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ARTICLE

Methods, Tools, and Technologies

Effects of tissue decomposition on stable isotope ratios and implications for use of stranded animals in research

Carl S. Cloyed[1,2] | **Che'Isha Johnson**[3] | **Kayla P. DaCosta**[1,2] | **Lauren R. Clance**[1,2] | **Mackenzie L. Russell**[1] | **Cristina Díaz Clark**[1] | **Elizabeth E. Hieb**[1] | **Ruth H. Carmichael**[1,2]

[1]Dauphin Island Sea Lab, Dauphin Island, Alabama, USA

[2]Department of Marine Science, University of South Alabama, Mobile, Alabama, USA

[3]Department of Biology, Talladega College, Talladega, Alabama, USA

Correspondence
Carl S. Cloyed
Email: ccloyed@disl.org

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Abstract

Stable isotope analysis (SIA) provides ecological data that can be safely and efficiently collected on endangered, threatened, and cryptic species. Marine mammals are an ecologically important group for which economical and logistical constraints can make data collection challenging. Stranded marine mammals are often used in research, but the causes of strandings and subsequent tissue decomposition could affect SIA. We conducted a three-part study to test the validity of using $\delta^{13}C$ and $\delta^{15}N$ values from tissues of stranded bottlenose dolphins (*Tursiops truncatus*) and West Indian manatees (*Trichechus manatus*) for ecological studies. First, we quantified isotopic overlap using ellipses based on 95% of the data to compare isotope values in skin between stranded and live-captured animals. Second, we compared stable isotope values from liver, skin, and muscle of animals that had stranded and were sampled at different decomposition stages. Third, we experimentally exposed each tissue to environmental conditions and sampled tissues as they decomposed. For both dolphins and manatees, isotopic ellipses from skin of stranded carcasses were similar to live-captured individuals. Among individuals recovered at different decomposition stages, more advanced decomposition affected $\delta^{13}C$ values in dolphin liver and skin but not in manatee tissues and had no effect on $\delta^{15}N$ values in any tissue for either species. In the experimental manipulation, decomposition resulted in depleted $\delta^{13}C$ values, enriched $\delta^{15}N$ values, and increased C:N in liver for both species. Skin and muscle from stranded dolphins and manatees are representative of their corresponding live populations and can be used for SIA with appropriate caution. To facilitate the use of tissues from stranded animals, tissues should be dried or frozen for storage as soon as possible after sampling. We recommend liver from stranded animals only be used for SIA when researchers need tissues with short turnover times and can access fresh samples. Without consideration of decomposition effects on isotope values, ecologists may make inaccurate inferences about

habitat use, diet, and community structure. Careful use of SIA on tissues from stranded animals can help researchers provide better quality information for managers and policy makers.

KEYWORDS
accumulated degree-days, bottlenose dolphins, carbon, decomposition code, marine mammal stranding network, nitrogen, standard ellipse area, West Indian manatees

INTRODUCTION

Stable isotopes from organismal tissues are an essential tool for providing insights into ecological processes and critical data that are necessary for species conservation and management plans (Boecklen et al., 2011; Newsome et al., 2007; Post, 2002). Ratios of heavy-to-light stable carbon isotopes (^{13}C:^{12}C expressed as $\delta^{13}C$) vary predictably among different photosynthetic pathways and habitat types (Boecklen et al., 2011; Newsome et al., 2007). Ratios of stable nitrogen isotopes (^{15}N:^{14}N expressed as $\delta^{15}N$) increase from one trophic level to the next, relative to basal sources, and are often used to measure trophic position (Perkins et al., 2014; Post, 2002). The predictable variation in $\delta^{13}C$ and $\delta^{15}N$ values allows ecologists to quantify elements of organismal niches and community dynamics (Jackson et al., 2011; Layman et al., 2007; Newsome et al., 2007). Stable isotopes, therefore, provide data on dietary information that can also be applied to define habitat use and organismal movements such as migration and dispersal. Stable isotope analysis (SIA) can be particularly useful for species that are rare, cryptic, or otherwise difficult to sample because it is easier for researchers to obtain ecological information from SIA compared with traditional methods, such as direct feeding observation or stomach content analysis (Newsome et al., 2010; Shipley et al., 2017).

SIA may be particularly useful for marine mammals, an ecologically diverse and key group of species, because they are often difficult and costly to study (Bowen, 1997; Kiszka et al., 2015; Newsome et al., 2010). Most marine mammal species are top predators that significantly contribute to the structuring of ecological communities and frequently indicate ecosystem health (Bowen, 1997; Kiszka et al., 2015). Ecological research on marine mammals can, therefore, reveal the underlying state of ecosystems and is important for understanding and managing these ecosystems (Moore, 2008). Marine mammals, however, typically spend much of their time underwater in areas where they are unobservable and inaccessible to researchers, and many species are rare, threatened, or endangered (Avila et al., 2018), making biological sampling difficult or impossible. Most countries also have regulations that greatly restrict access to marine mammals for research (McHugh, 2013). As a result, marine mammal researchers frequently use data collected from dead-stranded animals as an alternative to invasive sampling of live animals (Hanson et al., 2018; Horstmann-Dehn et al., 2012; Yamamuro et al., 2004).

Although valuable insights into marine mammal ecology and subsequent management decisions have been derived from studies using stable isotopes in stranded animals, the effects of stranding and subsequent decomposition on stable isotope values in tissues are not well understood and may lead to accidental, erroneous ecological inferences (Perkins et al., 2013; Tarroux et al., 2010). Researchers have used SIA on a wide range of stranded marine mammals, including cetaceans, pinnipeds, and sirenians (Cloyed, Balmer, et al., 2021; Hanson et al., 2018; Horstmann-Dehn et al., 2012; Yamamuro et al., 2004). Stranded individuals, however, may be sick, have altered behavior, or been affected by the environment in ways that may alter their isotope values so they do not reflect the values of the source population. Tissue decomposition that occurs in the time between an animal's death and sample collection can also potentially alter stable isotope values (Burrows et al., 2014; Keenan & DeBruyn, 2019; Payo-Payo et al., 2013; Perkins et al., 2018; Yurkowski et al., 2017). During tissue decomposition, microbial processes create a variety of volatile compounds like carboxylic acids, benzene, methenamine, and carbon disulfide (Forbes & Perrault, 2014; Paczkowski & Schütz, 2011). Isotopic fractionation that occurs as these compounds are generated likely leads to changes in isotopic values of the remaining tissues. Studies that sample tissues as they decompose have produced mixed effects. For example, $\delta^{13}C$ values have increased, decreased, or been unaffected by decomposition (Burrows et al., 2014; Keenan & DeBruyn, 2019; Payo-Payo et al., 2013; Perkins et al., 2018; Yurkowski et al., 2017). The reported effects of decomposition are clearer and more consistent on $\delta^{15}N$ values, and most studies found $\delta^{15}N$ values increased with decomposition or were unaffected by it (Burrows et al., 2014; Keenan & DeBruyn, 2019; Payo-Payo et al., 2013; Perkins et al., 2018; Yurkowski et al., 2017). In addition to being inconclusive, these experiments did not directly compare live-captured and dead-stranded marine mammals nor connect their results

to field observations such as carcass condition on recovery. It is, therefore, difficult to know the extent to which these experimental findings may be relevant to SIA on stranded animals from wild populations.

