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Characterizing stable isotope relationships between green turtle (*Chelonia mydas*) skin and unhatched eggs

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ABSTRACT

RATIONALE: Stable isotope analysis is used to understand the foraging habits and movements of a diverse set of organisms. Variability in stable isotope ratios among tissues derived from the same animal makes it difficult to compare data among study results in which different tissue types are evaluated. Isotopic relationships between two green turtle (*Chelonia mydas*) tissue types, skin and unhatched egg contents are unknown. Similarly, few data exist to evaluate the influence of time elapsed after oviposition (as a proxy for decomposition) on isotopic variability among unhatched eggs within the same nest.

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METHODS: Skin and unhatched egg contents were collected from 69 adult female green turtles and associated nests at the Archie Carr National Wildlife Refuge in Florida, USA. Values of $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$ were measured for both tissue types using a continuous flow isotope ratio mass spectrometer. Standardized major-axis (SMA) regression was used to generate conversion equations of carbon, nitrogen, and sulfur isotope ratios between the two tissue types. Model selection frameworks consisting of single-factor linear models were employed per isotope ratio to assess how egg time-in-nest affected intraclutch isotopic variability.

RESULTS: Conversion equations for all three isotope ratios indicated significant relationships between skin and unhatched egg values, although model fits were lower than found in some studies examining similar patterns in other marine turtle species. The probability of increased intraclutch variability was significantly higher among eggs collected at longer intervals after deposition.

CONCLUSIONS: This study reports the first-ever $\delta^{13}C$ and $\delta^{15}N$ conversion equations between skin and unhatched eggs for green turtles, and the first $\delta^{34}S$ conversion equation for any marine turtle species. SMA regression was used to directly convert tissue values bidirectionally, unlike equations generated using ordinary least-squares regression. Issues with increased intraclutch variability at later excavation dates highlight the importance of collecting unhatched eggs as soon as possible after hatchling emergence.

Keywords: Carbon-13, Nitrogen-15, Sulfur-34, marine turtles, conversion equations

INTRODUCTION

Stable isotope analysis (SIA) is applied broadly to investigate habitat use and trophic ecology across a wide range of taxa. Stable isotope ratios exhibit spatiotemporal patterns across landscapes produced by differential fractionation in various biogeochemical processes and

elemental cycling as part of physiochemical changes in dynamic systems[1,2]. Over time, a consumer incorporates isotope ratios into tissues through its diet which is influenced by isotopic values at the base of the local food web. Factors including substrate availability, nutritional stress, foraging strategy, and method of protein synthesis can alter isotope ratios as food resources are incorporated into consumer tissues[2–4].

The disparity in stable isotope ratios between prey and consumer, known as discrimination, differs among the consumer's tissue types due to variation in nutrient routing and amino acid composition[4]. Within a consumer, there are tissue-specific turnover rates that are largely controlled by metabolic activity[5,6]. Multiple tissue types from the same individual with differing turnover rates can therefore serve as intrinsic markers for identifying foraging location over different time scales[1,7]. Tissues with slow metabolic turnover (and thus, slow isotopic turnover rates; e.g., months – chelonian red blood cells[8,9]), or those whose isotopic values are metabolically inert (e.g., keratin[2]), provide long-term information about an organism's foraging location at the time of tissue formation[7]. Tissues with rapid metabolic turnover (e.g., days – mammal liver[5]) can provide information about short-term changes in foraging strategy. Variability in stable isotope ratios among tissues within the same animal makes it difficult to compare data among studies from which different tissue types were evaluated.

Stable isotope analysis has been used in many marine turtle studies to identify prior foraging locations of adult females sampled at nesting beaches[10–15]. These studies analyzed bulk tissues with slow isotopic turnover rates (skin, red blood cells, fresh and unhatched eggs) that reflect isotope ratio profiles incorporated at foraging areas prior to migration to the nesting beach[8,9,16]. Most often, tissue values of $\delta^{13}C$, $\delta^{15}N$, and (less frequently[15,17]) $\delta^{34}S$ are used to separate females within isotopic space, which should generally reflect differences in geographic locations of foraging areas. However, actual isotopic discrimination differs between tissue types[9,18], and tissue-specific discrimination factors vary among different marine turtle species[19]. Foraging area isotope ratio data for these females is often derived solely from tissues acquired at the nesting beach, with little to no information directly from environmental or prey item

sampling at these often-distant foraging sites. It is therefore crucial that studies expand knowledge of both the relationships among commonly-collected tissues and the mechanisms underlying potential shifts in isotope ratios within these tissues.

