Feeding preferences of West Indian manatees in Florida, Belize, and Puerto Rico as indicated by stable isotope analysis

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ABSTRACT: The endangered West Indian manatee *Trichechus manatus* has 2 recognized subspecies: the Florida *T. m. latirostris* and Antillean *T. m. manatus* manatee, both of which are found in freshwater, estuarine, and marine habitats. A better understanding of manatee feeding preferences and habitat use is essential to establish criteria on which conservation plans can be based. Skin from manatees in Florida, Belize, and Puerto Rico, as well as aquatic vegetation from their presumed diet, were analyzed for stable carbon and nitrogen isotope ratios. This is the first application of stable isotope analysis to Antillean manatees. Stable isotope ratios for aquatic vegetation differed by plant type (freshwater, estuarine, and marine), collection location, and in one instance, season. Carbon and nitrogen isotope ratios for manatee skin differed between collection location and in one instance, season, but did not differ between sex or age class. Signatures in the skin of manatees sampled in Belize and Puerto Rico indicated a diet composed primarily of seagrasses, whereas those of Florida manatees exhibited greater regional variation. Mixing model results indicated that manatees sampled from Crystal River and Homosassa Springs (Florida, USA) ate primarily freshwater vegetation, whereas manatees sampled from Big Bend Power Plant, Ten Thousand Islands, and Warm Mineral Springs (Florida) fed primarily on seagrasses. Possible diet–tissue discrimination values for ^{15}N were estimated to range from 1.0 to 1.5‰. Stable isotope analysis can be used to interpret manatee feeding behavior over a long period of time, specifically the use of freshwater vegetation versus seagrasses, and can aid in identifying critical habitats and improving conservation efforts.

KEY WORDS: Stable isotope · West Indian manatee · Seagrass · Aquatic plants · ^{13}C · ^{15}N · Feeding ecology · Mixing model

INTRODUCTION

The West Indian manatee *Trichechus manatus* is 1 of only 2 extant sirenian species (*T. manatus* and *T. senegalensis*) that occupy marine, estuarine, and freshwater habitats (Hartman 1979, Best 1981). This herbivorous species has 2 recognized subspecies, the Florida manatee *T. m. latirostris* and the Antillean manatee *T. m. manatus*. Relatively little is known about manatee feeding ecology and habitat use outside of Florida since they often occupy shallow, turbid water. Even in Florida, most feeding studies have been con-ducted in captivity (e.g. Marshall et al. 2000) or in clear waters near natural springs (e.g. Hartman 1979). Reliable population estimates for both subspecies remain elusive (Lefebvre et al. 1995, UNEP Caribbean Environment Programme 1995, USFWS 2001), and a better understanding of manatee feeding ecology and its relation to habitat use is essential in order to improve conservation efforts.

Although there are few morphological differences between the 2 subspecies (Antilleans tend to be smaller in body size than Florida manatees and have differing skull features; Converse et al. 1994), there are

many other differences in habitat, climate, and population size that have unique effects on the success of each subspecies. Both subspecies appear to require regular year-round access to a freshwater source (Lefebvre et al. 1989, Olivera-Gomez & Mellink 2005), a requirement that is likely a result of osmoregulatory constraints (Ortiz et al. 1998, 1999). Winter habitat use by Florida manatees is primarily influenced by water temperature, whereby physiological constraints require that they aggregate at natural (springs) and/or artificial (power plant outflows) warm water sources when water temperatures drop below 20°C (Irvine 1983, Worthy et al. 2000, Bossart et al. 2003). This pattern is unnecessary for Antillean manatees since their range lies in the tropics where water temperatures are more constant. Population estimates for the Florida manatee suggest counts as high as 3807 individuals (FWC 2009), whereas Antillean manatees are thought to number fewer than 100 individuals in most countries (summarized by O'Shea & Salisbury 1991). The largest Antillean manatee population is in Mexico and is currently estimated at around 1000 to 2000 individuals (Quintana-Rizzo & Reynolds 2007). A 2002 aerial survey in Belize indicated a population of 338 individuals (Auil 2004), and recent evidence suggests a population in Belize of between 400 and 700 manatees (Quintana-Rizzo & Reynolds 2007). In Puerto Rico, manatee distribution is patchy, and population counts suggest a population of about 250 individuals (Slone et al. 2006).

Manatees are considered to be generalist feeders and are known to consume approximately 60 different species of vegetation in marine, estuarine, and freshwater habitats (Hartman 1979, Best 1981, Bengtson 1983). Florida manatees appear to exhibit regional and possibly seasonal differences in diet composition (Reich & Worthy 2006). Because of the previously mentioned habitat and climate differences between the 2 subspecies, seagrasses tend to make up a much larger portion of the manatee diet in Puerto Rico than freshwater and/or estuarine vegetation (Mignucci-Giannoni & Beck 1998). Very little is known of the feeding habits of manatees in Belize, but seasonal differences in lagoon water levels may lead to changes in habitat use (Morales-Vela et al. 2000, Auil et al. 2007) and consequently diet composition. Little research has been done on the Antillean subspecies, and expanded effort is crucial to provide measures on which to base conservation plans.

It can be challenging to evaluate feeding behavior and habitat use in marine mammals since they are difficult to observe directly and may have expansive migration patterns. A variety of methods have been used, including satellite tracking (e.g. Laidre et al. 2003), stomach content analysis (e.g. Spitz et al. 2006), fecal analysis (e.g. Sinclair & Zeppelin 2002), and fatty acid signature analysis (e.g. Iverson et al. 1997). Some complications involved in these methods include logistics, cost, invasiveness, difficulty in identifying and estimating the proportion of diet components, and the inability to assess long-term feeding history. The application of stable isotope analysis to ecological research has expanded in recent years (reviewed by Kelly 2000) and has some advantages over these techniques. Isotopic ratios of local food webs are incorporated into the tissues of the consumer and can be used to predict diet composition, the trophic level at which the consumer is feeding, and even habitat use and migratory patterns (e.g. Deniro & Epstein 1978, 1981, Fry 1981, Peterson & Fry 1987, Hobson 1999). Tissues such as hair and skin can be easily sampled from live animals, and both recent and long-term feeding history can be assessed by analyzing tissues that incorporate nutrients at different rates. To accurately interpret isotopic results, it is important to know the diet–tissue discrimination value (the difference in isotope ratios between the diet and consumer tissue) as well as the turnover rate (the amount of time required to incorporate isotopes from the diet into the tissue). Previous studies have calculated or estimated diet–tissue discrimination values and turnover rates for carbon and nitrogen isotopes in manatee skin (Ames et al. 1996, Reich & Worthy 2006, Alves-Stanley & Worthy 2009), allowing for more accurate interpretation of stable isotope data.

