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Effect of RNA preservation methods on RNA quantity and quality of field-collected avian whole blood

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Abstract

The transcriptome comprises all RNA molecules in a sample, tissue, or organism. A limitation of comparative transcriptomic studies which compare gene expression between individuals often under some differing exposure or treatment, of wild avian populations continues to be sample preservation of high-quality RNA (i.e., ribonucleic acids that transfer, translate, and regulate the genetic code from DNA into proteins). Field sampling of wild bird blood provides challenges as RNA degradation progresses quickly, due in large part to the high nuclease content of avian blood and because cryopreservation is often not feasible at remote locations. The introduction of commercial buffers for preservation of RNA enables field-collected studies as these buffers deactivate nucleases which degrade target nucleic acids. We seek to compare the effectiveness of widely available RNA stabilizing buffers, RNAlater (Ambion) and DNA/RNA Shield (Zymo) at varying concentrations along with a dry ice-based flash freezing method to determine optimal preservation methods for field-collected avian blood samples. To determine optimal preservation methods, we assessed RNA quantity and quality metrics: RNA integrity numbers (RINe), rRNA ratios, and total RNA concentration. Nucleated red blood cells, a characteristic common across non-mammalian vertebrates, provide sufficient transcriptionally active material enabling the identification of potentially active gene regions from small and non-lethal samples (~ 20 µl). A protocol was also optimized for total RNA extraction from avian blood samples with small starting volumes enabling sampling of birds with a minimum threshold of 5 g body mass. We found that RNA preservation buffers, RNAlater and DNA/RNA Shield at all concentrations provide sample protection from RNA degradation. We recommend that caution be exercised when using dry ice-based flash-freezing alone for sample preservation as these samples resulted in lower quality measures then samples in preservation buffer. Total RNA concentration was generally not affected by preservation treatment and may vary due to differences in initial sample volumes.

Keywords

Bird, degradation, field sampling, nucleated blood, RNA extraction, RNA integrity number

Key terms

RNA-Seq: high-throughput sequencing which profiles RNA molecules in a sample at the collection timepoint. Provides content and abundance information and may identify novel genes and isoforms. Prior to sequencing, library preparation may either target mRNA or total RNA with the use of enrichment or depletion steps.

Transcriptome: all RNA transcripts, both coding and non-coding, in a cell, tissue or organism. The transcriptome includes all coding RNA: messenger RNA (mRNA), and non-coding RNA: ribosomal RNA (rRNA), transfer RNA (tRNA), and micro RNA (miRNA), among others.

rRNA ratio (28S/18S): commonly used ratio of 28S and 18S ribosomal RNA (rRNA) (band sizes or peak area ratio) that provides information about the integrity of RNA. Appropriate ratios vary by taxonomic groups and associated rRNA sizes. Acceptable ratios of 28S to 18S are approximately 2.0 for eukaryotes such as birds.

Electropherogram: a quantitative distribution of fragment sizes.

Qubit 4 Fluorometer: fluorescence-based quantification of RNA molecule concentration using fluorescent intercalating dyes which bind to target molecules.

Agilent 4200 TapeStation: high-throughput analyzer which uses microfluidics, microcapillary electrophoresis, and fluorescence detection that allows for size determination of the isolated molecules. Used for quality control and quantification of DNA and RNA samples for downstream methods. Reports metrics to assess sample (DNA, RNA, protein) integrity and quantification, which includes:

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electropherogram, gel-like image, concentration values, 28S/18S rRNA ratios, and an algorithmically determined integrity score (RIN^e). Provides a more complete characterization of the measured samples than previous analyzers for downstream analysis.

Fast zone: area between small RNAs and the 18S rRNA fragment (~200 bp to 1.8 kb), where the degradation of the 18S and 28S peak fragments accumulate.

RIN^e: RNA integrity number (RIN equivalent) is a quality score that is determined by quantitative measurements of total RNA degradation. Measurements are based on features of the electropherogram which assess degradation or lack of degradation by measuring the ratio of area in the fast zone to the 18S peak signal. An RIN score of 10 signifies the highest quality intact RNA while zero is the worst score indicating a low-quality degraded sample.