Unlike skin, blood, and scute samples which require access to individual turtles and some level of technical training and animal handling, unhatched eggs (eggs that never began the process of embryonic development or in which an embryo died before hatching) are commonly found in nest excavations used to evaluate reproductive success. These unhatched eggs are an advantageous tissue type to collect, as they represent a non-invasive, non-destructive proxy for acquiring stable isotope ratios of nesting females[13,14,16,20]. Ceriani et al[16] recommended using unhatched eggs in marine turtle isotopic studies conducted at nesting beaches. In contrast to other tissue types (e.g., skin and blood) that are collected from live turtles and preserved immediately, unhatched eggs may be collected from nests anywhere from a few days to a few weeks after hatchling emergence; eggs therefore should be considered as a common currency for stable isotope studies of nesting marine turtles. Frankel et al[21] found that isotopic relationships between nesting female loggerhead turtles (*Caretta caretta*) and their hatchlings changed significantly if hatchling samples were collected from freshly-dead individuals *vs* those that had undergone some level of decomposition. No direct decomposition rates are known for unhatched eggs. Anecdotally, unhatched eggs observed during nest excavations soon after hatchling emergences exhibit a range of visual cues indicating decomposition. As the time between hatchling emergence and nest excavation increases, an increasing proportion of unhatched eggs exhibit indicators of extensive decomposition. Ranges in unhatched egg collection time-after-deposition suggest that decomposition could influence the isotope ratios of individual egg components as well as intraclutch variability for eggs collected at various times after hatchling emergence.

To understand isotopic relationships among tissues derived from the same individuals, it is necessary to develop models that convert isotope ratios of one tissue into corresponding ratios in another, referred to as “conversion equations”. These equations have been generated for

several tissue type comparisons in the loggerhead turtle[13,16,20–24], leatherback turtle (*Dermochelys coriacea*)[25] and green turtle (*Chelonia mydas*)[26].

In this study, we developed the first green turtle-specific conversion equations between skin and unhatched eggs from individual females for $\delta^{13}C$ and $\delta^{15}N$ values, and the first comparison between these two tissues for $\delta^{34}S$ values in any marine turtle species. Green turtle-specific conversion equations between tissues will aid studies that use stable isotope ratios to investigate adult turtle migratory connectivity and trophic ecology. We also evaluated unhatched green turtle eggs to assess the potential impact of time since oviposition (a proxy for level of decomposition) on intraclutch isotopic variability.

METHODS

Adult Female Sampling

In 2013 and 2014, 50 adult female green turtles were sampled each year (100 individual turtles) from June through September while nesting along the 21-km Brevard County portion of the Archie Carr National Wildlife Refuge (ACNWR), located in Melbourne Beach, FL, USA. Turtles were flipper tagged (Inconel Style 681; National Band and Tag Company, Newport, KY, USA) using standardized protocols[27] to prevent resampling. Two skin biopsies were obtained from nesting turtles after oviposition using a sterile 4-mm biopsy punch. In 2013, one shoulder biopsy was obtained from the right shoulder midway between the neck and flipper, and another skin sample was acquired from a rear flipper. In 2014, two shoulder biopsies were obtained from sampled turtles. Similar anatomical sampling locations were used in loggerhead turtles with no significant differences in the isotopic ratios of skin collected at each site (Ceriani unpublished data). Skin samples from 2013 were stored frozen in a –20°C non-frost-free freezer until processing. Samples from 2014 were stored in 70% ethanol at room temperature. Hobson et al[28]

suggested storage in 70% ethanol as a viable alternative to freezing, with Barrow et al[29] finding no significant effect of ethanol storage on the stable isotope ratios of turtle skin.

Nest Marking, Excavation, and Egg Sampling

Following oviposition, sampled females' nests in both 2013 and 2014 were marked for post-hatching nest content evaluation. Following guidelines used in Brost et al[30], all nests were monitored for hatchling emergence and excavated/sampled at least 72 hours after emergence, or at least 70 days after egg deposition if no emergence was observed. If available, up to five unhatched whole eggs were collected from each sampled nest and stored at –20°C in a non-frost-free freezer until processing for stable isotope analysis. To reduce the risk of aberrant stable isotope ratios, eggs were collected only if they appeared to have a minimal level of decomposition, were not punctured or damaged externally, and did not contain a large embryo.

