1 **Title:** Library preparation method and DNA source influence endogenous DNA recovery from

2 100-year-old avian museum specimens

3 **Running title:** WGS of historical avian museum specimens

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14 **Abstract**

15 Museum specimens collected prior to cryogenic tissue storage are increasingly being used as
16 genetic resources, and though high throughput sequencing is becoming more cost-efficient,
17 whole genome sequencing (WGS) of historical DNA (hDNA) remains inefficient and costly due
18 to its short fragment sizes and high loads of exogenous DNA, among other factors. It is also
19 unclear how sequencing efficiency is influenced by DNA source. We aimed to identify the most
20 efficient method and DNA source for collecting WGS data from avian museum specimens. We
21 analyzed low-coverage WGS from 60 DNA libraries prepared from four American Robin
22 (*Turdus migratorius*) and four Abyssinian Thrush (*Turdus abyssinicus*) specimens collected in
23 the 1920s. We compared DNA source (toepad versus incision-line skin clip) and three library
24 preparation methods: 1) double-stranded, single tube (KAPA); 2) single-stranded, multi-tube
25 (IDT); and 3) single-stranded, single-tube (Claret Bioscience). We found that the multi-tube
26 ssDNA method resulted in significantly greater endogenous DNA content, average read length,
27 and sequencing efficiency than the other tested methods. We also tested whether a predigestion
28 step reduced exogenous DNA in libraries from one specimen per species and found promising
29 results that warrant further study. The ~10% increase in average sequencing efficiency of the best
30 performing method over a commonly implemented dsDNA library preparation method has the
31 potential to significantly increase WGS coverage of hDNA from bird specimens. Future work
32 should evaluate the threshold for specimen age at which these results hold and how the
33 combination of library preparation method and DNA source influence WGS in other taxa.

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37 **Introduction**

38 Museum specimens collected prior to cryogenic tissue storage have long been used as
39 genetic resources to address questions in ecology, evolutionary biology, and conservation (Habel,
40 Husemann, Finger, Danley, & Zachos, 2014; Wandeler, Hoeck, & Keller, 2007). Genetic studies
41 using these specimens have increased with the advent of high throughput sequencing methods,
42 which in comparison to prior Sanger sequencing methods, drastically increase the proportion of
43 degraded DNA that is sequenced (Burrell, Disotell, & Bergey, 2015). Now museum specimens
44 commonly facilitate genomic studies via reduced representation (Bi et al., 2013; Linck, Hanna,
45 Sellas, & Dumbacher, 2017; McCormack, Tsai, & Faircloth, 2016) and even whole genome
46 sequencing (e.g. van der Valk, Díez-del-Molino, Marques-Bonet, Guschanski, & Dalén, 2019;
47 Wu et al., 2022). Despite its increasing prevalence and dropping cost, sequencing whole genomes
48 of museum specimens remains expensive because of the degraded nature of the historical DNA
49 (hDNA).

50 Historical DNA tends to consist of short fragment lengths (McDonough, Parker,
51 McInerney, Campana, & Maldonado, 2018; Straube et al., 2021; Tsai, Schedl, Maley, &
52 McCormack, 2020) that are smaller than the recommended library sizes for the most cost-
53 efficient sequencing setups (i.e., Illumina NovaSeq 6000 S4 flowcell, 200 or 300 cycles). As a
54 result, many sequencing cycles are directly wasted by a lack of base pairs to sequence or
55 indirectly wasted on adapter read through (Straube et al., 2021). Historical DNA libraries also
56 tend to consist of low proportions of DNA from the focal specimen (hereafter endogenous DNA).
57 The rest of the library may consist of exogenous DNA from (1) microbes that have colonized the
58 museum specimen or (2) other environmental microbes, (3) contaminating DNA from researchers
59 or (4) other museum specimens, and (5) more recent DNA samples (Fulton & Shapiro, 2019).

60 Altogether, the degraded nature of hDNA results in the recovery of lower proportions of
61 endogenous DNA sequence data (Burrell et al., 2015) and necessitates increased sequencing
62 effort per specimen to recover similar WGS coverage to modern, high quality DNA libraries.
63 This inefficiency limits the use of historical DNA from museum specimens to address population
64 genomic questions that require larger sample sizes in addition to sufficient coverage to address
65 questions about selection and demography (Lou, Jacobs, Wilder, & Therkildsen, 2021).

66 Ancient DNA researchers have identified that single-stranded (Bennett et al., 2014;
67 Gansauge & Meyer, 2013; Wales et al., 2015) and single-tube library preparation methods (Carøe
68 et al., 2018), and those that ligate adapters to unmodified DNA molecule ends (Kapp, Green, &
69 Shapiro, 2021), increase the amount of degraded ancient DNA molecules that are converted into
70 genomic libraries. However, the most implemented ancient library preparation methods are non-
71 proprietary (Gansauge et al., 2017; Henneberger, Barlow, & Paijmans, 2019), thus requiring a
72 high level of startup effort. Early ssDNA methods were also more expensive to implement than
73 double-stranded DNA (dsDNA) libraries, and their improvement in sequencing efficiency did not
74 warrant the additional effort and cost to implement for all but the most degraded ancient DNA
75 samples (Wales et al., 2015). That is perhaps why only two studies to date have evaluated the
76 influence of ssDNA versus dsDNA library preparation on shotgun sequencing of historical
77 specimens. Sproul and Maddison (2017) found that ssDNA libraries–in comparison to dsDNA
78 libraries–prepared from 16 whole beetle specimens resulted in more retained reads following
79 quality filtering, but no difference in endogenous DNA content. Similarly, Hahn et al. (2022)
80 recently found no difference in endogenous DNA content or insert length between ssDNA and
81 dsDNA libraries prepared from twelve taxonomically diverse wet collection vertebrate
82 specimens. Additional studies of the influence of library preparation on WGS of museum

83 specimens that control for taxonomy, locality, and collection age of specimens will be valuable
84 moving forward. Early ssDNA methods have been modified to reduce costs and ease
85 implementation (Gansauge et al., 2017) and ssDNA methods are now commercially available as
86 kits facilitating further study of their impact on hDNA sequencing efficiency.

87 Thus far, the majority of research investigating how to maximize the recovery of genetic
88 data from non-ancient museum specimens has focused on the influence of DNA source or
89 extraction method on DNA yield (Hahn et al., 2022; Hawkins, Flores, McGowen, & Hinckley,
90 2022; McDonough et al., 2018; Pacheco et al., 2022; Straube et al., 2021; Tsai et al., 2020; Zacho
91 et al., 2021). However, DNA yield does not necessarily predict sequencing success or efficiency
92 (McDonough et al., 2018; Straube et al., 2021; Zacho et al., 2021) because it is not possible to
93 estimate the proportions of endogenous versus exogenous extracted DNA. For example, a recent
94 study of hundreds of historical genomic DNA libraries built from samples of museum bird
95 specimens found that those built from specimens of smaller species (which generally produce
96 smaller samples) unintuitively had a higher proportion of endogenous sequence data (Irestedt et
97 al., 2022). Moreover, a few studies have shotgun sequenced DNA from multiple sources on the
98 same specimen and found differences in endogenous DNA content across sampling sites in fluid-
99 preserved garter snake specimens (Zacho et al., 2021), prepared mammal skins (McDonough et
100 al., 2018), and formalin-fixed specimens of a dozen vertebrate taxa (Hahn et al., 2022). Despite
101 research indicating that differences in hDNA sourced from toepads versus incision-line clips in
102 bird specimens could influence high-throughput sequencing results (Tsai et al. 2020), no studies
103 have evaluated their difference in endogenous DNA content and sequencing efficiency.

104 Bird study skin specimens have been an especially prolific source of hDNA research
105 (Billerman & Walsh, 2019) in part due to preservation methods (i.e., skin drying) that are not

106 catastrophic to DNA preservation relative to methods such as formalin-fixation. Bird study skins
107 have been the foci of some of the earliest studies of hDNA (Mundy, Unitt, & Woodruff, 1997),
108 the source for some of the first implementations of reduced representation, high throughput
109 sequencing methods using museum specimens (Linck et al., 2017; McCormack et al., 2016), and
110 some of the largest studies implementing WGS of hDNA to date (Irestedt et al., 2022). In this
111 study we aim to maximize the potential of hDNA from bird study skins by identifying whether
112 DNA source and library preparation method influence the endogenous DNA content and
113 sequencing efficiency of hDNA libraries, and by introducing a pre-digestion step prior to DNA
114 extraction to reduce exogenous DNA.