Introduction

Ribonucleic acids (RNA) are common to all living organisms and play a critical role in biological processes that include gene expression, regulation, and the synthesis of proteins. The transcriptome is the complete set of RNA within a cell, tissue or organism at a given time. Therefore, transcriptomics enables the identification and quantification of the transcripts present, allowing for a comprehensive view of the molecular processes. Transcriptomic studies may be used to examine gene expression, gene fusions, splice variants, novel transcript discovery and examine coding (mRNA) and non-coding sequences (including miRNA, sRNA, and other RNA classes). The implementation of RNA-Seq, a high throughput sequencing method to study the transcriptome, allows investigation of genome-wide (i.e., exploring the complete set of genetic material of an organism) responses to environmental stressors. Preservation of ribonucleic acids (RNAs) is an essential step in all transcriptomic studies that use field-collected samples.[1,2] Determining appropriate methods for RNA sample preservation of nucleated blood for variable field collecting conditions is essential to facilitate increased usage of wild RNA-Seq studies and improve our understanding of responses to environmental stressors in populations of free-living birds.[3] While most studies of RNA in wildlife animal research have been based on destructive sampling using high-concentrated RNA tissues, such as brain, heart, muscle, or liver.[4,5] there is little information on the performance of such buffers with suboptimal animal tissues, such as blood samples. However, field collection of samples is difficult due to the rapid rate of degradation in RNAs and because the options for cryopreservation, including tight temperature control and access to liquid nitrogen, are often not available in remote locations.[1,6–8] Appropriate and timely preservation of samples is necessary for accurate and unaltered characterization of the *in vivo* state of cells and subsequent associated downstream RNA expression (e.g., output data generated by sequencing).[9]

Ribonucleases (RNases) cause cleavage and rapid degradation of sample RNA which can distort RNA-Seq read coverage, alter gene expression profiles, and cause the loss of rare transcripts.[10] The fragility of RNA is due to both the abundance of RNases and the structure of RNA which makes it sensitive to hydrolysis. These degradation processes result in increasing numbers of degraded RNAs, which can be identified by an overaccumulation of degraded fragments in small RNA regions. Stabilization of RNA samples, halting RNase activity and hydrolysis, requires timely preservation following collection and planning that arises as the result of good study design. Immediate freezing in a -80°C or snap-freezing in liquid nitrogen has been considered the gold standard for RNA stabilization and preservation, particularly in clinical and laboratory settings.[11] However, while snap-freezing is still used in controlled experimental settings, the use of commercial agents for stabilization has increased greatly and facilitates use in non-controlled settings including field settings. Commercial RNA fixatives, which permeate cells and denature RNases, have previously been found to prevent degradation of RNA and stabilize expression levels in non-fluid tissues as the source material for RNA.[6,12,13] These commercial products use proprietary reagents to stabilize RNA: RNAlater (Ambion, Invitrogen, Waltham, MA, USA) according to its Material Safety Data Sheet, uses ammonium sulphate to stabilize proteins, whereas DNA/RNA Shield (Zymo Research, Irvine, CA, USA), according to a safety declaration, uses guanidine salt which induces protein unfolding. Thus far a single study has tested preservation of RNA from nucleated blood samples collected in the field.[7] This study tested the use of Tri Reagent BD (Molecular Research Center, Cincinnati, OH, USA) and Animal Protect Blood Tubes (Qiagen, Hilden, Germany) as preservation storage buffers and found both to be effective, although for differing effective periods of time. Tri Reagent was limited to less than three days when stored at 4-10°C, while Animal Protect Tubes allowed storage at 2–8°C for up to two weeks.

The majority of avian transcriptomic studies have relied on destructive sampling in order to obtain targeted organ sampling (e.g., spleen, brain, liver, heart, pancreas, muscle;[4,14–16]). For wild avian studies, blood sampling allows for a non-lethal and minimally invasive method of sample collection facilitating the study of wild avian transcriptomes.[2] Birds and all non-mammalian vertebrates have nucleated red blood cells, meaning red blood cells as well as leukocytes are transcriptionally active.[7,17] The high quantity of nucleated blood cells reduces the amount of blood needed for transcriptome studies compared to similar studies of mammals that have non-nucleated red blood cells. Non-destructive blood sampling from passerines can be a valuable tool for conservation studies, particularly for investigating factors such as inbreeding depression, genetic diversity, as well as pathogen and disease prevalence. The small amount of nucleated blood needed for RNA-Seq should enable increased studies of critically endangered avian species, species at risk, and small-bodied species. Thus far transcriptomic studies of vulnerable and endangered species have so far been limited, but are improving the genomic resources for kiwi (*Apteryx* spp.) to aid in captive management and improving genomic tools for understanding species in extreme environments such as saker falcons (*Falco cherrug*;[17–19]). However, nucleated blood is also high in DNA and nuclease content which rapidly degrade RNA, making preservation and isolation of RNA from nucleated blood samples challenging.[7]

