Nutritional and Physiological Status in Cold-Stunned Juvenile Sea Turtles: insight from stable isotope analysis in muscle, skin and blood tissues in Kemp’s ridley sea turtles (*Lepidochelys kempii*).

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Abstract:

Sea turtles annually migrate to the coasts of the Northeastern United States, venturing farther north every year along the Gulf Stream into the Gulf of Maine. During the warmer months, sea turtles utilize the nutrient-rich waters of New England before migrating south as Fall approaches. However, many of these individuals cannot navigate out of Cape Cod Bay due to the geographic configuration of Cape Cod’s eastern shorelines, which create a land barrier and prevent sea turtles from accessing open waters. These sea turtles, particularly the critically endangered Kemp’s ridley (*Lepidochelys kempii*), become cold-stunned as water temperatures decline. The cold-stunned sea turtles that do not survive provide valuable opportunities for scientific research that allows for a better understanding of how these animals are interacting with Cape Cod habitats.

Literature elaborating the biochemistry of sea turtles is very limited, despite the crucial value of this information in understanding the implications of cold-stunning on the physiology of sea turtles. In order to better understand how cold-stunning affects the biochemistry of sea turtles, we used $^{13}$Carbon and $^{15}$Nitrogen stable isotopes to analyze the pectoral muscle, skin and blood (whole and lipid-extracted) tissues of 24 dead, cold-stunned Kemp’s ridley turtles from the 2017 stranding season. All tissues, with the exception of whole blood, were lipid-extracted to reduce bias. In data analysis, turtle weight was used as a proxy for age. For $\delta^{13}$C analyses, there was not a significant trend between $^{13}$C content in tissues and turtle weight or fat thickness. Skin was the most enriched of the tissues by a range of about 3‰, while lipid-extracted blood, whole blood and pectoral muscle exhibited very similar $\delta^{13}$C composition. Variation was observed in blood samples of younger, smaller turtles which indicates the presence of a physiological component associated with turtle age. From $^{15}$N stable isotope analysis, we found that age and growth play a significant role in the integration of $^{15}$N and that smaller, younger turtles might have a slower integration rate than larger, older individuals, who turn over $^{15}$N more frequently. We observed a minimal offset between the $\delta^{15}$N of skin, blood and pectoral muscle tissues, although pectoral muscle exhibited the most dramatic trend with age. In smaller, younger turtles between 1-2.5kg, pectoral muscle $\delta^{15}$N became more enriched by about 2.5‰ before plateauing in turtles >2.5kg at 10‰. Holistically, the analysis of stable isotopes in pectoral muscle, skin, and blood tissues suggests a large physiological transition occurring in small, young turtles (<2.5kg). These findings are useful in the purview of sea turtle conservation efforts, providing a more thorough understanding of the biological drivers of cold-stunning and stable isotope composition in sea turtles.

**Keywords:** Cold-stunning, Stable Isotopes, Kemp’s ridley, pectoral muscle, skin, whole blood, lipid-extracted blood.
**Introduction:**

Sea turtles are widely distributed along coastlines of the Northeastern United States, venturing farther north every year along the Gulf Stream into the rapidly warming waters of the Gulf of Maine. Various species of sea turtles migrate into the productive coastal areas of New England during the warm summer months in search of abundant seagrass, crabs and jellyfish. Spending their entire lives at sea, sea turtles are elusive and difficult to study intimately, especially juveniles who spend this cryptic life stage in almost constant trans-oceanic migrations. These northern migrations make sea turtles susceptible to hypothermic conditions as the early winter months approach and water temperatures plummet.

Kemp’s ridley sea turtles are the most critically endangered species of sea turtle in the world, with a population that continues to struggle against anthropogenic presence in Gulf of Mexico waters. Legislation in 1991 requiring the installation turtle exclusion devices (TED) on all shrimp trawlers in the Gulf of Mexico allowed Kemp’s populations to recover until 2010, when the catastrophic Deepwater Horizon oil spill inhibited Kemp’s Ridley population recovery. Kemp’s Ridley nesting sites are limited to the gulf coasts of Mexico and Texas, with the vast majority of these nests recorded in Rancho Nuevo (Shaver et al. 2016). Kemp’s ridleys navigate energetically demanding migrations every year between their nesting grounds in Rancho Nuevo and foraging areas in the Northeastern United States, using the Gulf Stream as a conduit between locations.

