STABLE ISOTOPE DYNAMICS IN COWNOSE RAYS (*RHINOPTERA BONASUS*) WITHIN THE NORTHWESTERN GULF OF MEXICO

A Thesis

by

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ABSTRACT

The cownose ray (*Rhinoptera bonasus*) is a durophagous mesopredator that exerts for top-down control on commercial shellfish stocks along the Atlantic coast. Although the trophic ecology of this elasmobranch has been the subject of extensive investigation, there is limited information available on feeding patterns of cownose rays in the northwestern Gulf of Mexico. Stable isotope analysis has been used to study the foraging ecology of various species, but only recently applied to elasmobranchs. Therefore, this study conducted a controlled feeding trial to determine incorporation rates and diet-tissue discrimination factors for $\delta^{13}C$ and $\delta^{15}N$ in cownose ray epidermal tissue. Additionally, this study investigates $\delta^{13}C$ and $\delta^{15}N$ variability in cownose rays captured via entanglement nets, from surveys along the Texas coast from 2009 – 2012.

This is the first study to report $\delta^{13}C$ and $\delta^{15}N$ incorporation rates in elasmobranch epidermal tissue; estimated $\delta^{13}C$ and $\delta^{15}N$ incorporation rates were 0.0018 ± 0.0003 $days^{-1}$ and 0.0059 ± 0.0022 $days^{-1}$, respectively. Isotopic incorporation rates were highly variable amongst individuals but did not vary significantly with ray size (disc width or weight). Isotopic equilibrium was not reached between the epidermal tissue and the dietary treatment levels; therefore, estimated diet-tissue discrimination factors ($\Delta^{13}C$ = 4.26‰ and $\Delta^{15}N$ = 0.69‰) could not be applied for analyses of wild populations.

Relative size of Bayesian ellipses, denoting the isotopic niche of cownose rays, varied seasonally in the lower Laguna Madre, with Summer 2012 significantly smaller than all other sampling periods. Female mean $\delta^{13}C$ signatures were significantly

enriched compared to those of males, indicating that female rays are foraging over longer periods of time within inshore habitats. Isotopic niche size was comparable across the Texas bay systems in 2012, with only the lower Laguna Madre (Spring) significantly smaller. However, mean $\delta^{13}C$ and $\delta^{15}N$ in cownose rays varied spatially across bay systems along the Texas coast. This initial exploration into the trophic ecology of cownose rays within the northwestern Gulf of Mexico provides evidence of temporal and spatial variability in isotopic signatures, potentially aiding scientists in the management of this species.

DEDICATION

For my parents, Robert and Miriam St. Clair

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NOMENCLATURE

AB	Aransas Bay complex
ANOVA	Analysis of variance
ASE	Accelerated Solvent Extractor
CR	$\delta^{13}C$ range
DTDF	Diet-tissue discrimination factor
DW	Disc width
GB	Galveston Bay complex
GOM	Gulf of Mexico
HSD	Honest significant difference
LLM	Lower Laguna Madre
LMB	Lavaca – Matagorda Bay complex
NR	$\delta^{15}N$ range
SEA	Standard ellipse area
SEA_c	Corrected standard ellipse area
SIA	Stable isotope analysis
SIBER	Stable Isotope Bayesian Ellipses in R
STFERL	Sea Turtle and Fisheries Ecology Research Laboratory
TA	Total area
VPDB	Vienna Pee Dee Belemnite
YOY	Young-of-the-year

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CHAPTER I

INTRODUCTION

The cownose ray (*Rhinoptera bonasus*) is a euryhaline, semi-pelagic species found throughout the western Atlantic Ocean, from New England to Brazil, and within the Gulf of Mexico (GOM) (McEachran and Fechhelm 1998). Cownose rays are gregarious, forming large aggregations (*i.e.* hundreds to thousands of individuals) during seasonal migrations. Schwartz (1965, 1990) documented two distinct stocks of cownose rays within their natural range, where the western Atlantic population migrates along the Atlantic coast between the United States and Brazil in schools of up to 150,000 individuals and the GOM population migrates clockwise from Florida to the Yucatan Peninsula in schools of up to 10,000 individuals. However, cownose rays within some portions of the GOM may simply move to deeper thermostable waters when coastal water temperatures drop significantly, instead of large-scale migrations (Smith and Merriner 1987; Collins et al. 2007).

Studies on the life history of cownose rays have documented differences between the western Atlantic and GOM populations, where cownose rays in the GOM matured at a faster rate and at a smaller disc width (DW) than conspecifics in the Atlantic (Smith and Merriner 1986, 1987; Neer and Thompson 2005; Pérez-Jiménez 2011; Fisher et al. 2013; Poulakis 2013). However, despite regional differences in life history, all of the aforementioned studies agree that cownose rays are a K-selected species with low fecundity (usually one pup per reproductive cycle) and a long gestation period (~11 – 12

months) (Smith and Merriner 1986, 1987; Neer and Thompson 2005; Pérez-Jiménez 2011; Fisher et al. 2103; Poulakis 2013).

Cownose rays have been targeted by the commercial shellfish industry, especially in the Chesapeake Bay region, as the reason for declining commercial shellfish stocks since the late 1970s (Smith and Merriner 1979). Orth (1975) reported drastic reduction of eelgrass (*Zostera marina*) beds in Chesapeake Bay, due to cownose ray foraging, with decreases in invertebrate richness and density within the resulting fragmented habitats. Subsequent claims of exponential increases in cownose ray populations along the Atlantic coast and ray predation at oyster reef restoration sites has led to calls for a commercial cownose ray fishery (Myers et al. 2007; Fisher 2010; Fisher et al. 2013). However, in a study of cownose ray stomach contents collected from both fishery-independent and -dependent methods, Fisher (2010) identified 52 prey items and determined that oysters and clams were not dominant prey items in Chesapeake Bay.

Cownose rays are durophagous mesopredators that forage primarily on benthic invertebrates. To enable the consumption of hard-shelled prey, the jaws of cownose rays possess several modifications to the typical elasmobranch jaw structure including fused symphyses between the mandible and the palatoquadrate (Summers 2000), hypertrophied jaw adductor and coracomandibular muscles (González-Isáis 2003) and multiple layers of tesserae on the surface of the cartilage throughout the jaw, which provides reinforcement to the overall jaw structure (Summers et al. 1998). The result of these modifications is the production of two to four times the amount of bite force compared to the general elasmobranch jaw model (Summers 2000). Additionally,

cownose rays have molariform dentition, divided into hexagonal subunits set within an elastic dental ligament, allowing for greater flexibility and force dispersal during the crushing of prey (Summers 2000). Excavation and manipulation of prey is accomplished via hydraulic suction processes, facilitated by the buccopharyngeal cavity, gill slits, spiracles and paired cephalic lobes (Sasko et al. 2006). The accumulation of these biomechanical adaptations and feeding behaviors has enabled cownose rays to effectively forage on infaunal and epifaunal invertebrate prey.

Smith and Merriner (1985) initially determined that cownose rays were stenophagous in their consumption of bivalve species, with the soft shell clam (*Mya arenaria*) dominating the stomach contents collected from Chesapeake Bay, VA. Peterson et al. (2001) found that cownose rays in North Carolina caused severe declines in populations of bay scallops (*Argopecten irradians concentricus*) in localized areas. These studies promoted the assumption that cownose rays function as hard prey specialists on weak shelled bivalves. However, recent studies in the GOM have shown that cownose rays may be opportunistic generalists that exploit localized abundant prey resources including sedentary polychaetes and crustaceans (Collins et al. 2007) and amphipods (Ajemian and Powers 2012). Continued foraging studies are therefore necessary to provide information about regional differences in trophic dynamics in cownose ray populations to inform fishery managers.

Stomach content analysis has been used extensively to determine this species' foraging patterns (Ajemian and Powers, 2011; Collins *et al.*, 2007; Fisher, 2010) but is restricted to providing only a snapshot of an individual ray's diet and, oftentimes,

traditional sampling methods (*i.e.* stomach lavage or examination of stomach contents from dead specimens) yield stomachs devoid of contents in elasmobranchs (Fisher 2010; Wetherbee et al. 2012). In contrast, stable isotope analysis (SIA) can be less invasive and uses inherent variations of stable carbon and nitrogen isotopes (*i.e.* $^{13}C/^{12}C$ and $^{15}N/^{14}N$) within food webs as dietary tracers to reconstruct an individual's diet. An individual's tissue will reflect the isotopic ratios of the prey it consumes, where the heavier isotopes (^{13}C and ^{15}N) are preferentially assimilated into the consumer's tissues due to the fractionation of the isotopes that occurs during metabolic processing (Martínez del Rio et al. 2009). As a result of this enrichment in a consumer's tissues compared to its diet, the analysis of $\delta^{13}C$ and $\delta^{15}N$ within a species' tissues can be utilized to identify trophic position and movement patterns across disparate habitats (Peterson and Fry 1987). However, isotopic incorporation rates, the rate at which an isotope is incorporated into the tissues of a consumer, varies both across species and within different tissues of a species (Tieszen et al. 1983). Furthermore, the diet-tissue discrimination factor (DTDF), or the difference between the isotope value of the prey and that of the consumer, can be affected by a multitude of factors, including but not limited to growth rates (Reich et al. 2008), diet quality and quantity (Gaye-Siessegger et al. 2004; Robbins et al. 2005; Martínez del Rio 2009), and tissue type (Hobson and Clark 1992). Therefore, it is essential to utilize species- and tissue-specific isotopic incorporation rates and DTDFs to accurately interpret stable isotope data in wild populations (Hussey et al. 2010).

The analysis of $\delta^{13}C$ and $\delta^{15}N$ can be utilized to identify a species' movement patterns across isotopically distinct habitats and trophic position with a food web

(Peterson and Fry 1987). Natural gradients of $\delta^{13}C$ within marine ecosystems can be used to establish the primary source of organic matter. These $\delta^{13}C$ gradients are driven by differences in productivity, as a result of the dominant primary producer (ex. phytoplankton, seagrass, kelp) within a given habitat, leading to distinct $\delta^{13}C$ signatures (Hobson 1999; Graham et al. 2010). Clementz and Koch (2001) identified the following trend in $\delta^{13}C$ signatures where seagrass and kelp habitats are enriched in $\delta^{13}C$ compared to phytoplankton driven habitats and nearshore waters are enriched in $\delta^{13}C$ compared to offshore waters. Trophic position can be determined by the inherent gradient of $\delta^{15}N$ within a food web, with $\delta^{15}N$ increasing from primary producer to consumer and then with each successive consumer (Cabana and Rasmussen 1994; Post 2002).

Research Objectives

The trophic ecology in cownose rays has not been well studied within the northwestern Gulf of Mexico. Stable isotope analysis provides a minimally invasive and non-lethal technique to determine isotopic variability and habitat use patterns within cownose ray populations. However, there are no published isotopic incorporation rates or diet-tissue discrimination factors to provide baseline data for this species. In light of these data needs, the following research objectives were identified:

(1) To determine isotopic incorporation rates and discrimination factors in cownose ray epidermal tissue via a controlled feeding trial.

(2) To characterize $\delta^{13}C$ and $\delta^{15}N$ variability in cownose rays within the northwestern Gulf of Mexico.

CHAPTER II

ISOTOPIC INCORPORATION RATES AND DIET-TISSUE DISCRIMINATION FACTORS IN EPIDERMAL TISSUE OF COWNOSE RAYS

Introduction

Stable isotope analysis has been used to examine trophic dynamics and migration patterns in a myriad of animals (Hobson 1999; Kelly 2000; Rubenstein and Hobson 2004; Dalerum and Angerbjorn 2005). Inherent variations in carbon and nitrogen stable isotope ratios (*i.e.* $^{13}C/^{12}C$ and $^{15}N/^{14}N$) within food webs enable the use of isotope signatures as dietary tracers between prey and consumer tissues. These variations are a result of metabolic processing, during which carbon and nitrogen isotopes are fractionated and the heavier isotopes (^{13}C and ^{15}N) are preferentially assimilated into a consumer's tissues (Martínez del Rio et al. 2009). Natural gradients of carbon and nitrogen isotope values across spatial scales (*i.e.* marine versus freshwater, inshore versus offshore) create unique signatures within an animal's tissues, which can then be used to track movements across isotopically distinct habitats (Hobson 1999). Additionally, when applied to ecosystems with defined isotopic baselines, stable isotopes of carbon and nitrogen can be used to assess trophic positions of consumers and define the trophic structure of a food web (Post 2002). However, due to a suite of factors affecting stable isotope dynamics and processing within an animal, tissue and species-specific turnover rates and discrimination values must be incorporated into analysis in order to accurately interpret stable isotope data in relation to trophic structure and habitat

use patterns (Gannes et al. 1997; Martinez del Rio et al. 2009; Wolf et al. 2009; Hussey et al. 2010).

Isotopic incorporation

The isotopic incorporation or turnover rate is the rate at which the isotope (such as carbon or nitrogen) signature of assimilated prey is adopted by tissues of the consumer. These rates vary across species and amongst tissues within the same species, such that multiple tissues yield multiple temporally distinct isotopic records, measured in average residence times, for that species (Tieszen et al. 1983). Incorporation rates vary by species and are affected by body size, protein turnover and growth rates, with ectotherms generally having much slower incorporation rates than endotherms (Martínez del Rio et al. 2009). For example, the average residence time of $\delta^{13}C$ in muscle of Japanese quail (*Coturnix japonica*) is 18 days in contrast to 108 days for leopard sharks (*Triakis semifasciata*) (Hobson and Clark 1992; Malpica-Cruz et al. 2012). Within a species, metabolically active tissues (*i.e.* liver) will have faster isotopic incorporation rates than less metabolically active tissues (*i.e.* cartilage). For example, in leopard sharks, average residence time of $\delta^{13}C$ can range from 21 days in liver to 112 days in cartilage (Malpica-Cruz et al. 2012).

Studies investigating isotopic incorporation have been conducted on a variety of vertebrates (Dalerum and Angerbjorn 2005), but there has been limited work involving elasmobranchs. A review paper by Hussey et al. (2012) summarizes the current knowledge of the application of stable isotope analysis to elasmobranchs, focusing on the various methodologies used, tissues analyzed and application of stable isotope data

to natural populations. At this time, there have been four studies that have experimentally measured isotopic incorporation in elasmobranchs. Kim et al. (2012b) and Malpica-Cruz et al. (2012) reported carbon and nitrogen incorporation rates of various tissues in adult and young-of-the-year leopard sharks, respectively. Logan and Lutcavage (2010) performed a diet-switch experiment on sandbar sharks (*Carcharhinus plumbeus*) and reported carbon and nitrogen isotopic incorporation rates for blood (0.007 – 0.009 day^{-1} and 0.010 – 0.014 day^{-1}, respectively) and muscle (0.005 – 0.006 day^{-1} and 0.007 – 0.009 day^{-1}, respectively). Finally, a diet-switch experiment performed on ocellate river stingrays (*Potamotrygon motoro*), a freshwater species, represents the only study that investigates isotopic turnover of $\delta^{15}N$ in stingrays, with liver (0.015 day^{-1}) turning over at a faster rate than cartilage (0.002 day^{-1}) (MacNeil et al. 2006).

Due to the multitude of factors that influence isotopic incorporation rates, it is imperative that species-specific and tissue-specific incorporation rates are applied to natural populations (Gannes et al. 1997; Martínez del Rio et al. 2009; Wolf et al. 2009). Amongst the elasmobranchs, the dorso-ventrally flattened body and fusion of the enlarged pectoral fins of batoid fishes is a unique morphology, enabling a relatively sedentary, demersal life history strategy. However, members of family Myliobatidae, including the cownose ray, are considered free-swimming, tied only to the benthic substrate during foraging. As a result of this disparity in the life history strategies, there are physiological differences between cownose rays and demersal stingray species. Grim et al. (2012) found that cownose rays possessed higher hemoglobin concentrations, gill surface area and heart mass than the demersal Atlantic stingray, *Dasyatis sabina*.

Therefore, the metabolic activity and, subsequently, the isotopic incorporation rates of cownose rays may be more akin to sharks than demersal stingrays.

Epidermal tissue has primarily been used in stable isotope studies of marine mammals (Todd et al. 1997; Ruiz-Cooley et al. 2004; Alves-Stanley and Worthy 2009) and sea turtles (Seminoff et al. 2006; Reich et al. 2008) and, at this time, there are no published studies using epidermal tissue for stable isotope studies in elasmobranchs. As in other vertebrates, epidermal tissue in elasmobranchs is composed of a metabolically active epithelium and additional structural components such as keratin, elastin and collagen fibers (Meyer and Seegers 2012). Additionally, dermal denticles are embedded within elasmobranch skin and, as a distinct tissue that differs in structure from epidermal tissue, the carbon and nitrogen stable isotope signature of skin will potentially reflect a mixture of the two (Hussey et al. 2011). Due to aforementioned components, epidermal tissue has a much slower turnover rate (*i.e.* weeks to months) in contrast to tissues such as liver and blood that have a fast turnover rate (*i.e.* days to weeks). However, epidermal tissue is advantageous as it can be easily collected from individuals using a biopsy punch and is minimally invasive.

Diet-tissue discrimination factors

Carbon and nitrogen signatures within an organism's tissues are enriched in a predictable step-wise progression due to stable isotope fractionation, as the isotopes are reallocated within a food web, from primary producers to higher-level consumers (DeNiro and Epstein 1978, 1981; Fry 1988). Carbon fractionation is due in part to both the assimilation of dietary components from the bulk diet into consumer tissues and the

loss of isotopically light CO_2 during respiration (Peterson and Fry 1987; Gannes et al. 1997). Additionally, the assimilation of carbon from the bulk diet is complicated by isotopic routing, where there is a differential distribution of carbon from various dietary components (proteins, carbohydrates and lipids) into different consumer tissues (Schwarcz 1991). Nitrogen fractionation is due in part to the preferential excretion of ^{14}N in nitrogenous wastes (Peterson and Fry 1987; Gannes et al. 1997). This enrichment of the heavier isotopes (^{13}C and ^{15}N) between the diet and consumer's tissues is defined as the diet-tissue discrimination factor (DTDF) or trophic fractionation value, where $\Delta^{b}X_{tissue-diet} = \delta^{b}X_{tissue} - \delta^{b}X_{diet}$ for a specific isotope (Hussey et al. 2012) with the standard denotation for carbon and nitrogen DTDFs as $\Delta^{13}C$ and $\Delta^{15}N$, respectively.

