

Preserving avian blood and DNA sampled in the wild: a survey of personal experiences

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Abstract

Collecting and storing biological material from wild animals in a way that does not deteriorate data quality for analyses using DNA is instrumental for research in ecology and evolution. Our aims were to collect methods commonly used by researchers for the field collection and long-term storage of blood samples and DNA extracts from wild birds and gather reports on their effectiveness. Personal experiences were collected with an online survey targeted specifically at researchers sampling wild birds. Many researchers experienced problems with blood sample storage but not with DNA extract storage. Storage issues generated problems with obtaining adequate DNA quality and sufficient DNA quantity for the targeted molecular analyses, but were not related to season of blood sampling, access to equipment, transporting samples, temperature and method of blood storage. Final DNA quality and quantity were also not affected by storage time before DNA extraction or the methods used to extract DNA. We discuss practical aspects of field collection and storage and provide some general recommendations, with a list of pros and cons of different preservation methods of avian blood samples and DNA extracts.

Keywords

birds, blood sampling, long-term storage, DNA, molecular ecology

Introduction

Ecological and evolutionary processes shaping natural animal populations typically occur over multiple years or decades at least. Consequently, research in ecology and evolutionary biology often requires data extending over long periods of time and the accumulation of records of individuals over their entire lifespans (Clutton-Brock and Sheldon, 2010). Long-term individual-based studies have proven extremely valuable in gaining insight into the demographic and life-history traits of wild populations, for instance by making sense of aspects of breeding, survival, mate choice and lifetime reproduction (Bouwhuis, 2018; Mills et al., 2016). Over the past three decades, molecular tools have become easier to use and widely applied to multiple disciplines such as population ecology/biology (Deyoung & Honeycutt, 2005), biogeography (Riddle et al., 2008), conservation genetics (Primmer, 2009) or behavioural ecology (Bengston et al., 2018) and have led to the emergence of new fields such as landscape genetics, molecular quantitative genetics and population genomics (Black IV et al., 2001; Jensen et al., 2014; Manel et al., 2002). These techniques add to the size and richness of biological archives in recent years, spanning several decades and thousands of individuals. The wide array of cost-efficient molecular tools available nowadays and the increasing computational power able to handle large amounts of data allow researchers to reliably perform a variety of analyses on biological material from wild populations. However, sample storage conditions remain a key issue, and can limit the ability to generate high quality genetic data from animal blood or other tissues. At the time of logistical planning of a long-term study, an informed decision concerning sample preservation has to be made. Inadequate preservation might compromise sample quality and research scope, for example leading to the degradation of nucleic acids (Conrad et al., 2000; Kilpatrick, 2002; Seutin et al., 1991; Zimmermann et al., 2008). In this light, *storage method* and *temperature* are fundamental aspects of sample preservation. Because of their interactive influence on final sample quality, their effects – and limitations – have to be simultaneously taken into consideration before undertaking sampling for a specific project and in light of future applications that may arise with the progression of the study.

Storage method

Direct sample freezing is viewed as the method of choice for long-term storage, since enzymatic and other chemical activities decrease with lower temperatures (Wong et al., 2012). However, other storage methods which require a liquid preservative or physical support (e.g. paper-based substrate) might provide advantages over freezing, such as a reduction in space and energy consumption, no power outage risks, lower long-term costs, and easier transfer and shipping of samples. For example, blood can be stored in either 95-100% ethanol, in lysis buffers such as Queen's buffer and Longmire's buffer, or dried on filter paper, such as FTA® cards (Longmire et al., 1997; Seutin et al., 1991; Smith and Burgoyne, 2004). Such storage methods can differentially impact molecular assays that will be performed on the biological samples. For instance, in a PCR diagnostics study for avian and human malaria, lower accuracy of the test was associated with samples stored in a lysis buffer (containing sodium dodecyl sulfate or SDS) compared to a buffer lacking SDS (Freed and Cann, 2006). SDS may have been the cause since it releases endonucleases and creates extra cellular debris. There is also evidence that relative telomere length (RTL) measurements differ significantly depending on storage method: Reichert et al. (2017) showed how RTL of samples stored on FTA® cards at room temperature was significantly shorter than in samples preserved as frozen whole blood or frozen DNA. Different storage methods have also been shown to affect stable isotope signatures: in a study by Bugoni et al. (2008), a significant enrichment in $\delta^{13}\text{C}$ was detected in ethanol-preserved blood samples in comparison with dry and frozen blood samples. Samples preserved in ethanol also showed variation in $\delta^{13}\text{C}$ values according to the brand and even the batch of preservative.

Storage temperature

When directly freezing samples, temperatures of -80°C , or as low as possible, are recommended to maximise DNA preservation (Jackson et al., 2012; Wong et al., 2012). In case of biological material collected from birds, storage at -20°C was deemed sufficient to prevent DNA degradation, since avian red blood cell nuclei were considered metabolically inactive (Seutin et al., 1991). However, recent evidence (Stier et al., 2013) reports that avian blood cells do have

functional mitochondria involved in respiratory cellular metabolism. Consequently, lower storage temperature (e.g. -80°C) may still offer better preservation conditions than freezing at -20°C. When deep-cold storage is not feasible or practical, ethanol, lysis buffer and filter paper form a valuable alternative, since they are routinely stored at room temperature (Kilpatrick, 2002; Seutin et al., 1991; Smith and Burgoyne, 2004). However, multiple studies have reported poor stability of DNA extracted from whole blood samples stored at room temperature and better DNA yields from samples kept at +4°C or lower (Madisen et al., 1987; Richardson et al., 2006; Visvikis et al., 1998). Moreover, filter paper is known to generate different DNA yields depending on storage temperature: Hollegaard et al. (2011) showed how storing dried blood spots (DBS) samples, also known as Guthrie cards, at +4°C negatively affected DNA concentration, which increased when samples were stored at -20°C. Mei et al. (2011) reported similar results on DBS tested for Toxo-specific immunoglobulin-M: reduced recovery was observed in DBS stored at room temperature compared with specimens stored at -20°C.

