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Contribution to the Theme Section 'Effects of the Deepwater Horizon oil spill on protected marine species'

Assigning stranded bottlenose dolphins to source stocks using stable isotope ratios following the *Deepwater Horizon* oil spill

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ABSTRACT: The potential for stranded dolphins to serve as a tool for monitoring free-ranging populations would be enhanced if their stocks of origin were known. We used stable isotopes of carbon, nitrogen, and sulfur from skin to assign stranded bottlenose dolphins *Tursiops truncatus* to different habitats, as a proxy for stocks (demographically independent populations), following the *Deepwater Horizon* oil spill. Model results from biopsy samples collected from dolphins from known habitats (n = 205) resulted in an 80.5% probability of correct assignment. These results were applied to data from stranded dolphins (n = 217), resulting in predicted assignment probabilities of 0.473, 0.172, and 0.355 to Estuarine, Barrier Island (BI), and Coastal stocks, respectively. Differences were found west and east of the Mississippi River, with more Coastal dolphins stranding in western Louisiana and more Estuarine dolphins stranding in Mississippi. Within the Estuarine East Stock, 2 groups were identified, one predominantly associated with Mississippi and Alabama estuaries and another with western Florida. $\delta^{15}N$ values were higher in stranded samples for both Estuarine and BI stocks, potentially indicating nutritional stress. High probabilities of correct assignment of the biopsy samples indicate predictable variation in stable isotopes and fidelity to habitat. The power of $\delta^{34}S$ to discriminate habitats relative to salinity was essential. Stable isotopes may provide guidance regarding where additional testing is warranted to confirm demographic independence and aid in determining the source habitat of stranded dolphins, thus increasing the value of biological data collected from stranded individuals.

KEY WORDS: Carbon · Nitrogen · Sulfur · *Tursiops truncatus* · Stock structure · Gulf of Mexico

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INTRODUCTION

Information collected from stranded marine mammals can serve as a tool for monitoring free-ranging populations (Peltier et al. 2012). For example, the species composition of strandings may reflect the biodiversity of living populations in adjacent waters (Pyenson 2010, Byrd et al. 2014). The relative number or composition of strandings can be used to monitor long-term (Peltier et al. 2013) or cyclical (Evans et al. 2005, Peltier et al. 2014) shifts in distribution or anomalous strandings, such as unusual mortality events (UMEs; NMFS-OPR 2016), that indicate the presence of disease, high levels of fishery interactions, or other events or factors affecting the population (Byrd et al. 2008, Yang et al. 2008, Wilkin et al. 2012, Hohn et al. 2013, Litz et al. 2014). Samples from stranded marine mammals can be used to obtain data for estimating demographic parameters of species or populations for which data are otherwise difficult to obtain (Hohn et al. 1996, Lockyer & Kinze 2003, Mattson et al. 2006, Schwacke et al. 2017, this Theme Section). In these ways, robust stranding programs have substantially contributed to our knowledge of marine mammal populations. One important issue for interpreting data from strandings is accounting for spatial and temporal variation in the probability of animals becoming stranded and then the stranding being reported (Peltier et al. 2014). While we do not deal with this question here, a second major issue in using strandings to reflect free-ranging populations, and the motivation for this paper, is that the source population for stranded marine mammals is often unknown.

The more complex the population structure, the more complex the challenge to identify source populations of stranded animals. Recent research has demonstrated that common bottlenose dolphins *Tursiops truncatus* (hereafter bottlenose dolphins or dolphins) exhibit extensive population structuring, meeting the definition, for management purposes, of stocks as demographically independent populations (Moore & Merrick 2011). Along the US Atlantic coast, Rosel et al. (2009) identified at least 5 genetically differentiated coastal and estuarine populations, confirming demographic independence despite the absence of hard barriers, such as isolated estuaries. Genetic analyses, coupled with photo-identification (photo-ID) results, have identified structuring of bottlenose dolphin populations over relatively small coastal ranges such as for *T. truncatus* in Hawaii (Baird et al. 2009, Martien et al. 2012), Brazil (Fruet et al. 2014), South Africa (Natoli et al. 2005), Spain (Fernández et al. 2013), and the Mediterranean Sea (Gaspari et al. 2015), as well as for *T. australis* in southern Australia (Charlton-Robb et al. 2015). Further, while estuaries are porous with regard to movements of dolphins, significant genetic differentiation has been shown to occur among bay, sound, or estuarine (BSE) stocks and between BSE and their adjacent coastal stocks (Sellas et al. 2005, Möller et al. 2007, Rosel et al. 2009). Genetics has additionally shown that structuring can occur on a finer scale, with more than 1 population within close proximity of one another within an estuary (Rosel et al. 2009, Mirimin et al. 2011, Ansmann et al. 2012, Richards et al. 2013, Fruet et al. 2014).

Bottlenose dolphins are the most abundant coastal cetaceans from the US mid-Atlantic states to Texas. Along the northern Gulf of Mexico (GoM) coast of the US, 36 stocks are recognized under the Marine Mammal Protection Act, 31 of which are defined as BSE stocks and 3 as coastal stocks (Vollmer & Rosel 2013, Waring et al. 2015). Photo-ID and telemetry results support the concept of resident estuarine stocks of dolphins, from Florida (Wells & Scott 1990, Fazioli et al. 2006, Balmer et al. 2008, Conn et al. 2011, Bassos-Hull et al. 2013) through Mississippi (K. D. Mullin et al. unpubl. data) and Louisiana (Wells et al. 2017, this Theme Section) to Texas (Bräger et al. 1994, Irwin & Würsig 2004). These long-term photo-ID studies are limited to relatively few areas, and are primarily conducted in and of greatest value for stocks inhabiting estuarine or other semi-enclosed areas. Genetic studies have not been conducted at most of the photo-ID sites to confirm demographic independence or whether genetic assignment to stock is possible.

The assessment of injury to bottlenose dolphins following the *Deepwater Horizon* (DWH) oil spill required estimating injury by stock, necessitating assignment of stranded dolphins to their source stock (DWH NRDAT 2016). From March 2010 to July 2014, much of the northern GoM experienced a UME (Carmichael et al. 2012a, Litz et al. 2014). While some mortalities occurred in the months prior to the spill, the largest and most prolonged increases in mortality occurred after the spill and were primarily attributed to the spill (Venn-Watson et al. 2015a,b, Colegrove et al. 2016, DWH NRDAT 2016). Prior to the DWH oil spill, few studies had been conducted on the stock structure of bottlenose dolphins in the coastal and estuarine areas that were most heavily oiled during the DWH event, from western Louisiana through the western Panhandle of Florida (Michel et al. 2013, DWH NRDAT 2016). The available studies, however, suggested site fidelity and independent estuarine stocks. Photo-ID surveys in the Mississippi Sound

(from the mid-1980s) provided evidence for long-term estuarine residency with seasonal changes in abundance (Hubard et al. 2004). Results from a survey in Barataria and Caminada Bays, in southern Louisiana, indicated a small, closed population with some seasonal variation in sighting patterns (Miller 2003).

In the immediate aftermath of the DWH oil spill, photo-ID or genetics results available to identify the source stocks of stranded dolphins remained limited relative to the spatial extent of strandings. As a result, another method was needed to assign individual stranded dolphins to coastal or estuarine stocks. Stable isotopes have been used across a range of taxa as dietary tracers and spatial markers (Crawford et al. 2008, B. S. Graham et al. 2010, Newsome et al. 2010, Ben-David & Flaherty 2012). In the marine environment, stable isotopes of carbon indicate linkages to initial sources of primary productivity (e.g. seagrass, salt marsh, phytoplankton; Michener & Schell 1994, Currin et al. 1995), nitrogen reflects trophic level and the influence of different nutrient sources (Peterson & Fry 1987, McClelland et al. 1997, Rossman et al. 2013), while sulfur can further distinguish marine from freshwater- or terrestrial-derived sources relative to variation in dominant microbial processes (Peterson & Fry 1987, Connolly et al. 2004). The link between sulfur values and salinity has been well demonstrated in marine species, including along the northern GoM (Fry 2002, Fry & Chumchal 2011, MacAvoy et al. 2015). Thus, different combinations of stable isotope composition correlate with variation in environmental conditions, providing a tool to define populations and track large-scale movement patterns associated with migratory or foraging behavior (Lee et al. 2005, Hobson et al. 2010, Fry & Chumchal 2011).

