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RESEARCH ARTICLE

Diet induced differences in carbon isotope fractionation between sirenians and terrestrial ungulates

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Abstract Carbon isotope differences ($\Delta^{13}C$) between bioapatite and diet, collagen and diet, and bioapatite and collagen were calculated for four species of sirenians, *Dugong dugon* (Müller), *Trichechus manatus* (Linnaeus), *Trichechus inunguis* (Natterer), and the extinct *Hydrodamalis gigas* (Zimmerman). Bone and tooth samples were taken from archived materials collected from populations during the mid eighteenth century (*H. gigas)*, between 1978 and 1984 (*T. manatus*, *T. inunguis*), and between 1997 and 1999 (*D. dugon*). Mean $\Delta^{13}C$ values were compared with those for terrestrial ungulates, carnivores, and six species of carnivorous marine mammals (cetaceans = 1; pinnipeds = 4; mustelids = 1). Significant differences in mean $\delta^{13}C$ values among species for all tissue types were detected that separated species or populations foraging on freshwater plants or attached marine macroalgae ($\delta^{13}C$ values $< -6‰$; $\Delta^{13}C_{bioapatite-diet}$ ~14‰) from those feeding on marine seagrasses ($\delta^{13}C$ values $> -4‰$; $\Delta^{13}C_{bioapatite-diet}$ ~11‰). Likewise, $\Delta^{13}C_{bioapatite-collagen}$ values for freshwater and algal-foraging species (~7‰) were greater than those for seagrass-foraging species (~5‰). Variation in $\Delta^{13}C$ values calculated between tissues and between tissues and diet among species may relate to the nutritional composition of a species' diet and the extent and type of microbial fermentation that occurs during digestion of different types of plants. These results highlight the complications that can arise when making dietary interpretations without having first determined species-specific $\Delta^{13}C_{tissue-diet}$ values.

Introduction

Sirenians (i.e., manatees and dugongs) are the only extant marine mammals that subsist on an herbivorous diet (Husar 1978). The types of aquatic vegetation consumed by sirenians are surprisingly diverse (Table 1) and include virtually all species of marine angiosperms (i.e., marine seagrasses), several species of marine algae (e.g., *Hypnea* spp., *Ulva* spp., *Gracilaria* spp.), and a variety of freshwater and riparian plant species (e.g., *Vallisneria* spp., *Eichhornia crassipes, Typha* spp.) (Husar 1978; Best 1981; Ledder 1986). Information on sirenian diets has largely been based on stomach content analysis and field observation, but there is growing interest in using the stable isotope composition of sirenian tissues as a record of feeding habits for both extant (Ames et al. 1996) and extinct species (MacFadden et al. 2004). Mean carbon isotope ($\delta^{13}C$) compositions of freshwater vegetation (−27‰), marine

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Table 1 Reported $\delta^{13}C$ values for dietary resources exploited by sirenian populations analyzed in this study

Sampling region	Primary producer	Taxa	$\delta^{13}C \pm 1$s.d. (‰)	Mean $\delta^{13}C \pm 1$ s.d. (‰)
Queensland and Torres Strait, Australia[1,5]	Seagrass	*Halodule uninervis*	−10.0 ± 2.4	−10.6 ± 2.1
		Halophila ovalis	−11.0 ± 3.2	
		Cymodocea rotundata	−9.7 ± 1.3	
		Thalassia hemprichii	−8.4 ± 0.5	
		Enhalus acoroides	−8.7 ± 1.2	
Florida, USA[2,8,9]	Algae	*Gracilaria* sp.	−19.8 ± 4.1	−18.6 ± 2.9
		Ulva lactuca	−17.8 ± 0.4	
		Hypnea spp.	−17.6 ± 2.8	
		Caulerpa spp.	−18.6 ± 1.8	
	Seagrass	*Halodule wrightii*	−10.8 ± 1.2	−11.2 ± 3.2
		Halophila decipiens	−10.1 ± 1.6	
		Syringodium filiformes	−10.9 ± 3.9	
		Thalassia testudinum	−13.6 ± 3.1	
		Ruppia maritime	−14.0 ± 0.5	
	Terrestrial C3	*Cladium jamaicense* (Sawgrass)	−25.4 ± 1.2	−26.6 ± 1.1
		Hydrocotyle sp.	−27.0 ± 0.5	
		Panicum spp. (C3 species)	−27.4 ± 2.7	
	Terrestrial C4	*Panicum spp.* (C4 species)	−11.8 ± 0.8	−12.5 ± 1.0
		Spartina spp.	−13.2 ± 1.4	
	Freshwater	*Vallisneria* sp. (submerged)	−25.2 ± 0.7	−27.4 ± 2.4
		Eichhornia crassipes	−28.2 ± 0.4	
		Pistia stratiotes	−27.7 ± 1.9	
		Chara sp.	−29.0 ± 3.6	
		Hydrilla sp.	−26.0 ± 3.6	
		Typha sp.	−26.1 ± 1.7	
Bering Island, North Pacific[3,4,6]	Algae	*Laminaria solidungula (5)*	−20.1 ± 0.4	−19.4 ± 1.1
		L. lonigrururis (5)	−20.0 ± 0.6	
		L. longipes	−18.2 ± 1.3	
		L. dentinegra	−18.3 ± 0.5	
		Laminaria sp.	−18.0 ± 1.8	
		Agarum cribosum (5)	−20.1 ± 0.3	
		Alaria fistulosa (24)	−20.8 ± 3.6	
		Alaria sp. *(5)*	−19.4 ± 0.5	
South Florida Aquarium[11]	Fruit and vegetables	Romaine Lettuce (~97%)	−27.8	−27.4
		Apple, Broccoli & Sweet Potato (<1%)	−24.6 ± 1.0	
	Dietary supplements	Monkey Chow (~3%)	−19.8	
		Elephant Chow (<<1%)	−26.5	
Mote Marine Laboratory, Sarasota, FL[11]	Fruit and vegetables	Romaine Lettuce (~85%)	−27.8	−27.9
		Kale (~11%)	−30.7	
		Carrot, apple & beet (<1%)	−26.4 ± 1.6	
	Dietary supplements	Monkey Chow (3%)	−21.3	
Instituto Nacional de Pesquisas da Amazõnia, Brazil[7,10]	Freshwater	*Cabomba* spp. (~50%)	−31.5 ± 0.5	−30.0
	Terrestrial	*Panicum* sp. (~50%)	−27.4 ± 2.7	

[1] Fry et al. (1983), [2] Fry (1984), [3] Simenstad et al. (1993), [4] Hobson et al. (1994), [5] Loneragan et al. (1997), [6] Wainright et al. (1998), [7] Medina et al. (1999), [8] Chanton and Lewis (2002), [9] Anderson and Fourqurean (2003), [10] Fellerhoff et al. (2003), [11] MacFadden et al. (2004)

algae (−18.5‰) and seagrass (−11‰) are statistically distinct and capable of labeling consumer tissues sufficiently to allow researchers to deduce their relative contribution to diet (Osmond et al. 1981; Fry 1984; Boon and Bunn 1994; Clementz and Koch 2001; Raven et al. 2002). Depending upon the type of tissue analyzed, this isotope label can reflect an individual's diet over a timescale of weeks to years, making it a valuable supplement to other methods of diet analysis, which often only record the most recent meal.