115 In this study we test three library preparation methods that vary in 1) the number of
116 cleanups and tube transfers that occur before the library amplification step (one vs two) and 2)
117 whether they convert single or double-stranded DNA into library molecules. Each cleanup and
118 tube transfer is an opportunity to lose DNA molecules of the target length (greater than number
119 of sequencing cycles) due to the inherent imprecision of SPRI bead cleanups in addition to
120 pipette error. Methods that are optimized with one, in comparison to two, tube transfers should
121 transform more DNA molecules of target length into library molecules, thus maximizing library
122 complexity and sequencing efficiency. Double-stranded DNA library preparation methods cannot
123 convert ssDNA into library molecules, though as described above, hDNA is expected to consist
124 in some proportion of single strand molecules due to degradation. Methods that convert both
125 ssDNA and dsDNA molecules into library should increase the total number of input hDNA
126 molecules that are converted into library. Thus, we expect that dsDNA libraries prepared from
127 hDNA will have reduced sequencing efficiency and possibly endogenous DNA because a larger
128 content compared to that of ssDNA libraries.

129 We also test the influence of DNA source—toepads versus incision-line skin clips—on
130 endogenous DNA content and sequencing efficiency. A previous study indicated that toepads
131 consist of longer DNA fragments than skin clips (Tsai et al., 2020), another possible source of
132 hDNA from birds (Töpfer, Gamauf, & Haring, 2011). Libraries prepared from samples consisting
133 of longer DNA fragments should maximize the sequencing capacity, resulting in longer read
134 lengths on average and greater sequencing efficiency. To test these expectations, we prepared
135 shotgun DNA libraries from a toepad and skin clip from eight approximately 100-year-old bird
136 specimens via three methods: 1) double-stranded, single tube (KAPA HyperPrep Kit); 2) single-
137 stranded, multi-tube (IDT xGen™ ssDNA & Low-Input DNA Library Prep Kit); and 3) single-
138 stranded, single-tube (Claret Bioscience SRSLY® NanoPlus Kit). We sought to reduce
139 exogenous DNA by implementing a predigestion step modified from existing aDNA methods, to
140 our knowledge for the first time on bird specimens. To qualitatively evaluate the influence of the
141 predigestion we also prepared libraries from replicate toepad and skin clip DNA extractions not
142 subjected to predigestion from two of the eight specimens (Figure 1).
143
144 **METHODS**
145 **2.1. Sampling**
146 We sampled eight bird specimens: four Abyssinian Thrush (*Turdus abyssinicus*; hereafter
147 thrushes) and four American Robin (*Turdus migratorius*; hereafter robins; Table 1). We chose the
148 thrush specimens based on their inclusion in another ongoing project and chose to bolster our
149 sample size for this study with the robin specimens because they are a closely related, similar
150 species that is well-represented in North American natural history collections. Moreover, the
151 thrushes were collected in the tropics and the robins were collected in a temperate region which

could influence the drying time of the study skins and in turn, possible degradation due to rot or the microbial load within dried skin (Irestedt et al., 2022). We chose specimens collected within one year of each other to control for DNA degradation due to time since specimen preparation.

We collected two samples from each specimen—a toepad and a skin clip from the incision-line through the pectoral apterium (following Tsai et al., 2020)—to evaluate whether tissue source differed in proportion of endogenous DNA. Toepads and skin clips were approximately 3 x 3 mm (mean, M = 2.25 mg; standard deviation, SD = 1.01) and 5 x 5 mm (M = 2.33, SD = 1.91), respectively, though exact sizes varied so we weighed each sample to standardize our measures of DNA yield below (Table S1). We also took replicate samples from one specimen of each species (Table 1) to qualitatively evaluate the effect of sample predigestion prior to DNA extraction on the exogenous DNA load. The experimental design is summarized in Figure 1.

We followed stringent sampling precautions to limit the introduction of contaminant DNA: We (1) wore surgical masks and gloves throughout sampling, (2) took samples in the collections away from any specimen preparation laboratory, (3) did not enter any modern molecular DNA or specimen preparation laboratory prior to sampling, (4) prepared the work surface and all other supplies (e.g., forceps, optivisor, writing utensil) by cleaning with freshly prepared 10% bleach followed by 70% isopropanol or ethanol, and (5) immediately placed samples in sterile microcentrifuge tubes that were unpackaged in a sterile lab and not opened prior to beginning molecular lab work. We used a fresh pair of gloves and sterile scalpel blade for each sample to minimize contamination between samples.

2.2. Molecular laboratory work

174 We followed stringent ancient DNA clean lab protocols to minimize contamination
175 during molecular laboratory work (Fulton & Shapiro, 2019). We completed all pre-PCR steps in
176 an ancient DNA facility in the Department of Human Genetics at the University of Chicago in a
177 non-human specific room. We performed each step prior to PCR in a maximum batch size of 12
178 samples, introduced a negative control in each batch of extractions and library preparations, and
179 then carried these controls through the remaining steps of lab work.

180 Prior to DNA extraction we wanted to apply a pretreatment to remove exogenous DNA
181 from the sample surface. Several pretreatments to reduce exogenous DNA from teeth and bone
182 powder have been developed in the fields of forensics and aDNA, including but not limited to : 1)
183 scraping the surface from the sample, 2) wiping the sample surface with dilute bleach, 3) mixing
184 the sample in its entirety in a diluted bleach or phosphate buffer for fifteen minutes followed by
185 rinsing (Korlević et al., 2015), 4) enzymatic predigestion in the digestion buffer for a reduced
186 time and temperature (Damgaard et al., 2015), and 5) a combination of the previous methods (e.g.
187 Boessenkool et al., 2017). We were cautious in applying one of these treatment to toepads and
188 skin clips for the first time because these samples are dried skin and flesh and thus more delicate
189 compared to the powdered bone, bone fragments, or teeth these pretreatment are typically applied
190 to by aDNA researchers. Also, the starting material for each toepad and skin clip is much smaller
191 than that of a typical sample taken from bone. Given these considerations we chose a
192 predigestion rather than a more aggressive chemical or mechanical pretreatment. Previous
193 research on predigestion of bone powder and teeth has shown an increase in endogenous DNA
194 content after as little as 15 – 30 minutes of predigestion and that longer predigestion times can
195 reduce endogenous DNA content and drive down library complexity (Damgaard et al., 2015).
196 The standard protocol suggests considering input amount and preservation when choosing a

197 predigestion time between 15 and 30 minutes (Schroeder, Damgaard, & Allentoft, 2019). Again,
198 given the delicate nature and smaller size of our samples, we predigested each sample for up to
199 15 minutes in 180 uL of digestion buffer and 12 mAU proteinase K at 37C while rotating at 1000
200 rpm. To prevent significant digestion of the sample, we checked each predigesting sample
201 regularly and removed samples beginning after the first minute. We applied this predigestion
202 treatment to all but the four replicate samples described above (Figure 1).

203 We extracted DNA from all samples via a phenol-chloroform protocol followed by
204 ethanol precipitation as in Tsai et al. (2020) with minor modifications: 1) we used a digestion
205 buffer prepared in house and comprising 30mM Tris-HCl, 10mM EDTA, 1% SDS instead of the
206 Qiagen Buffer ATL, 2) we began samples digestion with 40 uL rather than 20 uL of proteinase
207 K, 3) we did not include DTT in our digestion solution, and 4) we did not mash tissue during
208 digestion. We performed an NEB PreCR DNA repair treatment following the sequential reaction
209 protocol on each DNA extraction. This treatment repairs DNA damage from hydrolysis and
210 oxidative stress, among other mechanisms, that result in deaminated cytosines, nicks, and other
211 DNA damage incurred with age. A previous study found that a different NEB repair kit
212 optimized for formalin-fixed specimens increased the yields of libraries prepared from historical
213 beetle specimens by approximately 30% (Sproul & Maddison, 2017). Following the damage
214 repair treatment, we performed a Qiagen MinElute column cleanup and resuspended the DNA in
215 50 µL of PCR-grade water. Next, we measured the DNA yield and distribution of DNA fragment
216 sizes using the Qubit High Sensitivity dsDNA (Thermo Fisher Scientific) and Agilent
217 Bioanalyzer High-Sensitivity DNA Kit assays, respectively, following the DNA extraction and
218 again following DNA repair and cleanup. We performed the same assays for each extraction
219 negative control to monitor for contamination.