In cetaceans, a number of studies found among-group differences in stable isotopes that were used to define stocks. The predominant elements used have been carbon and nitrogen (Witteveen et al. 2009, Ohizumi & Miyazaki 2010, Fernández et al. 2011, Aurioles-Gamboa et al. 2013, Borrell et al. 2013, Giménez et al. 2013). For bottlenose dolphins along the GoM coast of Florida, mean stable isotope ratio values of carbon, nitrogen, and sulfur differed between estuarine and coastal and/or offshore groups, with sulfur isotope values increasing seaward (Barros et al. 2010). Other stable isotope studies in marine mammals have been coupled with independent indicators, such as contaminants, trace elements, or fatty acids, to define stock structure (Borrell et al. 2006, Wilson et al. 2012, Quérouil et al. 2013, Browning et al. 2014a, Ansmann et al. 2015). Hence, stable isotopes in tissues of stranded dolphins could provide an alternative approach to assign stranded dolphins to stocks, pending more direct measures of demographic independence.

The goal of the current study was to test whether stable isotopes of carbon, nitrogen, and sulfur could be used to assign dolphins stranded after and in areas most affected by the DWH oil spill to estuarine or coastal source habitats, as a proxy for stocks. Under the current National Marine Fisheries Service stock definitions (Waring et al. 2015), barrier island and estuarine animals are included as part of the BSE stock. Due to the growing body of evidence from telemetry (Wells et al. 2017, K. D. Mullin et al. unpubl. data) and genetics (Rosel et al. 2017, this Theme Section), indicating that dolphins from barrier islands form a distinct group, we treated animals from barrier islands as a potential stock. The approach for stock assignment was to first develop a model from samples collected from known source populations ('Estuarine,' 'Barrier Island,' and 'Coastal') via biopsy sampling and then to apply those model results to predict the stocks of origin for stranded animals.

MATERIALS AND METHODS

Sample collection

Stable isotope analysis was conducted on skin samples collected from live animals, via remote biopsy or during capture–release health assessments, or from stranded animals. Remote biopsy sampling occurred during vessel-based surveys in 2006 and from 2010 to 2013 using biopsy tips measuring 7 × 25 mm or 10 × 25 mm (Sinclair et al. 2015). Collection data, including location and date, were recorded for each biopsy sample on a '*Deepwater Horizon* Oil Spill Response Biopsy Sheet' or a 'Marine Mammal Sighting Sheet' (see Appendices A and B in Sinclair et al. 2015). Biopsy samples were collected during health assessments in 2011 and 2013 following accepted capture–release health-assessment protocols (Wells et al. 2004, Schwacke et al. 2014). Samples from stranded dolphins were collected from 2010 to 2013 following stranding-response protocols detailed by Litz et al. (2014). Skin collected via biopsy sampling included up to 25 mm depth of the epidermis and dermis; skin collected from strandings was influenced by state of decomposition but also included the full thickness of available epidermis and upper layers of dermis. For all samples, attached blubber was removed.

Skin samples collected via biopsy sampling were placed in cryovials and stored in a liquid nitrogen vapor shipper in the field until transferred to an ultracold freezer for long-term storage at −80°C (small vessels) or were placed directly in an ultracold freezer carried on larger vessels (Sinclair et al. 2015). Skin samples collected from stranded animals initially frozen at close to −20°C were later transferred to −80°C. All samples remained frozen until stable isotope analysis.

Given the large number of strandings following the DWH oil spill (Litz et al. 2014, Venn-Watson et al. 2015b), a subset of available skin samples from stranded animals was selected for stable isotope analysis using several criteria: (1) the strandings occurred after the beginning of the DWH oil spill (on or after 20 April 2010), (2) the stranding occurred within the geographic range most affected by the DWH oil spill (Louisiana to the western Panhandle of Florida; Michel et al. 2013, DWH NRDAT 2016), (3) the total length of a stranded animal was >170 cm to exclude perinatal and young of the year (Fernandez & Hohn 1998) to avoid ontogenetic effects (Knoff et al. 2008, Riccialdelli et al. 2013), and (4) carcasses were fresh dead or exhibited only moderate decomposition (condition code ≤3 [Smithsonian Institution Coding System] Geraci & Lounsbury 2005) to reduce effects of decomposition, except for 11 carcasses of advanced decomposition (condition code 4) used to test for effects of decomposition.

Sample preparation and stable isotope analysis

Analysis of skin samples for stable isotope ratio composition was conducted by IsoForensics (Salt Lake City, UT; http://isoforensics.com/) in-house and at the Stable Isotope Ratio Facility for Environmental Research (SIRFER; http://sirfer.utah.edu). Samples were processed and analyzed as detailed by Valenzuela et al. (2012). In summary, skin samples were freeze-dried overnight or up to 24 h (FreeZone Freeze Dry System), lipid-extracted with a 2:1 chloroform:methanol mixture for 48 h using a Soxhlet extractor, and then cut into fragments. For analysis of carbon and nitrogen isotope ratios, 1.00 mg (± 10%) of dried, delipified skin was weighed into tin capsules (3.5 × 5 mm, Costech Analytical); for analysis of sulfur isotope ratios, 2.00 mg (± 10%) was weighed into tin capsules. Every 20th sample was analyzed in duplicate, except for 1 sample for which there was insufficient tissue and a different sample was analyzed in duplicate.

Carbon and nitrogen isotope ratio measurements were made at IsoForensics using a MAT 253 isotope ratio mass spectrometer (Thermo Finnigan) operated in continuous flow mode; tin capsules were loaded into an autosampler (Costech Analytical) interfaced with an elemental analyzer (EA; Thermo Finnigan) through a Conflo IV interface. Sulfur isotope ratio measurements were made at SIRFER using a Delta S isotope ratio mass spectrometer (Thermo Finnigan) operated in continuous flow. At SIRFER, tin capsules were loaded into a zero-blank autosampler (Costech Analytical) interfaced with an EA (Carlo Erba) through a Conflo III interface. In the EA, samples were flash combusted to produce CO_2, N_2, and SO_2 for carbon, nitrogen, and sulfur isotope analysis, respectively. Resultant gases were chromatographically separated and carried to the mass spectrometer via He stream.

The samples were analyzed alongside a series of laboratory reference materials. For each measurement, 2 of the reference materials (i.e. the 'normalization' reference materials) were used to (1) correct for potential memory, time ('drift'), and/or voltage effects and (2) normalize measured data to the international isotope scales. The other reference materials were used for quality control (QC) to verify corrections and normalization. Reference materials used for carbon and nitrogen stable isotope analysis included ground keratins that had been calibrated using the international isotope reference materials USGS40 and USGS41 to give reference values relative to VPDB of $\delta^{13}C_{VPDB}$ = −23.12‰ and −11.9‰ and $\delta^{15}N_{AIR}$ = +1.38‰ and +10.69‰ for normalization reference materials DS (dall sheep horn) and ORX (oryx antelope horn), respectively. Analytical precision, defined as the standard deviation of the QC reference material, powdered keratin (POW), was +0.02‰ for C (n = 71, $\delta^{13}C_{VPDB}$ = −24.03) and +0.04‰ for N (n = 71, $\delta^{15}N_{AIR}$ = +5.96‰). For sulfur stable isotope analysis, reference materials included a silver sulfide and zinc sulfide (normalization) and 2 ground keratins (QC). The normalization reference materials had been calibrated to the international isotope reference materials IAEA-S-1, -2, and -3 to give reference values on the Vienna Canyon Diablo Troilite (VCDT) scale of $\delta^{34}S_{VCDT}$ = +17.9‰ and −31.9‰ for reference materials UU-S-1 and UU-S-2, respectively. Analytical precision was 0.3‰ based on replicate measurements of POW (n = 50, $\delta^{34}S_{VCDT}$ = +4.2‰) and ground eider duck down (n = 94, $\delta^{34}S_{VCDT}$ = +16.7‰).