The present study is the first to apply stable isotope analysis to Antillean manatees and further expands the assessment of the feeding ecology of Florida manatees. The objectives of this study were to (1) analyze δ^{13}C and δ^{15}N values in epidermal samples collected from free-ranging manatees in Florida, Belize, and Puerto Rico, (2) compare signatures in the skin to those of freshwater, estuarine, and marine vegetation within the presumed manatee diet, and (3) assess possible differences in feeding preferences by region, sex, age class, and season.

MATERIALS AND METHODS

Sample collection. Samples of aquatic vegetation were collected within Florida (Table 1, Fig. 1) during the summer of 2001, the fall, winter, and spring of 2004 and 2005 (Charlotte Harbor: n = 15; Crystal River: n = 5; Indian River Lagoon: n = 21, St. Johns River near Blue Spring: n = 29; Tampa Bay: n = 16; and Ten Thousand Islands: n = 15) and from Belize (Table 1, Fig. 1) during the summer of 2002 (Drowned Cayes: n = 18). Plant collections from Puerto Rico were not possible due to study constraints. Each vegetation sample consisted of 2 to 3 whole plants or 20 to 30 seagrass blades for each available species at each site. Collection sites in the St.

Table 1. Aquatic plant species (freshwater, estuarine, and seagrasses) collected in the present study. Sites: SJ: St. Johns River, CR: Crystal River, IR: Indian River, CH: Charlotte Harbor, TB: Tampa Bay, TI: Ten Thousand Islands, DC: Drowned Cayes

Species	Florida							Belize
	Fresh	Estuarine		Seagrass				Seagrass
	SJ	CR	IR	CH	IR	TB	TI	DC
Alternanthera philoxeroides (alligatorweed)	X							
Chara sp. (stonewort)		X						
Eichhornia crassipes (water hyacinth)	X							
Gracilaria sp. (red alga)			X		X			
Halodule wrightii (shoal grass)			X	X	X	X	X	X
Halophila engelmannii (star grass)							X	
Hydrilla verticillata (hydrilla)		X						
Hydrocotyle sp. (pennywort)	X							
Lemna valdiviana (duckweed)	X							
Myriophyllum aquaticum (parrotfeather)	X							
Myriophyllum spicatum (watermilfoil)		X						
Nuphar luteum (spatterdock)	X							
Pontederia cordata (pickerel weed)	X							
Pistia stratiotes (water lettuce)	X							
Syringodium filiforme (manatee grass)			X	X	X	X	X	
Thalassia testudinum (turtle grass)			X	X	X	X	X	X
Unknown grass	X							

Johns River were sampled repeatedly during different months to assess potential seasonal variability.

Skin samples from free-ranging manatees in Florida (n = 118), Belize (n = 68), and Puerto Rico (n = 23) were collected during 2002 to 2005 as part of a larger study examining genetic relatedness. Sampling locations in Florida included Crystal River, Homosassa Springs, Tampa Bay (Big Bend Power Station, Tampa), Ten Thousand Islands (Port of the Islands, Naples), and Charlotte Harbor (Warm Mineral Springs; Fig. 1). Most Florida manatees (n = 113) were sampled during the fall and winter months when they often congregate in large numbers at natural or artificial warm water sources. Sampling locations in Belize included the Northern Lagoon, Southern Lagoon, Western Lagoon (part of the Southern Lagoon), and Drowned Cayes (Fig. 1). Locations in Puerto Rico included Cabo Rojo, Ceiba, Guayanilla, and Salinas (Fig. 1). Samples from Antillean manatees in Belize and Puerto Rico were collected opportunistically over multiple years. For seasonal comparisons, winter was defined as December through February, spring as March through May, summer as June through August, and fall as September through November.

Epidermal tissue was collected from the edge of the paddle using a cattle ear notch tool. Sex was determined by observing the position of the urogenital slit, and standard body length was measured as the straight distance from snout to paddle. Florida manatees were categorized into 3 age classes based on body length measurements (adults: >275 cm; subadults/late juveniles: 176–275 cm; and
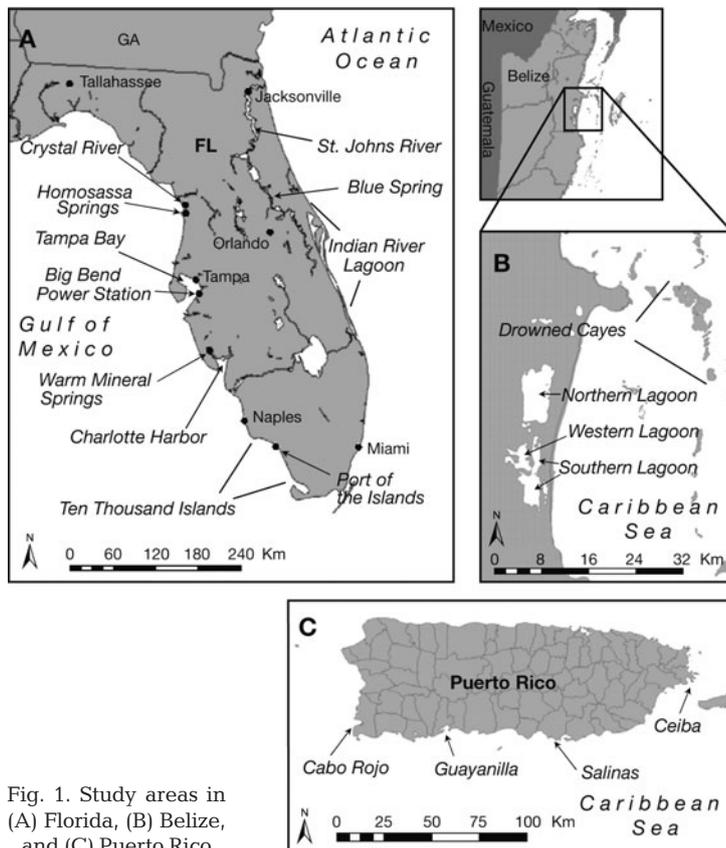

Fig. 1. Study areas in (A) Florida, (B) Belize, and (C) Puerto Rico

calves: <176 cm; O'Shea et al. 1985). Antillean mana-
tees were also categorized into age classes, although
these classes were defined by different body length
measurements since Antillean manatees are slightly
smaller (adults: >225 cm; subadults/late juveniles:
176–225 cm; calves: <176 cm; Mignucci-Giannoni et
al. 2000).

Sample preparation and analysis. All manatee tis-
sue and plant samples were frozen within 2 h of collec-
tion and held at –20°C until time of analysis. Samples
were then rinsed with distilled water and oven dried at
60°C for 24 h. Lipids were removed using petroleum
ether in a Soxhlet extractor for 24 h to remove the
potential effect of lipids on $\delta^{13}C$ values (Rau et al.
1992). Samples were then oven dried at 60°C for 24 h
to remove any remaining solvent. Samples were
ground and homogenized using a SPEX 8000 Mixer/
Mill (CertiPrep) or a Wig-L-Bug Amalgamator (Cres-
cent Dental Manufacturing), or they were chopped by
hand using a scalpel. Approximately 1.0 mg of mana-
tee tissue or 2.5 mg of plant materials were transferred
to 5 × 9 mm tin capsules and analyzed for carbon and
nitrogen stable isotope ratios by mass spectrometry
(Thermo Finnigan DELTAplus and DELTA C) at the
Stable Isotope and Ecology Lab, University of Georgia,
Athens, GA. Seagrasses were analyzed with and with-
out epiphytes when possible. Seagrasses analyzed
with epiphytes were prepared as discussed previously
for other aquatic plants, with the exception that they
were rinsed in saltwater instead of distilled water to
prevent further removal of epiphytes.