Post (2002) initially reviewed the stable isotope literature and provided mean discrimination values for $\Delta^{13}C$ and $\Delta^{15}N$ of 1.0 and 3.4 ‰, respectively, per trophic level across a variety of invertebrate and vertebrate taxa. These values had been commonly accepted as standard DTDFs and used to interpret trophic relationships in ecological field studies. However, recent reviews of the stable isotope literature have documented a high degree of variability in $\Delta^{13}C$ and $\Delta^{15}N$ among species and tissues with values ranging from -8.8 to 6.1‰ and -3.2 to 9.2‰, respectively (McCutchan et al. 2003; Vanderklift and Ponsard 2003; Reich et al. 2008; Caut et al. 2009; Wolf et al. 2009). Furthermore, factors such as diet quality and quantity (Gaye-Siessegger et al. 2004; Robbins et al. 2005; Martínez del Rio et al. 2009; Florin et al. 2011), diet isotopic ratio (Caut et al. 2008), age (Overman and Parrish 2001) and location of tissue samples (Waddington and MacArthur 2008) can also affect DTDFs. Therefore, it is vital to use

species- and tissue-specific diet-tissue discrimination factors in field studies and experimentally evaluate how the aforementioned conditions influence DTDFs.

As with tissue isotopic incorporation rates, controlled laboratory feeding trials enable researchers to experimentally determine DTDFs. After a dietary switch, a DTDF may be calculated once the tissue(s) in question has reached an isotopic equilibrium with the current diet. In their review of the recent literature concerning variation in DTDFs, Caut et al. (2009) cautions that it is imperative for DTDFs to be derived from tissues that are in equilibrium with the diet in order to reduce experimental bias.

There is a considerable lack of data examining DTDFs in elasmobranchs. At this time, there are only three studies that have experimentally determined DTDFs in elasmobranchs. Hussey et al. (2010) conducted a semi-controlled experiment with three sand tiger sharks (*Carcharias taurus*) and one lemon shark (*Negaprion brevirostris*) and reported DTDFs for liver ($\Delta^{13}C$: 0.2 – 0.6‰; $\Delta^{15}N$: 1.5 – 2.0‰), muscle ($\Delta^{13}C$: 0.9 – 1.3‰; $\Delta^{15}N$: 2.3 – 2.8‰), and vertebral cartilage ($\Delta^{13}C$: 3.8 – 4.1‰; $\Delta^{15}N$: 1.5 – 2.0‰). A second study determined DTDFs for blood and muscle tissues in leopard sharks based on three individuals fed a monotypic diet of squid (*Doryteuthis opalescans*) for more than 1000 days (Kim et al. 2012a). In their review of these two studies, Hussey et al. (2012) pointed out that DTDFs in elasmobranch species are variable not only across tissues but within tissues, as evident by the disparate $\Delta^{15}N$ and $\Delta^{13}C$ of muscle tissues reported [$\Delta^{15}N$: 2.3 ‰ ± 0.2 and $\Delta^{13}C$: 0.9 ‰ ± 0.3 in Hussey et al. (2010); $\Delta^{15}N$: 3.7 ‰ ± 0.2 and $\Delta^{13}C$: 1.7 ‰ ± 0.5 in Kim et al. (2012a)]. Lastly, Malpica-Cruz et al. (2012) documented carbon and nitrogen DTDFs in multiple tissues (liver, blood, muscle, fin

and cartilage) of neonate leopard sharks after a dietary switch and noted that there was larger range in carbon DTDFs (0.13 – 1.98 ‰) than in nitrogen DTDFs (1.08 – 1.76 ‰) across the tissues.

Objectives

Although stable isotope analysis has been increasingly used in elasmobranch trophic studies, questions still remain regarding the isotope incorporation dynamics and DTDFs of epidermal tissue, especially in larger, mature individuals (Hussey et al. 2012) In light of these informational gaps, a captive feeding trial was conducted to address the following objectives:

(1) To determine the isotopic incorporation rates of $\delta^{13}C$ and $\delta^{15}N$ in epidermal tissue of cownose rays.

(2) To examine the influence of age/size and sex of individuals on isotopic incorporation rates.

(3) To determine the isotopic diet-tissue discrimination factors for $\delta^{13}C$ and $\delta^{15}N$ in epidermal tissue of cownose rays.

(4) To examine the influence of diet quality, age/size and sex of individuals on diet-tissue discrimination factors.

Hypotheses

The following hypotheses, as well as the above objectives, are addressed in this chapter:

(1) $\delta^{13}C$ and $\delta^{15}N$ in epidermal tissue will have an average residence time of approximately 6 months.

(2) Size will influence isotopic incorporation rates.

(3) Sex will influence isotopic incorporation rates.

(4) $\delta^{13}C$ and $\delta^{15}N$ diet-tissue discrimination factors will be equal to 1.0 and 3.4 ‰, respectively.

Materials and Methods

Cownose ray capture

Entanglement netting of cownose rays was conducted within the Lavaca-Matagorda Bay complex (LMB), ~ 50 m off Indianola Beach, in Calhoun County, TX from 25 – 27 June, 2012 (Fig. 1). The sampling location was adjacent to a recreational area with a shell hash shoreline and approximately 24 km inshore from Pass Cavallo, which separates Matagorda Island and Matagorda Peninsula. Water depth at the sampling location was 2.5 m and the substrate consisted of soft mud and shell hash. Two large-mesh, entanglement nets (91.4 m long and 3.6 m deep, with 17.7 cm bar mesh of #9 twisted nylon) were set in tandem perpendicular to the beachfront. One vessel was primarily responsible for checking the nets every 20 minutes or more frequently as

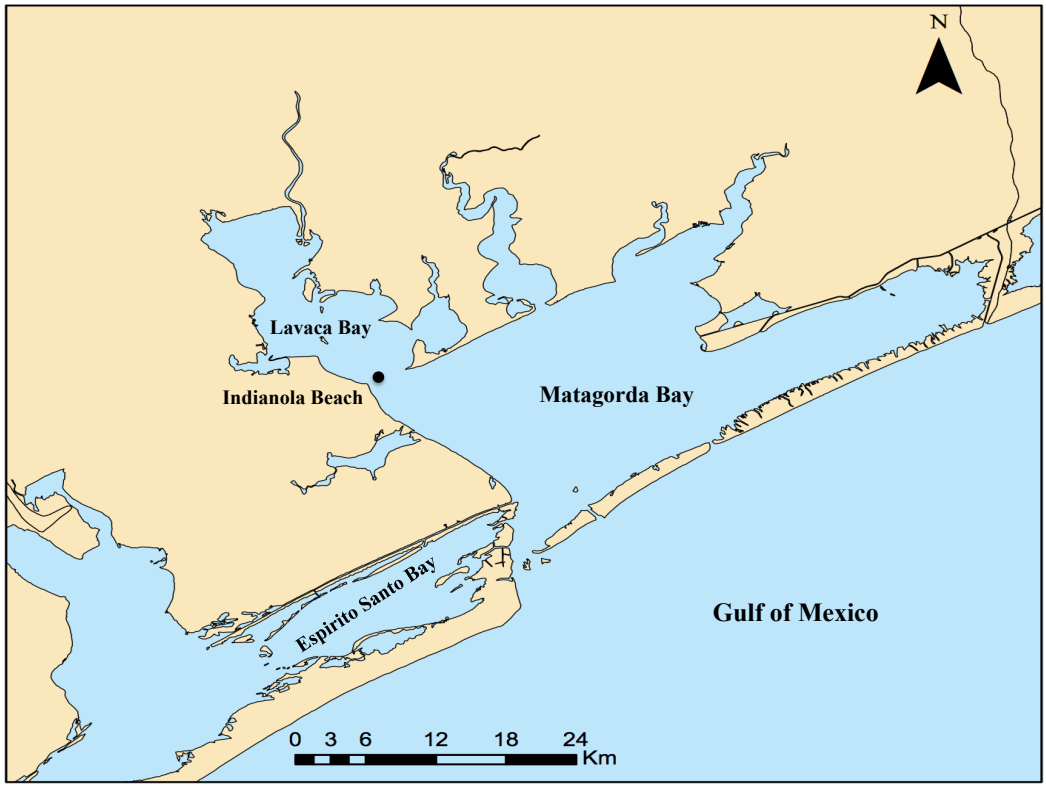

Fig. 1. Map of Lavaca-Matagorda Bay complex showing cownose ray capture location off of Indianola Beach.

splashes or other signs of potential capture dictated in order to minimize risk of injury to cownose rays. A second vessel with an aerated holding tank was used to run cownose rays from the nets upon capture to a transport truck located on the Indianola Beach shoreline. The transport truck held two 1.5-m diameter round tanks and requisite life support system equipment, with each tank able to house up to six cownose rays. Over the course of three days, a total of thirty-one cownose rays were transported to the quarantine facility at Moody Gardens, Galveston, Texas.

Feeding trials

Twenty-four cownose rays were selected from the original pool of thirty-one rays based on two criteria: 1) disc width (DW) greater than 60 cm and 2) good health condition and acclimation to captive conditions as determined by Moody Gardens biologist staff. The twenty-four rays were partitioned into two treatment groups (each, n = 12), with each group housed in round fiberglass Red Ewald tanks (5.8-m diameter, 1.8-m total depth with 1.5-m water depth). Each holding tank was equipped with a Triton II model TR410 rapid sand filter, 4 biological contact chambers, 1.5 Hp main centrifugal circulation pump and RK2 model RK75 foam fractionators. The cownose rays were acclimated to the experimental tanks for 50 days. Ambient O_2 at air-saturation was maintained through water circulation and air stones. Temperature (mean 25.3 °C ± 0.7 standard deviation, SD) and salinity (32.7 ‰ ± 2.5) were measured daily throughout the 201-day experiment.

Before initiating the feeding trial, all of the cownose rays were tattooed on their pectoral fin with silver nitrate ($AgNO_3$) for identification purposes per standard

aquarium practices. Approximately two months into the feeding trial ultrasound screening was done by Dr. Eileen Clark to determine the reproductive status of the female rays. Due to time constraints, nine female rays were selected (6 out of 12 individuals from treatment group #1; 3 out of 11 individuals from treatment group #2). The rays were netted out of the experimental tanks individually and placed into a small round tank containing 60 ppm MS-222 (2:1 sodium bicarbonate:MS-222). When the ray was sufficiently sedated, it was placed on a scoop net and an ultrasound was performed to determine the reproductive state. After the ultrasound was completed, the ray was placed onto a recovery tray in their respective experimental tank and allowed to swim back into the experimental tank on its own accord. One out of the six rays screened from treatment group #1 and all three rays from treatment group #2 were gravid (Fig. 2).

Treatment group #1 was fed a monotypic diet of peeled shrimp ($\delta^{13}C$: -21.3 ‰ ± 1.7; $\delta^{15}N$: 11.0 ‰ ± 2.2) and treatment group #2 was fed a monotypic diet of debeaked and depenned squid ($\delta^{13}C$: -18.8 ‰ ± 0.6; $\delta^{15}N$: 13.9 ‰ ± 0.9, respectively) throughout the duration of the study. Both treatment groups were fed ~ 3 – 4% of each tank's total biomass, six days per week as a maintenance diet. The diets for both feeding trials were purchased from Superior Gulf Shrimp, Inc. (Alvin, Texas) and stored at -22 ºC. Diet sub-samples (n = 48) were collected throughout the duration of the study and analyzed for $\delta^{13}C$ and $\delta^{15}N$ to identify any temporal variation in the isotopic signatures of the diets. At the end of the feeding study, ownership of all cownose rays was transferred to Moody Gardens under Texas Parks and Wildlife Department guidelines.

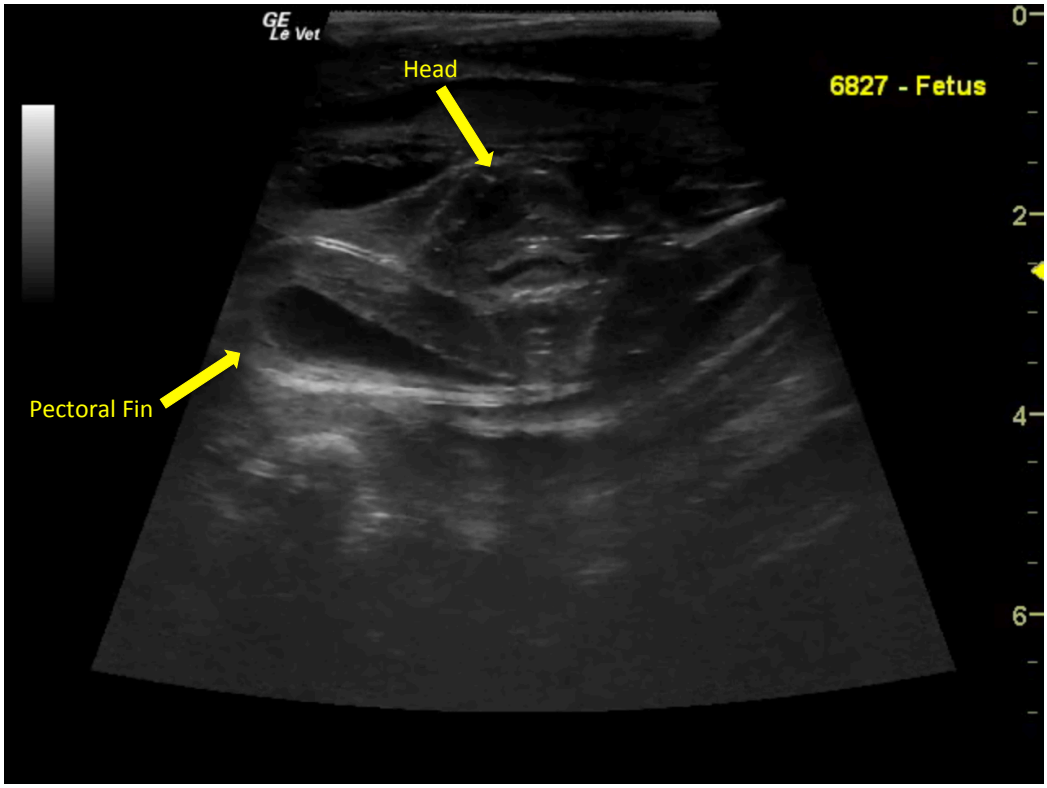

Fig. 2. Ultrasound of female cownose ray #6827 (Treatment Group #2), showing developing embryo with folded pectoral fins in utero.

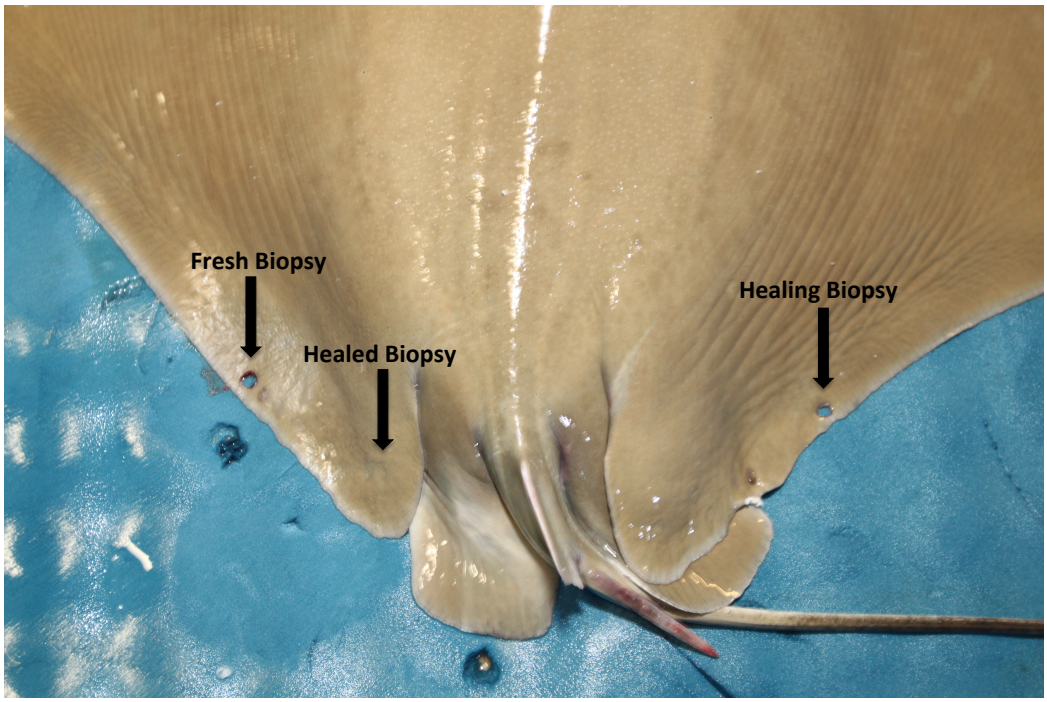

Fig. 3. Cownose ray epidermal biopsy sampling sites on trailing edge of pectoral fins, highlighting different stages of tissue regrowth.

