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Effects of Processing Time on Whole Blood and Plasma Samples from Loggerhead Sea Turtles (*Caretta caretta*) for ^{1}H-NMR-based Metabolomics

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***Abstract.*—The use of metabolomics in veterinary medicine is growing, but still tends to be limited to laboratory or hospital settings. In this study, we examined the effects of delayed processing time for Loggerhead Sea Turtle (*Caretta caretta*) whole blood and plasma samples under field conditions. We froze aliquots of heparinized whole blood samples immediately post-collection and held them to be processed for plasma at four time-points (median 22, 39, 56, and 72 min) post-collection. There were several significant differences between *C. caretta* whole blood and plasma samples with no apparent processing time association. These differences were largely related to erythrocyte metabolism and the intracellular concentration of certain metabolites within erythrocytes. Variability in results from plasma samples did not increase with time of delay to processing within the first three time-points of collection. The goals of an individual study and processing requirements should be considered to determine if whole blood or plasma is most appropriate to answer the study questions. While we do not recommend purposefully waiting to process samples collected in the field for nuclear magnetic resonance (NMR)-based metabolomics studies, if samples are consistently processed at a time within approximately 40–50 min of collection, their usefulness should not be disregarded due to processing time.**

Key Words.—nuclear magnetic resonance spectroscopy; NMR; sample handling; sample processing

Introduction

Metabolomics is an emerging discipline that studies the status of metabolites, the small molecules involved in the physiology of metabolism. These compounds include sugars, amino acids, fatty acids, and other small molecules critical to homeostasis, growth, and adaptation to environmental conditions. The use of metabolomics in veterinary medicine is relatively rare (Jones and Cheung 2007; Kirwan 2013; Schock et al. 2013) and tends to be represented by studies using animal species as models for human disease (Whitfield et al. 2005; Zhang et al. 2008; Banerjee et al. 2012). As awareness of the techniques has grown, clinical and laboratory studies using metabolomic techniques to answer domestic animal and wildlife research questions have begun to appear, but in general rely on captive individuals and/or sampling occurring in a controlled, laboratory setting (Viant et al. 2003; Lin et al. 2007; Tikunov et al. 2010;

Dove et al. 2012). An important challenge to using metabolomic techniques effectively to answer questions about free-ranging wildlife is to understand how robust the data and information obtained are to variability that can be introduced under the constraints of collecting samples under field conditions.

The biological end products of metabolism degrade after a sample is collected from a living animal (Ross et al. 2007). For example, Tikunov et al. (2013) showed that the slope of the change in lactate concentration in rat liver samples is greater than 1.0 mM/sec/g from 0 to 120 seconds until freezing. Given the sensitivity of nuclear magnetic resonance (NMR)-based metabolomics, efficient sample handling and processing is a concern with regards to accuracy and reproducibility of results (Beckonert et al. 2007).

Processing techniques for metabolomic studies that are effective in the laboratory or clinic are not necessarily practical in a field setting. Limited sample processing, such as centrifugation, may not be feasible and/or may be delayed due to a variety of reasons. It is commonly understood that after a sample is collected, metabolism should be rapidly suppressed because changes can occur on a millisecond scale (Beckonert et al. 2007). This is referred to as quenching the sample and is typically accomplished by immediately freezing samples in liquid nitrogen (Beckonert et al. 2007). However, due to its low boiling point, weight, and the need for a vacuum flask, liquid nitrogen can be logistically difficult to use in the field.

In this study, we examined the effects of delayed processing time for Loggerhead Sea Turtle (*Caretta caretta*) plasma samples and whole blood samples requiring minimal processing under field conditions, using dry ice to quench the samples. We hypothesized that there would be significant differences between whole blood samples and plasma samples and that as processing time increased, the metabolic differences between the whole blood sample and the plasma samples would become more pronounced. We also hypothesized that variability among replicate plasma samples would increase with delays in processing time.