Additional factors influencing sample stability

Technical assessments of specific protocols (Kilpatrick 2002; Michaud and Foran, 2011; Seutin et al., 1991; Zimmermann et al., 2008) have considerable value but may be limited due to specific focus and carefully controlled conditions. The diversity of conditions under which samples are collected and the variety of purposes for which they have been used across many researchers may reveal additional limitations. Indeed, some specific constraints of field collection and long-term storage of biological material might have an impact on sample quality and research outcomes that is not necessarily captured in laboratory assessments of DNA degradation. For instance, collecting samples in spring or summer means that biological material may be challenged by high temperatures, with negative consequences on DNA quality/integrity. Access to equipment in the field, such as a fridge or a freezer, might have a positive effect, ensuring sample stability before the long-term storage in the laboratory. Different ways of transferring samples from the field to the laboratory might also affect sample integrity if samples are exposed to high temperatures during transportation or experience delays in shipping. Moreover, storing

samples as blood or DNA extracts might have different outcomes on DNA integrity in the long run, so during logistical planning it might be necessary to take into consideration storage time before DNA extraction. In this light, we asked scientists directly about what experiences have informed their decisions regarding the long-term storage of blood and DNA, and about their perceived assessment of the efficacy of their storage procedures. Importantly, personal and first-hand experiences may shed light on how often problems that arise from sample collection or storage can affect DNA quality, thus impacting the final sample size and the quality of published research.

Here, we present the results from an online survey designed for researchers involved in the collection and storage of blood and DNA from wild birds. We circulated the survey among ecologists and field biologists in order to (i) review practices commonly used for field collection and storage of avian blood and DNA extracts, (ii) assess if any procedural or methodological gaps exist in current knowledge of avian blood and DNA storage and (iii) improve existing guidelines for the long-term storage of avian blood and DNA.

Methods

Survey

An online survey entitled “Preserving avian DNA from the wild: Your experience of blood sampling, DNA extraction and storage” was created on the Survey Monkey platform (Supporting Information 1). It comprised 3 sections (blood sample collection, blood storage, and DNA extraction and storage) with 52 questions, mostly multiple choice. 14 of these were open questions, where more detailed answers were required. Out of the 52 questions, 25% (n=13) were designed with a Likert scale, here a five-point rating scale which allows respondents to express how much they agree or disagree with a particular statement (Derrick and White, 2017). The survey was disseminated from summer 2018 until early spring 2019, specifically targeting researchers working with wild birds in the fields of evolution, ecology, and conservation biology. The survey was advertised on social media using Twitter, by email to colleagues known to have

collected avian samples with kind requests for further forwarding, on evolution and ecology international and national mailing lists and dissemination websites (EvoIDir, EvoIFrance, the Ornithological Societies of North American newsletter, and zmihor.blogspot.com) and during conferences (i.e. International Society for Behavioural Ecology 2018, International Ornithology Conference 2018 and Polish Evolutionary Conference 2018). Participation in the survey was anonymous, but respondents could leave their contact information.

Data analysis

Data analysis was carried out in R 4.1.0 (R Core Team, 2021). Chi-squared tests of independence were used to test the relationship between having experienced storage issues and problems with obtaining adequate DNA quality (yes/no) or quantity (yes/no). Storage issues (i.e. problems with storage which might have negative consequences on DNA integrity) were coded as a yes/no variable. Fisher's exact and chi-squared tests of independence were used to test the relationship between storage issues and having changed storage method for blood samples (yes/no) or DNA extracts (yes/no). Because it is known that filter paper leads to DNA degradation, a Fisher's exact test was run to specifically investigate this, by creating an additional variable from the open answers regarding DNA degradation for the different storage methods. Fisher's and chi-squared tests were also used to investigate the relationship between problems with obtaining adequate DNA quality (yes/no) or quantity (yes/no) and several aspects of sample collection and preservation. The investigated aspects were: season of blood sampling (tested as two separate explanatory variables with a separate test: either coded in 4 categories -Spring, Summer, Autumn, Winter- or coded as 2 categories -Dry and Wet season- as respondents could choose only one option), access to equipment in the field (yes/no), means of transportation of blood samples to the laboratory (6 categories: Airplane, Car, Courier, Boat, Train, Other), storage temperature of blood samples (5 categories: Room temperature, +4°C, -20°C, -80°C, Other), type of molecular analysis performed on the samples (twelve categories: Gene expression, Methylation assay, MHC characterization, Microsatellite assay, Molecular sexing, mtDNA analysis, Parasite DNA analysis, RAD sequencing, SNP chip, SNP genotyping, Telomere length,

Whole genome sequencing) and DNA extraction method (6 categories: Ammonium acetate, Chelex, Commercial column kit, In-house protocol, Phenol-chloroform, Other). To test whether some storage methods of blood samples were more likely to be associated with DNA quality/quantity problems further downstream, generalized linear models assuming quasibinomial error distribution (to correct for overdispersion) were employed. Occurrence of problems with obtaining adequate DNA quality (yes/no) or quantity (yes/no) were fitted as response variables and storage method for blood samples (7 categories: Ethanol, Lysis buffer, Direct freezing, Filter paper, TE buffer, RNA^{later}, Other) as fixed categorical explanatory variable. Similar models were run to test whether storage time before DNA extraction mattered, with occurrence of problems with obtaining adequate DNA quality (yes/no) or quantity (yes/no) as response variables and storage time (4 categories: Up to 6 months, Up to 1 year, More than 1 year, No standard time frame) as fixed categorical explanatory variable. Generalized linear models were employed, instead of mixed models accounting for respondent id as random effect, due to lack of convergence of the mixed models (only 7% of responses were not independent, since the same respondents filled the survey twice or more times).

Results

Blood sample collection

A total of 219 responses to the survey were collected. All anonymized answers are available in Supporting Information 2. 209 responses on wild birds and molecular analyses were kept. Overall, researchers taking part in the survey worked on 123 species of wild birds, encompassing 53 families and 20 orders, with blue tit (*Cyanistes caeruleus*) and great tit (*Parus major*) being the most represented (Table S1-S3 in Supporting Information 3). Of the 155 responses on affiliation, 85% (n=131) were research institutions located in North America and Europe, with the greatest contribution from the USA, France, UK and Poland, in decreasing order (Figure 1A). However, the experience of respondents in field blood sampling covered 53 countries and territories across the globe, with half of responses from the USA, France, Canada, Spain, Poland and Sweden, in decreasing order (Figure 1B).