Sample collection and processing

Skin biopsy samples were taken every 10 days from a subset (n = 6) of each treatment group and all individuals from both treatments sampled every 30 days for stable isotope analysis. Skin samples were taken from the trailing edge of the pectoral fins, and were alternated between the left and right pectoral fin for each individual's sampling event (Fig. 3). The sample site was cleaned using a 70% isopropyl alcohol swab prior to collection. All skin samples were collected using a 5-mm sterile biopsy punch and stored in a vial of 70% ethanol for subsequent stable isotope analysis. The storage of epidermal samples in 70% ethanol does not significantly alter $\delta^{13}C$ and $\delta^{15}N$ signatures (Barrow et al. 2008). Individuals were weighed to the nearest 0.01 kg and measured for disc width (DW) to the nearest 0.1 cm concurrent to the collection of tissuesamples. Skin samples were cleaned using 70% ethanol and rinsed with deionized water and diced using a #21 scalpel blade. The skin samples were dried to a constant weight at 60 ºC for 24 hours. Diet samples were dried to constant weight at 60 ºC for 24 hours and then homogenized using a mortar and pestle. To reduce the variability of $\delta^{13}C$, lipids were extracted from the epidermal tissue and diet samples using a Dionex Accelerated Solvent Extractor (ASE), with petroleum ether serving as the solvent (Reich et al. 2008). All samples (tissue and diet) were weighed to 600 µg (± 50 µg) into precleaned tin capsules and sent to the Light Stable Isotope Lab at the University of Florida, Gainesville, FL for analysis. Samples were combusted in a COSTECH ECS 4010 elemental analyzer interfaced via a Finnigan-MAT ConFlow III device (Finnigan MAT, Breman, Germany) to a Finnigan-MAT DeltaPlus XL (Breman, Germany) isotope

ratio mass spectrometer. Resulting stable isotope signatures were expressed in standard delta (δ) notation, as follows:

Equation 1. $\delta = [(R_{sample}/R_{standard}) - 1] [1000]$.

R_{sample} and $R_{standard}$ refer to the ratios of heavy to light isotopes ($^{13}C/^{12}C$ and $^{15}N/^{14}N$) in the sample and standard, respectively. The R standard for ^{13}C was the Vienna Pee Dee Belemnite (VPDB) limestone formation. The R standard for ^{15}N was atmospheric N_2. Units for both $\delta^{13}C$ and $\delta^{15}N$ are defined as parts per thousand (‰).

In order to determine if the isotopic signature of the dermal denticles within the epidermal tissue could be removed to avoid potential contamination of the carbon signature, a subset (n = 12) of lipid-extracted skin samples from treatment group #1 were acid washed and re-analyzed to compare the isotopic signatures from each preparation method (non-acid washed and acid washed). A drop of 0.1 M HCl was added to a vial of skin tissue and allowed to evaporate off for 12 hours. The samples were rinsed three times with dionized water and then dried for 24 hours at 60 ºC. Each sample was weighed and packaged in pre-cleaned tin capsules and sent to the Light Stable Isotope Lab at the University of Florida. Although, the weights of the acid washed samples (250 µg ± 87; range: 104 – 368 µg) sent off for analysis were less than the standard 600 (± 50) µg used for the previous skin isotope analysis (because these samples consisted of the remaining tissue left over from the initial stable isotope analysis run), no issues arose during the sample run.

Masses of C and N in each diet sample were derived by integrating the area under the curve of the thermal conductivity detector in the COSTECH ECS 4010

elemental analyzer and then a regression was created by measuring standards with known %C and %N and known C and N in grams (g) to determine the percent weight (%) of C and N of the unknown samples. Carbon (C) and nitrogen (N) masses were converted to percent by dividing the mass of C and N in the sample by the sample weight and multiplying by 100.

Statistical analyses

The age/size of the cownose rays within the treatment groups was represented by two parameters, DW (cm) and weight (kg). T-tests were used to examine any significant differences between the treatment groups for disc width, weight and initial $\delta^{13}C$ and $\delta^{15}N$ signatures. Another t-test was performed to examine any differences in disc width between males and females used in the feeding trial. A t-test was used to determine if the isotopic signatures of the shrimp and squid were isotopically distinct.

Isotopic incorporation. The fractional rate of isotopic incorporation, k (in $days^{-1}$), was estimated using the following equation in a non-linear fitting procedure (JMP®):

Equation 2. $\delta X_{tissue}(t) = \delta X_{diet}(\infty) + (\delta X_{tissue}(0) - \delta X_{diet}(\infty))e^{-kt}$

where $\delta X_{tissue}(t)$ is the isotopic composition of the tissue at time t, $\delta X_{diet}(\infty)$ is the asymptotic, equilibrium isotopic composition of the diet, $\delta X_{tissue}(0)$ is the initial isotopic composition of the tissue, and k is the fractional rate of isotope incorporation of a tissue (O'Brien et al. 2000; Martínez del Rio et al. 2009; Reich et al. 2008). Linear regression analyses were performed to determine if there was any relationship between cownose ray size (either disc width or weight) and isotopic incorporation rates. A Kruskal – Wallis test was used to detect if significant differences occurred in the isotopic

incorporation rates across three size classes (DW: 60.0 – 69.9 cm, 70.0 – 79.9 cm and 80.0+ cm) within each treatment group. Average residence time for $\delta^{13}C$ and $\delta^{15}N$ was calculated using the following formula:

Equation 3. Average residence time (days) = 1/k.

A t-test was used to examine any significant differences between the carbon and nitrogen signatures of non-acid washed and acid washed skin samples from treatment group #1.

Diet-tissue discrimination factors. Isotopic discrimination of carbon ($\Delta^{13}C$) and nitrogen ($\Delta^{15}N$) was calculated using the following formula:

Equation 4. $\Delta^{b}X_{tissue - diet} = \delta^{b}X_{tissue} - \delta^{b}X_{diet}$

where $\Delta^{b}X_{tissue - diet}$ is the DTDF for a specific isotope, $\delta^{b}X_{tissue}$ is the isotopic signature of the tissue and $\delta^{b}X_{diet}$ is the isotopic signature of the diet. DTDFs were calculated for all individual rays within each treatment group. $\Delta^{13}C$ and $\Delta^{15}N$ (mean ± SD) were then calculated for each treatment group. Multiple regression analysis was used to test if the disc width or weight of the rays within each treatment group significantly predicted their respective $\Delta^{13}C$ and $\Delta^{15}N$ values. A t-test was used to examine any significant sex-specific differences in the squid treatment group for $\Delta^{13}C$ and $\Delta^{15}N$.

A t-test was used to determine if there was any significant difference in the total percent carbon and nitrogen (C and N) of the shrimp and squid diets. A Kruskal-Wallis test was used to determine if there was any significant difference in the carbon:nitrogen (C:N) ratio, a proxy for diet quality, of the two diets. All statistical analyses used an alpha value of 0.05, using JMP software (Version 9.0.0, SAS Institute Inc.).

Results

Treatment group #1 (shrimp diet) consisted of all female individuals, ranging in disc width from 66.2 to 89.8 cm (DW; mean ± SD = 77.0 ± 8.4 cm) and weighing 4.5 to 12.9 kg (mean ± SD = 8.0 ± 2.4 kg). Treatment group #2 (squid diet) consisted of seven females and five males, ranging in disc width from 64.4 to 83.0 cm (mean ± SD = 75.9 ± 6.1 cm) and weighing 4.5 to 10.7 kg (mean ± SD = 8.0 ± 2.0 kg). A single female ray from treatment group #2 died during the feeding trial due to inanition and the failure to thrive in a captive environment and all statistical analyses therefore exclude this individual. There was no significant difference in the initial DW or weight between the two treatment groups ($t_{21} = -0.37$, $p = 0.71$, $n = 23$ and $t_{21} = 0.0134$, $p = 0.99$, $n = 23$, respectively). The isotopic signatures of the shrimp and squid diet were isotopically distinct ($\delta^{13}C$: $t_{40} = 6.50$, $p < 0.01$; $\delta^{15}N$: $t_{40} = 5.44$, $p < 0.01$).

There was also no significant difference in either the weight or disc width of the individuals in treatment group #1 between the first and last sampling event during the feeding trial (weight: $t_{22} = -0.61$, $p = 0.55$, $n = 24$; DW: $t_{22} = 0.28$, $p = 0.80$, $n = 24$). A similar result was found for the weight and disc width of individuals in treatment group #2 (weight: $t_{20} = -0.65$, $p = 0.53$, $n = 22$; DW: $t_{20} = 0.36$, $p = 0.72$, $n = 22$). As there was no significant growth in either treatment of rays during the course of the feeding trial, growth was not factored into the nonlinear fitting procedure that determined the fractional rate of isotopic incorporation of $\delta^{13}C$ and $\delta^{15}N$.

There was no significant difference between the initial signatures of $\delta^{13}C$ and $\delta^{15}N$ of the two treatment groups ($\delta^{13}C$: $t_{21} = -1.31$, $p = 0.21$, $n = 23$; $\delta^{15}N$: $t_{21} = 0.98$, $p =$

0.34, n = 23). The initial mean signatures of $\delta^{13}C$ and $\delta^{15}N$ in the skin of treatment group #1 was -16.39 ± 0.71 ‰ and 11.51 ± 0.88 ‰, respectively. The initial mean signatures of $\delta^{13}C$ and $\delta^{15}N$ in the epidermal tissue of treatment group #2 were -16.71 ± 0.41 ‰ and 11.87 ± 0.85 ‰, respectively.

Isotopic incorporation

Isotopic equilibrium between the ray skin tissue and diet item was not reached in either treatment group because the isotopic signatures of the ray skin tissue did not approach the asymptote of the diet isotopic signatures (Fig. 4 and Fig. 5). However, an estimate of isotopic incorporation, k, was calculated using Equation 2. For treatment group #1, there was a significant difference between the mean $\delta^{13}C$ signature of the shrimp diet and the final mean $\delta^{13}C$ signature of the ray skin tissue ($t_{31} = 8.58$, $p < 0.01$, n = 33). However, there was no significant difference between the mean $\delta^{15}N$ signature of the shrimp diet and the final mean $\delta^{15}N$ signature of the ray skin tissue ($t_{31} = 0.41$, $p = 0.686$, n = 33). The fractional incorporation of carbon for treatment group #1 was estimated to be 0.0018 ± 0.0003 $days^{-1}$ (mean ± SE; Fig. 4), with a range of average residence times of 467 – 651 days. However, the fractional incorporation rate of nitrogen could not be calculated, due to variation in the mean $\delta^{15}N$ signature of the shrimp during the course of the feeding trial in relation to the $\delta^{15}N$ signature of the ray skin tissue.

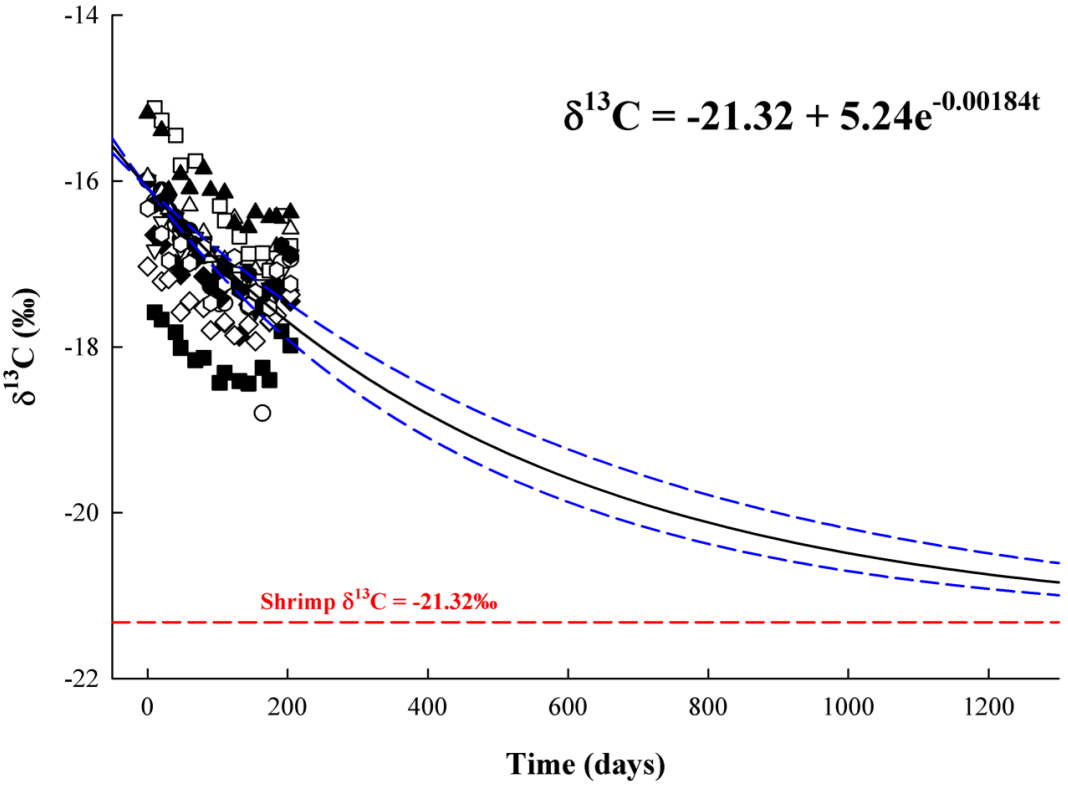

Fig. 4. Changes in $\delta^{13}C$ in cownose rays (Treatment Group #1) after a diet change. Cownose rays are identified by individual symbols. Average nonlinear model fit to individual ray values used to estimate $\delta^{13}C$ incorporation rate (k) is represented by a *solid black line*. *Dashed blue line* represents nonlinear model fit using k ± SE. $\delta^{13}C$ value of shrimp diet represented by a *dashed red line*.

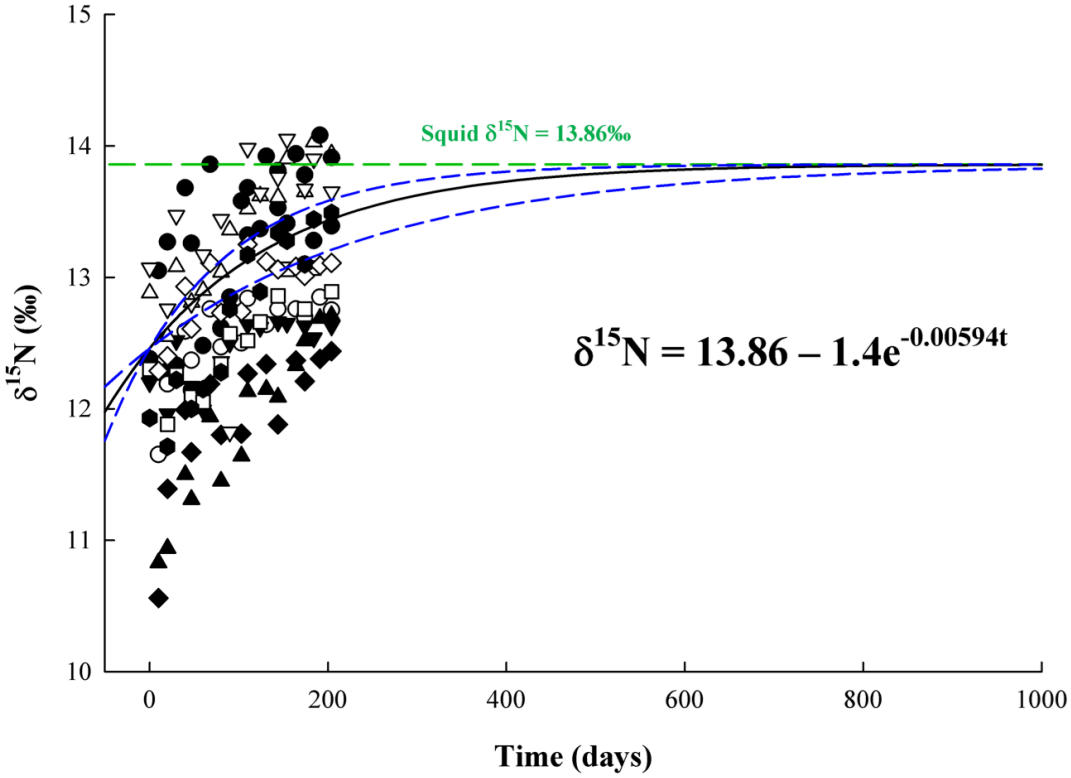

Fig. 5. Changes in $\delta^{15}N$ in cownose rays (Treatment Group #2) after a diet change. Cownose rays are identified by individual symbols. Average nonlinear model fit to individual ray values to estimate $\delta^{15}N$ incorporation rate, k, is represented by a *solid black line*. *Dashed blue line* represents nonlinear model fit using k ± SE. $\delta^{15}N$ value of squid diet represented by *dashed green line*.

For treatment group #2, there was a significant difference between the mean $\delta^{13}C$ and $\delta^{15}N$ signatures of the squid diet and the mean $\delta^{13}C$ and $\delta^{15}N$ signatures of the ray epidermal tissue ($\delta^{13}C$: $t_{30} = 13.8$, $p < 0.01$, $n = 32$; $\delta^{15}N$: $t_{30} = -2.42$, $p = 0.02$, $n = 32$). Variation in the mean $\delta^{13}C$ signature of the squid diet in relation to the $\delta^{13}C$ signature of the ray skin prohibited the estimation of the rate of fractional incorporation of carbon in treatment group #2, similar to the inability to estimate the fractional incorporation rate for nitrogen in treatment group #1. However, determining the rate of fractional incorporation for nitrogen for this treatment group was possible and estimated to be 0.0059 ± 0.0022 days^{-1} (mean ± SE; Fig. 5), with a range of average residence times of 123 – 265 days.

Incorporation rates of both $\delta^{13}C$ and $\delta^{15}N$ were highly variable amongst the individuals within each treatment group (Table 1 and Table 2, respectively). There was no relationship between ray weight or disc width and the carbon isotopic incorporation rate (weight: $r^2 = 0.003$, $p = 0.87$; DW: $r^2 = 0.04$, $p = 0.53$). Similarly, there was no relationship between ray weight or disc width and the nitrogen isotopic incorporation rate (weight: $r^2 = 0.0004$, $p = 0.96$; DW: $r^2 = 0.005$, $p = 0.84$). There was no significant difference in the isotopic incorporation rate of carbon ($F_{2,9} = 0.26$, $p = 0.77$, $n = 12$) or nitrogen ($F_{2,8} = 0.36$, $p = 0.71$, $n = 11$) amongst the three size classes (DW: 60.0 – 69.9 cm, 70.0 – 79.9 cm and 80.0+ cm) within each treatment group (Treatment group #1: n = 2, n = 3, n = 7, respectively; Treatment group #2: n = 1, n = 5, n = 5, respectively). Furthermore, there was no significant difference in $\delta^{15}N$ incorporation rates due to sex ($t_9 = 0.34$, $p = 0.74$, $n = 11$). As there were only females in treatment group #1,

Table 1. Individual cownose ray $\delta^{13}C$ incorporation rates for epidermal tissue based on data shown in Fig. 4.

