable isotope analysis

Key words: *Stable isotope analysis, Ontogenetic Shift, Elasmobranch, Stingray, Mangroves*

Abstract

Two species of stingray in the western Atlantic, *H. americanus* and *S. schmardae*, are considered data deficient and their habitat use and dietary patterns across ontogeny are poorly understood. To address this, stable isotope analysis of carbon and nitrogen was used with breakpoint analysis to delineate the size at which ontogenetic shifts may occur. Carbon isotope ratios suggest that a shift out of mangrove habitats occurs at approximately 705 and 568 mm disc width for *H. americanus* and *S. schmardae* respectively. A second breakpoint in $\delta^{13}C$ in *S. schmardae* indicated a return to mangroves occurring at 815 mm disc width, which aligned with a breakpoint in $\delta^{15}N$ values signifying a concurrent transition to higher trophic level prey. The number of breakpoints identified varied with tissue type analysed: four breakpoints were evident in white muscle, blood detected two and barb only detected one ecological shift. Future studies should analyse multiple tissues to provide a more comprehensive overview of shifts that may occur at varying temporal scales. The insights of ontogenetic changes in habitat use for *H. americanus* and *S. schmardae* demonstrated in the present study contribute to the ecological knowledge base for these two data deficient stingray species. Effective conservation must account for transitions in a species' ecology across different life stages.

Introduction

Stable isotope analysis in ecology

Stable isotope analysis (SIA) is increasingly being used in the field of ecology (Newsome *et al.*, 2007; Middelburg, 2014; Newton, 2016; Katzenberg and Waters-Rist, 2018). The proportion of certain stable isotopes within the body tissues of an organism can reflect ecological patterns of habitat use and foraging preferences (Inger and Bearhop, 2008). Ratios of the lighter nitrogen isotope (^{14}N) to the heavier form (^{15}N) provide an indicator of diet due to excretion of lighter isotopes of nitrogen and concurrent enrichment of heavier isotopes with trophic increment (Kelly, 2000; Fry, 2006). Ratios of carbon isotopes are associated with geographical location indicating terrestrial to offshore sources of carbon from primary production (Fry, 1983; Lin *et al.*, 1991; Hill *et al.*, 2006). It is important to consider multiple tissues when inferring the ecology of an organism (Bearhop *et al.*, 2004) as their isotopic signatures will differ with metabolic turnover rate (amongst other factors), reflecting different temporal scales. Highly metabolic tissues such as whole blood would likely demonstrate a shorter isotopic turnover rate and therefore timescale than muscle or a tissue with minimal metabolic activity such as cartilage (MacNeil *et al.*, 2005; Trueman *et al.*, 2012).

The use of SIA to investigate ecology is particularly useful for animals with cryptic life stages or that occupy challenging environments to study (Olson *et al.*, 2010; Churchill *et al.*, 2015). A single biological sample collected for SIA can indicate the location and diet of an organism across a wide temporal range (Hussey *et al.*, 2012). SIA has been used to gain insights into the ecology of a range of elasmobranch species (Estrada *et al.*, 2006; Dale *et al.*, 2011; Hussey *et al.*, 2012; Burgess *et al.*, 2018; Bird *et al.*, 2018). A large proportion of stingrays are categorised as data deficient by the IUCN redlist, and many of their ecological

features remain unknown. SIA could reveal aspects of stingray ecology which are currently poorly understood.

Ontogenetic shifts and the application of SIA

Marine species are not restricted to a single environment as many terrestrial species are, and it is common for mobile marine species to transition between habitats as a function of ontogeny (Nagelkerken *et al.*, 2000; Grol *et al.*, 2014). A classic, if extreme, example is the long distance migration of Pacific salmon species from saltwater to freshwater at the onset of sexual maturity (Ueda, 2011). Although ontogenetic habitat shifts are common, especially during the transition of juvenile fish from nursery habitats (Dahlgren and Eggleston, 2000), dietary shifts can also occur with life stage. Graham *et al.* (2007) observed a rapid diet shift in juvenile yellowfin tuna (*Thunnus albacares*), switching from a diet of planktonic larvae to teleosts within a narrow size range. It is therefore imperative to consider distinctions that may be dependent on life history stage to fully understand the complete ecology of a species.

The use of mangrove habitats as juvenile nursery grounds is not uncommon in stingrays, and the migration of sub-adults to offshore environments has been described previously (Aguiar *et al.*, 2009; Grubbs, 2010; Dale *et al.*, 2011). Kimirei *et al.* (2013) proposed that the drivers of ontogenetic change were multifaceted; dietary requirement, reproduction and trade-offs between food availability and predation pressure may contribute to the shifts undertaken by juvenile fish in tropical environments. Stable isotope analysis could prove an effective methodology for determining ontogenetic shift; carbon and nitrogen isotopes reflect habitat use and diet respectively, these are the suggested core ecological factors concerned in ontogenetic shift studies (Werner and Gilliam, 1984).

Estrada and colleagues (2006) analysed stable isotopes in the vertebra of white sharks (*Carcharodon carcharias*) and demonstrated a correlation between trophic level and body size. Other studies have alluded to the use of stable isotopes to detect ontogenetic shifts through comparisons with body size (Dale *et al.*, 2011; Kiszka *et al.*, 2015). Authier *et al.* (2012) used change point analysis in conjunction with SIA to detect ontogenetic shifts in elephant seals; by detecting the size at which there was a shift in mean of the longitudinal isotope data, they could estimate the size at which an ontogenetic shift occurred. A similar methodology could be applied in stingrays to determine changes in ecology with body size. The present study aims to use SIA to detect ontogenetic shifts in two species of stingrays, the southern stingray (*Hypanus americanus)* and the Caribbean whiptail ray (*Styracura schmardae)* using tissue types reflecting various timescales of isotopic assimilation (whole blood, white muscle and cartilage).

Methods

Study location

Stingray biopsy sampling took place at 23 sites over ~250 km of coastline around Cape Eleuthera and the Exuma Cays, The Bahamas between January 2015 and June 2017 (Figure 1). The capture locations of stingrays were categorised into mangrove habitats – locations within 200 m of a mangrove creek system and sandbar/beach habitats – locations which were offshore or more than 200 m from a mangrove creek system (See supplementary materials). Stingrays were captured using spot seining, this involved locating and encircling a stingray on foot towards a 10 m seine net whilst in shallow water (<1 m; see supplementary materials). The stingray was then secured in a 1 m diameter dip net before being restrained using puncture proof gloves and the venomous barb

sheathed using cloth and Velcro straps (see O'Shea *et al.* 2017 for detailed methodologies).

Tissue sampling and sample processing

Morphometric measurements including disc width were first taken using a flexible tape measure. Following this, samples of white muscle, blood, and cartilage (barb) were taken. White muscle samples (~1 cm^2) were taken from the left pelvic fin using sterilized scissors, 1 ml of blood was extracted from the caudal vein using an 18-gauge hypodermic needle, and finally a cartilage clipping (< 1 cm^2) was taken from the tip of the barb using sterilized scissors. The samples were kept on ice and frozen during transportation from the field and temporarily stored in the lab. To preserve the samples during storage they were oven dried at 70°C for 24 hours and additionally freeze-dried following overseas travel. In preparation for SIA, samples were ground to a fine powder using a pestle and mortar and weighed into tin cups to 0.70 mg ± 0.05 mg (for $\delta^{15}N$ and $\delta^{13}C$ analysis, see below). Shipley *et al.* (2017) concluded that neither lipid nor urea affected stingray stable isotopes so chemical extraction did not occur. Stable isotope ratios in muscle tissue were measured using continuous flow isotope ratio mass spectrometry, using an Elementar Pyrocube purge-and-trap elemental analyser (EA) interfaced with an Isoprime VisION stable isotope ratio mass spectrometer (IRMS) (after Fourel *et al.*, 2014). Stable isotope ratios in blood and barb samples were measured in an Elementar Pyrocube purge-and-trap elemental analyser run in NC mode, interfaced with a Thermo Fisher Scientific Delta XP Plus IRMS. The IRMS measures the ratio of nitrogen and carbon isotopes in relative succession in the same sample. Isotope ratios are expressed as $\delta^{15}N$ and $\delta^{13}C$ against international references (AIR and V-PDB respectively)

where the international references is defined as 0‰ in each case (Brand *et al.*, 2014).

