BIOPRESERVATION AND BIOBANKING
Volume 18, Number 5, 2020
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DOI: 10.1089/bio.2020.0026

Testing the Stability of Plasma Protein and Whole Blood RNA in Archived Blood of Loggerhead Sea Turtles, *Caretta caretta*

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Sample storage conditions can affect accuracy and reproducibility of biological measurements. Storing samples rapidly at the lowest available temperatures is considered ideal but is not always feasible when sampling in remote and logistically challenging field conditions, as is often the case with sea turtles. The objective of this study was to examine the stability of plasma proteins and quality of whole blood RNA from loggerhead sea turtle samples collected as part of an eighteen-year-long curated specimen collection. These biological variables are often used to assess sea turtle health; therefore, it is necessary to maintain the integrity of these components during storage. Protein electrophoresis was conducted on heparinized plasma from individual turtles collected in 2018 ($n=3$), 2008 ($n=3$), and 2001 ($n=3$). Plasma was also pooled from four turtles sampled in 2018 and subjected to various storage temperatures. Whole blood was collected in blood collection tubes containing sodium heparin or PAXgene tubes with an RNA preservative. These were subjected to different storage treatments that can possibly occur during logistically difficult field sampling. Following various treatments, plasma proteins showed minor differences across collection years and no differences among storage treatments were observed, even when exposed to 38°C for three hours. RNA quality was assessed from whole blood using an RNA integrity number (RIN). RINs were poor from sodium heparin tubes that were frozen and from PAXgene tubes after an extended thaw. High-quality RNA was obtained from sodium heparin tubes that were never frozen and from PAXgene tubes with freezing delayed by up to 11 days. Overall, these results indicate that plasma proteins remain stable over time and when exposed to undesirable storage conditions, and RNA degrades rapidly in sea turtle blood after freezing and when not properly preserved. These aspects are important to consider when planning sampling protocols and logistics for optimal long-term sample preservation.

Keywords: plasma protein electrophoresis, RNA quality, biorepository, marine turtle, reptile, preanalytical variables

Introduction

Preservation of tissues at cryogenic temperatures (< −150°C) is considered a best practice for long-term storage for certain biological and chemical analyses.[1] Storing blood at this temperature, achieved with liquid nitrogen, arrests metabolic activity and preserves existing biological molecules and other contents, including environmental contaminants. Careful curation of large numbers of frozen samples in a biorepository setting allows for optimal sample preservation as a basis for future retrospective studies to carefully select particular samples based on defined

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criteria (e.g., sex, age class, temporal, or spatial groups). In this way, biorepositories provide high-quality samples to answer unique biological and epidemiological questions.

Cryogenic storage (< −150°C) is rarely available to researchers. For sea turtle biologists, even −80°C freezers are less available than −20°C freezers, and conditions while sampling sea turtles at sea or on remote islands create logistical challenges for ideal sample handling and storage. The National Institute of Standards and Technology (NIST) in Charleston, South Carolina, has archived blood or tissues from endangered and threatened sea turtle species since 1999 at ≤ −20°C. Loggerhead sea turtles (*Caretta caretta*) are classified as either endangered or threatened, depending on the population segment, by the Endangered Species Act.[2] Due to their protected status, research to better understand their diseases and stressors is important. Loggerheads provide an economic benefit through ecotourism in places such as Costa Rica, the United States, and Australia.[3] Humans have become a major threat to sea turtle populations through vessel strikes and pollution,[4] including ingestion of plastic marine debris.[5] Research on loggerhead sea turtle health and disease can help guide conservation efforts, but optimal sample quality is essential and must be an important consideration.[6]

Biorepositories serve an important role in archiving samples, using best practices to preserve fragile components used as indicators of species health. RNA and plasma proteins are important biomarkers of sea turtle health. Measuring concentrations of plasma proteins by electrophoresis in sea turtles can help in diagnosing disease, choosing additional diagnostic testing, or choosing treatments during rehabilitation of individual turtles, and can be useful in assessing population health.[7] For example, chronically debilitated loggerhead sea turtles have significantly lower concentrations of plasma protein fractions compared with healthy turtles.[8] In fact, plasma albumin is one of the best prognostic indicators during rehabilitation. Likewise, RNA is increasingly used for the evaluation of gene expression from blood transcriptomes.[9–11] Storage temperature can affect concentrations of plasma proteins,[12,13] and RNA degrades rapidly if not properly stored.[14] However, few studies have assessed stability of whole blood RNA and plasma proteins of reptiles.[15,16] Therefore, when archived biospecimens are used, it is imperative to know how storage conditions may have affected the stability of targeted blood components in that particular taxon.