We determined the validity of using SIA on stranded marine mammals and defined the effects of decomposition on $\delta^{13}C$ and $\delta^{15}N$ values in two marine mammal species, bottlenose dolphins (*Tursiops truncatus*) and West Indian manatees (*Trichechus manatus*), hereafter referred to as dolphins and manatees, respectively. First, to determine if data from dead-stranded (dead) animals were similar to live-captured (live) animals, we compared isotopic values in skin between dead and live dolphins and manatees, from sites along the northern Gulf of Mexico (nGOM). Second, to determine if stable isotope values in samples differed relative to decomposition stage at the time of carcass recovery, we compared isotopic values in liver, skin, and muscle of individuals that stranded in and around Mobile Bay (MOB), Alabama and varied in decomposition stages from fresh dead to advanced decomposition at the time of sampling. Third, to determine the effects of decomposition on isotopes in dolphin and manatee tissues under a common set of natural environmental conditions, we sampled liver, skin, and muscle at regular intervals while they were exposed to environmental conditions and underwent decomposition. We refer to these approaches as the stranded versus live study, the decomposition stage study, and the decomposition experiment, respectively.

MATERIALS AND METHODS

Study region

Dolphins are a common cetacean species in nearshore waters and the most commonly stranded marine mammal species in the nGOM, comprising 89.4% of all marine mammal strandings at the Alabama Marine Mammal Stranding Network (ALMMSN; Russell et al., 2022). Manatees are a migratory, seasonally resident species in the nGOM where sightings and strandings have increased in recent years (Hieb et al., 2017). Dolphins stranded or were live captured along the nGOM, USA, including the coasts near Barataria Bay (BAR), Louisiana and MOB, Alabama. Manatees stranded or were live captured along the coasts of Mississippi, Alabama, and northwestern Florida. This subtropical region typically experiences winter water temperatures of ~5° to ~15°C and summer water temperatures of ~24° to ~32°C. Relative humidity on the nGOM coast is on average >60% throughout the year. Shipping and boating are ubiquitous across the region; the Intracoastal Waterway runs along the nGOM coast, and recreational and commercial boating are common in both BAR and MOB. Many of the embayments in the nGOM are freshwater dominated, and both BAR and MOB can experience prolonged freshwater incursions.

Sample collection

Samples from stranded animals

We collected samples of skin from dolphins ($n = 14$) that stranded in BAR during 2011, 2013, and 2014 and skin ($n = 53$), muscle ($n = 39$), and liver ($n = 38$) from dolphins that stranded in MOB during 2011–2015 and 2017–2020. We collected skin ($n = 19$), muscle ($n = 31$), and liver ($n = 31$) from manatees that stranded along the nGOM coast during 2008–2011 and 2013–2021. Samples were obtained from the Louisiana Department of Wildlife and Fisheries (LDWF) for dolphins that stranded near BAR and from the ALMMSN at the Dauphin Island Sea Lab for dolphins stranded in MOB and manatees stranded along the nGOM coast. All samples were stored at −20°C.

We categorized dolphin tissues into decomposition stages based on the standard condition codes assigned as part of the National Oceanic and Atmospheric Administration (NOAA) National Marine Fisheries Service documentation protocol for stranded cetaceans: 1, live; 2, fresh dead with little to no decomposition (fresh); early 3 (E3), little to moderate decomposition (early); late 3 (L3), moderate to late decomposition (late); and 4, advanced decomposition (advanced) (Geraci & Lounsbury, 2005). For BAR dolphins, skin samples were available for fresh, early, and late ($n = 14$) codes, but due to uneven representation among codes, comparisons were limited to stranded (all codes combined) versus live animals only. For dolphins that stranded in MOB, samples included skin: fresh ($n = 10$), early ($n = 17$), late ($n = 16$), and advanced ($n = 10$); muscle: fresh ($n = 9$), early ($n = 12$), late ($n = 14$), and advanced ($n = 4$); liver: fresh ($n = 9$), early ($n = 14$), late ($n = 12$), and advanced ($n = 3$). Manatee decomposition stages, which are documented differently than for dolphins, were designated as fresh, early, late, and advanced decomposition based on condition assignments in necropsy reports prepared by the ALMMSN and modified from previous methods (Bonde et al., 1983). For stranded manatees, we collected skin: fresh ($n = 3$), early ($n = 9$), late ($n = 15$), and advanced ($n = 1$); muscle: fresh ($n = 3$), early ($n = 12$), late ($n = 14$), and advanced ($n = 2$); liver: fresh ($n = 5$), early ($n = 11$), late ($n = 12$), and advanced ($n = 2$).

Samples from live animals

Skin samples from live dolphins were collected using a combination of biopsies during health assessments and dart biopsies from research vessels as described in detail

elsewhere (Barratclough et al., 2019; Gorgone et al., 2008; Sinclair et al., 2015). Samples were collected in BAR in 2011, 2013–2014, and 2017–2018 ($n = 59$) and in MOB during 2018–2020 ($n = 130$). Samples were immediately stored in liquid nitrogen in the field before being transferred to a −80°C freezer, except during 2018 when samples were transferred to a −20°C freezer. Skin samples from live manatees were collected during health assessments and rescues between 2009 and 2017 ($n = 14$). For health assessments, single manatees were located with an aerial observer, captured in a net deployed from a specialized capture boat, and released at the capture location, as described in detail elsewhere (Bonde et al., 2012; Cloyed et al., 2019). Samples were stored at −20°C.

Decomposition experiment

To determine the direct effects of decomposition on $\delta^{13}C$ and $\delta^{15}N$ values, we performed an experiment in which liver, skin, and muscle tissues were regularly sampled while exposed to known ambient environmental conditions. We obtained tissue samples from one stranded dolphin and one stranded manatee, selected based on decomposition stage at the time of necropsy and availability of all target tissues from a single individual. The sampled dolphin (Field ID = 11DISL022619) stranded in 2019, was 248 cm straight length, and had undergone little decomposition (fresh). The sampled manatee (Field ID = MSN012321.02), stranded in 2021, was 277 cm straight length and had undergone little decomposition (fresh). We placed portions of liver, skin, and muscle from each animal in separate polyester, 17 × 17 cm mesh bags, with a mesh size of approximately 3 mm. The mesh bags were suspended inside 36 × 33 × 11 cm rigid plastic-coated wire mesh cages, and the cages were placed inside 92 × 71 cm fine nylon mesh bags with <1 mm mesh. The bagged cages were hung on a clothesline approximately 1.5 m above ground level (Appendix S1: Figure S1). The suspended rigid cage prevented scavenging (e.g., raccoons, foxes, rats), and the mesh bags ensured that insects could not consume or lay eggs in the tissues. This design allowed us to focus on microbial-driven decomposition and its effects on stable isotope values. We left the tissues outside, exposed to ambient environmental conditions, for 11 days and sampled approximately 25 g of each tissue on days 0 (just before the samples were placed in cages), 1, 3, 6, and 10. We chose this progression of sample days because, in our experience, the majority of marine mammals that are recovered in our shallow nearshore areas are estimated to have been found within about two weeks of death, with the greatest changes to carcass condition occurring early in decomposition (R.H. Carmichael and M.L. Russell, personal observation, 2018–2022). In addition, controlled feeding experiments have found that most isotopic change occurs soon after the isotopic source changes (Cloyed et al., 2015; Martínez del Rio et al., 2009). Hence, we choose to mimic this setup that has been used in other decomposition studies (e.g., Payo-Payo et al., 2013), with greater concentration of sampling days early in the study to most likely detect changes of isotopic values during decomposition and maximize the applicability to inferences made using data from tissues of stranded animals. Large changes early in decomposition will have the largest effect on inferences made using data from tissues of stranded animals. This experiment was conducted in early June 2021, when high/low air temperatures averaged 31/22°C, air relative humidity ranged from 63% to 86%, and it rained on 3 of the 11 days that tissues were exposed. Tissues sampled from each day were subsequently split into three subsamples, resulting in three pseudoreplicates per day for each tissue from both the dolphin and manatee ($n = 45$ per species).