Analytical methods

Values of $\delta^{15}N$ and $\delta^{13}C$ from *H. americanus* and *S. schmardae* biopsy samples were tested for normality. Potential influencing factors of disc width were analysed using a Generalised Linear Mixed Model, including species, sex and region as fixed effects. The data was tested for linearity and models were created to display possible ontogenetic relationships between disc width and isotope distribution for each species in each tissue. Possible breakpoints were then calculated for the models of each tissue in *H. americanus* and *S. schmardae* for both nitrogen and carbon isotopic distributions. Breakpoint analysis pinpoints the change point (if one is present) in a longitudinal trend, the size (and indirectly the level of maturity) at which the mean isotopic signature of the population switches from one value to another. These were computed using the R package 'strucchange' (Version 1.5-1; Zeileis *et al.*, 2002; Zeileis *et al.*, 2003). All statistics were carried out in R statistical software (Version 3.4.2) and plotting was carried out using 'ggplot2' (Version 2.2.1; Wickham, 2016).

Ethics

All work (including stingray capture and tissue sampling) was undertaken under permits from the Bahamas Department of Marine Resources (DMR), and complied with the University of Exeter Research Ethics framework and ethical policy, and was approved by the College of Life and Environmental Sciences (2016/1546(rev2), 2016/1543(rev2)).

Results

Catch data

A total of 94 Caribbean whiptail rays (*Styracura schmardae*) and 110 southern stingrays (*Hypanus americanus*) were captured. *Styracura schmardae* ranged from 228 to 1472 mm disc width and 49 were female and 45 were male. Individuals captured in the Exuma Cays accounted for 35.1 % of the *S. schmardae* sample and the other 64.9% were captured around the coast of Eleuthera. *Hypanus americanus* stingrays ranged from 342 to 1102 mm disc width and 90 were female and 20 were male. Individuals captured in the Exuma Cays accounted for 1.8 % of the *H. americanus* sample and the other 98.2% were captured around the coast of Eleuthera. A total of 314 biopsy samples were successfully analysed by SIA for this study, of these 61 were cartilage (barb), 56 were whole blood and white muscle accounted for the final 197 samples.

Ontogenetic effects on isotopic distribution

Breakpoint analysis was carried out because data were not suitable for least square linear regression, thus relationships between disc width and isotopic distribution were likely non-linear or non-existent.

For $\delta^{15}N$ isotopic values there were no breakpoints as disc width increased, the only exception was a breakpoint at disc width 911 mm (95% confidence interval 770 to 1201 mm) in white muscle samples of *S. schmardae* (Figure 2a & b). At 911 mm there was a shift of almost 2‰ in $\delta^{15}N$ values, from a mean of 4.55‰ to 6.24‰. For $\delta^{13}C$ distribution breakpoints were identified in tissue samples of both species as disc width increased (Figure 2c & d). In *H. americanus*, blood samples showed a breakpoint at 580 mm (95% confidence interval 468 to 609 mm), with a mean decreasing from -9.3‰ before the breakpoint to -11.93‰ after. White muscle samples also exhibited a breakpoint

in $\delta^{13}C$ distribution with increasing disc width, at 705 mm (95% confidence interval between 595 and 745 mm) there was a shift in mean from -8.49‰ to -9.66‰. There was no significant breakpoint in $\delta^{13}C$ distribution along the range of disc widths for barb samples of *H. americanus*. In *S. schmardae* significant breakpoints in $\delta^{13}C$ distribution along disc widths were evident in all tissues. In white muscle samples two significant breakpoints were identified - at 568 mm (95% confidence interval 490 to 666 mm) the mean $\delta^{13}C$ value decreased from -9.34‰ to -10.44‰, then at 815 mm (95% confidence interval 745 to 1042 mm) the mean increases to -8.30‰. Barb samples showed a breakpoint in $\delta^{13}C$ distribution at 503 mm (95% confidence interval 475 to 581 mm), shifting from a mean of -8.24‰ to -9.98‰. Blood samples had a mean $\delta^{13}C$ value of -11.24‰ in disc widths smaller than 487 mm (95% confidence interval 451 to 501 mm), which decreased to -12.41‰ in disc widths larger than 487 mm.

Relationships between disc width and other factors

The sampled *H. americanus* individuals were significantly larger than *S. schmardae* individuals, median disc width 660 mm (±176 mm s.d.) versus 543 mm (±284 mm s.d.) respectively (Figure 3; Wilcoxon rank sum; $W_{96,102} = 5946$, $p < 0.01$). There were notable numbers of *S. schmardae* that were larger than the interquartile range, despite this the variances in disc width between species were equal (Fligner-Killeen; $X^2 = 0.0454$, df = 1, $p = 0.831$). Sex had a significant interaction with species to predict disc width ($t_{3,191} = 3.28$, $P < 0.01$) -female *H. americanus* were significantly larger (median 719 mm ±173 mm s.d.) than males (median 517 mm ±67 mm s.d.; Wilcoxon rank sum; $W_{18,83} = 1270$, $p < 0.01$), however, there was no sex specific difference in *S. schmardae*. Homogeneity of variance tests found that male *H. americanus* had less variation in disc width

compared to females (Fligner-Killeen; $X^2 = 11.1$, df = 1, $p < 0.01$). Disc width was not significantly predicted by region of capture in either *S. schmardae* (GLMM: $X_1 = 0.107$, P = 0.744) or *H. americanus* (GLMM: $X_1 = 0.0138$, P = 0.906). However, the variance in disc widths between *S. schmardae* captured in each region were heterogeneous, individuals from the Exuma Cays display a significantly greater range of disc widths than those captured around Eleuthera (Fligner-Killeen; $X^2 = 28.4$, df = 1, $p < 0.01$).

Discussion

This study is among the first pieces of research to analyse the ecology of stingrays within shallow water environments of offshore and mangrove habitats in the western Atlantic Ocean. It must be borne in mind that the sample of stingrays captured were biased towards shallow water, and that no stingrays were captured from environments deeper than 1 m. Potentially, there is a population of our study species which occupy deeper water in The Bahamas that are excluded from analysis in this study.

Shifts in isotopes

In this study novel change-point analysis was used to attempt to suggest the body size at which ecological transitions occur for the two study species. However, there is much variation in break point analysis and the suggested 'size at change' are only mathematical estimates based upon variable individual SIA data. Although breakpoint analysis occurred using a fairly large sample size of individuals, this may not be robust enough to infer absolute transitions and will likely only suggest vague estimates of the 'size at change'. The existence of breakpoints in both $\delta^{15}N$ and $\delta^{13}C$ values for *S. schmardae*, but only $\delta^{13}C$ values for *H. americanus* suggests that a habitat shift may occur in both *S. schmardae* and *H. americanus*, an additional dietary shift may also occur in *S. schmardae*.

