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SHORT COMMUNICATION

DEAD OR ALIVE: USE OF ELEMENTAL ANALYSIS TO DETERMINE STATUS OF STRANDED PERINATE BOTTLENOSE DOLPHINS (*TURSIOPS TRUNCATUS*)§

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INTRODUCTION

In the northern Gulf of Mexico (GOM) there has been an increase in perinate bottlenose dolphin (*Tursiops truncatus*) mortalities in recent years, evidenced in elevated numbers of late–term pregnancy failures compared to reference populations and historical stranding data from the region (Carmichael et al. 2012, Venn–Watson et al. 2015, Colegrove et al. 2016). Perinate mortality can occur *in utero*, during or just after birth (Colegrove et al. 2016). Determining at what stage a perinate dolphin died (*in–utero* or following parturition) is the first step to better understand the cause of pregnancy failure. For example, *in utero* death can occur as a result of uterine infection or abnormalities, fetal abnormalities, dystocia, or fetal distress, compared to a live birth with failure to thrive post–parturition. Understanding the causes of death in perinates is integral to stock assessments and population management.

Straight length, histology, and stomach contents can be used to define perinate status after stranding. Straight length (SL) is used to define age class (Read et al. 1993), whereas histological evaluation of lung tissue can help determine if a perinate dolphin took a breath following birth, and milk in the stomach indicates successful nursing (Turan et al. 2013). However, tissue loss from scavenging and decomposition can hinder SL determination and prevent accurate histological evaluation (Cummings et al. 2011), and a lack of recent nursing may result in an empty stomach despite successful prior nursing. Hence, there is a need for additional approaches to aid in the determination of live or dead status of stranded perinates.

Elemental analyses have potential to help determine live or dead status of perinate dolphins by tracking differences in nutritional status with age. Carbon to nitrogen ratios (C:N), for example, tend to reflect changes in diet quality throughout life, with C:N typically lower in high protein diets and often used as a proxy for relative lipid content in tissues (Post et al. 2007, Newsome et al. 2010). Stable isotope (SI) analysis in marine mammals has been used to study the transfer of maternal nutrients to a fetus during gestation, the shift to a lipid–rich milk–based diet after birth, and weaning of calves (Polischuk et al. 2001, Valenzuela et al. 2010, Borrell et al. 2016). Although isotope turnover rates vary among taxa, dietary discrimination and tissue–specific turnover rates are relatively consistent within species under common metabolic conditions (Michener and Kaufman 2007, Vander Zanden et al. 2015). The metabolic differences among tissues provide an opportunity to trace changes in nutritional status on different time scales, with muscle reflecting a longer–term diet (weeks) and organs such as skin and liver reflecting recent diet (days) (Browning et al. 2014, Vander Zanden 2015, Rode et al. 2016). Previous work to understand the effects of post–mortem decomposition on SI values in cetaceans found, after 62 days at ambient temperature, muscle and skin from striped dolphins (*Stenella coeruleoalba*) had no statistical change in $\delta^{13}C$ or $\delta^{15}N$ values (Payo–Payo et al. 2013). Therefore, elemental analyses may be useful to trace age–specific nutritional status that can indicate death *in utero* or following parturition even among moderately decomposed stranded perinates.

The objective of this study was to test the use of elemental ratios (C:N, $\delta^{13}C$, $\delta^{15}N$) to determine if stranded perinate bottlenose dolphins were born dead or alive based on expected diet shifts from *in utero* (fetus) to nursing (calf). We hypothesized that C:N would be higher and $\delta^{13}C$ lower in calves and perinates born alive that have successfully nursed compared to fetuses and dead perinates that would not have transitioned to a lipid–rich milk–based diet, and that this response would be most detectable in liver due to its higher lipid content and rapid turnover rate compared to other tissues (skin, muscle). In addition to these differences among young animals, we hypothesized that $\delta^{15}N$ would be higher in all young animals measured in this study (fetus to calf) compared to adults due to the maternal contribution to food

§ The first author conducted this research as part of the Dauphin Island Sea Lab's Research Experience for Undergraduates program.

sources *in utero* or via nursing.