Stable isotope analysis

All samples from dead and live dolphins in BAR and MOB during 2018 were lipid extracted using a modified Folch technique because lipid-rich tissues are often depleted in $\delta^{13}C$ (Cloyed et al., 2020). For live dolphins from MOB in 2019 and 2020, we used a mathematical lipid correction specifically determined for dolphins in our study area (Cloyed et al., 2020). Manatee samples were not lipid extracted or corrected because lipid extraction had no effect on the $\delta^{13}C$ values in manatee tissues (Cloyed et al., 2020). All samples were dried in an oven at 60°C for 48–56 h, homogenized with a mortar and pestle, weighed to approximately 1 mg, and packed into 3 × 5 mm tin capsules.

Stable isotope analyses were performed at the University of California, Davis Stable Isotope Facility (https://stableisotopefacility.ucdavis.edu). Isotopic values were expressed using delta notation (δ) in parts per thousand (‰), where $\delta X = (R_{sample}/R_{standard} - 1) \times 1000$, with R_{sample} and $R_{standard}$ representing the molar ratios of C^{13}/C^{12} and N^{15}/N^{14} of the sample and standard reference material, respectively. The reference material was Vienna-Pee Dee belemnite for carbon and atmospheric N_2 for nitrogen. Repeated analysis of in-house reference material (chitin, amaranth, caffeine, alanine, glutamic acid, keratin, and nylon powder) showed precision (SD) for $\delta^{13}C$ and $\delta^{15}N$ were 0.03‰ and 0.04‰.

Statistical analyses

To determine if there were differences in $\delta^{13}C$ and $\delta^{15}N$ values between skin samples from stranded and live animals, we used stable isotope Bayesian ellipses in R (SIBER) (Jackson et al., 2011). These ellipses represent variation in two-dimensional space, and because we set our ellipses to include 95% of the data, they are analogous to 95% credible intervals (Jackson et al., 2011). To determine if we could group isotopic data from different years, we first fit ellipses to data by year for dolphins and determined if ellipses among years differed in size and proportional overlap. If ellipses were similar among years, we grouped them for the stranded versus live analysis. We did not have enough manatee samples per year to run a SIBER analysis by year, therefore we grouped all stranded and live manatees. For dolphins in BAR, we only considered stranded versus live and did not differentiate among decomposition stages for stranded animals because we could not independently corroborate the consistency of assigned codes. For dolphins in MOB and for manatees, we compared ellipses from stranded animals with ellipses from live animals, separated by decomposition stage.

We used generalized linear models to test if decomposition stage affected $\delta^{13}C$ and $\delta^{15}N$ values and to determine if $\delta^{13}C$ and $\delta^{15}N$ values changed through the course of the decomposition experiment. For the decomposition stage study, $\delta^{13}C$ and $\delta^{15}N$ values were response variables and decomposition stage was the explanatory variable. For the decomposition experiment, $\delta^{13}C$, $\delta^{15}N$, and C:N values were response variables, and sample day and tissue type were explanatory variables. To make our analysis comparable across a wider range of decomposition studies, we also calculated accumulated degree-days (ADDs) (Megyesi et al., 2005) and ran the model with ADDs as the dependent variable and sample day and tissue type as explanatory variables. ADD is the sum of the averaged daily minimum and maximum temperatures above some threshold temperature value. We set our threshold value at 0°C because microbial activity is stopped below this temperature (Keenan & DeBruyn, 2019). Data were tested for homogeneity of variance using Levene's test and all models were validated graphically. All analyses were performed in R (R Core Team, 2013).

RESULTS

Stranded versus live animals

There was considerable overlap among years of the ellipses of skin samples from stranded and live dolphins in BAR and MOB (Appendix S1: Figures S2 and S3, Tables S1–S5). The lowest overlap (0.17) and thus greatest difference among ellipses for all stranded and live animals were between dolphins stranded during 2014 and 2011 in MOB (Appendix S1: Table S5), but most proportional overlaps were >0.5 (Appendix S1: Tables S2–S5). We therefore combined data from all years to compare ellipses between stranded and live animals of each species.

When comparing skin samples from stranded and live animals for all years combined, the sizes of ellipses were often different (Appendix S1: Tables S6–S10), but the ellipses always overlapped and had similar ranges (Figure 1A,B, Table 1; Appendix S1: Tables S11–S13).

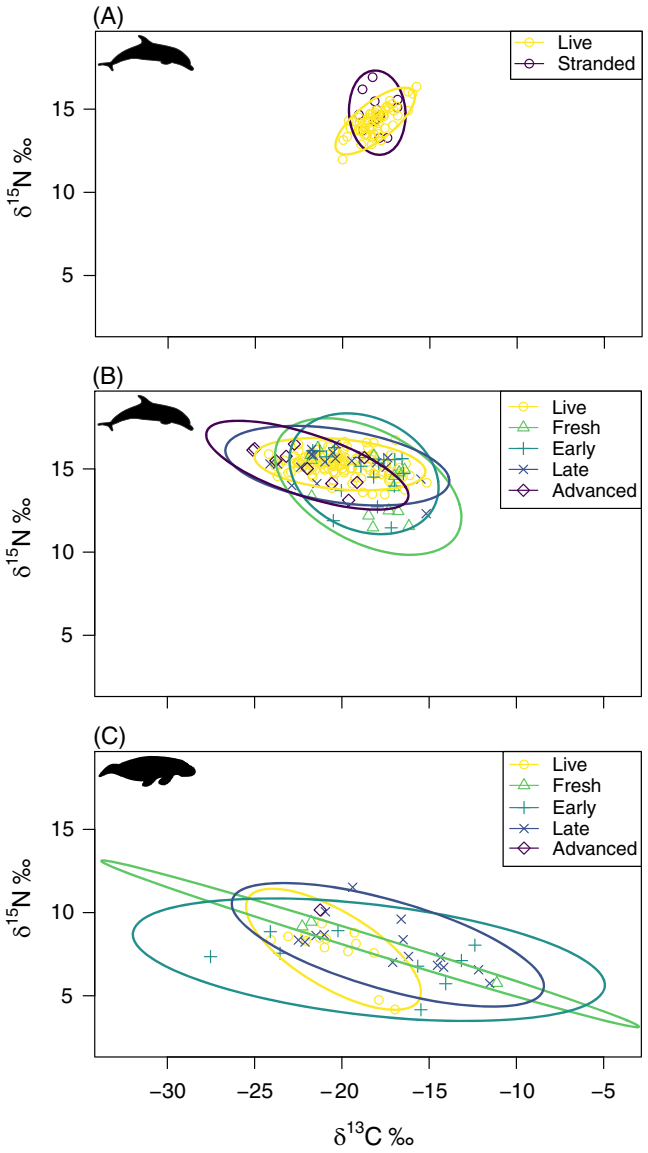

FIGURE 1 The 95% ellipse areas corrected for small sample sizes for live and stranded bottlenose dolphins in Barataria Bay (A), for live and stranded dolphins categorized by decomposition stage in Mobile Bay (B), and for live and stranded West Indian manatees along the northern Gulf of Mexico (LA to FL panhandle), categorized by decomposition stage (C).