Materials and Methods

Whole blood and plasma sample processing.—We used whole blood samples, collected for diagnostic purposes from five *C. caretta* of undetermined sex undergoing routine examination prior to release from the Karen Beasley Sea Turtle Rescue and Rehabilitation Center, Topsail Island, North Carolina, USA in this study. We collected blood from the dorsal cervical sinus into heparinized syringes (sodium heparin). All of the turtles had been fed a mixture of fish and squid at approximately 0700–0800 the morning their blood was drawn.

We immediately divided each sample into a 0.2–0.3 mL whole blood aliquot and four aliquots of approximately 0.4–0.6 mL to be processed for plasma. Each whole blood aliquot was immediately placed in a cryogenic vial, put on dry ice, and recorded as the A sample. We placed the remaining four aliquots from each animal in Eppendorf microcentrifuge tubes and left at ambient temperature. We then processed these at each of four processing time-points, intended to be 15 min (B), 30 min (C), 45 min (D), and 60 min (E) after blood collection. We processed aliquots by centrifugation at 3,000 × g for three minutes just prior to each time-point. The plasma was then separated, placed in a cryogenic vial, put on dry ice, and the time recorded.

We determined packed cell volume (PCV) by centrifuging heparinized blood in a capillary tube with no additives. We determined total protein (TP) concentration with a refractometer using the plasma fraction from the centrifuged capillary tube. Sampling occurred on 23 May 2012 from approximately 1100 to 1500. The maximum air temperature that day was 28° C (Sandy Run National Oceanic & Atmospheric Administration Station, North Carolina, USA). We transported the samples on dry ice, transferred them the same

day to an ultra-low freezer (-80$^{\circ C}$), and stored them until analysis approximately 12 weeks later.

NMR data acquisition.—We soaked Multi-Screen filter plates with Ultracel-10 membranes (Millipore Ltd., Carrigtwohill, Ireland) in distilled water for two days with water changes every 24 h and then washed by filtering Milli-Q water (Merck KGaA, Darmstadt, Germany) through the plate four times to remove the glycerol preservative. Plates were kept moist with Milli-Q water until use. We prepared samples for NMR analysis by pipetting 50 μL of sample into a pre-prepared filter plate. We centrifuged the plate with samples at 3,000 $\times$ g for 60 min at 21° C. We added Milli-Q water (100 μL) and we repeated centrifugation under the same parameters. The filtrate was lyophilized and resuspended in 50 μL deuterium oxide with 20 mM phosphate buffer, 0.1 mM trimethylsilyl propionate (TSP), and 1 mM formate. We used formate as an additional reference standard, as TSP can bind to plasma proteins affecting the resultant signal (Beckonert et al. 2007). We used a Varian Inova 600 MHz multinuclear INOVA NMR spectrometer (Varian Medical Systems, Palo Alto, California, USA) equipped with a Protasis microcoil NMR probe (Protasis Corporation, Marlboro, Massachusetts, USA) to obtain 1D, 1H-NMR spectra at 25° C with a 1.1 sec acquisition time. The sweep width of 7,195 Hz acquired 8,191 complex points and 1,024 transients (Macomber 1998; Viant et al. 2008).

Data processing, analysis, and interpretation.—We processed NMR spectra using ACD Labs 12.0 1D NMR Processor (Advanced Chemistry Development, Toronto, Ontario, Canada). We zero-filled spectra to 16,000 points and Fourier transformed it. The baseline and phasing was corrected automatically and adjusted by hand, if necessary. We referenced all spectra to the internal standard TSP peak at 0 ppm (Hz/MHz) chemical shift. We identified metabolites by comparison to reference data available through the Human Metabolome database (Wishart et al. 2009) and Chenomx NMR Suite 7.7 (Chenomx, Edmonton, Alberta, Canada).