The lack of breakpoint in $\delta^{15}N$ values for *H. americanus* is itself a notable result, because nitrogen denotes the trophic position of prey and the results suggest that *H. americanus* feeds at the same trophic level throughout its life (Kurle and Worthy, 2001; Inger and Bearhop, 2008). Breakpoint analysis on the same trend in *S. schmardae* suggests a dietary shift towards consuming higher trophic level prey in individuals larger than 911 mm. To attempt to suggest the size at which the shift generally takes place, individual tissue turnover rate and growth rate must be taken into consideration (Trueman *et al.*, 2005). MacNeil *et al.* (2006) carried out a diet switching study on freshwater ocellate stingrays (*Potamotrygon motoro*), where they estimated that white muscle tissue turnover of ^{15}N took 98 days to assimilate 50% of a new diet equilibrium. On this basis, *S. schmardae* in the present study should begin to switch to the higher trophic level diet at a smaller size than 911 mm. There are no studies reporting on the growth rate of *S. schmardae*, but using parameters produced by Vaudo *et al.* (2018) we can estimate that the growth rate for female *H. americanus* of ~900 mm disc width to be 76.6 mm per year. This approximation is made from individuals that have been fed a supplemental diet at a tourist site, and is therefore likely to be higher than natural growth rates of wild stingrays. Extrapolating from this, the actual disc width of *S. schmardae* at diet shift should be approximately 890 mm. For further breakpoints in the present study we will only refer to the size at isotopic detection, although the size at the ecological shifts suggested from this data is likely a centimetre or two less than the disc width stated, as detailed above.

Although size of sexual maturity remains unknown in this species, 911 mm is likely too large to represent a dietary shift among the juvenile population. This change in diet aligns with a breakpoint detected in $\delta^{13}C$ values of white muscles samples at 815 mm, which appears to represent a shift in habitat for *S.*

schmardae towards more inshore habitats (Fry, 1983; Hill *et al.*, 2006). Thus, while S. schmardae of this body size change habitat, they may also change their diet. This breakpoint follows an earlier decrease in $\delta^{13}C$ mean occurring at 568 mm, which most likely signifies a shift from mangroves into more pelagic zones for sub-adult *S. schmardae* (Grubbs, 2010). We propose that following an ontogenetic shift as juveniles, *S. schmardae* exhibit a secondary shift returning to mangroves at a later life history stage.

There a number of ecological mechanisms that could be driving this additional ecological shift, either acting solely or in conjunction with one another. One option could be that the secondary shift represents female adults returning to mangroves during gestation and pupping. Stingrays are known to utilise the warm and relatively sheltered mangrove environments to maintain the energy investment required in gestation and pupping (Jirik and Lowe, 2012). However, this secondary habitat shift is also detected in male *S. schmardae*. There is the possibility of *S. schmardae* utilising mangroves as the location for breeding aggregations. Other stingray species have been suggested to undergo mass aggregations for reproduction (Gray *et al.*, 1997; Vaudo and Lowe, 2006; Semeniuk and Rothley, 2008). Short-tail stingrays (*Dasyatis brevicaudata*) congregate in nursery areas for their annual mating aggregation (Le Port *et al.*, 2012), perhaps a similar event occurs within the nursery habitats of *S. schmardae* in The Bahamas. Another potential ecological function which could be at play here is sized-based foraging competition. Meadows (In Prep.) discussed the importance of mangrove habitats to *S. schmardae* throughout all life history stages, it is likely that mangrove systems are their default habitat. Bahamian mangrove creeks can be generally characterised by relatively narrow and convoluted tidal channels (Buchan, 2000), for large solitary feeding macro fauna

such as *S. schmardae* optimal foraging locations within mangroves would be a limited resource (Tilley *et al.*, 2013b). The initial ontogenetic shift could even be partly driven by size-based competition, with individuals leaving mangroves when they are too large to forage within mangrove prop roots, yet still too small to compete for sought after creek bed positions. The return to mangroves occurs when an individual reaches a size where it can compete with other adults for these sites. Most stingrays are solitary feeders (Semeniuk and Rothley, 2008), it is possible that adult stingrays occupy certain territories within mangroves, occupying and defending a preferred foraging location. This theory is also supported by the fact that *S. schmardae* do not show size-based sexual dimorphism like other stingray species, with mature males attaining similarly large body sizes to mature females (Last *et al.*, 2016; O'Shea *et al.*, 2017). Instead, size-based competition might explain the morphological similarity of the sexes, such that males can compete with females for optimal foraging zones if at a large size. The apparent concurrent alignment of habitat shift with diet shift suggests that adult *S. schmardae* returning to mangroves avoid competition with juveniles by foraging at a different trophic level. Earlier data (Meadows In Prep) suggested that *S. schmardae* adults within mangroves undergo trophic niche expansion rather than a direct diet shift. However, the results of the present study indicate that adult *S. schmardae* may instead switch to a higher trophic level. Niche contraction may even be occurring whilst *S. schmardae* adults specialise foraging behaviours to target higher trophic prey (Mahe *et al.*, 2007; Grubbs, 2010). It is imperative when adults and juveniles occupy the same habitats that competition does not adversely affect either group, dietary distribution is a method of resource partitioning between conspecifics at different life stages (Ebert, 2002). It is interesting to note that the secondary shift suggesting the return to mangroves

actually shifts the mean $\delta^{13}C$ to a value higher than that during juvenile sizes (-8.30‰ vs -9.34‰). Indeed, perhaps there is more competition between juvenile *H. americanus* and adult *S. schmardae* than between *S. schmardae* adults and juveniles. This discrepancy could also represent a more transient nature of the higher trophic prey which *S. schmardae* feeds upon on their return to mangroves (Sheridan and Hays, 2003). The diet shift may not only be a function of ontogenetic competition, larger *S. schmardae* may also require access to higher trophic sources to satisfy nutritional demands of reproduction or even growth required by size-based competition (Wirsing *et al.*, 2006; Kowalczyk *et al.*, 2014).

Tissue influences

Blood displayed shifts in $\delta^{13}C$ mean at smaller sizes than either white muscle or barb. The faster the metabolic rate of a tissue, the quicker the isotopic turnover rate should be within that tissue (MacNeil *et al.*, 2005; Busst and Britton, 2018). Buchheister and Latour (2010) recommended that due to rapid turnover, blood should be used to detect short term ecological changes in summer flounders (*Paralichthys dentatus)* whilst reducing confounding effects. Our data showed that blood can detect significant shift in habitat before other tissues, however there was no breakpoint detected in blood samples of either species for $\delta^{15}N$ values. In *H. americanus,* no breakpoint was detected in any tissues, so the likely conclusion is that no significant trophic shifts in diet may occur within an individual *H. americanus'* lifetime. However, in *S. schmardae*, there was a breakpoint detected in white muscle samples, which thus should have been evident in blood at a smaller disc width if blood represented a shorter time period. The data did not show a breakpoint in blood, however, this may be because relatively few individuals of a larger disc width were sampled for blood compared

to white muscle. The decrease in $\delta^{13}C$ mean of blood samples for *H. americanus* was over double the amount that was detected in white muscle samples, whereas in *S. schmardae* they differed by only 0.07‰. As blood is reported to be proficient at detecting fine-scale shifts in diet/habitat change, it should be more likely to also detect individual variation in feeding behaviours (Bearhop *et al.*, 2004; Dalerum and Angerbjörn, 2005). This creates a more variable range of isotopic signatures within a sample of stingrays, even of the same demographic. White muscle was also the only tissue to present the secondary shift in $\delta^{13}C$ values in *S. schmardae.* It has been suggested that the metabolic turnover of white muscle may be too low to reveal fine-scale insights (MacNeil *et al.*, 2006), our data found that stable isotopes of white muscle were able to predict significant shifts in ecological parameters. Barb samples only identified one (the initial habitat shift of *S. schmardae*) of the four total isotopic shifts detected in the present study. It is interesting that barb is able to detect the ontogenetic shift within *S. schmardae* but not *H. americanus*. The shift in habitat in *S. schmardae* may be dramatic enough that even slow metabolic tissues such as barb (cartilage) can reflect it. This shift produced breakpoints across tissues which were strongly concentrated in a size range (between 487 mm and 568 mm). It would be expected that blood would show a shift first followed by muscle and then barb, however barb detects the change before muscle in this instance. It is worth bearing in mind that the smaller sample size of barb could skew the data when directly compared with white muscle samples.