Inadvertent exposure to less than optimal storage conditions can leave researchers wondering if their samples are still useful for various analyses. The limited availability of samples from protected wildlife species can prompt researchers to make hard decisions regarding the inclusion of samples in a research study. The objective of this study was to examine the stability of plasma proteins and quality of whole blood RNA from loggerhead sea turtle samples collected as part of an eighteen-year-long curated specimen collection. Intentionally exposing samples to different temperature treatments is essential to understanding the effects of storage conditions on protein concentrations and RNA quality. Cray et al.[17] empirically studied the effects of temperature treatments on rat serum total protein and albumin concentrations, by storing at 4°C and −20°C frost-free freezers. Similar studies assessed the effects of storage temperature on psittacine plasma protein electrophoresis[18] and human blood RNA quality.[19] Similarly, the current study aimed to test loggerhead sea turtle plasma protein concentrations and whole blood RNA degradation at different storage conditions and over time.

Materials and Methods

Sample collection, selection, and processing

Since 2000, researchers from the South Carolina Department of Natural Resources have captured free-ranging juvenile loggerhead sea turtles (*Caretta caretta*) in summer months using trawl nets without turtle excluder devices for 30 minutes off the shores of South Carolina to Northern Florida.[20] Blood was collected within 16 minutes of capture from the dorsocervical sinus using double-ended needles and collected directly into 10-mL glass vacutainer blood collection tubes containing sodium heparin (Becton Dickinson, Franklin Lakes, NJ). Blood tubes were cooled in a refrigerator or insulated in a cooler not physically touching the enclosed ice or frozen gel packs. Turtles were treated humanely according to standards of current ethical animal research practice, which complied with local and national permits: NFMS Section 10(A)(1)(a) permit 19621, 1245 and 1540, and Georgia Department of Natural Resources Scientific Collection Permit CN 21303.

Whole blood tubes collected between 2000 and 2013, intended for long-term archival at NIST, were previously centrifuged aboard the vessel to separate plasma from red blood cells. The whole blood tubes were frozen standing upright at −20°C aboard the ship and later moved to NIST for storage between −20°C and −80°C. Blood tubes from three individual turtles captured in 2001 (Turtles 1–3) and from three different turtles captured in 2008 (Turtles 4–6) were selected from the archive for plasma protein electrophoresis (Supplementary Table S1). In 2018, these tubes were thawed standing upright in a refrigerator overnight. The plasma was carefully transferred using a glass pipette into a glass culture tube, avoiding the hemolyzed bottom layer, vortexed, and transferred into plastic cryovials. One plasma aliquot of 0.25 mL to 0.5 mL from each individual turtle was submitted through overnight shipping with a frozen gel pack for plasma protein electrophoresis to compare protein concentrations in individual turtles over long periods of time stored in the NIST Biorepository. Remaining blood components were transferred into additional cryovials and stored in liquid nitrogen vapor freezers (< −150°C) at the NIST Biorepository for long-term archival.

In 2018, blood was sampled from seven additional turtles for exposure to various storage conditions (Supplementary Table S1). The complex experimental design is summarized in Figure 1. In addition to sodium heparin vacutainer tubes, blood samples were also collected into PAXgene Blood RNA tubes (PreAnalytiX, Hombrechtikon, Switzerland) from four turtles. PAXgene tubes were stored at room temperature for the time recommended by the manufacturer (between 2 and 72 hours) before either moving to cooler temperatures or left unfrozen. All analyses were performed in summer 2018. Detailed sample collection and storage times and temperatures are provided in Supplementary Tables S2 and S3.

Plasma storage conditions and protein measurements

Pooled plasma samples were used for the storage temperature experiment to remove individual turtle variability.