For initial analysis, we selected preliminary dark regions from -5 to 0.5 ppm, 4.75 to 4.8 ppm, and 9 to 15 ppm. These dark regions eliminate areas without metabolites and the water peak from analysis. We applied more restrictive dark regions (Appendix A) prior to statistical analyses to minimize counting any statistically significant differences in bins containing only noise. We used intelligent bucket integration to divide the spectra into bins with a width of 0.04 ppm. We normalized individual samples by dividing each bin value by the sum of the bins of the sample. We calculated median values for each bin at each time-point and we compared bin differences between time-points using a two-tailed, paired-comparison, exact permutation tests (α = 0.1).

Results

Sample processing.—We divided each sample with the intent of freezing an aliquot of whole blood immediately (A) and aliquots of plasma 15 min (B), 30 min (C), 45 min (D), and 60 min (E) after collection from the animal. However, the difficulty of processing samples during routine clinical activity under field conditions did not permit the desired precision. As a result, though we froze the initial whole blood aliquot of each sample immediately after collection (within approximately one minute) to represent the first (A) time-point, subsequent aliquots were frozen at median times of B = 22 min (20–22 interquartile range [IQR]), C = 39 min (39–40 IQR), D = 56 min (55–57 IQR), and E = 72 min (70–73 IQR) after blood collection.

The turtles had PCVs and TP concentrations that ranged from 28–37% and 5.2–6.4 g/dL, respectively. Complete blood counts and plasma biochemistry panels identified values outside of the expected baseline range in some animals, particularly plasma calcium concentrations and

Table 1. List of metabolites identified in the ^{1}H-NMR spectra of whole blood and plasma samples from *C. caretta*. Time-point A represents whole blood results, while subsequent time-points represent plasma samples (median times after whole blood collection) at: B = 22 min (20–22 interquartile range [IQR]), C = 39 min (39–40 IQR), D = 56 min (55–57 IQR), and E = 72 min (70–73 IQR).[a] Identified in a single individual at this time-point.

Metabolite	A	B	C	D	E
Acetate	◇	◇	◇	◇	◇
Adenosine	◇				
Alanine	◇				
Creatine phosphate	◇	◇	◇	◇	◇
Formate	◇	◇	◇	◇	◇
Glucose	◇	◇	◇	◇	◇
Glutathione	◇				
Hydroxyaetone		◇	◇	◇	◇
Lactate	◇	◇	◇	◇	◇
Leucine	◇	◇	◇	◇	◇
Malonate	◇	◇	◇	◇ [a]	◇
Methanol	◇				
Myo-Inositol	◇				
Taurine	◇				
Trimethylamine	◇				
Trimethylamine N-oxide		◇	◇	◇	◇
Valine	◇	◇	◇	◇	◇

aspartate aminotransferase activity (Craig Harms, unpubl. data). However, careful consideration of these variations did not identify impacts that were germane to our analysis considering the scope of this study.

Limited qualitative metabolite profiling.— Spectra from whole blood samples had more metabolite diversity than the spectra of plasma samples (Table 1). The most noteworthy of these differences was the absence of glutathione in the plasma samples (Fig. 1).

Statistical analysis.—Examining the median integral value of each normalized bin (n = 218) by sample group after accounting for our initial dark regions demonstrated results similar to the qualitative analysis. Each of the plasma sample time-points appeared to have relatively little variation, while there were several regions where the whole blood sample group appeared to be different (Fig. 2). The major metabolites in these regions of difference included valine, lactate, glutathione, creatine phosphate, taurine, and glucose (Fig. 3).

Using our restrictive dark regions, the number of bins (n = 84) with significant differences ($P < 0.1$) between whole blood and plasma samples was 11, 13, 13, and 16 for time-points B, C, D, and E, respectively. Major metabolites identified in these statistically different areas include glutathione (RBC > plasma), creatine phosphate (RBC > plasma), and glucose (RBC < plasma). A slight decrease in plasma glucose over time (Fig. 3) was noted. The number of bins with significant differences ($P < 0.1$) for time pairings did not change consistently with time, with zero, one, seven, and one significant differences for the pairing B/C, B/E, C/E, and D/E, respectively. The B/D and C/D pairings each had 15 different bins ($P < 0.1$).