Data analysis. Stable isotope ratios were expressed
in ppt (‰) using delta notation:

$$\delta x = (R_{sample}/R_{standard} - 1) \times 1000 \qquad (1)$$

in which R_{sample} and $R_{standard}$ are the absolute isotope
ratios of the sample and standard (bovine tissue),
respectively, and x is ^{13}C or ^{15}N. Quality assurance of
stable isotope ratios was tested by running 1 known
standard sample for each 12 unknown (manatee or
plant) samples. Analytical errors for the bovine tissue
were ±0.1 SD for $\delta^{13}C$ and $\delta^{15}N$.

Statistical analysis. All statistical analyses were
judged to be significant at p < 0.05. Data were tested
for normality using the Shapiro-Wilk (n < 50) and
Kolmogorov-Smirnov (n > 50) tests. Levene's F and
Box's M were used to test homogeneity of variance
between factors and homogeneity of covariance,
respectively. Differences in $\delta^{13}C$ and $\delta^{15}N$ values were
tested using parametric and non-parametric analyses
as appropriate. Differences in $\delta^{13}C$ and $\delta^{15}N$ of mana-
tee skin were tested using MANOVA with the follow-
ing main and interaction effects: sex, age, season, loca-
tion, sex and location, sex and age class, and sex and
season. Data are presented as means ± SE.

Diet modeling. Recent developments in multiple
source isotope mixing models allow for the calculation of
relative proportions of different prey in the diet. IsoError
version 1.04 is a Microsoft Excel™ worksheet that calcu-
lates proportions of source contributions to a mixture (in-
cluding variances, standard errors, and confidence inter-
vals) while accounting for variability in stable isotope
signatures (see Phillips & Gregg 2001 for a full descrip-
tion and discussion). Using this mixing model and pub-
lished diet–tissue discrimination values (Alves-Stanley &
Worthy 2009), proportions of freshwater vegetation, es-
tuarine vegetation, and seagrasses contributing to the
Florida manatee diet were estimated.

RESULTS

Diet analysis

Florida aquatic plants

Because turtle grass *Thalassia testudinum* often has a
large amount of epiphytic algae and other encrusting or-
ganisms attached to the blades, $\delta^{13}C$ and $\delta^{15}N$ values for
clean and epiphytic blades were initially compared.
Paired *t*-tests indicated no significant difference in $\delta^{13}C$
($t = 2.15$, df = 10, p = 0.057) or $\delta^{15}N$ values ($t = 1.13$, df =
10, p = 0.29) between clean blades (mean $\delta^{13}C = -11.9 \pm$
0.8‰, mean $\delta^{15}N = 2.1 \pm 0.4$‰) and those with epiphytes
present (mean $\delta^{13}C = -12.9 \pm 0.8$‰, mean $\delta^{15}N = 2.4 \pm$
0.5‰), so samples were grouped for further analyses.

Stable isotope signatures of aquatic vegetation were
compared between collection locations in order to exa-
mine potential distinctions between freshwater plants,
estuarine plants, and seagrasses. Welch analysis of
variance (ANOVA) results indicated that $\delta^{13}C$ values
differed significantly ($F_{6,27} = 189.27$, p < 0.001). Fresh-
water plants were the most depleted in ^{13}C, while sea-
grasses were the most enriched. Plants that had inter-
mediate $\delta^{13}C$ values, those collected from Crystal River
and marine algae from the Indian River Lagoon, were
categorized as estuarine vegetation (Table 2, Fig. 2).
Regardless of collection location, $\delta^{13}C$ values differed
between all 3 plant types (Tamhane's T2: all p-values <
0.021). Welch ANOVA results also indicated that $\delta^{15}N$
values differed significantly between collection loca-
tions ($F_{6,34} = 38.19$, p < 0.001). Regardless of collection
location, all seagrasses were significantly depleted in
^{15}N compared to freshwater and estuarine vegetation
(Tamhane's T2: all p-values < 0.002), but $\delta^{15}N$ values
did not differ between freshwater and estuarine vege-
tation (all p-values > 0.61, Table 2, Fig. 2).

Within seagrass samples, $\delta^{13}C$ values differed signif-
icantly between collection locations (Welch ANOVA:
$F_{3,27} = 5.51$, p = 0.004) with seagrasses from Tampa Bay

Table 2. Stable isotope ratios of aquatic plants collected in Florida and Belize

| Plant type	n	δ^{13}C (‰)			δ^{15}N (‰)		
		Mean ± SE	Minimum	Maximum	Mean ± SE	Minimum	Maximum
Florida							
Freshwater vegetation							
St. Johns River	29	−28.1 ± 0.3	−31.6	−25.0	7.3 ± 0.5	1.9	10.3
Estuarine vegetation	13	−22.1 ± 0.6	−25.1	−18.8	6.3 ± 0.2	5.4	7.6
Crystal River	5	−22.3 ± 0.8	−24.2	−20.1	6.0 ± 0.3	5.4	7.1
Indian River Lagoon	8	−21.9 ± 0.9	−25.1	−18.8	6.4 ± 0.3	5.5	7.6
Seagrasses	59	−13.1 ± 0.4	−19.6	−6.6	1.6 ± 0.3	−2.9	5.4
Charlotte Harbor	15	−11.0 ± 0.5	−15.5	−8.3	1.4 ± 0.3	−0.7	4.4
Indian River Lagoon	13	−13.8 ± 1.2	−19.6	−6.6	1.1 ± 0.7	−2.0	5.3
Tampa Bay	16	−14.8 ± 0.8	−19.5	−9.2	2.5 ± 0.4	0.2	5.4
Ten Thousand Islands	15	−12.9 ± 0.5	−16.9	−9.5	1.1 ± 0.6	−2.9	3.8
Belize							
Seagrasses							
Drowned Cayes	18	−7.2 ± 0.5	−11.9	−2.9	−0.6 ± 0.5	−5.1	2.6
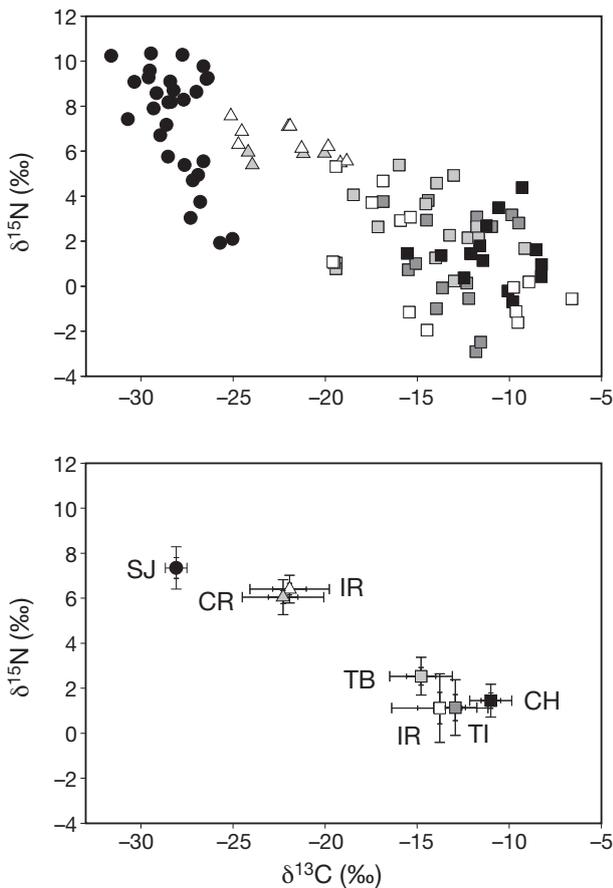
Fig. 2. Stable isotope values (scatterplot and mean ± SE and 95% CI) for aquatic plants in Florida. Circles represent freshwater vegetation, triangles represent estuarine vegetation, and squares represent seagrasses. Location abbreviations as in Table 1

