Molecular Ecology Resources

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RESOURCE ARTICLE

Unrecognised DNA Degradation in Flash-Frozen Genetic Samples in Natural History Collections

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Received: 19 August 2024 | **Revised:** 4 April 2025 | **Accepted:** 22 April 2025

Handling Editor: Brent Emerson

Funding: This work was supported by Division of Environmental Biology, DEB 0423286, DEB 0641023, DEB 1257610, DEB 9322600, DEB 9625873, DEB 9984496 Division of Biological Infrastructure, DBI 1950610, DBI 2029955.

Keywords: buffers | cryo-collection | DNA preservation | field expeditions | flash-frozen | tissue preservation | vertebrates

ABSTRACT

Optimal preservation of tissues from the field to long-term cryo-storage is paramount to securing genetic resources for research needs. DNA preservation techniques vary, with flash freezing currently considered the gold standard in tissue preservation. However, flash freezing tissue samples in the field presents challenges, necessitating a more comprehensive understanding of the quantity and quality of preserved DNA from different techniques in archival collections. We compared metrics from DNA extractions from field-collected amphibian, squamate and bird tissues from archival collections that were flash-frozen in liquid nitrogen or fixed in either ethanol or tissue lysis buffer prior to archival cryopreservation. We also included DNA extracted from tissues of known liquid nitrogen tank failures to provide a baseline of DNA degradation under the very worst-case scenario. Flash-frozen tissues often preserved higher yields of DNA, but peak fragment size, the percentage of fragments larger than 10 kb and DNA integrity numbers were all significantly reduced compared to tissues first preserved in fixative buffers. This pattern was observed across independent samples and between flash-frozen and buffer-preserved pair replicates. Degradation seen in flash-frozen tissues was also distinct to tissues from known tank failures. We suggest that degradation in flash-frozen tissues occurred during shipping, sample sorting/accession or during subsequent subsampling when tissues may partially or fully thaw, exposing DNA to damaging freeze–thaw processes. By contrast, tissues in fixative buffers were likely protected from freeze–thaw damage. This study highlights that using multiple field preservation methods and minimising freeze–thaw cycles for flash-frozen tissues may provide the most robust protection against the DNA degradation sources encountered by field collections.

1 | Introduction

With the development and advances of molecular biology in the 1960s and 70s, the collection of genetic samples for studies using proteins, allozymes and eventually DNA, had become routine field practice by the 1980s (Johnson et al. 1984; Martin 2006; Schindel and Cook 2018). This widespread collection of genetic resources has resulted in many institutions and research labs establishing archival genetic resource collections to support internal research, and often, the needs of the broader scientific community (Edwards et al. 2005; Martin 2006; Radin 2015). The rationale for collecting and archiving genetic resources is unquestionable. However, there are still uncertainties in how to best preserve samples, especially under field conditions at remote collecting sites. There are a number of potential options to preserve genetic resources in the field that range from flash freezing (with liquid nitrogen) to chemical fixation, all of which affect the concentration and fragmentation of DNA (Camacho-Sanchez et al. 2013; Dahn et al. 2022; Mulcahy et al. 2016; Nagy 2010). Given the rise of genomic technologies which require long fragment lengths (> 100 kb) to produce high-quality assemblies (Dahn et al. 2022; Wong et al. 2012), it has thus become imperative to empirically determine how different preservation techniques used in the field impact DNA degradation (Raxworthy and Smith 2021).

DNA degradation occurs rapidly, starting within minutes after an organism dies or is sampled from a living individual. After death, cellular repair mechanisms stop functioning and the DNA is exposed to numerous factors that threaten its stability, including digestion by intracellular nucleases and microorganisms and oxidative and hydrolytic damage (Allentoft et al. 2012; Dabney et al. 2013; Graham et al. 2015; Lindahl 1993). Under certain conditions (e.g., extreme cold, anoxia), the impact of these degradation mechanisms may be inhibited or slowed (Allentoft et al. 2012; Briggs et al. 2007; Hofreiter et al. 2015). It is well established that nucleic acids degrade with increased temperature and time (Allentoft et al. 2012; Dabney et al. 2013; Guo et al. 2018; Hofreiter et al. 2015; Schroeder et al. 2006) and at ultra-low temperatures (below −80°C), it is assumed many of these degradation forces are arrested (Nagy 2010; Wong et al. 2012) and therefore cryopreservation (typically storing at −80°C in an ultracold freezer or at −135°C to −196°C in vapour phase or liquid nitrogen) has been established as the best way to preserve tissues long-term. However, because cryogenic preservation presents logistical challenges under remote field conditions, samples are often non-cryogenically preserved in a buffer such as ethanol, isopropanol, dimethyl sulfoxide (DMSO), lysis buffers, or RNA-later. When left at room temperature, non-cryogenically preserved tissues have been shown to have significant DNA degradation after only weeks to months compared to cryopreserved tissues (Camacho-Sanchez et al. 2013; Dahn et al. 2022; Dawson et al. 1998; Frampton et al. 2008; Kilpatrick 2002; Michaud and Foran 2011; Mulcahy et al. 2016; Nagy 2010; Oosting et al. 2020; Reiss et al. 1995; Seutin et al. 1991). As liquid nitrogen availability has improved, and tanks and dry shippers have become more affordable and accessible, the use of flash freezing in the field has become routine.

Although cryogenically preserving a genetic resource immediately after collection (flash freezing) is considered the gold standard for both DNA (Anchordoquy and Molina 2007; Dahn et al. 2022; Frampton et al. 2008; Kilpatrick 2002; Oosting et al. 2020; Reiss et al. 1995; Seutin et al. 1991; Wong et al. 2012) and RNA (Camacho-Sanchez et al. 2013; Cheviron et al. 2011; Gorokhova 2005; Riesgo et al. 2012; Wang et al. 2006), numerous issues are faced under field conditions. Obtaining and maintaining adequate levels of liquid nitrogen is often logistically difficult or impossible. Even when the logistics can be arranged, airlines or freight/courier services may delay the transport of material. These factors can increase the risk of samples being lost or degraded should a tank or dry shipper fail or go dry, or get delayed or rejected during international shipping (Johnson et al. 1984). There are also practical considerations of flash-frozen material. Unlike controlled lab settings, the collection of genetic resources often varies among individual samples due to a host of reasons, with some samples being processed and frozen up to hours later. Also, flash-frozen field samples are transferred, organised and subsampled, exposing them to repeated cycles of non-freezing temperatures and possible thawing. Alternatively, non-cryogenic preservation methods such as buffers or EtOH may be used to fix tissues prior to long-term storage in a cryogenic facility. Laboratory studies have upheld the gold-standard status of flash freezing tissues (Dahn et al. 2022), but it has also been suggested short-term non-cryogenic storage can be sufficient even for long-read sequencing (Minich et al. 2023), and even that putting tissues into buffers before cryopreservation may actually be better than flash freezing (Mulcahy et al. 2016). No studies we are aware of have considered the field implications of these findings or have made direct comparisons of DNA degradation of field-collected archival samples across a diversity of DNA preservation methods.