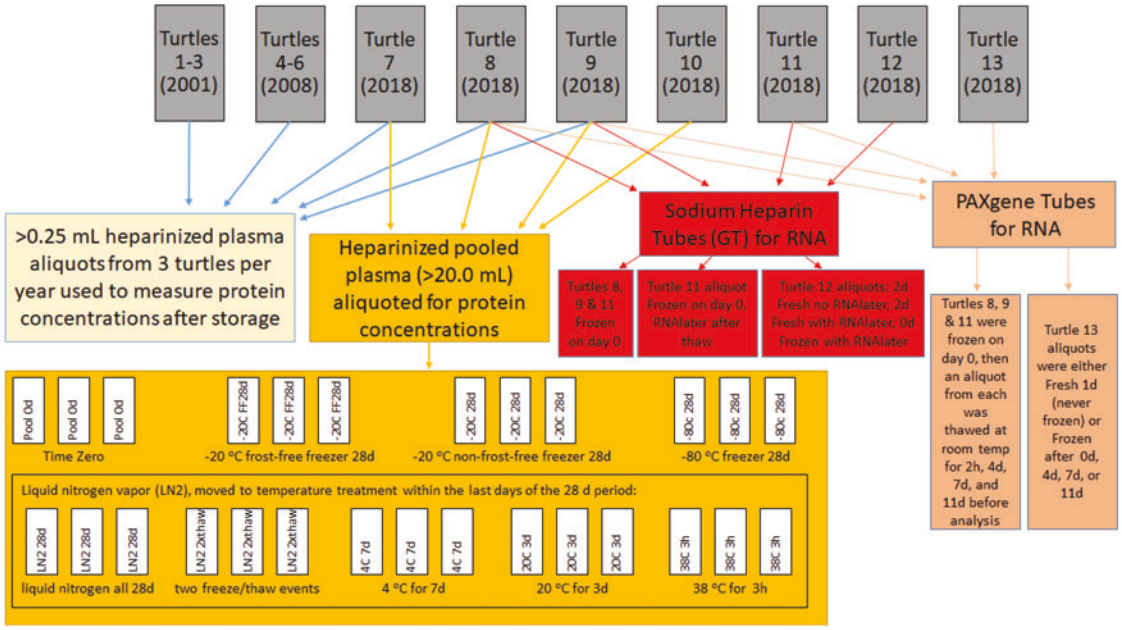

FIG. 1. Flow diagram of sample processing for experiments assessing storage conditions on loggerhead sea turtle plasma protein electrophoresis and whole blood RNA quality. Color images are available online.

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Pooled plasma aliquots were exposed to 10 different temperature treatments before plasma protein electrophoresis (Fig. 1). Dates and times of sample handling are provided in Supplementary Table S2. Blood samples from three turtles sampled in 2018 (Turtles 7–9) were thawed overnight from −80°C to 4°C. Plasma (0.25 mL) from these sodium heparin glass vacutainer tubes was transferred into cryovials. Remaining plasma from four turtles sampled in 2018 (Turtles 7–10) was pooled into one 50-mL centrifuge tube, which was vortexed and distributed into 27 2-mL cryovials, each containing 0.25 mL of pooled plasma. Three pooled replicates (pool 0d) and the three individual turtle plasma aliquots (Turtles 7–9) were sent on frozen gel packs overnight for analysis to serve as time zero (0 day). Plasma samples had no to mild hemolysis (Supplementary Table S1). Hemolysis was visually evaluated on a scale of 0 to 3 according to Stacy and Innis.[21] Three aliquots of the pooled plasma were stored for 28 days in each of the following treatments: liquid nitrogen vapor phase freezer (LN_2 28d), −80°C freezer (−80C 28d), −20°C nonfrost-free freezer (−20C 28d), and −20°C frost-free freezer (−20C FF 28d). Shorter-duration treatments were stored in liquid nitrogen vapor-phase freezers until their treatment began. Shorter treatments included storage in liquid nitrogen vapor with two freeze/thaw events over 28 days (LN2 2x thaw), 4°C for seven days (4C 7d), 20°C for three days (20C 3d), and 38°C (100°F) for 3 hours (38C 3h). After storage treatments, these aliquots of pooled plasma ($n=24$) and archived samples from 2000 ($n=3$) to 2008 ($n=3$), processed as described above, were thawed and shipped overnight with frozen gel packs.

Total protein, using the biuret method, and plasma protein electrophoresis were performed on day of receipt at the University of Miami Avian and Wildlife Laboratory, using methods previously described.[8] Shipping, handling, and analysis of all aliquots submitted for plasma protein electrophoresis were standardized so that these storage conditions were controlled across all experiments.

RNA storage condition treatments

Whole blood samples were exposed to 13 different treatments to assess RNA quality (Fig. 1, Supplementary Fig. S1, Supplementary Table S3). Sodium heparin glass vacutainer tubes (GT) from Turtles 8, 9, and 11 sampled in 2018 were refrigerated, frozen within 6 hours of collection at −20°C, transferred to −80°C one to three days later, thawed four to six days after collection, and processed for RNA isolation (GT 0d Frozen). An additional aliquot (0.5 mL) of heparinized blood from Turtle 11 that was frozen after collection was thawed in 1.3 mL of RNAlater® (Sigma-Aldrich, St Louis, MO) (GT 0d Frozen, RNAlater after thaw). RNAlater is a standard RNA preservative for tissues, intended to be used immediately at sample collection, and has been used for transcriptomic studies of reptilian nucleated red blood cells.[11] Heparinized blood from Turtle 12 was divided into three treatments (no replicates per treatment) immediately after collection. Six milliliters were removed and split equally between two vials each containing 7.5 mL of RNAlater. The remaining whole blood (1 mL) was left in the sodium heparin tube, wrapped in a paper towel, kept on ice, and never frozen until RNA isolation 48 hours after collection (GT 2d Fresh, no RNAlater). Of the two samples containing RNAlater, one was frozen (GT 2d Frozen, RNAlater immediate), whereas the other was left on ice until processing (GT 2d Fresh, RNAlater immediate).