being significantly depleted in ^{13}C by an average of 3.8‰ compared to those from Charlotte Harbor (Tamhane's T2: p = 0.003, Table 2, Fig. 2). No other significant differences were calculated for δ^{13}C values between any other collection locations (Tamhane's T2: all p-values > 0.26). δ^{13}C values did not differ between seagrass species regardless of how locations were grouped (ANOVA: F < 1.1, all p-values > 0.37). Finally, δ^{15}N values for seagrasses did not differ between collection locations (Welch ANOVA: $F_{3,26}$ = 1.79, p = 0.17, Table 2, Fig. 2) or species (ANOVA: $F_{2,53}$ = 2.34, p = 0.11). Star grass *Halophila engelmannii* was collected at only 1 location (Ten Thousand Islands), preventing inclusion in this analysis.

Freshwater plants from St. Johns River were collected during the months of April, July, and December, allowing for a seasonal comparison. Main effects for month and plant species were tested using multivariate ANOVA (MANOVA). There was a significant effect of month (Wilks' lambda: $F_{4,28}$ = 4.22, p = 0.009), but there was no effect of species ($F_{16,28}$ = 1.63, p = 0.13). The δ^{13}C values of freshwater plants did not differ between months (ANOVA: $F_{2,23}$ = 2.83, p = 0.080), but δ^{15}N values were significantly different ($F_{2,23}$ = 12.73, p < 0.001). Plants collected in July were depleted in ^{15}N by an average of 3.9‰ compared to those collected in December and April (both p-values < 0.002, Tukey HSD).

Belize aquatic plants

Samples of turtle grass *Thalassia testudinum* and shoal grass *Halodule wrightii* were collected opportunistically from the Drowned Cayes in Belize (Fig. 1).

Table 3. Stable isotope ratios in manatee skin from free-ranging animals in Florida, Belize, and Puerto Rico

Location	n	$\delta^{13}C$ (‰)			$\delta^{15}N$ (‰)		
		Mean ± SE	Minimum	Maximum	Mean ± SE	Minimum	Maximum
Florida							
Riverine manatees	88	−20.4 ± 0.3	−27.0	−14.6	7.0 ± 0.1	2.7	10.1
Crystal River (CR)	67	−20.0 ± 0.3	−24.9	−14.6	7.1 ± 0.2	3.8	10.1
Homosassa Springs (HS)	21	−21.4 ± 0.6	−27.0	−16.3	6.5 ± 0.3	2.7	9.2
Coastal manatees	30	−14.2 ± 0.5	−20.4	−9.3	4.6 ± 0.3	2.0	8.1
Big Bend Power Station (BP)	10	−13.5 ± 0.5	−15.4	−10.9	5.8 ± 0.6	2.0	8.1
Port of the Islands (PI)	14	−13.8 ± 0.7	−17.6	−9.3	4.0 ± 0.2	3.0	6.2
Warm Mineral Springs (WS)	6	−16.3 ± 1.2	−20.4	−13.1	4.0 ± 0.5	2.8	5.6
Belize							
Drowned Cayes (DC)	13	−7.5 ± 0.4	−11.0	−5.9	3.4 ± 0.2	2.1	4.7
All lagoons	55	−12.7 ± 0.2	−16.0	−9.2	1.6 ± 0.3	−2.3	5.7
Northern Lagoon (NL)	8	−13.4 ± 0.4	−15.5	−12.4	4.1 ± 0.3	2.6	5.3
Southern Lagoon (SL)	37	−12.4 ± 0.3	−16.0	−9.2	1.0 ± 0.3	−2.3	5.7
Western Lagoon (WL)	10	−13.3 ± 0.4	−15.1	−11.5	1.5 ± 0.7	−2.3	4.1
Puerto Rico							
Cabo Rojo (CA)	7	−9.6 ± 0.5	−10.9	−7.9	4.9 ± 0.2	3.7	5.7
Ceiba (CE)	6	−10.1 ± 0.6	−12.8	−8.8	4.6 ± 0.3	3.8	5.4
Guayanilla (GU)	9	−9.6 ± 0.4	−11.0	−7.5	6.2 ± 0.2	5.2	6.9
Salinas (SA)	1	−10.4	−10.4	−10.4	5.7	5.7	5.7

The $\delta^{13}C$ values ranged from −11.9 to −2.9‰ and $\delta^{15}N$ values ranged from −5.1 to 2.6‰ (Table 2). Species comparisons were not undertaken due to small sample size, but $\delta^{13}C$ and $\delta^{15}N$ values for *H. wrightii* fell within the range of those of *T. testudinum* (Table 2). There was no effect of collection month (July–September, MANOVA: Wilks' lambda: $F_{4,28} = 0.97$, p = 0.44) between seagrasses in Belize.

Free-ranging manatees

Florida manatees

The $\delta^{13}C$ values for manatee skin increased from very depleted signatures for skin collected in freshwater regions to more enriched signatures for skin collected in coastal regions. The $\delta^{15}N$ values for manatee skin decreased from enriched signatures for skin collected in freshwater regions to depleted signatures for skin collected in coastal regions (Table 3, Fig. 3).

MANOVA was run for $\delta^{13}C$ and $\delta^{15}N$ values using the following main and interaction effects: sex, age class, location, sex and age class, and sex and location. No significant effects were noted for sex (F test: $F_{2,99} = 0.28$, p = 0.76), age class (Wilks' lambda: $F_{4,198} = 0.76$, p = 0.55), or either of the interaction effects ($F < 0.66$, both p-values > 0.73), but there was a significant effect for location (Wilks' lambda: $F_{8,198} = 13.49$, p < 0.001).