In this study, we investigated the quantity and quality of DNA extracted from frozen field tissues ($N = 190$) from the Ambrose Monell Cryo Collection (AMCC) at the American Museum of Natural History (AMNH) of various preservation types: (1) flash-frozen in liquid nitrogen; (2) preserved in ethanol before freezing; (3) preserved in tissue lysis buffer before freezing; and (4) flash-frozen tissues from tanks and shippers that failed in the field or during shipping. This included tissue pairs ($N = 36$), where the same individual had both a flash-frozen tissue and either a tissue put into ethanol or tissue lysis buffer before cryopreservation, allowing direct comparisons of preservation methods. To deduce whether the degradation seen in flash-frozen tissues was likely due to failures of field or shipping cryopreservation, the quality of DNA extracted from tissues from known tank and dry shipper failures was also examined.

2 | Materials and Methods

2.1 | Sample Acquisition

We extracted 190 field-collected flash-frozen tissues that are accessioned at the American Museum of Natural History (AMNH) and the University of Michigan Museum of Zoology (UMMZ). These samples are from multiple taxa from the herpetology and ornithology collections, from 21 expeditions, and include three different preservation types: two types of tissues stored in buffer before cryogenic storage (95% ethanol or tissue lysis buffer; 1.2% SDS, 127 mM EDTA, 5 mM Tris HCl, 86 mM NaCl) and tissues

TABLE 1 | Frozen tissue sample numbers from each preservation condition and broad taxonomic groups. Sample numbers in brackets are the subset of frozen tissues that are part of the tissue pair dataset. Detailed collection information about each sample is provided in Table S1.

Tissue preservation	Group	Number of samples
Flash-frozen	Birds	21 (21)
	Squamates	10 (10)
	Amphibians	34 (6)
Ethanol	Birds	31 (21)
	Squamates	9
	Amphibians	44
Tissue lysis buffer	Birds	0
	Squamates	10 (10)
	Amphibians	12 (5)
Failed LN2 tank	Birds	15
	Squamates	4
	Amphibians	0

that were flash-frozen in liquid nitrogen (Table 1 and S1). Tissue samples were stored in the AMNH's Ambrose Monell Cryo Collection (AMCC) facility, a dedicated cryogenic collection facility (using liquid nitrogen vapour vats) and −80°C freezers at UMMZ and AMNH until the time of the study.

To deduce whether degradation patterns seen in flash-frozen tissues could be explained by liquid nitrogen tank failures, 19 tissues from known tank failures during expeditions to Madagascar in 1995 and Brazil in 2018 were added to this larger group of samples. For the Madagascar expedition, the tank lost its vacuum seal in the field, resulting in the tissues thawing to ambient temperature for about a day before being subsequently frozen in a −20°C freezer for 3 months prior to a −80°C freezer. For the Brazil expedition, the tank was not refilled with liquid nitrogen in due time, allowing the tissues to thaw to ambient temperature; subsequently, the tissues were stored in a −80°C freezer. The herpetological field samples were collected in Madagascar between 1995 and 2016 by 12 AMNH expeditions and 1 UMMZ expedition, the latter of which suffered from a liquid nitrogen failure in the field. The ornithological specimens were collected by eight AMNH expeditions in Vanuatu in 2014, Brazil in 2018, and six different times in North America between 2008 and 2021.

For the second phase of our investigation, we considered a subset of this larger collection which consisted of tissue pairs where the same individual had been sampled for two tissue preservation types. This subset included 21 tissue pairs across three species of birds from an expedition to Vanuatu in 2014 (Andersen et al. 2017), where the same individual had been sampled for ethanol and a flash-frozen tissue. Further, this subset also included 15 tissue pairs across multiple species of mantellid frogs and chameleons from two expeditions to Madagascar in 2002 and 2003, where the same individual had been sampled for a tissue lysis buffer and flash-frozen tissue.

2.2 | DNA Extraction

DNA extraction protocols vary in cost per sample, user-ease and stability, and are optimised to target different DNA fragment sizes. For example, high-molecular-weight (HMW) kits required for long-read sequencing size select for large fragments of DNA (e.g., 100–200 kb), whereas kits more suitable for sub-genomic and resequencing applications yield smaller fragments (e.g., 100 bp to 50 kb). A main aim for our study was to quantify patterns of DNA degradation that would be observable in fragments < 50 kb; therefore, our experimental design used a kit that was both highly stable and would capture patterns of degradation that would be expected in archival material, Qiagen DNeasy Blood & Tissue kit. We supplemented our pairwise comparisons with DNA extracted from a subset of samples using an HMW extraction kit (Qiagen Magattract HMW DNA kit) to determine if the same general patterns were observable across extraction kit types.

Tissue samples from heart, limb, or pectoral muscle were cut and weighed prior to extraction (Table S1). To reduce the potential impact of ethanol carryover, ethanol tissues were washed twice in ddH_2O prior to extraction. First, tissues were minced with a razor blade and whole genomic DNA was extracted from all samples using a Qiagen DNeasy Blood & Tissue kit following the manufacturer's protocols, with a double elution procedure, starting with 200uL AE buffer and followed by a second elution with either 100 or 200 μL of AE buffer for a total of 300 or 400 μL of eluted DNA. Next, a subset of the paired tissues were randomly selected—32 tissues in total; nine individuals with ethanol and flash-frozen tissue pairs, and six individuals with tissue lysis buffer and flash-frozen tissue pairs—for extraction with a Qiagen Magattract HMW DNA kit to compare the performance of tissues across different extraction protocols (Table S2). As for the standard Qiagen DNeasy Blood & Tissue kit, ethanol tissues were first washed twice in ddH_2O to minimise potential ethanol carryover. Up to 25 mg of tissue was cut, weighed and minced with a scalpel. Whole genomic DNA extracted using a Qiagen Magattract HMW DNA kit following manufacturer's protocols for animal tissues, with a second elution step with 100 μL of AE buffer for a total of 300 μL of extract.

2.3 | DNA Quantity and Quality Assessment

To measure DNA yield, fluorescence was measured using a High Sensitivity Assay Qubit, Invitrogen, Waltham, MA, using 1-μL aliquots. Final concentrations were then standardised by volume and input tissue weight to calculate ng of DNA extracted per mg of tissue. DNA fragment length distributions were analysed using Genomic DNA ScreenTapes on an Agilent 4150 TapeStation System, also calculating the proportion of fragments greater than 10 kb. DNA degradation through DNA Integrity Numbers (DINs; Kong et al. 2014) was also estimated using Genomic DNA ScreenTapes.

2.4 | DNA Library Preparation and Sequencing

To further facilitate fine scale DNA degradation quantification, libraries were prepared for all tissue pairs and failed nitrogen tank tissues and sequenced. Firstly, extracts with low DNA quantities were concentrated to approximately 50 μL using an Eppendorf 5301 Vacufuge Concentrator System (Hamburg, Germany). DNA from extracts was then fragmented using a Covaris ME220 (Covaris LLC, Woburn, MA) machine, using four microTUBE-50 screw-cap PN 520166 kits (Covaris LLC, Woburn, MA). As tissues from failed tanks were already highly fragmented, this fragmentation step was skipped. DNA libraries from fragmented DNA were then constructed using the BEST protocol (Carøe et al. 2018), with modifications as per Mak et al. (2017). Briefly, 16 μL of extracted DNA was combined with 0.2 μL of T4 DNA polymerase (3 U/μL), 0.6 μL of T4 PNK (10 U/μL), 2 μL of 10× T4 DNA Ligase Buffer, 0.2 μL of dNTP (25 mM) and 1 μL of a reaction enhancer (containing 25% PEG-4000, 2 μg/μL BSA and 400 mM NaCl). The reaction was then incubated at 20°C for 30 min followed by 65°C for 30 min. Ligation of adapter sequences was performed by the addition of 1 μL of BEDC3 adapter sequence (20 mM stock) and mixed followed by the addition of 3 μL of 50% PEG 4000, 0.5 μL of T4 DNA Ligase Buffer (10×) and 0.5 μL of T4 DNA ligase (400 U/μL). The reaction was then gently mixed by pipette mixing and incubated at 20°C for 30 min followed by 10 min at 65°C. Fill in reactions were then performed by adding 0.2 μL of dNTP (25 mM), 1 μL of 10× Isothermal Amplification Buffer, 0.8 μL of Bst 2.0 Warmstart Polymerase (8 U/μL) and 3 μL of molecular biology grade water. The final reaction was then incubated at 65°C for 20 min followed by 80°C for 20 min.