Blood transcriptomes remain underutilized in studies of free-living birds.[3] Transcription is tissue specific making the choice of RNA target material dependent on the research question. However, peripheral blood circulates around the body and informs of system-wide gene expression. When specificity of different tissue transcriptomes has been compared, the blood transcriptome demonstrated lower specificity,[20] indicating the utility of blood transcriptomes as environmental mediators. We only found eight studies that have used blood as the source material in studies of wild birds using RNA-Seq,[18,21–27] including captive/experimental manipulation studies of wild-caught taxa. Of these eight previous studies using blood samples from wild birds only two used an RNA preservative buffer, three used Trizol or Trizol/EDTA, two studies used liquid nitrogen or flash freezing by returning birds to a lab setting before taking samples, and one study did not detail the storage or preservations method used. Blood transcriptomes have been demonstrated to be informative for studies including immunologic, detoxification, lipid metabolism responses as well as recovering a high proportion of genes expressed across the genome and tissue types.[20,22,28–30] High quality and quantities of RNA are necessary for accurate *in vivo* characterization of blood transcriptomes.

To determine best practices for stabilization of whole avian blood from field-collected samples, we compared various widely available RNA preservation methods. Specifically, we compared two commonly used and commercially available RNA reagent buffers: RNAlater at 1:2 and 1:3 concentrations, and DNA/RNA Shield at 1:5 and 1:10 concentrations to a dry ice-based flash freezing method (Isopropanol 99% and dry ice mixture, -109°C). We predicted that the dry ice-based flash freezing method would outperform preservation buffers and perform similarly to flash freezing with liquid nitrogen. To compare preservation methods and dilutions, we isolated total RNA using an optimized extraction protocol and compared measures of quality (e.g., RIN^e, rRNA ratio) and quantity (e.g., total RNA concentration). The quality and quantity metrics were compared across preservation methods to determine the recommended RNA preservation protocols for field-collected avian blood samples to be used in downstream RNA sequencing studies.

Methods

Blood sampling and preservation

We captured adult birds opportunistically with a mist-net in Clearwater County, Minnesota, USA. Individual blood samples ($n = 8$) were collected from three species: Brown-headed Cowbirds (*Molothrus ater*; $n = 3$), Red-winged Blackbirds (*Agelaius phoeniceus*; $n = 1$), and Rose-breasted Grosbeaks (*Pheucticus ludovicianus*; $n = 4$). All birds were sampled under US Federal Bird Banding Permit #23623 and a University of Connecticut Institutional Animal Care and Use Protocol #A18-005. We collected a total of 100 µL of whole blood, via brachial venipuncture with sterile 26-gauge needles and collection via heparinized glass capillary tubes, from each individual adult bird shortly after capture. Specifically, 20 µL of blood was collected into five pre-measured capillary tubes for precise measurement. Each capillary tube of blood was then immediately expelled using a sterile air blower bulb (VSGO, Shanghai, China), into five different pre-aliquoted treatment cryogenic tubes with different preservation reagents × dilutions in random order. The five treatments included: 1) DNA/RNA Shield 1:2 (20 µL blood + 20 µL DNA/RNA Shield) , 2) DNA/RNA Shield at 1:3 (20 µL blood + 40 µL DNA/RNA Shield), 3) RNAlater at 1:5 (20 µL blood + 80 µL RNAlater), 4) RNAlater 1:10 (20 µL blood + 180 µL RNA later), and 5) dry ice + isopropanol bath based flash freezing mixture (-109°C) (20 µL blood only). Samples were then returned to the laboratory at the University of Minnesota Itasca Biological Station, in Lake Itasca, MN where the samples in RNAlater and DNA/RNA Shield were vortexed for 10 seconds, placed in a 4°C refrigerator for 24 hours, then moved to a -80°C freezer. The dry ice-based flash frozen samples were placed immediately into a -80°C freezer. After one week, all samples were shipped (next day air) on dry ice (-78.5°C) to laboratory facilities at University of Connecticut in Storrs, CT and stored at -80°C until processed.