Due to the short-term unavailability of the mass spectrometer at SIRFER, biopsy samples collected in coastal waters of Mississippi in 2006 were sent to the Center for Stable Isotope Biogeochemistry (CSIB) at

the University of California, Berkeley, for sulfur isotope analysis. For the sulfur stable isotope analysis conducted at CSIB, POW was used as the reference material, and measured $\delta^{34}S$ values matched the long-term statistics on POW analyzed at SIRFER, which included all samples from coastal waters of Louisiana. There was no significant difference in the mean $\delta^{34}S$ values between the 2 sets of coastal samples (n = 21 from Louisiana and n = 14 from Mississippi, *t*-test, p = 0.26, mean $\delta^{34}S_{SIRFER}$ = +14.96 [±2.3 CI], mean $\delta^{34}S_{CSIB}$ = +14.36 [±2.8 CI]).

Stable isotope values are reported in δ-notation, reflecting the ratio of the heavy to light isotope relative to the standard material, using the following standard expression:

$$\delta E\,‰ = [R_{sample} / R_{standard} - 1] \times 1000 \quad (1)$$

where E is the isotope (^{13}C, ^{15}N, ^{34}S) and R is the molar ratio of the heavier to lighter isotope for the sample and its corresponding standard, respectively. Comparative values of stable isotopes are expressed as higher or lower, heavier or lighter, and enriched or depleted, with respect to the relative value, in ‰, of the heavier isotope (Campbell et al. 2014). For example, +15‰ is greater, more positive, more enriched than +10‰.

Data analysis

Training data

A subset was selected from the full set of biopsy samples by choosing samples from geographically distinct locations that excluded areas of likely spatial overlap of stocks, creating a training subset of data. Given differences in the estuarine systems west and east of the Mississippi River Bird's Foot Delta (hereafter referred to as the MS River Delta), west and east sides were subsampled separately to create the training dataset. West of the MS River Delta (hereafter referred to as 'West'), the training dataset included samples collected (1) in the upper estuary of Barataria Bay (Estuarine), (2) near Grand Isle and Isle Grande Terre (Barrier Island [BI]), and (3) in coastal waters from 2 km off the beach to the 20 m isobath offshore of Barataria Bay (Coastal). East of the MS River Delta (hereafter referred to as 'East'), the training dataset included samples collected (1) near the mainland (upper estuary) in Mississippi and Chandeleur Sounds (Estuarine), (2) very near the barrier islands of Mississippi Sound, including Horn, Petit Bois, and Dauphin Islands (BI), and (3) in coastal waters offshore of BI (Coastal). Coastal animals from West were assumed to belong to the Western Coastal Stock while those from East were assumed to belong to the Northern Coastal Stock (Waring et al. 2015).

Stock assignment analysis

To describe the relationship between stable isotopes and stocks, we conducted a recursive partition analysis (also called a classification and regression tree, CART; Sutton 2005), using the rpart library (version 4.1-9) in the statistical software R (version 3.1.3; Therneau & Atkinson 2015). This analysis proceeded in 2 phases. First, a tree was built by splitting the data according to binary partitions of the candidate covariates. Candidate covariates included the factor variables region (East/West) and sex (male/female), and the continuous variables $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$. Temporal variables (year and month/season) were also considered, but they were confounded with stock because the Coastal stock was only sampled in the summer and East Coastal samples were collected in a year when no Estuarine or BI samples were collected. Each split was on a single covariate, and was chosen to minimize the misclassification rate of observations or some estimator of the unknown population misclassification rate in each branch. Second, the tree was pruned by removing branches that contributed the least; this pruning was achieved by reference to a measure of model complexity. Our analysis closely followed the suggestions of Therneau & Atkinson (2015) for tree building and pruning. In choosing splits, we used the Gini rule (Therneau & Atkinson 2015), with a minimum of 5 observations per group and per terminal node (or leaf), and prior probabilities that were proportional to observed data frequencies with a 0/1 loss. In pruning branches, we used the complexity parameter, and chose the structure that minimized this quantity over 10-fold cross validation. Results from the CART analysis were applied to the stranding samples to assign those samples to stocks (Estuarine, BI, or Coastal). Summary statistics and comparison tests were performed using SAS software (version 9.4); following convention, the α level used for statistical significance was 0.05.

RESULTS

Training dataset

In total, 335 biopsy samples were collected (Fig. 1, and see Table S1 in the Supplement at

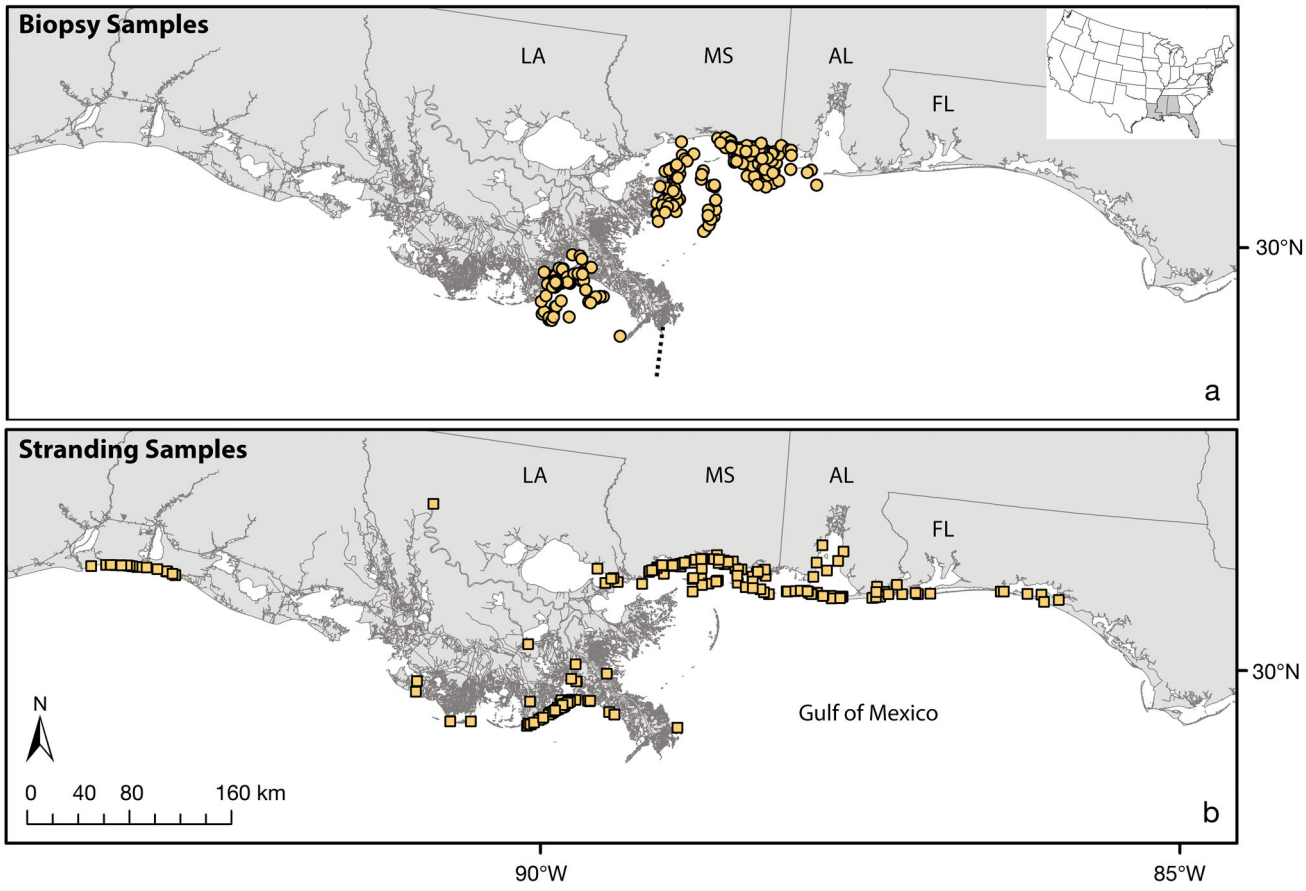

Fig. 1. Geographic distribution of (a) biopsy and (b) stranding samples of common bottlenose dolphins *Tursiops truncatus* analyzed for stable isotope ratios. In (a), the dashed line extending approximately southward from the tip of the Mississippi River Bird's Foot Delta indicates the demarcation between the Northern and Western Coastal Stocks (Vollmer & Rosel 2013). The shaded states in the USA map inset were included in the current analysis. LA: Louisiana, MS: Mississippi, AL: Alabama, FL: Florida