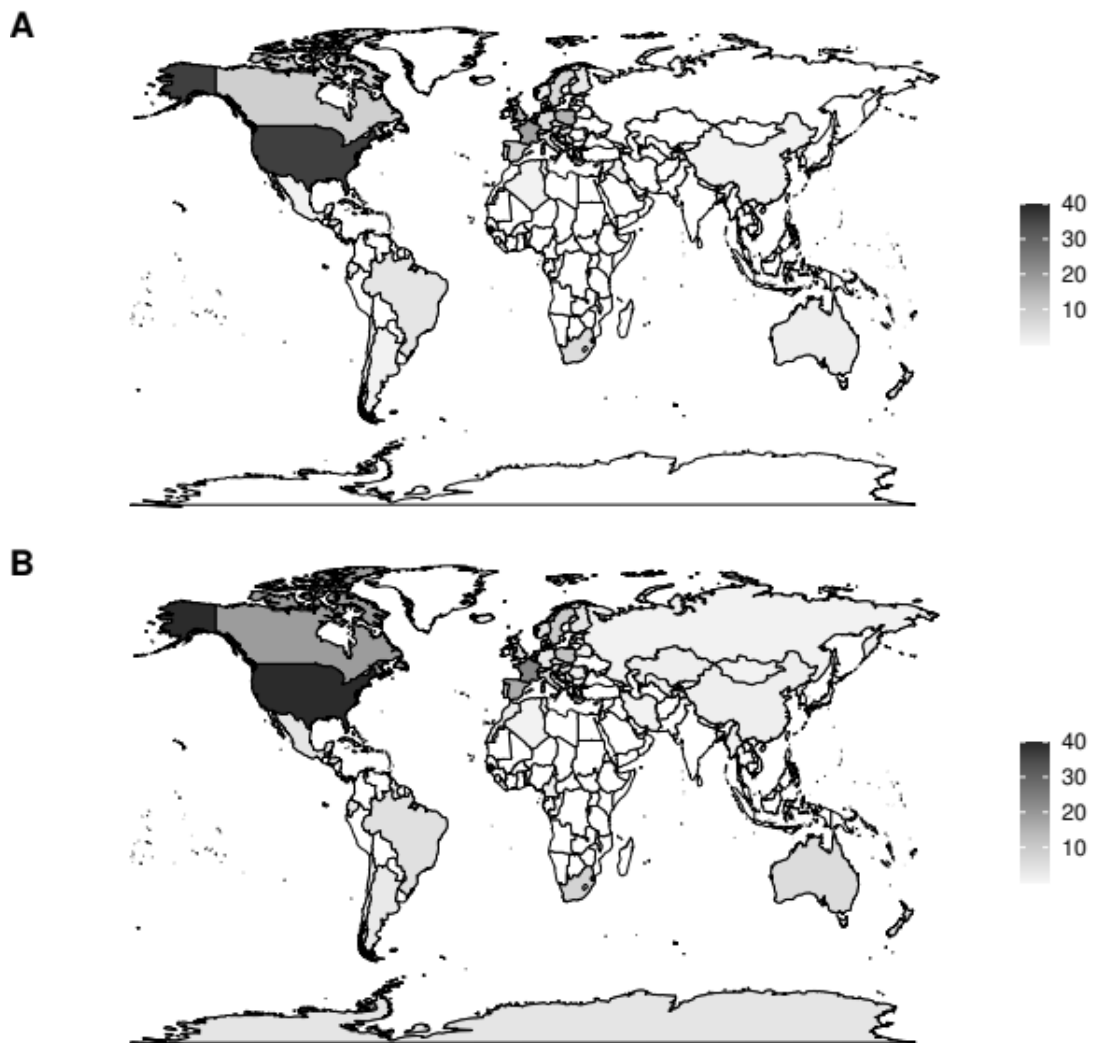


Figure 1 – World map reporting (A) countries of affiliation when researchers responded and (B) countries where fieldwork and blood sampling occurred.

Spring and summer were reported in 80% (165/203) of responses to the question “When are blood samples on this project most often collected?” (Figure 2A). Access to equipment in the field, such as a centrifuge, a fridge or a freezer, was reported in 68% (132/195) of responses (Figure 2B shows number of responses, with percentage of the total, for the different types of equipment).