Following preparation of libraries, DNA was cleaned using a Qiagen MineElute Reaction Cleanup Kit following the manufacturer's instructions and eluting in 22.5 μL of EB buffer with 0.05% Tween 20. Double-indexed Illumina adapters were added to DNA libraries through a round of indexing PCRs using PCR primers complementary to the BEDC3 adapter. P5 and P7 adapter sequences contained barcode sequences to allow multiplexing during subsequent high-throughput sequencing. Two technical replicates of each PCR of a library were performed to minimise PCR bias and maintain library complexity. Each PCR totalled 50 μL and contained 1× Gold buffer, 2.5 mM MgCl2, 1 mM dNTPs, 0.5 mM each indexing primer, 0.1 U AmpliTaq Gold DNA Polymerase and 6 μL of library DNA. Cycling conditions were 94°C for 12 min, 20 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 45 s, followed by 72°C for 10 min. PCR replicates were then pooled for each library and cleaned using AxyPrep magnetic beads (Axygen) following the manufacturers protocols and eluting in 30 μL of H_2O and quantified with a Qubit fluorometer using a Qubit dsDNA high sensitivity assay kit. DNA libraries were pooled and sent for shotgun sequencing on an Illumina HiSeq X Ten at Novogene Co.

2.5 | Data Processing

Adapter sequences were removed and paired-end reads merged using ADAPTER REMOVAL v2.3.2 (Schubert et al. 2016), trimming low-quality bases (<Phred20 –minquality 4) and discarding merged reads shorter than 25 bp (–minlength: 25). Read quality was visualised before and after adapter trimming using fastQC v0.11.9 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) to ensure efficient adapter removal. Filtered reads are available on the European Nucleotide Archive (Salis et al. 2024). Reads were then mapped against a mitochondrial reference genome of the corresponding species and a nuclear reference of the closest related species (Table S1) using BWA mem v0.7.17-r1188 (Li and Durbin 2009). Reads with mapping a Phred score less than 25 were removed using SAMTOOLS v1.12 (Li et al. 2009) and PCR duplicates were removed using MARKDUPLICATES from the Picard package v2.26.0 (http://broadinstitute.github.io/picard/).

2.6 | Statistical Analysis

The effects of different variables on the quantity and quality of DNA extracted from frozen tissues were evaluated using the generalised linear models in the R statistical platform v 4.3.1 (R Core Team 2024). Five response variables—DNA yield per unit of mass of tissue, peak DNA fragment size, proportion of fragments greater than 10 kb, DIN and estimated endogenous content—were modelled separately. The response variables DNA yield and peak DNA fragment size were square root transformed to meet assumptions of normality. Sequences from samples without a close reference genome were excluded because, without a close reference, estimates of endogenous content could not be made. Estimated endogenous content will still likely be an underestimate due to the large genetic distance from the reference genome in some taxa. The impact of this bias would be less for the tissue pairs, as the bias should be the same for each tissue pair. For all models, preservation method, taxonomic group and collection year, and their potential interactions were included as explanatory variables. Post hoc comparisons were performed using the R package emmeans v 1.10.3 (Lenth 2024) to test for differences between different levels of each factor.

Paired sample t-tests were run using a native library in R for tissue pairs to directly compare whether preservation method had a significant impact on the same measures of DNA quantity and quality across the same individual. For herpetological samples, this was flash-frozen vs. tissue lysis buffer, while for ornithological samples, this was flash-frozen vs. ethanol.

3 | Results

Our generalised linear models show that preservation treatment had a significant impact across all measures of DNA quantity and quality (Table 2: all p-values <0.001). Taxonomic group (whether bird, squamate, or amphibian) also had a significant impact on all response variables (Table 2: all p-values <0.05). Collection year had no significant impact on any of the response variables except when in interaction with preservation treatment in the peak DNA fragment size model (Table 2, p-value 0.03591).

3.1 | DNA Quantity

Overall, bird samples preserved more DNA per unit mass of tissue than amphibians or squamates (Figure S1; Table 2: p-value <0.001, mean difference 240 ng/mg). Flash-frozen tissues preserved more DNA in bird samples (mean difference 325 ng/mg),

TABLE 2 | *p*-Values of generalised linear models across the five measures of DNA quantity and quality (response variables) *p*-values below 0.05, 0.01 and 0.001 are indicated by *, ** and *** respectively. Effect sizes measured as partial eta squared are given in brackets for each measure in the model.

Explanatory variable	DNA yield	Peak fragment size	% fragments > 10 kb	DNA integrity number (DIN)	Endogenous content
Preservation	**2.56E-10*** (0.22)**	**2.00E-16*** (0.43)**	**2.20E-16*** (0.37)**	**2.96E-08*** (0.20)**	**1.10E-05*** (0.22)**
Taxonomic group	**1.53E-13*** (0.27)**	**0.01945* (0.04)**	**2.11E-03** (0.07)**	**9.88E-04*** (0.08)**	**2.13E-05*** (0.12)**
Collection year	0.724 (0.02)	4.15E-01 (0.004)	0.316 (0.006)	0.837 (0.000)	0.061 (0.06)
Preservation: Taxonomic group	**3.80E-05*** (0.1)**	0.112 (0.04)	**0.024* (0.06)**	0.168 (0.03)	0.425 (0.01)
Preservation: Collection year	0.212 (0.01)	**0.036* (0.04)**	0.143 (0.02)	0.146 (0.02)	0.313 (0.09)
Collection year: Taxonomic group	0.468 (0.01)	0.780 (0.003)	0.818 (0.002)	0.980 (0.000)	0.954 (0.000)

while in amphibians and squamates no significant difference in the DNA yield was observed between flash-frozen, ethanol and lysis buffer-preserved tissues (Figure S1). In all groups, tissues from failed liquid nitrogen tanks, as expected, had very low amounts of preserved DNA (Figure 1).

3.2 | DNA Quality

Remarkably, across all taxonomic groups, tissue lysis buffer and ethanol tissues preserved significantly higher peak fragment sizes, percentage of fragments over 10 kb, and DINs than flash-frozen tissues and the tissues from failed liquid nitrogen tanks (Figure 1), indicative of DNA degradation in the later tissues. As expected, tissues from failed liquid nitrogen tanks had vastly smaller fragment sizes than all other tissue types (mean 190 bp vs. 19 kb; Figure 1). In terms of endogenous content—the proportion of sequenced reads mapping to a reference genome—preservation method had no significant impact except in tissues from failed liquid nitrogen tanks that showed lower endogenous content on average.