Interpretation of $\delta^{13}C$ data from sirenian material has relied upon calculated carbon isotope differences between tissues and diet (i.e., $\Delta^{13}C_{tissue-diet} = \delta^{13}C_{tissue} - \delta^{13}C_{diet}$) for terrestrial ungulates (MacFadden et al. 2004). For example, a $\Delta^{13}C_{enamel-diet}$ value of 13.8 ± 0.6‰ between tooth enamel and diet has been reported for most large, terrestrial herbivores (Cerling and Harris 1999) including proboscideans, which are the closest living relatives of sirenians (Ozawa 1997). Like proboscideans, sirenians are all non-ruminant, hindgut

fermenters with most digestion occurring in the cecum and proximal colon of the large intestine (Murray et al. 1977; Burn 1986). Sirenians differ from other hindgut fermenters, however, in their extremely high digestive efficiencies with respect to cellulose (~64 to 97%) (Burn 1986; Goto et al. 2004), as well as in having basal metabolic rates that are 15–33% of predicted values based on body size relationships for terrestrial mammals (Irvine 1983; Kleiber 1975). In light of these physiological differences, it is possible that sirenian $\Delta^{13}C$ values between bioapatite and diet or other tissues could be significantly different from those calculated for terrestrial ungulates and therefore warrant independent calculation. The objectives of this study were: (1) to calculate $\Delta^{13}C_{bioapatite-diet}$ and $\Delta^{13}C_{collagen-diet}$ values for three extant and one extinct species of sirenian (*Dugong dugon*, *Trichechus inunguis*, *Trichechus manatus*, and *Hydrodamalis gigas*, respectively); (2) to find out how different aquatic diets impact the magnitude and variation in these $\Delta^{13}C$ values; and (3) to determine whether or not $\Delta^{13}C_{bioapatite-diet}$, $\Delta^{13}C_{collagen-diet}$, and $\Delta^{13}C_{bioapatite-collagen}$ values for sirenians differ significantly from those of carnivorous marine mammal species or terrestrial ungulates.

Materials and methods

Specimen collection

We obtained *Dugong dugon* (Müller) samples from a single population in the Torres Strait between September 1997 and November 1999. Tusk samples from 13 individuals were collected (Appendix 1; 7 females and 6 males) that ranged in age from 6 to 15-years old based on counts of growth layer groups in the tusks (Kwan 2002). Bone samples were available from eight of these individuals. In addition, ten molars from separate, unrelated individuals of unknown age or sex were collected from the same population. Dietary information for this dugong population was based on stomach content data reported for 128 deceased individuals collected within the Torres Strait (Kwan 2002; André et al. 2005). The stomach contents for these animals were comprised almost entirely of seagrass, mainly rhizomes. *Thalassia hemprichii*, *Syringodium isoetifolium*, and *Cymodocea* spp. were the dominant seagrass species found in the stomach contents (80–100%). Small quantities of the seagrasses *Halophila ovalis*, *Halophila spinulosa*, *Halodule uninervis*, *Enhalus acoroides* and *Thalassodendron ciliatum* were also found. Only a small percentage of the volume (≤5%) was non-epiphytic macroalgae.

Trichechus manatus (Linnaeus) samples were collected from eight Florida counties representing several habitat types, including marine, estuarine, and freshwater ecosystems. We accessed 17 specimens that had died between the years of 1978 and 1984 (Appendix 1; 7 females, 9 males, and 1 of unknown sex) and are now housed at the Florida Museum of Natural History in Gainesville, FL, USA. Specific age information for these individuals was unavailable, but all were either adult or sub-adult based on individual sizes. Bone and tooth samples were collected from each individual. All 17 specimens had been independently selected for stomach content analysis (Table 2) so detailed information

Table 2 *Trichechus manatus*. Percent composition of stomach contents

Specimen	%SG	%MA	%FW	%C3 Plants	% C4 Plants	% AM	% UI	Diet Group
UF15110	41.8	57.2	0.0	0.0	0.0	0.0	1.0	Mixed
UF15114	83.0	15.0	0.0	0.0	0.0	0.0	2.0	SG
UF15115	32.8	2.2	6.8	11.8	23.0	0.0	23.4	SG
UF15120	13.8	0.6	80.4	0.0	0.0	0.0	5.2	SG
UF15122	0.0	0	100.0	0.0	0.0	0.0	0.0	FW
UF15141	89.2	4.8	0.0	0.0	0.0	0.0	6.0	Mixed
UF15160	0.0	0.0	0.0	100.0	0.0	0.0	0.0	FW
UF15172	100.0	0.0	0.0	0.0	0.0	0.0	0.0	SG
UF15173	50.0	0.0	50.0	0.0	0.0	0.0	0.0	Mixed
UF15176	0.0	0.0	50.0	50.0	0.0	0.0	0.0	FW
UF15195	0.0	0.0	100.0	0.0	0.0	0.0	0.0	FW
UF15196	20.0	0.0	80.0	0.0	0.0	0.0	0.0	SG
UF19134	50.0	0.0	50.0	0.0	0.0	0.0	0.0	FW
UF19135	100.0	0.0	0.0	0.0	0.0	0.0	0.0	Mixed
UF20602	20.8	1.0	0.0	0.0	2.8	35.6	39.8	Mixed
UF20773	0.0	0.0	80.0	20.0	0.0	0.0	0.0	FW
UF23993	0.0	0.0	100.0	0.0	0.0	0.0	0.0	FW

Plant type categories include seagrass (SG); attached macrophytic marine algae (MA); freshwater vegetation (FW); C3 terrestrial/marsh plants (C3 plants); C4 terrestrial/marsh plants (C4 plants); animal matter (AM); and unidentified contents (UI). Diet groups defined by combination of stomach content, observational, and isotope data as described in the text

on the last meals for these manatees had been determined at or near the time of death. Digested material was grouped into seven categories: seagrasses, marine algae, C3 plants, C4 plants, freshwater vegetation, animal, and unidentifiable. Seagrasses were found in the majority of stomachs (82.3%) and averaged the largest percentage of stomach content volume (49.3%), followed by freshwater vegetation (25.6%) and terrestrial grasses (13.0%). Individual manatees were grouped into seagrass, freshwater and mixed diet categories based on stomach content analysis, field data, and $\delta^{13}C$ values.