Cape Cod observes large turtle stranding events every year within Cape Cod Bay due to cold-stunning, resulting in the mortality of hundreds of endangered and threatened sea turtles. Effective management of threatened sea turtle species requires understanding of habitat utilization. Kemp’s ridley juveniles constitute the largest cohort of cold-stunned sea turtles every
year in Cape Cod. It is unknown why Kemp’s Ridleys are the most prolific during cold-stunning events, although, being the smallest among sea turtle species, we hypothesize that size is a significant factor in stranding frequency. Availability of cold stunned turtles provide the opportunity to assess how cold-stunning affects sea turtle physiology, as temperature has a significant role in deciding sea turtle fitness. Since sea turtles are ectotherms, their body temperature is regulated by the ambient temperature of the ocean; therefore, when ocean temperatures drop, sea turtles become incapacitated and lethargic, which causes loss of mobility and the ability to find prey (Avens et al. 2012). This change in feeding habits, and consequent physical degradation, is predictably reflected in the biochemistry of cold-stunned individuals and provides useful information that will contribute to an understanding of how cold-stunning affects these animals (Doi 2017).

Stable isotope analysis has become an increasingly prevalent tool in environmental studies. Analysis of stable isotopes presents a quantitative expression of isotopic ratios within a sample, expressing the ratio of heavy $^{15}$N isotopes to light $^{14}$N ($^{15}$N:$^{14}$N), and heavy $^{13}$C isotopes to light $^{12}$C isotopes ($^{13}$C:$^{12}$C) relative to a standard. Isotopic ratios are expressed in parts per thousand ($\%$) as $\delta^{13}$C or $\delta^{15}$N = $[(R_{\text{sample}}/R_{\text{standard}})/ R_{\text{standard}}] \times 1000$, where R is the ratio of $^{13}$C :$^{12}$C or $^{15}$N:$^{14}$N, respectively. The analysis of stable isotopes provides valuable insight, tracing the origins of organic substances, and indicating diet change and habitat use. In marine studies, stable isotope analysis provides an understanding of the nutritional, spatial and temporal ecology of marine vertebrates, especially migratory species.

Consumers fractionate carbon and nitrogen in predictable ways that allow for diet reconstruction (DeNiro and Epstein 1978). $\delta^{13}$C describes the carbon source and metabolic pathways from which an organism’s $^{13}$C signature is derived, usually residing between $-14\%$ and
-28‰ within marine systems (O’Leary 1988). $\delta^{13}C$ tends to be relatively consistent and well-conserved with very little enrichment by trophic level, therefore exhibiting a similar $\delta^{13}C$ between a predator and its prey. In higher organisms, Nitrogen isotopes also exhibit this trend with the caveat that $\delta^{15}N$ is enriched by about 3‰ between trophic levels, primarily due to the faster speed of fractionation of light nitrogen isotopes into membranes as well as the excretion of light nitrogen isotopes in urine (Peterson and Fry 1982). Because of this preferential excretion of $^{14}N$, and consequential $^{15}N$ enrichment, the trophic position and diet reconstruction of an organism can be determined. Isotopic fractionation and the integration of nitrogen and carbon within tissues differs by tissue type, being that metabolically active tissues, particularly blood and muscle, fractionate faster with lower $\delta^{13}C$ values than other tissues, such as skin (Fry and Arnold 1982; Vander Zanden et al. 2015). Turnover times are ultimately affected by the components of each respective tissue, depending on how fast these components are acquired, assimilated and utilized by the organism for energy. Therefore, different tissues are capable of providing a temporal resolution of dietary variation over a certain timescale (Gannes et al. 1998).

Growth and age also has an impact on stable isotope signatures, indicating that discrimination factors between tissues are lowest during periods of highest growth. Therefore, younger individuals assimilate more nitrogen to growth, and thus fractionate less than fully-grown individuals (Sears et al. 2008). This differential allocation of nutrients due to growth influences how isotopic signatures are reflected in analyses, with the additional caveat that diet selection also may change among organisms with age. Another important consideration in the analysis of tissue isotopic signatures is the potential for post-depositional catabolism and the effect of starvation on stable isotope ratios. In periods of fasting or starvation, higher organisms will begin to draw energy by catabolizing its own internal protein and lipid reserves, resulting in
a significant increase in $^{15}$N isotope incorporation (Doi 2017).