3.3 | Tissue Pair Comparisons

For the individuals with both an ethanol and a flash-frozen tissue, paired t-tests revealed that although flash-frozen tissues preserved higher quantities of DNA (Figure 2: p-value 0.028), ethanol tissues preserved higher peak fragment sizes (p-value < 0.0001) and showed fewer signs of DNA degradation, with a higher percentage of fragments above 10 kb (p-value < 0.0001) and higher average DIN values (p-value $= 0.0026$). However, no significant difference was observed in the percentage of mapped reads (p-value $= 0.087$). Regarding DIN values, it is evident that ethanol-preserved tissues are more consistent in DIN values when compared to flash-frozen, which showed a wide variability in DIN values, with both high and very low values, where low values are indicative of DNA degradation (Figure 2).

For individuals with both a tissue preserved in lysis buffer and flash-frozen, strikingly similar results were found. DNA yield was highest in flash-frozen samples (Figure 3: p-value $= 0.033$). However, tissues preserved using lysis buffer consistently had higher peak fragment sizes (p-value $= 0.035$), a higher percentage of fragments greater than 10 kb (p-value $= 0.018$) and higher DIN values (p-value 0.018), with flash-frozen again showing large variability in DIN values, indicative of DNA degradation in many of these tissues (Figure 3). Again, no significant difference was found in the percentage of mapped reads (p-value $= 0.9$).

The results of both tissue pairs (lysis and ethanol vs. flash-frozen), which represent three separate expeditions—two expeditions to Madagascar in 2002 and 2003 and an expedition to Vanuatu in 2014—show significant signs of damage in flash-frozen tissues.

3.4 | Failed Liquid Nitrogen Tank Tissues

Tissues from failed liquid nitrogen tanks showed extreme signs of degradation with significantly lower DNA quantity, fragment

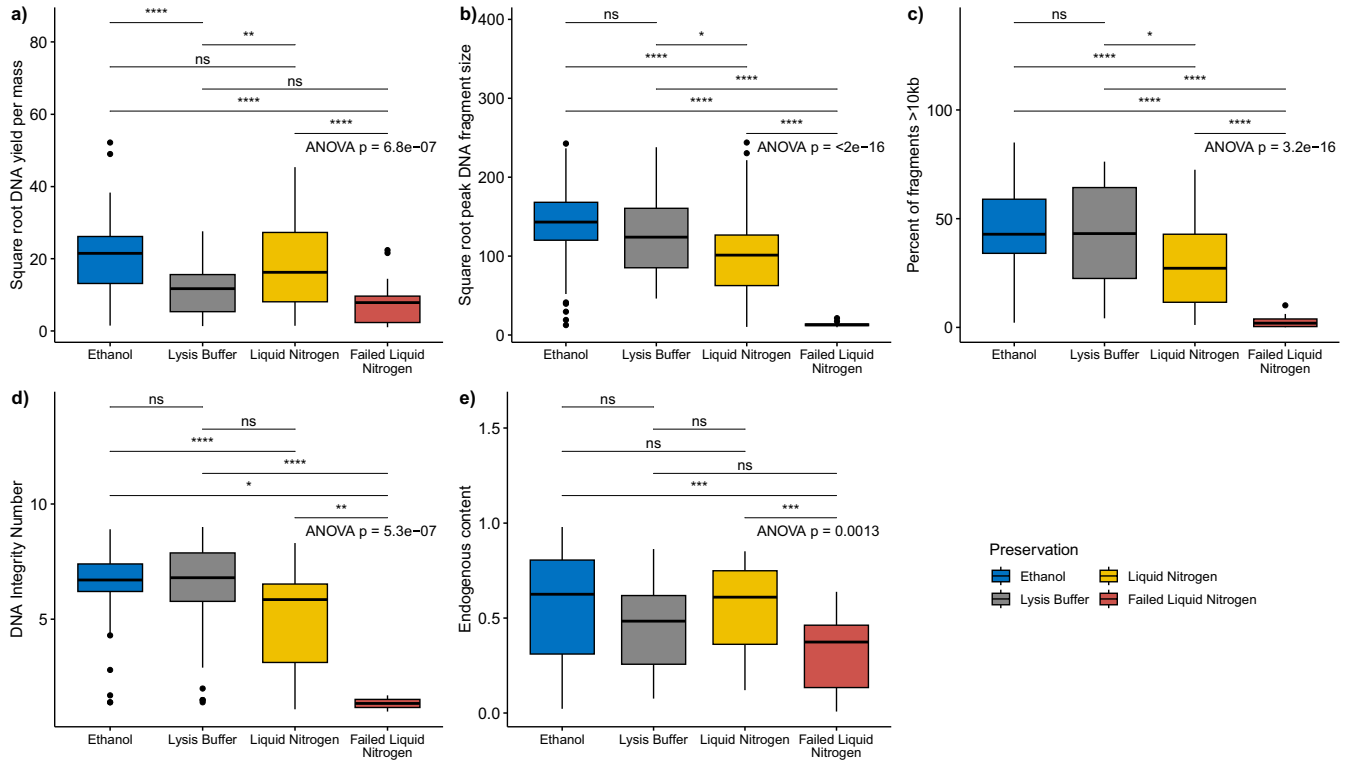

FIGURE 1 | Effect of tissue preservation method on measures of DNA quality and quantity. (a) DNA yield per unit mass of tissue, (b) DNA peak fragment size, (c) percentage of DNA fragments greater than 10, (d) DNA degradation as measured by DNA integrity numbers and (e) endogenous content, the fraction of sequenced reads mapping to the reference genome. Significant relationships from post hoc comparisons are shown as connecting bars with the following significance levels: Ns $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

size and DIN values than any other tissue type (Figure 1). Fragment sizes from tank failures were routinely under 1000 bp, but showed no signs of DNA deamination patterns at the ends of DNA fragments (Figure S3). Notably, our flash-frozen tissues produced significantly higher yields of DNA and higher-quality DNA than tissues from known tank failures, indicating that the degradation patterns seen in flash-frozen samples are not from tank failures.

3.5 | High-Molecular-Weight Extractions

When using a HMW kit, DNA yields were significantly lower in both buffer-preserved (p-value = 0.00059) and flash-frozen tissues (p-value = 0.00073) compared to when using the more general DNeasy kit (Figure S4). Buffer-preserved tissues extracted with the HMW kit tended to produce larger fragment sizes, a higher percentage of fragments > 10 kb and higher DNA integrity values; however, none of the comparisons were significantly different (Figure S4.1). For the flash-frozen tissues, the HMW kit produced significantly larger fragment sizes (p-value = 0.0021), more fragments > 10 kb (p-value = 0.029) and higher DNA integrity values (p-value = 0.25) compared to when using the DNeasy kit (Figure S4.2). When comparing the performance of buffer-preserved and flash-frozen tissue pairs extracted using the HMW kit, the same pattern of larger fragments (p-value = 0.01), a higher percentage of fragments > 10 kb (p-value 0.015) and higher DNA integrity values (p-value = 0.016), as when using the DNeasy kit, was maintained (Figure 4). However, contrary to the DNeasy results, DNA yield was highest in buffer-preserved tissues, not flash-frozen (p-value = 0.0074). This pattern was seen across both types of tissue pairs (Figure S5).