Samples were also obtained from three captive West Indian manatees (*Trichechus manatus*) housed at the Mote Marine Laboratory in Sarasota, Florida and the Parker Manatee Aquarium of the South Florida Museum in Bradenton, Florida and two captive *Trichechus inunguis* (Natterer) kept at the Instituto Nacional de Pesquisas da Amazónia in Manaus, Brazil. Tooth samples (i.e., molars) were recovered from tanks after being shed by the manatees (MacFadden et al. 2004). No bone material was available for analysis. The diet of captive *T. manatus* was composed largely of lettuce with some vegetable and protein supplements (Ames et al. 1996; MacFadden et al. 2004), whereas that of the captive *T. inunguis* was composed of grass (*Panicum* sp.) and freshwater plants (*Cabomba* sp.) (Domning and Hayek 1984).

Samples of the extinct marine sirenian *Hydrodamalis gigas* (Zimmerman) were collected from individuals butchered by fur traders along the coasts of Bering Island during the mid eighteenth century. Remains are now stored at the Smithsonian National Museum of Natural History (Appendix 1). We analyzed only bone from ten individuals, as the species was edentulous. The gender of all specimens is unknown. Based on the size of the bones sampled, all individuals were adults or large sub-adults. Estimated diet for these individuals was based on written observations recorded by Steller during his stay on Bering Island (Brandt 1846; Domning 1978). However, much of the plant material consumed by *H. gigas* had not yet been formally described, so Steller's descriptions of the material are subject to some interpretation. At present, the best interpretations of these plants include several species of marine brown algae (*Agarum* sp., *Nereocystis luetkeana*, *Alaria esculenta*) and red algae (*Dumontia fucicola, Constantinea rosa-marina*) (Domning 1978).

Samples from one species of cetacean (harbor porpoise, *Phocoena phocoena*), one species of mustelid (California sea otter, *Enhydra lutris*), and four species of pinniped (harbor seal, *Phoca vitulina*; northern fur seal, *Callorhinus ursinus*; California sea lion, *Zalophus californianus*; northern elephant seal, *Mirounga angustirostris*) were collected from beached specimens of populations foraging in waters off the coast of central California between 1999 and 2000. Bones and teeth were sampled from individual specimens of each species (Appendix 1). The species sampled forage in different foodwebs, including kelp ecosystems (*E. lutris*), nearshore habitats (*P. phocoena, P. vitulina, Z. californianus*) and offshore habitats (*C. ursinus, M. angustirostris*).

Isotope composition of dietary resources

Estimates of the $\delta^{13}C$ values of dietary resources of wild populations, extinct *Hydrodamalis gigas*, and captive *Trichechus inunguis* were made using data from the literature (Table 1). Carbon isotope values for plants fed to captive *Trichechus manatus* were reported by MacFadden et al. (2004). Primary producers were limited to species either identified from stomach content analysis or from historical records of observed feeding habits for these populations. Stable isotope measurements of these species were compiled from studies in the region from which sirenian samples were collected. When studies reported different values for the same plant species, the mean and standard deviation were calculated from these values. For marine carnivores, diet $\delta^{13}C$ values were estimated from collagen $\delta^{13}C$ values assuming a standard $\Delta^{13}C_{collagen-diet}$ of 5‰ (Ambrose and Norr 1993; Tieszen and Fagre 1993; Jim et al. 2004).

Stable isotope preparation and analysis

We sampled bone and teeth from a minimum of five individuals per species to provide an estimate of the mean and variance for populations (Clementz and Koch 2001). Bone samples (~200 mg) were collected from either the ribs or vertebrae for analysis of the organic (collagen) and mineral (bioapatite) phases of the bone. Among the three sirenian species with teeth, only manatee teeth had sufficient enamel for sampling, so dentin was collected from dugong tusks and molars.

Analysis of the carbonate component of bone and tooth bioapatite required removal of organic material via soaking in 0.5 ml of 2–3 wt% NaClO for 1 (enamel) to 3 days (bone/dentin). Following rinsing (5×, deionized water), samples were immersed overnight in 0.5 ml 1.0 N acetic acid buffered with calcium acetate to pH 5.3 to remove non-lattice bound carbonates, then rinsed again (5×, deionized water) and lyophilized overnight to dryness (Koch et al. 1997).

Collagen was prepared by removal of all adhering muscle or adipose tissue with a scalpel, followed by decalcification in ~5 ml of 0.5 N HCl for three to five days under refrigeration. Samples were then rinsed (5×, deionized water) and lipid extracted by immersion and sonication in ~6 ml of organic solvent (2 parts methanol: 1 part chloroform: 0.8 part water). Samples were lipid extracted three times, rinsed (5×, deionized water), and then lyophilized overnight (Tuross et al. 1988).

All stable isotope analyses were done using the Micromass Optima gas source mass spectrometer at the UCSC Stable Isotope Laboratory, which was linked to either an ISOCARB preparation system for bone/tooth carbonate or a Carlo Erba Elemental Analyzer for bone collagen. Approximately 1.5 mg of bone/tooth powder per sample was dissolved in 100% phosphoric acid bath at 90°C. After dissolution, the CO_2 produced was channeled to the Optima mass spectrometer for measurement. For collagen, ~1.5 mg of sample was combusted under a steady stream of O_2 producing CO_2 and N_2O for isotopic analysis.