TABLE 1 Bayesian ellipse areas (95% CIs) corrected for small sample size of skin samples from live and stranded dolphins and manatees in Barataria (BAR) and Mobile (MOB) Bays.

Species	Location	Status (code)	*N*	Ellipse area (95% CIs)	~δ^{13}C range (‰)	~δ^{15}N range (‰)
Dolphin						
	BAR					
		Live (1)	59	5.072 (2.962–8.803)	−21 to −16	12–16
		Stranded (2–4)	14	4.030 (1.261–2.145)	−20 to −17	12–17
	MOB					
		Live (1)	130	9.486 (7.960–11.236)	−25 to −16	14–17
		Fresh (2)	10	24.630 (13.639–42.851)	−24 to −13	10–18
		Early (E3)	17	18.914 (11.071–30.512)	−23 to −15	11–19
		Late (L3)	16	17.749 (10.471–28.506)	−27 to −14	13–18
		Advanced (4)	10	13.512 (7.835–29.527)	−28 to −17	13–18
Manatee						
	MOB					
		Live	14	7.011 (3.750–12.137)	−25 to −16	4–12
		Fresh	3	23.352 (3.648–115.175)	−34 to −3	3–13
		Early	9	22.690 (10.106–47.029)	−32 to −5	4–11
		Late	15	12.690 (7.524–22.543)	−26 to −8	5–12
		Advanced	1	NA	NA	NA

Abbreviation: NA, not applicable.

For dolphins from BAR, the size and ranges of the ellipses for stranded and live dolphins were similar ($p < 0.82$; Figure 1A, Table 1), and the ellipses overlapped >0.50 (proportional overlaps = 0.66 and 0.54 for the stranded ellipse on the live ellipse and for the live ellipse on the stranded ellipse, respectively). For dolphins from MOB, the ellipses from stranded dolphins at different decomposition stages were all similar in size and had similar ranges (Figure 1B, Table 1; Appendix S1: Table S10) and were larger than the ellipse for live dolphins. Despite these differences in ellipse sizes, most proportional overlaps were >0.40 between stranded dolphins at different decomposition stages and live dolphins (Figure 1B; Appendix S1: Table S11). Like dolphins, the ellipse of each decomposition stage from stranded manatees was larger than the ellipse for live manatees, and most ellipses had proportional overlaps >0.50 (Figure 1C; Appendix S1: Tables S12 and S13).

Decomposition stages

Decomposition stage at the time of carcass recovery had limited effect on δ^{13}C and δ^{15}N values in dolphin and manatee tissues (Figure 2, Table 2). For dolphins, we found that δ^{13}C values in liver and skin became depleted with greater decomposition (Figure 2A,C, Table 2), with late and advanced decomposition (codes = L3 and 4) samples depleted 3‰–4‰ compared with fresh and early decomposition (codes = 2 and E3) samples (Figure 2A,C, Table 2). Dolphin muscle was unaffected by decomposition stage (Figure 2E). For manatees, late decomposition liver samples were 5‰–7‰ more enriched compared with fresh and early decomposition samples (Figure 2B), and the pairwise comparison between fresh and late decomposition was statistically different ($t = 2.158$, $p = 0.040$), but the model as a whole was not significant (Table 2). δ^{13}C values of skin and muscle from stranded manatees were unaffected by decomposition stage (Figure 2D,F, Table 2). Decomposition stage had no effect on δ^{15}N values for either dolphins or manatees (Figure 3, Table 2).

Decomposition experiment

Decomposition by experimental manipulation primarily affected stable isotope values in liver of dolphins and manatees (Figure 4). The model δ^{13}C ~ sample day was significant for both dolphins and manatees (Figure 4A,B; dolphins: $F_{5,41} = 543.5$, $p < 0.001$, $R^2 = 0.98$; manatees: $F_{5,41} = 387.2$, $p < 0.001$, $R^2 = 0.98$), and δ^{13}C values in liver became ~0.5‰ depleted for both dolphins and manatees (Table 3, Figure 4A,B). δ^{13}C values in skin and

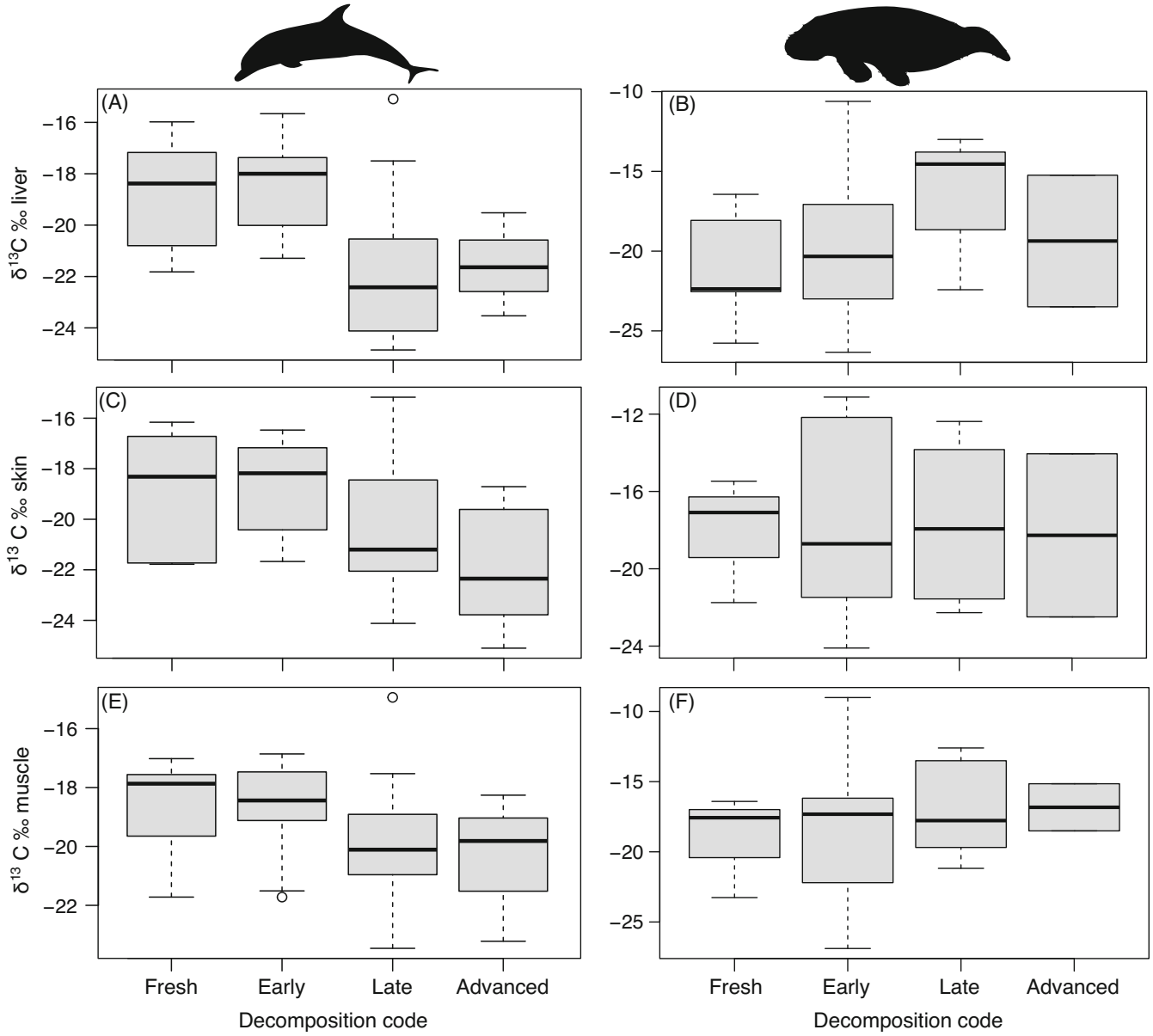

FIGURE 2 The $\delta^{13}C$ values in liver (A, B), skin (C, D), and muscle (E, F) from bottlenose dolphins and West Indian manatees at different stages of decomposition at the time of carcass recovery. For dolphins, liver and skin from late and advanced decomposition samples became significantly depleted in $\delta^{13}C$. For manatees, liver from fresh samples was significantly depleted compared with late decomposition.