Individual	k (day^{-1})	r^2
6831	0.00150±0.00014	0.651
6832	0.00196±0.00029	0.397
6825	0.00186±0.00009	0.908
6833	0.00105±0.00012	0.538
6829	0.00417±0.00067	n.d.
6835	0.00071±0.00025	0.369
6836	0.00292±0.00024	n.d.
6837	0.00280±0.00041	n.d.
6838	0.00032±0.00017	0.234
6839	0.00148±0.00016	n.d.
6840	0.00146±0.00015	0.475
6790	0.00185±0.00021	n.d.
Mean	**0.00184±0.00030**	0.170

Data are means ± standard error.
n.d., not determined

Table 2. Individual cownose ray $\delta^{15}N$ incorporation rates for epidermal tissue based on data shown in Fig. 5.

Individual	k (day^{-1})	r^2
6819	0.00487±0.00095	0.646
6823	0.00130±0.00051	0.312
6824	0.00048±0.00042	0.093
6830	0.01179±0.00166	0.752
6826	0.02395±0.00841	0.279
6827	0.00144±0.00062	0.320
6820	0.00126±0.00086	n.d.
6801	0.00386±0.00053	0.601
6828	0.00110±0.00105	n.d.
6788	0.01166±0.00389	0.211
6822	0.00363±0.00118	0.480
Mean	**0.00594±0.00217**	0.189

Data are means ± standard error.
n.d., not determined

differences in $\delta^{13}C$ incorporation rates due to sex could not be tested.

Carbon and nitrogen signatures in the acid washed skin samples were depleted in comparison to the non-acid washed samples ($\delta^{13}C$: $\bar{x}$ = -17.5 and $\bar{x}$ = -17.1, respectively; $\delta^{15}N$: $\bar{x}$ = 11.1 and $\bar{x}$ = 11.3, respectively). There was a significant difference in $\delta^{13}C$ (t_{22} = -2.54, p = 0.02, n = 24) but no significant difference in $\delta^{15}N$ (t_{22} = -0.62, p = 0.54, n = 24) between the two sample preparation methods.

Diet-tissue discrimination factors

As noted previously, the cownose ray epidermal tissue did not reach equilibrium in either of the two diet treatments. The $\delta^{15}N$ of the epidermal tissue of the shrimp treatment group did not approach equilibrium with the diet due to variability of $\delta^{15}N$ in the shrimp throughout the trial. This problem also occurred with the $\delta^{13}C$ of the epidermal tissue and squid diet. The variability of the $\delta^{15}N$ in shrimp and $\delta^{13}C$ in squid were a result of using additional batches of diet items due to unforeseen circumstances during the feeding trials. Therefore, DTDFs could not be calculated for either of these parameters. However, as $\delta^{13}C$ and $\delta^{15}N$ in the ray epidermal tissue in the shrimp and squid diet treatment group, respectively, were approaching the asymptote for the respective diets, DTDFs were estimated using Equation 4. Mean $\Delta^{13}C$ was determined to be 4.26 ‰ ± 0.44 in the shrimp diet treatment group. Mean $\Delta^{15}N$ was determined to be 0.69 ‰ ± 0.53 in the squid diet treatment group. Individual ray $\Delta^{13}C$ and $\Delta^{15}N$ for both diet treatment groups are presented in Table 3.

Neither disc width (β = 0.12, p = 0.11), weight (β = -0.49, p = 0.11) nor disc width x weight (β = 0.004, p = 0.65) were significant predictors of $\Delta^{13}C$ in the shrimp

diet treatment group. The overall model fit was $R^2 = 0.29$. Similarly, disc width (β = 0.11, p = 0.10), weight (β = -0.39, p = 0.09) and disc width x weight (β = -0.003, p = 0.91) were not significant predictors of $\Delta^{15}N$ in the squid diet treatment group. There was also no significant difference in $\Delta^{15}N$ in the squid diet treatment group due to sex (t_9 = -0.55, p = 0.59).

There was a significant difference in total C between the shrimp and squid diet (t_{40} = 1.73, p < 0.01; Table 4) but no significant difference in total N between the two diets (t_{40} = 4.27, p = 0.09). The shrimp diet C:N ratio was slightly lower than that of the squid diet and there was a significant difference in the C:N ratio between the two diets (H = 18.61, 1 d.f., p < 0.01).

Discussion and Conclusions

Isotopic incorporation

Based on review of the literature, it appears that this study is the first to report on the isotopic incorporation of $\delta^{13}C$ and $\delta^{15}N$ in skin of an elasmobranch. Previous studies on isotopic incorporation of $\delta^{13}C$ and $\delta^{15}N$ in elasmobranchs focused on turnover rates for liver, blood (whole, plasma and red blood cells), muscle, cartilage and fin (MacNeil et al. 2006; Logan and Lutcavage 2010; Kim et al. 2012a; Malpica-Cruz et al. 2012). In the absence of directly comparable tissue results from other studies (*i.e.* skin in other elasmobranchs), a comparison can be made between the turnover rates for $\delta^{13}C$ and $\delta^{15}N$ in cownose ray skin and other elasmobranch tissues with similar turnover rates (*i.e.* muscle, fin and cartilage).

Table 3. Individual cownose ray diet-tissue discrimination factors (Δ) for epidermal tissue.

Animal ID	Sex	Diet	Final DW, cm	Final Weight, kg	$\Delta^{13}C$, ‰	$\Delta^{15}N$, ‰
6825	Female	Shrimp	77.4	5.80	3.95	n.d.
6836	Female	Shrimp	84.7	8.30	3.87	n.d.
6838	Female	Shrimp	85.9	7.45	4.94	n.d.
6840	Female	Shrimp	66.0	4.05	4.43	n.d.
6829	Female	Shrimp	70.0	4.55	3.34	n.d.
6839	Female	Shrimp	87.1	8.45	4.43	n.d.
6833	Female	Shrimp	68.2	3.50	4.74	n.d.
6832	Female	Shrimp	70.6	4.45	4.38	n.d.
6835	Female	Shrimp	83.3	6.65	4.54	n.d.
6837	Female	Shrimp	80.5	7.50	3.95	n.d.
6790	Female	Shrimp	92.6	9.80	4.08	n.d.
6831	Female	Shrimp	82.3	7.55	4.41	n.d.
6826	Female	Squid	78.0	7.30	n.d.	-0.05
6828	Female	Squid	76.2	6.15	n.d.	1.15
6827	Female	Squid	80.7	7.10	n.d.	0.97
6823	Female	Squid	85.9	8.25	n.d.	1.11
6824	Female	Squid	72.7	4.50	n.d.	1.23
6788	Female	Squid	83.6	8.65	n.d.	0.21
6819	Male	Squid	67.5	4.25	n.d.	0.47
6821	Male	Squid	74.3	4.95	n.d.	0.75
6820	Male	Squid	83.3	5.60	n.d.	1.42
6830	Male	Squid	72.1	4.65	n.d.	-0.08
6822	Male	Squid	85.0	8.10	n.d.	0.37

n.d., not determined (see text).

Table 4. Stable isotope ratios of diets fed to captive cownose rays.

					$\delta^{13}C$, ‰			$\delta^{15}N$, ‰		
Diet	*N*	C:N	Total C, %	Total N, %	Mean ± s.e.	Minimum	Maximum	Mean ± s.e.	Minimum	Maximum
Shrimp	21	3.23	40.5 ± 0.8	12.5 ± 0.2	21.3 ± 0.4	-24.4	-18.9	11.0 ± 0.5	7.0	14.6
Squid	21	3.42	44.6 ± 0.6	13.1 ± 0.2	18.8 ± 0.1	-19.8	-17.5	13.9 ± 0.2	11.7	15.3

Total C and N are means ± s.e.

The incorporation rate of $\delta^{13}C$ in cownose ray epidermis was much slower than previously reported rates in any elasmobranch tissue (Logan and Lutcavage 2010; Kim et al. 2012a; Malpica-Cruz et al. 2012). This result is consistent with other studies that have shown that a tissue with low metabolic activity (*i.e.* skin) will have a lower incorporation rate than a metabolically active tissue (*i.e.* liver or blood) (Tieszen et al. 1983; Hobson and Clark 1992; Hobson et al. 1996). Compared to other elasmobranch "slow" turnover tissues (*i.e.* muscle, cartilage and fin), carbon turnover in cownose ray skin was 2.5 – 5 times slower. However, this lower turnover rate could be due to differences in the life history stages examined. The studies by Logan and Lutcavage (2010), Kim et al. (2012a) and Malpica-Cruz et al. (2012) utilized either neonate or juvenile individuals, whereas, the present study employed subadult (DW: 60.0 – 79.9 cm) and small adult (DW: 80.0+ cm) cownose rays, based on size at maturity as defined by Smith and Merriner (1987), and small, faster growing elasmobranchs are more likely to have faster incorporation rates than large, slower growing individuals.

Conversely, the incorporation rate of $\delta^{15}N$ in cownose ray skin was similar to $\delta^{15}N$ incorporation rates in cartilage (0.0041 ± 0.0004 days^{-1}) of ocellate river stingrays (MacNeil et al. 2006) and fin tissue (0.0065 ± 0.0002 days^{-1}) of neonate leopard sharks (Malpica-Cruz et al. 2012). However, the $\delta^{15}N$ incorporation in cownose ray skin was lower than in muscle (0.0093 ± 0.003 days^{-1}) and cartilage (0.0089 ± 0.001 days^{-1}) of neonate leopard sharks (Malpica-Cruz et al. 2012) and muscle (0.009 ± 0.001 days^{-1}) of sandbar sharks (Logan and Lutcavage 2010). While these tissues are not directly

comparable, the differences in incorporation rates highlight the variability of rates in slow turnover tissues across various elasmobranch taxa.

Metabolic rate, as a function of life history strategy, directly affects isotopic incorporation rates and it would be expected for taxa with disparate life history strategies to have different isotopic incorporation rates. Cownose rays and leopard sharks are coastal, benthic predators that undergo seasonal migrations (Smith and Merriner 1987; Castro 2011), while ocellate river stingrays are primarily demersal and restricted to the freshwater river basins of the Neotropics (Shibuya et al. 2009). However, all of the aforementioned $\delta^{15}N$ incorporation rates are relatively similar despite differences in life history strategies. Similar to the $\delta^{13}C$ incorporation rate, the $\delta^{15}N$ incorporation rate of subadult/adult cownose rays used in this feeding trial was slower than $\delta^{15}N$ rates reported in neonate leopard sharks, reinforcing the importance of both species-specific and life history stage-specific isotopic incorporation rates. Although, species-specific incorporation rates should always be used for studies involving wild populations, cownose ray incorporation rates (at least for $\delta^{15}N$) may be suitable for use in stable isotope analyses of other coastal elasmobranchs, such as eagle rays or bat rays species, in the absence of experimentally derived incorporation rates for those species.

It has been well documented that carbon and nitrogen incorporation rates are uncoupled and, therefore, not equal (Hobson and Bairlein 2003; Carleton and Martínez del Rio 2005). Assimilation rates of carbon and nitrogen differ from each other due to differences in biochemical fractions of the diet (*i.e.* carbohydrate, lipid and protein) that are integrated in the isotopic composition of a tissue and "isotopic routing", where there

is a differential allocation of biochemical fractions into various tissues (Schwarcz 1991; Gannes et al. 1997; Caut et al. 2010). Carbon incorporation rates for other elasmobranch species/tissues are reported to be 0.5 to 1.5 times faster than nitrogen incorporation rates (Logan and Lutcavage 2010; Kim et al. 2012a). However, in this study, the rate of carbon incorporation was approximately three times slower than the rate of nitrogen incorporation. This may be attributed to the estimation of incorporation rates without complete isotopic turnover in the skin tissue, different tissues used in the other studies (*i.e.* muscle, liver, cartilage) and/or the presence of dermal denticles within the skin biopsy samples analyzed in this study.

Dermal denticles, which are imbedded within the skin of elasmobranch species, are homologous structures to elasmobranch teeth and contain inorganic carbonate within the enameloid and collagen layers (Miyake et al. 1999; Sasagawa and Akai 1999). Vennemann et al. (2001) investigated the source of carbon for different layers within elasmobranch teeth and concluded that the $\delta^{13}C$ signature of the enameloid layer is derived from inorganic carbon and not from a dietary source. The presence of nondietary, inorganic carbonates may contaminate the $\delta^{13}C$ of the sample, as carbonates are more depleted than other biochemical fractions of carbon (DeNiro and Epstein 1978). Essentially, there were two distinct tissues within the sample (*i.e.* skin and dermal denticles) and the incorporation rate of dietary $\delta^{13}C$ in the sample may have been artificially lowered due to the effect of the dermal denticles' more depleted $\delta^{13}C$ signature. It may be possible to isolate the dietary $\delta^{13}C$ signature in the enameloid using HCl to demineralize the dermal denticles, similar to a procedure used to prepare bone

and teeth for isotopic analysis (Ambrose 1990). An analysis of a subset of cownose ray skin samples found that acid washing lipid-extracted skin with 0.1 M HCl does significantly affect the $\delta^{13}C$ signature, potentially eliminating the dermal denticles signature, but has no significant effect on the $\delta^{15}N$ signature of the skin. Further experimental studies are needed to investigate the effects of HCl on dermal denticles and a standard acid-washing procedure for elasmobranch skin samples needs to be developed to enable comparisons across studies. In regards to the $\delta^{15}N$ incorporation rate, the rate reported would potentially reflect an average between the two tissues but there would be no contamination of the $\delta^{15}N$, as most nitrogen is derived from dietary protein.

Elasmobranchs follow a pattern of determinate growth where there is a period of rapid growth as juveniles followed by slower growth coinciding with the onset of maturity (Hussey et al. 2012). Growth, catabolic turnover and metabolic turnover all influence isotopic incorporation rates in ectotherms, but studies have found that growth is the primary contributor, especially in early life stages (Fry and Arnold 1982; Tieszen et al. 1983; Hesslein et al. 1993; Reich et al. 2008). Previous studies determining isotopic incorporation rates in elasmobranchs used neonate and juvenile individuals (MacNeil et al. 2006; Logan and Lutcavage 2010; Kim et al. 2012b; Malpica-Cruz et al. 2012) and as such, growth would be the overriding contributor to the resulting isotopic incorporation rates. Nelson et al. (2011) reported that in adult gag, *Mycteroperca microlepis*, metabolism, not growth, significantly contributed to the turnover of $\delta^{13}C$. As noted previously, the present study utilized subadult and adult cownose rays and both treatment groups were fed a maintenance diet and there was no significant growth over

the course of the feeding trial. Thus, the isotopic incorporation rates reported in this study most likely reflect metabolic turnover of $\delta^{13}C$ and $\delta^{15}N$.

Incorporation rates of $\delta^{13}C$ and $\delta^{15}N$ were highly variable amongst individual rays in each respective treatment group. Nelson et al. (2011) documented similar individual variability in muscle isotopic incorporation rates of gag and suggested size, sex and life history stage of each individual as potential sources of variability. Individual size (weight or disc width) did not significantly affect incorporation rates of either isotope in this experiment. Additionally, in treatment group #2, sex did not significantly affect the incorporation rate of $\delta^{15}N$. Kim et al. (2012b) proposed that some aspect of variation amongst individuals will be growth independent but the life history stage and the associated growth rate of the captive individuals used to experimentally determine isotopic incorporations might alter the efficacy of the incorporation rates when applied to wild populations.

The reproductive state of the female rays may have also been a source of variation within the isotopic incorporation rates, as a result of the effects of gestation on an animal's metabolism (Pecquerie et al. 2010). Studies have shown that $\delta^{15}N$ signatures and, potentially $\delta^{13}C$ signatures, will fluctuate in response to metabolic stress (Hatch 2012). During the feeding trial, four out of nine females screened were determined to be gravid via ultrasound (1 individual from treatment group #1; 3 individuals from treatment group #2). However, due to the limited sample size and the inability to ultrasound all of the female rays within the treatment groups, statistical comparisons of isotopic incorporation rates cannot be made. Nonetheless, this topic warrants further

experimental study, as cownose rays have an 11 – 12 month gestation period (Smith and Merriner 1986; Neer and Thompson 2005) and may be a source of variation in isotopic values of wild populations.

Based on the results of this study, analysis of carbon and nitrogen stable isotopes in epidermal tissue of cownose rays will not effectively capture short term diet shifts or seasonal movements, because average residence times of carbon and nitrogen are greater than ~ 540 and 160 days, respectively. However, low incorporation rates of carbon and nitrogen in epidermal tissue may enable stable isotope analysis to access long-term dietary intake (Alves-Stanley and Worthy 2009). Bearhop et al. (2004) proposes using stable isotope analysis as a tool to determine trophic niches by using variances in carbon and nitrogen stable isotope ratios to differentiate between generalists and specialist feeders in natural populations. The application of stable isotope analysis for determining resource use in cownose rays using epidermal tissue will be explored in Chapter III.

Diet-tissue discrimination factors

As noted previously, there have been limited studies examining diet-tissue discrimination factors in elasmobranchs. Based on review of the literature, it appears that this is the first study estimating carbon and nitrogen DTDFs in a batoid species. However, it must be noted that the DTDFs generated in this study do not coincide with expected DTDFs estimates in elasmobranchs (Hussey et al. 2010; Kim et al. 2012a; Malpica-Cruz et al. 2012) nor those discrimination values reported by Post (2002). This result is directly attributable to the fact that the epidermal tissues in both treatment groups did not reach isotopic equilibrium with each treatment groups' respective diet.