All isotope values are reported in standard delta notation, where $\delta^{13}C = (({}^{13}C/{}^{12}C_{sample}/{}^{13}C/{}^{12}C_{standard}) - 1)*1000$. Carbon values are reported relative to the V-PDB standard. Precision for carbonates and collagen was assessed based on multiple runs of an in-house elephant enamel standard ($1\sigma\ \delta^{13}C = 0.1‰$, $n = 22$) and an in-house gelatin standard (PUGEL: $1\sigma\ \delta^{13}C = 0.2‰$, $n = 23$), respectively. As mentioned above, we defined $\Delta^{13}C$ as the difference in $\delta^{13}C$ values between two tissues (e.g., $\Delta^{13}C_{bone-collagen} = \delta^{13}C_{bone} - \delta^{13}C_{collagen}$) or between a specific tissue and diet (e.g., $\Delta^{13}C_{enamel-diet} = \delta^{13}C_{enamel} - \delta^{13}C_{diet}$). Furthermore, we use $\delta^{13}C_{bone}$ to refer to the $\delta^{13}C$ composition of bone bioapatite (mineral), $\delta^{13}C_{collagen}$ to refer to the $\delta^{13}C$ composition of bone collagen (organic), and $\delta^{13}C_{tooth}$ to refer to the $\delta^{13}C$ composition of tooth enamel (for most species) or dentin (for *D. dugon*).

Statistical methods

Statistical significance of differences in mean values among multiple groups of samples was assessed using a parametric, one factor analysis of variance (ANOVA) followed by a post-hoc Bonferroni test for pair wise comparisons between groups. When the parametric criteria necessary for ANOVA were not met (i.e., normality, equal variance), we employed a non-parametric test, Kruskal–Wallis ANOVA by Ranks (KWAR), followed by a post-hoc Dunn's Method for pair wise comparisons between groups. Statistical significance of differences in mean values between two groups was assessed using either a parametric Student's *t* test or non-parametric Mann–Whitney rank sum test. Evaluation of the significance of correlation between isotope data pairs was done using either a Pearson's Product Moment test (PPM) or linear regression. Statistically significant differences in variance between species were determined using an *F* test. All statistical analyses were conducted using the program SigmaStat v. 2.03 or Microsoft Excel 2000.

Results

$\delta^{13}C$ differences between tissues and diet

Specimens from two seagrass consumers, *Dugong dugon* and marine populations of *Trichechus manatus*, were analyzed (Table 2). Dugong diets were assumed to consist mostly of seagrass (André et al. 2005). Marine-foraging individuals of *T. manatus* were defined by the presence of identifiable seagrass digesta and little to no freshwater vegetation within stomach contents (Table 2). Primary seagrass foragers were then identified from these specimens via high tissue $\delta^{13}C$ values that fell within the range reported for *D. dugon*. Remaining individuals were grouped as mixed-marine consumers. Seagrass species from Florida ($-11.2 \pm 3.2‰$) were not statistically different from those reported from the Torres Strait ($-10.6 \pm 2.1‰$) (Table 1) and match reported global mean values for seagrass ($-10‰$ to $-11‰$; Hemminga and Mateo 1996). The mean diet $\delta^{13}C$ value for general marine consumers was estimated from the relative contribution of seagrass and other dietary items within the stomach contents of these specimens ($-14.6 \pm 3.2‰$; Table 2).

Carbon isotope values for *Dugong dugon* were extremely high for all tissue types, and consistently higher than those for all other marine mammals including seagrass-foraging *Trichechus manatus* (Appendix 1). $\Delta^{13}C_{tooth-diet}$ for *D. dugon* ($11.1 \pm 1.1‰$), seagrass *T. manatus* ($10.9 \pm 0.6‰$), and mixed-marine *T. manatus* ($10.7 \pm 1.3‰$) were not statistically distinct (One-way ANOVA, $P = 0.77$). Calculated $\Delta^{13}C_{bone-diet}$ values were significantly lower for *D. dugon* ($9.3 \pm 0.8‰$) and seagrass-foraging ($8.1 \pm 1.2‰$) and marine-foraging ($8.1 \pm 3.3‰$) *T. manatus*, but still higher than $\Delta^{13}C_{collagen-diet}$ (*D. dugon* = $4.1 \pm 0.9‰$; seagrass *T. manatus* = $1.8 \pm 1.4‰$; mixed-marine *T. manatus* = $1.8 \pm 3.7‰$).

Six enamel samples were analyzed from captive individuals of *Trichechus manatus* with known diet $\delta^{13}C$ values (MacFadden et al. 2004). The mean $\delta^{13}C$ value for five molars from Mote Marine Laboratory individuals

was $-13.7 \pm 1.0‰$, which produced a $\Delta^{13}C_{tooth-diet}$ value of $14.2 \pm 1.0‰$ (Appendix 1). A single tooth from the South Florida Museum yielded a $\delta^{13}C$ value of $-14.5‰$ and a $\Delta^{13}C_{tooth-diet}$ value of 12.9‰. Five molars from two individuals of *T. inunguis* had a mean $\delta^{13}C$ value of $-16.5 \pm 3.1‰$ and a calculated $\Delta^{13}C_{tooth-diet}$ value of $13.5 \pm 3.1‰$ (Appendix 1).

Six specimens of *Trichechus manatus* collected from the wild were identified as freshwater foragers based on the lack of seagrass or the high proportion of freshwater vegetation and/or terrestrial grasses in their stomach contents (Table 2). Using average $\delta^{13}C$ values for representative plant species from Florida localities (Table 1) and information on diet composition based on stomach contents, the mean diet $\delta^{13}C$ value for these individuals was estimated as $-25.6 \pm 1.6‰$. Enamel $\delta^{13}C$ values averaged $-11.9 \pm 1.2‰$ and the $\Delta^{13}C_{tooth-diet}$ was $13.9 \pm 1.3‰$. Bone ($-12.5 \pm 2.0‰$) and collagen ($-19.3 \pm 1.9‰$) had lower mean $\delta^{13}C$ and $\Delta^{13}C$ values ($\Delta^{13}C_{bone-diet} = 13.2 \pm 2.2‰$ and $\Delta^{13}C_{collagen-diet} = 5.9 \pm 1.8‰$).

Only one species of sirenian, *Hydrodamalis gigas*, specialized on a diet of marine algae. The mean $\delta^{13}C$ value of kelp species available as food items for *H. gigas* was calculated to be $-19.4 \pm 1.1‰$. Lack of teeth made it impossible to calculate a $\Delta^{13}C_{tooth-diet}$ value, but the difference between bone bioapatite ($-7.3 \pm 0.8‰$) and diet was $12.1 \pm 0.8‰$ and between bone collagen ($-15.2 \pm 1.0‰$) and diet was calculated at $4.1 \pm 0.9‰$ (Appendix 1).