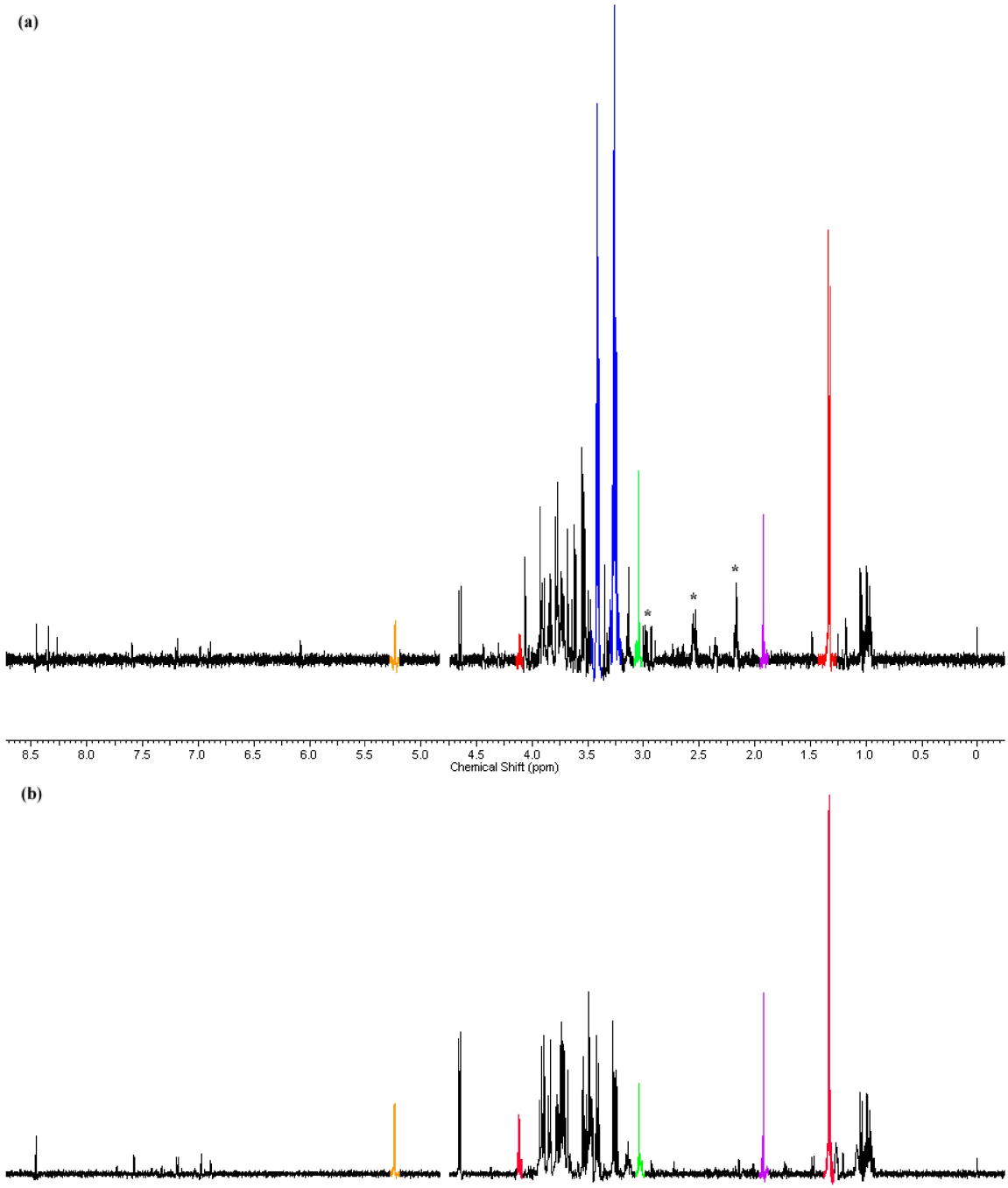

Figure 1. (a, upper) Representative ^{1}H-NMR spectrum of a whole blood sample from a 57.2 kg *Caretta caretta* of undetermined sex. Glutathione (*) was identified only in whole blood. Other peaks, for reference: lactate (red), acetate (purple), creatine phosphate (green), taurine (blue), and the alpha anomer peak of glucose (orange). The water peak has been removed. (b, lower) ^{1}H-NMR spectrum of a plasma sample from the same turtle, processed and frozen 22 min after the whole blood sample. Spectra were zero-filled to 16,000 points and Fourier transformed. The baseline and phasing was corrected automatically and adjusted by hand, if necessary. Both spectra were referenced to the internal standard TSP peak at 0 ppm (Hz/MHz) chemical shift. Formate (1 mM) was included as an additional reference standard. The y-axis is indicative of intensity.