In this study, we used $^{13}$Carbon and $^{15}$Nitrogen stable isotopes to determine the trophic status and habitat utilization of juvenile Kemp’s ridley ($Lepidochelys kempii$) sea turtles in Cape Cod coastal waters and also how cold-stunning may affect their physiological status. Seminoff (2006) has shown that sea turtle stable isotopes vary with the physiological status of the animal, and are affected by metabolism, age, nutrition and nutrition quality, and body temperature. Pearson (2017) elaborated in his review of stable isotope studies in sea turtle ecology that isotopic ratios change with environmental gradients and resource use. However, few studies have elaborated upon the stable isotope composition of multiple tissues within endangered sea turtle species, and even fewer in juveniles. This study expects tissue isotopic signatures to exhibit physiological condition of the turtles as they wash ashore cold-stunned, addressing questions about a) how the stable isotopes of pectoral muscle, skin and blood tissues differ, b) how stable isotope expression varies with fitness and physiological stress, c) how cold-stunned sea turtles are catabolizing energy reserves, d) the covariance of tissue fractionation. Therefore, this study focuses on quantifying differences in stable isotope composition among pectoral muscle, skin and blood tissues of dead cold-stunned sea turtles. Variation in tissue isotope analysis allows for a much more holistic view of a turtle’s life-history, providing an understanding of an individuals’ condition in the present and recent past (Seminoff 2006).

This paper will detail the analysis of carbon and nitrogen isotopic values (represented in permil) of three different tissue types from dead, cold-stunned turtles from the 2017 stranding season. These results are compared to variables of physiological status such as weight and fat thickness, using weight as a proxy for age.
Our findings indicate that age and growth significantly affects the expression of stable isotopes in different tissues, with some variation in $\delta^{13}C$ and $\delta^{15}N$ that is likely due to physiological stress. Similarly, analysis of accompanying data comparing turtle weight, water temperature and stranding data indicates that smaller turtles are more likely to cold-stun earlier and in warmer temperatures than larger turtles. This study provides valuable information in regards to sea turtle physiological response to cold-stunning, and how cold-stunning affects the biochemistry of turtles and overall sea turtle fitness, and will inform sea turtle conservation efforts as we gain a better understanding of how these animals are interacting with local habitats in the warmer months.

**Background:**

Cape Cod waters provide a nutrient-rich habitat for migratory sea turtles, especially Kemps Ridley turtles, which migrate farther north every year, following the Gulf Stream from nesting sites near Rancho Nuevo, Mexico to the Gulf of Maine during the warm summer months. The Gulf of Maine is among the most rapidly-warming ocean basins in the world, recording a temperature of 20.52°C (68.93°F) in August of 2018, according to a 2018 ocean surface temperature report by NASA’s Earth Observatory. While Cape Cod water temperatures are warm until mid-fall, these northern waters are susceptible to cold-snaps in which weather systems quickly alter conditions, causing ocean temperatures to plummet. Once ocean temperatures fall below the critical temperature of about 6°C (42°F), sea turtles become dangerously vulnerable to cold-stunning (Avens et al. 2012).
Sea turtle cold-stunning events on Cape Cod have grown since sea turtles were first spotted around Cape Cod in the early 1970’s. Since 2012, sea turtle cold-stunning has increased significantly along with ocean temperatures, reporting an all-time high of over 1,200 strandings in 2014. 2018 has proven to resemble the 2014 season with an exceptionally high number of strandings of around 700 turtles by the end of November 2018. A single stranding event in November brought in 220 dead turtles which washed ashore irreparably frozen and dead. Climate change is among the most pertinent causes of the increasing turtle cold-stuns, but the geographic configuration of Cape Cod is also a significant contributor to turtle mortality. The shape of Cape Cod, specifically the area extending from Dennis to Provincetown, traps sea turtles in Cape Cod Bay, preventing them from navigating south to warmer climates as the northern waters cool. The sea turtles that do not survive cold-stunning provide valuable opportunities for scientific research, allowing the ability to learn more about these enigmatic, highly endangered animals. While a solution to the cold-stunning issue is not immediately attainable, this research is essential for identifying conservation priorities and better preparing for large cold-stunning events.

**Methods:**

**Laboratory:**

*Collection of cold-stunned turtles and necropsies:*

Cold-stunned turtles were collected from beaches along Cape Cod Bay during the 2017 stranding season (Figure 1). Dead sea turtles were frozen to preserve for analysis. Sea turtle pectoral muscle, skin and blood tissues were obtained from necropsies conducted at Woods Hole Oceanographic Institution necropsy facility in the winter of 2018. Tissues were excised and
frozen at -80°C prior to analyses. This study focused on 24 dead, cold-stunned Kemp’s ridley sea turtles (*Lepidochelys kempii*) from the 2017 beach collections.

**Sample preparation:**

*a) Pectoral Muscle and Skin:*

All muscle and skin samples were removed from -80°C storage to thaw at least 30 minutes before being transferred into separate borosilicate tubes. The samples were freeze-dried for 24 hours. After 24 hours elapsed, samples were removed from the freeze-drier and manually homogenized with a mortar and pestle and razor to produce a fine powder of tissue. Homogenized muscle and skin samples were lipid extracted by ultrasonicating in three rounds of 2:1 chloroform:methanol. Samples were centrifuged, and lipid extract was removed into new vials to be archived in freezer for later studies. This process was repeated two more times in order to ensure that all lipids were extracted. We subsampled a target weight of 1-2 mg of dry tissue (~700mg C) for stable isotope measurements. Samples were packed and folded within 4x6 mm tin capsules for analysis on the Stable Isotope Mass Spectrometer system.