220 We prepared three shotgun sequencing libraries from each DNA extraction and negative
221 control. Each of the three libraries were prepared via a different method: 1) double-stranded,
222 single tube (KAPA HyperPrep Kit); 2) single-stranded, multi-tube (IDT xGen™ ssDNA & Low-
223 Input DNA Library Prep Kit); and 3) single-stranded, single-tube (Claret Bioscience SRSLY®
224 NanoPlus Kit). We largely followed manufacturer protocols with the following modifications:
225 during the KAPA adapter ligation step we ligated 25 µM iTru Stubs (Glenn et al., 2019) to each
226 library molecule. For all cleanups we used a homebrew SPRI bead-solution (Rohland & Reich,
227 2012) and for each cleanup step in the KAPA and IDT protocols we performed 1.2x SPRI
228 concentration cleanups. We indexed each library via amplification with 2.5 µM of a unique pair
229 of iTru5 and iTru7 indexed primers (Glenn et al., 2019) and KAPA HiFi HotStart Uracil+
230 ReadyMix. For amplification we split each adapter-ligated library into two replicates of 25 µL
231 each and ran a nine- to twelve-cycle PCR, depending on input DNA amount and method, on the
232 first replicate; then we estimated the yield of the first replicate via a Qubit High Sensitivity
233 dsDNA assay and ran a 10- to 12-cycle PCR on the second adapter-ligated library replicate. We
234 combined amplified replicates for each library and performed a final SPRI cleanup. Finally, we
235 measured the average molecule size and calculated the concentration of adapter ligated molecules
236 for each sample library via an Agilent Bioanalyzer High-Sensitivity DNA Kit assay and qPCR
237 with the KAPA Library Quantification Kit. We submitted a final library pool to Texas Tech
238 University Center for Biotechnology & Genomics for sequencing. They first checked that
239 libraries were sequencable with an Illumina MiSeq nano run followed by 100 base pair (bp)
240 paired-end sequencing on one Illumina NovaSeq SP flowcell.

241 **2.3. Bioinformatics**

242 We received demultiplexed sequence data as raw fastq files from the sequencing facility
243 and ran FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) to assess quality
244 and adapter contamination by library preparation method. We trimmed 10 bp from the beginning
245 of every IDT library read 2 via Seqtk *trimfq* (https://github.com/lh3/seqtk) to remove a low-
246 complexity polynucleotide tail that facilitates adapter ligation in this method. Duplicate reads
247 resulting from PCR were identified and removed via Super Deduper
248 (https://github.com/s4hts/HTStream). Then we used SeqPrep
249 (https://github.com/jstjohn/SeqPrep) to simultaneously identify adapter contamination and
250 overlapping paired reads, and then trim adapters and merge reads as necessary. We trimmed
251 bases from both read ends via four bp sliding window to a minimum quality of 15 and then
252 removed reads that were less than 30 bp long via Trimmomatic v2.X (Bolger, Lohse, & Usadel,
253 2014). Finally, we removed any remaining reads that were comprised of more than 50% of one
254 nucleotide via remove_low_complex.py (distributed as part of the NF-Polish sequence polishing
255 pipeline described in Irestedt et al. (2022)). We aligned cleaned sequencing reads to the Rufous-
256 bellied Thrush (*T. rufiventris*) reference genome (ASM1318643v1) via BWA 0.7.17 *mem* (Li,
257 2013) and indexed mapped reads with Samtools 1.9 *index* (Danecek et al., 2021; Li et al., 2009).
258 Following sequence cleaning and alignment we evaluated adapter contamination and sequence
259 quality via FastQC. We used MapDamage 2.0 (Ginolhac, Rasmussen, Gilbert, Willerslev, &
260 Orlando, 2011; Jónsson, Ginolhac, Schubert, Johnson, & Orlando, 2013) to estimate the
261 frequency of C to T and G to A misincorporations that result from a transition to uracil via
262 hydrolysis during DNA degradation over time. For each library we output sequencing metrics via
263 Samtools 1.9 *stats* and estimated sequencing efficiency and endogenous DNA content as the
264 proportion of raw and cleaned bases, respectively, that aligned to the reference genome. This

265 measure of endogenous DNA content is likely an underestimate of the true proportion of
266 endogenous DNA in our samples because some endogenous DNA sequences are lost during
267 bioinformatics processing due to the minimal sequence length that can be mapped with a given
268 algorithm and the genetic distance of the sample from the reference genome.

269 **2.4. Analyses**

270 We tested whether there were differences in DNA yield and mean DNA fragment size
271 between different sources (toepad vs. skin clip) via paired-t tests. We evaluated whether DNA
272 source, library preparation method, or an interaction between them resulted in differences in 1)
273 endogenous DNA content, 2) sequencing efficiency, and 3) mean read length via two-way,
274 repeated-measures ANOVAs. For any two-way ANOVA that resulted in a significant interaction,
275 we performed a one-way, repeated measures ANOVA for each method to evaluate whether there
276 were significant differences by DNA source. For any two-way ANOVA that resulted in a
277 significant effect of either independent variable, we performed subsequent pairwise, paired-t tests
278 between all library preparation methods. We accounted for multiple-testing in all post-hoc one-
279 way and paired-t tests by adjusting p-values via the BH method (Benjamini & Hochberg, 1995).

280 We expected that samples with larger mean DNA fragment sizes would also have longer
281 mean read lengths and as a result, greater endogenous DNA content and sequencing efficiency.
282 To test this hypothesis while controlling for any effect of DNA source and library preparation
283 method we defined two linear models for each of the following response variables: endogenous
284 DNA content, sequencing efficiency, and read length. Each null model included the library
285 preparation method and DNA source as fixed effects and sample as a random effect. The
286 alternative model also included mean DNA fragment length as a fixed effect. To test whether

287 mean DNA fragment length had a significant influence on each response variable we performed a
288 likelihood ratio test of the null and alternative model.

289 Finally, we sought to qualitatively evaluate the effect of predigestion on replicate
290 samples. To do so, we plotted the difference in each metric of interest between the replicate
291 samples. All statistical analyses were completed in R v4.1.0 (R Core Team, 2021). ANOVAs
292 and t-tests were conducted with the package *rstatix v0.7.0* (Kassambara, 2021), linear mixed
293 models were built in *lme4* v1.1-27.1 (Bates, Mächler, Bolker, & Walker, 2015), and we used the
294 suite of functions in *tidyverse* v1.3.1 (Wickham et al., 2019) for data parsing, manipulation, and
295 visualization.

296

297 **RESULTS**

298 **3.1. DNA Yield and Size**

299 All 60 DNA extractions were successful in terms of producing measurable amounts of
300 DNA with an average of 589.1 nanograms (ng) per sample and a minimum of 48.6 ng in the skin
301 clip control replicate from robin specimen 162188 (Table 1). All samples retained enough DNA
302 through the DNA repair and cleanup to progress to library preparation by each of the three
303 methods. In general, the DNA repair resulted in an upshift in the distribution of DNA fragment
304 lengths (Figures S1A and S2A). There was no statistical difference in DNA yield and size
305 between toepad and skin clips immediately following extraction or after the DNA repair and
306 cleanup (Figure 2). However, the lack of statistical difference in DNA size is driven by the large
307 variance in skin clips (Table 2). The mean size of DNA extracted from the toepad sample is
308 greater than that of the skin clip sample for all but two specimens: robin specimen 83114 and
309 thrush specimen 66823 (Table 1). For example, these two specimens bias the distribution of the

310 post-repair skin clip mean size (post-repair M = 315.63; median, Mdn = 65.49; SD = 651.92)
311 upwards, but not the toepad mean size (post-repair M = 83.94, Mdn = 83.94, SD = 7.13).