Therefore, the values reported herein only provide an initial DTDF estimate for this species and these DTDF values are not recommended for examining trophic relationships in wild cownose ray populations. Instead, potential sources of variations in DTDFs within and across the treatments will be discussed.

Variations in DTDFs are a result of a variety of factors including age, growth rate, size, diet quality and quantity, single versus multisource diet, nutritional stress and diet elemental composition (Minagawa and Wada 1984; Post 2002; Vanderklift and Ponsard 2003; Trueman et al. 2005; Caut et al. 2009; Dennis et al. 2010; Robbins et al. 2010). In this study, there was no evidence of disc width or size affecting the $\Delta^{13}C$ and $\Delta^{15}N$ in the shrimp diet and squid diet treatment group, respectively. Additionally, the consumer's sex did not affect the $\Delta^{15}N$ in the squid diet treatment group. The lack of a relationship between size and DTDF values may be due to only including subadult and adult cownose rays (Smith and Merriner 1987), thus restricting the size range of individuals (DW: 66.2 to 89.8 cm) utilized in both feeding trial treatment groups. Further experimental work should incorporate both juvenile and subadult/adult cownose rays to investigate any potential differences in DTDFs between life history stages.

Diet quality has been identified as a major determinant of DTDFs (Martínez del Rio et al. 2009; Dennis et al. 2010; Robbins et al. 2010). Carbon:nitrogen (C:N) ratios are often used to assess dietary quality, where a low C:N ratio is indicative of a high quality protein diet (Pearson et al. 2003). While this feeding study did utilize two diets with significantly different C:N ratios, comparisons of the resulting carbon and nitrogen DTDFs between the diets cannot be made due to variations in the shrimp diet nitrogen

and squid diet carbon isotopic signatures throughout the trial. Although the respective diet source was to remain consistent for each treatment group during the trial, extenuating circumstances resulted in different batches of shrimp and squid being used, and led to the variability in the aforementioned diet isotopic signatures.

Concluding remarks

Stable isotopes have been increasingly used to elucidate trophic interactions and habitat use patterns of various species, yet, there are still questions (*i.e.* effects of isotopic routing, growth versus metabolic turnover) that must be addressed experimentally to ensue proper interpretation of values. While captive husbandry of elasmobranchs can be challenging and cost-prohibitive for research institutions without the necessary infrastructure, partnerships with commercial aquaria offer researchers the opportunity to conduct long-term feeding studies that enable further exploration of stable isotope dynamics in elasmobranchs and can enhance the conservation of elasmobranchs through the refinement of husbandry practices and public outreach.

Although a variety of elasmobranch tissues and methods have been used for stable isotope analyses (Hussey et al. 2012), caution must be exercised when comparing studies that utilized different sample preparation protocols, especially when making inferences about wild populations. It is imperative that a standard protocol, in regards to tissue processing, be developed and disseminated to ensure compatibility across elasmobranch research programs, as has been initiated within the sea turtle (Arthur et al. 2010) and the marine mammal research communities (Reich personal comm.).

Several papers have called for researchers to focus experimental feeding studies on indicator species (Hussey et al. 2010; Logan and Lutcavage 2010; Hussey et al. 2012), as controlled studies on elasmobranchs often pose many difficulties. The ease at which cownose rays adapt to captive environments (Dehart 2004) makes them an ideal candidate to serve as an epibenthic durophagous indicator species. Therefore, future experimental studies should investigate isotopic incorporation and discrimination dynamics in multiple tissues (epidermis, muscle, liver, and blood) of cownose rays, as well as the effect of diet quality, life history stage and diet composition (single vs. multisource) on said incorporation rates and DTDFs.

CHAPTER III

$\delta^{13}C$ AND $\delta^{15}N$ VARIABILITY IN COWNOSE RAYS WITHIN THE NORTHWESTERN GULF OF MEXICO

Introduction

Recent studies have focused on the decline of marine apex predators as a result of differential fishing pressure and the potential for deleterious trophic cascades (Baum et al. 2003; Baum and Myers 2004; Myers et al. 2007; Heithaus et al. 2008; Ferretti et al. 2010). While the magnitude of these trophic cascades have been subject to much debate among researchers, there have been limited studies examining the role and impact of mesopredators within marine communities (Baum et al. 2005; Burgess et al. 2005a; Burgess et al. 2005b). Mesopredators serve as intermediaries between lower and higher trophic level predators and help stabilize the overall community structure (Ritchie and Johnson 2009; Tilley et al. 2013). Batoids have been identified as an important group of marine mesopredators that impact communities through foraging activities and bioturbation and yet, little is known about their trophic ecology (Orth 1975; Valentine et al. 1994; Vaudo and Heithaus 2011; O'Shea et al. 2013; Tilley et al. 2013).

Cownose rays occur throughout the western Atlantic basin from Brazil to New England and within the GOM (McEachran and Fechhelm 1998). They are a transient species, frequently found within inshore and nearshore waters, but are capable of undergoing extensive seasonal migrations (Smith and Merriner 1987; Blaylock 1989; Collins et al. 2008). Cownose rays are durophagous mesopredators that forage on

benthic invertebrates, primarily bivalves, polychaetes and crustaceans (Smith and Merriner 1985; Collins et al. 2007a; Fisher 2010). Shoals of cownose rays in North Carolina have been shown to decimate hyperabundant bay scallop (*Argopecten irridians concentricus*) populations, when available in localized areas (Peterson et al. 2001). Cownose rays have also been associated with declines in other commercial shellfish stocks including eastern oysters (*Crassostrea virginica*), surf clams (*Mya arenaria*) and hard clams (*Mercenaria mercenaria*) (Merriner and Smith 1979; Smith and Merriner 1985; Blaylock 1993). Furthermore, Myers et al. (2007) hypothesized that the collapse of the North Carolina bay scallop industry was the result of a trophic cascade involving an exponential increase in the cownose ray population within the western Atlantic Ocean, due to declines in top predatory elasmobranch stocks. However, recent stomach content studies have shown that cownose rays may operate within a continuum of opportunistic generalist and hard-prey specialist foraging strategies based on prey availability and densities (Collins et al. 2007a; Ajemian and Powers 2012). Understanding the trophic dynamics of cownose rays in coastal systems, including how foraging patterns vary spatially and temporally, is essential for the management of this species, as well as commercial shellfish stocks.

Hutchinson (1957) originally described a niche as the ecological space utilized by a species, which can be defined within an *n*-dimensional hypervolume. This hypervolume can be delineated on scenopoetic and bionomic axes, representing the environmental conditions experienced and resources used by a species, respectively (Hutchinson 1978). The niche concept has been integrated into isotopic ecology and

defined instead as δ-space, which is measured by $\delta^{13}C$ and $\delta^{15}N$ (Newsome et al. 2007). Stable isotopes are incorporated from prey to consumer tissues over a specific time period, depending on tissue type; therefore, they can represent an average of prey/resource use for that specified time period (Hesslein et al. 1993), where carbon isotopes are indicative of habitat (scenopoetic) and nitrogen isotopes reveal trophic position (bionomic) (DeNiro and Epstein 1978; Post 2002).

It is important to note that the isotopic niche of a species is not interchangeable with either ecological or trophic niches (Newsome et al. 2007; Martínez del Rio et al. 2009). However, quantifying the isotopic niche of a species can reveal patterns of dietary generalization or specialization, as well as establish general trophic relationships within a food web (Bolnick et al. 2003; Bearhop et al. 2004; Newsome et al. 2007; Martínez del Rio et al. 2009). Several metrics have been developed to quantify isotopic niches on both the individual and community-wide scale. Bearhop et al. (2004) suggested using the variance surrounding mean $\delta^{13}C$ and $\delta^{15}N$ signatures to measure niche width of a population. On a broader scale, Layman et al. (2007) developed a set of six community-wide metrics that quantify niche space within a $\delta^{13}C$- $\delta^{15}N$ bi-plot to elucidate trophic relationships within food webs. However, recent studies have shown that the Layman metrics are highly sensitive to sample size leading to problems when making comparisons across locations with differing sample sizes (Jackson et al. 2011; Syväranta et al. 2013). As a more robust alternative to the Layman metrics, the novel method of Jackson et al. (2011) used Bayesian multivariate ellipses to calculate isotopic niches, enabling comparisons within or across communities.

Objectives

The trophic ecology of cownose rays has been studied extensively along the Atlantic coast, particularly in Chesapeake Bay, and the eastern Gulf of Mexico but is not well described in the northwestern Gulf of Mexico. In light of these information gaps and the role of cownose rays as important elasmobranch mesopredators in the northwestern Gulf of Mexico, the following objectives were identified:

(1) To examine seasonal trends in $\delta^{13}C$ and $\delta^{15}N$ of cownose rays in the lower Laguna Madre.

(2) To examine the differences in $\delta^{13}C$ and $\delta^{15}N$ of cownose rays across bay systems along the Texas coast.

(3) To examine the influence of size and sex of individuals on $\delta^{13}C$ and $\delta^{15}N$ signatures.

Hypotheses

The following hypotheses, as well as the above objectives, are addressed in this chapter:

(1) Isotopic signatures of cownose rays in the lower Laguna Madre will exhibit seasonal variation.

(2) Isotopic signatures of cownose rays will vary across bays systems along the Texas coast.

Materials and Methods

Study areas

In-water entanglement netting of cownose rays was conducted along the Texas coast as part of a larger study documenting sea turtle abundance and distribution patterns in the following bay systems: the lower Laguna Madre (LLM), Aransas Bay complex (AB), Lavaca – Matagorda Bay complex (LMB) and Galveston Bay complex (GB) (Fig. 6). Sampling in LLM, the southernmost site, was located at Mexiquita Flats, adjacent to Port Isabel, Cameron County, TX, and approximately 3.5 km from the Brazos-Santiago Pass (Fig. 7). Sampling occurred at various seasonal intervals during 2009 – 2012, providing a multi-year dataset. The sampling site was adjacent to extensive beds of shoal (*Halodule wrightii*), manatee (*Syringodium filiforme*), and turtle grasses (*Thalassia testudinum*). Water depth ranged from 0.75 – 1.75 m.

All other locales were sampled solely in 2012. Netting sites in the AB complex were located west of Port Aransas, Nueces County, TX in the East Flats area of Corpus Christi Bay and Redfish Bay during August 2012 (Fig. 8). Both sites were approximately 9.0 km from Aransas Pass. Water depth at the East Flats sampling site ranged from 1.3 – 1.8 m and the site was dominated by turtle grass. Water depth at the Redfish Bay sampling site was 1.8 m and the area was dominated by shoalgrass. The LMB complex was sampled in June 2012 off of the Indianola Beach recreational area, southeast of Port Lavaca in Calhoun County, TX (Fig. 9). The sampling site was adjacent to a recreational area with a shell hash shoreline and approximately 24.0 km from Pass Cavallo. Water

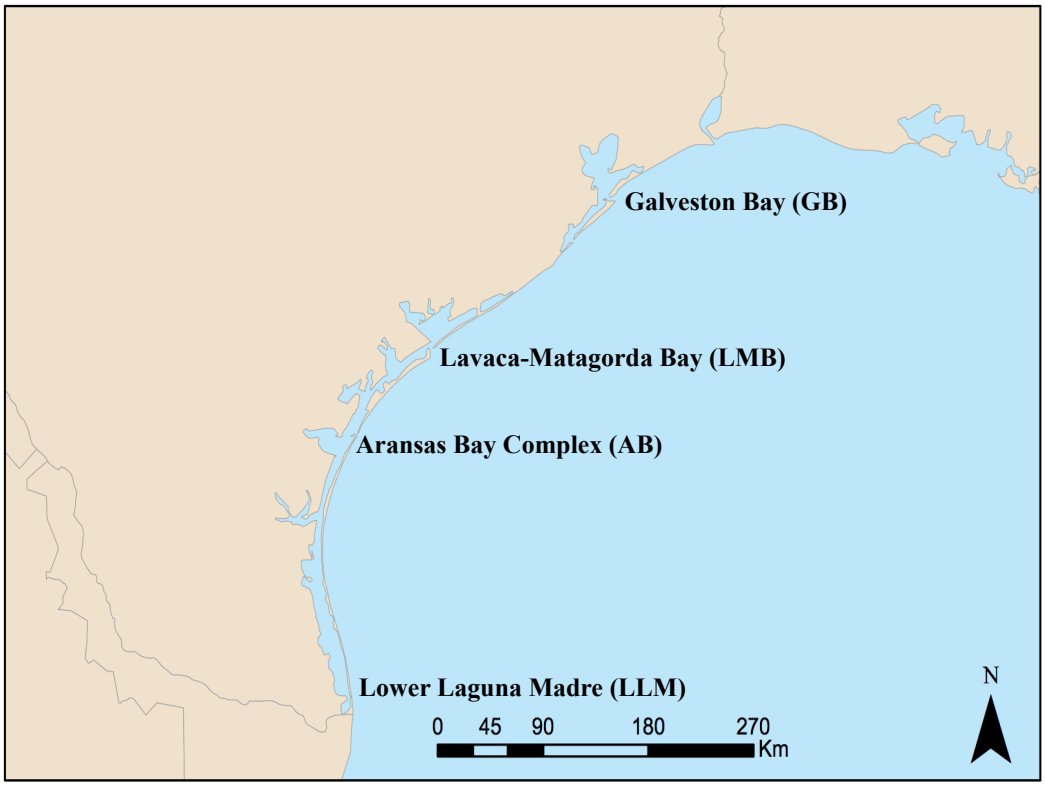

Fig. 6. Map of Texas coast showing major bay systems where cownose rays were sampled.

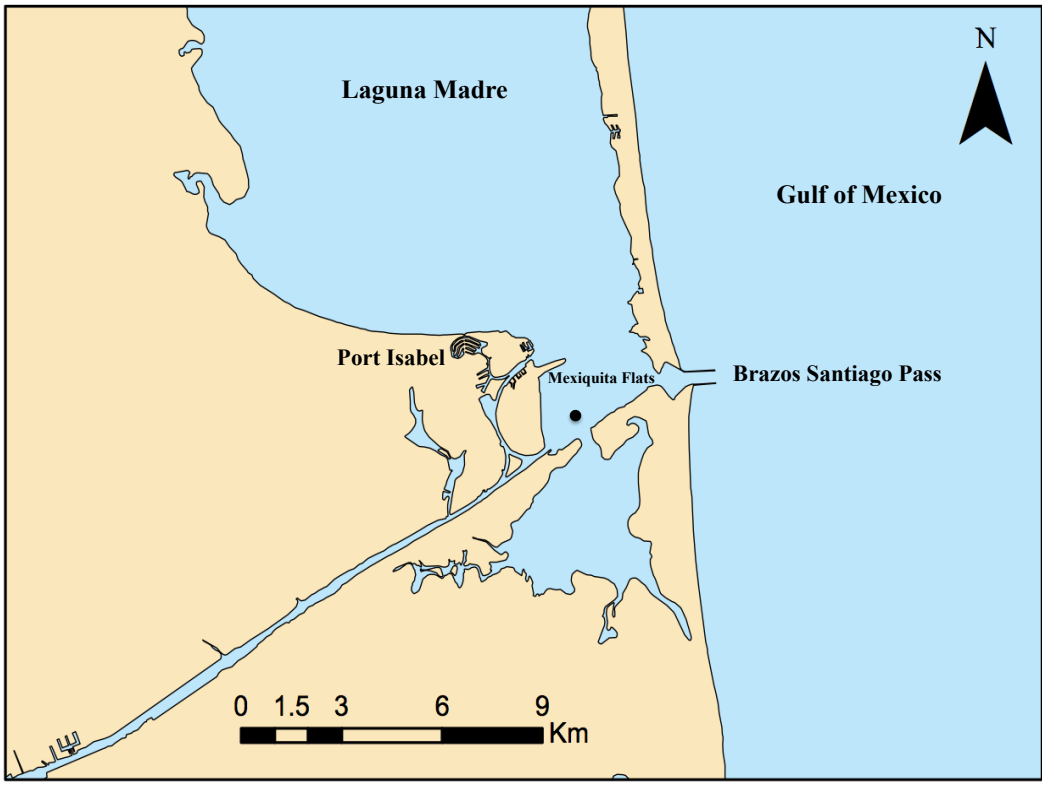

Fig. 7. Map of lower Laguna Madre showing general location of the Mexiquita Flats sampling site.

depth at the sampling location was 2.5 m and the substrate consisted of soft mud and shell hash. The GB complex, the northernmost site, was sampled in October 2012 in East Bay, northeast of Port Bolivar in Galveston County, TX (Fig. 10). The sampling site was adjacent to dredge spoil islands lined by cordgrass (*Spartina* spp.) and approximately 2.5 km from Rollover Pass. Water depth at the sampling location was 1.75 m and the substrate consisted of soft mud and small patches of eastern oyster (*Crassostrea virginica)*.

Cownose ray capture and collection

Large mesh entanglement nets (91.4 m long and either 3.6 m or 2.9 m deep, with 17.7 cm bar mesh of twisted #9 nylon) were used to capture cownose rays. The type of the net used depended on the water depth and current present at each netting location but all sampling was limited to sites with water depth of less than 3 m. Each sampling event utilized 2 – 4 nets set either in tandem or perpendicular to one another for 6 – 8 hours per day. Nets were checked every 20 minutes or more frequently as splashes or other signs of potential capture dictated in order to minimize risk of injury to cownose rays. Upon completion of each net check, any rays captured were sampled and returned to the wild within 5 minutes.