Materials and Methods

Tissue samples were collected from 46 dolphin carcasses that stranded on the Alabama coast from 2015–2019. Carcasses were categorized into 4 age classes based on SL and reproductive status (Read et al. 1993, Mattson et al. 2006): fetus (n = 12, < 95cm), perinate (n = 18, 96–115 cm), calf (n = 11, 116–169 cm), or adult (n = 5, >230 cm or evidence of lactation or pregnancy), including one lactating female and one late term pregnant female (mother = 229 cm, *in utero* fetus = 91cm). Perinates were further categorized as dead (n = 10) or alive (n = 8) at birth, based on histological evidence of aerated lungs or milk in the stomach. Tissue samples were collected from animals of condition codes 2 (fresh dead) and 3 (moderately decomposed), and included a combination of skin (n = 43), muscle (n = 42), liver (n = 33), depending on tissue condition; all three tissue types were sampled from 32 animals. For histological evaluation, lung tissue was fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 5µm, stained with hematoxylin and eosin, and evaluated by veterinary pathologists (University of Illinois College of Veterinary Medicine, Zoological Pathology Program). Code 2 carcasses had full histologic evaluation on all organ systems. Milk (n = 1) was collected from the lactating female. All tissues were stored at –20°C prior to processing.

For elemental analysis, tissue samples were rinsed with ultra–pure water and divided into 2 subsamples. One subsample was dried to a constant weight at 60°C (hereafter referred to as bulk samples). To allow SI analysis normalized for differences in lipid content among tissues, the other subsample was lipid extracted (LE) using a modified Sweeting–Folch method (Sweeting et al. 2006, Mintenbeck et al. 2008). Subsampled tissues were macerated in ultra–pure water using a handheld rotor–stator homogenizer (Waverly H100, Waverly Scientific, USA), sonicated in 2:1 chloroform:methanol, and centrifuged for 10 min at 3000g. The process was repeated as needed (1–2x) until the supernatant was clear, indicating lipids were removed, and samples were dried as before. Bulk and LE samples were ground to a powder using a mortar and pestle, packed in tin capsules and sent to the UC Davis Stable Isotope Facility for analysis by continuous–flow isotope ratio mass spectrometry (PDZ Europa 20–20), after combustion to CO_2 and N_2 by an online elemental analyzer, which allowed determination of N and C content. To detect potential handling effects, randomly selected pseudoreplicates were analyzed for 18% (n = 22) of the samples, resulting in mean reproducibility (± SD) of 0.1 ± 0.4‰ for $\delta^{13}C$ and 0.1 ± 0.1‰ for $\delta^{15}N$. These values are below the long–term reproducibility for the instrument (standard deviation 0.2‰ for $\delta^{13}C$ and 0.3‰ for $\delta^{15}N$; https://stableisotopefacility.ucdavis.edu).

Molar C:N values were determined from analysis of bulk tissues, and due to differences in SI values following extraction, $\delta^{13}C$ values in LE tissues and $\delta^{15}N$ values in bulk tissues were used for all SI comparisons and associated statistical analyses. Two–way Analysis of Variance (ANOVA) was used to compare C:N or SI values among age groups (fetus, dead perinate, alive perinate, calf, adult) and tissue types (muscle, skin, liver). Differences in SI ratios between bulk and LE tissues were compared to provide an estimate of relative lipid content among samples. Regression analysis was used to relate lipid content ($\delta^{13}C‰_{LE–bulk}$) to bulk C:N.

Results

The C:N in tissues ranged from 4–11 in muscle, 4–9 in skin and 4–13 in liver; although the main effects were not different, there was an interaction between age group and tissue type (2–way ANOVA: $F_{4,2,8} = 2.75$, $p < 0.01$). This pattern was driven by higher mean C:N in muscle from fetuses (< 95 cm) compared to live perinates (96–114 cm), calves (115–169 cm), or adults (>230 cm) (Tukey HSD: $p < 0.02$ for all comparisons; Figure 1, A–C). The C:N in muscle of dead perinates, however, was similar to fetuses and all