RNA isolation, quantification, and quality assessment

Tri-Reagent based phase separation followed by a column cleanup has previously been found effective for the extraction of total RNA from avian whole blood samples.[7,31] This two-step extraction method, via the phase separation and column cleanup, is effective in reducing the high proportion of proteins found in whole blood samples and the large quantity of nuclease associated with nucleated blood. Here, we optimized the phase separation and column-based extraction protocol for small avian whole blood samples (~20 µL). Isolation and extractions of RNA were completed in an RNase free environment. A complete step-by-step isolation and extraction protocol can be found at https://github.com/JAHarvey/RNA-Blood-preservation-extraction/blob/main/RNA_Isolation_Protocol.mkd. Total RNA was isolated from 20 µL of peripheral whole blood using a modified Tri-Reagent (Ambion, Invitrogen, Carlsbad, CA, USA) and Direct-zol RNA Miniprep Plus Kit (Zymo Research, Irvine, CA, USA) protocol. The samples were incubated at room temperature for 2 minutes and then centrifuged for 1 minute at 8,000 RCF to lightly pellet the blood. Preservation fluids (i.e., RNAlater, DNA/RNA Shield) were pipetted off leaving no more than ~15 µL of preservative and 500 µL of Tri-Reagent were added along with a sterile 5 mm stainless steel bead (Thomson, Radford, VA, USA). The samples were then vortexed for 30 seconds before adding an additional 500 µL of Tri-Reagent. The sample was then vortexed for 10 minutes at room temperature. The phase separation portion of the Tri-Reagent protocol was then followed, and the upper aqueous phase (500 µL) was transferred to new microcentrifuge tubes. We then used column purification with the Direct-zol RNA Kit, following manufacturer's protocol beginning with the RNA purification step and including DNase treatment. We eluted total RNA using 50 µL of DNase/RNase-Free water (Invitrogen, Waltham, MA, USA). We quantified total RNA concentration (ng/µL) using a Qubit RNA HS assay kit on a Qubit 4 Fluorometer (Thermofisher Scientific, Waltham, MA, USA). We used a 4200 TapeStation Analyzer System and High Sensitivity RNA ScreenTape assays (Agilent, Santa Clara, CA, USA) to determine rRNA ratio, total RNA concentration, and RNA integrity numbers (RIN^e).

Statistical Analysis

All statistical analyses were conducted, and figures were created, in R version 4.1.0 and RStudio (v1.4.1717). Generalized linear mixed models (GLMMs) were used to determine the effect of different preservation treatments on total RNA quantity (Tapestation and Qubit concentration) and quality (RIN^e, rRNA ratio ratio), with individual bird as a random effect. When the response variables did not pass the Shapiro-Wilk normality test, they were log transformed for the analyses. Analyses for GLMM were carried out using the lmer function with the lme4 package in R (Bates et al. 2015) and the ANOVA function with the car package in R (Fox and Weisberg 2011). Pairwise post-hoc comparisons were compared among treatments using Tukey's HSD (honestly significant difference) tests in the emmeans package in R (Lenth et al. 2018). Pearson r correlations were used to determine the strength of the relationships among measures of RNA quality and quantity.

Results

Preservation of samples at the time of collection is critical in all downstream sample preparation and sequencing steps. We found that preservation treatment affected both quality metrics (Table 1), RIN^e (Figure 1A; $\chi^2 = 37.59$, df = 4, P x< 0.0001) and rRNA ratio (Figure 1B; $\chi^2 = 19.50$, df = 4, P = 0.0006). For RIN^e, RNAlater had the highest values and the lowest variance (mean ± SD: 9.59 ± 0.14, Table 1), while dry ice-based flash frozen samples had the lowest scores (mean ± SD: 8.36 ± 0.84, Table 1). The RIN^e scores for dry ice-based flash frozen samples were significantly lower than samples preserved in preservation buffers at all concentrations (Tukey post-hoc test, all $P < 0.01$). Differences among RIN^e scores RNA preservation buffer treatments were not significant

Table 1. Average (mean ± standard deviation) of RNA quality metrics, RIN^e and rRNA ratio, and total RNA concentrations as measured by the Tapestation and Qubit, from RNA extracted from field-collected blood samples of individual free-living birds across different preservation treatments.