www.int-res.com/articles/suppl/n033p235_supp.pdf), of which 205 were selected for inclusion in the training dataset (Table 1, Fig. 2). The correct classification rate from the training dataset was 80.5% from 5 partitions (6 terminal nodes: 1 Coastal, 2 BI, and 3 Estuarine) using the predictor variables $\delta^{34}S$, region, and $\delta^{13}C$, respectively (Table 2, Fig. 3). To put this in context, the correct classification rate expected by chance from random assignment of animals to stock is 0.39, and the probability of achieving an 80.5% or better correct classification by chance is $<1 \times 10^{-6}$. The correct classification rate at each terminal node provided an assignment probability for samples with characteristics corresponding to that node, e.g. 28 of the 38 animals within Coastal were sampled within the Coastal training stratum, so the estimated probability of being Coastal for animals in that node is 28/38 or 0.736 (Fig. 3).

Mean stable isotope values across the 6 nodes ranged from −19.6 to −16.8‰ for $\delta^{13}C$ (overall range of −21.3 to −15.1), +13.8 to 15.9‰ for $\delta^{15}N$ (overall range of 12.0–17.0‰), and +10.8 to 15.6‰ for $\delta^{34}S$

Table 1. Sample sizes within each habitat or stock area for the training dataset derived from the full set of biopsy samples of common bottlenose dolphins *Tursiops truncatus*

Region	Stock	Sex		Total
		Female	Male	
East	Estuarine	28	53	81
	Barrier Island	8	18	26
	Coastal	4	10	14
West	Estuarine	10	12	22
	Barrier Island	22	19	41
	Coastal	7	14	21
Total		51	73	124

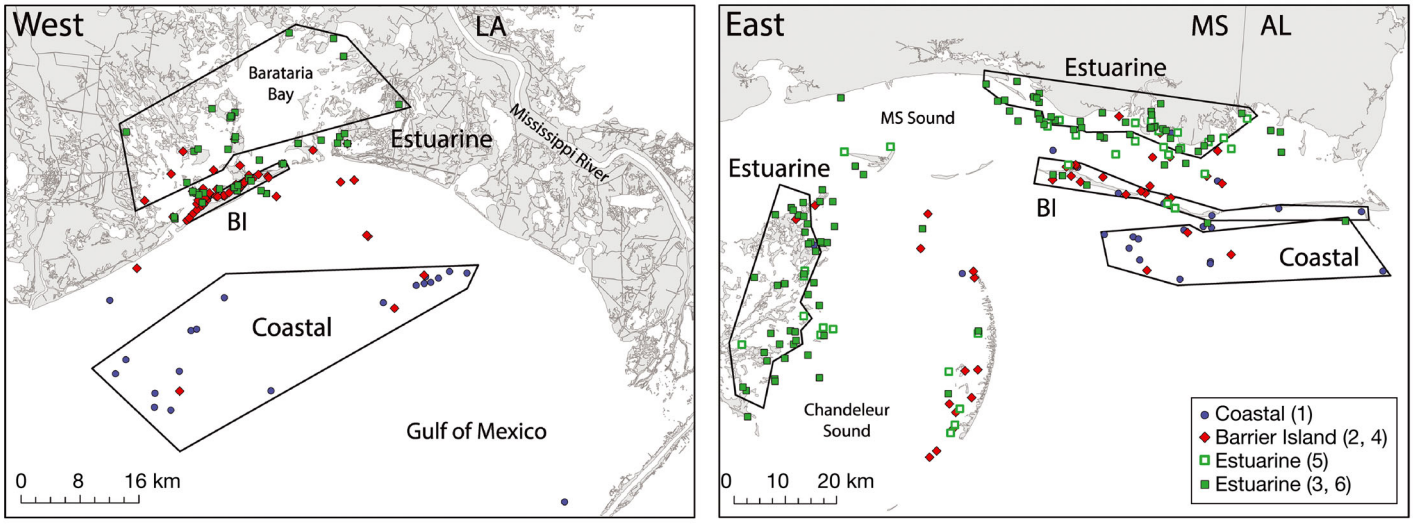

Fig. 2. Biopsy samples of common bottlenose dolphins *Tursiops truncatus* selected as part of the training dataset and the resultant predicted stock. Polygons encircle samples included in the training dataset, labeled to indicate 'observed' stock on the basis of location of sampling. Symbol shape and color indicate the predicted stock, Estuarine, Barrier Island (BI), or Coastal, resulting from the model output. For example, blue circles in the coastal polygon represent samples collected in the coastal area (observed Coastal) and assigned to the Coastal stock (predicted Coastal); red diamonds in the Coastal polygons represent samples collected in the coastal area but assigned to the BI stock. Biopsy samples external to the polygons were not included in the training dataset but could be assigned to stock on the basis of their $\delta^{13}C$ and $\delta^{34}S$ values. The numbers (in parentheses) associated with the node names correspond to the nodes in the tree diagram (see Fig. 3). LA: Louisiana, MS: Mississippi, AL: Alabama

(overall range of 6.9–18.1; Table S2 in the Supplement). Of 45 pairwise comparisons (6 nodes, 3 isotopes), 37 were statistically significant after adjusting for multiple comparisons (SAS Proc Multtest, p-values adjusted using bootstrapping, significant p-values from <0.0001 to 0.045; Table S3 in the Supplement). Overall, isotopic values for all 3 elements were sequentially greater from Estuarine to BI to Coastal and from West to East, with significant positive correlations (SAS Proc Corr, Spearman correlation coefficients, all $p < 0.001$); the relative increase from Estuarine to BI to Coastal was greater in West than East (Fig. S1 in the Supplement). Within nodes, however, a significant correlation between $\delta^{13}C$ and $\delta^{34}S$ occurred only for Coastal samples ($p = 0.04$). Two estuarine nodes were identified in East (Estuarine(5) and Estuarine(6)). Estuarine(5) values were higher for $\delta^{13}C$ and $\delta^{15}N$ and lower for $\delta^{34}S$ relative to Estuarine(6), but not different from East BI or Coastal. Overall in East, higher values occurred from BI to Coastal, but with no difference in $\delta^{13}C$ or $\delta^{15}N$ from Estuarine(5) to Coastal or from Estuarine(6) to BI.

Table 2. Within-sample confusion matrix for the training dataset of common bottlenose dolphin *Tursiops truncatus* samples. Rows are classification from the model, columns indicate the stock sampled. For example, 67 samples were collected from Barrier Islands (BI) while the model predicted 61 samples to be BI

Stock	Estuarine	BI	Coastal	Total predicted
Estuarine	91	14	1	106
BI	9	46	6	61
Coastal	3	7	28	38
Total observed	103	67	35	205

Stranding samples

Effects of decomposition

Carcasses exhibiting advanced decomposition (condition code 4) were distributed across 3 nodes, West BI (n = 6), West Estuarine(3) (n = 1), and East Estuarine(6) (n = 4). Although the sample size was small, there was no significant difference within node (West BI and East Estuarine(6)) in mean stable isotope values for any of the isotopes (Welch's *t*-test, p-values ranged from 0.183 to 0.998) as a function of condition code, and there were no outlying values for the code 4 samples relative to samples from strandings of condition codes 1 to 3 (Table S4 in the Supplement). Therefore, code 4 samples were retained for the analyses.