In contrast to sirenians, estimated diet $\delta^{13}C$ values for marine carnivores varied little, ranging from a low of $-19.5‰$ for *Callorhinus ursinus* to $-16.7‰$ for *Enhydra lutris* (Appendix 1). Calculated $\Delta^{13}C_{tissue-diet}$ values for marine carnivores were typically lower than those reported for all sirenian species. $\Delta^{13}C_{tooth-diet}$ values typically clustered between 7‰ and 10‰; only *C. ursinus* (6.6‰) and *Mirounga angustirostris* (5.4‰) had values that fell outside of this range (Appendix 1). $\Delta^{13}C_{bone-diet}$ values were likewise lower than those for sirenians, but the range was not as extreme as that for enamel. Values for all species fell between 7.0 and 10.1‰ with *Phocoena phocoena* (7.0‰) and *M. angustirostris* (7.3‰) possessing the lowest values.

Calculated $\Delta^{13}C_{tooth-diet}$ values were statistically distinct among sirenians, marine carnivores, and terrestrial ungulates (One way ANOVA, $F = 68.27$, $P < 0.01$) and a post-hoc analysis found significant differences between all pairings (Bonferroni test, $P < 0.01$). $\Delta^{13}C_{tooth-diet}$ values were highest for terrestrial ungulates at $13.8 \pm 0.6‰$ (Cerling and Harris 1999), followed by that of sirenians ($12.3 \pm 1.3‰$), and marine carnivores ($7.8 \pm 1.6‰$) (Appendix 1; Fig. 1). Statistically

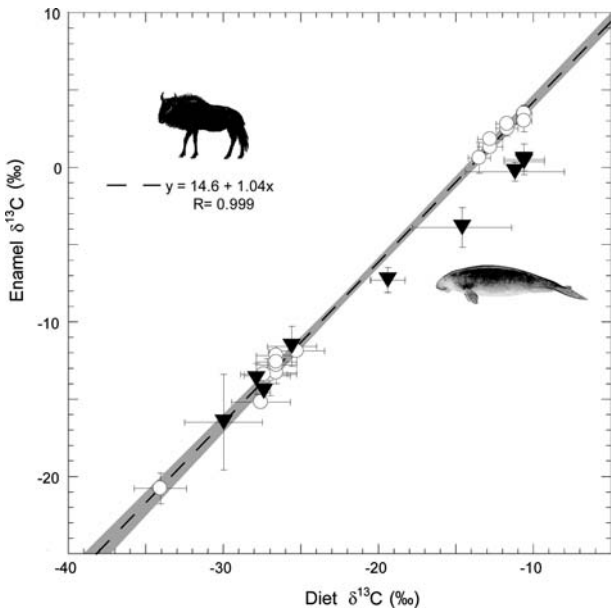

Fig. 1 Plot of estimated mean diet $\delta^{13}C$ values versus mean enamel or dentin $\delta^{13}C$ values for all sirenians (*black inverted triangles*) and terrestrial ungulates (*white circles*). Error bars represent ±1 SD. Linear regression for terrestrial ungulate (*dashed line*) data is plotted and *R* value is reported. *Gray shaded* region around linear regression line denotes estimated error around relationship (95% CI). Note strong overlap between sirenian and terrestrial ungulate values at low diet $\delta^{13}C$ values, which is lost as diet $\delta^{13}C$ values increase

significant differences in variance of $\Delta^{13}C_{tooth-diet}$ values were also detected among the groups. Calculated $\Delta^{13}C_{tooth-diet}$ values among different species and/or populations of terrestrial ungulates were less variable than those for species/populations of sirenians and marine carnivores (*F* test, $P < 0.01$). Among sirenian populations, $\Delta^{13}C_{tooth-diet}$ values for freshwater foraging and captive manatees (12.5–14.2‰) were highest and similar to those of terrestrial ungulates, whereas those of dugongs and seagrass foraging manatees were significantly lower (10.8–11.1‰) (Fig. 1).

Tissue-to-tissue differences in $\delta^{13}C$ values

Bone bioapatite and collagen $\delta^{13}C$ values were not available for terrestrial ungulates, so only sirenians and carnivorous marine mammals were compared. However, mean $\Delta^{13}C_{bone-collagen}$ values have been calculated for terrestrial herbivores ($6.8 \pm 1.4‰$), omnivores ($5.2 \pm 0.8‰$), and carnivores ($4.3 \pm 1.0‰$) (Lee-Thorp et al. 1989) and were used for comparison (Fig. 2). Mean $\Delta^{13}C_{bone-collagen}$ values were significantly higher for sirenians ($6.6 \pm 1.0‰$) than those for marine carnivores ($3.9 \pm 1.3‰$) (Student's *t* test, $t = 4.27$, $P < 0.01$) (Appendix 1). Mean $\Delta^{13}C_{bone-collagen}$ values for

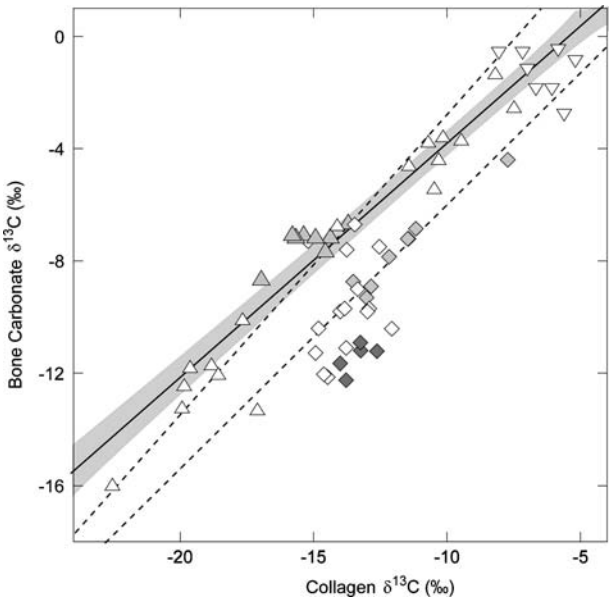

Fig. 2 Plot of individual collagen and bone bioapatite $\delta^{13}C$ values for sirenians (*white triangles*: *D. dugon* = *inverted*; *T. manatus* = *upright*; *gray triangles* = *H. gigas*), cetaceans (*dark gray diamonds*), sea otters (*light gray diamonds*), and pinnipeds (*white diamonds*). *Solid black line* is linear regression calculated for all sirenian values ($y = 0.833x + 4.39$, $R^2 = 0.929$, $P < 0.001$), *gray-shaded field* represents error around equation. *Long-dashed line* represents the equation for bone bioapatite–collagen spacings reported for terrestrial herbivores ($\delta^{13}C_{bioapatite} = 7.8 + 1.06 \times \delta^{13}C_{collagen}$) and *short-dotted line* that for carnivores ($\delta^{13}C_{bioapatite} = 3.4 + 0.94 \times \delta^{13}C_{collagen}$) (Lee-Thorp et al. 1989)