Treatment (*n*)	RIN^e	rRNA ratio	Tapestation (ng/μL)	Qubit (ng/μL)
Dry ice-based freezing[8]	8.36 ± 0.84	1.18 ± 0.40	104.6 ± 56.61	117.71 ± 52.45
DNA/RNA Shield 1:2[8]	9.26 ± 0.20	1.28 ± 0.24	75.23 ± 33.97	75.60 ± 29.05
DNA/RNA Shield 1:3[8]	9.23 ± 0.24	1.39 ± 0.23	104.76 ± 97.88	77.17 ± 39.59
RNAlater 1:5[8]	9.49 ± 0.18	1.45 ± 0.21	89.81 ± 72.29	72.63 ± 27.15
RNAlater 1:10[8]	9.59 ± 0.14	1.71 ± 0.15	80.34 ± 46.23	76.20 ± 30.34

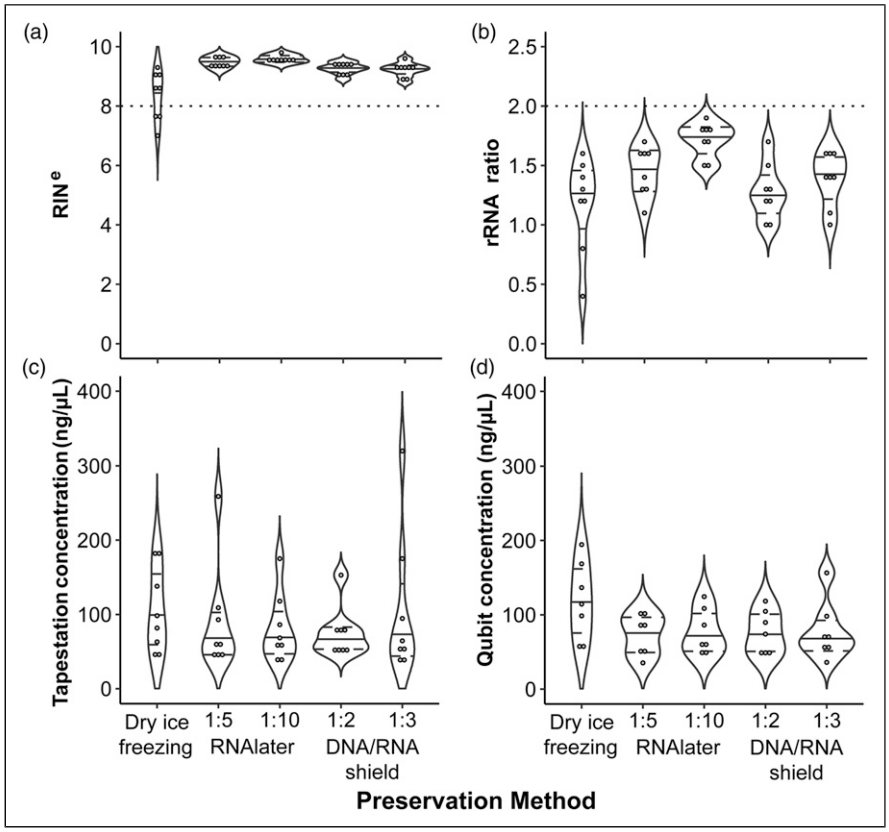

Figure 1. Effect of preservation treatment, on A) RINe, B) rRNA ratio, C) Tapestation RNA concentration (ng/μL), and (D) Qubit RNA concentration (ng/μL), where each point represents an RNA extract from field collected blood samples of individual free-living birds across different preservation treatments.