4 | Discussion

This study shows significant deviations in the quality of DNA between flash-frozen tissues and tissues stored in buffers such as lysis buffer or ethanol before cryopreservation in the analysed field collections. Flash-frozen tissues appeared to preserve high quantities of DNA, but their fragment sizes were often smaller, had lower percentages of total fragments greater than 10 kb and DINs were often lower than DNA extracted from tissues preserved in buffer before cryopreservation in the analysed archival field collections. Notably, the patterns observed in all these metrics were maintained when using a specialised, HMW DNA extraction kit, except for DNA yield, where yields were instead lowered in flash-frozen tissues. These results are indicative of significant degradation in HMW DNA of field-collected flash-frozen tissues. In the case of the HMW kit, the lower yield of flash-frozen tissue—despite having higher yields when using a standard DNA extraction kit—further supports this with a larger proportion of low-molecular-weight DNA being lost in flash-frozen tissues during HMW extractions. Sequencing outcomes from the HMW extractions were not evaluated with long-read sequencing; however, short-read sequencing revealed that although

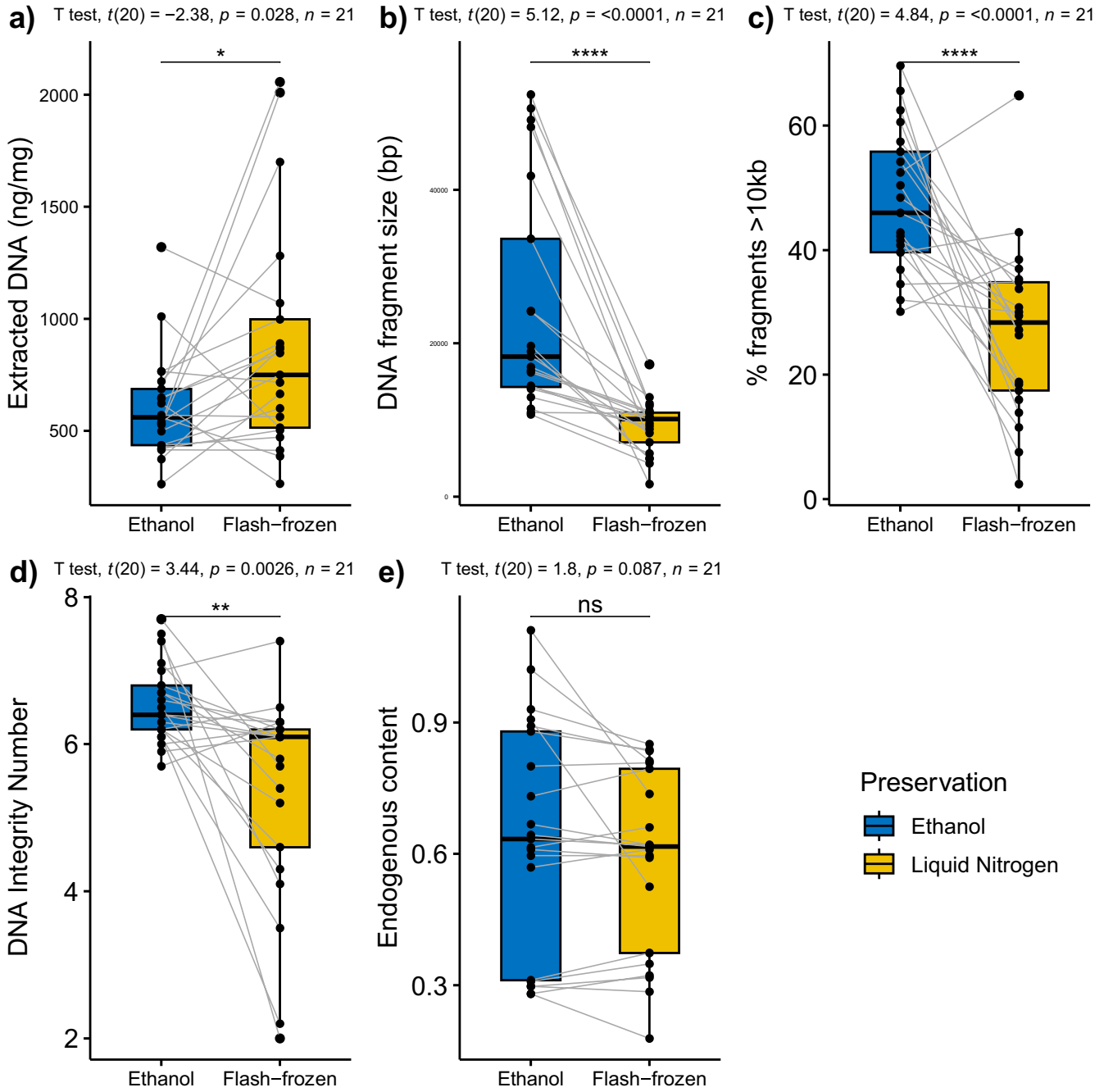

FIGURE 2 | Paired comparisons of flash-frozen and ethanol preserved bird frozen tissues taken from the same individual (21 pairs of comparisons) across five measures of DNA quality and quantity. Results of paired sample t-tests for each response variable is given above each panel. (a) DNA yield per unit mass of tissue, (b) DNA peak fragment size, (c) percentage of DNA fragments greater than 10 kb, (d) DNA degradation as measured by DNA integrity numbers and (e) endogenous content, the fraction of sequenced reads mapping to the reference genome. Significance levels of t-tests are as follows: Ns $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$.

flash-frozen tissues showed significant signs of degradation, no observable differences were found in the endogenous content of resulting extracts.

Recent experimental research by Dahn et al. (2022) has shown that flash freezing tissues in liquid nitrogen under lab or simulated field conditions is the 'gold standard' for DNA preservation and superior compared to first storing in ethanol or other storage buffers. Our findings analysing field-collected tissues under typical field conditions and long-term storage in a cryo-facility are in direct contrast to Dahn et al. (2022)—where *de novo* sampling was performed—with flash freezing samples with liquid nitrogen in the field showing significant degradation of DNA fragments compared to first storing in buffers such as ethanol or tissue lysis buffer. Our research is consistent with other research suggesting that submerging tissue in buffer prior to cryopreservation may be better than flash freezing for preservation of HMW DNA (Mulcahy et al. 2016). It is possible that the process of flash freezing tissues in liquid nitrogen could be physically damaging the