Future directions

Future studies should use empirical methods to examine habitat use by different sized individuals using the sizes at change suggested here to determine the validity of breakpoint analysis using SIA data in these species.

Although the absolute reliability of breakpoint analysis remains to be determined, the results from this study show clearly that mangroves play an integral part within life history events of both *H. americanus* and *S. schmardae*. However, it is apparent that mangroves offer fundamental services to both juvenile and adult *S. schmardae*. Mangroves are key habitats that provide a range of ecosystem services (Harborne *et al.*, 2006; Barbier *et al.*, 2011) whilst being under threat, with some predictions of a loss of at least one third of the world's population in the last 66 years (Alongi, 2002; Adeel and Pomeroy, 2002; Gilman *et al.*, 2008; Polidoro *et al.*, 2010). As a data deficient species the population status of the Caribbean whiptail ray remains unknown, however it is likely to be intrinsically linked to the status of mangroves. Although SIA can demonstrate the occupation of mangroves at multiple life history stages by *S. schmardae*, it can only provide a basis for theories as to precisely how these stingrays utilise mangrove creek systems. Further studies should investigate the habitat use dynamics of *S. schmardae* in mangroves, tracking tidal and diurnal use patterns across both juveniles and adults.

Conclusion

Stable isotope analysis was able to pick up on ontogenetic shifts in habitat by both species of stingray through breakpoint analysis. However, similar research on ontogenetic shifts in these species using different methods such as movement and activity space studies (Lowe *et al.*, 1996; Franks, 2007) are needed to fully explore this. Stable isotope analysis also picked up on a second

ecological shift (in both habitat and diet) representing a return to mangrove habitats by *S. schmardae* at later life. The different tissues in the present study revealed insights into a range of temporal periods, and thus future studies should seek to integrate multiple tissues.

Figures and Tables

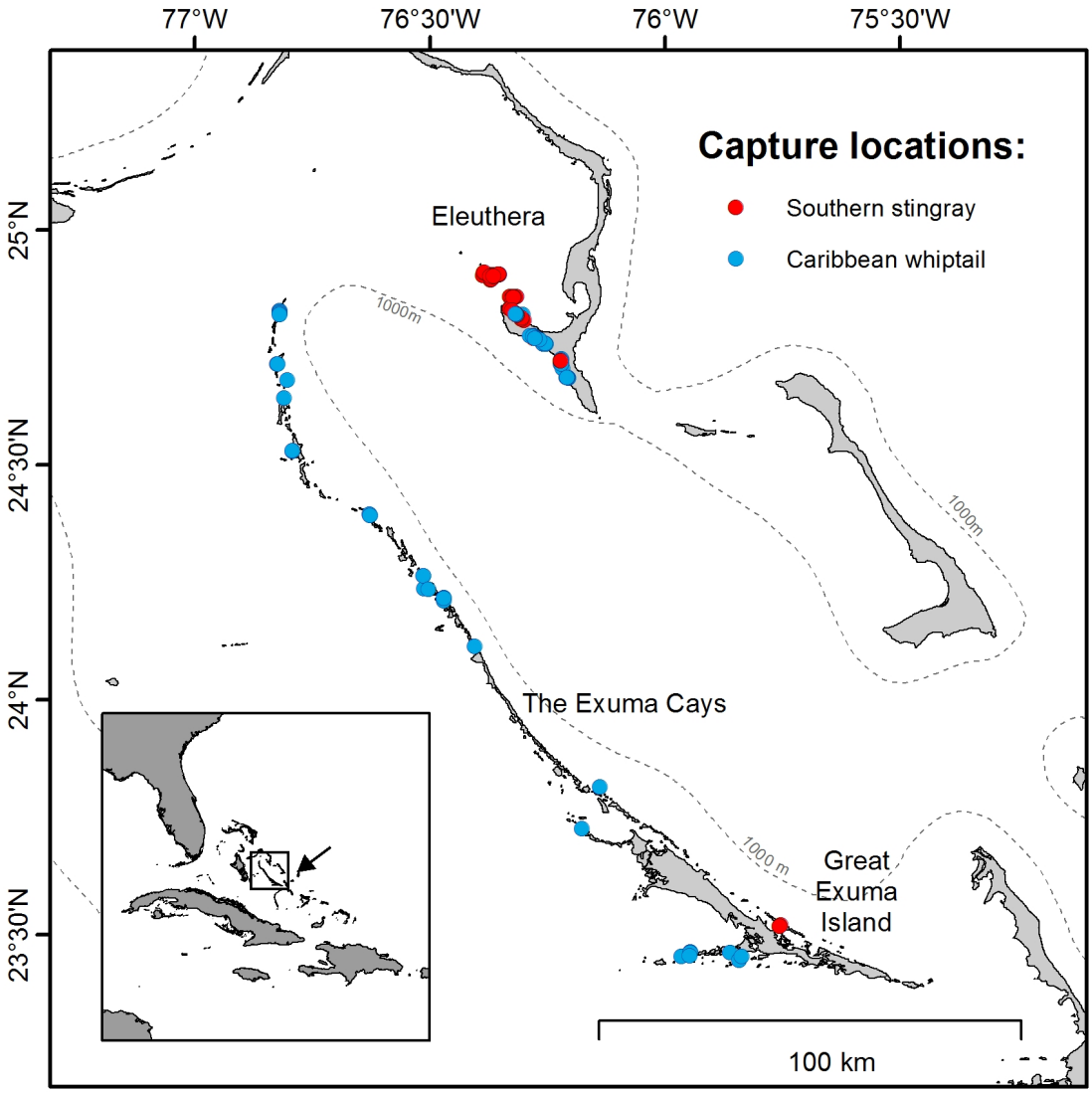

Figure 1: Map showing the location of 23 sites where stingray biopsy samples were collected, across the Exuma Cays and Southern Eleuthera within the Bahamas archipelago. Sampling locations of *S. schmardae* are displayed by blue circles and *H. americanus* by red.