Sample Processing and Stable Isotope Analysis

Underlying connective tissue was removed from the epidermal layer (*stratum corneum*) of skin samples; this epidermal layer (hereafter referred to as "skin") was then sliced into the smallest possible pieces using a scalpel blade. These pieces were placed in a freeze drier for 12 hours. Lipids were removed from skin samples in a soxhlet device for 24 hours using petroleum ether as solvent. Generally, it is difficult if not impossible to distinguish egg components (e.g., albumin and yolk) in post-emergence unhatched eggs, unlike with fresh eggs. Therefore, we included the entire egg contents (excluding egg shell) in our stable isotope analyses, per Ceriani et al[16]. Each unhatched egg was opened, and egg contents were placed into individual freezer bags (one egg per bag). Egg contents from up to three of the least-decomposed unhatched eggs per nest were freeze-dried for 48h. The dried contents were homogenized with a mortar and

pestle and a subsample of each egg was lipid-extracted using a soxhlet device for 24h with petroleum ether as solvent.

Approximately 0.5 to 1.0 mg of each tissue sample was placed into a tin capsule and analyzed for $\delta^{13}C$ and $\delta^{15}N$ values. Values are expressed in delta (δ) notation as: $\delta X = [(R_{sample}/R_{standard}) - 1]$ where X is the heavy isotope of the element in question (e.g., ^{13}C), and R is the heavy to light isotopic ratio (e.g., ^{13}C:^{12}C), expressed as per mil (‰). Carbon and nitrogen isotope ratios and elemental concentrations were measured at the University of South Florida College of Marine Science Stable Isotope Biogeochemistry Laboratory (St. Petersburg, FL, USA) on a ThermoFinnigan Delta+XL continuous flow isotope ratio mass spectrometer (Thermo Fisher Scientific, Bremen, Germany), and are scaled to VPDB ($\delta^{13}C$ values) and AT-Air ($\delta^{15}N$ values)[31] using a secondary reference (1577b Bovine liver)[32]. The measurement uncertainties for skin samples, expressed as ±1 standard deviation of n = 25 measurements of the laboratory reference material, were ± 0.23‰ for $\delta^{13}C$ values and ± 0.10‰ for $\delta^{15}N$ values. The measurement uncertainties for unhatched eggs, expressed as ±1 standard deviation of n = 32 measurements of 1577b Bovine liver were ± 0.14‰ for $\delta^{13}C$ values and ± 0.09‰ for $\delta^{15}N$ values.

For $\delta^{34}S$ analyses, approximately 3 mg of tissue was placed into a tin capsule and sent to the Washington State University Stable Isotope Core Laboratory (Pullman, WA, USA). These samples were analyzed with a ThermoFinnigan Delta PlusXP continuous flow isotope ratio mass spectrometer (Thermo Fisher Scientific)[33]. Sulfur isotopic ratios are reported relative to VCDT by assigning a value of –0.3‰ to IAEA S-1 silver sulfide[34]. The skin measurement uncertainty, expressed as ±1 standard deviation of n = 8 measurements of a laboratory reference material (IAEA S-1 silver sulfide), was ± 0.09‰ for $\delta^{34}S$ values. The unhatched egg measurement uncertainty, expressed as ±1 standard deviation of n = 9 measurements of an IAEA S-1 silver sulfide, was ± 0.29‰ for $\delta^{34}S$ values. For all nests that contained at least 2–3 unhatched eggs that were sampled for SIA, we calculated the mean and standard deviation (SD) for each nest per isotope ratio.

Isotope Conversion Equations

All statistical analyses for this study were conducted using program R[35]. An α value of 0.05 was used to determine significance. To avoid model bias caused by high isotopic differences between eggs in the same clutch, we used a Tukey's test on the distribution of egg SD to determine a threshold for each isotope ratio, above which a nest would be identified as an outlier in terms of intraclutch variability. From there, the aberrant egg within the nest was eliminated from the data set. If, after eliminating that egg, the recalculated SD for that nest was still above the threshold, or there were no longer at least two eggs, that nest was removed from the analyses. We then used samples from the remaining individuals to construct standardized major-axis (SMA) regression models using the package "smatr"[36] in R for each isotope ratio comparing skin and mean unhatched egg SI values. Previous marine turtle conversion equations were based on generalized linear models (specifically, ordinary least-squares regression), wherein an independent variable is used to predict a dependent variable. Due to their asymmetrical structure, these equations should not be used to "back-predict" variables in the opposite direction because of differences in error structure, slope, and interpretation[37]. Standardized major-axis regression is more useful for developing conversion models. Equations generated using this technique can convert delta values from one tissue into values from the other in both directions[37,38]. For comparisons with previous studies, we have included additional conversion equations for each isotope ratio generated using OLS regression as supplementary information (supporting information 1).