Both $\delta^{13}C$ (ANOVA: $F_{4,113} = 43.39$, p < 0.001) and $\delta^{15}N$ values ($F_{4,113} = 20.15$, p < 0.001) for manatee skin differed significantly based on location. Skin from
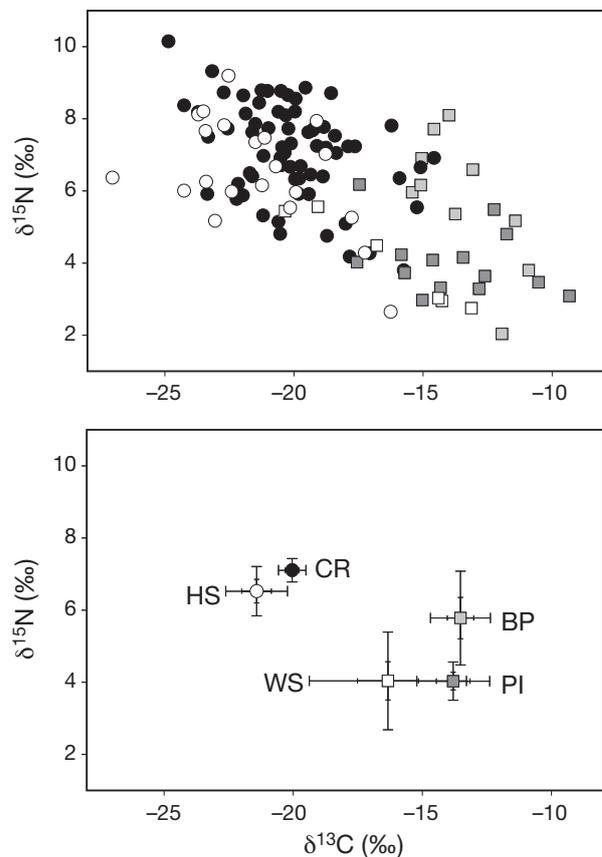

Fig. 3. *Trichechus manatus latirostris*. Stable isotope values (scatterplot and mean ± SE and 95% CI) for manatee skin collected from animals in Florida. Circles represent riverine manatees and squares represent coastal manatees. Location abbreviations as in Table 3

manatees sampled in Crystal River and Homosassa Springs was depleted in ^{13}C by an average of 6.2‰ compared to skin from manatees at the Big Bend Power Plant, Port of the Islands, and Warm Mineral Springs (Tukey HSD: all p-values < 0.010, Table 3, Fig. 3). The δ^{15}N values differed significantly between skin from manatees at the Big Bend Power Plant and Port of the Islands by an average of 1.8‰ (Tukey HSD: p = 0.020, Table 3, Fig. 3). The δ^{15}N values did not significantly differ between skin from manatees at any other locations (Tukey HSD: all p-values > 0.10). Because there were no significant differences in δ^{13}C or δ^{15}N values in skin from manatees sampled in Crystal River and Homosassa Springs, data from these 2 Florida locations were pooled ('riverine manatees') for further analyses (Table 3, Fig. 3). In addition, because there were no significant differences in δ^{13}C values in skin from manatees at the Big Bend Power Plant, Port of the Islands, and Warm Mineral Springs, data from these 3 Florida locations were pooled ('coastal manatees') for further analyses.

Age class comparisons were further investigated since sample distribution did not allow testing for an interaction effect between age class and location. Within riverine manatees, all skin samples were from calves or subadults. MANOVA showed no effect for age class (F test: $F_{2,85}$ = 0.09, p = 0.91). For skin from coastal manatees, δ^{13}C values did not differ between any of the 3 age classes (ANOVA: $F_{2,27}$ = 3.27, p = 0.054). Regardless of how locations were grouped for coastal manatees, δ^{15}N values of skin also did not differ between age classes (all test statistics < 1.9, all p-values > 0.082).

Month of skin sample collection was further investigated as a possible effect on carbon and nitrogen stable isotope ratios. For skin from riverine manatees, MANOVA failed to show an effect for month (October to February, Wilks' lambda: $F_{8,160}$ = 0.72, p = 0.67). For skin from coastal manatees, δ^{13}C values did not differ between months (January, December, and April, ANOVA: $F_{2,27}$ = 1.57, p = 0.23). The δ^{15}N values of skin also did not differ between months, regardless of how the locations were grouped for coastal manatees (F < 0.2, all p-values > 0.82).

Antillean manatees (Belize)

The δ^{13}C values for Antillean manatee skin ranged from depleted for skin collected from lagoon regions to more enriched for skin collected from the Drowned Cayes (Table 3, Fig. 4). Differences in δ^{13}C values were tested using ANOVA with the following main and interaction effects: sex, age class, season, location, sex and location, sex and season, and age class and season.
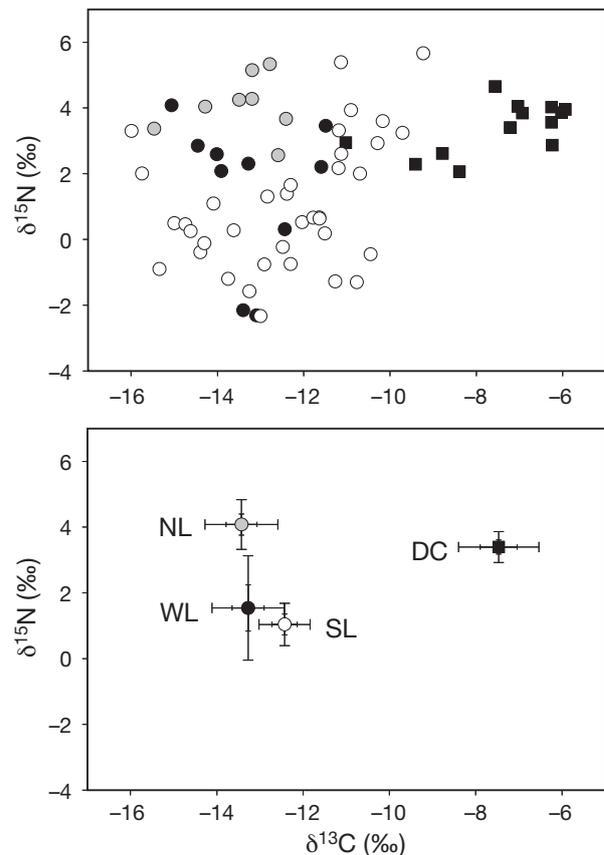

Fig. 4. *Trichechus manatus manatus*. Stable isotope values (scatterplot and mean ± SE and 95% CI) for manatee skin collected from animals in Belize. Circles represent lagoon manatees and squares represent manatees from the Drowned Cayes. Location abbreviations as in Table 3

There were significant effects for location ($F_{3,71}$ = 21.41, p < 0.001) and season (F test: $F_{2,71}$ = 3.77, p = 0.028), but no other effects were significant (F < 2.00, all p-values > 0.13).