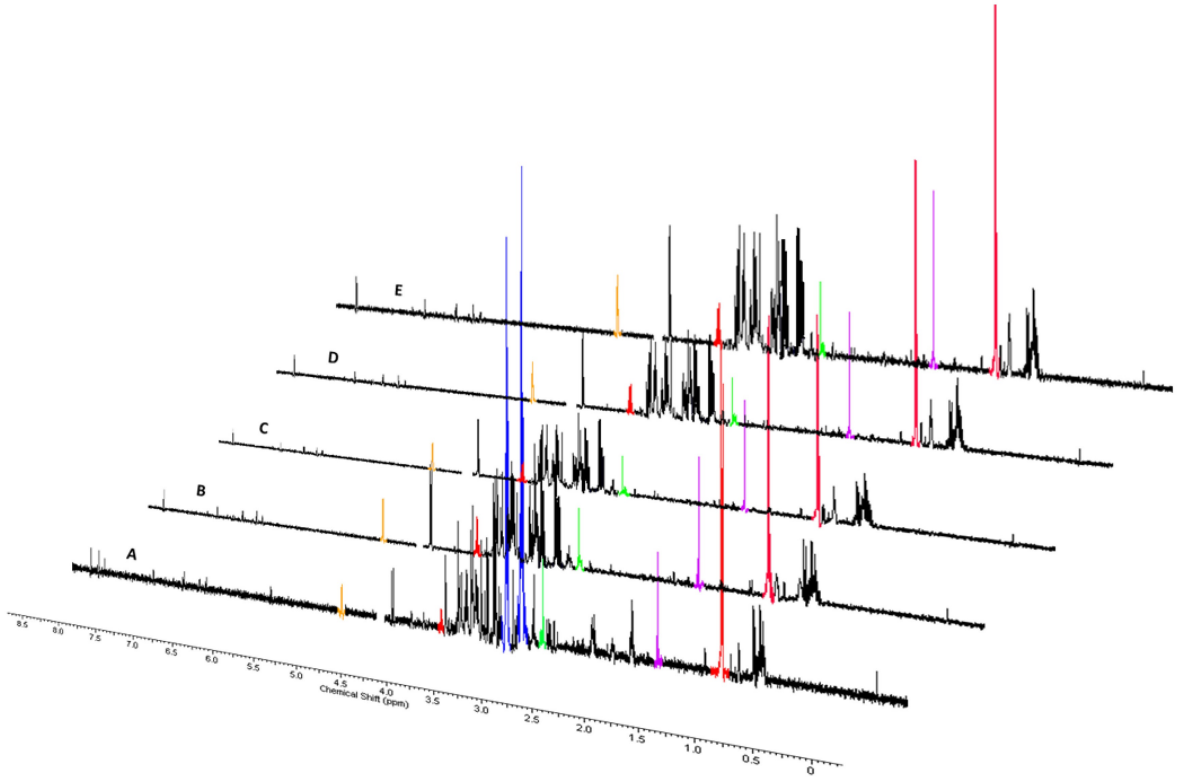

Figure 2. Representative ^{1}H-NMR spectra from a 57.2 kg *Caretta caretta* of undetermined sex over time. Time-point A represents whole blood results, while subsequent time-points represent plasma samples (median times after whole blood collection) at: B = 22 min (20–22 interquartile range [IQR]), C = 39 min (39–40 IQR), D = 56 min (55–57 IQR), and E = 72 min (70–73 IQR). Labeled peaks, for reference: lactate (red), acetate (purple), creatine phosphate (green), taurine (blue), and the alpha anomer peak of glucose (orange). Spectra were zero-filled to 16,000 points and Fourier transformed. The baseline and phasing was corrected automatically and adjusted by hand, if necessary. All spectra were referenced to the TSP peak at 0 ppm (Hz/MHz) chemical shift. Formate (1 mM) was included as an additional reference standard. The y-axis is indicative of intensity.

Discussion

We examined the metabolic effects of delayed processing time for *C. caretta* plasma samples and whole blood samples under field conditions. Our results demonstrate the promising application of dry ice as a quenching method, some of the difficulties of using whole blood samples, and the difficulty of processing samples in a timely fashion in the field, especially when personnel or equipment may be limiting. The study also showed the metabolic profile of *C. caretta* whole blood is different from plasma and provided initial evidence that the possible clinical application of plasma samples should not be discounted simply due to processing delays common in field situations.