muscle were unaffected by decomposition (Figure 4A,B, Table 3). The model $\delta^{15}N$ ~ sample day was significant for both dolphins and manatees (Figure 4C,D; dolphins: $F_{5,41} = 216.7$, $p < 0.001$, $R^2 = 0.96$; manatees: $F_{5,41} = 108.1$, $p < 0.001$, $R^2 = 0.92$), and $\delta^{15}N$ values in liver samples became more enriched with decomposition for manatees and marginally more enriched for dolphins (Figure 4C,D, Table 3). $\delta^{15}N$ values in skin and muscle were unaffected by decomposition (Figure 4C,D, Table 3). The model C:N ~ sample day was significant (Figure 4E,F; dolphins: $F_{5,41} = 1001.00$, $p < 0.001$, $R^2 = 0.99$; manatees: $F_{5,41} = 59.17$, $p < 0.001$, $R^2 = 0.86$). C:N in dolphin and manatee liver samples increased with decomposition (Figure 4E,F, Table 3), but skin and muscle were unaffected (Figure 4E, Table 3). Since the response data were the same, the model using ADD had the same statistical values as sampling day but the parameter estimates, particularly slope, differed (Appendix S1: Table S14).

DISCUSSION

The effects of decomposition on stable isotope ratios in stranded dolphins and manatees were tissue and element

TABLE 2 General linear model statistics comparing the effect of different decomposition stages on stable isotope values in liver, skin, and muscle from stranded dolphins and manatees.

Species	Isotope	Tissue	*F*	df	*p*	R^2
Dolphin						
	$\delta^{13}C$					
		Liver	**5.72**	**3, 34**	**0.003**	**0.28**
		Skin	**4.90**	**3, 49**	**0.005**	**0.18**
		Muscle	1.55	3, 35	0.218	0.04
	$\delta^{15}N$					
		Liver	1.62	3, 34	0.204	0.05
		Skin	0.93	3, 49	0.432	0.00
		Muscle	1.39	3, 35	0.261	0.03
Manatee						
	$\delta^{13}C$					
		Liver	1.968	3, 26	0.144	0.09
		Skin	0.014	3, 15	0.998	0.00
		Muscle	0.403	3, 27	0.752	0.02
	$\delta^{15}N$					
		Liver	0.151	3, 26	0.928	0.00
		Skin	0.827	3, 15	0.499	0.00
		Muscle	1.032	3, 27	0.394	0.00

Note: Rows in boldface indicate statistically significant differences among decomposition stages. Statistics correspond to Figures 2 and 3.

specific and overall limited in scope. First, variation in isotopic space through time and among decomposition stages was similar between stranded and live dolphins and manatees, and there was no difference in stable isotope values between stranded and live animals. These data indicate that the ranges of stable isotope values in stranded animals were reflective of their living counterparts. The ranges of ellipses were quite large, particularly for dolphins from MOB and manatees, and any potential effects of decomposition were quite small compared with this natural variation. Although ellipses from live animals were smaller, this is likely because live individuals were captured within relatively small spatial areas while the origins of stranded individuals are often unknown and probably represent a larger spatial scale. Second, while we found some differences in stable isotope values with decomposition stage at the time of carcass recovery and experimentally, these differences were primarily in liver and to a lesser extent in skin at late and advanced stages of decomposition. The sample size for advanced decomposition in manatees was low; therefore, it is possible that additional sampling will better distinguish isotopic change from decomposition. These findings indicate that not all tissues were equally affected by decomposition and effects were greater with increasing level of decomposition. It is likely that decomposition had a greater or more rapid effect on isotope ratios in liver because liver is highly metabolic and decomposes more quickly than skin and muscle (Vass, 2001). Hence, liver may be functionally less fresh at any given decomposition code than other tissues. Similarly, these effects were more pronounced for $\delta^{13}C$ than for $\delta^{15}N$, particularly in liver, a highly fatty and carbon-rich tissue (Waterlow, 2006), suggesting that $\delta^{13}C$ values are more sensitive to decomposition than $\delta^{15}N$, and $\delta^{13}C$ values need to be interpreted more carefully. The effects of decomposition on isotopes may be slower in skin and muscle because these tissues decompose at a slower rate. This difference may explain why we do not see an effect on stable isotope ratios in skin during the decomposition experiment but do see an effect among decomposition stages if late and advanced stages have decomposed to a greater extent than the decomposition experiment samples as of day 10. Overall, these findings indicate that SIA on stranded animals can be used in ecological research to understand living populations if used strategically, with consideration of tissue-specific levels of decomposition.

While marine mammals can strand for many reasons, our results suggest that in most cases the causes of strandings are less likely than decomposition to affect stable isotope values in tissues and subsequent ecological inferences. Marine mammals often strand because of disease (dolphins and manatees), human interactions such as fisheries entanglements and vessel collisions (dolphins and manatees), prolonged freshwater exposure (dolphins), and cold stress (manatees) (Aragones et al., 2017; Duignan et al., 1995; Greig et al., 2005; Rogan et al., 2001). Disease may alter body condition in ways that sometimes affect isotope values (Ben-David et al., 1999), but these factors typically affect $\delta^{15}N$ and not $\delta^{13}C$ values (Hobson et al., 1993; Hobson & Clark, 1992), opposite of the pattern we documented. Other common causes of mortality such as human interactions and vessel collisions are acute, traumatic events that are unlikely to alter isotope values (Dunshea et al., 2013). Prolonged exposure to freshwater should result in depleted $\delta^{13}C$ values in animals that strand due to freshwater exposure (Cloyed, Wilson, et al. 2021; Fry, 2002), but these values should not differ from live animals occupying these same freshwater-influenced habitats. Comparison of isotopic values between animals that strand due to freshwater exposure and their live counterparts should consider the spatial and temporal scale of freshwater influence alongside the metabolic turnover rates of specific tissues. Among animals in this study, most manatees that stranded in the winter died from cold stress, a cause of stranding that is most likely to alter $\delta^{15}N$ values because these manatees are typically malnourished and in a