Skin from manatees sampled in the Drowned Cayes was significantly enriched in ^{13}C by an average of 5.2‰ compared to samples from the 3 lagoons (Tukey HSD: all p-values < 0.001, Table 3, Fig. 4), but there were no differences in δ^{13}C values between lagoon samples (all p-values > 0.37). The δ^{15}N values also differed significantly between manatee skin from different locations (Welch ANOVA: $F_{3,22}$ = 17.86, p < 0.001). There was a difference in δ^{15}N values between manatees sampled in the Drowned Cayes and Southern Lagoon (Tamhane's T2: p < 0.001). Additionally, δ^{15}N values for skin samples from the Northern Lagoon differed significantly from those of the Southern (p < 0.001) and Western Lagoons (p = 0.036, Table 3, Fig. 4).

Samples in Belize were collected during fall and spring (rainy and dry seasons, respectively). Results of *t*-tests indicated that manatee skin collected in the spring was

significantly enriched in both ^{13}C (Drowned Cayes, t = 5.18, df = 11, p < 0.001; Southern Lagoon, t = 2.95, df = 35, p = 0.006) and ^{15}N (Drowned Cayes, t = 2.32, df = 11, p = 0.041; Southern Lagoon, t = 2.41, df = 33, p = 0.022) compared to skin collected in the fall. There was no seasonal difference in δ^{13}C values for manatee skin collected in the Western Lagoon (t = 0.50, df = 8, p = 0.63), but skin collected in the spring was significantly depleted in ^{15}N compared to that collected in the fall (t = 6.22, df = 8, p < 0.001). Manatees from the Northern Lagoon were sampled exclusively during the spring.

Antillean manatees (Puerto Rico)

δ^{13}C values for manatee skin ranged from –12.8‰ for an animal from Ceiba to –7.5‰ for an animal from Guayanilla. δ^{15}N values ranged from 3.7‰ for an animal from Cabo Rojo to 6.9‰ for an animal from Guayanilla (Table 3, Fig. 5).

There was a significant effect of location (F test: $F_{2,11}$ = 4.22, p = 0.043), but no other effects were signif-

icant (F < 2.60, all p-values > 0.11). Only 1 skin sample was analyzed from Salinas, so it was not included in these analyses. There were no significant differences in δ^{13}C values (ANOVA: $F_{2,19}$ = 0.32, p = 0.73) between the 3 locations (Guayanilla, Cabo Rojo, and Ceiba), but there was a significant difference in δ^{15}N values ($F_{2,19}$ = 11.76, p < 0.001). Samples from Guayanilla were enriched in ^{15}N by an average of 1.4‰ compared to those from both Cabo Rojo and Ceiba (Tukey HSD: both p-values < 0.01, Table 3, Fig. 5).

Diet modeling

Carbon and nitrogen signatures for freshwater, estuarine, and marine vegetation (seagrasses) were plotted with a polygon connecting their mean values as possible diet sources for manatees in Florida (Fig. 6). The diet source mean values for δ^{13}C were corrected for diet–tissue discrimination by 2.8‰ as measured by Alves-Stanley & Worthy (2009) and consistent with Reich & Worthy (2006). To fit the isotopic signature of the consumer within the diet source polygon, possible nitrogen diet–tissue discrimination values ranging from 1.0 to 1.5‰ were used.

IsoError analysis suggested that freshwater and/or estuarine vegetation was the main diet component for Florida riverine manatees, while seagrasses were the main diet component for coastal manatees regardless of the nitrogen diet–tissue discrimination value (Table 4). IsoError results are especially sensitive to
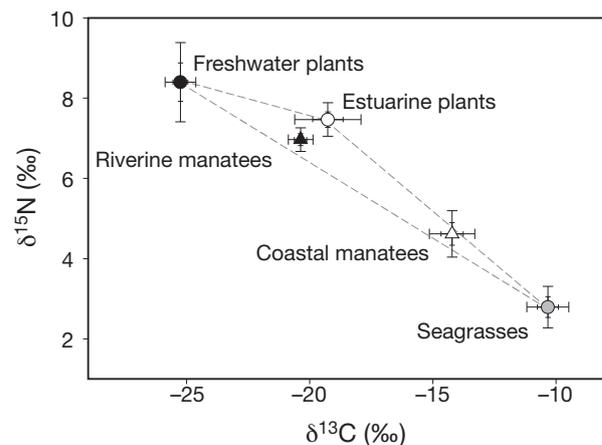
Fig. 6. *Trichechus manatus latirostris*. Estimation of diet–tissue discrimination in the skin of free-ranging Florida manatees. δ^{13}C and δ^{15}N values (mean ± SE and 95% CI) for aquatic vegetation and manatee skin were compared, and the 3 possible diet sources (corrected for diet–tissue discrimination) were connected by a polygon. The accepted enrichment values for manatee skin necessary for the means to fall within the polygon were 2.8‰ for carbon and ranged from 1.0 to 1.5‰ for nitrogen (1.2‰ was plotted)
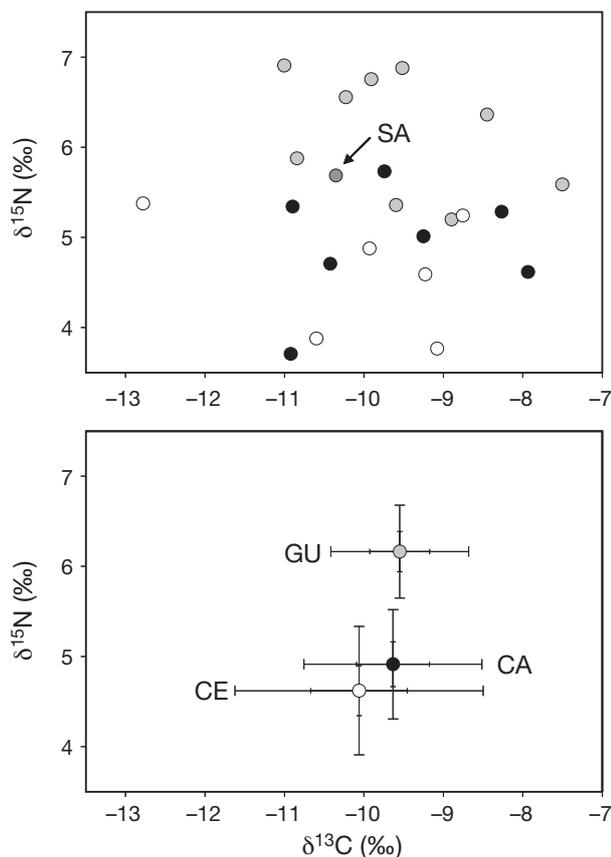
Fig. 5. *Trichechus manatus manatus*. Stable isotope values (scatterplot and mean ± SE and 95% CI) for manatee skin collected from animals in Puerto Rico. Location abbreviations as in Table 3