While use of dry ice in the field was safe and convenient, we have found that our method of freezing samples within their cryogenic vials could have been improved. We noted that samples did not freeze immediately, even when pipetted directly into a plastic tube kept on dry ice. We have now adopted a method initially described

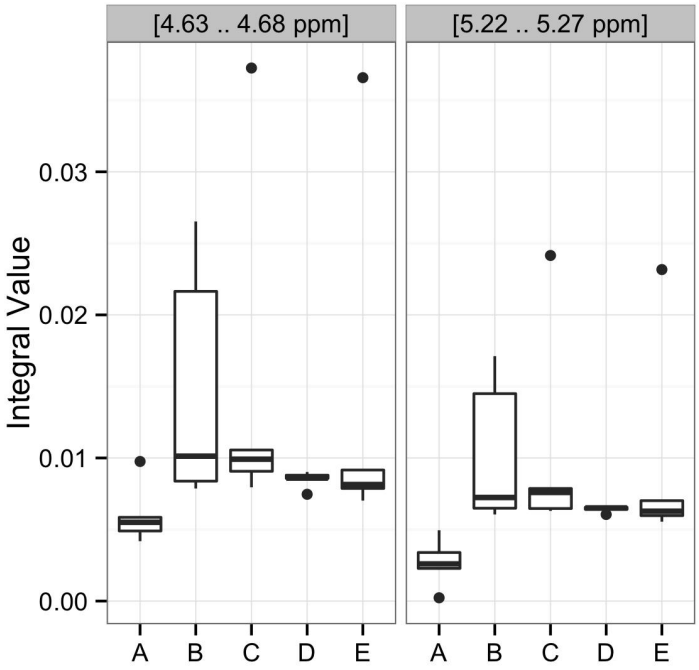

Figure 3. Integral values of two normalized bins of glucose from five *Caretta caretta* categorized by time-point. Intelligent bucket integration was used to divide the spectra into bins with a width of 0.04 ppm. Individual samples were normalized by dividing each bin value by the sum of the sample's bins. Integral values correlate to metabolite concentration. Time-point A represents whole blood results, while subsequent time-points represent plasma samples (median times after whole blood collection) at: B = 22 min (20–22 interquartile range [IQR]), C = 39 min (39–40 IQR), D = 56 min (55–57 IQR), and E = 72 min (70–73 IQR).

in the field of theriogenology (Nagase and Niwa 1964; Krause and Grove 1967), which involves freezing the sample directly within a well drilled into a slab of dry ice. Biofluid samples placed in direct contact with dry ice freeze spherically and are easily removed from the well with forceps. A secondary benefit is that fluid samples frozen in this way remain separated in their sample tubes and can be stored with multiple aliquots (from the same subject) per tube and/or fractured to obtain an aliquot without the need to thaw the remainder of the sample.