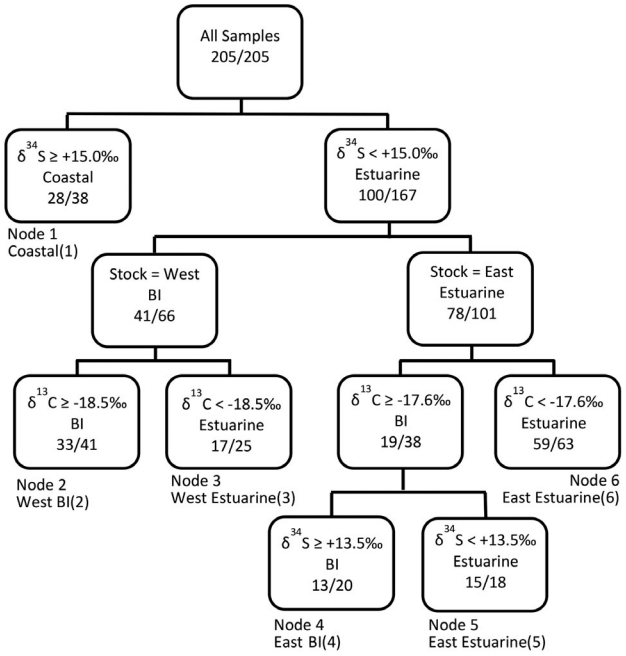

Fig. 3. Classification results from the recursive partition analysis using the training dataset of common bottlenose dolphins *Tursiops truncatus*. Within each box, the top row shows the splitting criterion, the second row indicates the primary stock membership (Estuarine, Barrier Island [BI] or Coastal), and the third row indicates the sample size, with the numerator giving the number of observations predicted to occur in that node and the denominator indicating the number of observed values from the training dataset. The 6 terminal nodes have been assigned node numbers and names to facilitate tracking of results in the text, tables, and other figures

Probability of Estuarine, BI, or Coastal

Stranding samples were collected from animals in areas that extended from western Louisiana to Choctawhatchee Bay, Florida (Fig. 1). Of the 217 stranded animals meeting the required criteria for analyses (n = 118 East and n = 99 West), the model predicted 104, 41, and 72 to be Estuarine, BI, and Coastal, respectively, with estimated assignment probabilities of 0.473, 0.172, and 0.355, respectively, for the combined sample. The distribution of predicted Estuarine, BI, and Coastal was not uniform. In particular, strandings in the westernmost area were predominantly Coastal. The habitat changes from west of Vermilion Bay, Louisiana, to the Texas–Louisiana border, where the estuaries are smaller with limited access to the coast, there are no barrier islands, and only a single, small, estuarine population of dolphins occurs. Thus, for purposes of capturing these differences in the prediction of source habitat for strandings, West was further stratified into western Louisiana (n = 23; west of Vermilion Bay, Louisiana, to the Texas–Louisiana border) and central Louisiana (n = 76; Vermillion Bay east to the MS River Delta). The highest probability of a stranded animal belonging to Estuarine occurred in the East and the highest probability of belonging to Coastal occurred in western Louisiana (Table 3, Fig. 4).

Table 3. Probability of a stranded common bottlenose dolphin *Tursiops truncatus* being assigned to Estuarine (p(Estuarine)), Barrier Island (p(BI)), or Coastal (p(Coastal)) stock for the total sample, for the stranding sample stratified by region (East or West), and with West further stratified into central and western Louisiana (LA). Strandings assigned to the Coastal stock presumably belong to the Western Coastal Stock (West) or the Northern Coastal Stock (East)

Region of stranding	N	p(Estuarine)	p(BI)	p(Coastal)
Total East and West	217	0.36	0.17	0.47
East only	118	0.67	0.15	0.19
West – central LA	76	0.27	0.65	0.08
West – western LA	23	0.17	0.45	0.38

Patterns among nodes

Mean stable isotopes values across the 6 nodes ranged from −19.6 to −16.3‰ for $\delta^{13}C$, +14.6 to 16.5‰ for $\delta^{15}N$, and +10.9 to 16.9‰ for $\delta^{34}S$ (Table S5 in the Supplement). While constrained by parameters determined from the training dataset, some trends occurred in the stranding samples. Similar to the training dataset, $\delta^{13}C$ and $\delta^{15}N$, but not $\delta^{34}S$, were significantly positively correlated overall (SAS Proc Corr, Spearman correlation coefficients, $p < 0.001$). Within node, however, significant correlations, all positive, occurred only within Coastal ($\delta^{13}C$ and $\delta^{15}N$) and BI (West BI: $\delta^{13}C$ and $\delta^{15}N$, $\delta^{15}N$ and $\delta^{34}S$; East BI: $\delta^{13}C$ and $\delta^{15}N$ only) nodes. In contrast to the training dataset, mean $\delta^{13}C$ values for Coastal were intermediate between Estuarine and BI samples.

Some patterns also emerged within the defined East BSE stocks (Table 4). All strandings from Choctawhatchee and Pensacola Bays and half of the samples from Perdido Bay, the easternmost bays, were assigned to Estuarine(5), with those samples representing 60% of that node and 75% of strandings from those 3 BSE stocks. All of the Estuarine strandings in Mobile Bay, the MS River Delta, and Lake Pontchartrain, and 89% of those in Mississippi Sound, all locations west of the previously noted bays, were assigned to East Estuarine(6). Only Estuarine strandings from Perdido Bay, between the eastern and western