Whole blood collected in PAXgene tubes from three turtles in 2018 (Turtles 8, 9, and 11) was refrigerated and then frozen at −20°C after 3 to 6 hours as per the manufacturer's recommendations (Supplementary Table S3). Portions of blood from these tubes (1.8 mL each) were processed 2 hours after thawing (PAX 0d Frozen, Thawed 2h), 4 days after thawing (PAX 0d Frozen, Thawed 4d), 7 days after thawing (PAX 0d Frozen, Thawed 7d), and 11 days after thawing (PAX 0d Frozen, Thawed 11d) at room temperature. PAXgene whole blood was collected from Turtle 13

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and maintained at room temperature for 4 hours. Portions of blood from this tube (1.8 mL each) were either never frozen (PAX Fresh 1d) or frozen at −80°C at time points 0 day (PAX 0d Frozen), 4 days (PAX 4d Frozen), 7 days (PAX 7d Frozen), and 11 days (PAX 11d Frozen) after collection. RNA isolation of the fresh (never frozen) aliquot was performed 24 hours after collection. The frozen aliquots were allowed to thaw at room temperature for 2 hours before RNA isolation.

RNA isolation and analysis

RNA was isolated from whole blood using standard protocols similar to methods developed for blood samples (L.M. Komoroske, unpublished data; https://github.com/lkomoro/Molecular_Laboratory_Protocols/blob/master/RNA-WB_extraction). RNA isolation was completed with TRI Reagent® (Sigma) followed by DNase treatment with an RNase-Free DNase Set (Qiagen) and the RNase inhibitor RNasin (Promega). An additional cleanup step was added using the Qiagen RNeasy Mini Kit. Samples were eluted in 30 µL of RNase/DNase-free water. When low concentrations were suspected, the final eluate was concentrated by placing the initial eluate from the first elution back on the column and repeating the elution step, resulting in a final volume of 30 microliters.

The isolated RNA was evaluated for RNA quality values by measuring the RNA integrity number (RIN) using a 2100 Bioanalyzer (Agilent) following standard protocols. The RIN is a calculated value of the 28s and 18s rRNA subunits fitted to a scale between 0 and 10,[22] where higher scores indicate better quality samples. However, the RIN algorithm was developed based on expected marker regions in mammalian tissues and it has been shown that high-quality RNA from other organisms may not conform to these expectations.[16] Therefore, we complemented our quantitative RIN analysis with qualitative visual RNA quality analyses to identify treatments that would yield RNA appropriate for quantitative PCR and RNA-sequencing library preparation. We compared our results with analogous traces of RNA extracted from blood run on a Fragment Analyzer (Agilent) that were previously shown to produce high-quality Illumina libraries.[23]

Statistical analyses

All statistical tests were performed using JMP software (SAS, Cary, NC) and significance was determined at a p-value <0.05. Concentrations of each protein class were checked for normality and homogeneity of variance. If both assumptions were met, an analysis of variance (ANOVA) was used to determine differences (1) among samples stored since 2001, 2008, and 2018; and (2) among 10 storage condition treatments. If an assumption was violated, the nonparametric Kruskal/Wallis test was used. The Tukey/Kramer multiple comparison test was used among years for only alpha 1-globulins (both assumptions were met). Wilcoxon all-pairwise comparisons were used among storage conditions.

A repeated measures ANOVA followed by a Tukey/Kramer test was used to compare RIN values for RNA quality across five treatments of blood. Both assumptions were checked as above. The data were not normally distributed for two of the five treatment groups even after attempting five different data transformations, so we relaxed this assumption and carried on with parametric statistics. A Spearman correlation was used to examine a relationship between RIN values and days before freezing samples from a PAXgene tube from turtle CC0922.

Results

Plasma protein concentrations

Plasma protein concentrations from each sample are shown in Supplementary Table S4. No significant differences were found among all various temperature treatments of pooled samples (all p-values >0.05) (Fig. 2). When comparing individual turtle samples stored for different