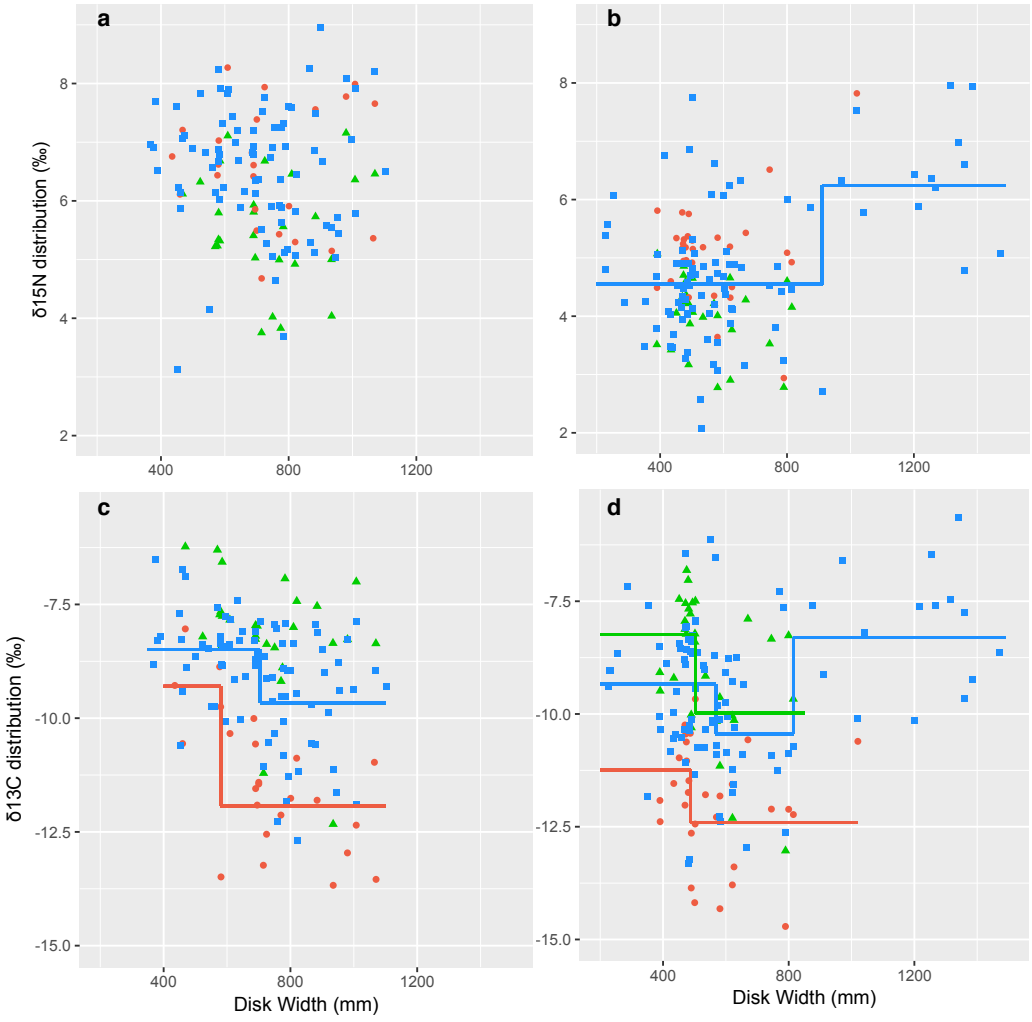

Figure 2: Scatterplots showing isotopic distribution (Top row: Nitrogen, Bottom row: Carbon) and disc width for *H. americanus* (Left) and *S. schmardae* (Right). Each tissue type is denoted in a different shape and colour: white muscle (blue squares), blood (red circles), and barb (green triangles). Breakpoints are demonstrated by solid lines indicating a change point (disc width point where the mean isotopic value shifts).

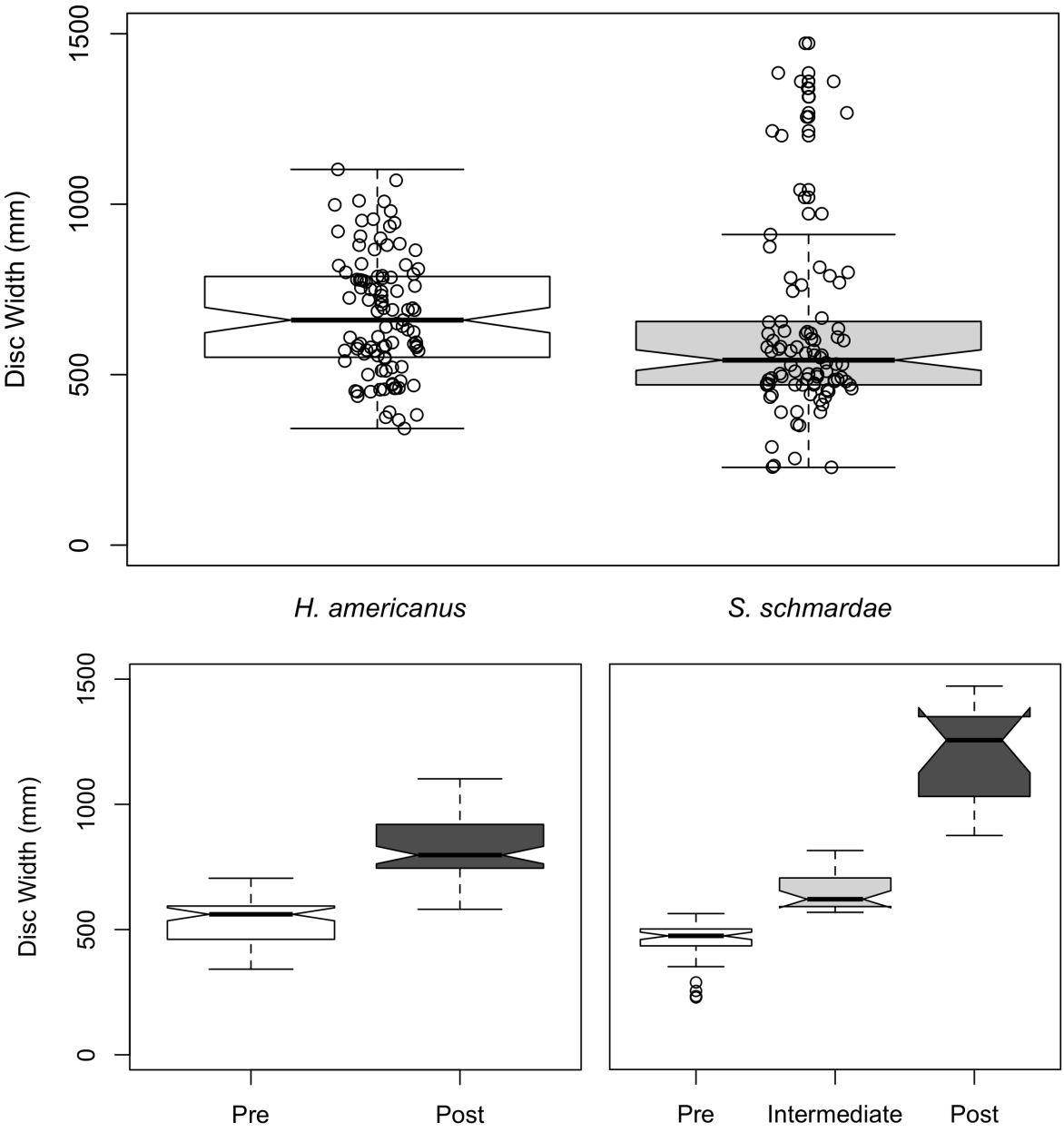

Figure 3: Boxplots of disc widths (mm) for a) all *H. americanus* (white) and *S. schmardae* (light grey); b) *H. americanus* before (white) and after (dark grey) the breakpoint in $\delta^{13}C$ of white muscle samples; c) *S. schmardae* before (white), during an intermediate size (light grey) and after (dark grey) the breakpoints in $\delta^{13}C$ values of white muscle samples. Horizontal lines indicate median values, boxes show interquartile range and whiskers represent range (minimum and maximum values) with outliers (points more or less than 1.5 times the upper or lower quartile segments) indicated by circles.

General Discussion

The data revealed in this study contributes to the limited knowledge base of these species, and these new insights about their ecology, especially in respect to mangroves, could be used to further conservation proposals of these species in the Bahamas. Mangrove habitats face a number of threats, both anthropogenic and natural (Gilman *et al.*, 2008). Globally they are being reduced at a substantial rate due to issues such as deforestation, sea level rise and pollution (Alongi, 2002; Hoegh-Guldberg and Bruno, 2010; Hamilton, 2013; Govers *et al.*, 2014; Stephenson and Jones, 2017). Despite this, they offer an abundance of valuable and arguably irreplaceable ecosystem services such as coastal protection, water filtration and fish nursery habitats (Barbier, 2006; Barbier, 2011; Lee *et al.*, 2014; Sandilyan and Kathiresan, 2015), and are estimated to be of high economic worth (Salem and Mercer, 2012).