*b) Blood:*

About 0.5mL of blood sample was lipid extracted using a modified Folch extraction method (see appendix A for detailed method). Briefly, we lysed blood cells using ultrasonication and extracted lipids using a 2:1 chloroform:methanol solution. All lipid extracted samples were placed in a Savant centrifugal evaporator until mostly dry and resuspended back to 0.5mL volume using MilliQ water. About 50µL from each lipid extracted sample was transferred into pre-weighed 5x9mm tin capsules. About 25µL of whole blood samples were also transferred into pre-weighed 5x9mm tin capsules. All samples were dried on a hot plate from 60°C-80°C, weighed (to verify dry material weight of 1-2mg) and submitted for isotope analysis.
**Stable isotope analyses:** Subsamples were analyzed at MBL’s Stable Isotope Lab using a Europa 20-20 CF-IRMS interfaced with the Europa ANCA-SL elemental analyzer.

**Results:**

**$^{13}$Carbon:**

In preliminary analyses, $\delta^{13}$C did not significantly vary by turtle weight (Figure 2). $\delta^{13}$C exhibits well-conserved trends for each tissue type, with skin exhibiting the heaviest carbon signature with a range between -14.6 and -16.1‰ for all turtle individuals (Figure 2). Whole blood ($\delta^{13}$C = -17.4 to -18.7‰), pectoral muscle ($\delta^{13}$C = -17.5 to -18.5‰), and lipid extracted blood ($\delta^{13}$C = -17.6 to -18.8‰), exhibited almost identical $\delta^{13}$C ranges (Figure 2). Whole and lipid extracted blood also exhibited very similar $\delta^{13}$C with slight variation (Figure 3). Taking the difference between whole blood and lipid extracted blood, there was a predictably non-random relationship, with the most variation viewed between small turtles between 1-2.5kg and a range between 0.6 and -0.1‰ (Figure 4).

In order to more closely investigate the relationship between pectoral muscle and blood samples, we plotted the difference and found that there was about a 1‰ difference between turtles of 1-2.5kg between pectoral muscle and whole blood, as well as lipid extracted blood (Figure 5; Figure 6). Larger turtles plateaued around 0, indicating that the pectoral muscle and blood $\delta^{13}$C were very similar in larger turtles (Figure 5; Figure 6). There did not appear to be any significant differences in the distribution of $\delta^{13}$C when compared to hip pad thickness, resembling the trend observed with turtle weight (Figure 7). Each tissue remained well-conserved across all fat thicknesses ranging from 4.0-17.0mm (Figure 7).
\textit{Nitrogen:}

$\delta^{15}\text{N}$ displayed variability in fractionation when compared to turtle weight. $\delta^{15}\text{N}$ of the pectoral muscle differed by a maximum difference of 2.5‰ and a continuous trend of enrichment with age, while skin exhibited a maximum difference of 3.1‰, lipid extracted blood 1.9‰ and whole blood a maximum difference of 2.1‰ (Figure 8). We observed an outlier of skin $\delta^{15}\text{N}$, identified as turtle K252, reporting a $\delta^{15}\text{N}$ skin value of 11.3‰, which is 1.3‰ greater than the next largest skin $\delta^{15}\text{N}$ value. The most variation in $\delta^{15}\text{N}$ was observed within turtles between 1-2.5kg (Figure 8). Pectoral muscle exhibited a significant enrichment of 2.5‰ in turtles between 1-2.5 kg (Figure 9). Whole and lipid extracted blood also exhibited very similar $\delta^{15}\text{N}$ with slight variation (Figure 10). Again, taking the difference between whole blood and lipid extracted blood, there was a non-random relationship, with the most variation viewed between small turtles between 1-2.5kg and a range between 0.6 and -0.5‰ (Figure 11). When separated into further weight classes, we observed that in comparing average $\delta^{15}\text{N}$ for pectoral muscle, skin, lipid-extracted blood and whole blood, that the smallest weight class of 1.0-1.5kg exhibited the least overall variation between tissues (SD=±0.19) while the largest weight class of 2.6-6.2 exhibited the largest overall variation (SD=±0.82) (Figure 12).