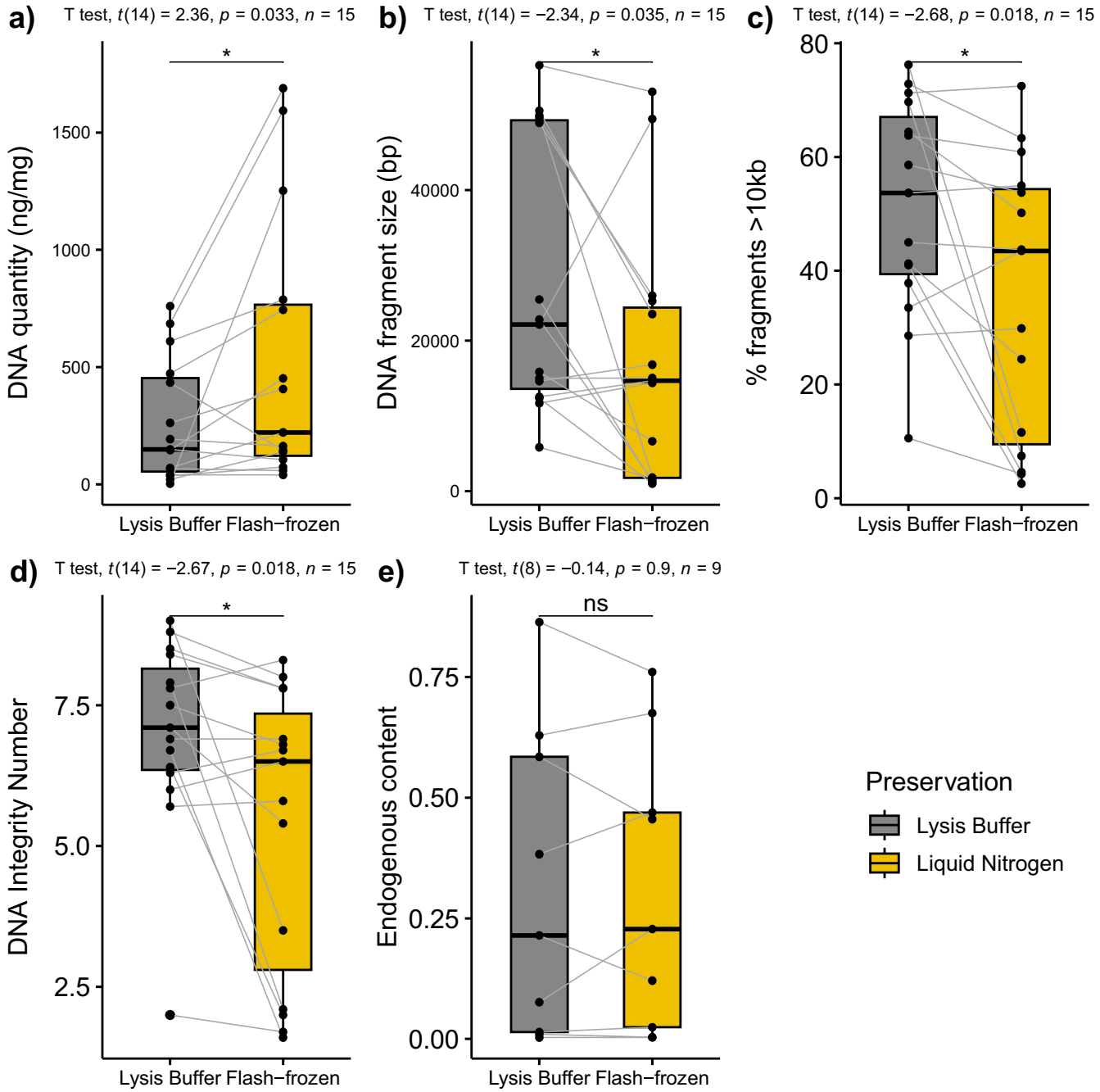

FIGURE 3 | Paired comparisons of flash-frozen and tissue lysis buffer-preserved herpetological frozen tissues taken from the same individual (15 pairs of comparisons) across five measures of DNA quality and quantity. Results of paired sample *t*-tests for each response variable is given above each panel. (a) DNA yield per unit mass of tissue, (b) DNA peak fragment size, (c) percentage of DNA fragments greater than 10 kb, (d) DNA degradation as measured by DNA integrity numbers and (e) endogenous content, the fraction of sequenced reads mapping to the reference genome. Significance levels of *t*-tests are as follows: Ns $p > 0.05$, *$p < 0.05$. Results split by taxonomic group are available in Figure S2.

DNA (e.g., physically shearing the DNA) and that first storing a sample in ethanol may buffer some of these damaging effects of liquid nitrogen. Mechanisms of physical damage from rapid freezing, or DNA cryolysis, have been proposed (Lyscov 1969; Lyscov and Moshkovsky 1969). However, experimental research suggests that the flash freezing process does not cause physical damage to the DNA and that the damage is occurring after (likely years) the flash freezing process (Dahn et al. 2022; Minich et al. 2023).

Flash-frozen samples may be subject to fluctuating temperatures through several mechanisms: thawing in dry shippers if delayed during transit, or exposure to room temperatures during handling, sorting, processing and subsequent subsampling. All these parts of the acquisition process, by potentially exposing samples to higher ambient temperatures, could result in flash-frozen samples degrading. If the temperature that samples are kept at fluctuates during the handling and sorting process, it is likely the tissues are allowed to undergo freeze–thaw cycles, which are known to