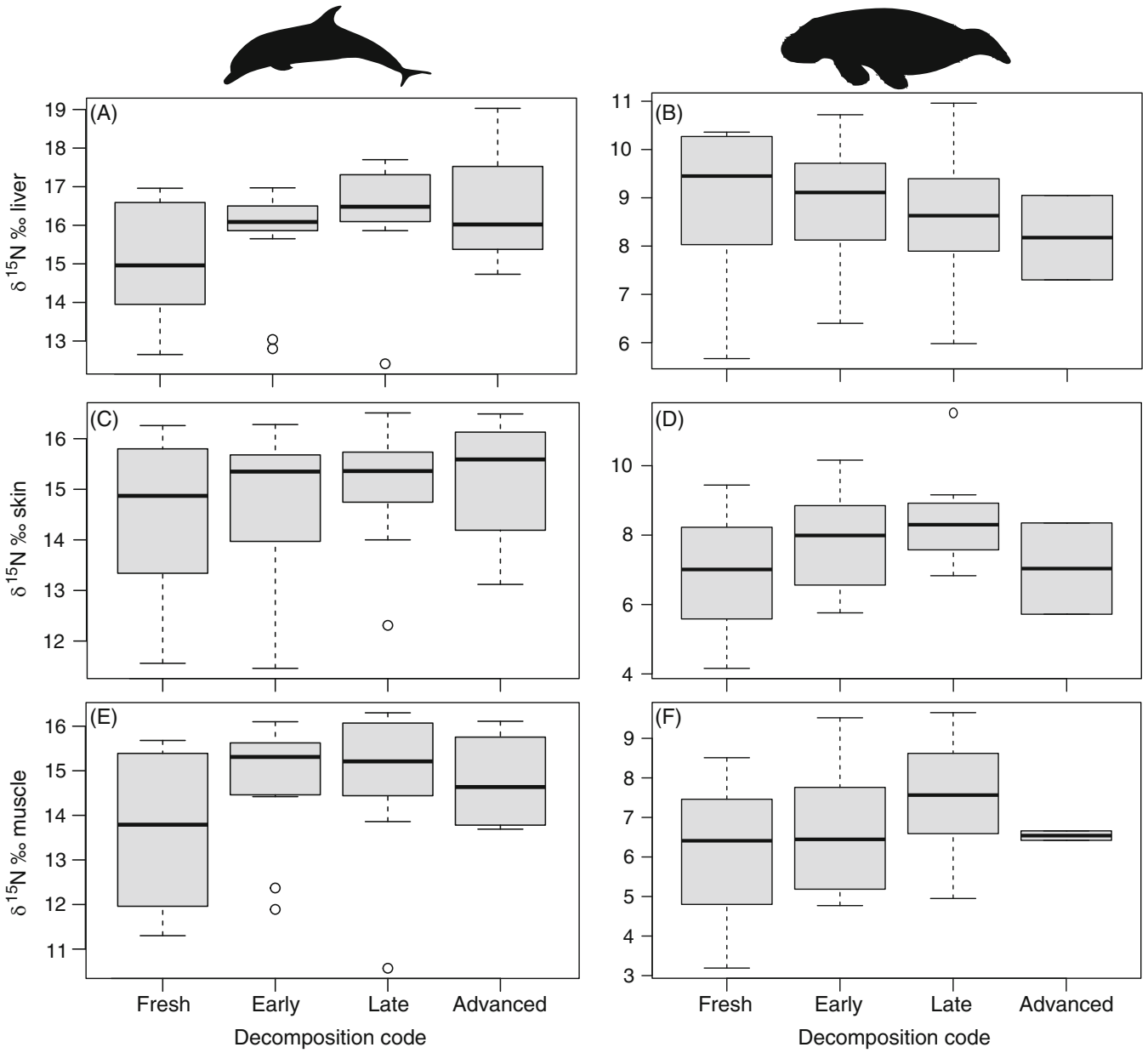

FIGURE 3 The $\delta^{15}N$ values among different stages of decomposition of liver (A, B), skin (C, D), and muscle (E, F) from bottlenose dolphins and West Indian manatees. There were no statistical differences in $\delta^{15}N$ values among decomposition stages.

catabolic state, which should result in enriched $\delta^{15}N$ values (Doi et al., 2017; Hobson et al., 1993). However, even stranded manatees did not have enriched $\delta^{15}N$ values compared with live manatees in this study. Although we did not directly test if certain causes of strandings can affect isotope values, our comparisons between stranded animals with known causes of death and live animals suggest that, in general, many common causes of strandings did not affect isotope values beyond effects that arise through decomposition.

Changes in $\delta^{13}C$ values in samples with late or advanced decomposition, especially in tissues like liver, kidneys, spleen, and pancreas that decompose quickly (Vass, 2001), can have serious implications in ecological research and associated conservation and management (Perkins et al., 2013; Tarroux et al., 2010). Reported effects of decomposition on $\delta^{13}C$ values vary considerably (Table 4) (Burrows et al., 2014; Keenan & DeBruyn, 2019; Payo-Payo et al., 2013; Perkins et al., 2018; Yurkowski et al., 2017). In other studies that performed decomposition experiments, the magnitude of effect is <1‰ (and in some cases not statistically different from 0) and therefore not ecologically meaningful, but the direction of the effects on $\delta^{13}C$ values varied considerably within and among studies with no obvious pattern (Table 4) (Burrows et al., 2014; Payo-Payo et al., 2013; Yurkowski et al., 2017). This seemingly random variation may emerge from patterns of fractionation between microbial communities and