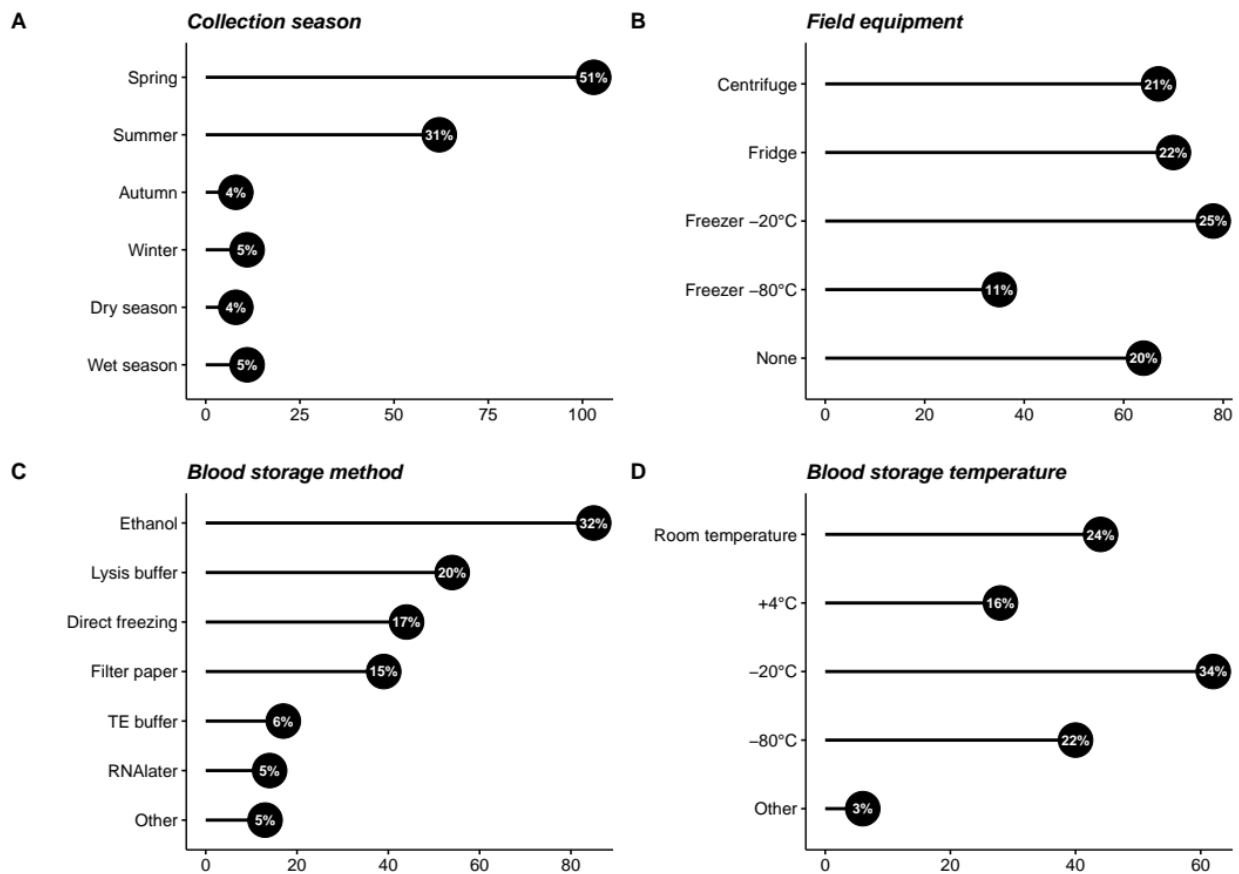


Figure 2 - Overview of aspects of collection and preservation of blood samples: season of blood sampling (A), equipment available in the field (B), storage method (C) and storage temperature for blood samples (D). On the x axis, number of responses, with percentages of the total, are shown; note that in (B) and (C) more than one response could be given. “Other” in (C) comprises: EDTA (3 responses), PBS (1), EDTA + PBS (1), a glycerol-based buffer (1), NBS buffer (1), TNE buffer (2), commercial buffer (3) and heparin buffered tubes (1). “Other” in (D) comprises: -35°C (1 response), -40°C (1), -50°C (1), -70°C (1) and liquid nitrogen (1).

Of the 284 responses to “How do you move blood samples from the field site to the permanent laboratory on this project?”, 53% (n=151) were by car, followed by airplane (23%; n=64), courier service (11%; n=32), train (6%; n=17), boat (3%; n=9), on foot (2%; n=5), bicycle (1%; n=3), bus (1%; n=2) and one response by helicopter. Of the 204 responses to the question “How large is your sample database on this project?”, 45% (n=91) were between 100 and 1,000 blood samples, followed by 34% (n=69) for 1,000-10,000 and 8% (n=17) for more than 10,000. Only 13% (n=27) of responses were for small sample sizes such as less than 100 samples.

Methods of blood storage used by field biologists

Ethanol, lysis buffer, direct freezing and filter paper (in decreasing order) were the methods of choice used to store blood samples (Figure 2C). TE buffer, RNA*later* and other mediums were used in the remaining 16% (44/266) of cases (Figure 2C). In terms of storage temperature, blood samples were stored at -20°C in 34% (62/180) of responses, followed by room temperature, -80°C and +4°C (Figure 2D). Table 1A shows storage methods for blood samples categorized by storage temperature, as reported in the survey.

Table 1 - Number of responses for each storage method by storage temperature for blood samples (A) and DNA extracts (B). Percentages within each storage method are shown in brackets. For blood samples, more than one response could be given. “RT” indicates Room temperature. “Other” comprises: EDTA (3 responses), PBS (1), EDTA + PBS (1), a glycerol-based buffer (1), NBS buffer (1), TNE buffer (2), commercial buffer (3) and heparin buffered tubes (1).

A. BLOOD SAMPLES						
	RT	+ 4° C	- 20° C	- 80° C	Other	Total
Ethanol	23 (29)	15 (19)	27 (34)	11 (14)	3 (4)	79
Lysis buffer	14 (29)	11 (22)	12 (24)	9 (18)	3 (6)	49
Direct freezing	0 (0)	0 (0)	18 (43)	22 (52)	2 (5)	42
Filter paper	16 (46)	5 (14)	5 (14)	7 (20)	2 (6)	35
TE buffer	1 (7)	3 (20)	7 (47)	4 (27)	0 (0)	15
RNA <i>later</i>	1 (7)	2 (14)	6 (43)	3 (21)	2 (14)	14
Other	0 (0)	2 (17)	3 (25)	6 (50)	1 (8)	12
Total	55	38	78	62	13	246
B. DNA EXTRACTS						
	RT	+ 4° C	- 20° C	- 80° C	Other	Total
TE	0 (0)	2 (3)	60 (80)	13 (17)	0 (0)	75
Water	0 (0)	3 (9)	21 (66)	8 (25)	0 (0)	32
Kit buffer	0 (0)	2 (10)	11 (52)	8 (38)	0 (0)	21

Tris	0 (0)	1 (5)	17 (77)	4 (18)	0 (0)	22
Total	0	8	109	33	0	150

Methods of DNA storage used by field biologists

Of the 165 responses to the question “How long after collecting blood samples do you usually extract DNA?”, 26% (n=43) were within 6 months of collection, 19% (n=32) within 1 year of collection and 19% (n=32) after 1 year. 35% (n=58) of responses were for “I don’t have a standard time frame”. Regarding DNA extraction, 57% (121/214) of responses were for commercial column kit, followed by phenol-chloroform, ammonium acetate, in-house protocol and other methods (Figure 3A). To preserve DNA extracts, TE was most frequently used, followed by water, a kit buffer and Tris (Figure 3B). DNA samples were most frequently archived at -20°C, followed by -80°C and rarely at +4°C (Figure 3C). Table 1B shows storage method categorized by storage temperature for DNA extracts.

Molecular analyses following DNA extraction

Of the 170 responses to the question “How long after DNA extraction do you usually perform analyses?”, 41% (n=70) were within 6 months of collection, 14% (n=24) within 1 year of collection and 10% (n=17) after 1 year. 35% (n=59) of responses were for “I don’t have a standard time frame”. Respondents performed a wide variety of analyses on the collected samples (Figure 3D). Microsatellite assay, parasite DNA screening, mtDNA analysis and RAD sequencing, in decreasing order, comprised 70% (286/410) of responses. The rest of the answers, in decreasing order, were: SNP chip, telomere length measurement, molecular sexing, gene expression analysis, methylation assay, sequencing, whole genome sequencing, MHC characterization and SNP genotyping (Figure 3D).