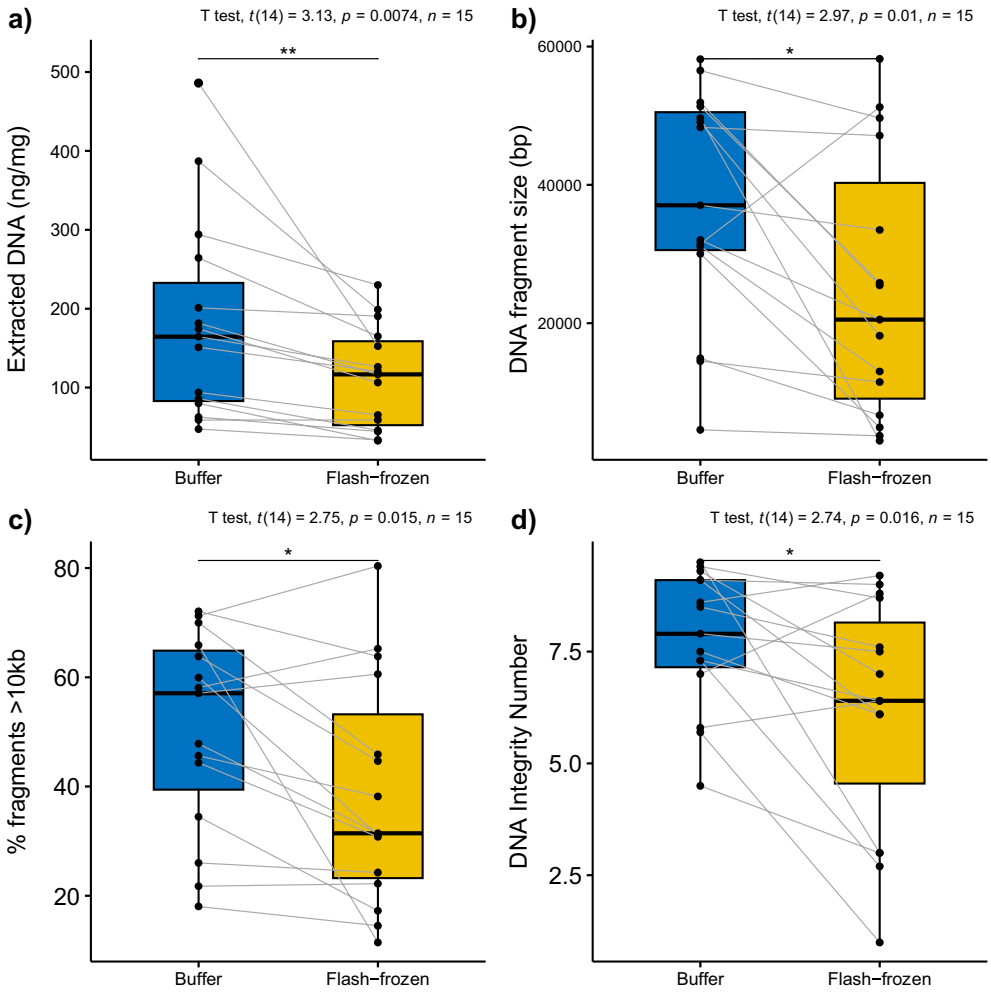

FIGURE 4 | Paired comparisons of flash-frozen and buffer-preserved (lysis buffer or ethanol) frozen tissues taken from the same individual (15 pairs of comparisons) extracted using a Qiagen Magattract HMW DNA kit across four measures of DNA quality and quantity. Results of paired sample t-tests for each response variable is given above each panel. (a) DNA yield per unit mass of tissue, (b) DNA peak fragment size, (c) percentage of DNA fragments greater than 10 kb, (d) DNA degradation as measured by DNA integrity numbers. Significance levels of t-tests are as follows: $^{*}p < 0.05$, $^{**}p < 0.01$. Results split by tissue pair type are available in Figure S5.

be highly damaging to nucleic acids (Cordsmeier and Hahn 2022; Davis et al. 2000; Hu et al. 2017; Ji et al. 2017; Lyscov 1969; Lyscov and Moshkovsky 1969; Shao et al. 2012). Freeze–thaw cycles have been known to damage biomolecules across various media types; for example, freeze–thaw cycles have been shown to damage DNA and fragment DNA, especially of larger, >100 kb, fragments (Cordsmeier and Hahn 2022; Matange et al. 2021; Shao et al. 2012).

There are typically as many as four steps where field frozen tissues could thaw during the acquisition and use process: (1) sorting and dividing vials into multiple collections in the field, such as between collaborating intuitions or for different export permits (e.g., CITES and non-CITES); (2) sorting vials into strict numerical sequence based on field numbers (such as from unsorted collections in dry shippers or liquid nitrogen tanks); (3) handling vials during cataloguing and renumbering into archival collections at museums or research labs; and (4) handling vials during subsampling for specific research use. In certain cases, such as in the case of smaller taxa or when whole organisms are frozen as vouchers for later identification and processing, handling of these samples during later identification may also result in full or partial thawing. Any or all of these stages could lead to the sample thawing as it is handled at room temperature.

Obtaining samples with HMW DNA is critical for keeping pace with long-read sequencing technologies, so our unexpected results showing degradation in flash-frozen genetic samples (i.e., lower molecular weight DNA) are significant for future research using these newer technologies and require explanation, although we did not evaluate long-read sequencing outcomes. Our findings could reflect idiosyncrasies associated with our samples that caused differences between flash-frozen samples versus approaches using fixatives. For example, this could happen if the dry shippers thawed or the samples were left out too long during processing. However, this seems highly unlikely for these herpetology and bird samples, in that there were no delays in exporting at the end of the expeditions; the samples were promptly processed. To our knowledge and observation, the samples arrived frozen in New York City. The consistency of our results across 21 independent expeditions also demonstrates the universality of our

findings. The other discussed possibility of physical shearing of DNA due to cold exposure is an interesting idea. We are not aware of any studies that have studied this effect, but if true, this presents a major challenge to collecting HMW DNA in the field.

The last possibility is that chemical-fixed samples are better protected against the environmental shifts that samples are routinely subject to. Tissue samples are inventoried and sorted at the end of an expedition and during their transfer into a cryo-collection, and they are subsampled when researchers want access to the genetic resource. The extreme temperature fluctuations during each of these times may lead to partial or complete thawing and potential substantial degradation. Storing tissue samples in ethanol or other buffers may protect the DNA from fluctuations in temperature during the shipping, accession and subsampling fulfilment process. Indeed, the simulated field conditions in Dahn et al. (2022), which mainly focused on different temperature treatments and duration before ultracold storage, did not include the possibilities of fluctuations in temperature during the shipping process or exposures to ambient temperatures when samples are first transferred to a cryo-collection and later subsampled for research projects. Therefore, even though flash freezing in liquid nitrogen may theoretically be a superior preservation method, variabilities during the shipping process from the field to the museum may make storing a tissue in buffer before freezing a more reliable method, especially for long-term storage and when sampling in more remote and inaccessible locations where liquid nitrogen tanks are more likely to fail and the shipping process is more complicated. Other research has also suggested that buffers may protect genomic resources from degradation (Minich et al. 2023; Mulcahy et al. 2016), although, as far as we are aware, our study is the first record in long-term archived tissue collections (including tissues from 1995). Our study helps to close the rather large knowledge gap on the efficacy of different preservation techniques under long-term archival storage and under uncontrolled field conditions, and suggests the need for future work in testing how DNA degrades under these field conditions and in archival cryogenic storage.

Only two tissue preservation buffers (ethanol and tissue lysis buffer) are investigated in this study; however, a variety of different buffers are used in the field, many of which may outperform ethanol or tissue lysis buffers. For example, Camacho-Sanchez et al. (2013) found that Nucleic Acid Preservation (NAP) buffer outperforms both ethanol and tissue lysis buffers in terms of DNA quantity and quality. Dimethylsulphoxide (DMSO) has also been shown to preserve high quantity and quality of DNA compared to other buffers (Anchordoquy and Molina 2007; Dahn et al. 2022; Dawson et al. 1998; Frampton et al. 2008; Kilpatrick 2002; Michaud and Foran 2011; Mulcahy et al. 2016; Oosting et al. 2020; Seutin et al. 1991), although the performance of DMSO compared to other buffers such as ethanol appears to be dependent on taxon (Mulcahy et al. 2016). Our results are not generalisable to these works, but suggest it is worth further investigating whether there are even better ways of collecting tissue samples in the field with sample stability through the shipping and handling process in mind. Further, although buffers such as tissue lysis buffer appear to be good for general genome sequencing applications, the nature of the buffer makes it unsuitable for downstream applications that require maintenance of enzymatic structures, chromatin structure and cell nuclei, such as proteomics, chromosome conformation technologies (e.g., Hi-C) and histological methods. Further, there are applications where only freshly collected or flash-frozen tissues will be suitable, such as metabolomics (Smith et al. 2020). If applications beyond genome sequencing are desired when collecting tissues for archival storage, it is imperative that these factors are taken into account.

For general applications, such as whole-genome resequencing, variability in DNA quality identified by this study is likely acceptable under most short-read sequencing applications. Optimal short-read Illumina DNA sequencing is observed with 350 bp fragments, with a significant reduction in sequencing quality in fragments above 500 bp (Tan et al. 2019). During library preparation, DNA is typically fragmented into smaller sizes, typically 300–600 bp. Long-read sequencing technologies such as those provided by Pacific Biosciences and Oxford Nanopore, however, are becoming increasingly popular in natural history research (Blom 2021; Blumer et al. 2022; Humble et al. 2020; Jebb et al. 2020; Kautt et al. 2020; Lind et al. 2019; Osipova et al. 2023; Shao et al. 2023; Winter et al. 2020). Long-read sequencing technologies can sequence fragments over 100 kb, allowing sequencing through traditionally difficult-to-sequence genomic features and easier identification of structural variants. Consequently, these technologies are far more sensitive to DNA quality, where significantly degraded DNA, such as that observed in our flash-frozen tissue samples, will result in lower average read sizes and a decrease in the chance of sequencing difficult regions, making successful *de novo* genome assemblies less likely.