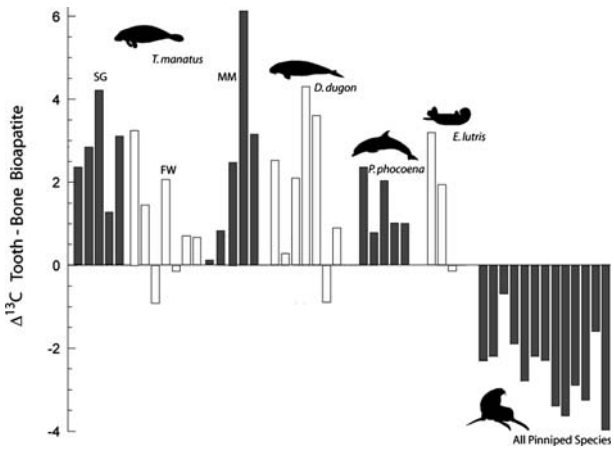

Fig. 3 Bar graph showing $\Delta^{13}C_{tooth-bone}$ values for three populations of *T. manatus* (seagrass *SG*; mixed marine *MM*; freshwater *FW*), *D. dugon*, *P. phocoena*, *E. lutris*, and four species of pinniped (*P. vitulina, M. angustirostris, Z. californianus*, and *C. ursinus*)

sirenians as a group and carnivorous marine mammals closely matched those reported for terrestrial herbivores and carnivores, respectively.

Within sirenians and marine carnivores, significant differences in $\Delta^{13}C_{bone-collagen}$ values were detected. Among sirenians, $\Delta^{13}C_{bone-collagen}$ values were statistically distinct (One Way ANOVA, $F = 6.34$, $P < 0.001$) and ranged from 5.2 ± 1.5‰ for *Dugong dugon* to 7.8 ± 0.7‰ for *Hydrodamalis gigas* (Appendix 1; Fig. 2). When compared to marine carnivores, however, only the $\Delta^{13}C_{bone-collagen}$ value of *H. gigas* was significantly different from those of the sea otter and most pinniped species. Mean $\Delta^{13}C_{bone-collagen}$ values for *Mirounga angustirostris* and *Phocoena phocoena* were the lowest for all groups and significantly different when compared to sirenian species and, at least for *P. phocoena*, other marine carnivores (Appendix 1).

Tooth and bone bioapatite $\delta^{13}C$ values were available for wild populations of two sirenian species (*Dugong dugon* and *Trichechus manatus*) and all species of marine carnivores (Fig. 3). Mean $\Delta^{13}C_{tooth-bone}$ values were positive for sirenians, harbor porpoises, and sea otters, ranging from 1.0‰ for freshwater manatees to 2.8‰ for seagrass-foraging manatees. Tooth bioapatite was consistently ^{13}C-enriched relative to bone. In contrast, calculated $\Delta^{13}C_{tooth-bone}$ values for pinnipeds were negative, ranging from −3.2‰ for northern fur seals to −0.8‰ for harbor seals, and showed that tooth materials were consistently ^{13}C-depleted relative to bones (Fig. 3).

Discussion

Analysis of sirenian bioapatite and collagen demonstrates that the biological fractionation of carbon isotopes in these species is different from that for terrestrial ungulates and carnivorous marine mammals. The difference between sirenians and marine carnivores is not surprising, but the lack of agreement with values for terrestrial ungulates is perplexing. Cerling and Harris (1999) found little variation in the $\Delta^{13}C_{enamel-diet}$ values (range: 12.5–14.6‰) for different clades of large herbivores (e.g., proboscideans, artiodactyls, and perissodactyls) despite variations in diet (browser vs. grazer) and digestive physiology (foregut ruminants vs. hindgut fermentation). In contrast, values for sirenian species are considerably more variable (range: 10.9–14.6‰) with the lowest values approaching those reported for marine and terrestrial carnivores. The low $\Delta^{13}C_{tooth-diet}$ values are significant and may relate to dietary, physiological, and/or metabolic differences between sirenians and terrestrial ungulates. However, uncertainties associated with the calculation of mean diet $\delta^{13}C$ values for wild populations could also account for some of this variation and must be addressed before interpreting these differences.

Estimates of diet $\delta^{13}C$ values for wild populations were based on reported values for vegetation sampled in each study region (Table 1). These vegetation samples were not collected at the same time or from exactly the same location as the sirenians, so temporal and/or spatial variation in vegetation isotope values could complicate estimates of diet $\delta^{13}C$ values. At present, we have no way to assess this error rigorously, but point out that even after averaging vegetation $\delta^{13}C$ values from multiple sources to maximize the potential spatial and temporal range in values (Table 1), calculated variances for sirenian diets are not significantly higher than those reported for terrestrial ungulates (Cerling and Harris 1999). In addition, we note that most populations exhibit low variance in tissue $\delta^{13}C$ values, which would be unlikely if strong regional or temporal $\delta^{13}C$ gradients were present or if subtle differences in plant type mixture were an important factor.

Along with environmental variation, the potential ingestion of epiphytes and epizoans (epibionts) can also complicate calculation of diet $\delta^{13}C$ values. A variety of organisms (e.g., algae, bryozoans, crustaceans, arthropods) attaches to or lives within the blades of freshwater and marine vegetation (Hall and Eiseman 1981; Virnstein and Carbonara 1985; Jensen and Gibson 1986). Epibiont $\delta^{13}C$ values can differ significantly from that of the plants on which they grow and, depending upon how much carbon they contribute to the total diet pool, can impact estimates of diet $\delta^{13}C$ values (Fry 1984; Bunn and Boon 1993). Dugongs and manatees have been observed to avoid consuming vegetation heavily covered with epibionts (Thayer et al. 1984), so the incidental contribution of epibionts to sirenian diets is probably minor. This interpretation is supported by analysis of sirenian stomach and mouth contents from Florida and northern Australia (Marsh et al. 1982; Ledder 1986; André et al. 2005), which typically contain only minor quantities of epibionts. Thus the uncertainty associated with epibiont consumption on estimated diet $\delta^{13}C$ values is most likely insignificant. Until controlled feeding experiments can be performed on captive sirenian species using natural diets, our calculated $\delta^{13}C$ values for the diets of wild sirenian populations will have to suffice.