When compared to hip pad fat thickness, $\delta^{15}\text{N}$ integration did not significantly differ by fat thickness, reflecting consistency among each respective tissue type of turtles with fat thicknesses varying from 4.0mm to 17.0mm (Figure 13). In order to reflect possible covariance among tissues, we plotted the residual $\delta^{15}\text{N}$ values of each tissue type against each other. Results indicate that there may be covariance with skin tissue, which exhibited significant correlations with pectoral muscle ($R^2=0.29$) and whole blood ($R^2=0.24$) (Figure 14). Lipid extracted blood did not fit well to this model ($R^2=0.06$). Turtle K252 was again observed as a possible outlier.
within the data, with large residual values due to its large δ¹⁵N skin value of 11.3‰ (Figure 14). Removing the outlier caused the significance of whole blood ($R^2 = 0.17$) and lipid-extracted blood to decrease ($R^2 = 0.02$), while pectoral muscle ($R^2 = 0.32$) increased in significance with the removal of the outlier, indicating that pectoral muscle may be the only tissue to exhibit true covariance with skin (Figure 15).

In order to emphasize the differences in tissue δ¹³C and δ¹⁵N, we plotted the average difference between each tissue δ¹³C and δ¹⁵N and skin δ¹³C and δ¹⁵N (Figure 16). For δ¹³C, pectoral muscle, whole blood and lipid extracted blood exhibited a consistent offset of about 3‰. There was less observable consistency for the δ¹⁵N, asserting that there is more variation among tissues in the integration of δ¹⁵N (Figure 16). However, pectoral muscle and skin show almost no difference ($\delta^{15}$N<sub>pectoral</sub> - $\delta^{15}$N<sub>skin</sub> = 0.01; SD=±0.65), thus suggesting a covariant relationship (Figure 16).

**Further Analysis:**

Our analysis also suggests that sea turtle fitness is predictably related to the conditions observed on the date of turtle stranding. While this data set is not a complete compilation of 2017 turtle strandings, it suggests that turtle weight is correlated ($R^2 = 0.32$) to water temperature on the day of stranding (Figure 17). Being that water temperature and stranding date is very significantly correlated ($R^2 = 0.95$), turtle weight also exhibited a similar correlation to stranding date ($R^2 = 0.34$) (Figure 18). In these analyses, strandings of this subset that occurred on November 26<sup>th</sup> and November 27<sup>th</sup>, 2017 were excluded due to a large storm system that occurred on these dates. Spatial distribution of individuals within this subset is displayed in Figure 1, with 67% of the strandings occurring between Eastham and Truro, along the eastern shorelines of Cape Cod Bay.
Discussion:

\textit{13}Carbon:

In this study, we found that the $\delta^{13}$C among tissues was very well conserved within each individual tissue type as a function of weight (Figure 2). Lipid extracted blood, whole blood and pectoral muscle exhibited almost identical $\delta^{13}$C values between -17.4‰ and -18.8‰, indicating that these tissues are lighter and similarly metabolically active (Figure 2). When taking a closer look at this relationship through the difference between pectoral muscle and whole blood, we observe a very significant $^{13}$C enrichment in turtles between 1-2.5kg while larger turtles exhibit almost no difference between pectoral muscle and whole blood and lipid extracted blood $\delta^{13}$C (Figure 5; Figure 6). Given that $\delta^{13}$C exhibited some variation between pectoral muscle and whole blood values for smaller turtles between 1-1.5kg (Average difference Pectoral $\delta^{13}$C - whole blood $\delta^{13}$C = 0.48‰) and less variation with larger turtles between 2.6-6.2kg (Average difference Pectoral $\delta^{13}$C - whole blood $\delta^{13}$C = 0.08‰), it is possible that we are observing differential selection of carbon source within younger turtles, or a difference that is due to growth and allocation of carbon since young turtles fractionate slower than older turtles (Reich et al. 2008).

The similarities observed among pectoral muscle and whole and lipid extracted blood is contrary to our expectation that blood would exhibit the fastest turnover time, as prior studies have indicated that blood is catabolized and accrued faster than other tissues (Pearson et al. 2017; Vander Zanden et al. 2015). Multiple studies on healthy juvenile sea turtles have found that whole blood $\delta^{13}$C resides between -20‰ and -22‰ (Seminoff et al. 2006; Reich et al. 2008). Our results reported an average of -18‰ (SD= ±0.36‰), suggesting that cold-stunning may be playing a role in the more enriched $\delta^{13}$C values due to reduced blood flow and circulation in the presence of cold temperatures, or the remobilization of proteins due to fasting in cold-stunned
turtles (Hoschield et al. 2002). Ultimately, since few studies have analyzed stable isotopes in sea turtle pectoral muscle, this finding indicates that both blood and pectoral muscle have similar biochemistry, likely due to the significant metabolic requirements of pectoral muscle which is used by sea turtles for swimming and enduring long migrations.