We recognise that we did not attempt to prepare long-read sequencing libraries or perform long-read sequencing on any of our samples, limiting the extrapolation of our results to such applications. The suitability of non-cryogenically ethanol preserved tissues for long-read sequencing has been demonstrated, often with comparable success to flash-frozen tissues under shorter time periods (Minich et al. 2023). The successful application of buffer-preserved tissues with long-read technologies (Hartke et al. 2019; Minich et al. 2023; Peona et al. 2021; Schneider et al. 2021), together with the observation that DNA fragment sizes tend to correlate with N50 lengths of assembled genomes (Minich et al. 2023), suggests that similar success may be seen in buffer-preserved tissues. We anticipate that many labs are currently experimenting with long-read technologies using samples that were not flash-frozen. Future research should validate the utility of archival tissues of differing preservation histories to long-read sequencing and whether certain buffers (e.g., tissue lysis buffers) may have negative impacts on long-read sequencing success. Ultimately, freshly collected tissues will yield the best results for long-read sequencing and archival tissue collections used where they are likely to be the only genomic resource available, for example, for rare, inaccessible, or recently extinct taxa.

While the use of archival tissue collections in research using long-read sequencing is becoming increasingly common, the original intended use of archival tissue collections—many of which predate the advent of long-read sequencing technologies—is towards more general and cost-effective techniques such as PCR-based approaches, genome resequencing using short-read

technologies, and reduced-representation sequencing (e.g., RADseq approaches) and is likely to be far more common.

Although previous generation DNA sequencing technologies show resilience to variations in DNA quality, it is well known that low quality DNA can lead to biases in read distributions across the genome, inconsistent coverage across samples, and inaccurate mutation detection (Anderson et al. 2010; Goswami et al. 2016; Sah et al. 2013). Low DNA quality, for example, can present issues for RADseq approaches, which rely on large enough fragment sizes for restriction enzymes to work efficiently, and are a popular sequencing method for non-model organisms that often lack high-quality reference genomes (Baird et al. 2008; Peterson et al. 2012). It has been shown that degraded samples result in reduction of the efficiency of such sequencing approaches, with an increase in missing loci (Graham et al. 2015; Guo et al. 2018). Therefore, for successful application of a range of technologies on non-model organisms, high-quality tissues that yield HMW DNA are extremely important.

Our study lays the groundwork for future examinations on tissue preservation methods and storage conditions in the field. We verified across multiple separate expeditions and three different vertebrate clades that DNA degradation is likely higher than expected using optimal approaches. Subsequent work is also necessary to understand the magnitude of DNA degradation and address any idiosyncrasies particular to our equipment or storage of our samples. We also note that we did not investigate the preservation of RNA in our tissue collections, which almost certainly has different sensitivities during the handling and sorting process of frozen tissue collection and is often key in creating high-quality genome assemblies. Therefore, our results are likely not applicable to RNA preservation and should be addressed in future research, although similar patterns have been suggested in avian blood (Harvey and Knutie 2023).

Almost all flash-frozen tissues collected in the field will inevitably go through thaw cycles, thus we recommend that field researchers use several preservation techniques to store samples, when possible. Duplicate samples will allow users to empirically determine which preservation technique has led to higher molecular weight DNA samples. They also will serve as back-ups if samples are lost or there is a tank failure. When flash freezing tissues, subsampling into smaller aliquots during accession, although logistically challenging, could help prevent possible damage due to repeated freeze–thaw cycles during subsampling request fulfilment. More generally, all handling steps that could result in tissue thaws should be carefully considered to minimise exposure to temperature fluctuations. These suggestions are particularly important for fieldwork that targets samples intended for long-term archiving, where genetic samples will be used multiple times over the course of decades.

5 | Conclusion

As the application of DNA sequencing technologies in natural history research continues to expand and researchers move towards approaches that require HMW and high-quality DNA, archiving well-preserved frozen tissues is paramount. Our study highlights that current gold-standard flash freezing of tissue samples under actual field conditions may result in significant degradation of DNA long-term with a reduction of HMW DNA, likely due to repeated freeze–thaw cycles. Storing in fixative buffers prior to cryopreservation may instead be protective against degradation forces. To help reduce the impact of degradation, we suggest duplicate samples from the same specimen using multiple preservation methods. Further, when using flash freezing methods, we recommend subsampling the tissue into smaller aliquots before being accessioned into a cryobank to eliminate the need for repeated freeze–thaw cycles from subsampling.

Author Contributions

A.T.S., B.T.S. and C.J.R. designed research; A.T.S., M.F., Z.W., N.A., S.K. and J.K. performed research; B.T.S., C.R.J. and N.R. contributed to new reagents/analytic tools; A.T.S., Z.W., B.T.S. and C.J.R. analysed data; A.T.S., Z.W., M.F., B.T.S. and C.J.R. wrote the paper with input from N.A., S.K., J.K. and N.R.

Acknowledgements

We would like to thank the American Museum of Natural History's Ambrose Monell Cryo Collection facility (Svetlana Katanova and McKenna Santiago Coyle) and the University of Michigan Museum of Zoology. In addition, we are grateful to the following individuals and institutions who helped in the collection of the ornithological specimens: Paul Sweet, Thomas Trombone, Alexandre Alexio, Lilly Fatdal, Michael Andersen and the Department of Environmental Protection and Conservation, Vanuatu. We would like to thank Gregory Thom for feedback and support during the initial stages of the project. Field research in Madagascar was made possible due to the assistance of the Ministère de l'Environnement et du Développement Durable *de* l'Écologie et des Forêts, Madagascar National Parks; the Faculté des Sciences (Mention: Zoologie et Biodiversité Animale), Université d'Antananarivo; and the Faculté des Sciences, de Technologies et de l'Environnement (Mention Sciences de la Vie et de l'Environnement), Université de Mahajanga. This research was funded by the U.S. National Science Foundation (DBI 2029955) awarded to C.J.R and B.T.S.; the U.S. National Science Foundation (DBI 1950610); and the Madagascar fieldwork supported by the U.S. National Science Foundation (DEB 1257610, 0641023, 0423286, 9984496, 9625873, 9322600 and BSR 9024505) to C.J.R. Support for the Science Research Mentoring Program at the American Museum of Natural History is provided by Christopher C. Davis, The Shelby Cullom Davis Charitable Fund; The Pinkerton Foundation; the Bezos Family Foundation; the Doris Duke Charitable Foundation; the Solon E. Summerfield Foundation Inc; and the Adolph and Ruth Schnurmacher Foundation.

Disclosure

Benefit-Sharing Statement: This research was in collaboration with scientists at different levels, from senior scientists to early career scientists (graduate, undergraduate and high school students), the latter of whom received extensive training in molecular techniques and bioinformatics as a part of the project. The study tested commonly used field-based methods for tissue preservation, which will help guide future field expeditions where tissues are being collected as a genetic resource. All data are freely accessible as described below.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Demultiplexed and filtered shotgun sequencing have been uploaded to the European Nucleotide Archive (study accession PRJEB79053).

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.