Aside from uncertainties associated with diet $\delta^{13}C$ values, differences in $\Delta^{13}C_{tooth-diet}$ values between sirenians and terrestrial ungulates may be explained by differences in the nutritional composition of terrestrial and aquatic vegetation. Total fiber content varies significantly among plant types, ranging from high content in terrestrial vegetation and marine macroalgae to lower levels in freshwater vegetation and seagrass (Appendix 2). High fiber diets have been correlated with increased production of methane (Jensen and Jorgensen 1994), which is extremely ^{13}C-depleted, as well as biogenic CO_2 that is strongly ^{13}C-enriched (Metges et al. 1990). The carbon source for bone and tooth bioapatite is blood bicarbonate (HCO_3^-), which is sourced by CO_2 produced via oxidation of whole diet (i.e., carbohydrates, fats, and lipids) in animal cells, as well as CO_2 generated by microbial activity in the gut. Several studies have suggested diffusion of this ^{13}C-enriched CO_2 from the gut to the blood stream explains why the $\delta^{13}C$ value of herbivore bioapatite shows a larger ^{13}C-enrichment relative to bulk diet than carnivore bioapatite (Hedges 2003; Passey et al. 2005). Consumption of low-fiber diets (i.e., seagrass) would reduce microbial methanogenesis and decrease the amount of ^{13}C-depleted methane expelled from the body. This could affect $\Delta^{13}C_{tooth-diet}$ values in seagrass consumers, producing values less than the 13.8‰ observed for terrestrial ungulates.

In addition to fiber content, differences in the ^{13}C content of the structural compounds within plants can also impact $\Delta^{13}C_{bioapatite-diet}$ values. Lignin is a primary constituent of plant cell walls that is consistently more ^{13}C-depleted than other plant compounds (Benner et al. 1987; Loader et al. 2003; Wedin et al. 1995). Lignin is difficult to digest and loss of it without assimilation can leave the assimilated dietary carbon pool ^{13}C-enriched, resulting in high $\Delta^{13}C_{tooth-diet}$ values. Compared to terrestrial grasses and freshwater plants, seagrasses contain very little lignin (Appendix 2). Combined with methanogenesis, variation in the lignin content of dietary plants is the most plausible explanation for the large range in $\Delta^{13}C_{tooth-diet}$ values observed for sirenians.

Support for these explanations also comes from study of the digestive system in living sirenians. Gas samples from the cecum and large intestine of dugongs yield significant quantities of CO_2, but little to no H_2 or CH_4, suggesting that either methanogenic bacteria are not part of the microflora of the dugong hindgut or that ingested fiber is insufficient to promote extensive methanogenesis (Murray et al. 1977; Marsh et al. 1978). Sampling of the bacteria in the dugong digestive system has found that methanogenic bacteria are present at low quantities (Goto et al. 2004), and when sufficient dietary substrates are present, they can produce significant quantities of methane (22% of total gas volume). Thus, the lack of methane in the dugong digestive tract is the result of low fiber consumption and reduced methanogenesis.

Calculated $\Delta^{13}C_{tooth-diet}$ values for most marine carnivores were similar to values reported for terrestrial carnivores and captive species raised on low fiber diets

(~9.5‰: Ambrose and Norr 1993; Tieszen and Fagre 1993). For the pinnipeds *Callorhinus ursinus, Mirounga angustirostris*, and *Zalophus californianus*, however, calculated values were 2–4‰ lower than those for other marine carnivores (Appendix 1). As discussed above for sirenians, two factors that might account for these differences are miscalculation of dietary $\delta^{13}C$ values and differences in diet composition or digestive physiology. Several controlled feeding experiments have shown that changes in the concentration and isotope composition of macronutrients within an animal's diet can significantly alter $\Delta^{13}C_{\text{collagen–diet}}$ values (Ambrose and Norr 1993; Tieszen and Fagre 1993; Hedges 2003; Jim et al. 2004). In particular, consumption of diets rich in ^{13}C-depleted lipids can reduce $\Delta^{13}C_{\text{collagen–diet}}$ values by as much as 2–3‰. If true for the marine species we analyzed, our calculation of diet $\delta^{13}C$ values using this 5‰ offset was incorrect. Use of a lower $\Delta^{13}C_{\text{collagen–diet}}$ value (+2 to 3‰) would bring calculated $\Delta^{13}C_{\text{bioapatite–diet}}$ values much closer to the average reported value of +9.5‰. Further support for this explanation is found in the $\Delta^{13}C_{\text{bone–collagen}}$ values, which is discussed in detail below.

Krueger and Sullivan (1984) and Lee-Thorp et al. (1989) were the first to report consistent differences in $\Delta^{13}C_{\text{bone–collagen}}$ values among terrestrial carnivores (4.3 ± 1.0‰), omnivores (5.2 ± 0.8‰), and herbivores (6.8 ± 1.4‰). The source of these differences is still debated, but is thought to stem from differences in the assimilation and routing of dietary components (i.e., carbohydrates, fats, and lipids) into the organic (i.e., collagen) and inorganic (i.e., bioapatite) portions of bone, as well as the impact of methanogenesis on herbivore bioapatite $\delta^{13}C$ values (Hedges 2003). As mentioned above, the carbon source for bone bioapatite is blood bicarbonate (HCO_3^-). In collagen, proteins are preferentially routed from the diet into the organic bone structure and typically less than half of the carbon originates from the synthesis of new amino acids via intermediates of the citric acid cycle (Tieszen and Fagre 1993; Ambrose and Norr 1993; Jim et al. 2004).

The differences in $\Delta^{13}C_{\text{bone–collagen}}$ values observed in terrestrial consumers are expected to be the same for carnivorous and herbivorous marine mammals. Prior to this study, however, very little research had been conducted to verify this assumption. Lee-Thorp et al. (1989) examined bone bioapatite and collagen from Cape fur seals (*Arctocephalus pusillus*) and found extremely low $\Delta^{13}C_{\text{bone–collagen}}$ values (mean offset = 1.3 ± 0.8‰). They also noted that these differences increased with age, correlating with a switch from milk-based (high lipid) diets for pups and juveniles to the fish and squid diets of adults.