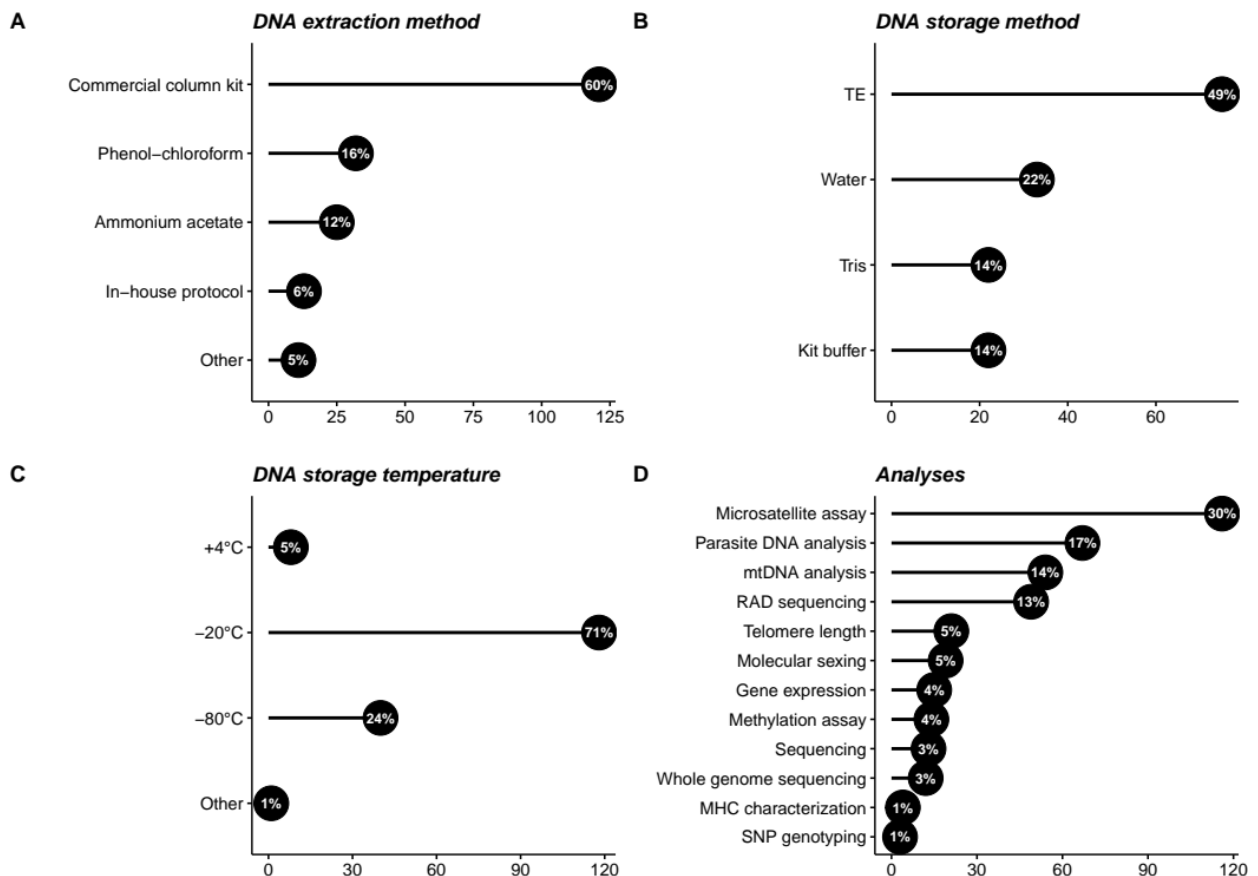


Figure 3 - Graphical summary of responses related to: DNA extraction method (A), storage method (B) and storage temperature for DNA extracts (C) and molecular analyses performed by respondents (D). On the x axis, numbers of responses, with percentages of the total, are shown; note that in (D) each respondent could provide multiple answers. “Other” in (A) comprises: Commercial magnetic bead kit (3 responses), salt extraction (3), CTAB (1), other types of commercial kits (3) and soda (1). “Other” in (C) refers to -50°C (1 response).

Table 2 shows type of molecular analyses performed depending on the type of storage method of blood (A) and DNA (B) samples.

Table 2 – Type of analyses for blood (A) and DNA (B) by sample storage method. Percentages within each storage method are shown in brackets. More than one response could be given.

A. BLOOD SAMPLES

	Microsatellite assay	SNP chip	RAD sequencing	Gene expression	Methylation assay	Parasite DNA analysis	mtDNA analysis	Molecular sexing	Sequencing	Telomere length	Whole genome sequencing	SNP genotyping	MHC characterization	Total
Ethanol	53 (27)	12 (6)	25 (13)	4 (2)	5 (3)	35 (18)	29 (15)	10 (5)	6 (3)	10 (5)	6 (3)	1 (1)	1 (1)	197
Lysis buffer	44 (34)	5 (4)	24 (19)	4 (3)	3 (2)	14 (11)	17 (13)	3 (2)	5 (4)	3 (2)	5 (4)	1 (1)	1 (1)	129
Filter paper	26 (29)	4 (4)	15 (16)	1 (1)	3 (3)	9 (10)	19 (21)	6 (7)	2 (2)	1 (1)	3 (3)	1 (1)	1 (1)	91
Direct freezing	15 (19)	3 (4)	8 (10)	2 (2)	3 (4)	17 (21)	9 (11)	6 (8)	3 (4)	10 (12)	2 (2)	2 (2)	0 (0)	80
RNAlater	8 (20)	3 (7)	3 (7)	5 (12)	1 (2)	9 (22)	6 (15)	0 (0)	1 (2)	2 (5)	1 (2)	0 (0)	2 (5)	41
TE buffer	7 (23)	2 (6)	2 (6)	4 (13)	2 (6)	6 (19)	3 (10)	0 (0)	1 (3)	1 (3)	1 (3)	1 (3)	1 (3)	31
Other	8 (26)	1 (3)	4 (13)	2 (6)	1 (3)	5 (16)	4 (13)	1 (3)	1 (3)	3 (10)	1 (3)	0 (0)	0 (0)	31
Total	161	30	81	22	18	95	87	26	19	30	19	6	6	600

B. DNA SAMPLES

TE	44 (27)	28 (17)	23 (14)	17 (11)	11 (7)	6 (4)	5 (3)	5 (3)	8 (5)	6 (4)	4 (2)	3 (2)	1 (1)	161
Water	18 (23)	15 (19)	14 (18)	13 (16)	0 (0)	5 (6)	2 (3)	1 (1)	3 (4)	2 (3)	5 (6)	0 (0)	1 (1)	79
Kit buffer	12 (28)	5 (12)	6 (14)	4 (9)	4 (9)	3 (7)	3 (7)	4 (9)	0 (0)	0 (0)	1 (2)	1 (2)	0 (0)	43
Tris	13 (30)	9 (21)	2 (5)	7 (16)	2 (5)	2 (5)	4 (9)	2 (5)	0 (0)	2 (5)	0 (0)	0 (0)	0 (0)	43
Total	87	57	45	41	17	16	14	12	11	10	10	4	2	326