Sample collection and processing

This study utilized epidermal tissue, which has an average residence time of approximately 540 and 160 days for $\delta^{13}C$ and $\delta^{15}N$, respectively (see Chapter II). Skin biopsy samples were taken from the trailing edge of the pectoral fin. The sample site was cleaned using a 70% isopropyl alcohol swab prior to collection. All skin samples were

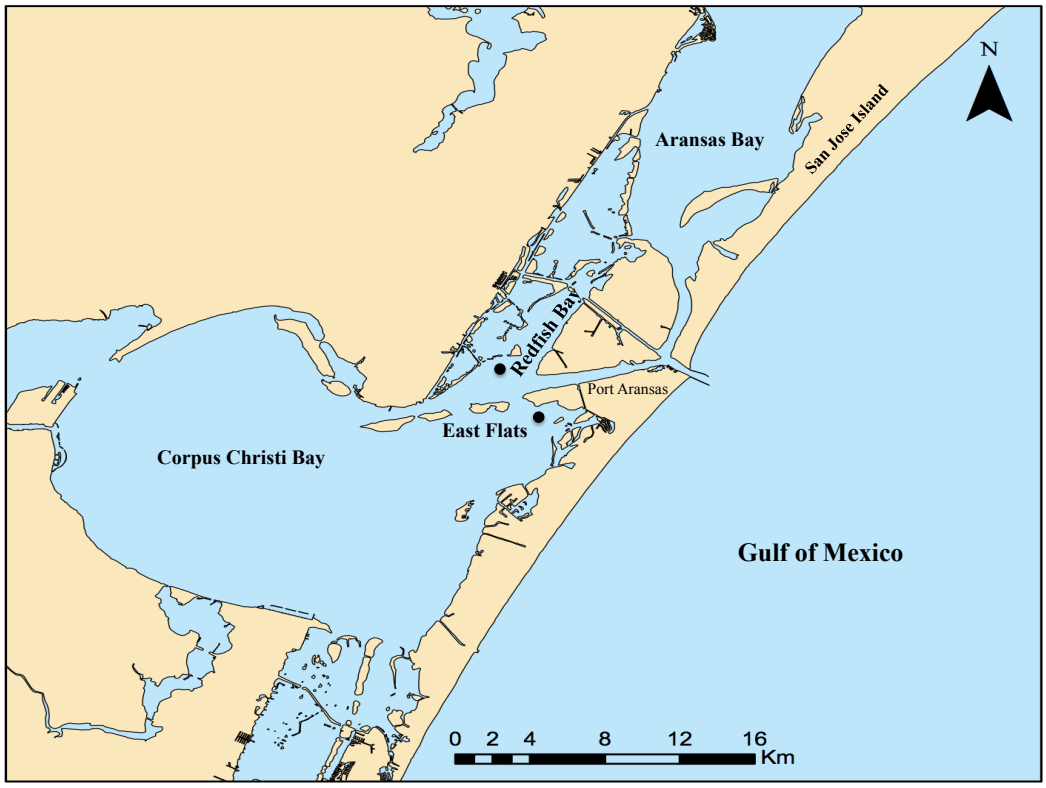

Fig. 8. Map of Aransas Bay complex showing general locations of sampling sites within East Flats and Redfish Bay.

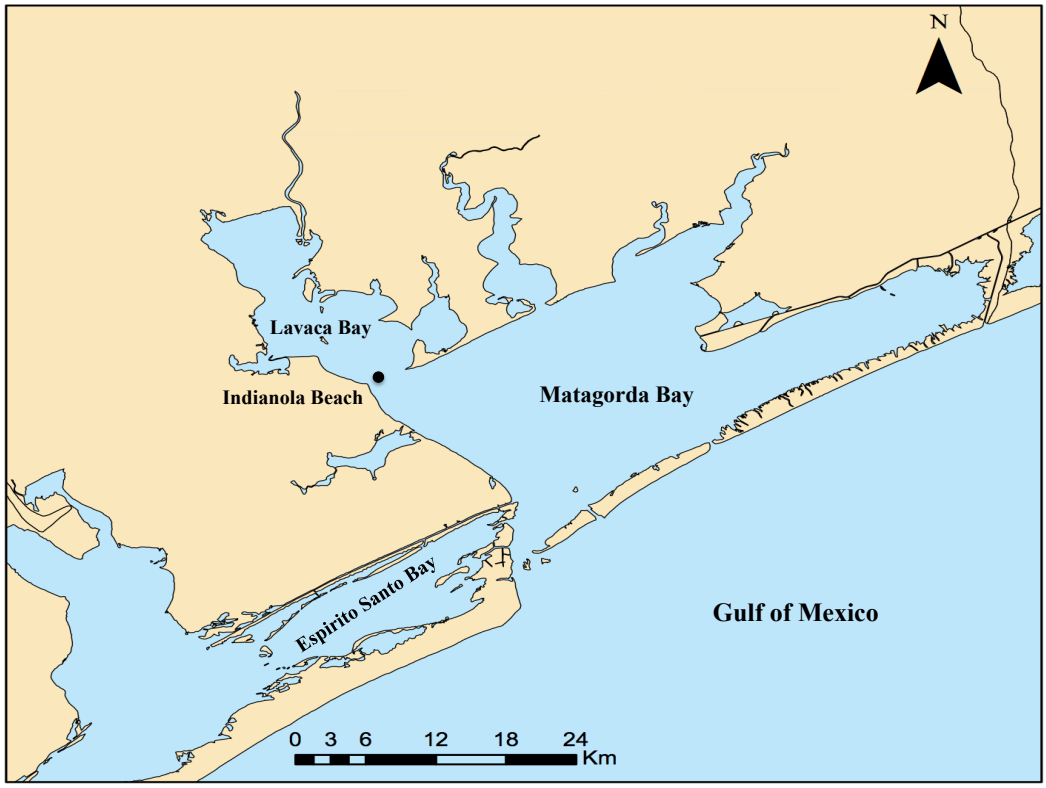

Fig. 9. Map of Lavaca-Matagorda Bay complex showing general location of sampling site off of Indianola Beach.

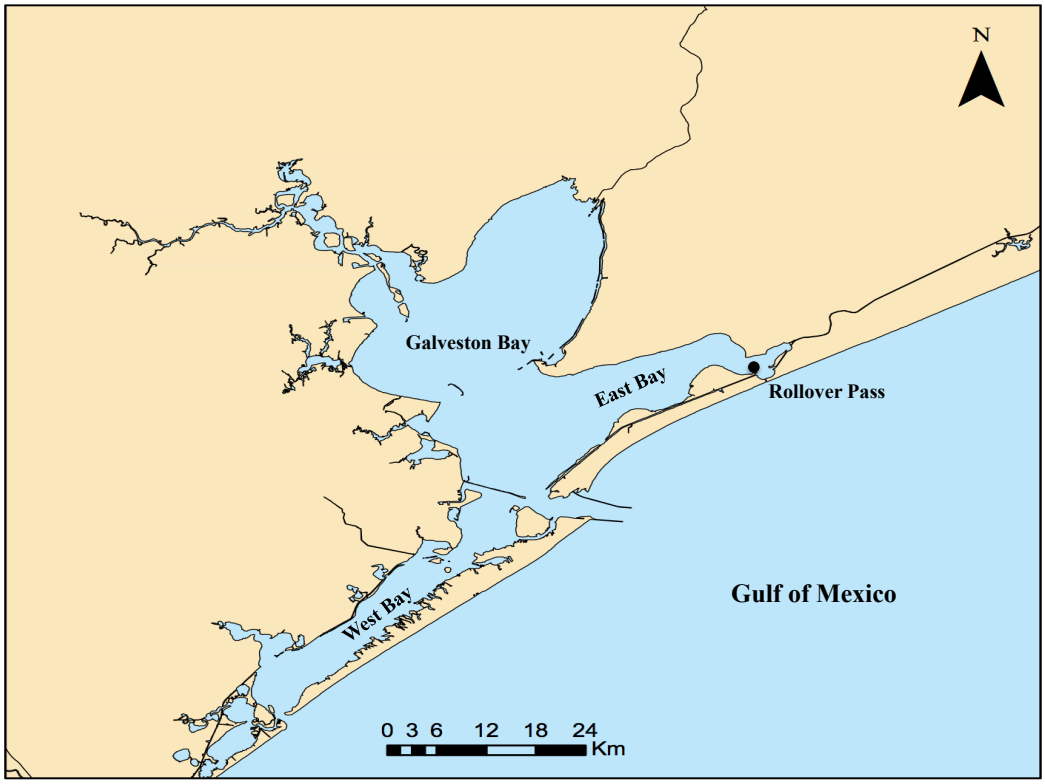

Fig. 10. Map of Galveston Bay complex showing general sampling location near Rollover Pass.

collected using a 6-mm sterile biopsy punch and stored in a vial of 70% ethanol for subsequent stable isotope analysis. Individuals were measured for disc width (DW) to the nearest 0.1 cm concurrent to the collection of tissue samples.

Skin samples were cleaned using 70% ethanol and rinsed with deionized water and homogenized using a #21 scalpel blade. The skin samples were dried to constant weight at 60 °C for 24 hours. To reduce the variability of $\delta^{13}C$, lipids were extracted from the epidermal tissue using a Dionex Accelerated Solvent Extractor (ASE), with petroleum ether serving as the solvent. All samples were weighed to 600 (± 50) µg into pre-cleaned tin capsules and sent to the Light Stable Isotope Lab at the University of Florida, Gainesville, FL for analysis. Samples were combusted in a COSTECH ECH 4010 elemental analyzer interfaced via a Finnigan-MAT ConFlow III device (Finnigan MAT, Breman, Germany) to a Finnigan-MAT DeltaPlus XL (Breman, Germany) isotope ratio mass spectrometer. Resulting stable isotope signatures were expressed in standard delta (δ) notation, as follows:

Equation 1. $\delta = [(R_{sample}/R_{standard}) - 1][1000]$.

R_{sample} and $R_{standard}$ refer to the ratios of heavy to light isotopes ($^{13}C/^{12}C$ and $^{15}N/^{14}N$) in the sample and standard, respectively. The R standard for ^{13}C was the Vienna Pee Dee Belemnite (VPDB) limestone formation. The R standard for ^{15}N was atmospheric N_2. Units for both $\delta^{13}C$ and $\delta^{15}N$ are defined as parts per thousand (‰).

Statistical analyses

Mean disc widths (DW; cm) were calculated for the entire Texas coast, as well as for each individual bay system sampled. The life history stage of individuals was

determined by disc width, using the categories defined by Smith and Merriner (1987). T-tests were used to determine if DWs were significantly different between the sexes in each of the bay systems.

One-way analysis of variances (ANOVA) were used to determine if significant differences occurred in mean $\delta^{13}C$ and $\delta^{15}N$ temporally in the LLM (2009 – 2012) and spatially among bays systems in 2012. If significant differences did occur, Tukey's Honestly Significant Difference (HSD) was used to make post-hoc comparisons. T-tests were used to determine if there were significant differences between either sex or life history stages, defined by size at maturity (Smith and Merriner 1987) across all sampling periods.

The isotopic niches of cownose rays were described using three of the metrics developed by Layman et al. (2007). These metrics are based on plotting the isotopic values of individuals on a $\delta^{13}C$ – $\delta^{15}N$ bi-plot, representing isotopic space. The first two metrics, $\delta^{13}C$ range (CR) and $\delta^{15}N$ range (NR), represent the distance between the two extreme values (*i.e.* minimum and maximum) within each isotopic signature. The total area (TA) is the area of a convex hull that incorporates the isotopic values of all individuals. It has been noted that the Layman metrics are highly sensitive to sample size and are not ideal for making comparisons within or across studies with unequal samples sizes (Jackson et al. 2011). Therefore, this study will use the alternative method developed by Jackson et al. (2011) utilizing Bayesian ellipses to compare isotopic niches within and across bay systems. Isotopic niches were quantified using Bayesian ellipses created within the R package 'Stable Isotope Bayesian Ellipses in R' (SIBER). Corrected

Standard Ellipse Areas (SEA_c) were used instead of Standard Ellipse Areas (SEA) to avoid underestimation of the isotopic niche of a population due to low sample size, as well as for comparisons within and among sites with unequal sample sizes (Jackson et al. 2011). Additionally, overlap between isotopic niches was quantified by comparing SEA_c to examine dietary similarity among and within cownose ray populations. All statistical analyses used an alpha value of 0.05.

Results

A total of 112 cownose rays (73 female, 39 male) were sampled across the Texas coast from 2009 to 2012 with disc widths ranging from 54.6 to 97.0 cm (mean DW: 75.6 ± 8.2 cm). Fifty-eight rays (47 female, 11 male) were sampled at the lower Laguna Madre in the Mexiquita Flats area from 2009 to 2012 with disc widths ranging from 56.5 to 97.0 cm (mean DW: 77.7 ± 7.7 cm). Twenty-one rays (15 female, 6 male) were sampled from 7 – 9 August, 2012 in the Aransas Bay complex (7 – 8 August at East Flats site; 9 August at Redfish Bay site) with disc width ranging from 54.6 to 90.4 cm (mean DW: 75.1 ± 9.4 cm). Thirty rays (9 female, 21 male) were sampled on 13 June, 2012 within the Lavaca – Matagorda Bay complex with disc widths ranging from 57.4 to 83.8 cm (mean DW: 71.1 ± 6.3 cm). Three rays (2 female, 1 male) were sampled on 12 October, 2012 in East Bay within the Galveston Bay complex with disc widths ranging from 80.5 to 87.5 cm (mean DW: 83.2 ± 3.8 cm). Disc widths were not statistically different between sexes for any of the sampling locations tested (LLM: $t_{55} = 1.34$, p =

0.19; AB: $t_{19} = -0.11$, $p = 0.91$; LM: $t_{28} = 0.45$, $p = 0.66$; GB: not tested due to insufficient sample size).

Seasonal trends in $\delta^{13}C$ and $\delta^{15}N$ within the lower Laguna Madre

The $\delta^{13}C$ and $\delta^{15}N$ values of skin tissue collected from LLM on 7 – 10 August 2010 were not statistically different (ANOVA $F_{2,24} = 0.92$, $p = 0.41$ and ANOVA $F_{2,24} = 2.98$, $p = 0.07$, respectively) and were therefore, collectively used as Summer 2010 samples for seasonal analyses. Seasons were defined as follows: Spring (April), Summer (June and August) and Fall (October). All other skin tissues for the seasonal sampling periods (Fall 2009, Spring 2011, Spring 2012 and Summer 2012) were collected in a one-day period within each respective season. Mean $\delta^{13}C$ and $\delta^{15}N$ values were significantly different across seasons (ANOVA $F_{4,52} = 11.53$, $p < 0.01$ and $F_{4,52} = 8.46$, $p < 0.01$, respectively) (Table 5). Spring 2011 yielded the most depleted $\delta^{13}C$ value ($\bar{x}$ = -15.91 ‰) with the most enriched signature occurring in Fall 2009 ($\bar{x}$ = -11.57 ‰). The reverse was found in $\delta^{15}N$ values with the most depleted and enriched signatures occurring in Fall 2009 ($\bar{x}$ = 7.78 ‰) and Spring 2011 ($\bar{x}$ = 11.88 ‰), respectively. Across all seasons there was no significant difference in either mean $\delta^{13}C$ and $\delta^{15}N$ values between subadult and adult rays ($\delta^{13}C$: $t_{54} = 0.19$, $p = 0.85$, $n = 55$; $\delta^{15}N$: $t_{54} = -0.32$, $p = 0.75$, $n = 55$) but there was a significant difference in both mean $\delta^{13}C$ and $\delta^{15}N$ values due to sex ($\delta^{13}C$: $t_{55} = 3.56$, $p < 0.01$, $n = 57$; $\delta^{15}N$: $t_{55} = -3.57$, $p < 0.01$, $n = 57$). The female rays mean $\delta^{13}C$ value ($\bar{x}$ = -13.81 ‰) was enriched compared to the male signature ($\bar{x}$ = -15.73 ‰) with the reverse found in $\delta^{15}N$ values, where females ($\bar{x}$ = 9.80 ‰) were depleted compared to males ($\bar{x}$ = 11.39).

The range of $\delta^{13}C$ and $\delta^{15}N$ values were similar in each of the respective sampling periods but varied across seasons with the largest range for both isotopes in Spring 2011. TA was highly variable across all seasons, ranging from 0.88 (Summer 2012) to 8.79 ‰ (Summer 2010). The SEA_c of cownose rays varied across seasons and ranged in size from 0.58 to 3.40 ‰ (Table 6). Relative size comparisons of the Bayesian ellipses (SEA) among the sampling periods showed that the isotopic niche of cownose rays in Summer 2012 were significantly smaller than all other periods (Fall 2009: $p = 0.05$; Summer 2010: $p = 0.02$; Spring 2011: $p = 0.01$; Spring 2012: $p = 0.05$) but all other sampling periods were comparable (Fig. 11). The highest area of niche overlap occurred between Summer 2010 and Summer 2012 (58.4%). Across all sampling periods, female rays utilized a larger NR and CR than male rays but the SEA_c of the two sexes did not significantly differ ($p = 0.63$) (Fig. 12). Similarly, subadult rays utilized a larger CR and NR than adult rays across all sampling periods. However, the SEA_c of adult rays was significantly smaller than the SEA_c of subadult rays ($p < 0.01$) (Fig. 13).

Trends in $\delta^{13}C$ and $\delta^{15}N$ along the Texas coast

Mean $\delta^{13}C$ and $\delta^{15}N$ values were significantly different across bay systems (ANOVA $F_{3,69} = 24.31$, $p < 0.01$ and $F_{3,69} = 24.48$, $p < 0.01$, respectively). Tukey's HSD revealed for $\delta^{13}C$ there were significant differences between the LLM and the rest of the bay systems and for $\delta^{15}N$ that each bay system was significantly different from the others (Table 7). Mean $\delta^{13}C$ and $\delta^{15}N$ values were not significantly different due to sex within any bay systems, with the exception of the mean $\delta^{13}C$ in LMB ($t_{28} = -2.33$, $p = 0.03$). Males ($\bar{x} = -16.82$ ‰; $n = 21$) in LMB were enriched compared to

Table 5. Mean (±1 SE) of $\delta^{13}C$ and $\delta^{15}N$ in cownose ray epidermal tissue measured in fall 2009 (*N* = 4), summer 2010 (*N* = 27), spring 2011 (*N* = 7), spring 2012 (*N* = 11) and summer 2012 (*N* = 8). *P* values from one-way ANOVAs are provided. Letters indicate pairwise comparisons based on posthoc Tukey's HSD (*P*>0.05).