In this study we established through SIA (including novel use of sulphur isotopes) that mangroves represent important habitats for both Caribbean whiptail and southern stingrays, and appear to constitute a primary habitat for Caribbean whiptail rays. Breakpoint analysis was used on stable isotope data to estimate the body sizes at which ontogenetic shifts may occur. We determined that both species of stingray likely exhibit ontogenetic habitat shifts from nursery habitats to more offshore environments at smaller, possibly juvenile sizes. Our analysis also detected a probable return to mangrove habitats by *S. schmardae* at a later life stage. Thus, the status of the poorly studies Caribbean whiptail ray is likely directly entwined with the status of mangroves, and their conservation will result in the protection of both the ecosystem and the stingray. Additionally, we suggest that trophic resource partitioning may occur between the two

sympatric species, with southern stingrays feeding at a higher trophic level than Caribbean whiptail rays.

Stable isotope analysis has offered useful insights that may not have been detected in conventional studies, however due to limitations in the interpretation of the data, support of the ecological patterns suggested in this thesis by other techniques with more empirical evidence would be recommended.

Alternative sampling methodologies should be carried out to ensure the entire extent of Bahamian habitat locations for *S. schmardae* and *H. americanus* populations are sampled, that there is no hidden 'deep' population which is being excluded from the dataset. In addition, as breakpoint analysis is used as a novel statistical technique here, further studies should look to utilise methodologies which produce intrinsic evidence of the ontogenetic shifts which are suggested by this study.

Supplementary Materials

Figure 1: Example photos of mangrove habitat (a) and sandbar/beach habitat (b) capture locations. Photo courtesy of Owen O'Shea (a) and Catherine Argyrople (b).

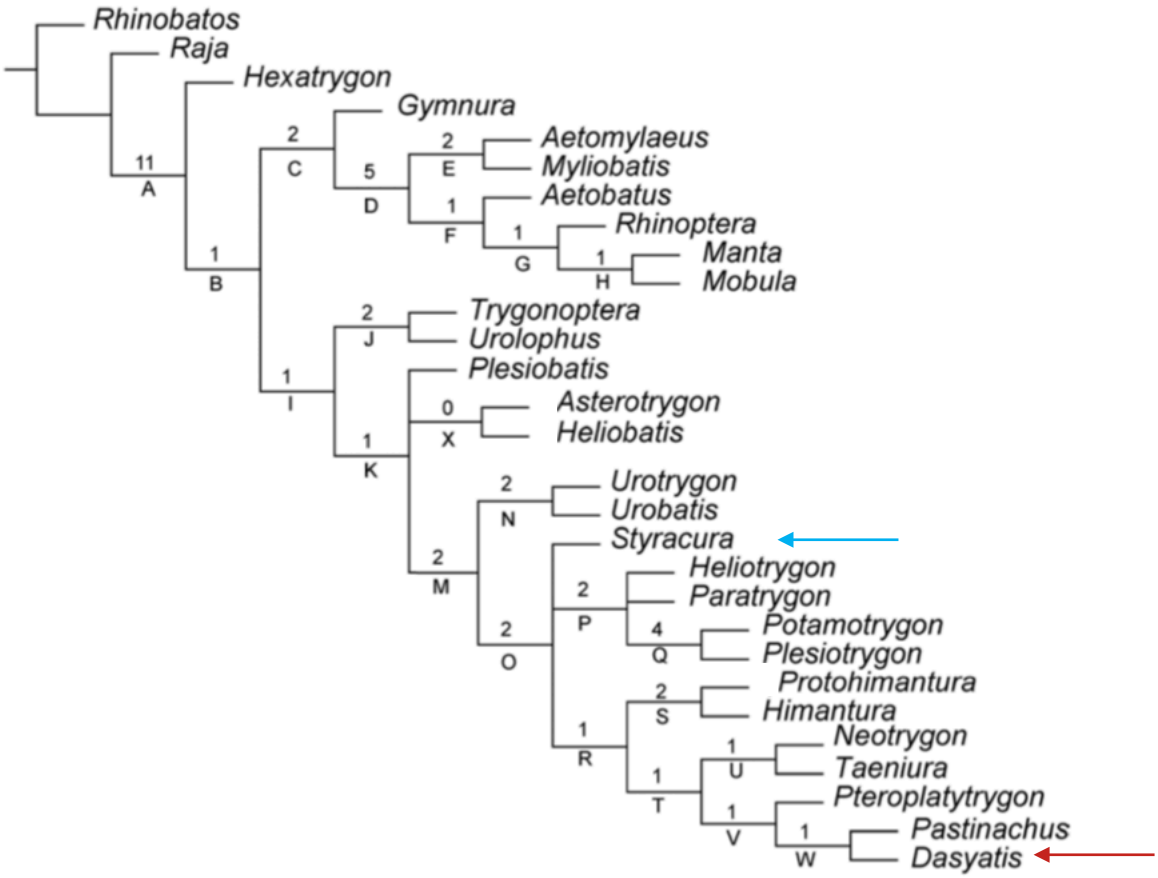

Figure 2: Phylogenetic tree showing relative positions of *S. schmardae* (blue arrow) and *H. americanus* (red arrow). Figure taken from Marrama *et al.* (2018), tree based on hypothetical relationships between 102 morphological characteristics of *Prohimantura vorstmani* within Myliobatiformes. Numbers on nodes indicate the Bremer support in their study. See Marrama *et al.* (2018) for further information.