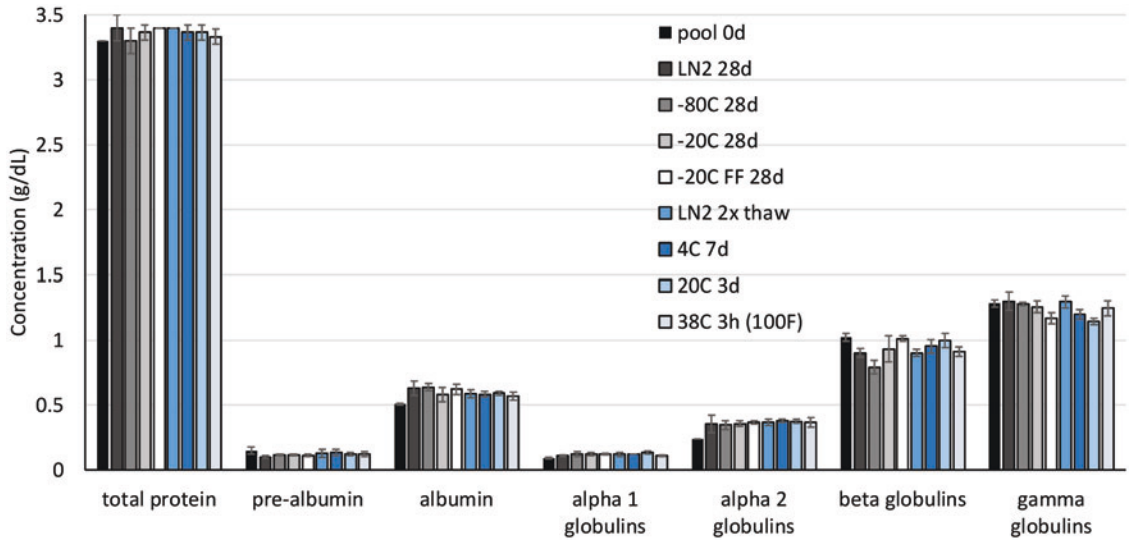

FIG. 2. Protein concentrations measured by electrophoresis from a loggerhead sea turtle plasma pool stored at nine different conditions. Averages and one standard deviation of triplicates are shown. No significant differences were observed among storage conditions within each protein class ($p > 0.05$). FF, frost-free freezer; LN2, liquid nitrogen vapor. Color images are available online.

lengths of time (17 years, 10 years, and 4 days) in the NIST Biorepository, concentrations of alpha 1 globulins (p-value = 0.0394) were significantly higher in 2008 samples compared with 2018, but the remainder of protein fractions were not significantly different across years (Fig. 3). The standard deviations are large, expectedly so, because of interindividual differences. Eighteen of 21 average protein concentrations were within ranges given for foraging loggerhead turtles from Deem et al.[24] (Fig. 3). Three average protein concentrations fell outside of these ranges: prealbumin (slightly higher in 2018) and alpha 2 globulin (higher in 2001 and 2008).

Whole blood RNA quality

RIN values from each sample are shown in Table 1. The repeated measures ANOVA model for RNA quality (measured by RIN values) indicated significant differences (p-value = 0.0013). The treatment, rather than the turtle, was the significant variable (p-value = 0.0005). RNA quality was significantly higher from the frozen PAXgene tubes processed only 2 hours after thawing (average RIN of 3 samples in treatment ''PAX 0d Frozen, Thawed 2h'' = 7.6) than blood from the same tubes processed 4 to 11 days after thawing (average RINs ranged from 3.1 to 4.0) and from the frozen sodium heparin tube (average RIN of three samples in treatment ''GT 0d Frozen'' = 2.4) (Fig. 4). The delayed freeze times from the PAXgene tube of Turtle 13 showed no significant relationship with RIN values that ranged from 7.2 to 7.6 (Fig. 5).

Heparinized whole blood combined with RNAlater as it thawed resulted in a low RIN (3.6). Furthermore, heparinized whole blood combined with RNAlater at collection still resulted in low RINs regardless if sample was frozen (RIN = 1.75) or not (RIN = 1.0) (Table 1). However, a RIN of 7.45 was obtained from heparinized whole blood left unfrozen with RNA isolation 48 hours after collection. This RIN was similar to that of a PAXgene tube never frozen (RIN = 7.35), however, qualitatively the background and relative 18S and 28S peaks differed between the two conditions (Supplementary Fig. S2). Additionally, even samples with higher RIN numbers generally displayed reduced 28S/18S peak ratios not necessarily related to degradation, congruent with earlier studies on RNA in other nonmammalian taxa,[16] as well as previous marine turtle blood samples extracted using the same methods that produced high-quality Illumina RNA-Seq libraries (Supplementary Fig. S2).[23]

Discussion

The current study is the first to assess the stability of plasma proteins and quality of whole blood RNA from loggerhead turtles after exposure to various storage conditions. The great interindividual variability in plasma protein concentrations (Fig. 3) may mask changes in protein concentrations due to storage instability. However, the one significant difference between the average concentration for alpha 1 globulin in 2008 and 2018 cannot be attributed to protein degradation since the concentration was higher in the older 2008 samples than the 2018 samples. The lack of other differences is encouraging for the use of archived loggerhead plasma samples for protein electrophoresis, including those stored in the 17-year-long NIST Biorepository. This conclusion is further supported by the observation that most of the protein concentration averages were within normal ranges for the species (Fig. 3) based on plasma samples from foraging loggerhead sea turtles off the coast of Georgia in 2000 to 2004.[24] These ranges were chosen for comparison because of similar analytical methodology used between the two studies.