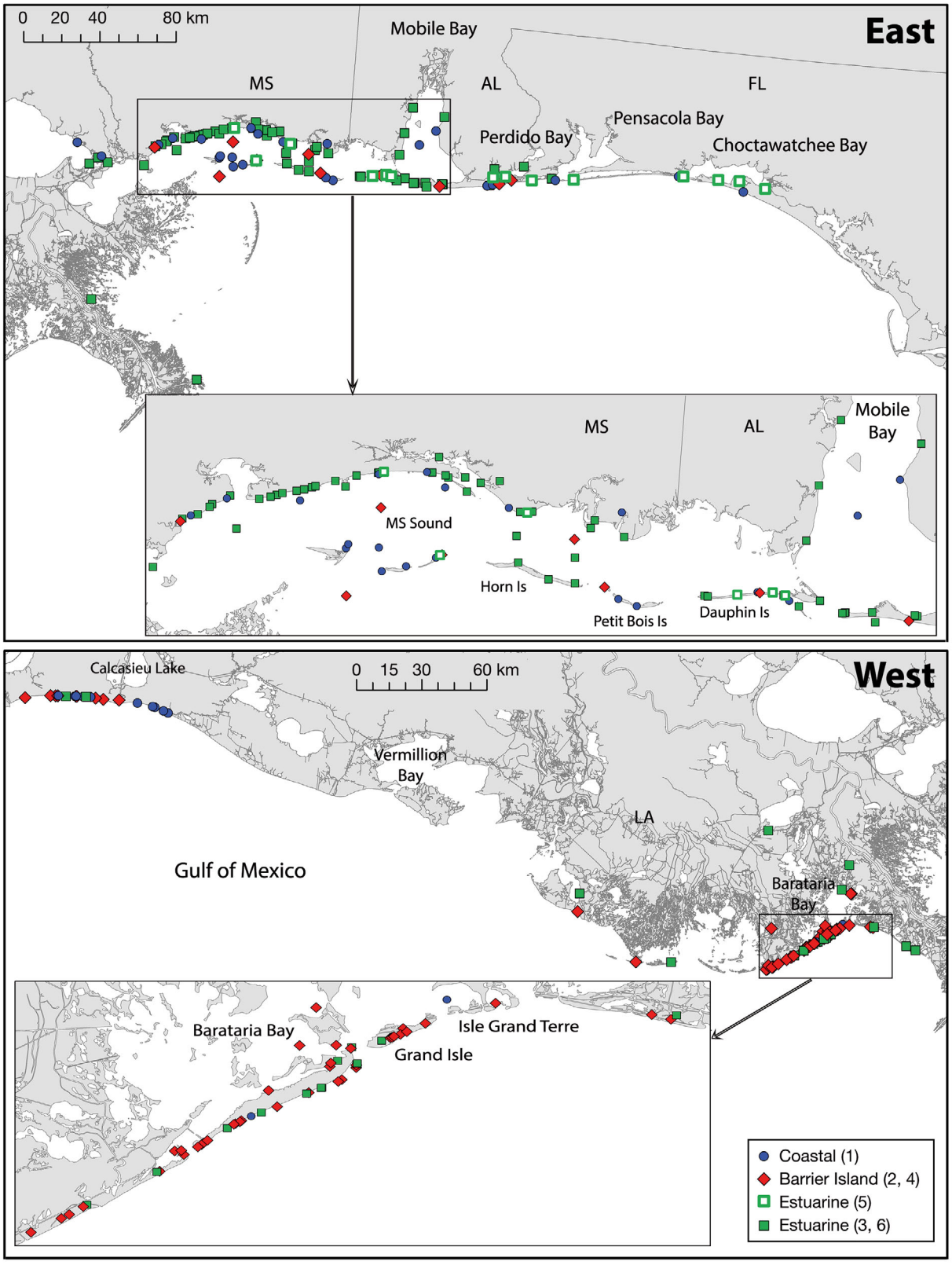

Fig. 4. Assignment of common bottlenose dolphin *Tursiops truncatus* strandings to Estuarine, Barrier Island, or Coastal stock predicted from the recursive partition analysis. The node names and numbers are as in Fig. 3

Table 4. Comparison of assigned stock for strandings of common bottlenose dolphins *Tursiops truncatus* that occurred east of the Mississippi River Bird's Foot Delta. The Stock Assessment Report (SAR) (Waring et al. 2015) designation assigns the stranding to stock on the basis of stranding location. The recursive partition analysis predicted the classification from stable isotope values, assigning strandings to Estuarine, Barrier Island (BI), or Coastal stock. For example, of 12 total strandings in Mobile Bay, 2 were predicted to be from the Coastal stock even though the stranding was in the estuary; there was agreement that the remaining 10 strandings were Estuarine. The bay, sound, and estuarine stocks are listed geographically from east to west. The Northern Coastal Stock comprises strandings that occurred outside of defined bays, sounds, and estuaries. The node name and number refer to the classification tree in Fig. 3 (e.g. BI(4) refers to Node 4 East)

SAR Stock	Node				
	Coastal(1)	East BI(4)	East Estuarine(5)	East Estuarine(6)	Total
Choctawhatchee Bay, FL			5		5
Pensacola Bay, FL			1		1
Perdido Bay, FL and AL		1	3	2	6
Mobile Bay, AL	2			10	12
Mississippi Sound, MS	14	6	5	42	67
Mississippi River Delta, MS				3	3
Lake Pontchartrain, MS	2			2	4
Northern Coastal	9	3	1	7	20
Total	27	10	15	66	118

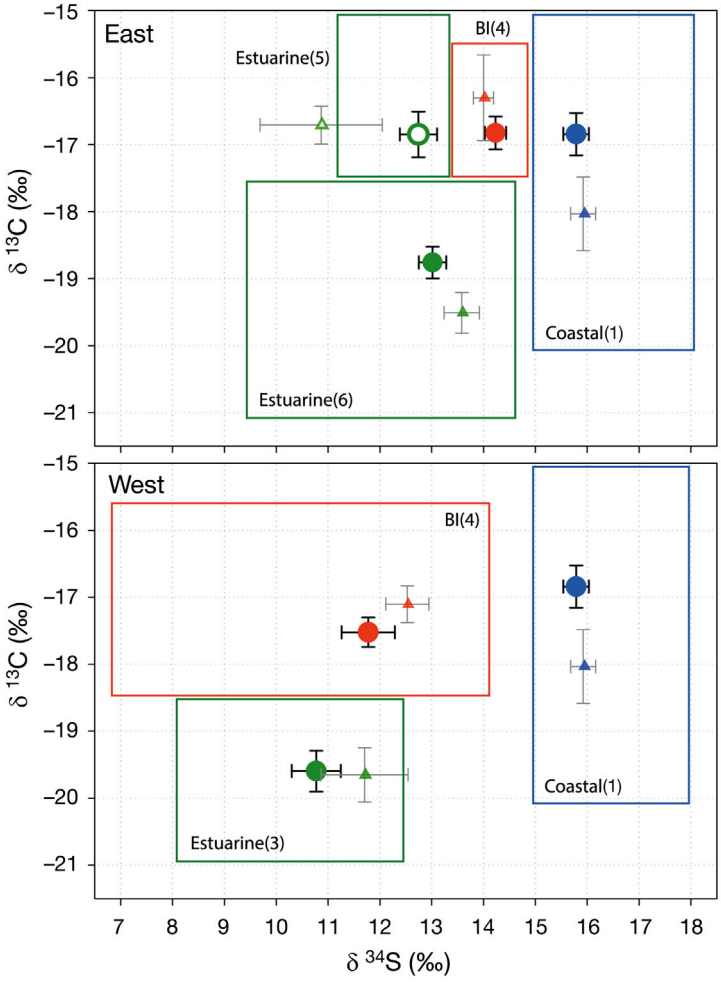

Fig. 5. Within-node comparison of stable isotope values between biopsy training samples and samples from stranded common bottlenose dolphins *Tursiops truncatus*. The mean is denoted with a symbol (circle for training subsample from biopsies; triangle for strandings); the error bars represent the upper and lower 95% confidence limits of the mean. The outline of the boxes indicates the isotope space, i.e. the range of stable isotope ratio values for carbon and sulfur, for each node from the training sample. The node names and numbers are as in Fig. 3

estuaries referenced above, were relatively evenly split between those 2 nodes, although the sample sizes were small.

Comparison of stable isotope values between live and stranded dolphins

Some variation occurred in isotopic values between samples from live (biopsy) and stranded animals (Fig. S2 in the Supplement). Within node, the range in values for all 3 stable isotope ratios was greater in stranded animals in 14 of 18 strata (3 isotopes, 6 nodes), as shown in the 2 elements, carbon and sulfur, that contributed to defining the stocks (Fig. S3 in the Supplement). Statistically significant differences occurred in the mean values between the training and stranding samples for 10 of 18 strata (SAS Proc Multtest, p-value adjusted using the bootstrap method, significant p-values from <0.0001 to 0.0285). Differences occurred most frequently for $\delta^{15}N$, occurring at 4 nodes, with a fifth node almost significant ($p = 0.052$), thus excluding only Coastal; in all but one of those 5 nodes, the stranding sample had higher $\delta^{15}N$ values than the biopsy sample, with differences ranging from 0.31 to 1.34‰ (of a total range in $\delta^{15}N$ across all samples of 5‰). Consistent differences between biopsy and stranding samples (e.g. stranding samples generally heavier or lighter) were not detected for $\delta^{13}C$ and $\delta^{34}S$ (Fig. 5). Overlap between West BI and East Estuarine(5) was sufficient to have caused misassignment to node in the absence of first accounting for region.