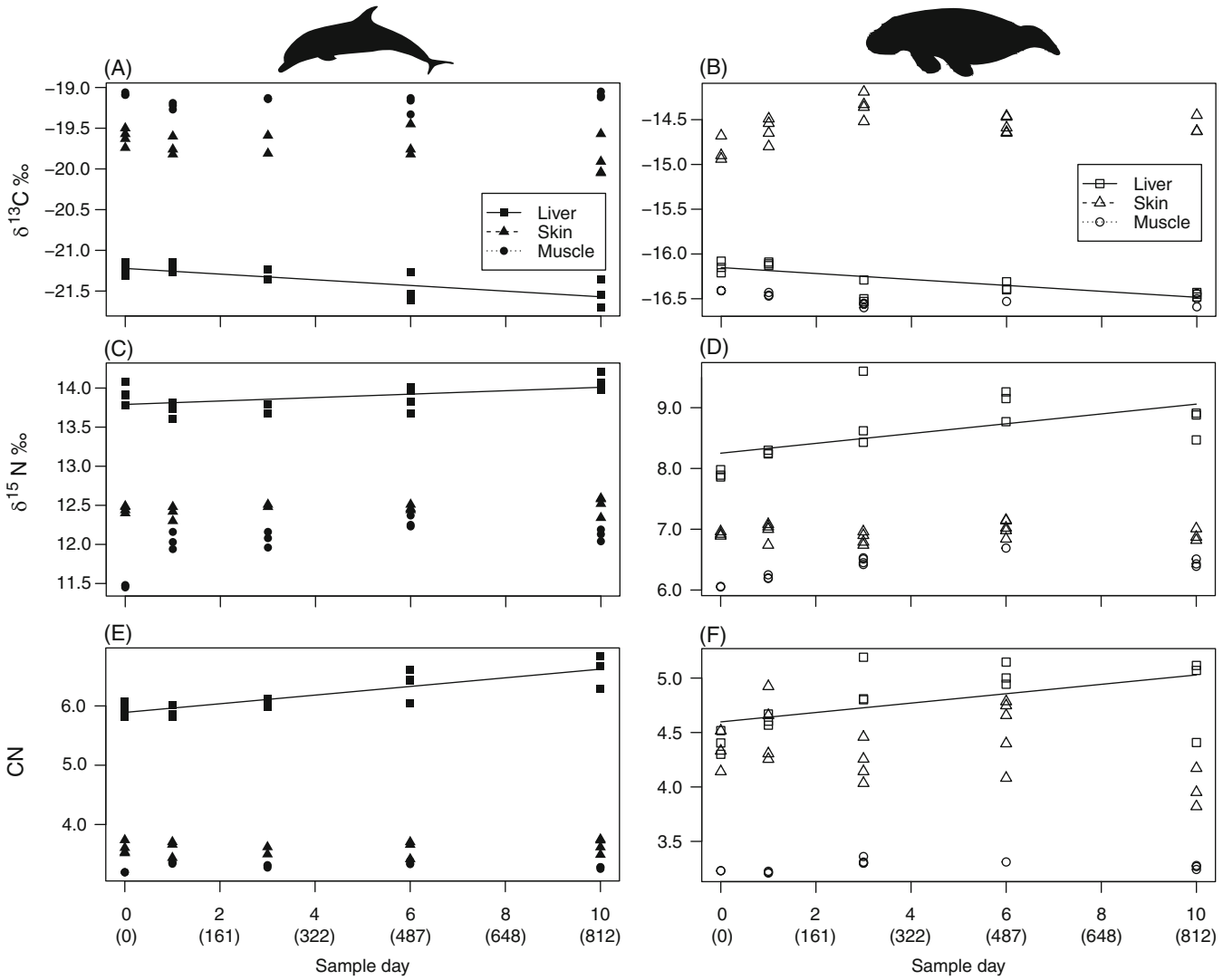

FIGURE 4 The $\delta^{13}C$ and $\delta^{15}N$ values in liver (A, B), skin (C, D), and muscle (E, F) from bottlenose dolphins and West Indian manatees through time during decomposition, with accumulated degree days in parentheses on the *x*-axis.

their host substrates. Aside from studies focused on particular chemical pathways that drive biogeochemical cycles, direct tests on isotopic fractionation between microbial communities and their substrates are rare. These studies, however, suggest carbon fractionation can vary immensely, from −40‰ to 70‰ (Londry & Des Marais, 2003; Miller et al., 2001), with the microbial composition on decomposing tissues driving the direction of the change in $\delta^{13}C$ values. Given the complexity and diversity of microbes present on decomposing tissues (Metcalf et al., 2016; Vass, 2001), it is likely that the microbes have a mixture of positive and negative fractionation values that ultimately determine the net change in isotopic values of decomposing tissues. Therefore, the direction of change in $\delta^{13}C$ values could be, in part, driven by the colonization of microbial species. The composition of microbial communities on decomposing bodies has followed predictable successional patterns and often originates from local substrates (e.g., Metcalf et al., 2016; Singh et al., 2018; Vass, 2001). The variability in the microbial community through the process of decomposition, therefore, may result in varying isotopic fractionation and an inconsistent pattern of effects of decomposition on $\delta^{13}C$ values. For dolphin liver and skin, samples at late (L3) and advanced (4) decomposition stages had isotopic changes that were sufficiently large (4‰–7‰) to confound identification of prey groups and habitat use in ecological studies (Fry, 2002; Newsome et al., 2007). If stable isotope data are used without consideration to these effects of decomposition when they occur, there is high potential for inaccurate ecological inferences about diet, habitat use, and community structure. The consequences of these inferences could be particularly important for threatened and endangered species if conservation and management decisions are based upon those inferences.

TABLE 3 Generalized linear model results comparing stable isotope and CN values through time during experimental decomposition of liver, skin, and muscle from dolphins and manatees.

Species	Isotope	Tissue	Intercept	Intercept 95% CIs	Slope	Slope 95% CIs
Dolphin						
	$\delta^{13}C$					
		Liver	**−21.222**	**−21.314 to −21.131**	**−0.035**	**−0.052 to −0.017**
		Skin	−19.632	−19.852 to −19.411	−0.023	−0.063 to 0.018
		Muscle	−19.159	−19.383 to −18.935	0.003	−0.039 to 0.044
	$\delta^{15}N$					
		Liver	**13.790**	**13.666–13.915**	**0.022**	**−0.001 to 0.045**
		Skin	12.435	12.135–12.735	0.007	−0.047 to 0.062
		Muscle	11.801	11.496–12.105	0.049	−0.008 to 0.105
	CN					
		Liver	**5.890**	**5.793–5.983**	**0.073**	**0.056–0.090**
		Skin	3.586	3.357–3.812	0.005	−0.036 to 0.046
		Muscle	3.281	3.048–3.509	0.003	−0.039 to 0.046
Manatee						
	$\delta^{13}C$					
		Liver	**−16.176**	**−16.285 to −16.068**	**−0.032**	**−0.052 to −0.012**
		Skin	−14.631	−14.888 to −14.374	0.014	−0.034 to 0.062
		Muscle	−16.476	−16.742 to −16.209	−0.006	−0.056 to −0.043
	$\delta^{15}N$					
		Liver	**8.251**	**8.041–8.462**	**0.081**	**0.042–0.120**
		Skin	6.932	6.435–7.429	0.002	−0.091 to 0.095
		Muscle	6.238	5.721–6.754	0.031	−0.064 to 0.112
	CN					
		Liver	**4.598**	**4.411–4.784**	**0.043**	**0.008–0.078**
		Skin	4.465	4.024–4.906	−0.029	−0.378 to 0.053
		Muscle	3.255	2.797–3.714	0.003	−0.111 to 0.102

Note: Text in boldface indicates significant or marginal results where 95% CIs of the slopes do not cross zero or cross zero by <0.001.

The effects of decomposition on $\delta^{15}N$ values in our study were consistent with other studies that performed similar experiments (Table 4) (Burrows et al., 2014; Keenan & DeBruyn, 2019; Payo-Payo et al., 2013; Perkins et al., 2018; Yurkowski et al., 2017). This consistency among studies suggests that it is typical for microbes to preferentially select for the lighter stable nitrogen isotopes, leaving heavier isotopes in the remaining tissues and enriching $\delta^{15}N$. Like carbon, fractionation of nitrogen between microbial communities and their substrates is not well known, but fractionation of various chemical pathways during nitrification, denitrification, and ammonia production can vary between −60‰ and 38‰ (Brunner et al., 2013; Casciotti et al., 2003; Hoch et al., 1992; Kobayashi et al., 2019). In our experiment, microbes appeared to deplete nitrogen more quickly than carbon as reflected in the increasing C:N values in liver during the experiment for both dolphins and manatees. This finding is unsurprising given that nitrogen is almost always a limiting nutrient compared with carbon (Rabalais, 2002). Thus, despite some variation in fractionation, the microbial chemical pathways involved in tissue decomposition appear to result in negative fractionation between −60‰ and −25‰ for nitrogen in most cases (Brunner et al., 2013; Hoch et al., 1992; Kobayashi et al., 2019), which can explain the increased $\delta^{15}N$ values observed in experimentally tested tissues in this study.

Experimental studies testing decomposition effects on stable isotope values have several caveats. First, the duration of most studies was relatively short, including our own. It is often unknown how long before sampling an animal died. For marine mammals, there is likely at least approximately 24 h before death and stranding because most marine mammals sink upon death, before gases

TABLE 4 Information and results from studies that tested effects of decomposition on isotopic values.