Loggerhead sea turtle plasma proteins appear to be stable long term and under various treatment conditions. Pooled plasma from 2018 was subjected to storage treatments that could occur during logistically challenging field sampling. None of the treatments, not even a 3 hours heat treatment at 38°C, affected any of the protein fractions, suggesting

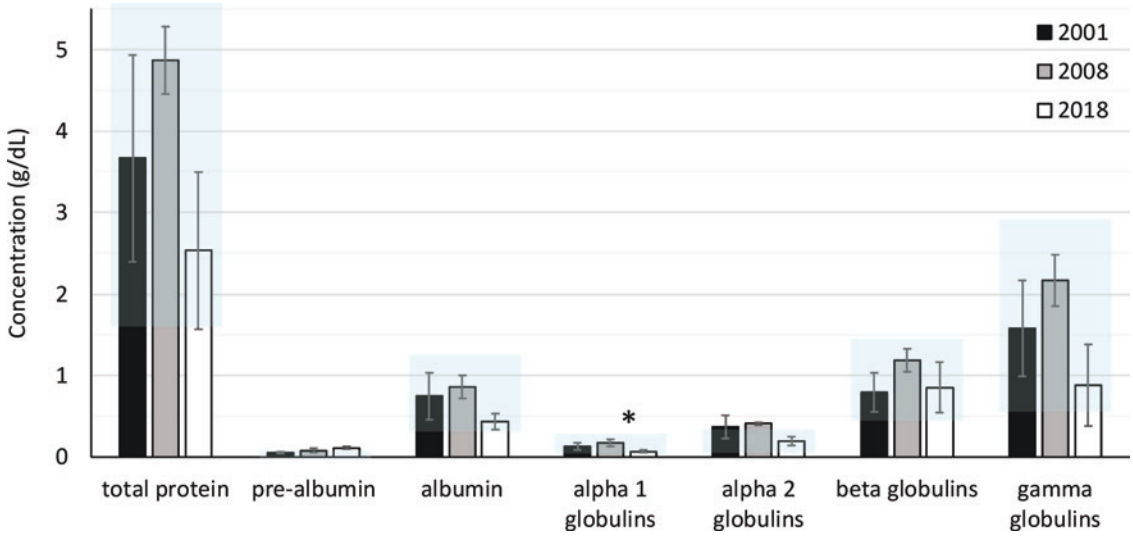

FIG. 3. Protein concentrations measured by electrophoresis from frozen archived loggerhead sea turtle plasma samples collected in three different years. Averages and one standard deviation from three individual turtles per year of collection are shown. *Blue* shaded regions represent ranges in foraging loggerhead turtles from Deem et al.[24] The *asterisk* indicates a significant difference between 2008 and 2018 for alpha 1 globulins (p = 0.0394). Color images are available online.

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Table 1. RNA Integrity Number Measured in Each Loggerhead Sea Turtle Whole Blood Sample

Turtle no.	*Turtle ID*	*Tube*	*Treatment*	*RIN*	*Statistics used*
8	CC0895	Green top Na Heparin (GT)	GT 0d Frozen	2.6	Repeated measures ANOVA
9	CC0904	Green top Na Heparin (GT)	GT 0d Frozen	1.9	Repeated measures ANOVA
11	CC0893	Green top Na Heparin (GT)	GT 0d Frozen	2.6	Repeated measures ANOVA
11	CC0893	Green top Na Heparin (GT)	GT 0d Frozen, RNAlater after thaw	3.6	
12	CC0914	Green top Na Heparin (GT)	GT 2d Fresh, no RNAlater	7.45	
12	CC0914	Green top Na Heparin (GT)	GT 2d Fresh, RNAlater immediate	1	
12	CC0914	Green top Na Heparin (GT)	GT 2d Frozen, RNAlater immediate	1.75	
8	CC0895	PAXgene	PAX 0d Frozen, Thawed 2h	7.4	Repeated measures ANOVA
9	CC0904	PAXgene	PAX 0d Frozen, Thawed 2h	7.7	Repeated measures ANOVA
11	CC0893	PAXgene	PAX 0d Frozen, Thawed 2h	7.8	Repeated measures ANOVA
8	CC0895	PAXgene	PAX 0d Frozen, Thawed 4d	5.3	Repeated measures ANOVA
9	CC0904	PAXgene	PAX 0d Frozen, Thawed 4d	4.8	Repeated measures ANOVA
11	CC0893	PAXgene	PAX 0d Frozen, Thawed 4d	2.1	Repeated measures ANOVA
8	CC0895	PAXgene	PAX 0d Frozen, Thawed 7d	3.9	Repeated measures ANOVA
9	CC0904	PAXgene	PAX 0d Frozen, Thawed 7d	3.5	Repeated measures ANOVA
11	CC0893	PAXgene	PAX 0d Frozen, Thawed 7d	3.9	Repeated measures ANOVA
8	CC0895	PAXgene	PAX 0d Frozen, Thawed 11d	3.2	Repeated measures ANOVA
9	CC0904	PAXgene	PAX 0d Frozen, Thawed 11d	3	Repeated measures ANOVA
11	CC0893	PAXgene	PAX 0d Frozen, Thawed 11d	3.1	Repeated measures ANOVA
13	CC0922	PAXgene	PAX Fresh 1d	7.35	
13	CC0922	PAXgene	PAX 0d Frozen	7.2	Spearman correlation
13	CC0922	PAXgene	PAX 4d Frozen	7.4	Spearman correlation
13	CC0922	PAXgene	PAX 7d Frozen	7.6	Spearman correlation
13	CC0922	PAXgene	PAX 11d Frozen	7.3	Spearman correlation

ANOVA, analysis of variance; RIN, RNA integrity number.