	Fall 2009		Summer 2010		Spring 2011		Spring 2012		Summer 2012		
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	*P* value
$\delta^{13}C$	-11.57[a]	0.55	-13.71[b]	0.25	-15.91[c]	0.85	-15.67[c]	0.33	-13.52[ab]	0.19	<0.0001
$\delta^{15}N$	7.78[a]	0.49	9.91[b]	0.23	11.88[c]	0.77	10.50[bc]	0.26	9.81[ab]	0.14	<0.0001

Seasons with similar superscript letters did not differ (*P* > 0.05) in mean isotopic signature (ANOVA, Tukey's HSD)

Table 6. Isotopic niche metrics for cownose rays in LLM during 2009 - 2012.

	NR	CR	TA	SEA	SEA_c	*N*
By season						
Fall 2009	2.25	2.21	2.00	2.27	3.40	4
Summer 2010	5.30	4.78	8.79	2.89	3.00	27
Spring 2011	6.43	7.10	1.29	0.81	0.97	7
Spring 2012	3.08	3.49	4.92	2.36	2.62	11
Summer 2012	1.08	1.77	0.88	0.50	0.58	8
Across seasons						
Female	8.26	8.49	21.14	3.76	3.84	47
Male	3.70	3.77	6.23	3.15	3.50	11
Subadult	8.26	8.49	21.42	4.94	5.09	34
Adult	2.25	4.67	5.76	1.74	1.82	23

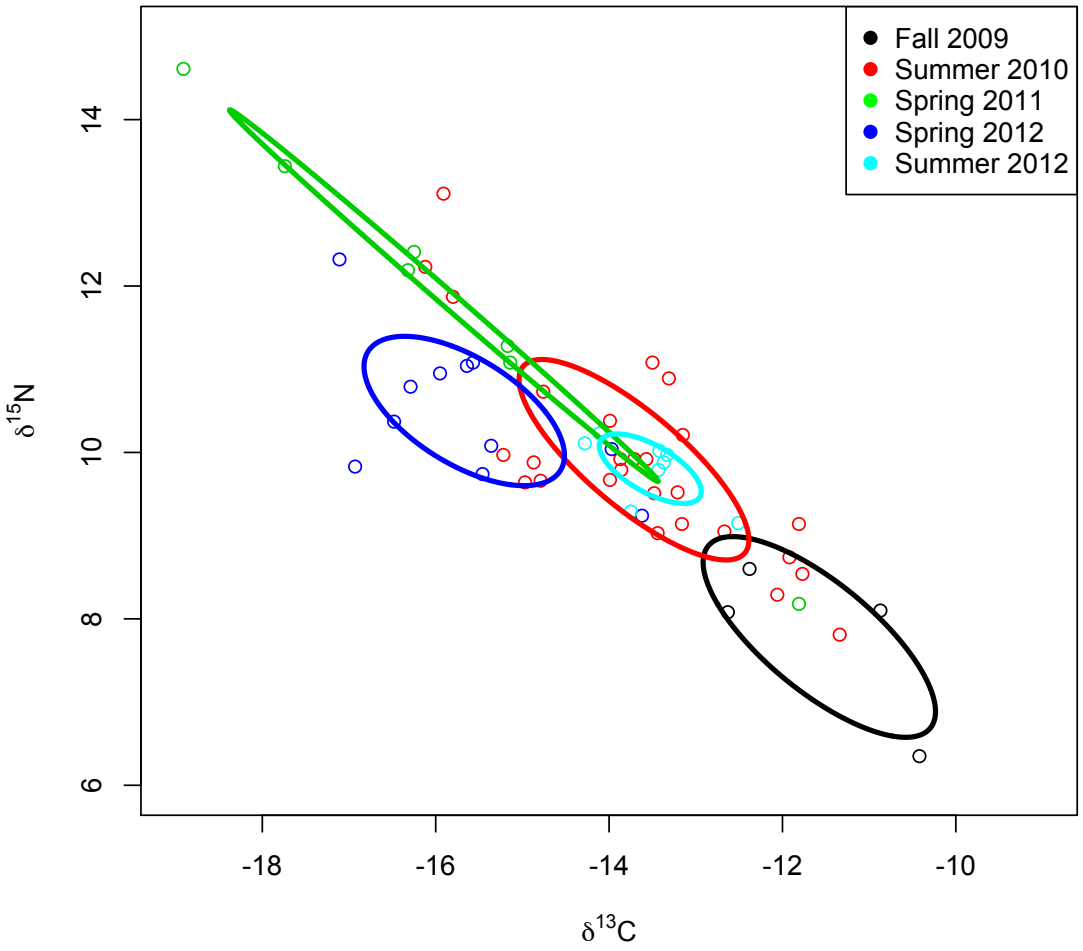

Fig. 11. Bivariate plot of δ-space depicting isotopic niches of cownose rays across seasons sampled in LLM.

female ($\bar{x}$ = -17.36 ‰; n = 9) rays. Mean $\delta^{13}C$ and $\delta^{15}N$ values were not significantly different due to life history stage within any bay systems, with the exception of the mean $\delta^{13}C$ in AB (t_{18} = 2.22, p = 0.04). Subadult rays ($\bar{x}$ = -16.90 ‰; n = 13) in AB were enriched compared to adult rays ($\bar{x}$ = -16.20 ‰; n = 7).

The SEA_c of cownose rays varied along the Texas coast in 2012 from 0.30 to 2.62 ‰ (Table 8). Relative size comparisons of the SEA across the bay systems showed that the isotopic niche of the cownose rays in the LLM – Summer was significantly smaller than in the LLM – Spring (p = 0.05) but that all other niches were comparable (Fig. 14). The highest area of niche overlap occurred between the AB and LMB (52.6%). There was no significant difference in the relative size of SEA_c due to sex or life history stage in either AB (p = 0.33 and p = 0.59, respectively) or LMB (p = 0.30 and p = 0.15, respectively). For AB, there was 99.3% and 61.7% niche area overlap between sexes and life history stages, respectively. For LMB, there was 40.1% and 89.0% niche area overlap between sexes and life history stages, respectively. Small sample size across life history stages and between sexes in both LLM and GB did not allow comparisons of isotopic niches.

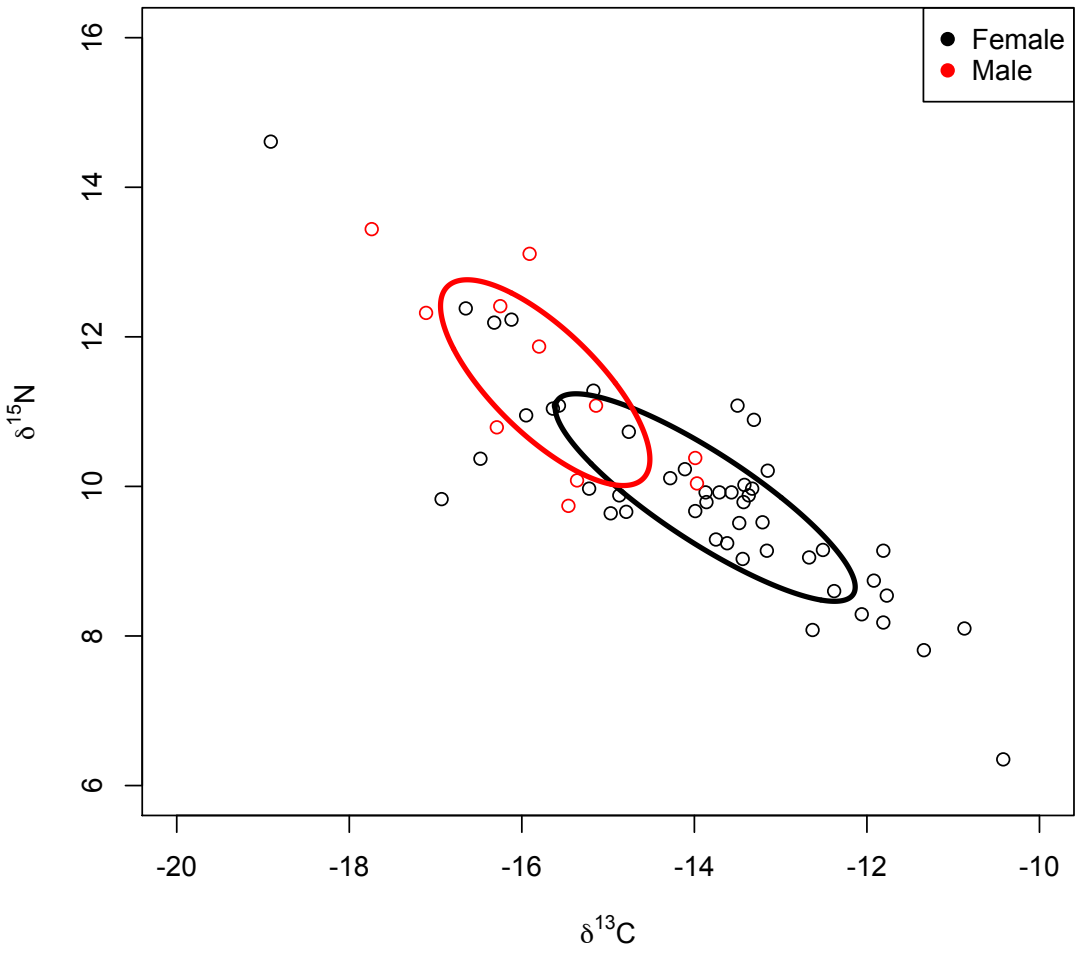

Fig. 12. Bivariate plot of δ-space depicting isotopic niches of female and male cownose rays in the LLM from 2009 - 2012.

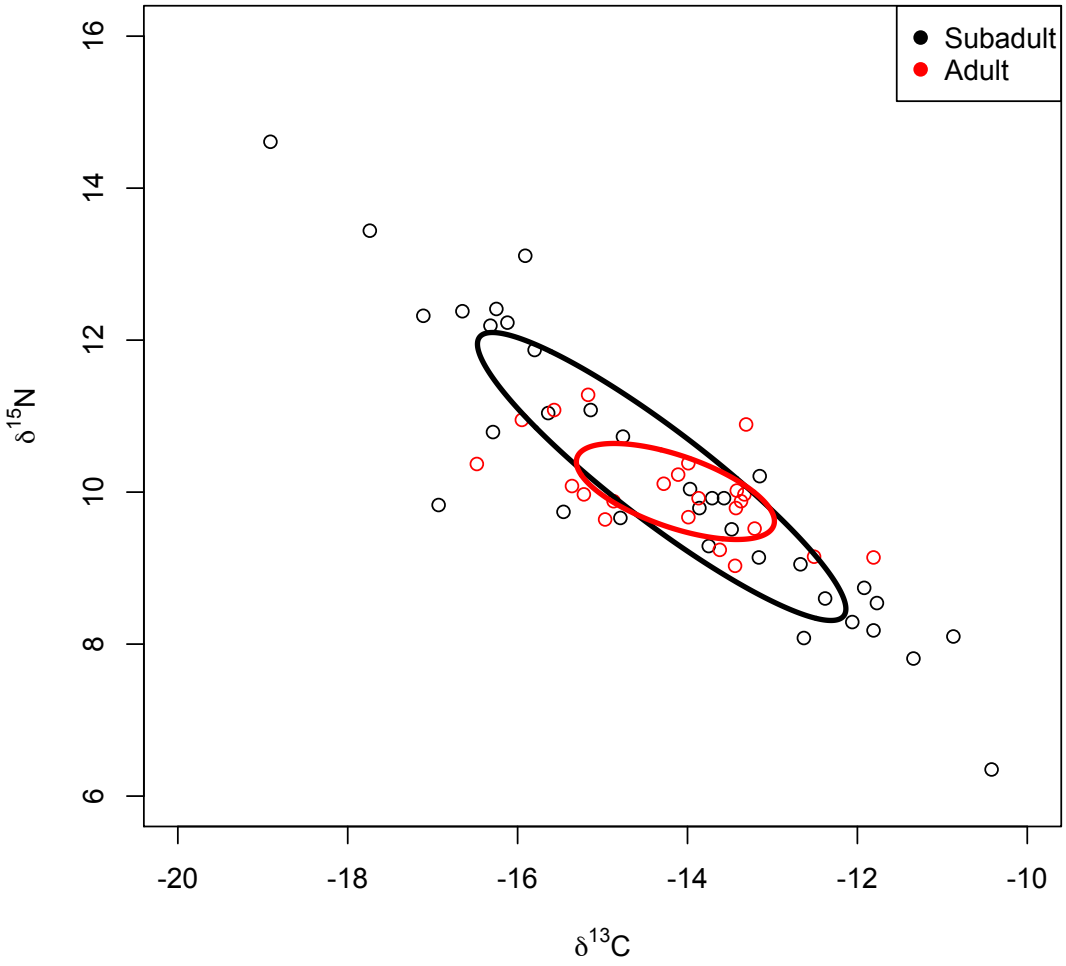

Fig. 13. Bivariate plot of δ-space depicting isotopic niches of subadult and adult cownose rays in the LLM from 2009 - 2012.

Table 7. Means (± 1 SE) of $\delta^{13}C$ and $\delta^{15}N$ in cownose ray epidermal tissue measure in LLM (*N* = 58), AB (*N* = 21), LMB (*N* = 30) and GB (*N* = 3). *P* values from one-way ANOVAs are provided. Letters indicate pairwise comparisons based on posthoc Tukey's HSD (*P*<0.05).

	LLM - Spring		AB - Summer		LM - Summer		LLM - Summer		GB - Fall		
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	*P* value
$\delta^{13}C$	-14.77^{b}	0.32	-16.74^{c}	0.17	-16.98^{c}	0.11	-13.52^{a}	0.19	-16.80^{c}	0.20	<0.0001
$\delta^{15}N$	10.21^{cd}	0.18	10.88^{c}	0.16	11.77^{b}	0.16	9.81^{d}	0.14	13.47^{a}	0.43	<0.0001

Locations with similar superscript letters did not differ (*P* > 0.05) in isotopic variable (ANOVA, Tukey's HSD)

Table 8. Isotopic niche metrics for cownose rays across the Texas coast in 2012.

	NR	CR	TA	SEA	SEAc	*N*
LLM						
All	1.08	1.77	0.88	0.50	0.58	8
AB						
Female	1.99	3.41	4.31	1.60	1.73	15
Male	2.16	1.50	1.97	1.50	1.97	6
Subadults	2.33	2.67	3.73	1.74	1.90	13
Adults	1.93	1.45	1.78	1.18	1.42	7
All	2.33	3.41	5.20	1.68	1.77	21
LMB						
Female	1.29	2.20	1.34	0.71	0.81	9
Male	3.94	2.21	1.44	1.52	4.65	21
Subadults	3.94	2.83	4.42	1.41	1.46	26
Adults	1.12	1.67	0.87	1.58	3.17	3
All	3.94	2.88	5.57	1.50	1.55	30
GB						
All	1.43	0.62	0.08	0.15	0.30	3

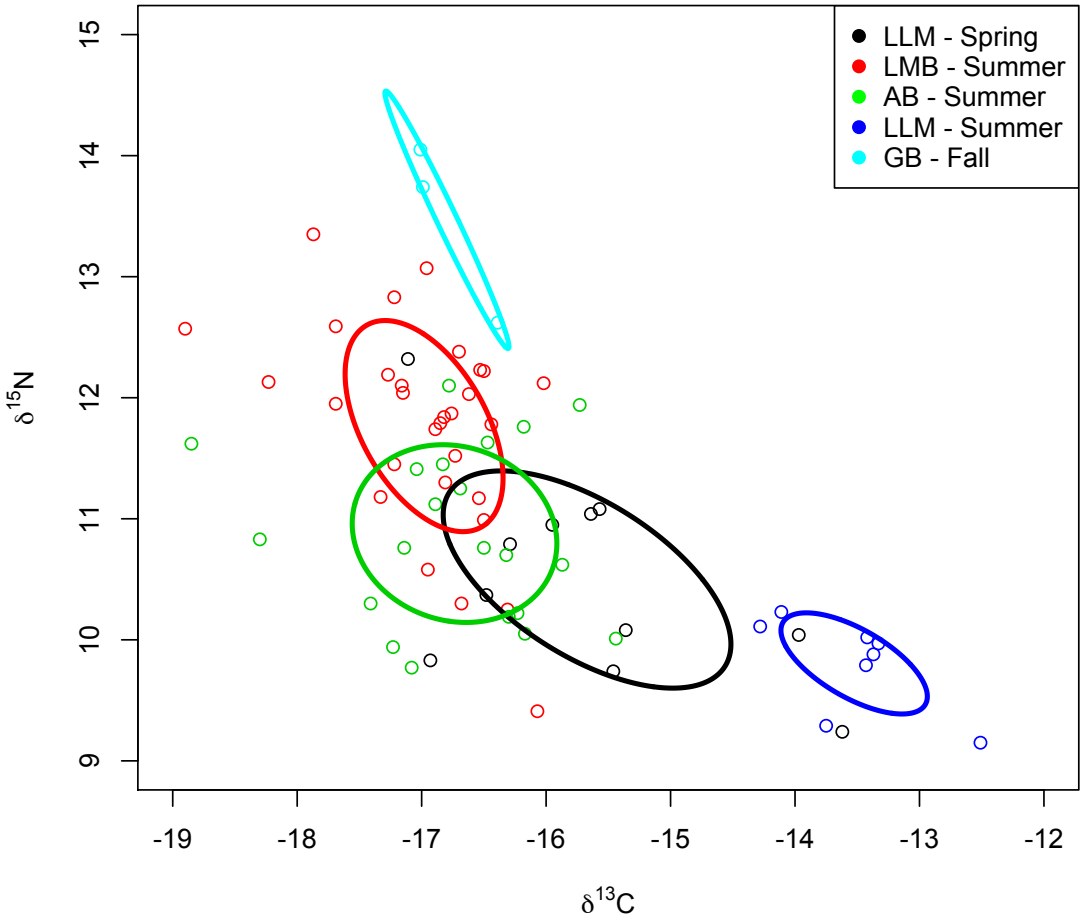

Fig. 14. Bivariate plot of δ-space depicting isotopic niches of cownose rays across the Texas coast in 2012.