Study	Species	Tissues	Isotopes	Study type	Effects	Experiment time frame (days)
Burrows et al. (2014)	*Orcinus orca*	Skin, blubber	$\delta^{13}C$, $\delta^{15}N$	Exp	• $\delta^{13}C$ and $\delta^{15}N$ in skin increased. • $\delta^{13}C$ in blubber decreased. $\delta^{15}N$ in blubber unaffected.	14
Keenan and DeBruyn (2019)	*Castor canadensis*	Fat, heart, liver, lungs, muscle, gut tissue, bone, hair	$\delta^{13}C$, $\delta^{15}N$	Exp	• $\delta^{13}C$ in tissues was unaffected. • $\delta^{15}N$ increased in all tissues except bone and hair.	24
Payo-Payo et al. (2013)	*Stenella coeruleoalba* and *Caretta caretta*	Skin, muscle	$\delta^{13}C$, $\delta^{15}N$	Exp	• $\delta^{13}C$ and $\delta^{15}N$ in tissues were unaffected.	62
Perkins et al. (2018)	*Babylonia areolate*, *Ephalopholis boenak*, and *Miyakea nepa* *Paphia amabilis*, *Portunus sanguinoletus*, and *Siganus canaliculatus*	Muscle	$\delta^{13}C$, $\delta^{15}N$	Exp	• $\delta^{13}C$ in *M. nepa*, *P. sanguinoletus*, and *S. canaliculatus* increased 0.2‰–0.4‰; $\delta^{13}C$ values decreased 0.3‰ in *B. areolate* and *P. amabilis*. • $\delta^{15}N$ increased in all species except *B. areolate* and *P. amabilis*.	5
Yurkowski et al. (2017)	*Pusa hispida*, *Salvelinus namaycush*, and *Somniosus microcephalus*	Muscle	$\delta^{13}C$, $\delta^{15}N$	Exp	• $\delta^{13}C$ increased in moist decomposition environments. • $\delta^{13}C$ decreased in dry decomposition environments for *S. namaycush* and *S. microcephalus*, but increased for *P. hispida*. • $\delta^{15}N$ increased in moist and dry decomposition environments. • Most changes occurred after 8 days.	256
This study	*Tursiops truncatus* and *Trichechus manatus*	Liver, skin, muscle		Obs, exp	• Isotopic ellipses between live and stranded *T. truncatus* and *T. manatus* were not different. • $\delta^{13}C$ values from fresh and less decomposed liver and skin for *T. truncatus* were enriched compared to advanced decomposed samples. $\delta^{13}C$ values from fresh liver samples were depleted for *T. manatus*. $\delta^{15}N$ values were unaffected by decomposition stage for both *T. truncatus* and *T. manatus*.	10

(Continues)

TABLE 4 (Continued)

Study	Species	Tissues	Isotopes	Study type	Effects	Experiment time frame (days)
					• $\delta^{13}C$ values of *T. truncatus* liver decreased with decomposition time, while $\delta^{15}N$ and CN values increased for both *T. truncatus* and *T. manatus*.	

Abbreviations: Exp, experimental; obs, observational.

from decomposition accumulate and cause the carcass to float, after which they may strand and can be reported to a stranding network. Other factors such as temperature and degree of scavenging will also influence how quickly tissues can decompose. Insect infestation occurs in almost all carcasses and drives many aspects of decomposition, potentially altering stable isotope values in ways that have not been tested (Metcalf et al., 2016; Singh et al., 2018; Vass, 2001). Additionally, most decomposition experiments removed tissues from bodies and then exposed them to either environmental or laboratory conditions, and tissues exposed to environmental conditions are exposed to more variable ambient conditions. In most dead and decomposing animals, tissues are encapsulated within the carcass, and decomposition generally starts from the inside. Skin and blubber can somewhat insulate internal tissues from outside conditions, and individual organs decompose at different rates depending on enzymatic function and composition (Clark et al., 1997). As such, experimental conditions can be quite different from the natural settings in which wild animals decompose. For example, in the decomposition experiment, the $\delta^{15}N$ values in liver became enriched but did not change in tissues from carcasses sampled at late and advanced decomposition stages. This difference may be explained if liver sampled from the decomposition stage study was relatively more protected by remaining in the carcass and therefore took longer to be affected by decomposition compared with the samples removed and directly exposed to the elements during the decomposition experiment. In our experiment, temperature and humidity were both high, with maximum temperatures ranging 29–31°C and relative humidity ranging 60%–85%, and providing environmental conditions conducive to microbial growth (Megyesi et al., 2005; Yurkowski et al., 2017). Additionally, decomposition may proceed differently when a carcass is partially buried in sand or submerged in water, which most marine mammal carcasses will have been for at least some time before washing ashore, and these effects have not been studied. Future work allowing whole bodies to decompose while sampling over a longer period of time (but see Keenan & DeBruyn, 2019) and comparing decomposition effects in different environments, for example, air versus water, will be informative to understand potential differences between most laboratory experiments and field conditions. Studies to establish the net effects of decomposition on stable isotope ratios in samples from stranded animals can help researchers make more accurate ecological inferences and better understand both the applications and limitations of opportunistically collected stranding data. Future work should be aimed at improving our understanding of these effects under natural conditions.

We found that stable isotope values in skin, on average, did not differ between dead and live dolphins and manatees. Muscle was unaffected in both the decomposition code study and experiment. As such, skin and muscle samples are recommended for use in SIA over tissues such as liver that decompose more quickly. To reduce the likelihood of decomposition effects on stable isotope ratios in any tissues, we recommend sampling tissues as soon as possible after an animal dies. If samples are obtained in the field, we recommend immediately placing samples on ice in coolers or to flash freeze with dry ice or liquid nitrogen. If samples are obtained in the laboratory during necropsy or similar examination, they should either be promptly dried and prepared for SIA or placed in a −20°C freezer for storage. Tissues from stranded animals can provide ecological information in the weeks to months leading up to death and may prove useful in determining if the animal had recently undergone a shift in diet or habitat. If research requires the use of tissues that have rapid turnover rates but decompose quickly, we recommend limiting analyses to the freshest samples possible and taking care to consider decomposition effects when interpreting data. We recommend researchers continue to perform decomposition studies that compare tissues across a range of decomposition stages observed in the field and experimentally measure the effects of decomposition on isotopes to inform ecological studies. If enough decomposition studies are

performed across a range of tissues, it may be possible to develop tissue- and condition-specific correction models from meta-analyses.

AUTHOR CONTRIBUTIONS

Carl S. Cloyed and Ruth H. Carmichael conceived and designed this project. Carl S. Cloyed aided in performing the studies and experiments, led data analyses, and wrote the initial manuscript. Che'Isha Johnson carried out the decomposition stage study and the decomposition experiment. Carl S. Cloyed, Kayla P. DaCosta, Lauren R. Clance, Mackenzie L. Russell, Cristina Díaz Clark, Elizabeth E. Hieb, and Ruth H. Carmichael all contributed tissue samples or in obtaining tissue samples. Mackenzie L. Russell, Cristina Díaz Clark, Ruth H. Carmichael, and Che'Isha Johnson contributed to experimental design, data analyses, and/or interpretation of results. All authors contributed to revising and editing the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interests.

DATA AVAILABILITY STATEMENT

Stable isotope data from the live and stranded dolphins from Barataria Bay (BAR), Louisiana as well as live captures in 2018 and strandings from 2011 to 2019 from Mobile Bay (MOB), Alabama are publicly available through the Gulf of Mexico Research Initiative Information & Data Cooperative (GRIIDC), https://data.gulfresearchinitiative.org/data/R6.x809.000:0020. Data from BAR in 2011 and 2013 can be found under the *Deepwater Horizon* Natural Resource Damage Assessment Data in the DIVER database, https://www.diver.orr.noaa.gov. Data from live dolphins in 2019–2020, stranded dolphins in 2019–2020 in MOB, and live and stranded manatees are at Dauphin Island Sea Lab Data Management Center, https://data.disl.edu/dataset/stable-isotope-values-of-live-and-stranded-dolphins-and-manatees-from-the-northern-gulf-of-mexico.

ORCID

Carl S. Cloyed https://orcid.org/0000-0001-6321-5808

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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