(Tukey post-hoc test, all $P \geq 0.57$). For rRNA ratio, the samples preserved in RNAlater at a 1:10 concentration had significantly higher values (mean ± SD: 1.71 ± 0.15, Table 1) than the dry ice-based flash frozen samples (mean ± SD: 1.18 ± 0.40, Table 1; Tukey post-hoc test, all $P = 0.004$). No other differences among treatments were significant for rRNA ratio samples (Tukey post-hoc test, all $P \geq 0.17$)

We found that preservation treatment had little effect on quantity metrics; differences in Qubit concentrations were significant (Figure 1D; $\chi^2 = 11.04$, df = 4, $P = 0.03$) but there was no significant effect for Tapestation concentrations (Figure 1C; $\chi^2 = 4.40$, df = 4, $P = 0.36$). Qubit RNA concentrations from the dry ice-based flash frozen samples were higher (117.71 ± 52.45, Table 1) than samples across all preservation treatments but difference among preservation buffer treatments were not significant (Tukey post-hoc test, all $P \geq 0.09$). Both quantity measures, Tapestation and Qubit, total RNA concentrations were highly correlated (Figure 2B; Pearson's r = 0.90), as were quality measures RIN^e and rRNA ratios (Figure 2A; Pearson's r = 0.73). However, neither quality value was related to either RNA quantity measure (Pearson's r < 0.30 for all pairs).

Discussion

Non-lethal sampling of peripheral avian blood can provide sufficient transcriptionally active RNA enabling increased usage of genome-wide transcriptome studies across passerines,[22,32] including at risk and endangered species.[18,21] Here we provide a comparison of RNA preservation methods for whole blood samples collected from free-living birds. We tested preservation methods in widely available commercial buffers at various concentrations as well as a dry ice-based flash freezing method in place of liquid nitrogen flash freezing. Both preservation buffers, RNAlater and DNA/RNA Shield, at all concentrations, produced extractions with high quality scores demonstrating that RNA preservation buffers, successfully preserved RNA from whole avian blood. The highest scores for RIN^e and rRNA ratios were yielded by samples preserved in RNAlater 1:10, while also demonstrating the lowest variance across treatments. We caution that handling time of samples prior to preservation as well as during downstream processing may increase degradation and increase bias of *ex vivo* effects on results. Additionally, we did not test extended storage periods of RNA-preserved samples at ambient or field temperatures as this has been found to lower RNA quality and impact expression profiles.[8,33] Our goal was to test RNA preservation methods for storage conditions commonly available to us in the field, conditions where liquid nitrogen is not available but cold storage, a freezer or refrigerator, is available. Often field-sampling conditions do not have cold storage immediately available, but 4°C can be achieved with a cooler and ice packs, and thus reagent preservation is necessary.

While flash freezing with liquid nitrogen (-190°C) is considered the standard for RNA preservation,[11] liquid nitrogen was not available at the field site and is commonly difficult to acquire at remote sites when field sampling. We expected our dry ice-based flash freezing method (-109°C) to provide a suitable substitute for liquid nitrogen flash freezing and be able to serve as the standard comparison for comparing the commercial stabilization buffers. Unfortunately, the dry ice-based flash freezing method performed less reliably. The dry ice-based flash frozen samples had lower and more variable quality scores than samples collected in either of the preservative buffers at any concentration. More than half of the RIN^e values for the dry ice-based flash freezing method were suitable for downstream RNA-Seq as they met the commonly accepted threshold of RIN > 8. Previous comparisons of methods have shown that flash freezing tissue samples in the field with liquid nitrogen yielded comparable quality RNA extractions and higher RNA concentrations as compared to RNAlater.[8,33] The performance of the dry ice-based flash freezing method may have been impacted by factors such as degradation from thaw/freeze cycles during transport or initial steps of the RNA extraction. Alternatively, the increased degradation may be due to uneven freezing of the blood samples impacting degradation activity. This method of dry ice-based flash freezing, or the use of dry ice alone are not recommended for field collection and preservations of avian blood RNA samples alone without the use of preservation buffers.

Lower rRNA ratios indicate higher degradation has occurred in the sample as the 28S peak is known to decrease faster than the 18S peak. However, rRNA ratios have previously been found to be inconsistent when run multiple times on the same samples suggesting caution against rRNA ration being solely used to characterize the quality of RNA