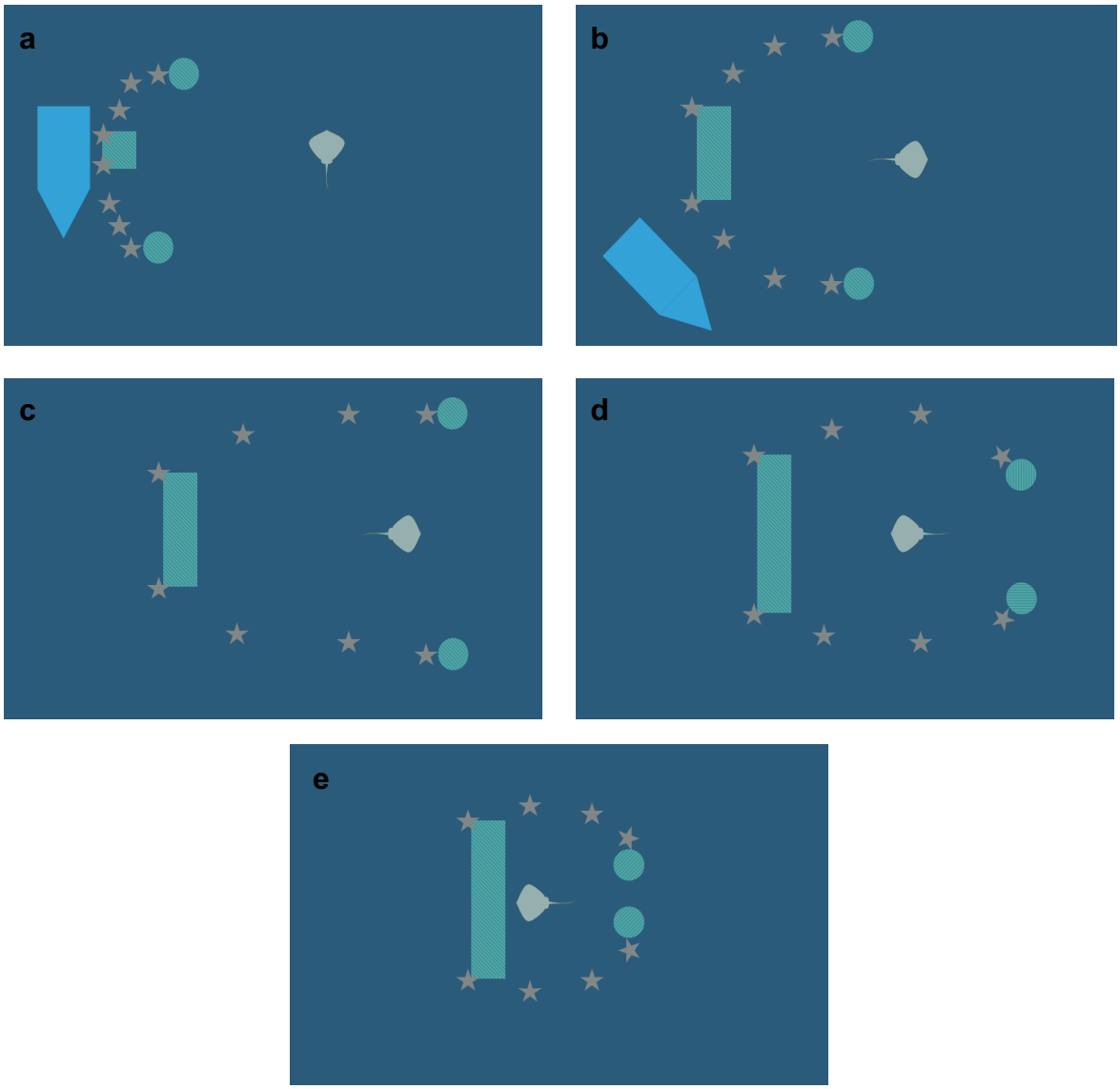

Figure 3: Sequence of events in capture methodology. a) Stage 1: The research team (depicted by grey stars) spot a stingray in shallow waters (<1 m) and disembark from the boat (represented by the blue polygon; alternatively, the research team may approach a stingray whilst walking in shallow water). b) Stage 2: Two members of the team wield large dip nets (green circles) & move either side of the stingray and run forwards in the direction in which the stingray is travelling. Two other members of the team unfurl the seine net (green rectangle) whilst moving forwards towards the stingray. c) Stage 3: The entire team moves at speed in the same direction, until the

dip net wielders overtake the stingray. A large berth must be maintained from the stingray at all times so as not to startle it. d) Stage 4: The dip net wielders now move towards one another and block the stingray's forward movement, causing the stingray to switch direction. At this point the seine net should be fully extended and should represent the largest 'gap' between the team members. e) Stage 5: The entire team should now maintain even distribution as they close in on the stingray and drive it towards the seine net, in an effort to dissuade it from trying to exit through any other gaps in the circle. Once the stingray moves into the seine net, one of the dip net wielders can safely scoop the stingray into the dip net for sampling.

Table 1: P-values and test statistic from GLMM, measuring factors other than species that could influence isotopic values, $\delta^{15}N$, $\delta^{13}C$ and $\delta^{34}S$. Bold text indicates statistical significance.

	$\delta^{15}N$		**$\delta^{13}C$**		**$\delta^{34}S$**	
	X_1	P-value	X_1	P-value	X_1	P-value
Sex	3.77	0.0522	1.64	0.200	0.0940	0.759
Season of capture	0.146	0.702	0.0465	0.829	0.0216	0.883
Island of capture	2.94	0.0866	**6.21**	**0.0127**	1.69	0.194
Disc width	**7.44**	**< 0.01**	2.72	0.0993	1.32	0.251

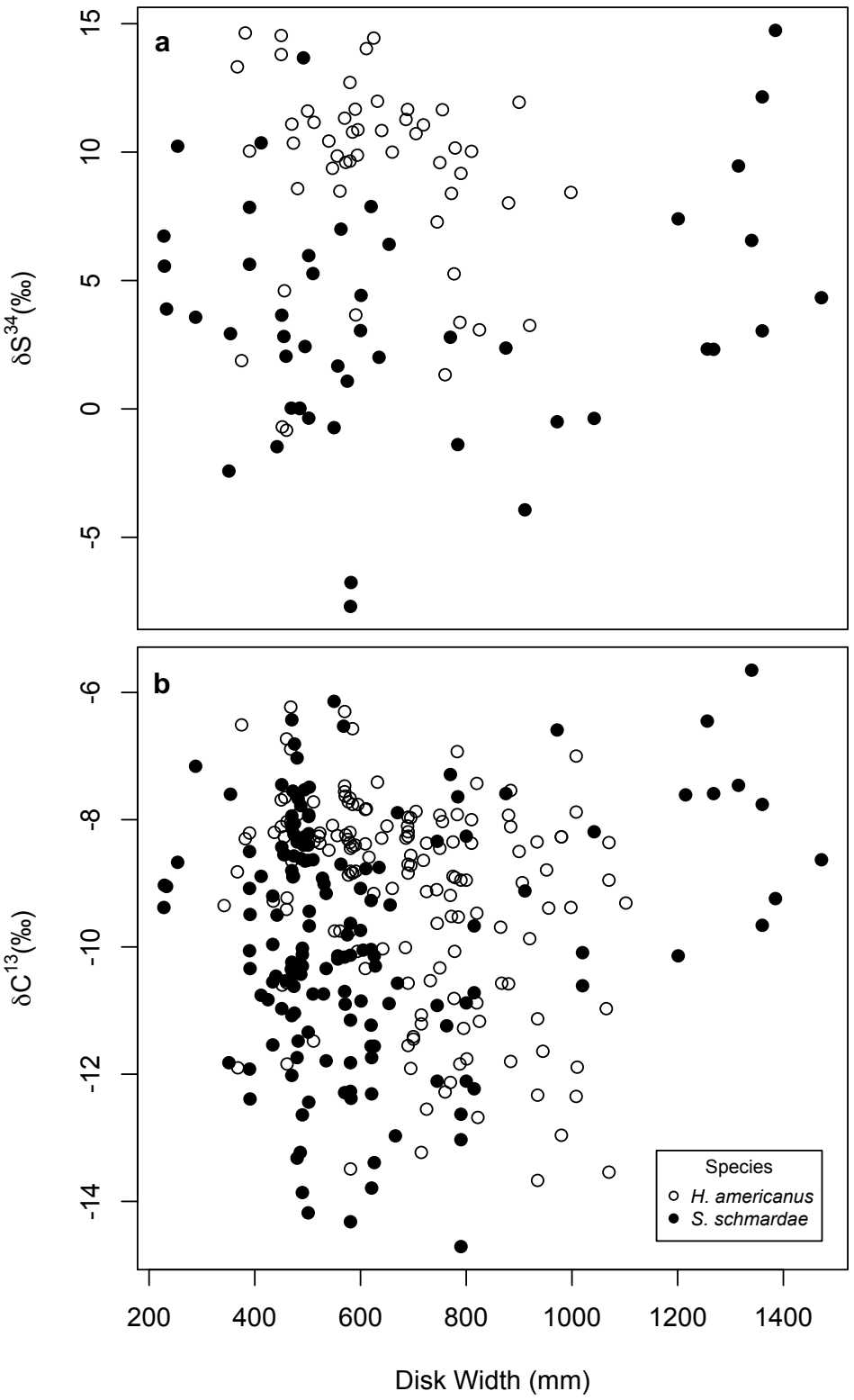

Figure 4: The relationship between individual stingray disc width (mm) and isotopic values of a) $\delta^{34}S$ (‰) and b) $\delta^{13}C$ (‰) in white muscle of all individual stingrays for species *H. americanus* (empty circles) and *S. schmardae* (solid black circles).