As variation in preservation method (70% ethanol *vs* freezing) may have an impact on the isotope ratios in our skin samples, we tested for differences between these two methods within our data using a model selection framework coupled with models constructed using the "smatr" package. A suite of single factor, additive, and multiplicative models was constructed for each isotope ratio, including overall relationship of skin to unhatched eggs, as well as preservation

method of the skin. Within each model selection scenario, AICc was used to compare models; models with a ΔAICc score of < 2.0 were considered indistinguishable.

Days Post-Deposition Analyses

Although decomposition was not directly measured in this study, the longer that marine turtle eggs remain in the ground, the more likely it is that tissue decay could alter stable isotope ratios[39]. As such, we used the number of days since oviposition that eggs were collected from nests (hereafter referred to as "days post-deposition" - DPD) as a proxy for decomposition to examine how it could affect intraclutch isotopic variability. As mentioned previously, although eggs were subjectively (rather than randomly) collected to select those with the least amount of apparent decomposition, each nest was sampled only once, with that bias applying equally across sampling. Intraclutch variability was determined for each nest by using the standard deviation of egg values for all three isotope ratios. We used a model selection framework to evaluate a suite of four single-factor linear models per isotope ratio. Intraclutch variability was the dependent variable in all models, while independent variables were the null model, DPD, log-transformed DPD, and a quadratic function of DPD. Within each model selection scenario, AICc was used to compare models; models with a ΔAICc score of < 2.0 were considered indistinguishable.

RESULTS

Isotope Conversion Equations

Of the 100 nests marked for this project, 69 contained at least two unhatched eggs that were collected for stable isotope analysis. The Tukey's test identified an intraclutch δ^{13}C-SD threshold of 0.39‰, a δ^{15}N-SD threshold of 0.48‰, and a δ^{34}S-SD threshold of 0.86‰. This resulted in nine original outlier nests for δ^{13}C values, 12 for δ^{15}N values, and five for δ^{34}S values.

The aberrant egg was removed from each of these nests; seven nests were fully removed from the dataset after this step because their recalculated intraclutch SD was still above the threshold for at least one isotope ratio (n = 5), or there was only one egg remaining (n = 2). Average intraclutch isotopic SD among the remaining 62 nests (2013: n = 33, 2014: n = 29) was 0.11‰ for $\delta^{13}C$ values, 0.12‰ for $\delta^{15}N$ values, and 0.25‰ for $\delta^{34}S$ values. The mean skin isotope ratios for these samples were –8.91 ± 1.38 for $\delta^{13}C$ values (2013: –9.26 ± 1.46; 2014: –8.52 ± 1.19), 7.26 ± 1.16 for $\delta^{15}N$ values (2013: 7.34 ± 0.83; 2014: 7.18 ± 1.45), and 9.09 ± 2.31 for $\delta^{34}S$ values (2013: 8.89 ± 2.14; 2014: 9.31 ± 2.50). The mean unhatched egg isotope ratios for these samples were –10.17 ± 1.27 for $\delta^{13}C$ values (2013: –10.30 ± 1.30; 2014: –10.02 ± 1.25), 6.13 ± 1.22 for $\delta^{15}N$ values (2013: 6.08 ± 1.01; 2014: 6.19 ± 1.44), and 8.57 ± 2.51 for $\delta^{34}S$ values (2013: 7.94 ± 2.19; 2014: 9.28 ± 2.69).

The model selection results indicated no significant effects of preservation method on $\delta^{15}N$ and $\delta^{34}S$ values for the skin-unhatched egg relationship. One model in the $\delta^{13}C$ model selection scenario indicated similar slopes for the skin-unhatched egg relationship but significantly different intercepts, with frozen skin samples having being more depleted in ^{13}C than those preserved in 70% ethanol. However, this model was within 2.0 AICc of the model which only included the skin-unhatched egg relationship, indicating they are statistically commensurate with one another. For both parsimony and interpretability, we include data from both preservation techniques together in conversion equations but note that this may impact the model fit for the $\delta^{13}C$ conversion equation.