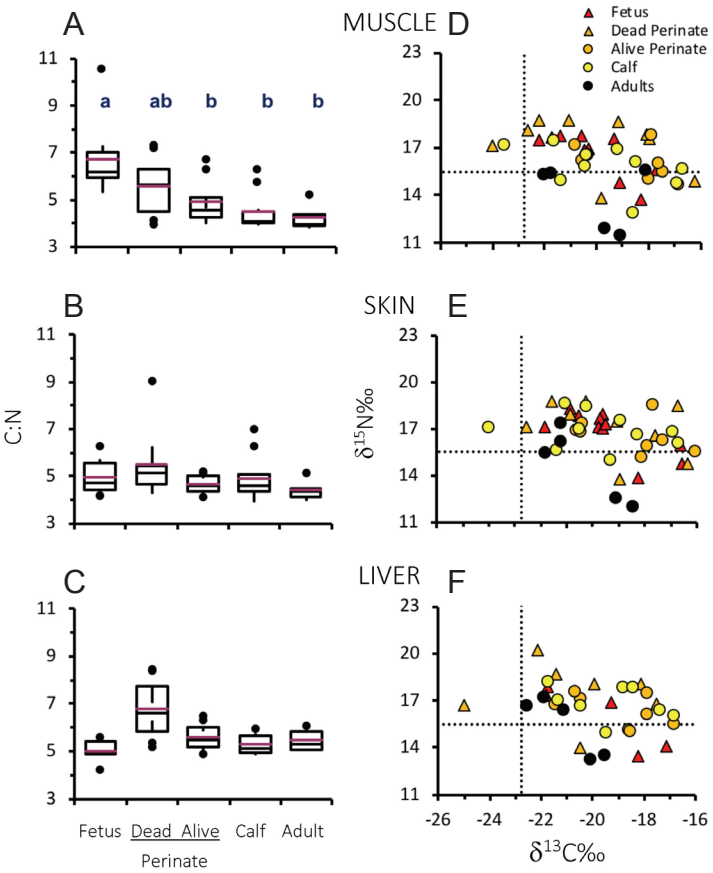

FIGURE 1. *Elemental ratios in tissue from bottlenose dolphins in different age groups (fetus, dead perinate, alive perinate, calf, adult). A. Carbon to nitrogen ratio (C:N) in muscle tissue. Lower case letters indicate significant difference (Tukey HSD, p<0.02) B. C:N in skin tissue. C. C:N in liver tissue. D. Stable isotope values in muscle tissue. E. Stable isotope values in skin tissue. F. Stable isotope values in liver tissue. In the box plots (A, B, C), the purple and black lines represent mean and median C:N values, respectively. In the scatter plots (D, E, F), the dashed lines show stable isotope values in milk from a lactating female. One value in calf liver (C:N=13.4) is not shown for clarity of scale among the remaining data.*

other age groups due to a logarithmic decrease in C:N with increasing body size found only in muscle ($y = -2.58\ln(x) + 17.57$, $R^2 = 0.33$, $F_{1,40} = 20.08$, $p < 0.001$).

The $\delta^{15}N$ values in muscle from all juvenile groups (fetuses, perinates, calves) were 2–4‰ higher than in adult muscle (ANOVA: $F_{4,2} = 2.45$, $p < 0.001$; Tukey HSD: $p < 0.01$ for all comparisons; Figure 1D). These differences were greatest (3–4‰) between adults (mean ± SD: 14 ± 2‰) and fetuses (17 ± 1‰, $p<0.01$) or dead perinates (17 ± 2, $p<0.001$). There were no other differences in $\delta^{13}C$ or $\delta^{15}N$ values among age groups or tissue types (Figure 1D–F). In all age groups $\delta^{13}C$ values in LE tissues ranged −16 to −25‰, consistent with a marine and estuarine based diet, and $\delta^{15}N$ values ranged 11 to 20‰ in bulk samples, consistent with life as a higher trophic level consumer. All juvenile age groups had mean $\delta^{13}C$ and $\delta^{15}N$ values higher than found in the available sample of milk (−22.8‰ and 15.5‰, respectively; Figure 1D–F, dashed lines).

Relative lipid content ($\delta^{13}C‰_{LE-bulk}$) increased with C:N ($y = 0.27x - 0.51$, $R^2 = 0.30$, $F_{1,116} = 49.62$, $p < 0.001$). However, one calf with severe hepatic lipidosis (diagnosed via histology) had an unusually high relative lipid content and corresponding C:N (13.4) in the liver. The relationship between C:N and relative lipid content was significant even with removal of the hepatic lipidosis outlier ($y = 0.21x - 0.21$, $R^2 = 0.16$, $F_{1,115} = 22.01$, $p < 0.001$; Figure 2). The low values for R^2 in the combined dataset (combined for simplicity in this study) reflect the variation in relative lipid content among tissues and highlight the value of analyzing tissues separately.