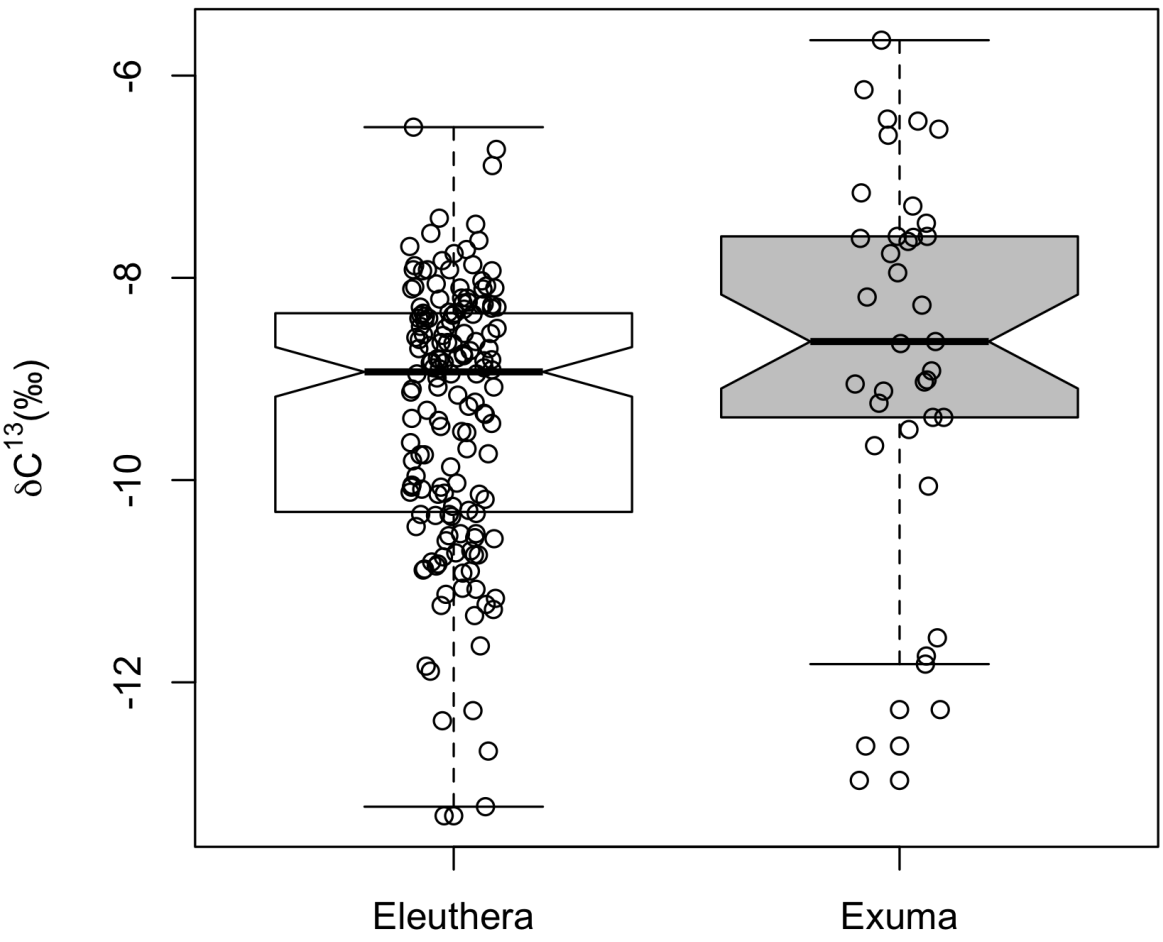

Figure 5: Boxplot displaying $\delta^{13}C$ isotopic values (expressed in ‰) of muscle samples of stingrays captured around Exuma Cays and Eleuthera island, Bahamas. In the boxplots, lines indicate median, boxes show upper (75%) and lower (25%) quartiles and whiskers represent range (minimum and maximum values) with outliers indicated by circles. These are defined as points more or less than 1.5 times the upper or lower quartile segments using R statistical software.

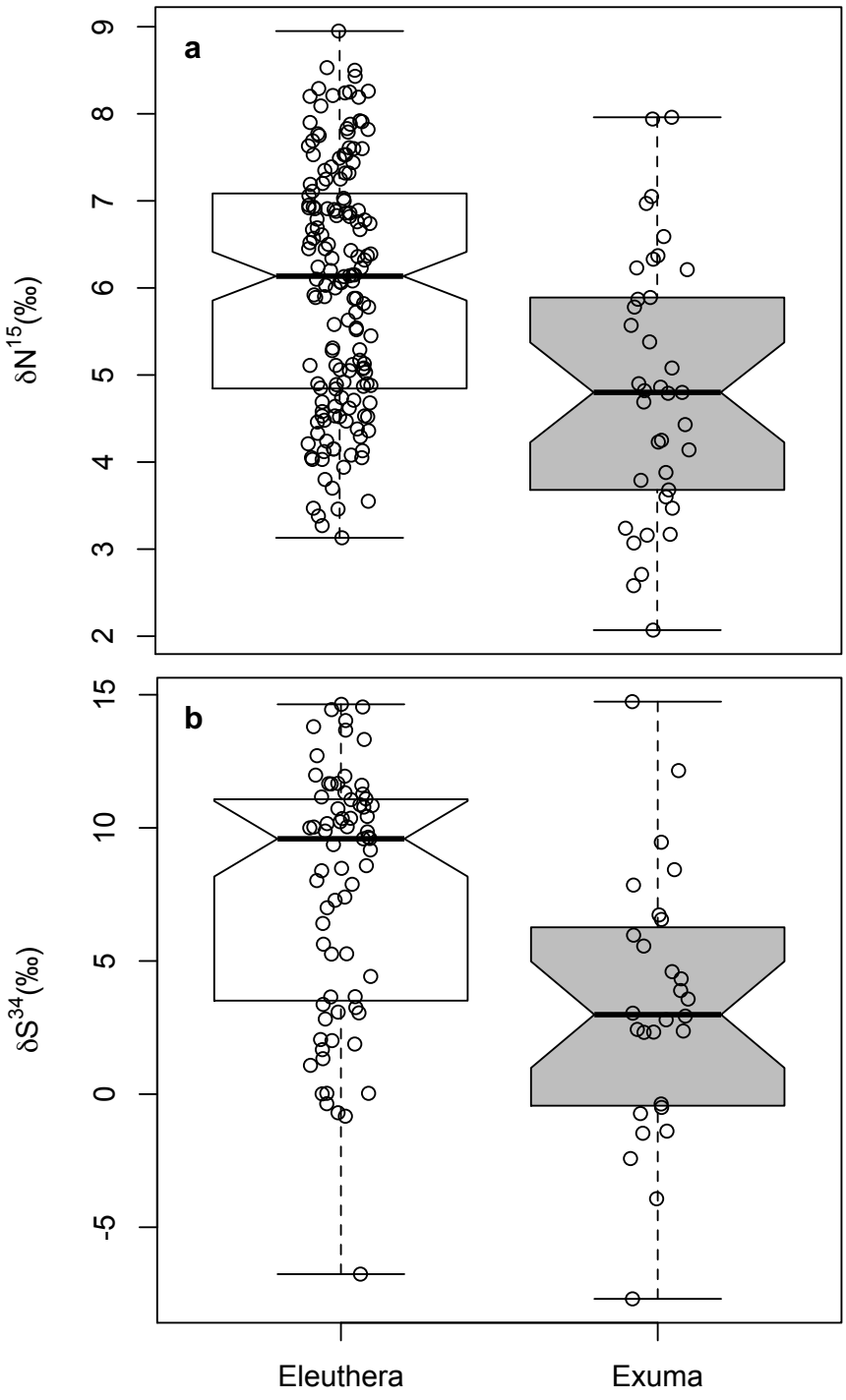

Figure 6: Boxplot displaying a) $\delta^{15}N$ and b) $\delta^{34}S$ isotopic values (expressed in ‰) of muscle samples of stingrays captured around Exuma Cays and Eleuthera island, Bahamas. In the boxplots, lines indicate median, boxes show upper (75%) and lower (25%) quartiles and whiskers represent range (minimum and maximum values) with outliers indicated by circles. These are defined as points more or less than 1.5 times the upper or lower quartile segments using R statistical software.

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