We used the remaining 62 nests to construct finalized skin to unhatched egg conversion models with equations (Figure 1) for each isotope ratio:

$$\delta^{13}C_{Unhatched} = 0.920*\delta^{13}C_{Skin} - 1.971 \quad \text{[Equation 1]}$$

$$\delta^{15}N_{Unhatched} = 1.053*\delta^{15}N_{Skin} - 1.520 \quad \text{[Equation 2]}$$

$$\delta^{34}S_{Unhatched} = 1.088*\delta^{34}S_{Skin} - 1.320 \quad \text{[Equation 3]}$$

The models for all three isotope ratios indicated significant relationships between skin and unhatched egg values ($p < 0.001$; $\delta^{13}C$ model $R^2 = 0.70$, $\delta^{15}N$ model $R^2 = 0.64$, and $\delta^{34}S$ model $R^2 = 0.64$).

Days Post-Deposition Analyses

Hatchling emergence typically occurred at 54 days ± 4 SD (range = 47–62 days), while average days post-deposition **(**DPD) at which eggs were collected was 64 days ± 8 SD (range = 50–84 days). All 69 clutches were evaluated for $\delta^{13}C$ and $\delta^{15}N$ values, whereas only 68 clutches were evaluated for $\delta^{34}S$ values. All non-null models indicated a significant increase in intraclutch SD with increasing DPD for $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$ values ($p < 0.05$ for all non-null models and all slope terms within each model; Figure 2). These models all had ΔAICc scores less than 2.0 among the various analytical scenarios and are considered equally informative. However, they explained little variation in the data (R^2 values for each non-null model < 0.09). For each of the three isotope ratios, model selection scenarios indicated the null model to be uninformative relative to the other three linear models.

DISCUSSION

This study reports the first $\delta^{13}C$ and $\delta^{15}N$ conversion equations between skin and unhatched eggs for green turtles, and the first $\delta^{34}S$ conversion equation for any marine turtle species. Our SMA regression conversion equations can be used to directly exchange skin and unhatched egg isotope ratios bidirectionally, unlike equations generated using ordinary least-squares regression. This also means that slope and intercept estimates for this study's models that compare skin and unhatched egg values of $\delta^{13}C$ and $\delta^{15}N$ are not directly comparable with those found in Ceriani et al[16], comparing maternal skin and unhatched egg content values. However, the method of evaluating model fit using R^2 values for SMA regression is commensurate with

the procedure for ordinary least-squares regression, so these values are comparable with those in other studies.

Fits for the $\delta^{13}C$ and $\delta^{15}N$ models in this study ($R^2 = 0.70$ and $R^2 = 0.64$, respectively) are lower than those for the skin to unhatched egg conversion equations generated by Ceriani et al[16] for loggerheads ($R^2 = 0.83$ and $R^2 = 0.86$) using the same processing and isotope analytical methods. Our study included skin samples that were frozen as well as samples that were preserved in 70% ethanol. Although we found no evidence that these mixed preservation techniques had any effect on our $\delta^{15}N$ and $\delta^{34}S$ conversion equations, they did appear to have some effect on the $\delta^{13}C$ conversion equation. Although a caveat of these results, conversion models incorporating skin preservation technique did not provide statistically more information than the one that did not. This probably indicates that although samples preserved using both methods were included together to construct the $\delta^{13}C$ conversion model, this did not considerably reduce the model fit. However, these results indicate that future studies should maintain caution when conducting stable isotope analyses with skin samples preserved using mixed techniques and should avoid doing so if possible. Our model fits are similar to those found in Kaufman et al[23] between loggerhead skin and frozen, lipid-extracted yolk from sacrificed fresh eggs ($R^2 = 0.70$ for $\delta^{13}C$ and $R^2 = 0.72$ $\delta^{15}N$). This, coupled with the high correlation of isotope ratios between fresh and unhatched eggs in Ceriani et al,[16] suggests that unhatched eggs may also be a viable, non-destructive proxy for fresh eggs in the green turtle, although this assumption should also be tested empirically.