DISCUSSION

For carbon, nitrogen, and sulfur, stable isotope values fell within expected ranges for secondary consumers, in this case bottlenose dolphins, in estuarine and nearshore habitats. $\delta^{13}C$ values were consistent with the range of values typically found in benthic micro- and macroalgae and marine microalgae; values at estuarine sites were lower in $\delta^{13}C$ compared to coastal sites, with BI values intermediate, suggesting spatial mixing of brackish and marine water masses and associated carbon sources at BI sites (Currin et al. 1995, Chanton & Lewis 2002, Garcia et al. 2007). The ranges of $\delta^{13}C$ values in dolphins were similar to those in skin samples from estuarine and nearshore bottlenose dolphins from other sites, e.g. Florida (Wilson et al. 2012, Browning et al. 2014a) and Australia (Ansmann et al. 2015). These $\delta^{13}C$ values were enriched compared to values found in oil-derived materials associated with the DWH oil spill, which are typically near −27‰, reflecting deltaic origins (Chung et al. 1992, W. Graham et al. 2010, Carmichael et al. 2012b). Given the 5 to 10‰ enrichment in dolphin skin compared to oil sources, it is likely that dolphins assimilated little or no dietary C from oil-derived sources. This finding does not mean that dolphins did not ingest oil materials but that it was not a significant component of their assimilated diet during the 6 to 8 wk (skin turnover rate, Browning et al. 2014b) prior to sampling. $\delta^{15}N$ values were consistent with the range of values found in other studies reflecting the trophic level of prey for coastal and estuarine bottlenose dolphins (Barros & Wells 1998, Gannon & Waples 2004, Wilson et al. 2013). However, $\delta^{15}N$ values vary with type of local nutrient loading (Macko & Ostrom 1994, Schlacher et al. 2005), which can confound interpretation of $\delta^{15}N$ values and may account for some of the differences among nodes (Rossman et al. 2013). $\delta^{34}S$ values were consistent with expected increases in salinity from freshwater to marine systems (Peterson et al. 1986, Fry & Chumchal 2011). Although $\delta^{34}S$ is less frequently used as a tracer than $\delta^{13}C$ or $\delta^{15}N$, the additional power of $\delta^{34}S$ to discriminate sources based on salinity was essential for assigning dolphins stranded after the oil spill to coastal or BSE stocks.

The high probability (80.5%) of correct assignment to Estuarine, BI, or Coastal stock indicates predictable variation in stable isotopes within each region and fidelity to habitat within the time frame represented in the skin sample. Stable isotope data have demonstrated habitat residency of invertebrates and fish across a number of studies in the northern GoM. Samples from 118 species of benthic fish from the estuarine waters of Barataria Bay, Louisiana, to lower saline waters further north in the river systems, formed 4 distinct isotopic groups also primarily based on stable isotope ratios of carbon and sulfur (Fry 2002). Using stable isotopes of sulfur, Fry & Chumchal (2011) were able to identify transience and residence in more than 60 species of fish, including known prey of bottlenose dolphins (Barros & Wells 1998), from less saline to more saline areas in Barataria Bay and Breton Sound, located west and east, respectively, of the MS River Delta. From 9 sites bordering Mississippi Sound, Comyns et al. (2008) identified differences in stable isotopes of carbon and oxygen, along with 5 trace elements, in spotted seatrout *Cynoscion nebulosus*. Assignment probability was greater than 90% for 6 of the sites and greater than 70% for the remaining 3 sites, with $\delta^{13}C$ and $\delta^{18}O$ showing the highest regional affinities. Adjacent sites were within tens of kilometers. On a finer scale, juvenile gray snapper *Lutjanus griseus* had a correct assignment probability of 80% to nursery sites within 10 km of each other in the Florida Keys, also using $\delta^{13}C$ and $\delta^{18}O$. Stable isotope values of carbon and sulfur from fish sampled across 10 sites in the eastern panhandle of Florida (among estuarine, nearshore, and offshore waters) resulted in a correct classification rate of 73% (Wilson et al. 2013). Using results from fish collected from known locations as training data, Wilson et al. (2013) applied the model to samples from dolphins, resulting in 89% of dolphins (16 of 18) sampled from one of the estuarine systems correctly classified to that estuary. The assignment probabilities for the training sample from the current study are similar to those for fish and dolphins from the same or nearby areas in the northern GoM.

Linear covariation between $\delta^{13}C$ and $\delta^{15}N$ is a characteristic of marine systems (Kelly 2000). More specifically, in the northern GoM, increasing values of $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$ from more estuarine to more coastal habitats have been reported in a number of fish studies (Clementz & Koch 2001, Wissel & Fry 2005, Fry & Chumchal 2011, Fulford & Dillon 2013, Olsen et al. 2014). Increases of more than 4‰ for $\delta^{13}C$ and $\delta^{34}S$ have been found in a range of species from 5 stations in Apalachicola Bay, Florida, spanning from the upper estuary of the Apalachicola River to mid-bay to a coastal reef site (Chanton & Lewis 2002). Fry (2002) reported enrichment in $\delta^{13}C$ and $\delta^{34}S$ in 118 species of benthic fish from the Mississippi River (freshwater) to the brackish waters of Barataria Bay (average increase in $\delta^{13}C$ of 6‰ and in

$\delta^{34}S$ of over 11‰). Sulak et al. (2012) compared $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$ in multiple species of fish from riverine freshwater habitats to marine habitats in western Florida; $\delta^{13}C$ increased from a range of −17.2 to −20.3‰ in estuarine species to −15.0 to −17.7‰ in coastal GoM species, and $\delta^{34}S$ increased from a range of about 11.0 to 20‰ from estuarine to coastal species, respectively. The linear covariation between $\delta^{13}C$ and $\delta^{15}N$ found in the current study was consistent with previous reports, although the magnitude of enrichment was lower (maximum differences of 3, 2, and 5‰ for $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$, respectively, between mean values) compared to fish studies. This difference may be due, in part, to fish sampled in very low salinity areas where bottlenose dolphins typically spend less time (Hornsby et al. 2017, this Theme Section).

In bottlenose dolphins from the GoM coast of Florida, similar trends were found in stable isotope values among habitats. Samples from coastal and offshore dolphins had higher $\delta^{15}N$ values than estuarine samples (+1.5 to 2.5‰), and $\delta^{34}S$ values were progressively higher from estuarine to coastal to offshore (by approximately 4 and 5‰, respectively; Barros et al. 2010). The magnitude of increase for Florida dolphins was similar to dolphins from the northern GoM coast for $\delta^{15}N$, while the range for $\delta^{34}S$ was much greater for Florida animals, reflecting relatively low $\delta^{34}S$ values in the Florida estuary, Sarasota Bay, relative to Barataria Bay and Mississippi Sound. Further, $\delta^{13}C$ was higher in Sarasota Bay relative to both coastal and oceanic samples from Florida and samples from the northern GoM coast. The relatively low $\delta^{34}S$ and high $\delta^{13}C$ values in Sarasota Bay were due to the influence of extensive seagrass habitat (Fry et al. 1982, Trust & Fry 1992, Hemminga & Mateo 1996, Connolly et al. 2004, Barros et al. 2010, Rossman et al. 2013). Seagrass habitats are common in Sarasota Bay and have a variable presence in some of the western Florida Panhandle bays (Yarbro & Carlson 2013) but are less common in both Mississippi Sound and Barataria Bay (Handley 2011). Nonetheless, along the GoM coast of Florida and the northern GoM, sulfur isotopes were the most diagnostic for distinguishing estuarine or nearshore and barrier island dolphins from coastal or offshore dolphins.