Discussion and Conclusions

Seasonal trends in $\delta^{13}C$ and $\delta^{15}N$ within the lower Laguna Madre

Cownose rays in the LLM exhibited seasonal variation in the relative size of isotopic niches. However, only the Summer 2012 isotopic niche was statistically smaller than the rest of the sampling periods and may potentially represent a shift to specialist foraging activities. A large variance in isotopic signatures within the tissues of a population is the result of foraging on prey items over a wide isotopic range. Therefore, smaller levels of variance in isotopic signatures would represent foraging on either prey items that are isotopically similar or on a single prey species (Bearhop et al. 2004). While the isotopic niche of cownose rays was reduced in Spring 2011, it is important to note that the largest CR and NR was documented within this sampling period. This large range in $\delta^{13}C$ and $\delta^{15}N$ values is indicative of cownose ray individuals foraging occurring across disparate habitats and multiple trophic levels, respectively (Peterson and Fry 1987). The entire state of Texas experienced a historic-level drought throughout 2011 (Nielsen-Gammon 2012). Freshwater inflow is critical to structuring benthic communities, especially for molluscan species, in estuaries (Montagna and Kalke 1996). Additionally, during past drought events in Texas, there were invasions of open-Gulf invertebrate species into estuaries, significantly altering benthic communities (Parker 1955). Therefore, the large ranges in isotopic values may reflect changes in prey assemblages in the LLM and/or inshore-offshore movements of individual rays, enabled by the proximity of the Mexiquita Flats site to the GOM via the Brazos-Santiago Pass, in search of adequate prey resources.

Overall, mean $\delta^{13}C$ signatures in cownose rays varied among seasons, with values depleted in the spring and enriched in the fall. The range of average residence times of $\delta^{13}C$ in cownose ray skin tissue is 467 – 651 days (Chapter II). Therefore, $\delta^{13}C$ signatures generated from spring, summer and fall sampling periods reflect foraging activities that occurred in the fall, winter and spring of the previous year. Fry and Parker (1979) reported $\delta^{13}C$ signatures for bivalves and polychaetes found within seagrass beds of the Laguna Madre (-13.7 to -16.1 ‰ and -9.8 to -11.7 ‰, respectively) and offshore (-17.8 to -19.5 ‰ and -17.8 to -18.2 ‰, respectively). Based on these $\delta^{13}C$ signatures of potential prey items in LLM, cownose rays may be foraging within inshore waters during late winter/spring, as the $\delta^{13}C$ signatures of rays sampled in the summer and fall were enriched (-11.6 to -13.7 ‰). Average water temperatures in the LLM do not drop below 15 °C except in January (Hervey and US DOC 2014). Therefore, cownose rays may not conduct large-scale seasonal migrations and may be present in the LLM throughout the year, similar to cownose ray populations in the Charlotte Harbor estuary system in Florida (Collins et al. 2007b), only moving to thermostable waters offshore if water temperatures drop below 15 °C inshore (Smith and Merriner 1987; Collins et al. 2007b). The $\delta^{13}C$ signatures of rays collected during the spring sampling period, representing foraging occurring in the fall, were depleted (-15.7 to -15.9 ‰), but as the cownose ray signatures are not as depleted as the potential offshore prey items, cownose rays may be foraging in both inshore and offshore habitats.

Across seasons in the LLM, the mean $\delta^{13}C$ signature was significantly different between the sexes with female rays ($\bar{x}$ = -13.81 ‰) enriched in $\delta^{13}C$, indicating that

female rays may spend more time foraging within inshore habitats ($\delta^{13}C$ range of potential inshore prey items: -10.6 to -15.1 ‰) than males ($\bar{x}$ = -15.73 ‰). Sexual segregation is well documented in elasmobranchs (Wearmouth and Sims 2008), including cownose rays (Smith and Merriner 1987). Collins et al. (2007b) tracked cownose rays using acoustic telemetry and noted that females remained within the estuarine study area longer than males. Ajemian et al. (2012) documented a similar trend in eagle ray (*Aetobatus narinari*) habitat use in Bermuda, where mature female rays spent significantly more time within inshore waters than male rays. Female cownose rays may be utilizing inshore waters over longer periods of time than males to forage on benthic prey during gestation. Cownose rays have gestational periods of 11 – 12 months (Smith and Merriner 1987; Neer and Thompson 2005; Poulakis 2013) and there were several instances of females giving birth to pups, in various developmental stages, during spring and summer sampling (*personal observation*). In this study, females dominated the catch throughout all seasons sampled, with males most abundant during spring sampling and this trend is corroborated by historical Sea Turtle and Fisheries Ecology Research Laboratory (STFERL) netting records from LLM (Metz and Landry, unpublished data). The increased presence of male cownose rays in April in the LLM may be attributed to the mating season. Poulakis (2013) documented mating wounds along the trailing edge of pectoral fins on mature female cownose rays, as well as mating behaviors between the two sexes, within the Charlotte Harbor system in Florida from October to June, with the highest occurrence between April and June.

Ontogenetic shifts in diet, as a result of increased body size, are common in elasmobranchs (Grubbs 2010) and, in batoids, changes in gape, in conjunction with an increase in bite force, enables a wider variety of benthic prey to be consumed (Babel 1967; Peterson et al. 2001; Fisher et al. 2011). Ajemian and Powers (2012) found differences in cownose ray diets with bivalves comprising the majority of young-of-the-year (YOY) and juvenile diets, with adult diets dominated by crustaceans. However, in their study, sampling locations typically yielded only one life history stage, with YOY and juveniles found predominately at estuarine locations and adults found predominately at open gulf locations. The isotopic niche of adults in the LLM across seasons was significantly smaller than that of the subadults across all seasons sampled. However, there was no significant difference in mean isotopic signatures between the life history stages and the isotopic niche of the adults fell completely within the subadult niche. Fisher et al. (2013) conducted an age and growth study on cownose rays in Chesapeake Bay and concluded that the rays grow at a very fast rate within the first few years. Furthermore, cownose rays in the Gulf of Mexico mature at a faster rate and smaller size than rays in the Chesapeake Bay system (Neer and Thompson 2005). For subadults within LLM, the isotopic signatures may incorporate foraging activities undertaken as juveniles, as average residence times for $\delta^{13}C$ and $\delta^{15}N$ in skin tissue are greater than 18 and 9 months, respectively, leading to the larger CR and NR utilized. Alternatively, the difference in isotopic niche size between subadult and adult rays may be the result of seasonal foraging in isotopically distinct habitats. Therefore, it is imperative to perform stomach content analyses across all life history stages and/or utilize a tissue with a

shorter turnover rate (*i.e.* liver), in conjunction with isotopic values of potential prey items, to establish spatial and temporal baselines within cownose ray foraging areas, to identify potential ontogenetic shifts within the LLM.

Trends in $\delta^{13}C$ and $\delta^{15}N$ along the Texas coast

There were differences in mean $\delta^{13}C$ and $\delta^{15}N$ in cownose ray populations, both seasonally within a bay system and across bay systems geographically. The Texas coastline covers approximately 590 linear kilometers and encompasses a semi-arid to humid climatic gradient from the southwest to the northeast, respectively, with the LLM (Lower Rio Grande Valley), AB (Post Oak Savanna), and LMB and GB (Gulf Coastal Plains) located within different zones (TWDB 2012). Natural gradients in $\delta^{13}C$ and $\delta^{15}N$ within coastal environments are heavily influenced by primary producers, which include but are not limited to phytoplankton, macroalgae, seagrasses and salt marsh plants, and freshwater inflow (Boutton 1991; McClelland et al. 1997), both of which are dictated by the regional climate and hydrology. The LLM is part of a shallow lagoon system that receives low levels of freshwater inflow annually and limited water exchange with the GOM, in comparison to other Texas estuaries that are classified as either neutral (AB) or positive estuaries (LMB and GB) (McKee 2008). Therefore, the differences in cownose ray mean $\delta^{13}C$ in the LLM (in both spring and summer 2012) versus signatures in rays within other bay systems may be linked to the effects of hydrology and climate on primary production and the resulting isotopic differences in prey assemblages, and/or the availability and abundance of prey resources within the LLM. This may also hold true

for mean $\delta^{15}N$, where cownose rays in the LLM and AB had similar values but all other ray populations had isotopically distinct values.

Isotopic niche size was relatively consistent across Texas bay systems in 2012. The lone exception occurred within the LLM, where the summer trophic niche was significantly smaller than the spring trophic niche. This reduction in the summer isotopic niche may possibly be the result of cownose rays preferentially foraging on an abundant prey source, to the exclusion of other prey resources. Ajemian and Powers (2012) examined the diet of cownose rays off of coastal Alabama and noted that over 50% of the stomach contents was comprised of one prey source. This led the authors to suggest that cownose rays can exhibit specialist foraging behavior by selectively foraging on prey that occur in high densities when available, which has also been documented in the Chesapeake Bay (Fisher 2010) and Charlotte Harbor estuarine system (Collins et al. 2007b).

Aggregate feeding behaviors are often seen in cownose rays with shoals segregated either by size (Smith and Merriner 1987; Blaylock 1993) or, in some cases, sex (Smith and Merriner 1987). Sex segregation was documented in each bay system with female and male rays dominating the population in AB and LMB, respectively. This sex bias within cownose ray populations in these bay systems is corroborated by historical STFERL netting records from 2006 – 2007 and 2010, with a female bias in AB and male bias in LMB, excluding 2007, during summer months (Metz and Landry, unpublished data). However, isotopic niche sizes were similar in male and female rays in both AB and LMB, indicating that cownose rays, regardless of sex, utilize prey

resources across a similar size range of isotopic values. Collins et al. (2007a) found that there were no significant differences in the diets of male and female rays in Charlotte Harbor but it must be noted that shoals were comprised of both sexes. Nonetheless, there is some indication of potential sex-related foraging differences in LMB, as there was a significant difference between male and female $\delta^{13}C$ values, similar to LLM. However, in contrast to LLM, male rays ($\bar{x}$ = -16.82 ‰) were significantly enriched compared to female rays ($\bar{x}$ = -17.36 ‰), although this may be an artifact of sample sizes (male: n = 21; female: n = 9).

There was a subtle but significant difference in mean $\delta^{13}C$ in AB subadult ($\bar{x}$ = -16.90 ‰) and adult ($\bar{x}$ = -16.20 ‰) rays, potentially indicative of differential foraging between life history stages. While studies have shown that maturity level does produce differences in cownose ray diet (Smith and Merriner 1985; Peterson et al. 2001; Ajemian and Powers 2012), SIA may not be able to identify differences in diet unless the prey items are isotopically distinct or if foraging occurs in isotopically disparate habitats. Ajemian and Powers (2012) noted a distinct difference in cownose ray habitat use/diet in coastal Alabama, according to life history stage, with immature and mature rays found predominately in inshore and open Gulf sites, respectively. This study utilized only subadult and adult cownose rays. Therefore, it is imperative for future studies to incorporate samples from YOY and juvenile, along with mature rays, to determine if there are ontogenetic shifts in diet along the Texas coast.

Concluding remarks

While this study provides valuable insight into the trophic dynamics of cownose rays populations along the Texas coast, there are still many uncertainties about cownose ray foraging and habitat-use patterns in the northwestern Gulf of Mexico that must be addressed. Stable isotope analysis is a powerful and minimally invasive tool, but the appropriate isotopic baselines for estuarine systems must be established. These baselines can be documented by sampling potential prey species for isotopic analysis. For cownose rays in the northwestern Gulf of Mexico, stomach content analysis is essential to identify prey species consumed, as well as to investigate dietary specialization and foraging strategies. At the same time, benthic surveys of invertebrates will provide integral information about prey abundances and availabilities for both inshore and offshore cownose ray foraging grounds.

Findings from this study demonstrate that SIA of epidermal tissue provides an isotopic profile of long-term dietary information. Examination of tissues sampled over a multi-year period showed variation in isotopic signatures across sampling periods in LLM that may be a result of seasonal inshore-offshore movements. Differences in isotopic signatures were also documented between the sexes, indicating that female rays may spend more time within inshore and coastal waters to forage on abundant benthic resources during gestation. However, slow turnover tissues, like epidermis, do not capture short-term habitat use patterns, such as selective foraging of seasonally abundant prey items. Therefore, future studies should use a multi-tissue approach, integrating tissues with both fast and slow turnover rates, enabling comparisons of habitat use over

both short- and long-term timescales. Additionally, using a multi-tissue approach would supplement the findings of this study by determining periods of transition between inshore and offshore habitats along the Texas coast.

By quantifying isotopic niches of cownose rays along the Texas coast, this study demonstrates that resource use in these populations is dynamic, both temporally and spatially. Continued analysis of foraging and habitat-use patterns will help define the role that cownose rays play, as mesopredators, within these communities in the northwestern Gulf of Mexico. Understanding the trophic dynamics of cownose rays will be essential for management practices, especially in light of potential increases in cownose ray populations due to increased fishing pressure on apex elasmobranch species.

CHAPTER IV

CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

This study is the first to explore stable isotope dynamics in cownose rays in the northwestern GOM. The application of SIA to elasmobranchs is a relatively new endeavor with limited research regarding isotope dynamics in elasmobranch tissues. Therefore, this study conducted a controlled feeding trial to establish incorporation rates and DTDFs of $\delta^{13}C$ and $\delta^{15}N$ in epidermal tissue of cownose rays. Once these values were established, analyses were conducted to explore the temporal and spatial variability of $\delta^{13}C$ and $\delta^{15}N$ in cownose ray populations along the Texas coast. Conclusions regarding the hypotheses presented in Chapter II and Chapter III are summarized in Table 9 and 10, respectively.

The cownose rays collected from LMB were divided into two treatment groups with each group fed an isotopically distinct diet (either shrimp or squid). Due to extenuating circumstances, the $\delta^{13}C$ incorporation rate (0.0017 ± 0.001 days^{-1}) could only be estimated for the shrimp treatment group and the $\delta^{15}N$ incorporation rate (0.00305 ± 0.00048 days^{-1}) could only be determined for the squid treatment group. Incorporation rates of both $\delta^{13}C$ and $\delta^{15}N$ were highly variable amongst the individuals within each treatment group and there was no relationship in ray weight or disc width with respect to incorporation rates. A subsequent analysis of acid washed versus non-

Table 9. Summary and evaluation of the hypotheses presented in Chapter II.

Hypothesis	Evaluation
(1) δ13C and δ15N in epidermal tissue will have an average residence time of approximately 6 months	Reject
(2) Size will influence isotopic incorporation rates.	Reject
(3) Sex will influence isotopic incorporation rates.	Reject
(4) δ13C and δ15N diet-tissue discrimination factors will be equal to 1.0 and 3.4 ‰, respectively.	Inconclusive

Table 10. Summary and evaluation of the hypotheses presented in Chapter III.

Hypothesis	Evaluation
(1) Isotopic signatures of cownose rays in the lower Laguna Madre will exhibit seasonal variation.	Accept
(2) Isotopic signatures of cownose rays will vary across bay systems along the Texas coast.	Accept

acid washed epidermal samples resulted in a significant difference in $\delta^{13}C$ signature and may have been a source of variability in the $\delta^{13}C$ incorporation rate. As the epidermal tissue in neither treatment group reached isotopic equilibrium with its respective diet, the DTDFs reported in this study did not agree with values previously reported for elasmobranchs (Hussey et al. 2010; Kim et al. 2012a; Malpica-Cruz et al. 2012). Potential factors that may influence DTDFs in epidermal tissues were investigated but neither ray size or sex significantly affected the resulting DTDFs.

Temporal and spatial variability in $\delta^{13}C$ and $\delta^{15}N$ within cownose rays in the northwestern GOM was examined using Bayesian ellipses. Seasonal variation in the relative size of isotopic niches was documented in the LLM. Potential sources of variation in $\delta^{13}C$ and $\delta^{15}N$ signatures include changes in benthic prey resources due to a historic drought that occurred in 2011 and movements between inshore and offshore habitats. Furthermore, female $\delta^{13}C$ signatures were enriched compared to male signatures, suggesting that female rays spend more time within inshore habitats, possibly to forage on available prey resources during gestation. Additionally, mean $\delta^{13}C$ and $\delta^{15}N$ in cownose ray populations varied spatially across bay systems along the Texas coast, possibly as a result of differences in regional climate and hydrology and their effect on primary production within each bay system.

Future Directions

While this study is the first to explore isotopic dynamics in cownose ray epidermal tissue and apply this knowledge to wild populations, there are several issues

regarding stable isotope dynamics that must be addressed to fully understand how SIA can be used to investigate the ecology of cownose rays. There are a multitude of factors that can affect isotopic incorporation rates and DTDFs and, at this time, there have been limited experimental trials, including this study, for elasmobranchs. Future studies should investigate topics such as growth, isotopic routing and diet quality, to determine how they affect stable isotopes within batoid tissues, as well as determine incorporation rates and DTDFs for other tissues in a batoid, such as muscle, liver and blood plasma.

Secondly, this study determined that epidermal tissue could be used to assess isotopic variability in cownose ray populations. However, as the average residence times of $\delta^{13}C$ and $\delta^{15}N$ in epidermal tissue are both greater than 12 months, epidermal tissue cannot capture seasonal or ontogenetic shifts, as well as short-term specialist feeding patterns due to abundant prey resources in cownose ray diet. Therefore, future studies should utilize a multi-tissue approach, incorporating tissues with both fast and slow turnover rates in order to effectively capture short and long term foraging patterns. Furthermore, potential cownose ray prey items in the northwestern GOM should be identified through stomach content analysis, as well as analyzed using stable isotope techniques to establish isotopic baselines for potential prey resources for both inshore and offshore habitats. Subsequent studies could then use stable isotope mixing models to determine the relative contributions of prey sources to the diet of a consumer (Parnell et al. 2013), which is of particular interest to fisheries managers, given the documented impacts cownose rays have on commercial shellfish stocks (Smith and Merriner 1979; Smith and Merriner 1985; Blaylock 1993; Peterson et al. 2001; Myers 2007).

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