No similar marine turtle study exists with which to compare tissue isotope conversion equations for sulfur. Although few $\delta^{34}S$ data are available for marine turtles[15,17,40], the $\delta^{34}S$ value is a potentially useful isotope ratio to incorporate into new research. Values of $\delta^{34}S$ shift more quickly across a smaller geographic space and outperform $\delta^{13}C$ and $\delta^{15}N$ values at separating producers in marine systems in certain circumstances[41]. Bradshaw et al[15] effectively used $\delta^{34}S$ values to assign nesting female green turtles to foraging areas that were isotopically indistinguishable using $\delta^{13}C$ and $\delta^{15}N$ values alone.

The lower explanatory power for the $\delta^{13}C$ and $\delta^{15}N$ models in this study versus loggerheads in Ceriani et al[16] suggests that it is possible that the relationship between isotope discrimination factors for skin and eggs is weaker in green turtles than in loggerheads. Adult green turtles in the Northwest Atlantic are predominantly herbivorous, feeding primarily on seagrasses and algae[42], unlike loggerheads which are generalist carnivores[43]. Turtle grass (*Thalassia testudinum*), one of the major contributors to the diet of adult green turtles in the Northwest Atlantic[42], experiences seasonal shifts in $\delta^{13}C$ and $\delta^{15}N$ values[44]. These seasonal changes may alter isotope ratios in the tissues of herbivores, as has been observed in moose and caribou[45], further confounding the isotope tracking technique. Green turtles, like other marine turtles species, lay multiple clutches of eggs during a nesting season[46]. After each successive clutch is laid, follicles rapidly develop into fully-formed eggs using endogenous resources derived from foraging areas[47]. Warner et al[48] identified experimentally that the multi-clutch lizard *Amphibolurus muricatus* produces egg components (i.e., lipids and proteins) using different relative contributions of energy sources (i.e., endogenous and recently acquired food). Although green turtles rely almost exclusively on endogenous energy sources for reproduction[49], if differential allocation of energy sources to lipids and proteins is present in marine turtle eggs, seasonal isotopic variation in foraging material could manifest as higher levels of variation between maternal tissue and unhatched egg contents. As generalist carnivores, this baseline seasonal variation may be dampened in loggerhead endogenous reserves, leading to more tightly coupled isotopic ratios between maternal tissue and egg contents.

The green turtle nesting season at the ACNWR typically occurs between late May and late September of each year, with females laying an estimated average of 3.0 ± 1.84 nests per year[50]. Changes in isotope ratios between sequential clutches throughout the nesting season could not be evaluated in the current study, as each nest was produced by a different individual. If significant changes do occur, this could influence the accuracy of a conversion model. Trends in egg isotope ratios among sequential clutches vary in significance and direction (increasing or decreasing) for $\delta^{13}C$ and $\delta^{15}N$ values across multiple species[10,16,20,25,51]. This makes it difficult to

infer whether shifts throughout the nesting season occur for marine turtles as a group. Replicating this study for other green turtle nesting populations would elucidate whether the conversion equations that we developed are population-, or species-specific. Factors including pre-migration female body condition, foraging during the nesting season, clutch frequency, and differences in the relative proportions of egg components could all influence these trends across sequential clutches.

Our method for eliminating aberrant eggs in this study is biased against high variation; approximately 10% of the originally sampled nests were removed using our protocol. The average intraclutch isotopic variability after removing aberrant eggs/nests in this study ($\delta^{13}C$ = 0.11‰, $\delta^{15}N$ = 0.12‰) is similar to values observed by Ceriani et al[16] ($\delta^{13}C$ = 0.13‰, $\delta^{15}N$ = 0.16‰), but lower than those observed by Zbinden et al[20] ($\delta^{13}C$ = 0.21‰, $\delta^{15}N$ = 0.20‰) using unhatched eggs collected from loggerhead nests. Of note is that Zbinden et al[20] collected eggs 14 days after hatchling emergence, which may have contributed to these higher levels of intraclutch variability. Collection dates after oviposition for this study were not standardized, nor were multiple dates for the same nests evaluated for isotopic variability between eggs, as these questions were not the primary foci for this study. Nevertheless, we found that DPD had a significant impact on intraclutch variability for all three isotope ratios. This is consistent with controlled laboratory experiments in which rotting significantly altered $\delta^{13}C$ and $\delta^{15}N$ values in *Drosophila melanogaster* tissue[39]. Especially for $\delta^{15}N$ values, bacterial and fungal metabolism within these eggs may have shifted isotope ratios as a result of trophic enrichment.