We had expected freezing whole blood to -80° C and thawing to result in complete or near complete hemolysis. However, examination of undiluted thawed, whole blood samples via hemocytometer had too many erythrocytes to count (Jennifer Niemuth, unpubl. data). Because the whole blood samples were frozen and filtered prior to analysis, the final analyzed sample was actually a partial whole blood lysate with plasma. We expect some of the variation we observed between samples could be due to differences in the proportion of red blood cells lysed in processing. Similarly, the absolute number of cells available to lyse would vary with the PCV. We recommend that studies measure PCV and TP at the time of sampling and use either mechanical homogenization or sonication to ensure that the cellular components of thawed samples are completely lysed to reduce variation between samples from different individuals or even time-points on the same individual. Evaluation of the lysed sample using a hemocytometer to confirm complete cell lysis prior to performing NMR analysis is also advisable.

The artificial environment, diet, and persistent physical abnormalities in some animals used in this study precludes consideration of these individuals as representative of normal wild *C. caretta*. Routine hematologic and biochemical panels identified values outside of the expected baseline range in some animals, particularly plasma calcium concentrations and aspartate aminotransferase activity (Craig Harms, unpubl. data). Considering this, we focused on subjectively evaluating spectra quality and major qualitative changes over time with only limited metabolite profiling and no comparisons between individuals. Additionally, 2-dimensional NMR experiments could be added in the future for additional confirmation of peak identification.

In general, the resultant spectra from whole blood samples had more metabolite diversity than the spectra of plasma samples. The most noteworthy of these differences, was the absence of glutathione in the plasma samples. This is not completely unexpected, as glutathione has been found to be concentrated as the major intracellular antioxidant in human erythrocytes (Rosenthal 2009). In concert with the pentose phosphate pathway, the glutathione defense system protects the red blood cell from hemolysis (Rosenthal 2009). Subjectively, changes over time in the plasma metabolic profile appeared minor.

The results of two-tailed, paired-comparison, exact permutation tests support the whole blood samples having significant differences compared to plasma samples at all time-points. However, unlike our initial hypothesis, there did not appear to be a trend of increasing differences associated with increasing sample processing time. This result and the sample median integral values suggests that while there are differences between whole blood and plasma, the differences over the first 3 time-points would be expected to affect interpretation of only a few metabolites, such as glutathione or taurine, at the level of clinical relevance. A future study examining time-paired whole blood and plasma samples may further elucidate these differences.

The slight decrease in plasma glucose over time was expected. Glycolysis in erythrocytes continues in vitro and longer sample handling times prior to processing for standard clinical pathologic tests results in lower glucose concentrations over time (Stockham and Scott 2008). Lower concentrations of glucose in the whole blood samples were unexpected and are likely an artifact of the processing methods. It is possible that intact erythrocytes continued to have some level of enzyme activity during freezing, while stored at -80° C, and after thawing. Retained enzymatic activity, as well as changes in minerals and metabolites, has been demonstrated during storage of rat and canine plasma and serum samples used for routine clinical pathologic testing (Thoresen et al. 1995; Cray et al. 2009). During sample preparation, which included 120 min of centrifugation at 21° C, contact between plasma and intact erythrocytes may be expected to decrease glucose concentrations by 5–10% per hour (Stockham and Scott 2008). Also, erythrocyte glucose concentrations are approximately 76% that of plasma (Stockham and Scott 2008) and the presence and/or lysis of erythrocytes during sample preparation may have contributed to the lower glucose concentrations in the whole blood samples.

Lactate, an end-product of anaerobic glycolysis in erythrocytes (Stockham and Scott 2008), was increased in the whole blood samples and supports our hypothesis that erythrocytes were metabolically active, either during storage and/or during sample preparation. Some hemolysis of the whole blood samples was observed and would also be expected to release erythrocyte enzymes, including lactate dehydrogenase, which could act to increase sample lactate concentrations (Stockham and Scott 2008).