Storage issues can result in low DNA quality and quantity

Of the 199 responses to the question on experiencing issues with sample storage, 31% (n=61) reported problems: 21% (n=41) were problems with storage of blood samples, one with DNA storage and 2% (n=5) with storage of both blood and DNA samples. 7% (n=14) of responses

were from researchers who experienced problems with sample storage but could not identify the issue (Figure 4A). 44 open responses explained what was the storage issue researchers faced, as follows: (i) use of anticoagulant (possibly due to an overuse of heparin) in collection devices interfering with PCR (5%; n=2), (ii) difficulties with DNA extraction due to lysis buffer, either because of too much blood for the amount of buffer or because of long storage time or lysis buffer interfering with telomere length assay (27%; n=12); (iii) DNA degradation when blood was stored on filter paper, especially in case of long storage time (23%; n=10); (iv) evaporation of ethanol from tubes and ethanol not good enough for PacBio sequencing (20%; n=9), (v) DNA degradation in TE buffer (9%; n=4), (vi) freezer failure (5%; n=2), (vii) misidentification of samples (2%; n=1), (viii) sample shipping (7%; n=3), (ix) DNA extraction (2%; n=1). DNA degradation, as reported in the open answers, was not more likely to occur in any of the four storage methods for blood samples indicated by respondents (filter paper, ethanol, lysis buffer, TE buffer; two-tailed Fisher's exact test, p-value = 0.136). Of the 61 responses reporting problems with sample storage, 84% (n=51) also reported a reduction in sample size of the project (Figure 4B), which for example led to a reduction in the geographic range of the sampling, exclusion of some target species, reduction in statistical power and left holes in paternity analyses.

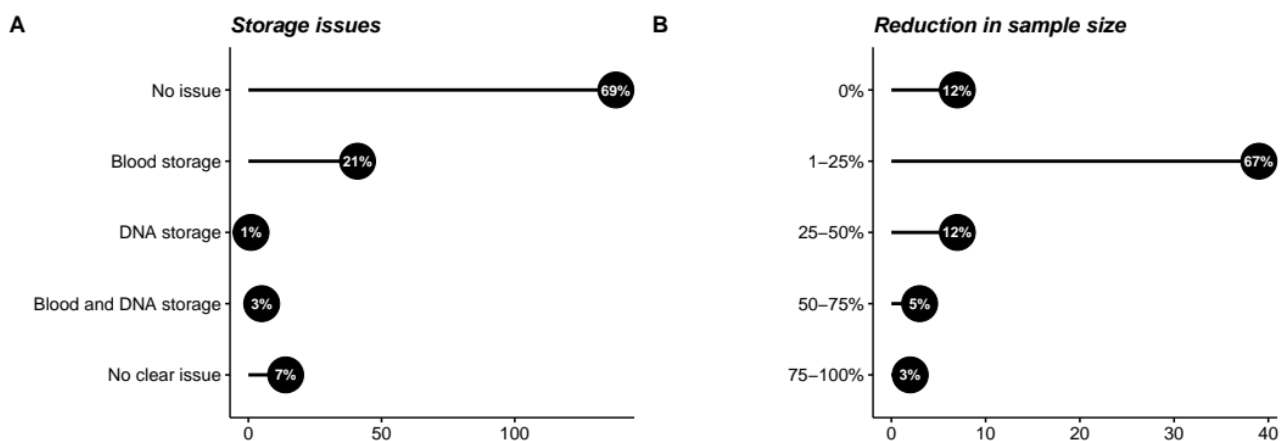


Figure 4 – Overview of problems with storage encountered by respondents (A) and reduction in sample size of the project due to storage issues (B). On the x axis, numbers of responses, with percentages of the total, are shown.

Consequently, 18% (n=11) of projects were not published and 8% (n=5) were published in a less prestigious journal. Storage issues were related to problems with obtaining adequate DNA quality ($\chi^2_1 = 28.596$, p-value = $8.915e-08$) and sufficient DNA quantity ($\chi^2_1 = 6.139$, p-value = 0.013; Table 3).

Table 3 – Number of responses for problems with sample storage (both blood samples and DNA extracts) with respect to problems with obtaining adequate DNA quality and sufficient DNA quantity. Percentages within rows are shown in brackets.

Storage problems	Problems with DNA quality			Problems with DNA quantity			
	Yes	No	Total	Yes	No	Not measured	Total
Yes	27 (48)	29 (52)	56	19 (35)	32 (59)	3 (6)	54
No	12 (11)	101 (89)	113	18 (16)	82 (73)	13 (11)	113
Total	39	130	169	37	114	16	167

No aspect of sample collection and storage influences DNA quality and quantity

The survey did not identify any association between season when blood samples were collected and problems with obtaining either adequate DNA quality (categories for season: spring, summer, autumn, winter: two-tailed Fisher's exact test, p-value = 0.219; categories for season: dry vs wet season: two-tailed Fisher's exact test, p-value = 0.319) or sufficient DNA quantity (categories for season: spring, summer, autumn, winter: two-tailed Fisher's exact test, p-value = 0.524; categories for season: dry vs wet season: two-tailed Fisher's exact test, p-value = 1). Having access to equipment in the field was not associated with problems with obtaining adequate DNA quality ($\chi^2_1 = 0.368$, p-value = 0.544) or sufficient DNA quantity ($\chi^2_1 = 0.076$, p-value = 0.783). There was also no association between way of transferring samples from field to laboratory and problems with obtaining adequate DNA quality (two-tailed Fisher's exact test, p-value = 0.160) or sufficient DNA quantity (two-tailed Fisher's exact test, p-value = 0.282). No

storage method for blood samples was more likely than others to generate problems with obtaining either adequate DNA quality (Table 4A) or sufficient DNA quantity (Table 4B).

Table 4 - Binomial generalised linear models explaining problems with obtaining adequate DNA quality (A) or sufficient DNA quantity (B) based on blood storage method. "Direct freezing" is the reference for parameter estimates.