The probability of increased intraclutch SD was significantly higher among eggs collected later (Figure 2). However, this trend is poorly estimated by model results, with some nests maintaining low intraclutch SD even with much later collection dates. Nests sampled at or after 70 DPD appear more likely to exhibit extremely elevated levels of intraclutch isotopic variability, especially for $\delta^{15}N$ values, whereas intraclutch variability begins to elevate even before 60 DPD for $\delta^{34}S$ values. Few nests were sampled between 62 and 70 DPD, making it difficult to determine when intraclutch variability begins to elevate for $\delta^{13}C$ and $\delta^{15}N$ values. No

discernable trends were evident when searching visually for effects of lay date and nest distance to the dune in relation to outlier nests with high intraclutch isotopic variability. Our delayed egg collection dates (in comparison with the hatchling emergence dates) in this study may have contributed to our need to remove a sizable number of aberrant eggs/nests from the dataset before constructing conversion equations. Based on these results, we suggest collecting eggs for SIA as soon as possible after hatchling emergence to reduce the chances of elevated intraclutch variability, and to measure stable isotope ratios of multiple eggs from the same nests to monitor within-nest variability. Future work incorporating collection date and resampling nests at different time intervals into a controlled, experimental approach would help elucidate the rate at which decomposition affects the stable isotopic ratios of nest contents.

Conclusion

Our conversion equations will allow researchers to accurately compare datasets using different tissues, facilitating greater understanding of green turtle movement and trophic status at larger spatial scales. Using unhatched egg stable isotope ratios for female foraging area assignment may be particularly useful in regions where many nests are marked for excavation and reproductive output assessments (e.g., southeastern USA, Brazil, Australia, El Salvador, Nicaragua, Indonesia, and Japan). Combining these two levels of isotopic data across a wide breadth of important nesting habitat would allow for stronger inference when studying the migratory ecology of the green turtle.

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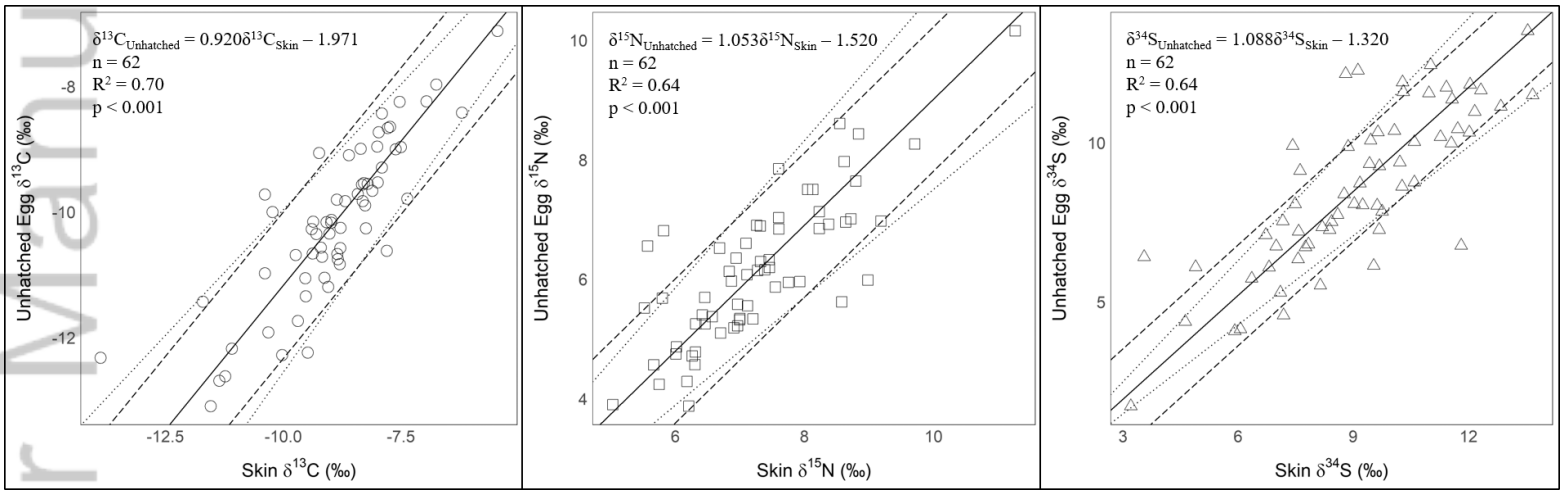
Figure 1: Common currency equations between isotopic values of green turtle skin and unhatched eggs for $\delta^{13}C$ (A), $\delta^{15}N$ (B), and $\delta^{34}S$ (C). Confidence interval estimates of model parameters cannot be represented in the same way on graphs of standardized major-axis regressions as they traditionally are for those of ordinary-least squares regression. Instead, in these panels, 95% confidence intervals of the intercept and slope are represented by dashed and dotted lines, respectively.