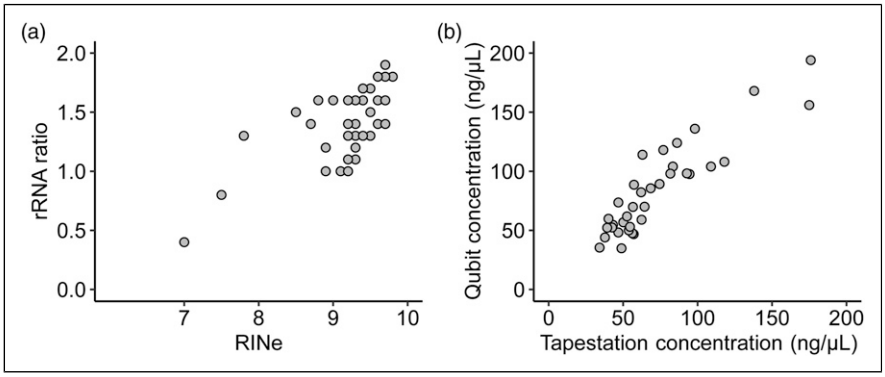

Figure 2. Relationships between the A) RINe and rRNA ratio (quality measures), and B) Tapestation and Qubit RNA concentrations (quantity measures) of RNA extracted from field-collected blood samples of individual free-living birds across different preservation treatments.

extractions (for a thorough discussion of rRNA ratios see[34]). However, contrary to this previous finding, we recovered highly correlated rRNA ratios and RIN^e scores. Preferentially, RNA quality is assessed wholistically using both RNA integrity number (RIN and RIN^e) and rRNA ratios to inform RNA quality prior to downstream sequencing, as the RNA integrity number provides the most complete characterization of RNA quality by measuring features of degradation.[35]

Tapestation and Qubit concentrations (ng/ μL) for all samples were sufficient for downstream sequencing. However, total concentration measures though highly correlated varied across analyzers. The Qubit measures florescence via target-specific dyes, while the Tapestation calculates the concentrations using the cumulative area of electropherogram sample peaks (28S and 18S) compared to the lower marker, which is a peak of standardized and calibrated concentration. Quantity measurements assess total RNA concentrations, including many small RNAs and when measured with a fluorometer this measurement may not be able to differentiate degraded RNA. The quantification measurement is therefore expected to vary when measured with different analyzer methods, such as the Qubit Fluorometer and the Tapestation, are used. Concentration of RNA extracted from avian and reptile blood (both nucleated blood cells) have been found to vary in concentrations despite the equivalent starting blood volumes for the extraction.[7] These differences in concentration may be due to differences in hydration levels of birds that were captured and sampled. Overall, total RNA quantity was generally not affected by preservation treatment and is not as important a limitation to downstream usage as RNA quality.

Future studies could increase the focal species and numbers sampled followed by creation of sequencing libraries and sequencing the samples to determine specific differences across methods. Library preparations may further impact the downstream sequencing results of peripheral avian blood and the selection of appropriate methods varies with the intended objective of sequencing. These options include removal of rRNA which comprises 90% of total RNA.[36] Removal of rRNA may be carried out via polyadenylated region selection via various commercial kits, which target transcripts with polyA tails present as in most mRNAs, or via rRNA depletion which binds or targets rRNA for subsequent removal. However, this is beyond the scope of this study.

Conclusion

Here, we demonstrate that field collection of high quality preserved peripheral avian blood RNA samples is possible with available commercial buffers. Our results will enable future study of avian blood transcriptomes in wild (free-living) avian populations. To this day, most avian transcriptome studies have been conducted in clinical and laboratory settings. Controlled experimental setups in captive species are informative and allow researchers to reduce the number of variables impacting study species e.g., disease, environment, resource availability, competition. However, captive studies do not describe the complex transcriptomic response of wild species in dynamic natural environments as the transcriptomic response has accurately been described as a "snapshot" of cellular response to its environment.[37] While we only examined preservation of passerine blood samples for RNA extraction, these preservation methods are applicable to all birds and other vertebrates with nucleated blood cells, i.e., amphibians, reptiles, and fish. The increased implementation of RNA-Seq studies in wild birds via non-lethal blood sampling will help to inform wild avian transcriptomic responses to environmental stressors and improve the genomic tools available to inform species' conservation and management.

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Author contributions

J.A.H. and S.A.K. conceived the idea, design, experiment; S.A.K. collected field data and edited the paper, J.A.H. conducted the research and wrote the paper, J.A.H. and S.A.K. analyzed the data. Both authors approved the manuscript before final submission.

Declaration of conflicting interests

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Ethics statement

All applicable national and institutional guidelines for the care and use of animals were followed. All bird handling and work was conducted according to approved University of Connecticut IACUC (Institutional Animal Care and Use Committee) protocols (No. A18- 005).

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