Skin tissue was offset by about 3‰ from the other tissues, residing between -14.6‰ and -16.1‰ (Figure 2). This indicates that skin tissue is the most $^{13}$C enriched tissue among pectoral muscle, whole and lipid extracted blood. These tissues do not appear to exhibit a very significant trend with size/age, although there is some variation observed in smaller turtles between 1-2.5kg, especially when comparing lipid extracted and whole blood (Figure 3). We observed some variation between whole and lipid extracted blood, although it is likely that these variations are not random and could be due to differential turnover times or how the blood is fractionating as a result of blood protein content, caused by the lack of lipoproteins in the lipid-extracted blood (Figure 4). Generally, $\delta^{13}$C among all tissues remain well-conserved across turtle weights, therefore indicating that there is generally no trend between the expression of $\delta^{13}$C in different tissues. When compared to hip pad thickness—another variable of physiological status in sea turtles—$\delta^{13}$C also remains well-conserved across all fat thicknesses which range from 4mm to 17mm, indicating that fat thickness likely does not play a role in the expression of $^{13}$C in turtle tissues (Figure 7).

$^{15}$Nitrogen:

Within our nitrogen isotope analyses, we observed much more variation among individuals, especially when considering sea turtle weight (Figure 8). Most of this variation occurs within turtles between 1-2.5kg, with each tissue exhibiting a maximum variation of ~2-3‰ (Figure 8). Sea turtles spend the duration of their juvenile life-stage in pelagic environments
before recruiting to neritic habitats when they reach sexual maturity. This pelagic stage can last about 12 years, making the juvenile period one of the longer ontogenetic life-stages that turtles experience; therefore, it is likely that within the span of 12 years, sea turtles undergo changes in diet selection due to age and growth that is reflected in these results (Bjorndal et al. 2000). In Figure 8, significant variation in δ15N values for smaller turtles was observed while heavier turtles exhibited more consistent trends, perhaps suggesting diet variation in the younger turtles. Reich et al. (2008) reported similar results in their data, indicating that the rate of nitrogen integration varied significantly among whole blood, skin and scute tissues. An exception to this trend throughout our δ15N analyses was turtle K252, which exhibited a heavier δ15N skin sample that was more enriched with 15N (δ15N= 11.13‰) than the skin δ15N average (Average= 9.34‰). It is possible that this turtle was feeding at a higher trophic level than the rest of the cohort, since δ15N is enriched; although it is also important to consider the turnover dynamics of the skin tissue. It is possible that skin turns over slower than the other tissues, thus the high δ15N of turtle K252 could possibly be depicting diet from a previous habitat. This turtle also may be displaying the beginnings of post-depositional catabolism in which internal protein or lipid reserves are being catabolized and affecting 15N enrichment; however, this hypothesis is less likely since the δ13C exhibits no unusual activity for this individual (Doi 2017). Particularly noticeable is the obvious 2.5‰ increase in pectoral muscle 15N enrichment in turtles between 1-2.5kg (Figure 9). This tissue exhibits the most dramatic relationship with turtle weight/age, and suggests that there is a significant physiological component affecting the representation of δ15N, likely associated with age. When compared to hip fat thickness, there was also no significant variation in δ15N that indicates that δ15N integration is affected by fat content of turtle individuals (Figure 13).
While our results reflected variation in individual δ\textsuperscript{15}N among tissues, an analysis of tissue δ\textsuperscript{15}N averages compared among weight classes indicates a distinct consistency in δ\textsuperscript{15}N enrichment in the smallest weight class of turtles (Figure 12). In turtles between 1.0-1.5kg, pectoral muscle, skin, whole blood and lipid extracted blood exhibited very similar δ\textsuperscript{15}N integration (Figure 12). Sea turtles within the largest weight class between 2.6-6.5kg were significantly more enriched, though exhibited the most variation between tissues, with pectoral muscle becoming significantly more enriched (Figure 12). Throughout all weight classes, however, whole blood and lipid-extracted blood remained relatively consistent while pectoral muscle and skin showed significant enrichment with age class. We are unsure of why this trend is exhibited, although some studies indicate that integration rate is affected by age (Reich et al. 2008; Sears et al. 2008). It has been found that young individuals fractionate less than fully grown individuals, indicating that younger, smaller turtles allocate nitrogen differently than the older, larger individuals. Specifically, Reich et al. (2008) concluded that sea turtle hatchlings recorded a longer residence time of \textsuperscript{15}N for skin (66.7 days ±7.36 days) and whole blood (35.7 days ±2.73 days) tissues than juvenile skin (44.9 days ±3.1 days) and whole blood (27.7 days ±3.5 days), quantifying the effect of age and growth on the representation of stable isotopes in analysis. Using weight as a proxy for age, we observe this trend within the 24 Kemp’s Ridley turtles, in which the smallest turtles are the least \textsuperscript{15}N-enriched among tissues, with a small range between 7.99‰ to 8.33‰ (Figure 12). Understanding that sea turtles exhibit allometric growth, our data shows that \textsuperscript{15}N integration is slower in small, young individuals predictably because more nitrogen is being assimilated for growth, while larger, older individuals turnover \textsuperscript{15}N faster and more frequently (Sears et al. 2008). We also observed consistent trends in lipid-extracted blood and whole blood with age, remaining well-conserved among turtle individual weight and
weight class (Figure 8; Figure 12). Looking closer at the relationship between lipid-extracted and whole blood, we observed a similar trend as in δ^{13}C, in which the two blood types are very similar in δ^{15}N enrichment (Figure 10). Again, we know that the variation we observe in this relationship is not random, likely due to the protein content in whole blood, driving the visible enrichment of whole blood in Figure 11.