Variable	χ^2	df	Pr(> χ^2)	Estimate \pm SE
A. DNA quality	5.703	5, 119	0.3362	
Ethanol				0.821 \pm 0.735
Filter paper				0.415 \pm 1.006
Lysis buffer				0.128 \pm 0.889
Other				0.174 \pm 1.262
TE buffer				1.897 \pm 0.930
B. DNA quantity	3.292	5, 105	0.655	
Ethanol				0.938 \pm 0.758
Filter paper				1.226 \pm 0.883
Lysis buffer				0.379 \pm 0.844
Other				0.245 \pm 1.277
TE buffer				0.091 \pm 1.267

When researchers were asked whether they changed storage method, a higher number of responses (23%; 43/191) were collected for blood samples compared to DNA extracts (8%; 13/170). Experiencing storage issues was associated with having changed storage method for blood samples ($\chi^2_1 = 10.424$, p-value = 0.001). Half (n=8) of the responses explaining why researchers changed storage method for blood samples indicated an increase in DNA yields and the other half (n=8) logistical reasons, either because of space constraints in the laboratory or because of issues during sample transport. There was no association between storage temperature of blood samples and problems with obtaining adequate DNA quality (two-tailed

Fisher's exact test, p-value = 0.482) or sufficient DNA quantity (two-tailed Fisher's exact test, p-value = 0.423). Storage time of blood samples before DNA extraction did not influence either obtaining adequate DNA quality (Table 5A) or sufficient DNA quantity (Table 5B).

Table 5 – Analysis of storage time of blood samples before extraction on problems with obtaining adequate DNA quality (A) or sufficient DNA quantity (B). Analysis used binomial generalized linear models and the category “Up to 6 months” was the reference for parameter estimates.

Variable	χ^2	df	Pr(> χ^2)	Estimate \pm SE
A. DNA quality	1.712	3, 158	0.634	
Up to 1 year				-0.233 \pm 0.588
More than 1 year				0.405 \pm 0.532
No standard time frame				-0.215 \pm 0.500
B. DNA quantity	2.514	3, 141	0.473	
Up to 1 year				0.811 \pm 0.609
More than 1 year				0.310 \pm 0.651
No standard time frame				0.707 \pm 0.550

DNA extraction method did not predict problems with obtaining either adequate DNA quality (two-tailed Fisher's exact test, p-value = 0.268) or sufficient DNA quantity (two-tailed Fisher's exact test, p-value = 0.614). However, respondents shared pros and cons of different extraction methods: for instance, phenol chloroform is more time consuming and more toxic than commercial column kits, but provides higher DNA yields and is less expensive. Salt precipitation is less toxic, faster, without risk of contaminating samples and gives comparable results in terms of DNA yields relative to phenol-chloroform. Respondents also suggested to extract DNA sooner after collection and expressed an interest in finding methods that might provide higher yields, for instance magnetic beads. In case of DNA extracts, there was no relationship between storage issues and having changed DNA storage method (two-tailed Fisher's exact test, p-value = 0.210). Among open answers given to explain the change, 4 reported a change from buffer to

water, so the sample was easily concentrated in case of necessity, 1 a change from -20°C to -80°C for logistical reasons and 1 because of issues with ethanol.

It was not possible to test whether some molecular analysis goals are more sensitive to problems from some storage methods. No molecular analysis was more likely associated with problems with obtaining either adequate DNA quality (two-tailed Fisher's exact test, p-value = 0.154) or sufficient DNA quantity (two-tailed Fisher's exact test, p-value = 0.871) than others. However, respondents provided recommendations regarding specific storage methods and assays: for instance, they suggested to use RNA*later* or direct freezing for Pac BIO sequencing or other NGS techniques and direct freezing for telomere length measurement, while ethanol, lysis buffer or FTA® cards are suitable for microsatellites, mtDNA analysis and other PCR-based assays. Table 6 reports some issues faced by respondents with specific methods of blood storage.

Table 6 – Examples of comments given by respondents explaining problems with some storage methods and assays.

Ethanol

“While good and sufficient for most things the DNA quality (average fragment size) is too small for optimal Pac Bio sequencing”

“We have never had any problems with genotyping, sex-typing etc but we now believe that storage of blood in ethanol has a progressive effect on the detection of telomeric sequence by qPCR.”

“The blood was put into 100% ethanol and stored long term. These samples were originally collected in 2007 and the tubes must not have been air tight seals as the ethanol evaporated and the blood became dried scabs”

Lysis buffer

“It was more difficult to achieve the minimum concentration for RADseq and whole genome with blood samples stored in lysis buffer.... sometimes (not always), but increasing the lysis incubation time and eluting with less buffer often did the trick.”

“Previous samples were collected into a lysis buffer and stored at RT. Over the years the DNA seems to be of lower quality than that collected recently and stored frozen.”

“Used lysis for several years because of ease of preservation, but switched back to freezing when it became apparent this wouldn't work for telomeres”

“Lysis buffers, including "Queen's buffer", have two serious problems: 1) DNA degrades quickly (potentially within months) if not extracted soon after collection; 2) freezing often creates a gel-like consistency that proteases cannot penetrate”

Direct freezing

“Our freezer failed overnight and so a small number of extracted DNA samples were damaged”

FTA® cards

“Storage of blood on paper filter during 5 years, sufficient for microsatellite analyses but too degraded for next-generation sequencing”

“We had issue to perform whole genome sequencing from blood samples stored on FTA cards. They generated significantly less DNA and less pair-ended reads (77 millions vs. up to 215 millions with blood preserved in ethanol). Furthermore, we were not able to generate mate pair libraries out of it because of the lack of DNA available.”

“Used to use FTA cards for microsat work, but have since switched to lysis buffer for whole genome and RAD sequencing as DNA quality is much higher in buffer compared to on filter paper”

Discussion

Proper archiving of biological samples collected in the wild is crucial for current and future research in ecology and evolution, as the way samples are collected and stored has implications for the outcome of the project in many different disciplines. Furthermore, proper archived material provides opportunities for subsequent and future investigations allowed by technical developments (Jackson et al., 2012; Wong et al., 2012). We report first-hand experiences of

ecologists and field biologists regarding worldwide practices for the field collection of blood samples from wild birds and the long-term storage of blood samples and DNA extracts in the lab. In terms of DNA storage, recommendations available in the literature overlap with the storing conditions most often reported in the present survey (see Morin et al., 2010; Prendini et al., 2002). DNA is usually archived dry, or in a neutral pH buffer with chelating agents such as EDTA at low temperatures (e.g., -20°C). Respondents also suggested using aliquots of valuable samples to reduce time in the fridge and limit freeze-thaw cycles, provided space is not an issue. Some respondents suggested that FTA® cards might be used to store DNA, if this method would prove efficient. While no clear signal emerged from the survey in terms of co-variation between specific storage methods and DNA quality and quantity output in downstream lab work, a critical point identified by the survey is that the outcome of molecular analyses often depends on storing conditions of blood samples and less of DNA extracts, possibly because clean DNA is easier to store and/or more resilient to damage. We integrate personal experiences of respondents and the available literature to provide general recommendations for blood storage and to ameliorate blood storing practices (Table 7).

