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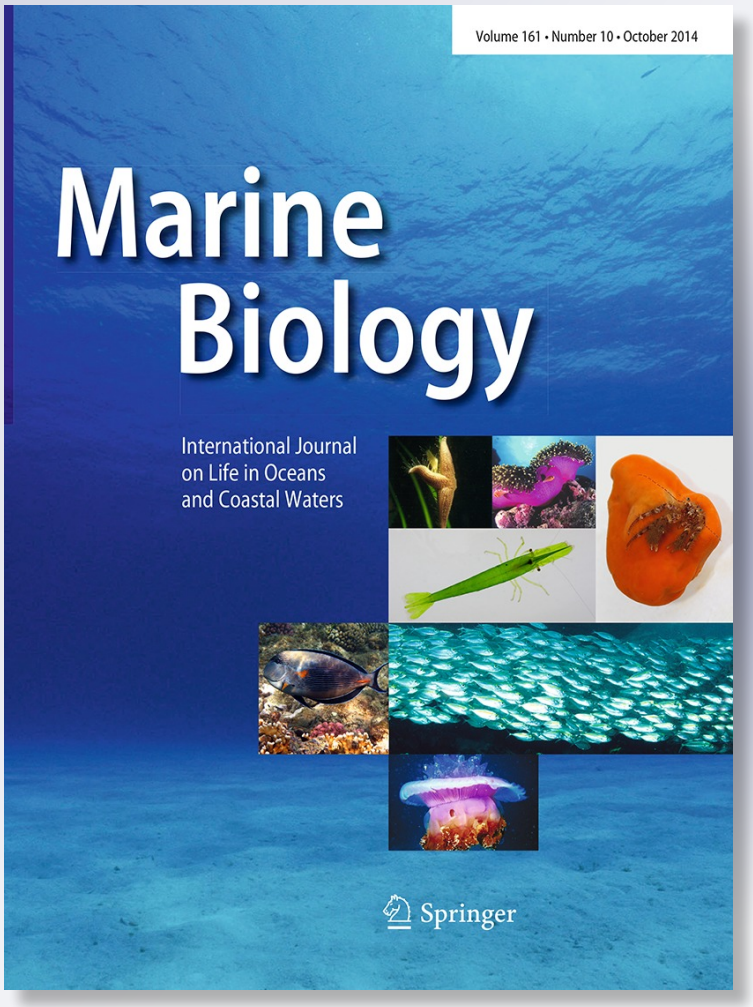

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ORIGINAL PAPER

Developing a common currency for stable isotope analyses of nesting marine turtles

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Abstract Understanding geospatial linkages is critical to the development of appropriate management and conservation strategies for migratory species. Stable isotope analysis is a powerful tool that is performed routinely across taxa to unravel migratory connectivity. Marine turtles are a highly migratory and widely distributed taxon, but are largely studied at breeding areas. Isotopic values of several slow turnover rate tissues have been used to identify often distant foraging areas. However, as more isotopic data from various tissues become available, the relationships between tissues need to be calculated to permit meta-analyses to elucidate isotopic patterns across broader spatiotemporal scales. We used several commonly collected tissues (blood, skin, fresh eggs and unhatched eggs) collected simultaneously from loggerhead turtles (*Caretta caretta*) to develop a common currency for stable isotope analysis studies conducted on the nesting beach. We found highly significant relationships between the tissue signatures (r^2 ranged from 0.83 to 0.96) and developed equations to convert isotopic values from one tissue to another. We examined inter- and intra-clutch isotopic variability and found that a single sampling event over the 4-month nesting season adequately defined the loggerhead female foraging area. Consequently, we propose using unhatched eggs as a common currency in stable isotope studies of nesting loggerheads. Unhatched eggs represent a noninvasive and nondestructive method that enables more extensive (both numerically and spatially) sampling. Given similar physiologies, analogous relationships might be derived in other sea turtle species.

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Introduction

Studying animal migration is challenging as migratory species often traverse vast distances and are often elusive. Nonetheless, identifying linkages between habitats used by migratory species during their lifecycle is necessary to understand their ecology, demography and evolutionary biology. There is an urgency to understand migratory connectivity because it is unknown how imperiled migratory species will respond to threats posed by climate change and habitat loss and degradation (Hobson and Norris 2008), which typically differ between foraging and breeding areas. Despite difficulties, our understanding of animal migration has seen tremendous improvements over the last two decades thanks to advances in genetics, stable isotope applications and tracking device technology (e.g., miniaturization, light geo-locators and solar-powered satellite tags). Multi-technique approaches have proven to be the most powerful at unraveling linkages between breeding, wintering and intermediate stopover sites used by migrants (Clegg et al. 2003; Roscales et al. 2011; Chabot et al. 2012).

Stable isotope analysis of light elements (C, H, N, O and S) is commonly used to identify migratory linkages. This

approach is based on the idea that the ratios of stable isotopes of naturally occurring elements vary across the landscape, often in systematic ways and at the continental scale due to a variety of biogeochemical processes. Patterns in stable isotope ratios at the base of food webs are transferred to various degrees (depending on the element) at higher trophic levels. Stable isotopes act as forensic recorders of animal migratory and foraging behaviors if organisms move between isotopically distinct landscapes and maintain quantifiable isotopic differences in one or more tissues, either permanently (e.g., feathers, hairs, whiskers and nails) or integrated over some time duration (e.g., blood solutes and skin), that can be linked to past locations (Hobson and Norris 2008). If these prerequisites are met, stable isotopes function as intrinsic markers that reflect the isotopic composition of the environment (location and food web) where the tissue under consideration was synthesized.

A growing body of literature has used stable isotope analysis of slow turnover rate (e.g., skin) and metabolically inert (e.g., feathers) tissues to investigate migratory connectivity (e.g., Hobson and Wassenaar 1997; Witteveen et al. 2009). In the majority of cases, migrants are intercepted either at foraging (e.g., whale sharks, Wilson et al. 2006) or at breeding areas (e.g., song birds, Norris et al. 2004) or stopover sites (e.g., song birds, Wilson et al. 2008) because only one of these locations is known or is logistically feasible to sample. Many organisms tend to aggregate during the breeding season (e.g., geese, Cooper 1978; humpback whales, Witteveen et al. 2009; salmons, Gross 1991). Sampling breeding aggregations are ideal because these aggregations (1) are often spatially and temporally predictable and (2) usually represent a mix of individuals coming from several isotopically distinct foraging areas. In addition, a variety of marine migratory organisms across taxa are tied to land for reproduction (e.g., penguins, sea lions, marine turtles and pelagic sea birds), which facilitates sampling. Having access to marine organisms during their reproductive stage on land has enabled a variety of questions to be addressed related to their reproductive physiology (Cherel et al. 2005), feeding ecology (Cherel 2008; Páez-Rosas et al. 2012) and migration routes (Roscales et al. 2011; Ceriani et al. 2012).

Stable isotopes have been increasingly used to study marine turtle migratory connectivity (Hatase et al. 2002), foraging ecology (Wallace et al. 2009; McClellan et al. 2010) and ontogenetic habitat shifts (Arthur et al. 2008). The technique has proven to be particularly effective to study migratory linkages for the adult life stage. The life history and reproductive biology of sea turtles make them ideal for stable isotope applications. Sea turtles are highly migratory and tend to move across broad geographic scales. Females embark on breeding migrations every 1–4 years between spatially distinct foraging grounds and breeding areas. Each female from a nesting aggregation typically forages in one of several geographically distinct foraging grounds (Schroeder et al. 2003; Girard et al. 2009; Hawkes et al. 2011; Ceriani et al. 2012; Foley et al. 2013). Individual females appear to show fidelity to both nesting and feeding area throughout adult life (Miller et al. 2003; Broderick et al. 2007; Tucker et al. 2014). Lastly, sea turtles are capital breeders (Plot et al. 2013), using energy stored at the non-breeding ground for reproduction (Stearns 1992), for whom maternal investment ends with egg deposition.

Researchers have collected a variety of tissue samples from nesting females for stable isotope analysis to assign the putative foraging area used during the non-breeding season. Most studies have used to some extent satellite telemetry to validate the use of stable isotopes to assign foraging areas. These studies have employed a variety of tissues and all but one (Caut et al. 2008) has used a single-tissue approach: skin samples (loggerheads, Reich et al. 2010; Pajuelo et al. 2012; leatherbacks, Seminoff et al. 2012; green turtles, Vander Zanden et al. 2013), red blood cells (RBCs) (leatherbacks, Caut et al. 2008; loggerheads, Ceriani et al. 2012), freshly laid eggs (loggerheads, Hatase et al. 2002; leatherbacks, Caut et al. 2008) and a combination of freshly laid and unhatched eggs (loggerheads, Zbinden et al. 2011). Despite the variety of tissues used and the fact that tissue turnover rates in adult sea turtles are unknown, all of the tissues examined so far have been shown to represent the foraging area used by the female, most likely because sea turtles are large bodied ectotherms and, as such, have slow metabolic and tissue turnover rates.

Given the success of this relatively inexpensive technique, studying migratory connectivity with stable isotope analysis is becoming widespread in sea turtle research. However, protocols, such as the choice of tissue to sample, are not standardized, which makes comparisons and meta-analysis problematic. Similar issues have been recently addressed with success in sea birds using blood and simultaneously grown feathers across 27 populations of 22 wild bird species (Cherel et al. 2014). Such extensive data are not available for sea turtles as the application of isotopic approaches to sea turtles is more recent. We initiated this study to (1) define the relationship between tissues that are commonly used to infer sea turtle non-breeding areas and (2) examine whether it is possible to develop a common currency for stable isotope analysis studies conducted on the nesting beach. In particular, we were interested on the feasibility of using unhatched eggs as they represent a noninvasive, non-destructive and easily collectable tissue. We hypothesized that a freshly laid egg yolk collected at time of clutch deposition and unhatched non-viable eggs collected from the same nests post-hatching emergence will be equivalent isotopically. Moreover, as sea turtles lay several clutches during a nesting season, we were interested on

testing whether a single, non-intrusive sampling event (collecting unhatched eggs from a single nest) over the course of the 3–4 month nesting season adequately represents the isotopic signature of the foraging area.

Materials and methods

Study site and sampling

We simultaneously collected multiple tissues (blood, skin biopsy and at least one unhatched whole egg retrieved during post-hatch clutch excavation) for stable carbon and nitrogen isotope analysis from $n = 80$ individual loggerheads nesting along the Atlantic coast of Florida between 2009 and 2012. Females included in this study were sampled in two locations: the Archie Carr National Wildlife Refuge (ACNWR) in east central Florida and Juno Beach in South Florida. These two beaches (total length = 28 km) are loggerhead hotspots in the western hemisphere and account for approximately 22 % (i.e., 27,000 nests in 2012) of all the nests laid in Florida each year (Florida Fish and Wildlife Conservation Commission, unpubl data). Nesting activity is monitored at both sites, and a subsample of females is encountered and tagged using both Inconel flipper tags and passive integrated transponders during night surveys. Blood samples (4 ml) were collected from the cervical sinus with a 20-gauge needle and syringe (Owens and Ruiz 1980) as soon as the turtle began to cover her nest. Blood was transferred to a non-heparinized container and placed in ice. Skin samples were collected from the right shoulder (the area between the neck and the front flipper) using sterile 4-mm biopsy punches. Thirty-six (of the 80) females included in this study were sampled in conjunction with telemetry projects investigating movement patterns during the inter-nesting period, post-nesting migration destinations or estimating demographic parameters such as annual survival (Ceriani et al. 2012; Ceriani et al. in review; Ceriani et al. unpubl data; Sasso et al. unpubl data). Foraging areas used by 19 of the 36 tracked loggerheads were known, but for the remaining 17 individuals, the tags failed prematurely and did not transmit the data as programmed, i.e., all at once 1 year after deployment. We obtained a freshly laid egg at deposition only from individuals equipped with tracking devices as it is sometimes impossible to retrieve unhatched eggs from a given clutch at post-hatching excavation due to beach erosion or predation. Blood, skin and a freshly laid egg were collected at time of clutch deposition. We recorded GPS coordinates of the nests laid by the 80 females and marked them using standard triangulation protocols used in sea turtle research (Florida Fish and Wildlife Conservation Commission 2007). The fate of these nests was followed throughout incubation and excavation of clutches occurred after hatchling emergence (3–14 days post-emergence) to determine hatching success and retrieve at least one unhatched eggs for stable isotope analysis.

Loggerheads lay several clutches (mean 4, range 2–8; Miller et al. 2003; Tucker 2010) over the course of the 4-month nesting season, at approximately 2-week intervals, and each clutch contains an average of 112 eggs (Miller et al. 2003). To examine inter-clutch variability in egg isotopic values, we collected a single freshly laid egg per nest from 11 (of the 36) females equipped with a tracking device that were encountered repeatedly over the course of the nesting season. To examine intra-clutch variability in egg isotopic values, we retrieved 5–10 unhatched eggs at time of post-hatching excavation from 19 (of the 80) nests. We used unhatched eggs to minimize the amount of destructive sampling because sea turtles are species of conservation concern and unhatched eggs are readily available in most nests, varying in number from one egg per clutch (or none in some rare cases) to the entire clutch (Ehrhart unpubl data). If several unhatched eggs were available, we retrieved whole undeveloped eggs; we did not collect broken eggs or those containing late-stage development fetuses. Samples were stored in ice until returning to the field station. Blood was separated into serum and cellular components by centrifugation (5,000 rpm × 10 min). Tissue samples (RBC, serum, skin, freshly laid egg and unhatched undeveloped eggs) were frozen at −20 °C until analysis.

Sample preparation and stable isotope analysis

To address our objectives, we measured the stable carbon and nitrogen isotope ratios of RBC, epidermis, fresh egg yolk, unhatched eggs and serum. Other than serum, these tissues are assumed to have slow turnover rates that should reflect an integration of diet and habitat at the foraging ground prior to breeding migration. Tissue turnover rates in adult sea turtles are unknown, but it has been estimated that RBC, epidermis and egg yolk reflect the foraging habits at least 4 months prior to sampling (Brace and Altland 1955; Ceriani et al. unpubl data; Hamann et al. 2003; Reich et al. 2008, 2010; Seminoff et al. 2007).

Samples were prepared following standard procedures. Skin samples were rinsed with distilled water and cleaned with 70 % ethanol. Epidermis (*stratum corneum*) was separated from the underlying tissue (*stratum germinativum*) and finely diced using a scalpel blade. Epidermal samples were then dried at −60 °C for 48 h. Fresh eggs were separated into egg components (albumen and yolk) using an egg separator. In the case of unhatched eggs, it is often not possible to distinguish between egg components so the entire egg content was used for analysis after removing the eggshell.

RBC, egg (egg yolk and unhatched egg) and serum samples were freeze-dried for 24–72 h before being homogenized with mortar and pestle. Lipids were removed from all tissues using a Soxhlet apparatus with petroleum ether as solvent for 12 h (RBC and serum) and 24 h (epidermis, egg yolk and unhatched egg). Subsamples of prepared tissues (0.4–0.7 mg) were weighed with a microbalance and sealed in tin capsules. Prepared samples were sent to the Paleoclimatology, Paleoceanography and Biogeochemistry Laboratory at the University of South Florida College of Marine Science (St. Petersburg, FL, USA), where they were converted to N_2 and CO_2 using a Carlo-Erba NA2500 Series 2 Elemental Analyzer (Thermoquest Italia, S.p.A., Rodano, Italy) and analyzed with a continuous flow isotope ratio mass spectrometer (Delta PlusXP, Thermofinnigan, Bremen). Stable isotope ratios were expressed in conventional notation as parts per thousand (‰) according to the following equation:

$$\delta X = \left[\left(R_{\text{sample}}/R_{\text{standard}}\right) - 1\right] \times 1{,}000$$

where X is ^{15}N or ^{13}C, and R is the corresponding ratio ^{15}N:^{14}N or ^{13}C:^{12}C. The standards used for ^{15}N and ^{13}C were atmospheric nitrogen and Peedee Belemnite, respectively. Estimates of analytical precision were obtained by replicate measurements of internal laboratory reference materials (1577b Bovine liver) and yielded a precision (reflecting ±1 SD) of 0.13 ‰ for $\delta^{13}C$ and 0.16 ‰ for $\delta^{15}N$.

Statistical analyses

Principal component analysis (PCA) and simple linear regressions were used to illustrate the relationships between tissues sampled: RBC, epidermis, fresh egg yolk, unhatched egg and serum. Since we collected a fresh egg only from females equipped with tracking units, we performed two sets of analyses. PCA was used to define the overall pattern in the 36 tracked loggerheads for which we had $\delta^{13}C$ and $\delta^{15}N$ values for the four different tissues (RBC, epidermis, fresh egg yolk and unhatched egg) that have been used in previous studies to assign females to non-breeding foraging areas. We did not include serum in the PCA. Even though tissue turnover rates are not known for adult loggerheads, it is commonly assumed that serum has a much faster turnover rate compared with the other tissues (Hobson 1999), and thus, it is not the tissue of choice for foraging area assignment.

We used PC-ORD v5 (2006) for the PCA and the R Statistical Package (R Development Core Team 2011) for the remainder of the analyses with an alpha level set to 0.05 for all statistical analyses. We were interested in developing predictive equations to derive isotopic values of unhatched egg from other tissues because only this tissue can be collected widely and noninvasively. Therefore, unhatched egg isotopic values were treated as the dependent variables in all comparisons. Although we collected up to ten unhatched eggs from each individual, when results for multiple unhatched eggs were available for a single clutch, we randomly chose one unhatched egg to include in the analysis. We conducted four simple linear regressions for each element, $\delta^{13}C$ and $\delta^{15}N$ ($n = 36$ for independent variables fresh egg and serum, which we had only from the tracked females; $n = 80$ for RBC and epidermis, which were sampled from all females); we applied the Bonferroni correction to account for the risk of inflating Type I error and set an adjusted α-level (α') to 0.006 ($\alpha = 0.05/8$). Lastly, we used a paired t test to compare stable isotope values ($\delta^{13}C$ and $\delta^{15}N$) between unhatched egg and fresh egg yolk from each female.

To determine whether a single sampling event occurring at any time during the nesting season provides an adequate isotopic representation of the foraging area used in the non-breeding season, we examined inter-clutch variation in fresh egg yolk isotopic values for females ($n = 11$ individuals) that were repeatedly sampled throughout the nesting season. We developed general linear models (GLMs) in which the dependent variable was each stable isotope ratio ($\delta^{13}C$ and $\delta^{15}N$) and the independent variables were the individual turtle (random effect) and the time in days since the female's first clutch was observed (time 0 corresponded to the day we observed the first clutch for each female). Normality of the dependent variables was evaluated prior to the analyses.

To examine intra-clutch variation, we used clutches for which we collected at least five unhatched eggs ($n = 19$ individual clutches from different females). We followed a Monte Carlo approach to calculate the mean isotopic values for each element ($\delta^{13}C$ and $\delta^{15}N$) as a function of number of eggs sampled from randomly drawn combinations of eggs for each of the 19 clutches. We used the overall standard deviation of the reference material (laboratory precision) to determine how many eggs per clutch should be sampled to maintain the representative level of isotopic variation for the clutch, keeping within the measurement precision of the laboratory.

Results

Isotopic relationships between tissues

Isotopic signatures of the five tissues examined (RBC, epidermis, fresh egg yolk, unhatched egg and serum) are summarized in Fig. 1. We found marginal differences and no significant difference in $\delta^{13}C$ and $\delta^{15}N$ between fresh egg yolk and unhatched egg from each female ($\delta^{13}C$: paired t test, $t_{35} = 1.97$, $P = 0.06$; $\delta^{15}N$: paired t test, $t_{35} = -1.01$,

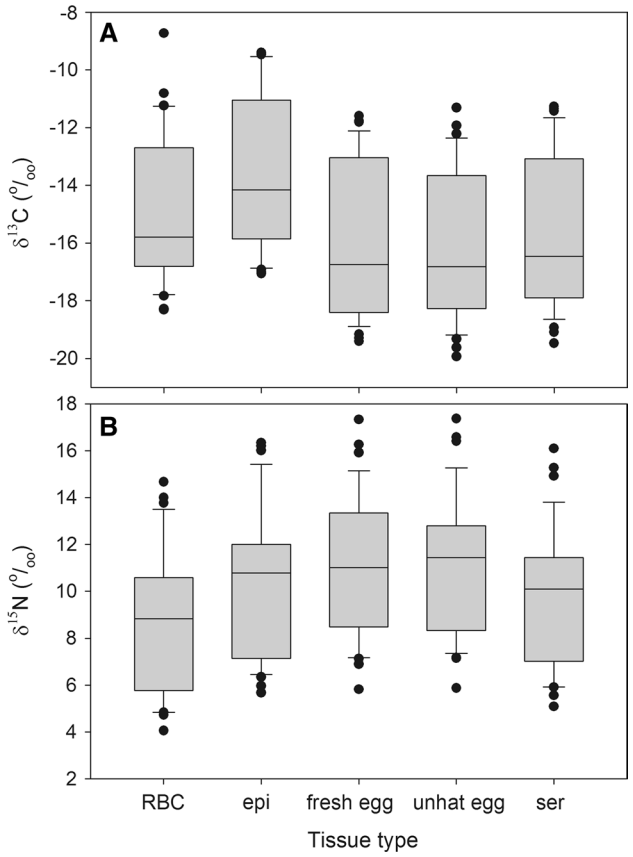

Fig. 1 Boxplot summarizing the distribution of $\delta^{13}C$ (**a**) and $\delta^{15}N$ (**b**) ratios found in five tissues (RBC, skin, fresh egg yolk, unhatched egg and serum) from 36 nesting loggerhead turtles. The box extends from the 25th to the 75th percentile; the *central line* indicates the median. The whiskers extend from the 10th to the 90th percentile. *Black circles* represent outliers. *RBC* red blood cells, *epi* epidermis, *fresh egg* fresh egg yolk, *unhat egg* unhatched egg, *ser* serum

$P = 0.32$), respectively. The relationship between the $\delta^{13}C$ and $\delta^{15}N$ for the different tissues is shown in Fig. 2.

We used PCA to reduce the number of inter-correlated variables, $\delta^{13}C$ and $\delta^{15}N$ values of several turtle tissues, into one or a few variables. The single significant PCA-derived variable (PC1), based on Jackson's "heuristic" approach to determine the number of significant axes (Jackson 1993 cited in McCune et al. 2002), explained 83.5 % of the variance of the isotopic values. The first two principal components (PC1 and PC2) accounted for 97.5 % of the variance. PC1 was highly positively correlated with $\delta^{15}N$ and highly negatively correlated with $\delta^{13}C$ (Table 1). Though all analyzed tissues have large differences in $\delta^{15}N$ and $\delta^{13}C$ correlation values, suggesting they discriminate among the isotopic signatures sufficiently, only one tissue (unhatched eggs) is non-destructive and noninvasive. PCA results showed distinct clustering of the PC scores for females using the three different foraging areas described in Ceriani et al. (2012) with only partial overlap between clusters (Fig. 3). The distribution of isotopic signatures from the combined tissues shows a similar grouping pattern to the latitudinal gradient found in Ceriani et al. (2012). As shown by the correlation matrix (Table 2), levels of a particular element were highly correlated among tissues. However, $\delta^{13}C$ was negatively associated with $\delta^{15}N$.

We found highly significant positive relationship between unhatched egg $\delta^{13}C$ and $\delta^{15}N$ and the corresponding RBC ($\delta^{13}C$: $r^2 = 0.91$, $F_{(1,78)} = 820.39$, $P < 0.001$; $\delta^{15}N$: $r^2 = 0.86$, $F_{(1,78)} = 494.81$, $P < 0.001$), epidermis ($\delta^{13}C$: $r^2 = 0.83$, $F_{(1,78)} = 451.91$, $P < 0.001$; $\delta^{15}N$: $r^2 = 0.86$, $F_{(1,78)} = 468.61$, $P < 0.001$), fresh egg yolk ($\delta^{13}C$: $r^2 = 0.95$, $F_{(1,34)} = 736.75$, $P < 0.001$; $\delta^{15}N$: $r^2 = 0.96$, $F_{(1,34)} = 861.69$, $P < 0.001$) and serum ($\delta^{13}C$: $r^2 = 0.78$, $F_{(1,34)} = 130.14$, $P < 0.001$; $\delta^{15}N$: $r^2 = 0.83$, $F_{(1,34)} = 147.59$, $P < 0.001$) values (Fig. 4).

Inter-clutch egg isotopic variability

We had fresh eggs from multiple clutches for 11 loggerheads equipped with tracking devices and used these females to investigate among-clutch variability in $\delta^{13}C$ and $\delta^{15}N$ egg values within a given nesting season. We were unable to retrieve unhatched eggs at post-hatching excavation from each clutch for all 11 turtles as some nests were lost due to beach erosion or raccoon depredation. Therefore, we used fresh eggs to examine inter-clutch variability in stable isotope ratios. Eggs from one female were sampled five times, eggs from another female were sampled four times, eggs from five females were sampled three times, and eggs from four females were sampled twice. The time of each laying event marginally affected $\delta^{13}C$ in fresh egg yolk, becoming progressively more depleted from one clutch to the next (rate of $\delta^{13}C$ change = −0.066 ‰, $F_{1,10} = 3.97$, $P = 0.074$; Fig. 5a), while $\delta^{15}N$ did not change significantly over time ($F_{1,10} = 0.0006$, $P = 0.98$; Fig. 5b).

Intra-clutch egg isotopic variability

Five or more unhatched eggs (range 5–10 eggs/clutch) were analyzed from a single clutch from 19 different females. The overall laboratory analytical uncertainty standard deviation was 0.13 ‰ for $\delta^{13}C$ (range 0.08–0.23 ‰) and 0.16 ‰ for $\delta^{15}N$ (range 0.10–0.23 ‰). The overall unhatched egg mean standard deviation within each clutch was 0.21 ‰ for $\delta^{13}C$ (range 0.04–0.53 ‰) and 0.20 ‰ for $\delta^{15}N$ (range 0.04–0.47 ‰). The Monte Carlo analyses showed (Fig. 6) the level of variability in $\delta^{13}C$ and $\delta^{15}N$ associated with the number of unhatched eggs with respect to the analytical precision of the laboratory. The probability that the clutch sample mean fell within the range of laboratory precision improved as the number of unhatched eggs analyzed per

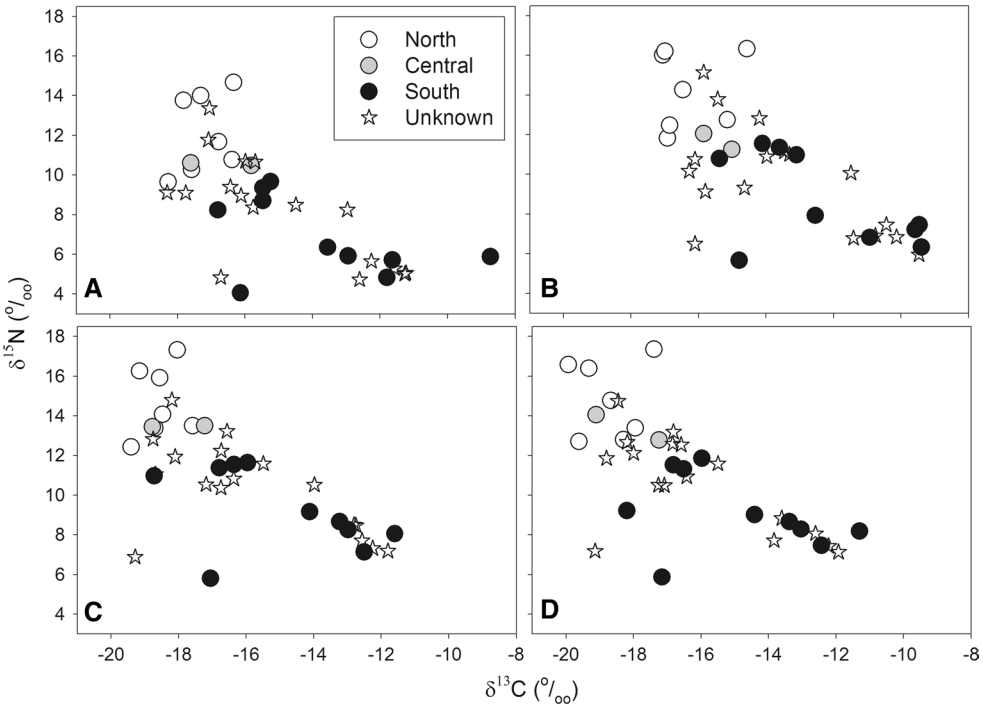

Fig. 2 Scatterplot of $\delta^{13}C$ and $\delta^{15}N$ values for the 36 nesting loggerheads sampled simultaneously for four tissues: **a** RBC, **b** epidermis, **c** fresh egg yolk and **d** unhatched egg. Foraging areas used by 19 of the tracked loggerheads were known to be north of the nesting area (*white circles*), near the nesting area in eastern central Florida (*gray circles*) or south of the nesting area (*black circles*; see Ceriani et al. 2012). *Stars* represent loggerheads whose foraging ground destinations were unknown

Table 1 Pearson and Kendall correlations (r) and relative weights (τ) of carbon and nitrogen stable isotope ratios by tissue on the first two principal component axes

Axis	1		2	
	r	τ	r	τ
$\delta^{13}C_{RBC}$	−0.905	−0.726	−0.386	−0.291
$\delta^{15}N_{RBC}$	0.924	0.737	−0.349	−0.187
$\delta^{13}C_{epidermis}$	−0.908	−0.719	−0.382	−0.270
$\delta^{15}N_{epidermis}$	0.924	0.732	−0.353	−0.199
$\delta^{13}C_{fresh\ egg}$	−0.915	−0.724	−0.384	−0.295
$\delta^{15}N_{fresh\ egg}$	0.915	0.781	−0.388	−0.168
$\delta^{13}C_{unhatched}$	−0.921	−0.748	−0.354	−0.253
$\delta^{15}N_{unhatched}$	0.900	0.748	−0.411	−0.195

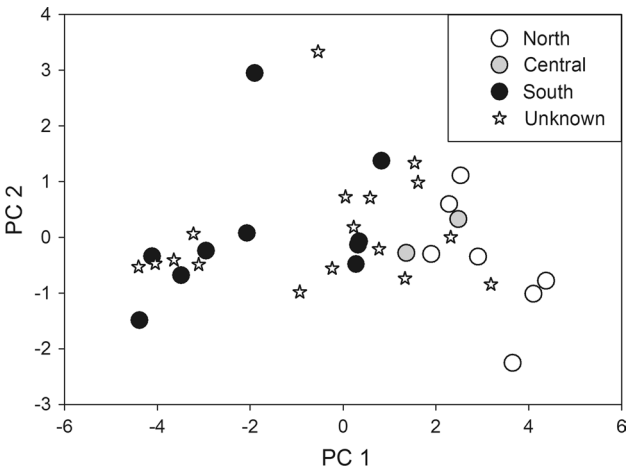

Fig. 3 Principal component analysis (PCA) ordination of $\delta^{13}C$ and $\delta^{15}N$ values of loggerhead tissues. Foraging areas used by 19 of the tracked loggerheads were known to be north of the nesting area (*white circles*), near the nesting area in eastern central Florida (*gray circles*) or south of the nesting area (*black circles*; see Ceriani et al. 2012). *Star* symbols represent loggerheads whose foraging ground destination was unknown

clutch increased up to three eggs. This sampling exercise suggests that three unhatched eggs sufficiently provide an accurate isotopic representation of the clutch as a whole.

Discussion

Isotopic relationship between tissues

We designed our study to investigate whether it is possible to (1) develop predictive equations to convert stable isotope results from one tissue into another one and (2) implement the use of a common tissue among researchers undertaking stable isotope studies on nesting marine turtles.

In the last decade, several researchers have used stable isotope analysis to investigate migratory connectivity in adult sea turtles (Hatase et al. 2002; Caut et al. 2008; Reich et al. 2010; Zbinden et al. 2011; Ceriani et al. 2012; Pajuelo et al. 2012; Seminoff et al. 2012). Researchers have used a variety of tissues and, in most cases, a single-tissue approach. We were interested in developing predictive

Table 2 Cross-products matrix with correlation coefficients among isotopes measured in the four tissue types included in the principal component analysis

	$\delta^{13}C_{RBC}$	$\delta^{13}C_{epi}$	$\delta^{13}C_{fresh}$	$\delta^{13}C_{unhat}$	$\delta^{15}N_{RBC}$	$\delta^{15}N_{epi}$	$\delta^{15}N_{fresh}$	$\delta^{15}N_{unhat}$
$\delta^{13}C_{RBC}$	1.000							
$\delta^{13}C_{epi}$	0.954	1.000						
$\delta^{13}C_{fresh}$	0.969	0.970	1.000					
$\delta^{13}C_{unhat}$	0.959	0.961	0.975	1.000				
$\delta^{15}N_{RBC}$	−0.695	−0.712	−0.715	−0.721	1.000			
$\delta^{15}N_{epi}$	−0.698	−0.699	−0.714	−0.725	0.971	1.000		
$\delta^{15}N_{fresh}$	−0.683	−0.684	−0.687	−0.699	0.973	0.977	1.000	
$\delta^{15}N_{unhat}$	−0.658	−0.656	−0.659	−0.695	0.964	0.966	0.982	1.000

RBC red blood cells, *epi* epidermis, *fresh* fresh egg yolk, *unhat* unhatched egg

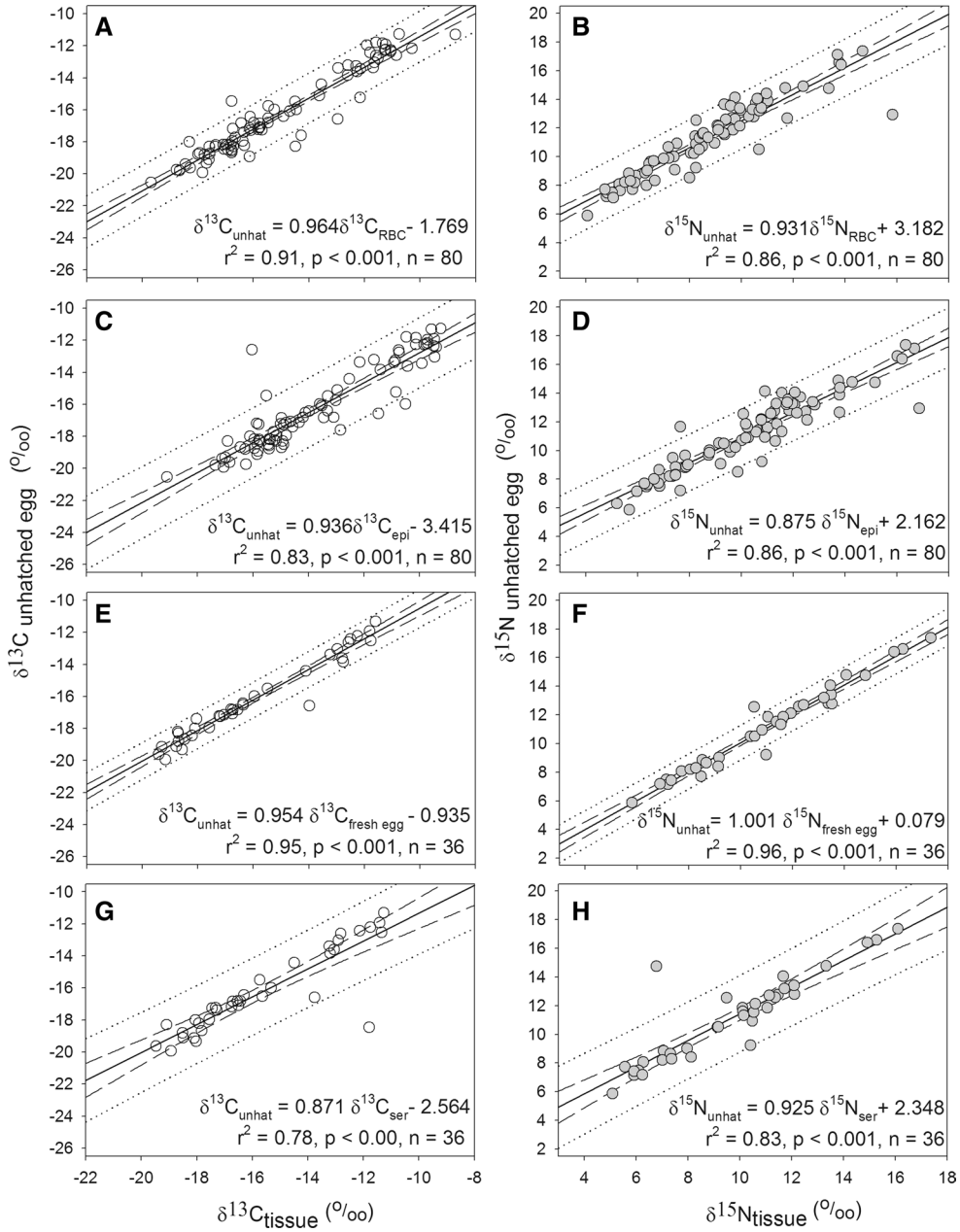

Fig. 4 Relationships between unhatched egg isotopic values and $\delta^{13}C$ and $\delta^{15}N$ values for red blood cells (**a**, **b**), epidermis (**c**, **d**), fresh egg yolk (**e**, **f**) and serum (**g**, **h**). *RBC* red blood cells, *epi* epidermis, *fresh egg* fresh egg yolk, *unhat egg* unhatched egg, *ser* serum

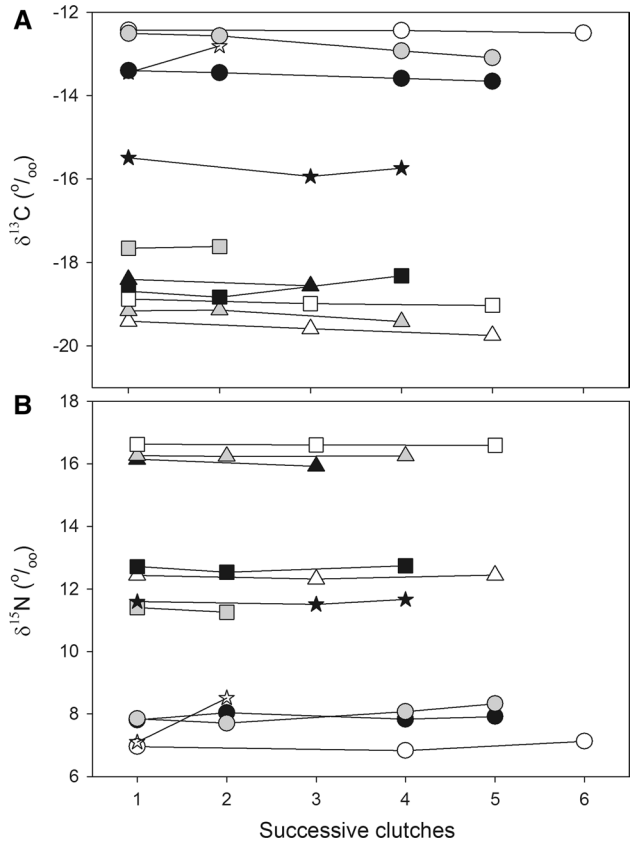

Fig. 5 Inter-clutch isotopic variability: trends in isotopic values for $\delta^{13}C$ (**a**) and $\delta^{15}N$ (**b**) in fresh egg yolk of 11 nesting loggerhead turtles. Each *point* represents a sampled clutch; each *line* connects successive clutches laid by one female. *Markers* correspond to specific females

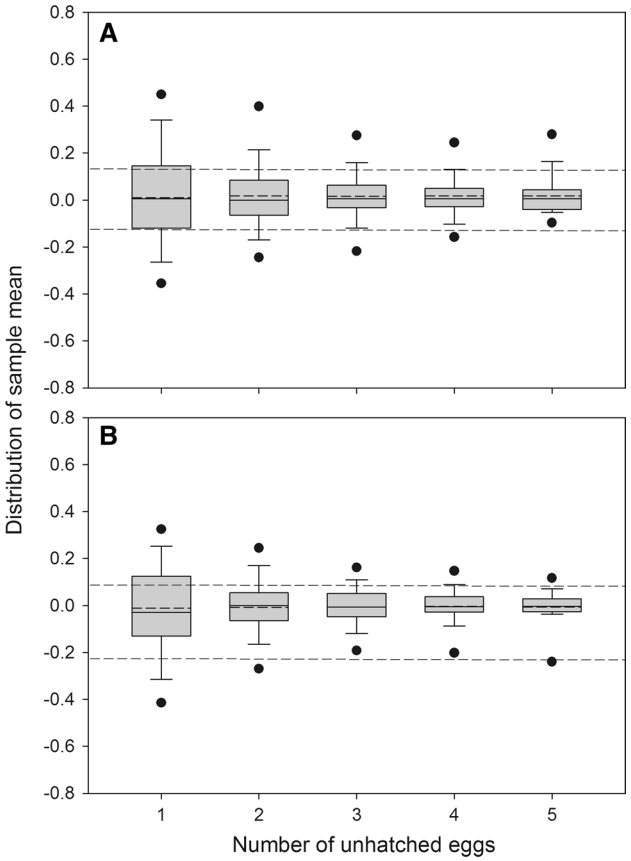

Fig. 6 Intra-clutch isotopic variability in $\delta^{13}C$ (**a**) and $\delta^{15}N$ (**b**) for the 19 clutches for which we collected more than 5 unhatched eggs. The boxplots summarize 100 Monte Carlo simulations and represent the distribution of the isotopic mean as a response of the number of unhatched eggs sampled per clutch. The solid line indicates the 50th percentile (median) and the *dashed line* the mean. The box encompasses the 25th to the 75th percentile. The whiskers extend from the 10th to the 90th percentile. *Black circles* represent the 5th and 95th percentiles. The *black horizontal dashed lines* represent the laboratory analytical precision of ±0.13 ‰ for $\delta^{13}C$ and 0.16 ‰ for $\delta^{15}N$

equations that could be used to integrate results from different studies and direct future work. Isotopic relationships between a few tissues have been investigated to some extent in previous studies but did not constitute their main scope. Caut et al. (2008) used fresh egg yolk and RBC stable isotope values to identify post-nesting migration destination of leatherbacks nesting in French Guyana. In doing so, they examined the relationship between fresh egg yolk and RBC values and found a positive and significant relationship between signatures ($\delta^{13}C$ and $\delta^{15}N$) of the blood and their fresh egg yolk. However, the relationship between $\delta^{15}N$ of fresh egg yolk and RBC explained only 64 % of the variation in the data (Caut et al. 2008; Table 1). Zbinden et al. (2011) used similar methods to assign putative non-breeding areas used by loggerheads nesting in Greece. Although they found a significant relationship between female carapace keratin and unhatched egg yolk $\delta^{15}N$ ($\delta^{15}N_{\text{unhatched egg yolk}} = 0.73 \times \delta^{15}N_{\text{carapace}} + 4.69$, $r = 0.92$, $P < 0.001$), they did not find a good fit in $\delta^{13}C$ between the two tissues (due to unequal distribution of data points across the range) and, thus, did not propose a predictive equation for $\delta^{13}C$.

We found that the relationships between unhatched egg isotopic values and RBC, epidermis, fresh egg yolk and serum were all highly significant and characterized by narrow confidence and predictive intervals. In all cases, the slope of the relationship was close to 1, indicating that the five tissues are isotopically equivalent and all represent the isotopic signature of the foraging area used by the female during the non-breeding season. Intercepts differed significantly from zero in all but one comparison (fresh yolk vs. unhatched egg) for both $\delta^{13}C$ and $\delta^{15}N$. These results suggest that diet–tissue discrimination (i.e., the difference between isotopic values of turtle tissues and its diet) differs between unhatched eggs and the other three tissues (RBC, epidermis, and serum). Eggs were the most ^{13}C-depleted of all the tissues and the most enriched in ^{15}N.

We expected to find highly significant relationships between unhatched egg, RBC, epidermis and fresh egg yolk isotopic values. In fact, previous studies have used satellite telemetry to validate the use of RBC (Ceriani et al. 2012; Pajuelo et al. 2012), epidermis (Seminoff et al. 2012), fresh egg yolk and unhatched eggs (Hatase et al. 2002; Caut et al. 2008; Zbinden et al. 2011) in both loggerhead and leatherback turtles and were able to assign post-nesting migration destinations based on values of $\delta^{15}N$ alone (Zbinden et al. 2011; Seminoff et al. 2012) or using a combination of $\delta^{15}N$ and $\delta^{13}C$ (Hatase et al. 2002; Caut et al. 2008; Ceriani et al. 2012; Pajuelo et al. 2012). However, we were surprised by the strength of the relationship between unhatched egg and serum isotopic values. In endotherms, serum is a fast turnover tissue (Hobson 1999) that is used to investigate short-term diet and habitat use. Even though there is increasing evidence suggesting that serum has a slow turnover rate in reptiles (summarized by Rosenblatt and Heithaus 2013; Table 2), there are no data available on tissue turnover rate in adult sea turtles. The significant positive relationship between $\delta^{13}C$ and $\delta^{15}N$ of serum and unhatched egg suggests that all the tissues we analyzed are in equilibrium with the diet of the female at the foraging ground.

Methodological validation: inter- and intra-clutch isotopic variation

The use of egg components (i.e., fresh egg yolk and unhatched egg) or egg products (i.e., hatchling) *in lieu* of other maternal tissues (i.e., RBC, epidermis and carapace) to study sea turtle trophic ecology and infer foraging grounds has been previously investigated to some extent (Caut et al. 2008; Zbinden et al. 2011; Frankel et al. 2012). Zbinden et al. (2011) examined the relationship between stable isotope ratios ($\delta^{13}C$ and $\delta^{15}N$) in fresh egg yolk and unhatched eggs and found what appeared a systematic enrichment of yolk at clutch excavation compared to fresh egg yolk, but the conclusions were obtained using a small sample size ($n = 5$ pairs). In contrast, we found no consistent differences in $\delta^{13}C$ and $\delta^{15}N$ values between unhatched egg and fresh egg yolk ($n = 36$ comparisons) and found a positive and highly significant relationship for both $\delta^{13}C$ and $\delta^{15}N$ in the two tissues, supporting the conclusion that fresh egg yolk and unhatched eggs are isotopically equivalent.

Marine turtles lay several clutches, each of which contains an average of 50–130 eggs (depending on the species, Miller et al. 2003); thus, we examined both inter- and intra-clutch isotopic variation in $\delta^{13}C$ and $\delta^{15}N$. We found that $\delta^{13}C$ values in egg yolk decreased slightly from one clutch to the next, becoming progressively more depleted, while $\delta^{15}N$ did not change significantly over time. We have no apparent explanation for the slight decrease in $\delta^{13}C$ over time. We predicted to find a change in $\delta^{15}N$ and not in $\delta^{13}C$. Changes in $\delta^{15}N$ may have been attributed to nutritional stress (if females were fasting) or different diet and change in $\delta^{15}N$ baseline (if females were foraging during the nesting season). Previous studies addressing inter-clutch isotopic variation found mixed results despite the common methodology. Hatase et al. (2002) found no significant difference in $\delta^{13}C$ and $\delta^{15}N$ values of fresh egg yolk among four serial clutches of the one loggerhead they examined but found a significant enrichment in $\delta^{15}N$ with the progression of the nesting season in one green turtle for which they sampled five serial clutches (Hatase et al. 2006). Caut et al. (2008) observed a significant decrease in $\delta^{13}C$ in fresh egg yolk in consecutive clutches of leatherbacks ($n = 23$ females) but no differences in $\delta^{15}N$, while Zbinden et al. (2011) found a small but significant decrease in $\delta^{13}C$ and $\delta^{15}N$ values with clutch order in unhatched eggs, with $\delta^{13}C$ and $\delta^{15}N$ on average becoming depleted by 0.14 and 0.13 ‰ with each successive clutch ($n = 14$ females). Despite a lack of agreement in results among studies, we agree with Zbinden's conclusion (2011) that the low magnitude of isotopic inter-clutch variability is not a concern if the purpose of the study is to use stable isotopes as intrinsic markers to infer origin of female foraging ground.

Caut et al. (2008) examined intra-clutch variability in fresh egg yolk $\delta^{13}C$ and $\delta^{15}N$ ($n =$ two fresh eggs from three clutches) and found that within-clutch variability was similar to laboratory measurement error. Thus, despite the small sample size, they concluded that a single egg yolk reflected the whole clutch. We found some level of isotopic variability in unhatched eggs, which may represent natural isotopic variation or may be affected by decomposition processes. Nests are excavated and unhatched eggs retrieved anywhere between 72 h (if hatching emergence is observed) and 2 weeks after expected emergence date (if no sign of emergence is noticed) depending on the protocol implemented by different research groups and permit guidelines. Thus, the effect of biological decomposition on unhatched eggs may vary and affect stable isotope values to different degrees and should be further investigated. Overall unhatched egg mean standard deviation within each clutch was 0.21 ‰ for $\delta^{13}C$ (range 0.04–0.53 ‰) and 0.20 ‰ for $\delta^{15}N$ (range 0.04–0.47 ‰). These values are comparable to the within-clutch unhatched egg isotopic variability reported by Zbinden et al. (2011). We found that analyzing three unhatched eggs is sufficient to obtain isotopic values that are representative of the whole clutch. However, such level of precision is perhaps unnecessary when the purpose of the study is to assign foraging ground used by the females because previous studies have shown that isotopic differences among distinct foraging areas are greater than 1 ‰ for at least one of the elements considered (Ceriani et al. 2012; Pajuelo et al. 2012; Seminoff et al. 2012).

Recently, Frankel et al. (2012) proposed to use nest content (hatchling epidermis tissue) retrieved at clutch excavation to derive the isotopic values of female foraging ground. They found that fresh dead hatchling $\delta^{15}N$ and $\delta^{13}C$ values were significantly correlated with the values of their mothers, but the relationship between hatchling and female epidermis $\delta^{13}C$ should be interpreted with caution due to the small range in $\delta^{13}C$ values and low r^2. Hatchlings that were sampled after being discovered dead in the nests (3 days after hatching emergence) had significantly different discrimination values from those of live hatchlings, suggesting decomposition affects the reliability of stable isotope ratios. In addition, they found little isotopic variation in live hatchling collected from the same clutch ($n = 5$ hatchlings from three distinct nests) and suggested collecting a skin biopsy from a single live hatching to obtain an acceptable estimate of the whole nest and derive an isotopic value for the mother. We recognize the potential of sampling live hatchlings; however, we believe that unhatched eggs are the preferred tissue to use to investigate female sea turtle migratory connectivity. Even though repeated sampling of skin did not affect growth rates and health status of hatching loggerheads raised in captivity (Bjorndal et al. 2010), collecting a skin biopsy from live hatchling is invasive, logistically more difficult (hatchlings must be intercepted when leaving the nest) and requires appropriate training and permits.

Conclusions and conservation implications

Based on our results, we conclude that (1) unhatched eggs are isotopically equivalent to RBC, epidermis, fresh egg yolk and serum, (2) inter-clutch variability in egg isotopic values is minimal and, therefore, a single sampling event during the nesting season adequately represents the foraging ground used by the female during the non-breeding season, and (3) three unhatched eggs should suffice to account for intra-clutch isotopic variability.

Being able to convert results obtained by different studies into a common tissue provides an opportunity to explore these datasets to undertake meta-analysis of stable isotope results to derive more general isotopic patterns at broader geographic scales. Moreover, our results provide empirical data supporting the choice of unhatched eggs as a common currency in stable isotope studies of nesting sea turtles. The use of unhatched eggs has profound practical and management implications. Unhatched eggs are an ideal tissue to sample because collecting unhatched eggs does not require encountering the nesting female. Few research groups intercept and work with nesting females, while morning nest monitoring programs are extremely common and in place worldwide. For example, Florida accounts for approximately 90 % of all the loggerhead nests laid in the southeast USA (2008–2012 nest number average = 62,867; Florida Index Nesting Beach Survey program), and it is debated whether it is the first or the second largest loggerhead aggregation in the world (Ehrhart et al. 2003). Despite its importance and the fact that Florida beaches are generally easy to access, only a handful of research groups encounter nesting females at night. On the other hand, the State of Florida—as well as the entire southeast USA and many other nesting beaches around the world—have very well established and comprehensive morning monitoring programs where sea turtle nests are counted daily or on a regular basis. Thus, retrieving unhatched eggs can be done much more simply and provide an opportunity to sample at a much larger scale (both numerically and geographically). Using unhatched eggs, in turn, provides an opportunity to (1) reduce sampling biases, (2) obtain information that is more representative at the population level, (3) investigate relative importance of foraging grounds on a yearly basis, (4) investigate how contribution from different foraging grounds varies over time and (5) elucidate remigration intervals and environmental parameters that may affect nesting patterns. Moreover, collecting unhatched eggs is a noninvasive and non-destructive sampling method, which is preferred in general and especially when dealing with threatened and endangered species. We recognize that it is not always possible to retrieve unhatched eggs at inventory because nests may be lost due to stochastic events such as storms, hurricanes and, consequent, beach erosion, or predation (e.g., raccoons, Barton and Roth 2008). Thus, while we advocate sampling unhatched eggs, we recommend collecting a fresh laid egg at time of deposition or a skin sample if an individual is particularly important for a specific study (i.e., the female is equipped with a tracking device).

While we have not compared isotopic values among tissues in other sea turtle species, we expect the strong relationships we found between tissues will be maintained across marine turtle species. This hypothesis is supported by the work Caut et al. (2008) conducted on leatherback turtles. Sea turtles are widely distributed species of conservation concerns, but our understanding of their life at sea is still relatively limited. The use of unhatched eggs as a common currency can be applied to loggerhead breeding aggregations worldwide if females forage in areas that are isotopically different and if their isotopic values fell within the data range of this study, assumptions supported by the studies that have already been conducted (Hatase et al. 2002; Zbinden et al. 2011; Ceriani et al. 2012; Pajuelo et al. 2012). Thus, the regressions we provide to mathematically correct loggerhead RBC, skin and fresh egg yolk $\delta^{13}C$ and $\delta^{15}N$ into unhatched egg isotopic values open new opportunities to conduct large-scale studies that can improve our comprehension of this species' ecology and migratory

connectivity. A better understanding of sea turtle migratory connectivity is particularly important given that many of the recently compiled research priorities for marine turtle conservation and management have a significant spatial component (Hamann et al. 2010; Wallace et al. 2011).

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