δ^{15}N data also indicates possible covariance with skin tissue. We observed relatively correlated relationships when plotting the residual values of pectoral muscle (R^2 = 0.29), whole blood (R^2 = 0.24) and lipid extracted blood (R^2 = 0.1) against the residual values of skin tissue, indicating that changes in skin δ^{15}N are correlated with changes in the δ^{15}N of pectoral muscle, whole blood and lipid-extracted blood (Figure 14). However, the skin outlier was likely affecting these data due to its high δ^{15}N value (δ^{15}N = 11.3‰) and thus, large residual value (Figure 14). In correcting for this outlier, correlations reduced significantly for whole blood (R^2 = 0.17) and lipid-extracted blood (R^2 = 0.02), but remained consistent for pectoral muscle (R^2 = 0.32, p<0.01), indicating that the most significant relationship of covariance is between pectoral muscle and skin tissue (Figure 15). Given the close relationship between ^{15}N enrichment in pectoral muscle and skin tissue, this covariance asserts the correlation between pectoral muscle and skin ^{15}N integration, indicating that they integrate at consistently similar rates (Figure 15).

Further Analysis:

From the accompanying necropsy data, we have a better understanding of how and when Kemp’s ridley turtles are stranding. Historical sea surface temperature data from 2017 indicates that water temperature and stranding date are significantly correlated (R^2 = 0.94) (Figure 17). When plotting stranding date and water temperature against stranded turtle weight, we excluded strandings that occurred on November 26^{th} and November 27^{th}, 2017 due to a large storm system.
that influenced the stranding of many turtles. We found that smaller turtles generally strand earlier in the season ($R^2 = 0.34$, $p<0.05$) and at warmer surface temperatures ($R^2 = 0.32$, $p<0.05$) than larger turtles (Figure 18). Therefore, sea turtles with a large surface area to body-mass ratio are more vulnerable to temperature change and hypothermic conditions. Body mass and size therefore likely explains the distinctive abundance of Kemp’s ridley strandings on Cape Cod, which dominated almost 90% of total sea turtle strandings in 2017.

**Conclusion:**

On November 24, 2018, Thanksgiving day, a weather system with high winds and temperatures that dipped to 23°F created conditions so frigid that icy slush floated along Cape Cod shorelines. Between Dennis and Truro, 227 sea turtles were found dead within three days, flash-frozen from the sudden cold-snap. 2018 is likely to see more than 800 turtles cold-stunned or dead, the majority of these being the critically endangered Kemp’s ridley. In order to effectively manage conservation efforts, it is essential to understand the biological drivers of cold-stunning. From $^{13}$C stable isotope analysis, this study found that skin is the most enriched of the tissues, while whole and lipid extracted blood and pectoral muscle are more depleted and exhibit very similar $\delta^{13}$C, perhaps due to the metabolic requirements of these tissues.

From $^{15}$N stable isotope analysis, we found that age and growth play a significant role in the integration of $^{15}$N and that smaller, younger turtles have a slower integration rate than larger, older individuals, who turn over $^{15}$N more frequently. This finding helps to explain the trend of Cape Cod cold-stunning in juvenile Kemp’s ridleys, given that these young individuals are about 7% of the size of an adult Kemp’s ridley (35kg) (Marquez 1994). Among the $\delta^{15}$N values of each tissue type, we found that pectoral muscle exhibits the most dramatic enrichment in $^{15}$N due to
age, increasing by about 2.5‰ before plateauing in larger, older turtles. Pectoral muscle and skin also exhibited the most significant relationship in $^{15}$N integration and covariance, indicating that changes observed in Kemp’s ridley skin $\delta^{15}$N can explain changes in the Kemp’s ridley pectoral muscle $\delta^{15}$N. When considering the effect of abiotic conditions on sea turtle cold-stunning, we found that smaller turtles generally experience cold-stunning earlier and at warmer temperatures than larger turtles, with almost 70% of strandings within our subset of turtles occurring along the ‘arm’ of Cape Cod in Eastham, Wellfleet and Truro (Figure 1). Further work should be done to refine our understanding of how age and size affects the biochemistry of cold-stunned sea turtles. It will also be useful to understand how consistently the trends we observed in our study are preserved across different sea turtle species and within different locations that experience cold-stunning, such as other northeastern shorelines and the Florida coast. While our results represent just a small subset of cold-stunned sea turtles, these data reveal significant trends that may help elucidate the phenomenon of cold-stunning and inform conservation efforts.