Table 4. IsoError results representing possible proportions of freshwater vegetation, estuarine vegetation, and seagrasses contributing to the manatee diet. Diet source means were corrected for diet–tissue discrimination by 2.8‰ for carbon. Model criteria allowed diet–tissue discrimination (enrichment) factors for nitrogen ranging from 1.0 to 1.5‰, so 3 possible solutions are shown for riverine and coastal manatees

Diet source	δ^{15}N enrichment = 1.0‰			δ^{15}N enrichment = 1.2‰			δ^{15}N enrichment = 1.5‰		
	Mean ± SE (%)	Lower 95% CI	Upper 95% CI	Mean ± SE (%)	Lower 95% CI	Upper 95%CI	Mean ± SE (%)	Lower 95% CI	Upper 95% CI
Riverine manatees									
Freshwater vegetation	41 ± 12	16	66	50 ± 13	24	76	64 ± 15	34	95
Estuarine vegetation	44 ± 19	5	82	28 ± 21	0	70	5 ± 25	0	55
Seagrasses	15 ± 8	0	30	21 ± 8	4	38	31 ± 10	10	51
Coastal manatees									
Freshwater vegetation	2 ± 17	0	36	11 ± 17	0	45	25 ± 18	0	61
Estuarine vegetation	40 ± 26	0	92	25 ± 26	0	77	1 ± 28	0	57
Seagrasses	58 ± 10	39	77	64 ± 10	44	84	73 ± 11	52	95

this diet–tissue discrimination value (or enrichment). When nitrogen enrichment was set to 1.0‰, the confidence interval of the model indicated that riverine manatees may not have had any seagrass component in their diet (lower 95% CI = 0). IsoError results for coastal manatees did not require any freshwater or estuarine component to the diet regardless of the assumed nitrogen diet–tissue discrimination value (lower 95% CI = 0).

DISCUSSION

Diet analysis

For aquatic plants collected in Florida and Belize, seagrasses were the most enriched in ^{13}C, while freshwater plants were the most depleted, and estuarine plants had intermediate values. This pattern is consistent with findings in previous studies (e.g. Reich & Worthy 2006, Alves-Stanley & Worthy 2009). Aquatic plants incorporate carbon from the dissolved inorganic carbon (DIC) in the surrounding water into their tissues. DIC in freshwater is depleted in ^{13}C compared to that of seawater due to the isotope discrimination that takes place upon the conversion of CO_2 into bicarbonate and the contribution of decomposing terrestrial matter (Boutton 1991). Freshwater and estuarine plants collected in Florida did not differ in their nitrogen signatures, but both plant types were significantly enriched in ^{15}N compared to seagrasses. Differences in nitrogen ratios have been shown to be based on nitrogen source, specifically in freshwater versus marine ecosystems (e.g. Bardonnet & Riera 2005); however, the understanding of nitrogen isotope patterns especially in aquatic plants is limited compared to those of carbon isotopes. The combined use of carbon and

nitrogen stable isotope analysis further distinguishes the 3 aquatic plant types in this study.

No significant differences were found in δ^{13}C or δ^{15}N values between *Thalassia testudinum* blades with or without epiphytes. Previous studies have shown conflicting results, in which some did find differences between isotope ratios in seagrasses versus epiphytes (e.g. Moncreiff & Sullivan 2001) while others did not (e.g. Fry et al. 1982). Typically, epiphytes are depleted in ^{13}C and enriched in ^{15}N compared to the seagrass blades from which they were removed. In terms of diet estimation, it is not likely that epiphyte signatures could cause seagrasses to be confused with signatures of freshwater plants. However, it is possible that the presence of epiphytes could cause carbon and nitrogen signatures of seagrasses to be more similar to those of estuarine vegetation.

Carbon and nitrogen stable isotope analyses were not precise enough to distinguish between aquatic plant species, but regional and seasonal differences in stable isotope ratios were found in Florida. Specifically, seagrasses collected from Charlotte Harbor were significantly enriched in ^{13}C compared to those from Tampa Bay. Variability in δ^{13}C values in seagrasses is mainly an effect of carbon source but can also vary with irradiance and temperature (Hemminga & Mateo 1996). Additionally, both carbon and nitrogen stable isotope ratios of seagrasses have been shown to exhibit seasonal and intra-annual variability (Anderson & Fourqurean 2003, Vizzini et al. 2003). Seagrasses from Charlotte Harbor were collected in late spring and those from Tampa Bay were collected during the summer. It is unknown whether differences in δ^{13}C between the 2 sites were due to location or a seasonal effect. In the St. Johns River, aquatic plants collected in July were significantly depleted in ^{15}N compared to those collected in December and April. Differences in

nitrogen signatures in plants may be due to seasonal variation in the amount of fertilizer runoff, wastewater discharge, and/or animal waste runoff into the aquatic system (Vizzini & Mazzola 2006). Additionally, seasonal differences in water temperature, streamflow, and dissolved oxygen concentrations have been shown to affect nitrogen balance in the St. Johns River (Kroening 2004). Temporal and geographical variability in stable isotope ratios for aquatic plants should be taken into consideration when estimating diet proportions for consumers.

Diet modeling

Reliance on stable isotope mixing model results to estimate short-term manatee diet and/or habitat use through analysis of epidermal tissue is problematic due to slow turnover rates in the skin. Alves-Stanley & Worthy (2009) calculated a mean half-life for ^{13}C turnover of 53 d, while mean half-life for ^{15}N turnover ranged from 65 to 72 d. Free-ranging manatees are known to switch diet sources (Best 1981, Lefebvre et al. 2000), and it is likely that as a result of these long turnover times, an intermediate isotope signature may be measured rather than one strictly representing a more recent diet. Rather than attempting to predict manatee diet composition involving all 3 aquatic plant types, it was thought to be more useful and more accurate to focus on the use of freshwater plants versus seagrasses. These vegetation types had very distinct δ^{13}C and δ^{15}N values, whereas the δ^{15}N values for estuarine plants did not differ from those of freshwater plants. Also, the slow turnover rate in epidermal tissue makes this analysis a better representation of average overall dietary intake over a long period of time rather than an indication of recent intake. Finally, when interpreting mixing model results, upper and lower 95% CI should be considered in addition to the mean values as a complete representation of possible diet source proportions (Phillips & Gregg 2003).

Seagrasses were the most frequently 'required' component present in the diets of Florida manatees. Due to slow turnover rates, seagrasses were not necessarily a recent diet component, but all Florida manatees in the present study likely fed on seagrasses sometime in the months prior to sampling. Seagrasses were an even more critical component in the diet of manatees sampled at the Big Bend Power Plant, Port of the Islands, and Warm Mineral Springs. For manatees from these locations, it was conceivable that they had consumed no freshwater vegetation in recent months. Manatees from Crystal River and Homosassa Springs had a more varied reliance on plant types, with freshwater vegetation most often being the main component.