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that these plasma proteins are quite stable. These results are supported by studies with bird, rat, and human proteins.[17,18,25] Few significant changes were seen in plasma protein electrophoresis of a pooled bird sample after 7 days of refrigeration or a single freeze/thaw cycle.[18] In rat serum, total protein or albumin concentrations did not significantly change after 7 days of refrigeration.[17] Furthermore, no significant differences were observed in albumin or beta globulin concentrations in human blood after 12 months of storage in the plastic BD Vacutainer® SST™ (serum separator) Plus Blood Collection Tubes at −80°C.[25] Altogether, these results indicate that long-term frozen archival at ultrafreezer temperatures is adequate and short-term storage challenges do not present a stability problem for these plasma proteins across diverse taxa.

Traditionally, RIN values ≥7.0 have been recommended for transcriptomic protocols,[26] with lower values not recommended for analysis due to high variation and possible loss of transcripts thorough RNA degradation that could result in the mischaracterization of gene expression. However, advances in library preparation and sequencing platforms are expanding capacity for successful analysis of lower quality input samples (depending on study goals).[27]

Adequately high RNA quality from loggerhead turtle whole blood can be obtained from either sodium heparin or PAXgene tubes with careful consideration of freezing and thawing. Sodium heparin tubes that are frozen before RNA isolation are unacceptable for gene expression research (Fig. 4 and Table 1), and the addition of an RNA preservative to heparinized blood either immediately after blood collection or before RNA isolation did not appear to adequately protect RNA quality ($n=1$, Table 1). Similar to findings with heparinized human blood,[19] loggerhead blood in sodium heparin tubes can produce high-quality

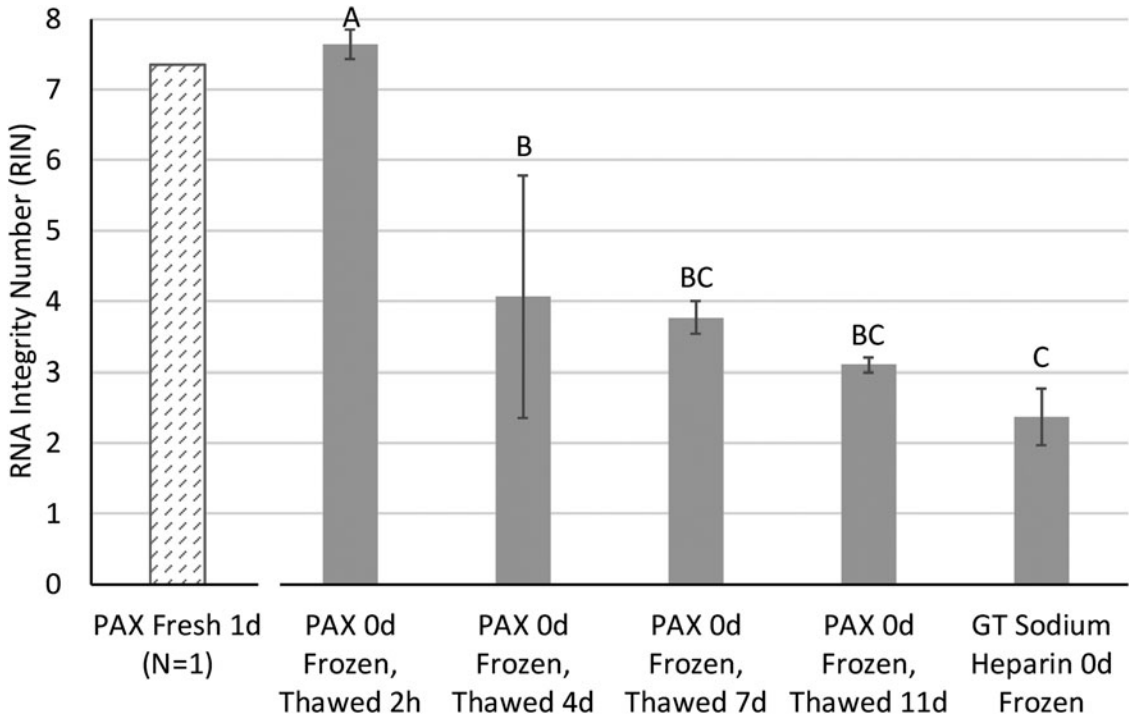

FIG. 4. RIN measured in loggerhead sea turtle whole blood samples collected and processed in different ways. The RIN from a nonfrozen blood sample from a single turtle (*dashed bar*) is shown for comparison, but was not included in statistics. Averages (*solid bars*) and one standard deviation from three individual turtles are shown. *Different letters* indicate significant differences among collection or thaw duration treatments (repeated measures analysis of variance, Tukey–Kramer test $p < 0.05$). RIN, RNA integrity number.