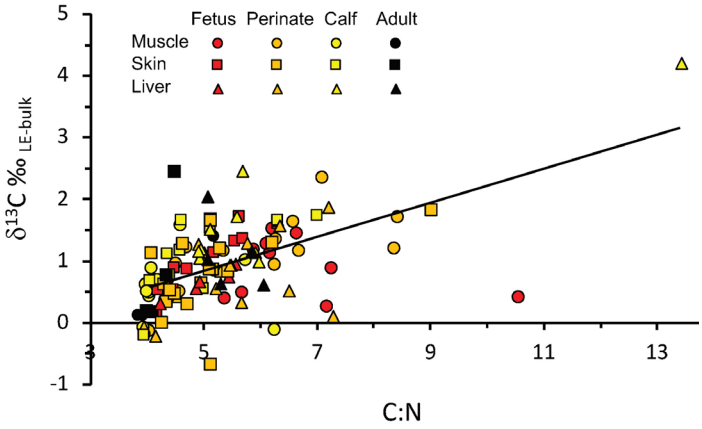

FIGURE 2. *The relationship between lipid extracted and bulk $\delta^{13}C$ values compared to carbon to nitrogen ratios (C:N) in tissues from bottlenose dolphin of different age groups (fetus, perinate, calf, adult). Data are combined for alive and dead perinates.*

Analysis of a pregnant female allowed direct SI comparison of a mother–fetus pair (Table 1). Fetus tissue was enriched in $\delta^{15}N$ compared to maternal tissues, with the greatest difference (2.4‰) in muscle. The $\delta^{13}C$ values in tissues were similar between mother and fetus.

TABLE 1. Stable isotope values in tissues from a known mother–fetus pair of bottlenose dolphins, and the difference (Diff.) between values.

	$\delta^{13}C‰$			$\delta^{15}N‰$		
Tissues	**Mother**	**Fetus**	**Diff.**	**Mother**	**Fetus**	**Diff.**
Muscle	−21.9	−21.7	0.3	15.2	17.7	2.4
Skin	−22.5	−21.8	0.7	16.6	17.3	0.7
Liver	−21.2	−20.9	0.3	17.3	18.3	1.0

Discussion

While neither C:N nor SI values could unambiguously distinguish live from dead perinates in this study, our results suggest that C:N in muscle may be useful to distinguish fetuses from dolphins born alive. Higher C:N and heavier $\delta^{15}N$ in young animals compared to adults and trophic similarity to milk among all young age groups can be explained by maternal–based nutrition derived from either *in utero* plasma or milk. Animals *in utero* or nursing are functionally consuming the mother's tissues and should appear relatively isotopically enriched (Newsome et al. 2010). This finding was validated in the mother–fetus pair, having N enrichment from mother to fetus in muscle that was consistent with a trophic step from food source to consumer (Michener and Kauffman 2007, Newsome et al. 2010). Overall, these findings suggest that C:N and SI ratios are indicative of age group–specific nutritional patterns that are independent of live or dead status and cannot be reliably distinguished in stranded dolphins because nutritional sources *in utero* and immediately post–birth are both of maternal origin.

The C:N was related to relative lipid content in muscle, liver and skin of all age groups. Lipid extraction of tissues was necessary prior to $\delta^{13}C$ analysis to ensure normalized comparison of SI values among age groups and tissue types, consistent with a previous study (Giménez et al. 2017). Higher lipid content and corresponding C:N in liver of an animal that was diagnosed with severe hepatic lipidosis (a common result of poor nutrition or starvation) demonstrates the potential for elemental analyses to also contribute to the identification of metabolic stress (Center et al. 1993, Choudhury and Sanyal 2004, Venn–Watson et al. 2012). The C:N, therefore, may be useful to corroborate possible metabolic stress in carcasses of any age that are too decomposed for confident histological evaluation alone. Future work could further test the reliability of elemental ratios to diagnose hepatic lipidosis and other related diseases.

A number of studies have shown ontogenetic shifts in elemental ratios in dolphins and in a variety of other marine mammals that are consistent with changes found among age groups in this study (e.g., Newsome et al. 2010, Browning et al. 2014, Borrell et al. 2016, Cherel et al. 2015). This study is unique, however, in extending these comparisons to animals based on evidence of live or dead condition at birth,

and in providing a first step to defining ontogeny–specific trophic ecology for dolphins throughout life in the northern GOM. As with any study on stranded animals, a lack of history about carcasses prior to stranding limits the ability to fully interpret some data. Additional studies on captive dolphins with known late–term fetus mortality or live birth with known duration of nursing could better resolve status–specific isotopic shifts. Analysis of additional mother–fetus pairs and high–quality plasma and milk samples could help set a baseline for future comparisons to stranded perinates of unknown origin to improve the application of elemental ratios to detect diet–related differences among fetuses and calves. Finally, our data can be combined with analysis of older calves and subadults to trace the shift to forage–based diet and, in turn, better define age of weaning and dependence on local fish and invertebrate stocks. Elemental ratios, therefore, have potential to support conservation of marine mammal resources and broader ecosystem–based management by defining trophic patterns and nutritive links to causes of mortality.

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