The present study has expanded the dataset for marine mammals by including three species of marine herbivores and six species of marine carnivores. As expected, marine herbivore $\Delta^{13}C_{\text{bone–collagen}}$ values were typically higher than those for carnivores. However, the difference between marine herbivore and marine carnivore $\Delta^{13}C_{\text{bone–collagen}}$ values decreased as marine herbivore diets shifted from freshwater vegetation and marine algae to seagrass (Fig. 2). This change in $\Delta^{13}C_{\text{bone–collagen}}$ values is most likely the result of decreased methanogenesis and reduced lignin content in diets (i.e., seagrass). As with tooth bioapatite, bone bioapatite $\delta^{13}C$ values correlate with the $\delta^{13}C$ composition of whole diet and blood bicarbonate (HCO_3^-) (Tieszen and Fagre 1993; Ambrose and Norr 1993). Observed $\Delta^{13}C_{\text{bone–diet}}$ values for sirenians also are 3–4‰ lower in seagrass consumers than in freshwater/marine algal foragers, which can account for the ~3‰ change in $\Delta^{13}C_{\text{bone–collagen}}$ values.

Whereas $\Delta^{13}C_{\text{bone–collagen}}$ values for most marine carnivores were similar to those for terrestrial carnivores, values for the pinniped *Mirounga angustirostris* and the cetacean *Phocoena phocoena* were consistently much lower, approaching the values reported by Lee-Thorp et al. (1989) for *Arctocephalus pusillus* pups and juveniles. Even though the diet of *M. angustirostris* typically consists of a greater proportion of squid relative to fish than that of *P. phocoena*, each species may be actively selecting lipid-rich prey as a high-energy food source. As mentioned above, consumption of foods high in ^{13}C-depleted lipids could reduce $\Delta^{13}C_{\text{bioapatite–diet}}$ values, which in turn would reduce $\Delta^{13}C_{\text{bone–collagen}}$ values (Jim et al. 2004). Controlled feeding experiments on captive marine mammals are needed to verify this interpretation, but our initial results support combined stable isotope analysis of bone bioapatite and collagen to gain additional insight into marine mammal diets.

An unexpected outcome of this project was the discovery that $\Delta^{13}C_{\text{tooth–bone}}$ values for sirenians, harbor porpoises, and sea otters were consistently positive, whereas those for pinnipeds were negative (Fig. 3). Few studies have examined bone and tooth bioapatite $\delta^{13}C$ values from the same individual, but prior work has reported differences between $\Delta^{13}C_{\text{bone–diet}}$ (+12 to 13‰) and $\Delta^{13}C_{\text{enamel–diet}}$ (+13.8‰) values from terrestrial ungulates (Sullivan and Krueger 1981; Lee-Thorp and van der Merwe 1987; Cerling and Harris 1999). Our results show a similar difference in these values for herbivorous marine mammals (Appendix 1). We suggest three factors that, when combined, may partially account for the differences we have reported: (1) timing and duration of bioapatite mineralization; (2)

seasonal changes in bulk diet $\delta^{13}C$ values; and (3) seasonal changes in the contribution of various dietary components (i.e., proteins, fats, carbohydrates) that differ in their relative $\delta^{13}C$ values.

Throughout an animal's life, both the inorganic and organic components of bone are constantly being remodeled during growth. Turnover rates for bone from terrestrial mammals have been estimated at ~4 years for bone bioapatite and ~7 years for bone collagen (Jim 2000). In contrast, the enamel cap of mammal teeth forms prior to eruption and, once deposited, is no longer remodeled by the body. Teeth contain material that formed over a relatively short time, whereas bone is constantly being reworked and reflects a running average of material ingested over a lifetime. As a consequence, differences in the $\delta^{13}C$ values of teeth and bone would be heavily influenced by ontogenetic and seasonal changes in diet. Foraging on dietary resources that differed in bulk $\delta^{13}C$ values (e.g., freshwater and marine vegetation) or macronutrient composition (e.g., protein vs. lipid) at different times of the year would create significant offsets between tooth and bone isotope records.

Our results partially support this explanation. For instance, the five manatees with mixed marine or seagrass diets had much greater $\Delta^{13}C_{tooth-bone}$ values than those for manatees foraging more or less exclusively in freshwater habitats (Fig. 3). Higher variation in bulk diet $\delta^{13}C$ values can thus accentuate the disparity in $\delta^{13}C$ values between these tissues. This, however, does not account for the consistently positive $\Delta^{13}C_{tooth-bone}$ values for most marine mammals or the consistently negative $\Delta^{13}C_{tooth-bone}$ values in pinnipeds. The only way to produce this would be if a species' teeth formed at the same time each year and if the timing of tooth formation was associated with a consistent shift to an isotopically distinct diet. For cetaceans and pinnipeds, tooth formation occurs largely in utero at approximately the same time each year. If females of these species change their diets towards the end of gestation while the teeth of the fetus are developing, then tooth $\delta^{13}C$ values could be consistently higher or lower than bone $\delta^{13}C$ values depending upon the $\delta^{13}C$ value of the new diet. For manatees, dugongs, and sea otters, on the other hand, development of the permanent dentition occurs mostly outside of the womb and is not known to occur at a specific season or time. At present, we cannot account for the consistently positive $\Delta^{13}C_{tooth-bone}$ values in these species.

Our results have expanded the ecological application of enamel and bone $\delta^{13}C$ values from terrestrial to marine ecosystems by quantifying $\Delta^{13}C_{tooth-diet}$, $\Delta^{13}C_{bone-diet}$, and $\Delta^{13}C_{collagen-diet}$ for marine mammals, particularly marine herbivores (i.e., sirenians). In contrast to terrestrial ungulates, sirenians show greater variation in $\Delta^{13}C_{tooth-diet}$ values, ranging from 10.8‰ for seagrass consuming species (*Trichechus manatus*, *Dugong dugon*) up to 14.2‰ for freshwater species and populations (*T. manatus*, *Trichechus inunguis*). Variation in the fiber content of sirenian diets and the associated decrease in methanogenesis during digestion may account for these differences. As in terrestrial ecosystems, marine carnivores and most aquatic herbivores were found to differ in $\Delta^{13}C_{bone-collagen}$ values. Again, diet composition (i.e., fiber content, lipid content) is thought to play a significant role in generating these values. These findings justify analysis of bone bioapatite and collagen material in tandem to provide additional information about the composition of marine mammal diets. Furthermore, application of these methods to the archaeological and fossil records could expand our understanding of how the diets and foraging preferences of mammals in marine and aquatic ecosystems have shifted over time.

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