Figure 2: Relationship between the number of days after oviposition at which eggs were collected and intraclutch isotopic standard deviation for $\delta^{13}C$ (A), $\delta^{15}N$ (B), and $\delta^{34}S$ (C). Model results indicate significant, increasing intraclutch standard deviations at greater number of days post-deposition for all three isotopes.



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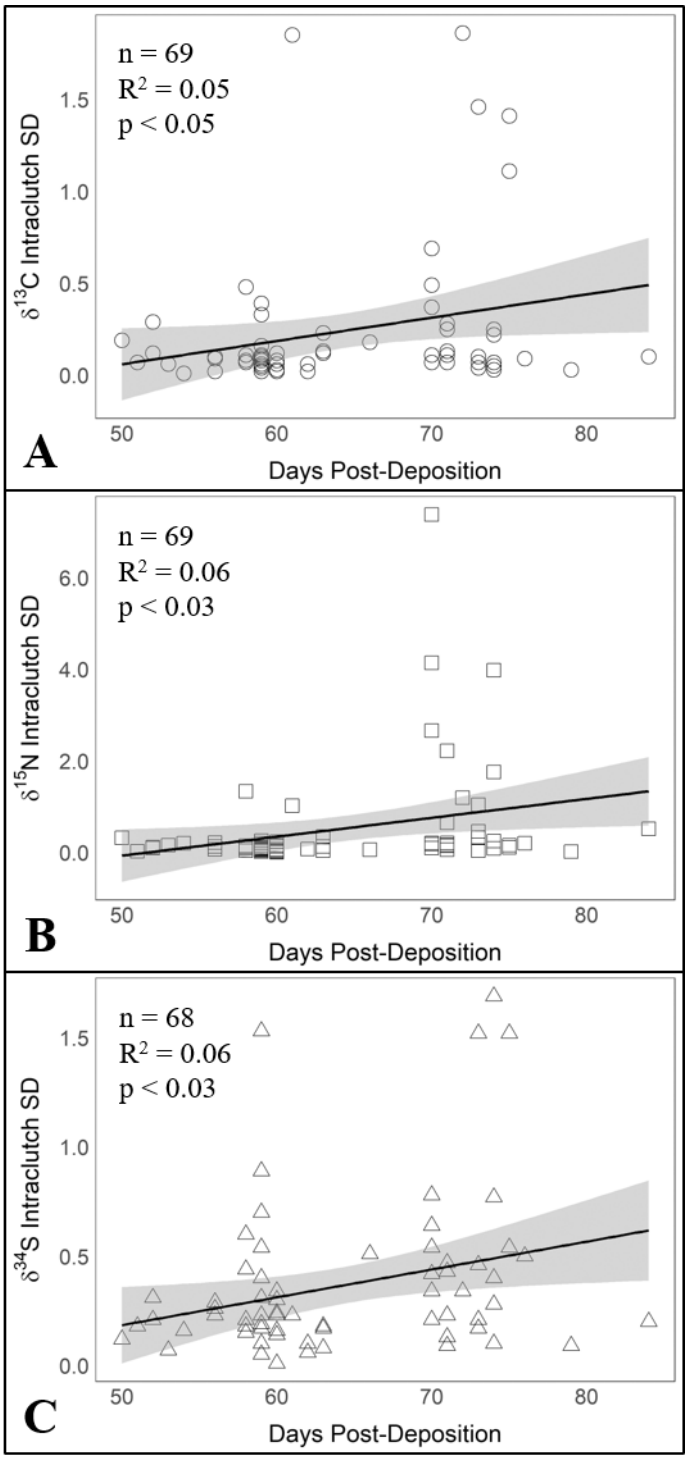

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