Creatine concentrations and creatinine metabolism are higher in erythrocytes versus serum due to a specific transporter (Jiao et al. 1998; Wyss and Kaddurah-Daouk 2000). This may explain the higher concentration in the whole blood samples. It has been suggested that

taurine plays a key role in avian osmoregulation based on avian erythrocyte taurine concentrations being approximately 100-fold greater than those in mammals (Shihabi et al. 1989). It is possible reptiles may have a similar mechanism, which would account for the higher concentration in whole blood samples.

With regard to our other hypothesis that variability would increase with increased processing time, comparing the number of bins with statistically significant differences between each possible time-point pairing for plasma samples failed to support the hypothesis. The majority of pairings had few to no statistically significant differences. The number of bins with statistically significant differences ($P < 0.1$) for the pairs did not change consistently with time. However, the B/D and C/D pairings each had 15 statistically different bins ($P < 0.1$) suggesting that some aspect of time-point D may have been contributed to this result. Plasma samples did not appear to become more significantly different as processing time increased. These results suggest that clinically relevant metabolomic results may still be obtained within approximately 40–50 min after blood collection.

***Conclusions.*—**We found that there were several significant differences between *C. caretta* whole blood and plasma samples analyzed for ^{1}H-NMR-based metabolomics. These differences were suspected to be largely related to continued metabolism of intact erythrocytes and intracellular concentration of certain metabolites within erythrocytes. The goals of an individual study, along with processing requirements, should be considered to determine which sample type may be most appropriate to answer the study questions.

This study contributes to our understanding of how rapidly samples collected for NMR-based metabolomics will degrade post-collection. In laboratory settings, where the researcher has greater control over study subjects, environmental conditions, and sampling conditions, we believe it is prudent to minimize the amount of time samples are held post-collection prior to processing. However, our results provide initial evidence that collection of samples under field conditions can still yield biologically useful data, even when plasma cannot be separated for up to approximately 40–50 min. While we do not recommend purposefully waiting to process samples collected in the field for ^{1}H-NMR-based metabolomics studies, if samples are consistently processed at a time within 40-50 min of collection, their clinical usefulness should not be disregarded due to processing time.

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Table A1. Restrictive dark regions applied prior to statistical analyses to minimize the effect of noise.

Region No.	Region (ppm)
1	-0.500–0.920
2	1.360–1.450
3	1.500–1.670
4	1.760–1.910
5	1.940–2.130
6	2.200–2.500
7	2.580–2.720
8	2.750–2.890
9	4.150–4.290
10	4.320–4.360
11	4.380–4.420
12	4.450–4.630
13	4.670–5.220
14	5.250–6.070
15	6.100–6.850
16	7.000–7.150
17	7.600–7.700
18	7.760–8.260
19	8.270–8.340
20	8.360–8.450
21	8.470–10.791

Jennifer N. Niesmuth is a doctoral candidate in North Carolina State University's (NCSU) Fisheries, Wildlife, and Conservation Biology program within the College of Natural Resources and College of Veterinary Medicine. She received her BS (Wildlife Ecology) and DVM from the University of Wisconsin-Madison. Her current work is focused on using NMR-based metabolomics to describe the physiologic changes of cold stun syndrome in sea turtles and determining the potential impact of climate change on this syndrome. (Photographed by Jennifer N. Niemuth).

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Michael K. Stoskopf directs the Environmental Medicine Consortium at NCSU and participates actively in the inter-college Fisheries, Wildlife, and Conservation Biology and Marine Sciences programs. He is a professor of wildlife and aquatic health in the College of Veterinary Medicine's Department of Clinical Sciences with appointments in Forestry (College of Natural Resources), Biomedical Engineering (College of Engineering), and Toxicology (College of Agricultural and Life Sciences). He received his DVM from Colorado State University, PhD (Environmental and Biochemical Toxicology) from Johns Hopkins University, and is a Diplomate, and former President, of the American College of Zoological Medicine. His research focuses on population, ecosystem and landscape approaches to health management of wildlife species broadly defined to include aquatic and marine species including invertebrates and vertebrates. (Photographed by Wendy Savage).