Reich & Worthy (2006) used IsoError to estimate diet source proportions using skin from stranded manatee carcasses, but comparisons between the present study and their results may not be appropriate. diet–tissue discrimination values used for carbon were consistent (2.8‰, present study; 3.0‰, Reich & Worthy 2006), but those for nitrogen were not (1.0 to 1.5‰, present study; 5.0‰, Reich & Worthy 2006). Computing an accurate diet–tissue discrimination value is essential when using mixing models to estimate proportions of a multiple source diet (Phillips & Koch 2002). Generally δ^{15}N diet–tissue discrimination values for whole body samples average 3‰ (Deniro & Epstein 1981, Minagawa & Wada 1984, Peterson & Fry 1987). Most individual tissues are enriched in ^{15}N by 2 to 5‰ compared to the diet (Peterson & Fry 1987, Kelly 2000), yet values ranging from <1 to 6‰ have been calculated for individual mammalian tissues (Vanderklift & Ponsard 2003). Hobson et al. (1996) calculated the only known diet–tissue discrimination value for nitrogen (2.3‰) for the skin of marine mammals (phocid seals). Further captive studies are needed to determine an accurate diet–tissue discrimination value for nitrogen in manatee skin and thus improve the accuracy of diet source estimation.

Another difference is that Reich & Worthy (2006) grouped manatees by much larger geographical regions. For example, the entire northwest Florida coast was grouped (including Tampa Bay). In the present study, manatees sampled in Crystal River and Homosassa Springs (northwest Florida) were grouped separately from those sampled at the Big Bend Power Plant (Tampa Bay) because stable carbon and nitrogen isotope ratios were significantly different in the skin. Consequently, it is likely too much of a generalization to categorize manatee feeding across larger geographical regions. Finally, it should be noted that no study involving manatee stable isotope analysis has used a concentration-dependent model such as that of Phillips & Koch (2002), which may further refine results.

Free-ranging manatees

Although the lack of freshwater and estuarine plant samples prevented mixing model analysis for Antillean manatees, general comparisons and interpretations were made based on known patterns. Skin from manatees sampled in the Drowned Cayes in Belize was enriched in ^{13}C compared to that of lagoon manatees, consistent with a coastal diet of predominantly seagrasses, while the lagoon animals probably relied more heavily on estuarine and/or freshwater vegetation. There were no differences in carbon signatures among manatees from the 3 lagoons. However, skin

from Northern Lagoon manatees was enriched in ^{15}N compared to that of the Southern and Western Lagoon manatees. The Western Lagoon is part of the Southern Lagoon, while the Northern Lagoon is a more distinct body of water (Fig. 1). It is possible that regional differences in nitrogen input from surrounding terrestrial sources had an effect on the nitrogen signatures of lagoon vegetation.

Enriched carbon ratios in the skin of manatees sampled in Puerto Rico indicate that seagrasses are the primary component of their diet. The lack of significant regional differences in carbon ratios suggests that this reliance on seagrasses is consistent among manatees in Puerto Rico. However, we found significant regional differences in nitrogen ratios in the skin of Puerto Rican manatees. As in the Belize analysis, it is possible that differences in coastal development, pollution, and agriculture had an effect on nitrogen input in the surrounding waters. The stable isotope results for free-ranging manatees agree with the hypothesis of Lefebvre et al. (2000) that due to thermally driven seasonal migrations, Florida manatees are less specialized grazers than manatees in Puerto Rico, for which seagrasses are the main diet component available.

There were no sex-based differences in isotopic composition of Florida or Antillean manatee skin. In the manatee social system, there are no separate breeding or feeding grounds as in other systems in which we may expect to see sex-based differences in diet and thus isotopic composition of tissues. Additionally, no differences were found in stable carbon or nitrogen isotope signatures in Florida manatee skin based on the collection month. Skin samples were collected mainly during the months of November, December, and January. This sampling period coincided with the large number of manatees aggregated at warm water sources. In general, all manatee skin sampling was opportunistic, so it does not necessarily mean that a manatee sampled in November arrived at a warm water refuge earlier than a manatee sampled in January. It is also unknown how long each manatee spent in the refuge or whether it left, traveling a long distance (giving it access to alternative diet sources) and then returned again. For these reasons, it is understandable that we do not see a progression towards a greater number of manatee skin signatures representative of the vegetation available near the refuge over time.

Seasonal differences in δ^{13}C and/or δ^{15}N values were found within the skin of Belize manatees; however, results for manatees from the Drowned Cayes and Western Lagoon should be interpreted with caution due to small sample sizes. Reported differences may be due to seasonal changes in manatee feeding, habitat, and/or seasonal variability in the stable isotope ratios of aquatic plants, the latter 2 of which have been pre-viously documented in Belize manatees (Self-Sullivan et al. 2003) and Florida aquatic plants, respectively (Anderson & Fourqurean 2003, present study).

There was no difference in δ^{13}C or δ^{15}N values in manatee skin between any of the 3 age classes for Florida or Antillean manatees. Calves (<176 cm) are considered nutritionally dependent, but manatees up to body lengths of 260 cm may nurse in addition to feeding on aquatic vegetation (O'Shea et al. 1985). Most calves that were sampled were <170 cm, near the calf/subadult division. It is likely that these manatees were already feeding on aquatic vegetation and possibly only nursing occasionally. Nursing animals, consuming milk derived from the mother's tissues, are in essence feeding at a higher trophic level (e.g. Fogel et al. 1989). Even skin from the 2 smallest manatees sampled (both body lengths = 130 cm, Florida) did not have enriched δ^{15}N values (5.8 and 4.3‰) compared to those of longer body lengths. Finally, variability in nitrogen signatures found for aquatic plants in Florida (–2.9 to 10.3‰) makes it difficult to assess possible differences in trophic level between nursing and weaned manatees. The age class division between subadults and adults is a gross estimate based on sexual maturity and is mainly used to assess manatee mortality rates and population structure (O'Shea et al. 1985). It has no relation to any specific feeding distinction.

Conservation implications

Fine-scale interpretation of our diet modeling results warrants caution; however, we can make general conclusions relevant to manatee conservation. Seagrasses were a required diet component for Florida manatees from all regions sampled in the present study and likely made up more than 50% of the diet of manatees wintering at the Big Bend Power Plant in Tampa Bay, Warm Mineral Springs near Charlotte Harbor, and Port of the Islands in Ten Thousand Islands. In an effort to provide more suitable habitat for Florida manatees, efforts should focus on reducing further losses of sea-grass beds especially near the regions mentioned above. The range of individual variation in δ^{13}C and δ^{15}N values in Florida manatee skin within a given location also suggests that warm water refuges are used by manatees from different geographic regions. Consequently, there may not be one single conservation solution regarding feeding habitat that caters to all manatees wintering at any of the specific locations in this study. It is also apparent that seagrasses are a critical component in the diet of Antillean manatees in Belize and even more so for those in Puerto Rico; therefore, regional management plans should emphasize protection of seagrass beds in those areas as well.

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