Differences were found in stable isotope values in samples from stranded dolphins relative to the biopsy samples. The most consistent difference was higher $\delta^{15}N$ values in skin from stranded dolphins. One proposed cause of $\delta^{15}N$ increase is protein catabolism resulting from nutritional stress, which might be expected for stranded animals nutritionally compromised before stranding. Following the DWH spill, animals were known to be in poor body condition (Schwacke et al. 2014), and it has been speculated that altered or reduced food resources due to the spill could have contributed to this (Carmichael et al. 2012a, Schwacke et al. 2014). It is also possible that poor condition and physiological impairment could have affected the ability to find or capture prey following the spill (Carmichael et al. 2012a). Both of these scenarios could result in catabolic conditions and concomitant increases in $\delta^{15}N$ values in stranded animals. However, the evidence for increases in $\delta^{15}N$ values due to starvation or nutritional stress is mixed. In a meta-analysis of published literature, Hertz et al. (2015) concluded that $\delta^{15}N$ values significantly increase due to nutritional restriction. In contrast, no significant differences in $\delta^{15}N$ values were found between stranded and bycaught harbor porpoises *Phocoena phocoena* in the North Sea exhibiting poor, moderate, or good body condition (Das et al. 2004), and no change was found in muscle from striped dolphins *Stenella coeruleoalba* with low lipid content in blubber (Gómez Campos et al. 2011). A significant but small decrease in $\delta^{15}N$ values was found in fin whales *Balaenoptera physalus* during the normal fasting that occurs during migration (Aguilar et al. 2014). Aguilar et al. (2014) suggested that the response to fasting by mammals with massive lipid reserves and limited access to drinking water, such as mysticetes, may represent a special case not directly applicable to birds and terrestrial mammals, while Gómez Campos et al. (2011) suggested that striped dolphins may undergo interim conditions of nutritional stress but do not experience strong, seasonal limitations in feeding that would lead to muscle catabolism. In contrast, BSE dolphins tend to be resident or make only relatively small or short-term movements (Wells et al. 2017), without a fasting stage. Thus, whether nutritional stress results in an increase in $\delta^{15}N$ values may depend on the underlying life history of a particular species. Additional studies may help confirm whether elevated $\delta^{15}N$ values in stranded BSE bottlenose dolphins was indicative of nutritional stress prior to stranding.

The increased variance in and range of isotopic values in stranded dolphins also suggests that some stranded animals originated from source populations not included in the training sample. While estuarine-resident bottlenose dolphins in the northern GoM generally exhibit site fidelity, movements beyond primary habitats have been documented. Paternity studies of bottlenose dolphins in Sarasota Bay, Florida, showed that approximately 15% of

calves were sired by non-resident males (Duffield & Wells 2002). Of 10 bottlenose dolphins radio-tagged in Matagorda Bay, Texas, 7 exhibited confined movements near the tagging site, while 3 (tag life 21–59 d) ranged into adjacent bays, with 1 animal spending about half its time outside Matagorda Bay (Lynn & Würsig 2002). Of 23 bottlenose dolphins radio-tagged near St. Joe Bay, Florida, 19 remained fully or predominantly in the focal survey region (Balmer et al. 2008), while 1 dolphin travelled about 100 km to Destin, Florida, and 2 were sighted in Mississippi Sound, more than 300 km from St. Joe Bay (Balmer et al. 2016). It was subsequently proposed that the latter 3 dolphins belonged to the coastal stock, despite having been tagged or photo-identified in an estuary. Seasonal fluctuations in abundance of dolphins in BSE along the GoM coast have been attributed to dolphins from coastal stocks periodically venturing into BSE waters (e.g. Hubard et al. 2004, Balmer et al. 2008, Conn et al. 2011), and the tagged dolphins from St. Joe Bay apparently support that hypothesis (Balmer et al. 2016). From photo-ID studies in Choctawhatchee and Pensacola Bays (easternmost bays within the area most affected by the DWH oil spill), 650 individual dolphins were identified; 88% were considered resident, and the remainder were considered transients on the basis of resightings (Shippee 2014). Of 44 satellite-tagged bottlenose dolphins in Barataria Bay, half ventured into nearshore waters of the GoM, spending, on average, 5.7% of their time outside the estuary (Wells et al. 2017). Such movements could affect the isotopic composition of dolphins, the degree to which would depend on where and for how long dolphins underwent inter-estuarine or coastal movements.

Underlying seasonal changes in the environment or seasonal changes in dolphin diet would also affect the stable isotope composition (Browning et al. 2014b, Ansmann et al. 2015). Seasonal variation in values of $\delta^{13}C$ and $\delta^{15}N$ has been reported in sediments, organic matter, and primary producers in cold- and warm-water estuaries (Ostrom et al. 1997, Vizzini & Mazzola 2003, Bergamino et al. 2014) and in lakes (Perga & Gerdeaux 2005). In a comparison of 2 tidal rivers along the central GoM coast of Florida, seasonal changes in $\delta^{13}C$ and $\delta^{15}N$ occurred in primary and secondary consumers, but not higher trophic levels (Olin et al. 2013). In Mississippi Sound, increases in both $\delta^{13}C$ (by about 4‰) and $\delta^{15}N$ (by about 2‰) occurred from summer to fall in spotted seatrout (Fulford & Dillon 2013). Seasonal differences found in several species of fish collected in Pensacola and Choctawhatchee Bays, Florida, were also reflected in skin samples from resident dolphins (Worthy et al. 2013). Perga & Gerdeaux (2005) reported that the seasonal changes in $\delta^{13}C$ and $\delta^{15}N$ of prey were reflected in liver tissue, with high turnover, but not in muscle, with lower turnover, of whitefish *Coregonus lavaretus* during autumn and winter when fish somatic growth was limited. Given the magnitude of some of the seasonal variation reported elsewhere, it is possible that incorporating season as a predictor variable in the current study would have increased the probability of correct assignment to stock for dolphins from the northern GoM coast, but the seasonal distribution of existing samples was insufficient. Future use of stable isotopes for similar studies may benefit from consideration of season during experimental design.

Two additional factors, tissue turnover rate and decomposition, may also affect the variability and range of stable isotope values in our samples. The stable isotope values in skin samples represent the time-averaged integration of assimilated stable isotopes. Browning et al. (2014b) were able to determine experimentally that the mean (±SE) half-lives of $\delta^{13}C$ and $\delta^{15}N$ in epidermis were 13.9 ± 4.8 and 15.3 ± 1.7 d, respectively. Thus, isotopic values in dolphin skin represent prey consumption for a relatively short period of time, 6 to 8 wk, especially given the mobility of the dolphins. For example, an individual dolphin moving between 2 habitats containing prey with different isotopic values would be expected to maintain an isotopic composition intermediate between the compositions of resident dolphins from either 2 habitats, if sampling occurred within the 6 to 8 wk turnover time of skin. The effect may be predicted assignment to the wrong habitat or a decrease in precision in assignment probabilities (Thomas et al. 2017, this Theme Section). Samples from stranded animals reflect a range of tissue quality as a result of decomposition. In skin-plus-muscle samples from 3 fresh-dead striped dolphins left outside to decompose from 0 to 62 d, during which time the tissue decomposed from condition code 2 (fresh) to code 4 (advanced decomposition; Geraci & Lounsbury 2005), no difference was found in the isotope composition of carbon or nitrogen (Payo-Payo et al. 2013). Our results support that decomposition may not be a large source of variability in stable isotope signatures, although sample sizes were small in both studies.

Despite the sources of variability and uncertainty, stable isotope compositions were useful for differentiating Estuarine, BI, and Coastal dolphins in the

northern GoM. The identification of separate BI and Estuarine groups in the current study is consistent with data from recent telemetry (Wells et al. 2017, K. D. Mullin et al. unpubl. data) and genetics (Rosel et al. 2017) studies. Overall, stable isotope ratios have proven useful to differentiate groups of dolphins at relatively fine scales among seasons and sites, even at some sites within the same estuaries (Worthy et al. 2013, Shippee 2014). Stable isotope values cannot address demographic independence, and, for many tissues, represent only a short-term signal of habitat preferences or prey. Nevertheless, stable isotope analyses are relatively inexpensive and fast, facilitating use of results to provide guidance regarding where genetic or other demographic testing is warranted. In addition, stable isotope ratios may aid in determining the source habitat of stranded dolphins, further increasing the value of biological data collected from strandings.

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