RNA if never frozen and extracted as soon as possible. However, it should be noted that gene expression is continuing in live cells *ex vivo* when whole blood is prepared in sodium heparin, which can alter the expression profiles. Increasing the ratio of RNA preservative to blood may help, but further research would be needed to confirm this hypothesis.

From PAXgene tubes, RIN values were surprisingly unaffected by extended room temperature storage up to 11 days before freezing (Fig. 5 and Supplementary Fig. S2). Therefore, the manufacturer's recommendations (maximum 72 hours) can be extended if field work logistics require it. This finding may be particularly informative for researchers working in remote locations without access to freezers, who may not currently attempt sampling for RNA due to assumptions that it cannot be stabilized long enough at room temperature. Upon thawing PAXgene tubes, we recommend isolating RNA within 2 hours (Fig. 4). It is also recommended that the PAXgene sample be processed all at once rather than subsampled and refrozen because RNA can rapidly degrade in samples that undergo a secondary freeze/thaw cycle (L. Komoroske, unpublished data). Collectively, these findings are informative to field researchers making decisions regarding how to collect and store samples for the best preservation and downstream data quality. For example, researchers may choose to leave samples collected in Paxgene tubes at room temperature after collection rather than freezing, when freeze/thaw is a potential risk due to required additional shipment or transport.

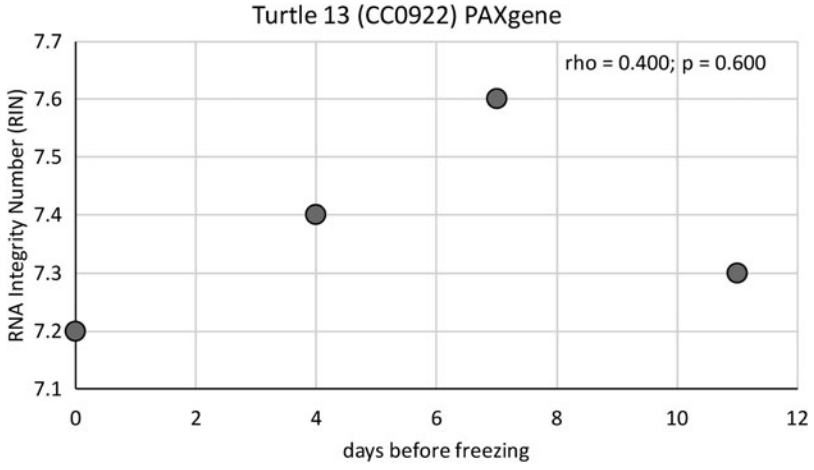

FIG. 5. Nonsignificant Spearman correlation between RIN measured in a loggerhead sea turtle whole blood sample collected in a PAXgene tube and frozen at different times after collection.

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The results from this study demonstrate that the NIST Biorepository of loggerhead turtle blood frozen in sodium heparin tubes since 1999 should not be used for RNA analysis, but can be used for plasma protein measurements. Future studies involving RNA should avoid using samples that have not been properly stored. However, studies involving plasma proteins have fewer constraints on which samples will yield accurate data. Data presented herein provide novel information on the effects of logistically difficult research situations on sample handling and storage. While the study focused on loggerhead sea turtles, the effects of these storage conditions on blood constituents may also be applied to other chelonian species. Furthermore, the importance of optimal storage conditions is highlighted by the goal of reducing preanalytical variables that may hinder interpretation of results.

Acknowledgments

The authors thank Dr. Michael Arendt and others from the South Carolina Department of Natural Resources for sample collection. They also thank Dr. Carolyn Cray, University of Miami Avian and Wildlife Laboratory for assistance with plasma protein analysis, and Debra Ellisor for reviewing.

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Author Disclosure Statement

No conflicting financial interests exist.

Funding Information

Funding for sample collection was provided by National Oceanic and Atmospheric Administration National Marine Fisheries Service Southeast Regional Office grant numbers: NA07FL0499 (2001), NA03NMF4720281 (2008), and NA13NMF4720182 (2018). This material is based on work supported by the U.S. National Science Foundation under Award No. DBI-1757899 for Kelly Townsend's participation in the Research Experience for Undergraduates Program.

Supplementary Material

Supplementary Table S1
Supplementary Table S2
Supplementary Table S3
Supplementary Table S4
Supplementary Fig S1
Supplementary Fig. S2

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