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Figures:

Figure 1. Cold-stunned Kemp’s ridley distribution in Cape Cod.

Figure 2. $\delta^{13}C$ (%) composition of Kemp’s ridley individuals by turtle weight (kg).

Figure 3. $\delta^{13}C$ (%) composition of whole blood and lipid extracted blood.
Figure 4. Difference in $\delta^{13}C$ (%) of whole and lipid extracted blood by turtle weight (kg).

Figure 5. Difference in $\delta^{13}C$ (%) of pectoral muscle and whole blood by turtle weight (kg).

Figure 6. Difference in $\delta^{13}C$ (%) of pectoral muscle and lipid-extracted blood by turtle weight (kg).
Figure 7. $\delta^{13}C$ (‰) composition of Kemp’s ridley individuals by hip pad thickness (mm).

Figure 8. $\delta^{15}N$ (‰) composition of Kemp’s ridley individuals by turtle weight (kg).

Figure 9. $\delta^{15}N$ (‰) composition in pectoral muscle of Kemp’s ridley individuals by turtle weight (kg).
Figure 10. $\delta^{15}N$ (%) composition of whole blood and lipid extracted blood.

Figure 11. Difference in $\delta^{15}N$ (%) of whole and lipid extracted blood by turtle weight (kg).

Figure 12. Average $\delta^{15}N$ (%) composition of turtle weight classes (kg).
Figure 13. $\delta^{15}N$ (%) composition of Kemp’s ridley individuals by hip pad thickness (mm).

Figure 14. $\delta^{15}N$ (%) residuals of pectoral muscle, lipid-extracted and whole blood by skin $\delta^{15}N$ (%) residual values.

Figure 15. $\delta^{15}N$ (%) residuals of pectoral muscle by skin $\delta^{15}N$ (%) residual values.
Figure 16. δ¹³C (‰) and δ¹⁵N (‰) isotopic differences in reference to skin.

Figure 17. Turtle weight (kg) by water temperature (°C) on date of stranding, excluding turtles stranded on Nov. 26th and 27th, 2017 due to large storm system.

Figure 18. Turtle weight (kg) and water temperature (°C) by date of stranding.
Appendix A:

Method for blood lipid extraction of turtle samples

Method is a modified Folch extraction based upon Klem et al. (PLOS 2012, Efficient and Specific Analysis of Red Blood Cell Glycerophospholipid Fatty Acid Composition).

Lyse blood cells:
- Put 0.5 ml whole blood (thawed) in 13 mm centrifuge tube with Teflon lined cap
- add 1 ml methanol + 1 ml 0.88% aqueous KCl. Shake to mix well.
- Ultrasonicate 5 min (22% intensity) to lyse blood cells
  (you should have a thick reddish-pink opaque mixture)

Extract lipids
- Add 2 ml Chloroform
  (you now have a mixture of ~1.5:1:2 aqueous salt:methanol:chloroform for the extract)

  - Shake vigorously (1 min) to extract lipids
  - Centrifuge 5 min at 1500 rpm to separate aqueous/organic layers and precipitate cell fragments + coagulated proteins

Remove lipids
(you should have a dark pellet of cell fragments + coagulated blood in bottom of centrifuge tube, a lower solvent layer (an azeotrope of chloroform :methanol:water), a pink interfacial layer, and an upper aqueous layer (an azeotrope of water:methanol)

-Using a pipet, carefully insert into solvent (lower) layer containing lipids and withdraw solvent layer. Discard solvent.

Backextract to remove remaining lipids
- Add 2 ml hexane and shake vigorously (1 min) to extract remaining lipids into hexane layer
- Centrifuge 5 min at 1500 rpm to separate layers and precipitate cell fragments + coagulated proteins
- Carefully remove hexane solvent (upper) layer and discard.

Concentrate
- Evaporate the remaining aqueous:methanol layer down to ~ 25% vol in the Savant, using a high heat setting
- This will take about 30 min
Citations:
