Inherent Variation in Stable Isotope Values and Discrimination Factors in Two Life Stages of Green Turtles

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ABSTRACT

We examine inherent variation in carbon and nitrogen stable isotope values of multiple soft tissues from a population of captive green turtles *Chelonia mydas* to determine the extent of isotopic variation due to individual differences in physiology. We compare the measured inherent variation in the captive population with the isotopic variation observed in a wild population of juvenile green turtles. Additionally, we measure diet-tissue discrimination factors to determine the offset that occurs between isotope values of the food source and four green turtle tissues. Tissue samples (epidermis, dermis, serum, and red blood cells) were collected from captive green turtles in two life stages (40 large juveniles and 30 adults) at the Cayman Turtle Farm, Grand Cayman, and analyzed for carbon and nitrogen stable isotopes. Multivariate normal models were fit to the isotope data, and the Bayesian Information Criterion was used for model selection. Inherent variation and discrimination factors differed among tissues and life stages. Inherent variation was found to make up a small portion of the isotopic variation measured in a wild population. Discrimination factors not only are tissue and life stage dependent but also appear to vary with diet and sea turtle species, thus highlighting the need for appropriate discrimination factors in dietary reconstructions and trophic-level estimations. Our measures of inherent variation will also be informative in field studies employing stable isotope analysis so that differences in diet or habitat are more accurately identified.

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Introduction

Stable isotope analysis is commonly used to investigate consumer foraging patterns in ecological studies. Dietary reconstructions through mixing models and trophic-level estimations rely on diet-tissue discrimination factors (the difference between stable isotope values of an organism's tissue and diet). More recent applications using carbon and nitrogen stable isotope compositions ($\delta^{13}C$ and $\delta^{15}N$, respectively) to examine trophic niche and specialization rely on measures of stable isotope variance within the population (Araujo et al. 2007; Layman et al. 2007*b*; Newsome et al. 2007; Vander Zanden et al. 2010). The isotopic niche is used as a proxy for ecological dimensions of resource use because the stable isotope ratios in the tissue of an organism represent the assimilated diet (Layman et al. 2007*a*; Vaudo and Heithaus 2011). Additionally, specialization can be inferred by examining the isotopic variation of a population or an individual through time. Low variation indicates specialization, while substantial variation indicates generalization (Bearhop et al. 2004; Martínez del Rio et al. 2009*a*; Newsome et al. 2009; Vander Zanden et al. 2010).

In many studies, isotopic variation is attributed to diet and habitat differences, but it can also result from variation in the isotopic composition within a prey species, inherent variation in the consumer, and measurement error (Bearhop et al. 2002; Matthews and Mazumder 2004; Phillips and Eldridge 2006; Barnes et al. 2008). Inherent variation in stable isotope values (hereafter referred to as "inherent variation") is a consequence of isotopic deviations that arise from individual differences in physiology despite consuming the same diet and experiencing controlled conditions. Although not often quantified, inherent variation could substantially affect conclusions based on stable isotope data. Inherent variation can depend on the species, life-history stage, and environment (Barnes et al. 2008), yet measurements of such variation from animals on controlled diets are sparse (Matthews and Mazumder 2004; Sweeting et al. 2005; Barnes et al. 2008; Seminoff et al. 2009). In one case, inherent variation made up a large portion of the isotopic variance measured in a wild population of sea bass *Dicentrachus labrax* (Barnes et al. 2008). Therefore, inherent variation should be considered when generating inferences about foraging patterns in wild populations. If it is assumed that all isotopic variation observed is due to differences in diet and habitat use, then the

resulting isotopic niche or level of generalization may be overestimated.

Diet-tissue discrimination is represented as $\Delta = \delta_{\text{tissue}} - \delta_{\text{diet}}$ and results from processes such as fractionation during metabolic transformations and isotopic routing (Martínez del Rio et al. 2009*b*). Accurate diet-tissue discrimination factors are essential to estimate trophic level and reconstruct diets, and variation in the discrimination factor should be accounted for in mixing models (Post 2002; Wolf et al. 2009). Many studies have used generalized discrimination factors because of the lack of species-specific values, yet the use of such values can lead to large errors or meaningless results in the output of mixing models (Caut et al. 2009).

Consumer tissues are often enriched in ^{15}N and ^{13}C compared with their diets (DeNiro and Epstein 1978; DeNiro and Epstein 1981; Post 2002), though discrimination factors may vary with life stage, environment, form of nitrogenous waste excretion, taxon, species, tissue, diet quality, and diet isotopic composition (Vander Zanden and Rasmussen 2001; Vanderklift and Ponsard 2003; Caut et al. 2009). The commonly used diet-tissue discrimination value for nitrogen ($\Delta^{15}N$) is 3.4‰ (DeNiro and Epstein 1981; Post 2002). Values of $\Delta^{13}C$ are typically much smaller than $\Delta^{15}N$ values, resulting in a reduced trophic shift in $\delta^{13}C$ values as nutrients are transferred through the food web (DeNiro and Epstein 1978).

The first objective of our study was to quantify the inherent variation in a captive population of green turtles *Chelonia mydas* fed a consistent diet. We examined the variation in stable isotope values—our measure of inherent variation—in four tissue types (epidermis, dermis, serum, and red blood cells) and two life stages (large juveniles and adults). We then compared our measure of inherent variation in epidermis with the isotopic variance observed in a wild population. The second objective of our study was to measure discrimination factors for each of the four tissues in both juvenile and adult green turtles maintained on an isotopically consistent diet. Furthermore, we incorporated the measure of inherent variation in our estimates of the discrimination factors. We also compared the discrimination factors found here with other sea turtle species.

Material and Methods

Study Conditions

Green turtles were housed at the Cayman Turtle Farm (CTF) in Grand Cayman, British West Indies. These turtles are descendants of a mixed breeding stock made up of turtles from at least four nesting populations (Wood and Wood 1980). Adults ranged from 10 to approximately 70 yr of age, from 92 to 110 cm curved carapace length (CCL), and from 75 to 186 kg. The large juveniles were approximately 4–6 yr of age and had been raised in captivity. Their size ranged from 64 to 92 cm CCL and 30 to 63 kg. At CTF, large juveniles grow at substantially higher rates (about 14 cm CCL/yr) than the same size class in the wild (Wood and Wood 1993; Bjorndal et al. 2000), and adults at CTF grow very little, if at all, after sexual maturity (Wood and Wood 1993).

The turtles were fed an extruded floating pellet diet manufactured by Southfresh Feeds at 0.5% body weight/d for 4 yr before sampling. The feed consists of at least 36% crude protein, 3.5% crude fat, 12% moisture, 6% crude fiber, and 1% phosphorus. A complete list of the feed ingredients is available in appendix A in the online edition of *Physiological and Biochemical Zoology*. The diet is highly digestible, and a similar diet (35% protein and 3.9% fat) had a dry-matter digestibility of 85.9% and a protein digestibility of 89.4% (Wood and Wood 1981). The turtles were assumed to be at isotopic equilibrium with the diet.

Juveniles and adults were maintained in tanks or an artificial pond. The water intake pipes for each were directed to create a slow circular current against which the turtles swam. They were almost constantly in motion during daylight hours, with resting periods at night. The maximum depth of the adult pond was 5.2 m, and there was an artificial beach available for females to lay eggs. The depth of the juvenile tanks was 0.9 m.

Sample Collection

During April and May 2010, tissue samples were collected from 30 adult female green turtles and from 40 large juvenile green turtles. Blood samples of 2–8 mL were drawn from the carotid arteries using sterile 16G × 2″ IV catheters (SURFLO I.V. catheters) and were immediately transferred to 9-mL draw CORVAC serum separator tubes. Serum and red blood cells were separated by centrifugation at 2,195 *g* and frozen separately at −20°C until analysis. Skin samples were taken with 6-mm Miltex sterile biopsy punches in the region between the front flipper and the head, just below the carapace, and placed in 70% ethanol. Isotope values of sea turtle epidermis preserved in ethanol were not different from those that were dried at 60°C, indicating that the preservation method does not affect the tissue stable isotope ratios (Barrow et al. 2008). At the time of sample collection, CCL was measured from the anterior midpoint of the nuchal scute to the posteriormost tip of the rear marginal scutes, and most individuals were weighed. Body-condition index was calculated as $(\text{mass}/\text{CCL}^3) \times 10^4$, with mass in kilograms and CCL in centimeters (Bjorndal and Bolten 2010). At the time of tissue sampling, two diet samples of approximately 100 g from the same commercial batch were set aside for stable isotope analysis. The manufacturer produces feed approximately once per month. The diet was specifically formulated for the CTF and is held as constant as possible by the manufacturer. Although there might be slight isotopic differences in different food lots, we are confident that these are minimal. Because all turtles are fed daily from the same lots, any differences we found in the captive population would not be a result of the different lots, as they would have experienced the variation equally. Samples were collected under University of Florida Institutional Animal Care and Use Committee protocol Z994/200801985.

Table 1: Bayesian Information Criterion (BIC) values for the 10 models

Hypotheses	Model parameterization		
	Mean and variance	Mean (with pooled variance)	Variance (with centered mean)
1. Null (all data in one group)	1,331.7	...	...
2. Life stage (two groups)	1,250.0	1,306.5	1,272.5
3. Tissue (four groups)	589.1	725.1	555.3
4. Life stage and tissue (eight groups)	365.3	589.2	286.5

Note. The data were grouped according to four hypotheses in which all data were considered together or were grouped by life stage, tissue, or both. Three model parameterizations were considered in which the mean, variance, or both were allowed to differ in maximizing the function. The first model could not be considered with alternative parameterizations.

Sample Preparation and Isotope Analysis

Serum and red blood cell samples were thawed, dried at 60°C for 24 h, and homogenized with a mortar and pestle to a fine powder. Skin samples were rinsed in distilled water, and the epidermis was removed from the dermis with a scalpel. A small portion of the dermis closest to the skin surface was subsampled to provide the dermis sample. Both dermis and epidermis samples were homogenized by dicing with a scalpel and then were dried at 60°C for 24 h. Diet samples were ground in a Wiley mill to a <1-mm particle size.

Tissue samples weighing 0.5–0.6 mg and diet samples ranging from 0.45 to 1.5 mg were analyzed for carbon and nitrogen isotopes at the University of Florida Department of Geological Sciences Light Isotope Lab. Samples were combusted in an ECS 4010 elemental analyzer (Costech) interfaced via a ConFlo III device to a Delta Plus XL isotope ratio mass spectrometer (ThermoFisher Scientific). The standards used for ^{13}C and ^{15}N were Vienna Pee Dee Belemnite and atmospheric N_2, respectively. Delta notation is used to express stable isotope abundances, defined as parts per thousand (‰) relative to the standard

$$\delta = \left(\frac{R_{\text{sample}}}{R_{\text{standard}}} - 1\right) \times 1{,}000,$$

where R_{sample} is the ratio of heavy to light isotopes ($^{13}C/^{12}C$ or $^{15}N/^{14}N$) in the sample and R_{standard} is the isotope ratio of the corresponding international standard. The reference material USGS40 (L-glutamic acid) was used as a calibration standard in all runs, with standard deviation (SD) = 0.12‰ for $\delta^{13}C$ and 0.14‰ for $\delta^{15}N$ ($n = 32$). Repeated measurements of a laboratory reference material, loggerhead sea turtle *Caretta caretta* scute, were used to examine consistency in a homogeneous sample with isotopic composition similar to that of the tissue samples in this study. The SD of the loggerhead scute was 0.07‰ for $\delta^{13}C$ and 0.25‰ for $\delta^{15}N$ ($n = 13$).

A subset of six dermis samples weighing approximately 1.0 mg plus diet samples weighing 3.0–4.5 mg was also analyzed for dry-mass percent carbon (C) and nitrogen (N) to calculate the C : N ratio. Lipids were extracted from a different subset of six dermis samples using petroleum ether in an accelerated solvent extractor (Dionex ASE300) and analyzed for carbon and nitrogen isotopes to examine the effect of lipids on the isotope composition and isotopic variation.

Data Analysis

Ten multivariate normal models were fitted to the carbon and nitrogen isotope data to examine how to best group the data while considering means and variances among groups (table 1). Four hypotheses were examined to determine whether the data were best described by considering (1) all samples together, (2) samples grouped by life stage (large juveniles, adults), (3) samples grouped by tissue type (epidermis, dermis, serum, red blood cells), or (4) samples grouped by both life stage and tissue type. Three model parameterizations were applied to each hypothesis (except the first) to create a total of 10 models (table 1). The second and third parameterizations were not applied to the first hypothesis because there was only one group, meaning that creating a pooled variance or centered mean had no effect.

In these parameterizations, the vector of means, the variances, or both were allowed to differ in the resulting multivariate normal likelihood function (Johnson and Wichern 2002) of the observed data. The maximum likelihood (ML) estimates also correspond to the vector of arithmetic means and the sample variance-covariance matrix (Johnson and Wichern 2002). The four tissues were assumed to be independent samples, and the analysis diagnostics (residuals) were examined to ensure that there were no major departures from the model assumptions.

The first hypothesis was a null model that assumed that the variability among all life stages and tissue types was best described with only one set of parameters: a single set of means and a single variance-covariance matrix. In the second hypothesis, the data were divided by life stage to determine whether adult and large juvenile samples were different in their isotopic values irrespective of tissue type. Thus, the samples were assumed to come from just two sampling multivariate normal models with different mean vectors and variance-covariance matrices. In the third hypothesis, each tissue type was considered separately, though adult and large juvenile values of the same tissue type were grouped. Hence, four different multivariate normal sampling models were needed to explain

Table 2: Mean and variance of $\delta^{13}C$ and $\delta^{15}N$ values

	Chelonia mydas (our study; adult $n = 30$, juveniles $n = 40$)		*C. mydas* ($n = 8$)[a]		*Dermochelys coriacea* ($n = 7$)[b]	
	$\delta^{13}C$	$\delta^{15}N$	$\delta^{13}C$	$\delta^{15}N$	$\delta^{13}C$	$\delta^{15}N$
Diet	−23.05 (.29)	2.49 (.05)	−19.03 (.97)	6.24 (.24)	−17.71 (.12)	8.64 (.22)
Diet (lipid extracted)			−18.64 (.20)	6.21 (.34)	−17.76 (.08)	8.59 (.53)
Adults:						
Epidermis	−21.44 (.08)	6.57 (.14)				
Dermis	−20.47 (1.14)	7.47 (.29)				
Serum	−22.80 (.08)	6.70 (.12)				
Red blood cells	−22.75 (.04)	5.01 (.07)				
Juveniles:						
Epidermis	−21.18 (.03)	6.31 (.11)	−18.54 (.04)[c]	9.00 (.32)[c]	−15.46 (.24)[c]	10.50 (.03)[c]
Dermis	−20.88 (.05)	6.69 (.16)	...	...	...	...
Serum/plasma	−21.89 (.02)	6.59 (.08)	−19.18 (.05)	9.14 (.03)	−18.35 (.21)	11.45 (.14)
Red blood cells	−22.54 (.03)	4.89 (.09)	−20.15 (.03)	6.52 (.04)	−17.31 (.05)	10.08 (.03)

Note. Mean $\delta^{13}C$ and $\delta^{15}N$ values (‰) and inherent variation (in parentheses) are reported for each of the four tissues in both life stages from our study and other juvenile sea turtle tissues at isotopic equilibrium reported from the literature. Diet sample mean $\delta^{13}C$ and $\delta^{15}N$ values and variance are also included. The values in our study were reported for serum, but plasma was used in the other studies. Because of the similarity in these two tissues, they are reported on the same line.

[a]Seminoff et al. 2006.

[b]Seminoff et al. 2009.

[c]Lipid-extracted tissue.

the data. In the fourth hypothesis, the samples were divided by both life stage and tissue type, creating eight groups. Thus, the joint likelihood of all the data needed for parameter estimation becomes the product of eight different multivariate normal probability density functions.

Model selection was carried out using Bayesian Information Criterion (BIC; Raftery 1995). Adding more parameters to a fixed model may improve the fit of the model, but the tradeoff is that it increases uncertainty in the estimation process. The BIC includes a term to penalize the ML score with a quantity proportional to the number of parameters used by the model. The BIC was calculated as

$$\text{BIC} = -2\ln(\hat{L}) + p\ln(q),$$

where $\hat{L}$ is likelihood function evaluated at the ML estimates, p is the number of parameters, and q is the sample size.

To evaluate whether there were differences in means and variances among the eight groups, pairwise comparisons were made between the mean vectors and variance-covariance matrices for a subset of all possible pairs. Each of the four tissue types were compared within the same life stage, but comparisons across life stages were made only for the same tissue type. In these pairwise comparisons, ML estimates and BIC values were calculated first assuming that the observed samples all came from a single sampling multivariate normal model and thus could be combined for the parameter estimation process and then assuming that the samples from the two different groups actually resulted from two separate multivariate normal sampling models where the means, variances, or both were assumed to differ. Differences in BIC values (ΔBIC) were calculated as $\text{BIC}_{\text{combined}} - \text{BIC}_{\text{separate}}$. A ΔBIC value greater than 2, 6, or 10 corresponds to positive, strong, or very strong evidence, respectively, for favoring the separate model over the combined model (Raftery 1995). Therefore, two groups were considered significantly different in their bivariate means or variances if $\Delta\text{BIC} > 2$. Negative values occurred when the $\text{BIC}_{\text{separate}}$ was larger than the $\text{BIC}_{\text{combined}}$.

Green turtle discrimination factors were calculated as $\Delta = \delta_{\text{tissue}} - \delta_{\text{diet}}$ for carbon and nitrogen. Variance from both the diet samples and the tissue was integrated into estimates of the discrimination factors through parametric bootstrapping. Normal distributions were used to represent the $\delta^{13}C$ and $\delta^{15}N$ values for the diet and each tissue for both life stages. The mean diet-tissue discrimination values ± SD were calculated by running 50,000 iterations.

The relationships between body-condition index and $\delta^{13}C$ dermis values were examined using Spearman's rank correlation. All statistical analyses were performed using R (R Development Core Team 2011). Annotated R code for the modeling is available in appendix B, available as a PDF in the online edition of *Physiological and Biochemical Zoology*.

Results

The variance in the stable isotope values, the inherent variation, of each tissue type and life stage differed among some tissues and life stages (tables 2, 3). The highest variance in the adult tissues occurred in dermis, which was significantly greater than the variance in other tissues, and the lowest variance in adult tissues was observed in red blood cells. For juvenile tissues, the

Table 3: Pairwise comparisons among bivariate variance-covariance matrices (above diagonal) and bivariate means (below diagonal)

	AEPI	ADERM	ASER	ARBC	JEPI	JDERM	JSER	JRBC
AEPI		30.7[a]	−9.9	−6.4	−2.1	...	...	...
ADERM	62.8[a]		33.1[a]	50.4[a]	...	68.5[a]	...	...
ASER	100.5[a]	93.7[a]		−6.4	...	...	7.0[a]	...
ARBC	132.3[a]	169.7[a]	111.3[a]		...	...	...	−6.4
JEPI	19.0[a]	...	...	...		−8.3	−8.9	−12.0
JDERM	...	97.2[a]	...	...	19.9[a]		3.4[b]	−6.1
JSER	...	...	126.2[a]	...	138.6[a]	178.4[a]		−9.9
JRBC	...	...	...	9.1[a]	208.8[a]	218.0[a]	178.3[a]	

Note. ΔBIC values >2 are considered significantly different (Raftery 1995). Comparisons that have no biological meaning are not included. A = adult; J = juvenile; EPI = epidermis; DERM = dermis; SER = serum; RBC = red blood cells.

[a]Strong or very strong evidence for a difference between the pair (ΔBIC values >6).

[b]Positive evidence for a difference between groups (ΔBIC values >2 but <6).

highest variance was also observed in dermis, with the lowest variance in serum.

The high variance in adult dermis was influenced by several points that exhibited high $\delta^{13}C$ values. Reanalysis of stable isotope ratios in those samples indicated that the values are accurate. To evaluate the influence of lipid content on variation or discrimination factors in dermis, the C : N ratio was measured and determined to be 2.8 in a subset of six samples. Additionally, three of the extreme dermis points and three randomly selected dermis samples were lipid extracted and compared with the nonlipid-extracted tissue using paired *t*-tests. There was no significant difference in either the $\delta^{13}C$ or $\delta^{15}N$ values ($\delta^{13}C$: $t = -0.36$, df = 5, $P = 0.73$; $\delta^{15}N$: $t = -0.86$, df = 5, $P = 0.43$). For all adult turtles, $\delta^{13}C$ values in dermis were significantly correlated with body condition (Spearman's rank, $\rho = 0.63$, $P < 0.001$). Condition indexes are often used as measures of health. While the condition index ranged from 0.5 to 1.9 in all turtles, the six adults with the highest dermis $\delta^{13}C$ values (>−20.2‰) also had high body-condition index measures (>1.4).

Pairwise comparisons between tissue means revealed significant differences among all tissues (table 3), which led to differences in discrimination factors among tissues. Discrimination factors between diet and turtle tissues (Δ) in adults ranged from 0.24‰ to 2.58‰ for carbon and from 2.48‰ to 4.93‰ for nitrogen (table 4). Discrimination factors in large juveniles ranged from 0.51‰ to 2.18‰ for carbon and from 2.36‰ to 4.15‰ for nitrogen, which were substantially larger than discrimination factors previously reported for juvenile green turtles (table 4). In comparison with the adults, juvenile $\Delta^{13}C$ values were larger in all tissues except dermis, and $\Delta^{15}N$ values were smaller in all tissues. Diet samples had a C : N ratio of 7.5 (mean C = 42.7%, mean N = 5.7%, $n = 4$). Mean $\delta^{13}C$ and $\delta^{15}N$ values of the diet were −23.05‰ and 2.53‰, respectively ($n = 12$; table 2).

To determine whether the data were best divided using both life stage and tissue type, four hypotheses with different data groupings were examined. BIC values decreased with the addition of more groups in each successive hypothesis, indicating an improvement in describing the data even though more parameters were estimated (table 1). Among the four hypotheses, the lowest BIC values were obtained when the data were grouped by tissue type and life stage, indicating that eight groups are most appropriate to divide the data (hypothesis 4; table 1).

Each of these hypotheses was examined with three different model parameterizations to examine the relative importance of the mean or the variance in reducing (thus improving) the BIC score. The first model parameterization included estimates of both the mean vector and the variance-covariance matrix. The four different groupings using the first parameterization are plotted with ML estimates and confidence ellipses in figures 1 and 2. In the second parameterization, the mean was estimated in each group using a pooled variance, and in the third parameterization, the variance was estimated for each group using a centered mean.

The first hypothesis could not be compared across parameterizations. For the other three hypotheses, the second model parameterization with pooled variance yielded higher BIC values than the first parameterization, indicating that a pooled variance among the groups does not perform as well in the model (table 1). The BIC values of the third parameterization compared with the first parameterization were higher for hypothesis 2 and lower for hypotheses 3 and 4. The lowest BIC value overall occurred in hypothesis 4 with the third parameterization, indicating that the variance is more influential in driving the group differences.

Discussion

Inherent Variation

Measures of inherent variation can be informative for field studies. Because the inherent variation differed among tissues, these measures can be used to select a tissue to minimize inherent variation and better understand variation in wild populations. For example, a population of resident juvenile green

Table 4: Discrimination factors ($\Delta^{13}C$ and $\Delta^{15}N$) measured in this study and for other sea turtle species reported from the literature

	Chelonia mydas adults (this study; $n = 30$)	*C. mydas* juveniles (this study; $n = 40$)	*C. mydas* juveniles ($n = 8$)[a]	*Dermochelys coriacea* juveniles ($n = 7$)[b]	*Caretta caretta* hatchlings ($n = 12$)[c]	*C. caretta* juveniles ($n = 12$)[c]
$\Delta^{13}C$:						
Epidermis	1.62 ± .61	1.87 ± .56	.17 ± .08[d]	2.26 ± .61[d]	2.62 ± .34[e]	1.11 ± .17[e]
Dermis	2.58 ± 1.19	2.18 ± .59	...	...	...	...
Serum/plasma	.24 ± .61	1.16 ± .56	−.12 ± .08	−.58 ± .53	.29 ± .20	−.38 ± .21
Red blood cells	.30 ± .58	.51 ± .56	−1.11 ± .17	.46 ± .35	−.64 ± .73	1.53 ± .17
$\Delta^{15}N$:						
Epidermis	4.04 ± .44	3.77 ± .40	2.80 ± .31[d]	1.85 ± .50[d]	1.65 ± .12[e]	1.60 ± .07[e]
Dermis	4.93 ± .59	4.15 ± .47	...	...	...	...
Serum/plasma	4.17 ± .41	4.06 ± .37	2.92 ± .08	2.86 ± .82	.32 ± .09	1.50 ± .17
Red blood cells	2.48 ± .35	2.36 ± .37	.22 ± .08	1.49 ± .76	−.25 ± .30	.16 ± .08

Note. All values are reported as means ± SD (‰). The discrimination factors in our study were reported for serum, though in all other studies the tissue used was plasma. Because of the similarity in the two tissues, they are reported on the same line. Discrimination factors were not measured for dermis in the other studies. Tissues were not lipid extracted unless noted.

[a]Seminoff et al. 2006.

[b]Seminoff et al. 2009.

[c]Reich et al. 2008.

[d]Lipid-extracted tissue and diet.

[e]Lipid-extracted tissue.

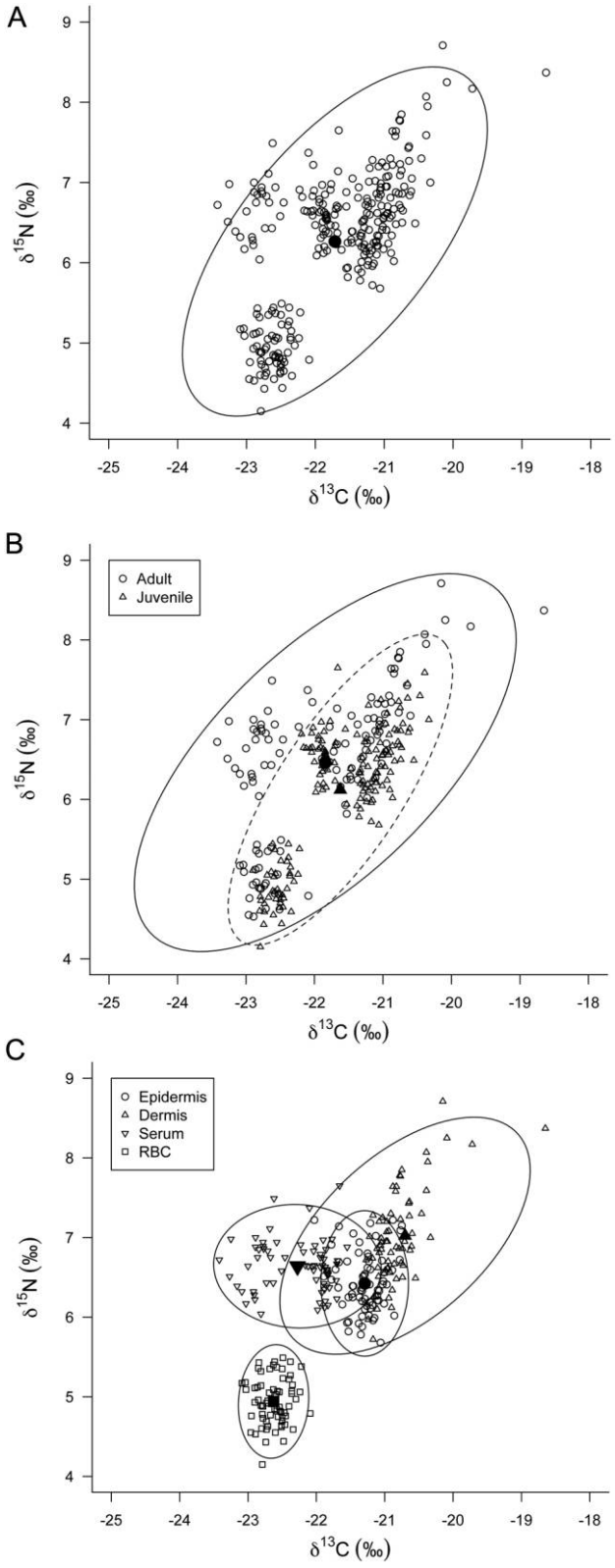

Figure 1. Results of three models using the first parameterization in which mean and variance are estimated. Filled symbols represent mean δ^{13}C and δ^{15}N values; open symbols represent individual measurements (*insets*). Bivariate 95% confidence ellipses are drawn for each group; a dotted ellipse is used for the juvenile group. The first model contains all data in one group (*A*), and then data are grouped by life stage (*B*) or tissue type (*C*). RBC = red blood cells.

turtles in a small foraging area in the Bahamas had a range of 5.4‰ in δ^{13}C and 6.6‰ in δ^{15}N values from epidermis samples (Bjorndal and Bolten 2010). In the captive population from our study, the epidermis ranges were 0.8‰ for δ^{13}C and 1.5‰ for δ^{15}N values. Moreover, the variance in both δ^{13}C and δ^{15}N values is much smaller in the captive population, as indicated by the size of the bivariate confidence ellipses (fig. 3). In this example, inherent variation does not form a large part of the isotopic variance in the wild population, as has been observed in other studies (Barnes et al. 2008). Therefore, it is unlikely that physiological differences in the wild population would create the observed variation in isotopic values; rather, individuals are probably using different diets or habitats or the prey species exhibit intraspecific variation. As additional studies begin to examine specialization in foraging through stable isotope consistency and isotopic niche space of distinct populations, these measures of inherent variation can be used to inform the baseline variation that is due to individual differences, and thus additional variation can be attributed to differences in diet and resource use with greater confidence.

We are uncertain about the cause of the wide range in dermis δ^{13}C values for adults in our study, but lipids do not appear to be responsible for the observed range. The measured C : N ratio of dermis in this study (2.8) falls below the cutoff of 3.5 for aquatic animals, indicating that lipid content is likely below 5% and would not influence the δ^{13}C values (Post et al. 2007). Also, removal of lipids did not result in significant differences for δ^{13}C values. We did observe a relationship between the dermis δ^{13}C values and the condition index. If the condition index is an indicator of health or if it changes with reproductive status, these factors may influence the range in adult dermis δ^{13}C values. Based on our results, we would discourage the use of dermis as a sampling tissue.

In our study, large juveniles generally had lower variance compared with the corresponding tissue in adults. Growth may affect the inherent variation, as it can also affect the discrimination factors (see "Life Stage"). The isotopic variation previously reported in juvenile green turtle tissues (Seminoff et al. 2006) is similar to our study (table 2). Additionally, inherent variation was quantified in a captive population of juvenile leatherbacks *Dermochelys coriacea* (Seminoff et al. 2009), and the measures of variance in the juvenile green turtles from our study were lower in all tissues (table 2).

Discrimination Factors

Mean discrimination factors of 0‰–1‰ for δ^{13}C and 3.4‰ for δ^{15}N were reported by early studies (DeNiro and Epstein 1978; Minagawa and Wada 1984) and confirmed by recent reviews (Vander Zanden and Rasmussen 2001; Post 2002). Nevertheless, discrimination factors have been observed to change with an array of variables. Because of a lack of species- or tissue-

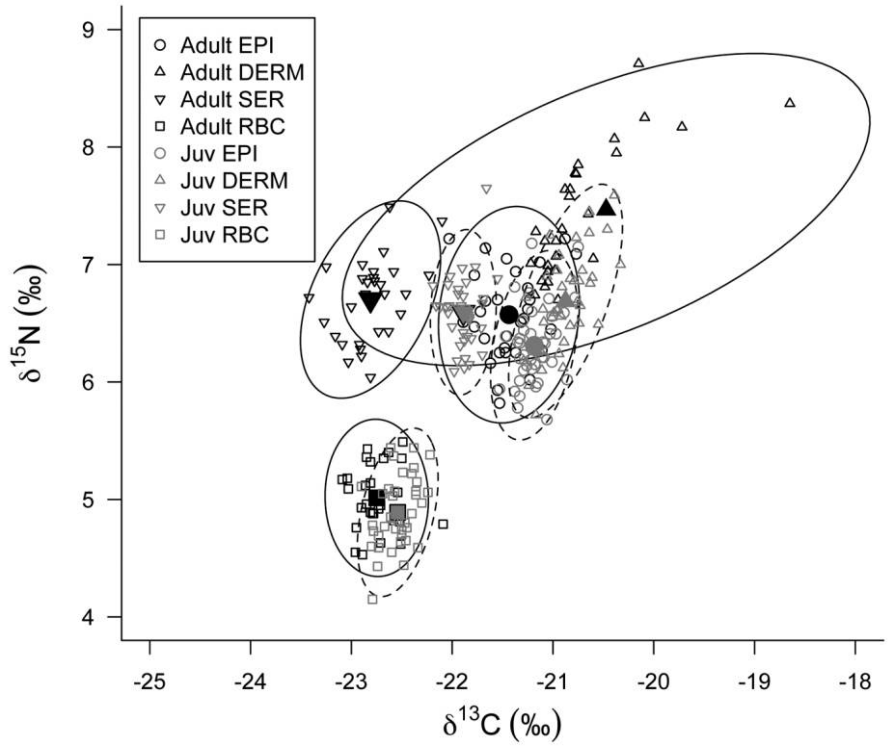

Figure 2. Results from hypothesis 4 using the first parameterization depicted separately to highlight the eight groups. Filled symbols represent mean $\delta^{13}C$ and $\delta^{15}N$ values; open symbols represent individual measurements (*inset*). Bivariate 95% confidence ellipses are drawn for adults (solid lines); dashed ellipse lines are used for juveniles. Data are grouped by life stage and tissue type. EPI = epidermis; DERM = dermis; SER = serum; RBC = red blood cells; Juv = juvenile.

specific discrimination factors available, these standard discrimination values continue to be applied to isotopic mixing models for dietary reconstructions or trophic-level estimations (Caut et al. 2009). Small changes in discrimination factors can lead to substantial differences in the output of these mixing models (Ben-David and Schell 2001); therefore, it is critical to provide species and tissue-specific measures. Stable isotope analysis has been increasingly used to investigate sea turtle foraging patterns because of the advantages of this technique to sample these long-lived migratory animals with cryptic life stages (Reich et al. 2007; Arthur et al. 2008; Vander Zanden et al. 2010). Additionally, mixing models have been applied to reconstruct sea turtle diets (Wallace et al. 2009; McClellan et al. 2010; Lemons et al. 2011).

Tissue. The means and variances of $\delta^{13}C$ and $\delta^{15}N$ are distinct among tissue types and life stages. These differences in tissue means translate into discrimination factor differences. The inherent variation observed in each tissue was also incorporated into the SDs of the discrimination factors. For example, dermis had the largest inherent variation among the four tissue types as well as the largest SDs in the estimates of discrimination values.

Consistent differences in $\delta^{15}N$ values have been observed for the same tissues across a variety of species, likely due to different metabolic properties that are used to create and maintain these tissues (Caut et al. 2009). Such differences might be caused by the amino acid content of each tissue (Martínez del Rio et al. 2009*b*). While some amino acids remain close to the isotopic composition of the diet, others are enriched through metabolic processes (McClelland and Montoya 2002; Popp et al. 2007), resulting in varying $\delta^{15}N$ values among amino acids. For example, in mammals, $\Delta^{15}N$ values of plasma > hair > red blood cells (Caut et al. 2009), similar to the pattern observed in this study with green turtle $\Delta^{15}N$ values of serum > epidermis (a keratin-based structure) > red blood cells. The mean difference between plasma/serum and red blood cell $\Delta^{15}N$ values was approximately 1.6‰ in mammals (Caut et al. 2009) and was 1.7‰ in this study, averaged between adults and large juveniles.

Differences among tissues in $\delta^{13}C$ values can be influenced by amino acid composition as well as lipid content, as lipids tend to be depleted in ^{13}C (DeNiro and Epstein 1977). Discrimination factors for carbon have been shown to vary with methods of sample preparation such as lipid removal or acidification (McCutchan et al. 2003). In our study, we did not remove lipids from either the tissue or diet samples, but in the subset of dermis samples for which lipids were removed, there was no effect on the stable isotope values of the tissue.

Isotopic routing is another factor that may affect both ni-

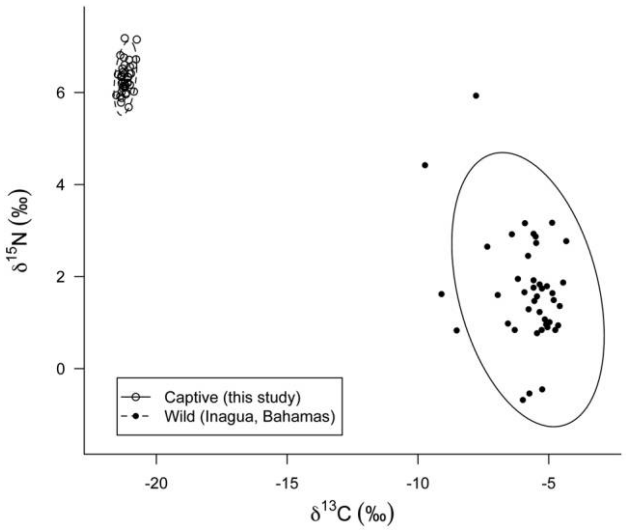

Figure 3. Comparison of isotopic variation in epidermis samples from juvenile green turtles. The inherent variation for the captive population ($n = 40$) is considerably smaller than the variation observed in the wild population resident on a small foraging area off Inagua, Bahamas, for at least 1 yr ($n = 42$). Bivariate 95% confidence ellipses are included for each group. Wild population data are modified from Bjorndal and Bolten (2010) for samples collected in 2002 and 2003.

trogen and carbon discrimination (Gannes et al. 1998). We are unable to evaluate the effects of isotopic routing in our study.

Life Stage. Nitrogen discrimination factors were larger in adults compared with the respective large juvenile tissues in our study. The differences between life stages may be due to protein balance differences rather than age, as animals with a positive protein balance should have lower Δ^{15}N values than animals that have a neutral or negative protein balance (Martínez del Rio and Wolf 2005). Protein balance is indicative of the efficiency of nitrogen deposition, measured as the ratio between protein assimilation and protein loss, and growing animals are expected to be in positive protein balance (Martínez del Rio et al. 2009*b*). Large juveniles grow rapidly, and adult growth is minimal (Wood and Wood 1993). The pattern in nitrogen discrimination factors in our study supports the predictions by Martínez del Rio and Wolf (2005). Studies comparing life stages or relative growth rates in other species have also reported patterns corroborating this prediction in red foxes *Vulpes vulpes* (Roth and Hobson 2000), Atlantic salmon *Salmo salar* (Trueman et al. 2005), and blue crabs *Callinectes sapidus* (Fantle et al. 1999).

Unlike nitrogen discrimination factors, there is no empirical prediction for the relationship between growth rate and carbon discrimination factors. The differences between adults and large juveniles were relatively small for epidermis, dermis, and red blood cells. The largest difference between Δ^{13}C values for the same tissue occurred in serum (0.93‰), probably as a result of higher lipids in adults. Females mobilize lipids for egg production, primarily vitellogenin (containing lipid triglycerides), which is synthesized in the liver and transported to the ovary in plasma (Hamann et al. 2003). Plasma triglyceride levels may increase up to 6 mo before the breeding season and remain high throughout the nesting season (Hamann et al. 2002). The adults in our study were all sexually mature females and were sampled just before the nesting season.

Intraspecific and Interspecific Comparisons. A negative trend between diet isotope values and discrimination factors has been observed across a wide range of taxa, though the trend was not examined in reptiles because of limited data (Caut et al. 2009). If this trend were sustained for reptiles, we would expect higher discrimination factors for juveniles in our study compared with those previously reported for juvenile green turtles (Seminoff et al. 2006) because of the lower δ^{13}C and δ^{15}N values in our diet (table 2). The method proposed by Caut et al. (2008, 2009) to apply a diet-dependent discrimination factor may be appropriate for reconstructing sea turtle diets through isotope mixing models. At this time, however, insufficient reptile data are available to calculate diet-dependent discrimination factors.

Nutritional content of the diet, particularly for nitrogen, may also affect the discrimination factor. A positive trend between diet C : N and Δ^{15}N values has been observed in a variety of species (Robbins et al. 2005). The feed used in our study had a higher C : N ratio than that used by Seminoff et al. (2006; 7.5 vs. 6.6). Consistent with the pattern observed by Robbins et al. (2005), the higher diet C : N ratio corresponded to a higher Δ^{15}N value. Yet further investigation of this pattern through varied diets in a single mammalian species yielded no relationship between C : N and Δ^{15}N values (Robbins et al. 2010). Rather, complementarity of amino acids and diets composed of a mixture of items may contribute to variation Δ^{15}N values (Robbins et al. 2010).

In comparison with other sea turtle species, the Δ^{15}N values measured in large juveniles from our study are higher than what has previously been reported (table 4). Besides possible dietary differences, growth rate differences are likely a major contributor to these discrimination value differences. The juveniles in our study were larger and likely had reduced growth rates, which would lead to larger Δ^{15}N values (Martínez del Rio and Wolf 2005).

The carbon discrimination factors were more variable among the sea turtle species. The largest Δ^{13}C value observed for epidermis was in leatherbacks (Seminoff et al. 2009), the largest Δ^{13}C value observed for serum/plasma was in green turtles from our study, and the largest Δ^{13}C value observed for red blood cells was in loggerheads (Reich et al. 2008; table 4). This may be due to differences in lipid concentration for each of the species, yet we are unable to make comparisons between potential lipid content, as C : N ratios were not measured in all studies.

Conclusions

In summary, we found that inherent variation is both tissue and life stage dependent, and these results can be useful for more accurately estimating the degree of specialization and isotopic niche width in wild populations. Inherent variation was apparently only a small portion of the variance in the stable isotope composition of a wild population. In addition, diet-tissue discrimination factors in sea turtles may vary with species, tissue type, diet, and growth rate, thus underscoring the need for appropriate discrimination values in mixing models and trophic-level estimations. We provide the first measure of discrimination factors for adult sea turtles. In juveniles, we believe the differences in discrimination factors compared with previous studies in sea turtles may be attributable to differences in diet and growth rate. Understanding the processes that influence isotopic discrimination and variance is fundamental to studies using stable isotope analysis to investigate foraging, behavior, and ecological roles of wild populations.

Acknowledgments

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 DOI: 10.1086/666902

Appendix A from H. B. Vander Zanden, K. A. Bjorndal, W. Mustin, J. M. Ponciano, and A. B. Bolten, "Inherent Variation in Stable Isotope Values and Discrimination Factors in Two Life Stages of Green Turtles"

(Physiol. Biochem. Zool., vol. 85, no. 5, p. 431)

Feed Ingredients

Green turtles in this study were fed an extruded floating pellet diet manufactured by Southfresh Feeds containing the following ingredients: plant protein products, processed grain by-products, grain products, animal protein products, fish meal, fish oil, dicalcium phosphate, calcium carbonate, vitamin A supplement, vitamin D supplement, vitamin E supplement, calcium pantothenate, niacin supplement, ascorbic acid (vitamin C), menadione dimethylprimidinol disulfate, pyridoxine hydrochloride, riboflavin supplement, thiamine mononitrate, vitamin B12 supplement, folic acid, zinc sulfate, ferrous sulfate, sodium selenite, copper sulfate, manganese sulfate, and ethylenediamine dihydriodide.

```
### APPENDIX B: ANNOTATED R CODE

### Vander Zanden et al. Inherent variation in stable isotope values and
 discrimination factors in two life stages of green turtles



### Analysis of the variability of C & N isotopes x age x tissue-type


#### Fitting Multivariate Normal models with parameterization 1 and 3:

# This function will allow for parameterization 1 (mean and variance can
 differ) or
# parameterization 3 (variance can differ with a centered mean),
 parameterization 2 is a separate function below
# This function has input of the data of carbon and nitrogen for any number
 of cases (individual turtles)
# belonging to the same group and outputs are the ML estimates for the mean
 vector and the variance-covariance matrix
# Parameterization 1: centered=FALSE and the mean and variance can differ
# Parameterization 3: centered=TRUE and only the variance can differ

library(mvtnorm)

MLmvn <- function(Xnp, centered=FALSE){

 dimX      <- dim(Xnp)
 n         <- dimX[1] #number of cases
 p         <- dimX[2] #number of variables (i.e. number of isotopes)
 S         <- var(Xnp) #unbiased
 Sigma.hat <- ((n-1)/n)*S  #to get ML estimate (biased)
 mu.hat    <- apply(Xnp,2,mean) #mean of the matrix by column (if used 1,
 by row)

 if(centered==TRUE){

    mus.mat <- matrix(rep(mu.hat,n),nrow=n,ncol=p,byrow=TRUE)
    Xcent   <- Xnp-mus.mat
    mu.hat  <- rep(0,p) # just to avoid numerical round-off errors, should
 be 0
    # no need to calc Sigma.hat again, it's the same...
 }


#lnLhat <- sum(dmvnorm(x=Xnp, mean=mu.hat,sigma=Sigma.hat,log=TRUE))
#a way to check calcs with library(mvtnorm)

#to get maximum of log likelihoods:
  lnLhat <- -(0.5*n*p)*log(2*pi) -(0.5*n*p)-(0.5*n)*log(det(Sigma.hat))

 return(list(mu.hat=mu.hat,Sigma.hat = Sigma.hat,lnLhat = lnLhat))
```

```
}

#### Fitting Multivariate Normal models with parameterization 2 (pooled
 variance and means can differ)
# Note: Pooled variance must be calculated prior to including it in the
 function

MLmvnpooled <- function(Xnp, Sp, centered=FALSE){ ##Sp is pooled variance

 dimX      <- dim(Xnp)
 n         <- dimX[1] #number of cases
 p         <- dimX[2] #number of variables (i.e. number of isotopes)
 Sigma.hat <- Sp  #to get ML estimate (biased)
 mu.hat    <- apply(Xnp,2,mean) #mean of the matrix by column (if used 1,
 by row)

 if(centered==TRUE){

    mus.mat <- matrix(rep(mu.hat,n),nrow=n,ncol=p,byrow=TRUE)
    Xcent   <- Xnp-mus.mat
    mu.hat  <- rep(0,p) # just to avoid numerical round-off errors, should
 be 0
    # no need to calc Sigma.hat again, it's the same...
 }


#lnLhat <- sum(dmvnorm(x=Xnp, mean=mu.hat,sigma=Sigma.hat,log=TRUE))
#a way to check calcs with library(mvtnorm)
#to get maximum of log likelihoods:

  lnLhat <- -(0.5*n*p)*log(2*pi) -(0.5*n*p)-(0.5*n)*log(det(Sigma.hat))

 return(list(mu.hat=mu.hat,Sigma.hat = Sigma.hat,lnLhat = lnLhat))

}


### Bayesian Information Criterion Function

BICcalc <- function(lnLhat, npars, samp.size){

 return(-2*lnLhat + npars*log(samp.size))

}

# Example parameterization 1 with hypothesis 1 (null model with all the
 data together)
Null.MLEs <- MLmvn(Xnp=The.data, centered=FALSE)
Null.BIC  <- BICcalc(lnLhat = Null.MLEs$lnLhat, npars=2+3, samp.size=len)
  #(npars is 1 mean vector and 3 elements in the var-cov matrix)
```

```
# Example of parameterization 2 (pooled variance) with hypothesis 2
 (groupings by life stage)
# First must calculate pooled variance
X.adult <- The.data[Tissues.data$AGE=="A",]
X.juv <- The.data[Tissues.data$AGE=="J",]
n1 <- length(X.adult[,1])
n2 <- length(X.juv[,1])
var.adult <- var(X.adult)*((n1-1)/(n1+n2-2))
var.juv <- var(X.juv)*((n2-1)/(n1+n2-2))
Sp <- var.adult+var.juv

M2.adult  <- MLmvnpooled(Xnp=X.adult, Sp=Sp, centered=FALSE)
M2.juv <- MLmvnpooled(Xnp=X.juv, Sp=Sp, centered=FALSE)

M2.npars <- 2*2 + 1*3 #(2 mean vectors and 3 elements in the var-cov matrix
 of every tissue)
Tot.loglike <- M2.adult$lnLhat + M2.juv$lnLhat
M2.BIC <- BICcalc(lnLhat=Tot.loglike,npars=M2.npars,samp.size=len)


# Example of parameterization 3 (centered mean) with hypothesis 3
 (groupings by tissue type)
X.epi <- The.data[Tissues.data$TISSUE=="EPI",]
X.derm<- The.data[Tissues.data$TISSUE=="DERM",]
X.rbc <- The.data[Tissues.data$TISSUE=="RBC",]
X.ser <- The.data[Tissues.data$TISSUE=="SER",]

M3.epi  <- MLmvn(Xnp=X.epi, centered=TRUE)
M3.derm <- MLmvn(Xnp=X.derm, centered=TRUE)
M3.rbc  <- MLmvn(Xnp=X.rbc, centered=TRUE)
M3.ser  <- MLmvn(Xnp=X.ser, centered=TRUE)

M3.npars <- 1*2 + 4*3 #(1 mean vector and 3 elements in the var-cov matrix
 of every tissue)
Tot.loglike <- M3.epi$lnLhat + M3.derm$lnLhat + M3.rbc$lnLhat +
 M3.ser$lnLhat
M3.BIC <- BICcalc(lnLhat=Tot.loglike,npars=M3.npars,samp.size=len)


### Pairwise comparisons

# Step 1: Pick two groups
# Step 2: Estimate the vector of means and the var-cov matrix for each group,
 get the BIC score
# Step 3: Pool the data for the same 2 groups and estimate a single vector
 of means and a single var-cov matrix and get the BIC score
# Step 4: Calculate difference between BIC scores

### Function for pairwise comparisons
  DBICs <- function(group1, group2){
  g1g2 <- rbind(group1, group2)
```

```
  g1g2.joint.est <- MVNpool(Xnp1=group1, Xnp2=group2)
  g1g2.pool.est <- MLmvn(Xnp=g1g2)
  ng1 <- dim(group1)[1]
  ng2 <- dim(group2)[1]
  g1est <- MLmvn(Xnp=group1, centered=FALSE)
  g2est <- MLmvn(Xnp=group2, centered=FALSE)
  sep.BIC <- -2*(g1est$lnLhat + g2est$lnLhat) + 5*2*log(ng1+ng2)
  joint.BIC <- -2*g1g2.joint.est$lnLhat + 7*log(dim(g1g2)[1])
  pool.BIC <- -2*g1g2.pool.est$lnLhat + 5*log(dim(g1g2)[1])
  varDBIC <- joint.BIC-sep.BIC
  meanDBIC <- pool.BIC-sep.BIC
  return(list(DBIC.mean=meanDBIC, DBIC.var=varDBIC))
}

# Example of pairwise comparison
DBICs(group1=X.aepi, group2=X.aderm)
```