312 **3.2. Endogenous DNA content and sequencing efficiency**

313 Sequencing returned a total of approximately 1.45 billion raw reads and, per library, an
314 average of 11.23 million raw reads (SD = 1.84) and 9.88 million mapped reads (SD = 9.78) per
315 library excluding non-predigested replicates. Detailed sequencing metrics for each library are
316 reported in the supplementary material (Table S1).

317 There was a significant difference across library-preparation methods, but not DNA
318 source in endogenous DNA content and sequencing efficiency with IDT outperforming SRSLY
319 and KAPA in both metrics (Table 3). Similar to the results described above for DNA size, the
320 toepad samples outperform the corresponding skin clip samples except for specimens 83114 and
321 66823 (Figure 3A, 3B) so we summarize the results by method and source (Table 4). The average
322 endogenous DNA content of IDT toepad and skin clip libraries is 88.7% (SD = 0.014) and 81.2%
323 (SD = 0.140) respectively, 0.06% and 1.4% greater than that of KAPA, and 2.4% and 1% greater
324 than that of SRSLY. The average sequencing efficiency of IDT toepad and skin clip libraries is
325 35.9% (SD = 0.045) and 29.8% (SD = 0.245), respectively. In comparison to IDT, KAPA toepad
326 and skin clip libraries are 11% and 1.8% less efficient, respectively, and SRSLY toepad and skin
327 clip libraries are 7.8% and 1.8% less efficient. There was also a significant difference among
328 methods in mean read length with IDT producing longer reads than KAPA and SRSLY with a
329 significant interaction between method and DNA source (Table 3, Table 4, Figure 3C). IDT
330 toepad libraries produced significantly longer reads than IDT skin clip libraries (Table 3, Table 4,
331 Figure 3C).

332 The tests of the influence of input DNA fragment size on sequencing outcomes produced
333 mixed results. Including DNA size significantly improved the fit of the linear models for
334 endogenous DNA content (χ_1^2 = 7.558, p = 0.006), sequencing efficiency (χ_1^2 = 45.771, p <
335 0.001), and read length (χ_1^2 = 25.308, p < 0.001). However, in the linear models including DNA
336 size, it was only a significant predictor of endogenous DNA content and read length based on
337 confidence intervals of the coefficient estimate. One to two toepad samples drive the relationship
338 between DNA size and sequencing efficiency to be negative for the IDT and KAPA methods and
339 also drive the relationship between DNA size and mean read length to be negative for IDT
340 (Figure S3).

341 Two of the 20 libraries exhibited low endogenous DNA content compared to all others.
342 The skin clip library of thrush specimen 83109 exhibited approximately 1% more differences
343 from the reference genome than all other samples (Table 1, Figure S4) in addition to a
344 comparatively low proportion of endogenous DNA content across preparation methods (IDT =
345 48.0%, KAPA = 47.7%, SRSLY = 51.9%). The replicate skin clip library of robin specimen
346 162188 that was not subjected to predigestion also had a low proportion of endogenous DNA
347 content across preparation methods (IDT = 17.4%, KAPA = 33.4%, SRSLY = 41.5%), but it was
348 similar to all other libraries in itsgenetic distance from the reference (Figure S4). The high
349 performing skin clips samples from specimens 83114 and 66823 that biased the skin clip
350 averages of most metrics upward also had similar genetic distances to the reference (Table 1,
351 Figure S4).

352 The influence of sample predigestion was unclear (Table 1, Figure S5). The differences in
353 DNA yield, mean DNA size, and endogenous DNA content were inconsistent between the

354 control and predigested replicates from robin 162188 and thrush 83114. The difference between
355 the replicates in sequencing efficiency and mean read length were marginal.

356

357 **4. Discussion**

358 **4.1. WGS of hDNA from 100-year-old bird study skins**

359 We have demonstrated via shallow sequencing of 60 hDNA libraries that ssDNA library
360 preparation methods outperform dsDNA methods in sequencing efficiency and, to a lesser extent,
361 in returning endogenous DNA content from WGS ~100-year-old bird specimens. In contrast to
362 our predictions, the IDT multi-tube, ssDNA method outperformed the Claret Biosciences single-
363 tube, ssDNA method and we discuss possible explanations below. We also confirm previous
364 research that suggested that toepads provide consistently larger DNA fragments (Tsai et al. 2020)
365 and demonstrate that hDNA from toepads rather than skin clips is a better source for WGS. We
366 show that, though skin clips may sometimes outperform toepads for a given specimen, toepads
367 have less variance and therefore less unexpected sequencing outcomes. Altogether, we've shown
368 that toepads are a better source of DNA and ssDNA library preparations are a better method for
369 collecting WGS from 100-year old bird specimens. Below we elaborate further on the nuances of
370 our findings and conclude with broader implications for WGS of historical DNA from museum
371 specimens in natural history collections.

372 **4.2. Library preparation method and DNA source influenced sequencing**

373 Library preparation method influenced all key metrics; but contrary to our predictions, the
374 SRSLY ssDNA, single-tube method did not outperform the other two methods. Instead, the IDT
375 ssDNA, multi-tube method resulted in greater endogenous DNA content, sequencing efficiency,

376 and read lengths than either of the other methods (Figure 3, Table 3). The margin of difference
377 between IDT and the other methods was much greater for sequencing efficiency and average read
378 length than endogenous DNA content (Table 4), suggesting that IDT produced more complex
379 libraries. The only other study to compare ssDNA and dsDNA methods for shotgun sequencing
380 of historical specimens prepared libraries from beetle specimens of various ages using the same
381 ssDNA, multi-tube method that we implemented and a different dsDNA, single-tube method
382 (Sproul & Maddison, 2017). Those results showed no difference in endogenous DNA content
383 between methods, and that ssDNA libraries maintained more sequencing reads through quality
384 filtering and trimming. We find this consistent with our results and suspect that controlling for
385 taxonomy and specimen age in our experimental design facilitated detecting the small difference
386 that library preparation method made in endogenous DNA content. That IDT outperformed
387 SRSLY makes some sense given that SRSLY was originally developed for cell-free DNA which
388 averages 30 bp long (Troll et al., 2019); though, the commercial kit provides several versions of
389 the protocol optimized for different purposes, and we implemented the version for moderate
390 length DNA inserts, less than 200 bp. That protocol for moderate length DNA inserts includes
391 two-sided SPRI cleanups following adapter ligation and indexing PCR, as compared to the IDT
392 ssDNA method which uses single-sided cleanups. We suspect that the two-sided cleanups in
393 SRSLY limited conversion of DNA molecules on the larger end of the DNA size distribution into
394 library molecules (Figures S1, S2). This is consistent with SRSLY producing shorter average
395 read lengths than IDT. Still, SRSLY outperformed the KAPA dsDNA method overall in
396 sequencing efficiency and outperformed IDT in sequencing efficiency for skin clips in a few
397 specimens (Figure 3A). It is possible that SRSLY may be the better method for samples that are
398 more degraded as a result of age or DNA source. More recently another ssDNA single-tube

399 method that builds upon SRSLY was developed specifically for ancient DNA samples (Kapp et
400 al., 2021); though the nonproprietary status limits ease of implementation. It may be worthwhile
401 to optimize the SRSLY cleanup steps to maximize conversion of the largest DNA fragments as
402 the IDT method costs 1.89× more than SRSLY per reaction.

403 Most statistical tests of the effect of DNA source on our metrics of interest were not
404 significant, though toepads seem to perform better for WGS when considering the influence of
405 the large variance in the skin clip metrics. Toepads had much smaller variance in DNA yield,
406 DNA size, endogenous DNA content, sequencing efficiency, and average read length than skin
407 clips. For six of the eight specimens, toepads clearly provided greater endogenous DNA content,
408 sequencing efficiency, and average read length than the corresponding skin clip. In general, this
409 result is reflected by the mean, and more so the median values, of these metrics for toepads as
410 compared to skin clips (Table 2, Table 4). That phenol chloroform extraction of toepads does not
411 yield more DNA but does result in longer DNA fragments than that of skin clips supports
412 previous research (Tsai et al., 2020). Our results are also in line with previous studies based on
413 five fluid-preserved garter snake specimens (Zacho et al., 2021) and three dried mammal skins
414 from different species (McDonough et al., 2018) that showed that different DNA sources have
415 differences in endogenous DNA content. It is possible that hDNA sampled from bird specimen
416 toepads produces better WGS data than that of skin clips because of their different structural
417 makeups. The keratinized, scaly skin of bird feet may provide a better environmental barrier to
418 water—which promotes DNA degradation via hydrolysis and also overall tissue degradation—
419 and to invading microbes that would increase exogenous DNA. Toepad cells may also have lower
420 innate water content due to desiccation during keratinization (Bengtsson et al., 2012). The role of
421 the keratin structure in maintaining better DNA for WGS is supported by the work of

422 McDonough and colleagues (2018) which showed that of bone, skin, and claw samples from
423 dried mammal specimens, claw samples had the highest or near highest proportion of endogenous
424 DNA and in a qPCR analysis, the highest copy number of nuclear genomic markers.

425 **4.3. Predigestion, high performing samples, and potential contamination**

426 Interpretation of the effect of predigestion on reducing exogenous DNA content and
427 increasing sequencing efficiency is limited by the small sample size for which we sequenced
428 replicate predigested and control samples. The lack of clear signal of predigestion effect in DNA
429 yield and size is unsurprising given the variation across all samples (Figure 2). Moreover, the
430 lack of any potential signal is unsurprising given that of the eight samples included as replicates
431 to evaluate predigestion, three were exceptions to the general trends identified by the larger
432 study. Both skin clips from thrush 83114 were among the few skin clip samples that performed
433 uncharacteristically better than all other samples and the control skin clip replicate from robin
434 from 162188 resulted in the lowest endogenous DNA content of all libraries (Table 1) indicating
435 high levels of exogenous DNA or contamination. Notably, the predigested skin clip replicate
436 sample from robin 162188 did not show signs of contamination suggesting that predigestion may
437 have reduced exogenous DNA in only two minutes of predigestion time. In contrast, the skin clip
438 from thrush specimen 83109 was one of four samples with the shortest, one-minute predigestion
439 and also showed a clearer signal of contamination based on low endogenous DNA content and a
440 larger genetic distance from the reference than other samples (Table 1, Figure S4). Finally, it is
441 likely that predigestion reduced total DNA yield, though enough DNA remained for all 20
442 samples to prepare three successfully sequenced libraries. Altogether we suggest this preliminary
443 investigation is a promising avenue to maximize endogenous hDNA from museum bird
444 specimens for WGS and warrants further research.

445 The primary source of the larger variance in most metrics for skin clips were two
446 specimens for which the skin clips not only outperformed the corresponding toepad from the
447 same specimen but all other samples. Robin 66823 and thrush 83114 returned the first and second
448 largest DNA yield, DNA size, endogenous DNA content, average read length, and highest
449 sequencing efficiency. Notably, thrush 83114 was one of the two specimens included in the
450 predigestion study, and both the predigested and non-predigested skin clips were high performing
451 samples and had consistent values across metrics. There are multiple explanations for why the
452 samples outperformed all others, the first being contamination. However, neither of these samples
453 show clear signs of contamination like the lower endogenous DNA content or large genetic
454 distance from the reference genome mentioned for the skin clip from thrush 83109. The only
455 explanation of contamination we consider plausible is contamination by modern DNA from the
456 same or a closely related species, perhaps from a more recently collected specimen in the same
457 drawer. This possibility highlights the need for stringent sampling procedures during sample
458 collection from museum specimens for hDNA purposes. Importantly, a population genomics
459 study involving deeper sequencing of these samples would allow assembly of mitochondrial
460 genomes that would enable identification of multiple individuals within one sequencing library.
461 Another possible explanation for the higher performance of these skin clips is that these
462 specimens received different treatments at the time of collection than the other specimens in the
463 study. At the time of collection it was common practice to treat bird (and mammal) skins with
464 arsenic-containing solutions for tanning as well as protection from pests (Marte, Péquignot, &
465 Von Endt, 2006), and arsenic has been demonstrated as a DNA polymerase inhibitor (Töpfer et
466 al., 2011). It is possible that the specimens with high performing skin clips were accidentally
467 skipped in some treatment that ultimately promoted DNA damage in the other skins. The last

468 and, in our opinion, most likely possibility is that this variation represents real variation in DNA
469 quality between specimens. Such large variations are not uncommon when working with hDNA
470 (e.g. McDonough et al., 2018) and ancient DNA (e.g. Wales et al., 2015) and highlights the value
471 of identifying methods and DNA sources that can consistently return an expected amount of
472 WGS data like we have found for toepads and the IDT ssDNA, single-tube method.
473
474 **5. Conclusion**

475 We have shown that for 100-year old museum bird study skin specimens, of those
476 combinations we tested, the combination of toepads and ssDNA library preparation, in this case
477 the IDT xGen™ ssDNA & Low-Input DNA Library Prep Kit, provide the best WGS data. Our
478 results regarding toepads, in combination with previous studies of endogenous DNA content in
479 other taxa, can be reasonably extended beyond birds to suggest that keratinous sources of hDNA
480 may be the best source for WGS and should motivate additional investigations of this hypothesis.
481 We also have shown that when comparing WGS from ssDNA and dsDNA methods, ssDNA
482 methods provide a larger increase in sequencing efficiency than endogenous DNA content,
483 suggesting that they successfully convert more hDNA molecules into sequenceable library
484 molecules and likely lead to more complex libraries better suited for WGS at the depth required
485 for population genomic studies. Further study of the impact of library preparation method on
486 sequencing efficiency that controls for variation among specimens and also evaluates the role of
487 age of the specimen is necessary to identify the threshold at which an ssDNA method is or is not
488 warranted. Also, it may be worthwhile to attempt to further optimize SRSLY cleanups to
489 minimize bias against converting larger fragment hDNA molecules into library molecules as
490 SRSLY is currently ~89% less expensive per reaction than the IDT method. Finally, our

inclusion of a predigestion step to reduce external exogenous DNA did not yield straightforward results, though it did provide some indication that it limited contamination in one of four samples for which we made a direct comparison. Importantly we showed that a brief (less than 15 minute), gentle (37C° incubation) predigestion step does not preclude successful library construction, and thus we will cautiously include this step in our own protocols moving forward. Altogether this study identifies how to maximize WGS data collected from 100-year old bird specimens and provides some general insights on how to increase the quality and quantity of WGS data recovered from hDNA of museum specimens overall.

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Conflict of interest statement

513 IDT has been a corporate sponsor of the Field Museum of Natural History as recently as 2020,

514 but no funds from IDT were used to directly support the research that is the subject of the

515 publication.

516

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667

668 **Data Accessibility and Benefit-Sharing**

669 Raw sequence reads will be deposited in NCBI Sequence Read Archive (SRA) (Settlecowski et
670 al. 2022). Code used in data analysis is available at: github.com/amiesett/WGS-hDNA-birds.
671 Benefit sharing: We are sharing all research benefits of this project by providing raw data,
672 bioinformatic scripts, and results in public databases. No monetary benefits are expected from
673 these research results.

674

675 **Author Contributions**

676 All authors contributed to project design and manuscript completion. JDM and BDM acquired
677 funding. AES performed lab work and data analysis, with support from JDM, and wrote the first
678 draft of the manuscript.

679 **Table 1.** Summary of extracted DNA and sequencing results for each sample.

Species	Sample	Post-extraction		Post-repair		Method	Type	Raw reads (millions)	Clean reads (millions)	Mapped reads (millions)	Mean read length	Dist.to ref.	Endog. DNA content	Seq. eff.
		Yield (ng/mg)	Mean DNA size (bp)	Yield (ng/mg)	Mean DNA size (bp)									
T. abyssinicus	83107					IDT	skin	10.54	3.38	2.96	71.99	0.018	0.851	0.193
T. abyssinicus	83107	400.03	57.36	136.10	68.02	KAPA	skin	60.29	15.17	12.45	59.75	0.014	0.820	0.119
T. abyssinicus	83107					SRSLY	skin	12.85	5.23	4.64	75.14	0.015	0.822	0.267
T. abyssinicus	83107					IDT	toepad	12.62	5.51	5.12	97.84	0.017	0.900	0.382
T. abyssinicus	83107	698.97	65.75	332.89	83.90	KAPA	toepad	85.06	28.05	25.05	78.48	0.012	0.889	0.224
T. abyssinicus	83107					SRSLY	toepad	8.81	3.36	2.89	67.02	0.017	0.853	0.213
T. abyssinicus	83109					IDT	skin	11.26	3.29	1.69	62.71	0.035	0.480	0.086
T. abyssinicus	83109	376.78	57.93	73.28	61.74	KAPA	skin	50.69	12.09	5.77	53.49	0.027	0.477	0.057
T. abyssinicus	83109					SRSLY	skin	10.08	3.74	2.13	65.56	0.030	0.519	0.119
T. abyssinicus	83109					IDT	toepad	10.95	4.56	4.18	88.02	0.018	0.896	0.323
T. abyssinicus	83109	858.85	66.68	338.13	85.08	KAPA	toepad	63.05	21.06	18.86	79.73	0.013	0.890	0.232
T. abyssinicus	83109					SRSLY	toepad	9.29	3.46	2.97	66.34	0.017	0.853	0.206
T. abyssinicus	83114					IDT	skin	46.55	32.78	30.90	100.17	0.009	0.916	0.640
T. abyssinicus	83114	1350.36	78.71	576.64	218.79	KAPA	skin	32.03	15.21	14.27	109.38	0.013	0.919	0.472
T. abyssinicus	83114					SRSLY	skin	9.56	5.06	4.69	104.24	0.017	0.903	0.503
T. abyssinicus	83114					IDT	toepad	24.48	10.02	8.80	89.44	0.016	0.856	0.309
T. abyssinicus	83114	800.00	53.96	55.17	98.74	KAPA	toepad	45.38	15.44	13.93	83.94	0.015	0.889	0.250
T. abyssinicus	83114					SRSLY	toepad	7.62	3.34	3.01	85.32	0.018	0.874	0.332
T. abyssinicus	83114†					IDT	skin	42.34	28.30	26.73	101.24	0.009	0.917	0.615
T. abyssinicus	83114†	4254.55	96.90	1872.73	211.98	KAPA	skin	34.26	15.74	14.75	107.05	0.013	0.919	0.447
T. abyssinicus	83114†					SRSLY	skin	9.35	4.80	4.44	101.67	0.017	0.902	0.474
T. abyssinicus	83114†					IDT	toepad	28.09	11.70	10.77	99.56	0.015	0.891	0.368
T. abyssinicus	83114†	1901.96	56.07	202.94	107.53	KAPA	toepad	62.51	22.23	20.22	84.52	0.013	0.899	0.266
T. abyssinicus	83114†					SRSLY	toepad	14.23	6.31	5.75	85.21	0.016	0.876	0.344

Species	Sample	Post-extraction		Post-repair		Method	Type	Raw reads (millions)	Clean reads (millions)	Mapped reads (millions)	Mean read length	Dist.to ref.	Endog. DNA content	Seq. eff.
		Yield (ng/mg)	Mean DNA size (bp)	Yield (ng/mg)	Mean DNA size (bp)									
T. abyssinicus	83115					IDT	skin	14.52	4.28	3.80	78.74	0.017	0.858	0.197
T. abyssinicus	83115	942.98	56.71	526.94	69.01	KAPA	skin	80.02	19.29	16.20	59.36	0.014	0.843	0.117
T. abyssinicus	83115					SRSLY	skin	11.55	3.30	2.65	54.80	0.015	0.803	0.120
T. abyssinicus	83115					IDT	toepad	43.02	17.60	16.22	94.73	0.013	0.893	0.345
T. abyssinicus	83115	564.00	59.43	217.47	83.13	KAPA	toepad	14.25	4.92	4.37	78.08	0.018	0.881	0.232
T. abyssinicus	83115					SRSLY	toepad	12.70	5.55	5.02	81.83	0.015	0.862	0.324
T. migratorius	162188					IDT	skin	15.77	4.77	3.97	60.97	0.018	0.822	0.146
T. migratorius	162188	375.19	52.58	597.41	58.71	KAPA	skin	2.14	0.74	0.60	60.52	0.018	0.803	0.162
T. migratorius	162188					SRSLY	skin	9.77	3.52	2.94	61.09	0.018	0.823	0.176
T. migratorius	162188					IDT	toepad	21.99	9.50	8.58	95.19	0.016	0.879	0.354
T. migratorius	162188	501.80	57.60	436.09	76.70	KAPA	toepad	10.85	4.53	3.96	78.38	0.019	0.866	0.275
T. migratorius	162188					SRSLY	toepad	8.36	3.60	3.15	76.19	0.019	0.868	0.279
T. migratorius	162188†					IDT	skin	6.56	4.11	0.86	80.05	0.020	0.174	0.100
T. migratorius	162188†	173.57	54.16	47.37	62.90	KAPA	skin	14.98	6.38	2.90	59.05	0.018	0.334	0.110
T. migratorius	162188†					SRSLY	skin	8.85	3.79	2.07	63.26	0.017	0.415	0.135
T. migratorius	162188†					IDT	toepad	9.14	3.95	3.55	89.65	0.019	0.875	0.333
T. migratorius	162188†	231.03	59.13	98.59	77.89	KAPA	toepad	31.17	12.31	10.86	78.33	0.017	0.873	0.263
T. migratorius	162188†					SRSLY	toepad	10.20	4.42	3.92	78.11	0.019	0.870	0.293
T. migratorius	175413					IDT	skin	8.18	2.72	2.31	78.30	0.019	0.807	0.208
T. migratorius	175413	197.25	50.97	59.33	62.37	KAPA	skin	15.67	4.99	3.86	60.91	0.018	0.770	0.144
T. migratorius	175413					SRSLY	skin	11.30	4.57	3.84	70.22	0.017	0.792	0.227
T. migratorius	175413					IDT	toepad	12.34	6.03	5.66	100.66	0.017	0.894	0.442
T. migratorius	175413	501.09	56.22	219.23	86.23	KAPA	toepad	32.87	13.07	11.71	81.68	0.016	0.882	0.280
T. migratorius	175413					SRSLY	toepad	7.51	3.53	3.24	86.20	0.017	0.868	0.372

Species	Sample	Post-extraction		Post-repair		Method	Type	Raw reads (millions)	Clean reads (millions)	Mapped reads (millions)	Mean read length	Dist.to ref.	Endog. DNA content	Seq. eff.
		Yield (ng/mg)	Mean DNA size (bp)	Yield (ng/mg)	Mean DNA size (bp)									
T. migratorius	175421					IDT	skin	6.68	2.31	1.97	64.10	0.019	0.840	0.180
T. migratorius	175421	463.63	52.32	111.02	62.95	KAPA	skin	56.18	17.57	14.70	63.39	0.015	0.833	0.159
T. migratorius	175421					SRSLY	skin	14.82	5.83	5.05	67.82	0.017	0.838	0.222
T. migratorius	175421					IDT	toepad	21.44	9.64	8.98	98.88	0.016	0.891	0.398
T. migratorius	175421	838.10	60.18	234.00	82.44	KAPA	toepad	51.02	19.65	17.51	78.30	0.015	0.879	0.259
T. migratorius	175421					SRSLY	toepad	7.29	3.33	3.03	83.03	0.018	0.868	0.343
T. migratorius	66823					IDT	skin	60.98	51.78	49.16	95.45	0.007	0.920	0.736
T. migratorius	66823	1150.86	387.24	950.45	1923.43	KAPA	skin	35.56	24.12	22.91	106.55	0.010	0.921	0.659
T. migratorius	66823					SRSLY	skin	9.88	6.23	5.87	105.58	0.017	0.917	0.605
T. migratorius	66823					IDT	toepad	5.13	2.24	2.03	85.15	0.020	0.885	0.321
T. migratorius	66823	223.70	53.26	47.36	75.28	KAPA	toepad	50.77	19.75	17.26	73.40	0.015	0.871	0.241
T. migratorius	66823					SRSLY	toepad	11.86	4.31	3.63	61.16	0.018	0.855	0.182

[†]Extraction replicate not subjected to predigestion

Table 2. Summary (median, mean, standard deviation) of DNA yield and mean DNA size for predigested toepads and skin clips.

	Yield (ng/mg)				Mean size (bp)			
	Post-extraction		Post-repair		Post-extraction		Post-repair	
Source	Mdn	M (SD)	Mdn	M (SD)	Mdn	M (SD)	Mdn	M (SD)
toepad (n=8)	631.49	623.314 (217.606)	226.62	235.042 (135.626)	58.52	59.136 (4.985)	83.51	83.937 (7.132)
skin (n=8)	431.83	657.135 (427.479)	331.52	378.896 (329.864)	57.04	99.227 (116.707)	65.49	315.628 (651.918)

Table 3. Summary of the statistical analyses of endogenous DNA content, sequencing efficiency, and mean read length, and their results. All the p-values presented for t-tests are corrected for multiple testing via the Benjamini and Hochberg (1995) method.

Response variable	Test	DF	Test-statistic	p-value
Endogenous DNA content	ANOVA - source	1,7	0.066	0.805
	ANOVA - method	2,14	6.241	0.012
	ANOVA - source:method	2,14	0.739	0.496
	T-test - IDT vs. KAPA	15	6.631	< 0.001
	T-test - IDT vs. SRSLY	15	2.476	0.026
	T-test - KAPA vs. SRSLY	15	-2.570	0.026
Sequencing efficiency	ANOVA - source	1,7	0.066	0.805
	ANOVA - method	2,14	6.241	0.012
	ANOVA - source:method	2,14	0.739	0.496
	T-test - IDT vs. KAPA	15	2.492	0.037
	T-test - IDT vs. SRSLY	15	2.765	0.037
	T-test - KAPA vs. SRSLY	15	1.283	0.219
Mean read length (bp)	ANOVA - source	1,7	1.333	0.286
	ANOVA - method	2,14	14.181	< 0.001
	ANOVA - source:method	2,14	6.778	0.009
	T-test - IDT vs. KAPA	15	3.879	0.004
	T-test - IDT vs. SRSLY	15	3.001	0.013
	T-test - KAPA vs. SRSLY	15	-0.184	0.856
Mean read length (bp) - IDT	ANOVA - source	1,7	7.179	0.096
Mean read length (bp) - KAPA	ANOVA - source	1,7	0.818	1.0000
Mean read length (bp) - SRSLY	ANOVA - source	1,7	0.002	1.0000

Table 4. Summary (median, mean, standard deviation) of sequencing metrics for predigested toepads and skin clips.

	Mean read length (bp)						Endogenous DNA content						Sequencing efficiency					
	IDT		KAPA		SRSLY		IDT		KAPA		SRSLY		IDT		KAPA		SRSLY	
Source	**Mdn**	**M (SD)**	**Mdn**	**M (SD)**	**Mdn**	**M (SD)**	**Mdn**	**M (SD)**	**Mdn**	**M (SD)**	**Mdn**	**M (SD)**	**Mdn**	**M (SD)**	**Mdn**	**M (SD)**	**Mdn**	**M (SD)**
toepad (n=8)	94.959	93.74 (5.598)	78.428	78.998 (3.059)	79.005	75.883 (9.771)	0.892	0.887 (0.014)	0.882	0.881 (0.009)	0.865	0.863 (0.008)	0.35	0.359 (0.045)	0.245	0.249 (0.021)	0.302	0.281 (0.072)
skin (n=8)	75.145	76.554 (14.799)	60.717	71.669 (22.587)	69.018	75.557 (19.097)	0.846	0.812 (0.14)	0.827	0.798 (0.14)	0.823	0.802 (0.123)	0.195	0.298 (0.245)	0.151	0.236 (0.212)	0.225	0.28 (0.179)

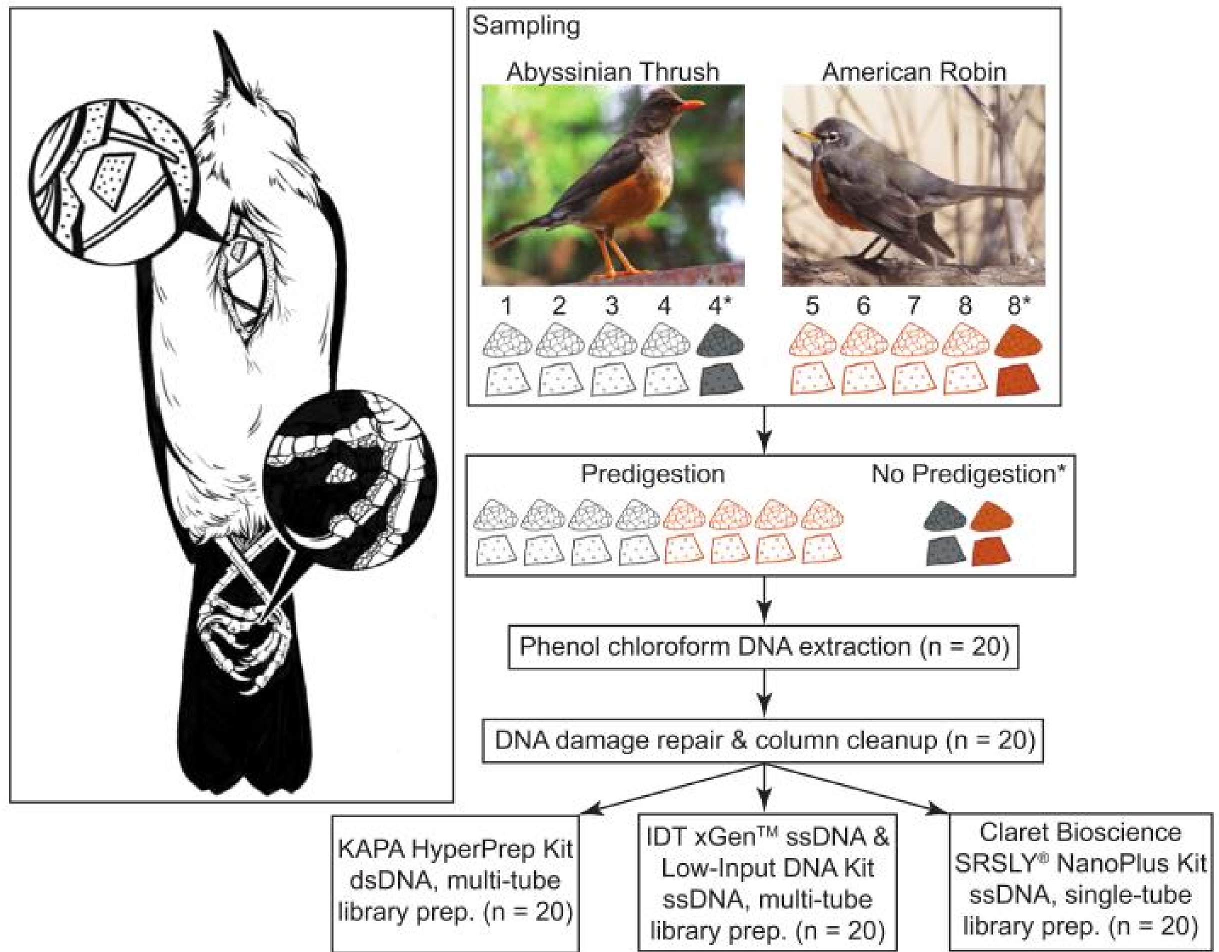

Figure 1. Study sampling design. The illustration on the left shows where incision-line skin clips and toepads are sampled from the specimens. The flowchart on the right is an overview of the experimental design and the laboratory procedures. Toepads and skin clips are depicted as triangular and trapezoidal shapes, respectively. Photographs by JDM and illustrations by L. Nassef.

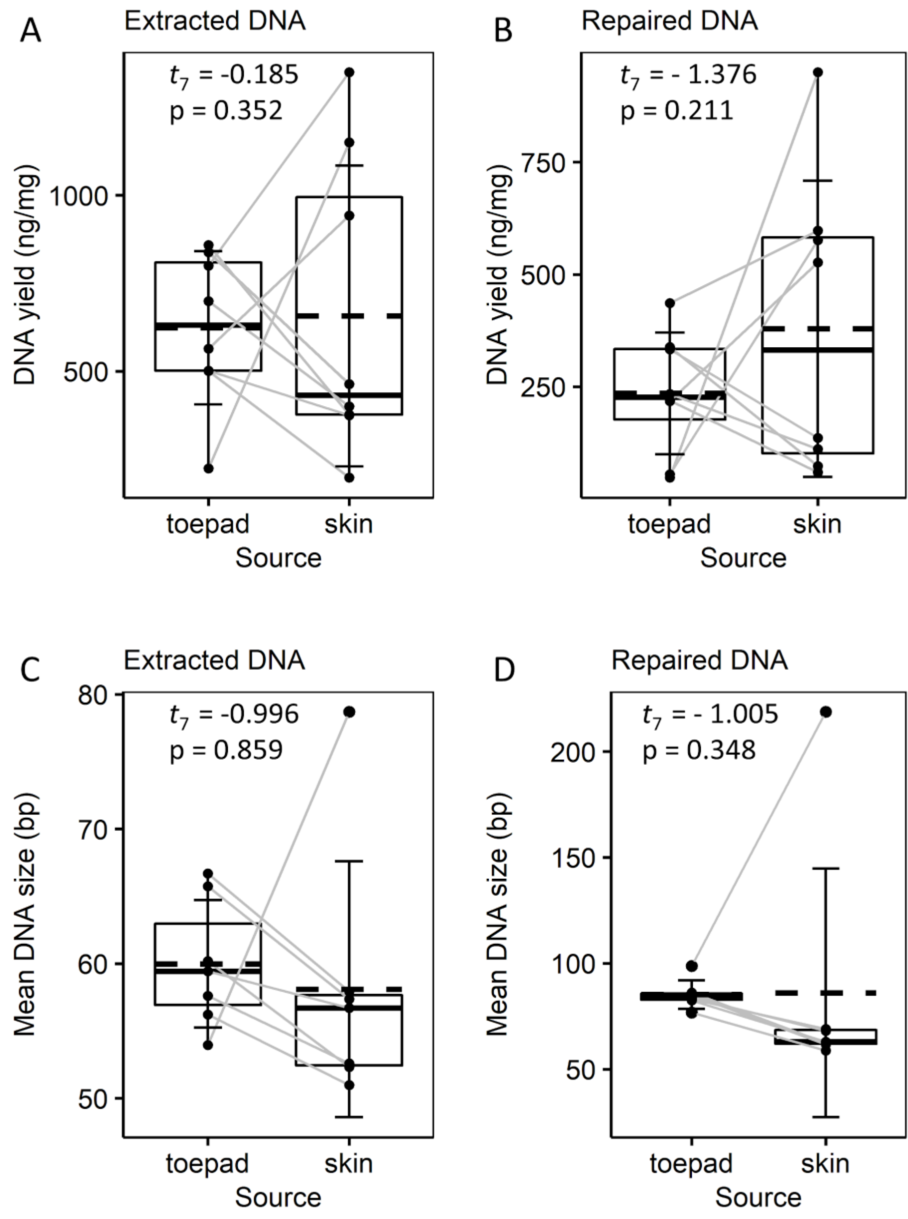

Figure 2. Source tissue impacts on DNA quantity and quality. For each sample, DNA yield (ng/mg) measured A) following DNA extraction and B) following DNA repair and cleanup is plotted by DNA source (toepad or skin clip). For each sample, mean DNA fragment size (bp) C) following DNA extraction and D) following DNA repair are plotted by DNA source. Samples from robin specimen 66823 are not plotted in C and D because the mean DNA size of its skin clip (Table 1) limits visualization of the variation in the size of the other samples. Gray lines connect toepad and skin clip data points from the same specimen. Summary statistics are also plotted by source: the mean and median are represented by dashed and solid lines, respectively, the upper and lower limits of the boxes represent the 75% and 25% quantiles, and the error bars represent the standard deviation.

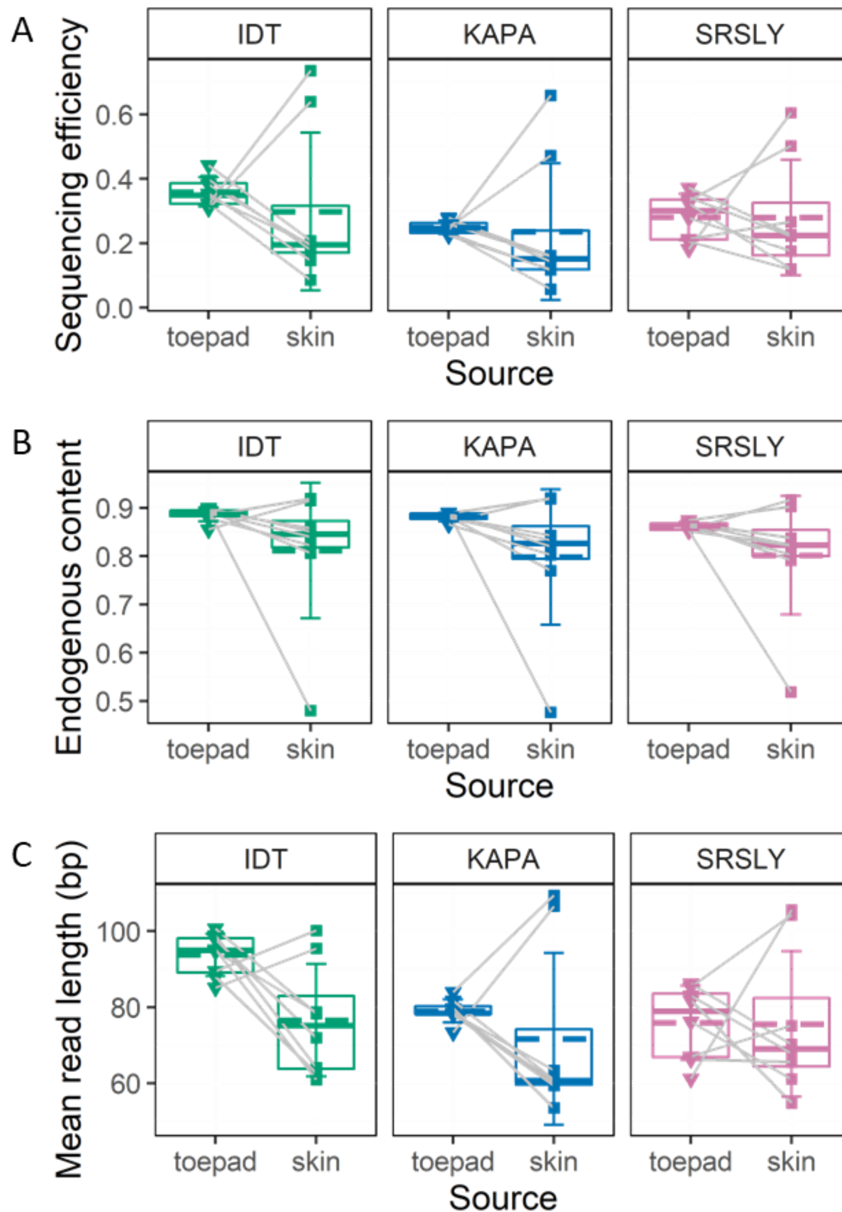

Figure 3. Association of sequencing library type and sequence data characteristics. For each library, A) sequencing efficiency, or the proportion of raw bases that uniquely mapped to the reference genome, B), endogenous DNA content, or the proportion of cleaned bases that uniquely mapped to the reference genome, and C) the mean read length (bp) is plotted by DNA source (toepad or skin clip). Gray lines connect toepad and skin clip data points from the same specimen. Summary statistics are also plotted by method and source: the mean and median are represented by dashed and solid lines, respectively, the upper and lower limits of the boxes represent the 75% and 25% quantiles, and the error bars represent the standard deviation.