Table 7. Storage methods, pros and cons, and recommended best practices for storing blood samples.

Storage method	Pro	Con	Best practices
Ethanol	Relatively inexpensive, readily available and easy to handle (this study)	Difficult to transport with some shipping companies and evaporation in low-quality tubes, leading to DNA degradation (this study) Evidence of DNA degradation over long periods of time at room temperature (not specifically blood) (Kilpatrick, 2002)	Optimal concentration between 95-100% (this study; Wong et al., 2012) Shaking tubes right after collection improves DNA yield (this study) NGS and telomere length measurement are likely to be negatively affected (this study) Remove all ethanol before extraction (this study) Ethanol-tissue (not specifically blood) ratio at least 3:1 (Wong et al., 2012).
Lysis buffer e.g. Longmire's, Queen's buffer	Relatively inexpensive, easy to handle and transport (this study)	Evidence of DNA degradation over long periods of time at room temperature (this study; Kilpatrick, 2002)	Avoid refrigeration (this study), despite recommendations for storage at +4°C or -20°C (Longmire et al., 1997; Seutin et al., 1991) NGS and telomere length measurement are likely to be negatively affected (this study) Lysis buffer is sensitive to changes in storage temperature (this study) Control amount of blood going into each tube of lysis buffer and collect duplicate tubes (this study). Blood to buffer ratio of 1:10 for Longmire's and Queen's buffer (Longmire et al., 1997; Seutin et al., 1991).
Freezing	-20°C, -80°C or liquid nitrogen provide minimal DNA degradation over long periods of time (Kim et al., 2011)	Difficult access to freezers, dry ice, or liquid nitrogen in remote field locations; difficult shipping of frozen samples; high costs and power consumption; high space requirements and chance of power loss and freeze-thaw cycles (this study)	Works well with all kinds of assays (this study) Setting ULT freezers at -70°C is energy saving compared to -80°C (https://www.freezerchallenge.org/resources.html).
Filter paper	Easy to handle and transport and minimal space requirements (this study)	Relatively expensive (this study) Routinely kept at room temperature, leading to DNA degradation (this study; Carpentieri et al., 2021; Hollegaard et al., 2011)	Long-term storage should be in a freezer (avoid fridge for risk of developing mildew) (this study; Carpentieri et al., 2021; Hollegaard et al., 2011) NGS and telomere length measurement are likely to be negatively affected (this study) Extract soon after collection (this study) up to 500 µL maximum total volume/card for Whatman® FTA® card technology (https://www.sigmaaldrich.com/NL/en/substance/whatmanftacardtechnology1234598765?context=product).

Overall, the responses collected by the survey (see examples in Table 6) are in agreement with the information currently available in the literature. However, respondents reported that “blood in lysis buffer annoyingly clogs up, whether storing in fridge or freezer” and “freezing often creates a gel-like consistency that proteases cannot penetrate” (Table 6). By contrast, previous literature recommends storing lysis buffer at +4°C or -20°C (Longmire et al., 1997; Seutin et al., 1991). These recommendations are targeted to facilitate avoiding problems with sample storage while setting up or introducing changes in biological sample libraries. This is of crucial importance, because, as shown by the personal experience of the surveyed researchers, sample storage issues were related to problems with obtaining adequate DNA quality and sufficient DNA quantity for the intended molecular analyses. Overall, 31% (61/199; Figure 4A) of the collected responses reported problems with storage of blood samples and/or DNA extracts, which, for the majority, led to a reduction in sample size of the project. Responses indicated that the blood storage issues and resulting DNA quality/quantity problems were not related to: (i) season of blood sampling, (ii) access to equipment in the field, (iii) means of sample transportation, (iv) storage time before DNA extraction and (v) DNA extraction methods. There was also no indication of storage method or temperature to affect DNA quality and/or quantity, despite previous studies reporting the opposite, similarly to first-hand accounts of respondents (Table 6). For instance, storing blood samples on FTA® cards at room temperature was reported to affect RTL measurements compared to frozen blood or DNA (Reichert et al., 2017); storing blood on Guthrie cards at +4°C affected DNA concentration relative to samples stored at -20°C (Hollegaard et al., 2011) and storing blood in ethanol was related to changes in stable isotope signatures (Bugoni et al., 2008). According to our survey, the effectiveness of a preservation method is not the only factor that should be considered when choosing how to collect and store biological material. Our respondents’ experiences show that planned or possible future analyses, storage time, logistics in the field, storage space and sample storage costs all contribute to influencing the choice of sample storage conditions. Respondents of the survey also reported that in most cases it is necessary to find cost-efficient solutions, often compromising between convenience in the field, storage space in the laboratory and costs for expensive preservation methods or for the maintenance of fridges and freezers. Consequently, logistical or funding reasons might force

researchers to adopt more convenient methods that still provide reasonable sample quality for the specific goal which they originally planned. The choices of storage methods are also often based on historical practices, therefore long-established methods might often be unsuitable for specific needs, especially for targets (e.g., telomeres) whose importance has emerged more recently. Accordingly, researchers planning new research goals that involve archived samples should make sure that the current storage method and temperature are suitable for the specific target assay, also considering new goals that may emerge later. For instance, according to respondents, the quality of DNA extracted from blood samples stored on filter paper were suitable for microsatellites but not for whole genome sequencing or RAD sequencing, which require higher quality DNA. For the latter analyses, blood samples should be stored in lysis buffer or ethanol, or even better, frozen or stored in *RNAlater*. Moreover, while storing samples in lysis buffer is more affordable and logistically easier than freezing samples, based on the experience of respondents, it might compromise results of telomere length assays. Lysis buffer samples are also quite sensitive to the quantity of blood used; typically, 20 μ l of blood in 1 mL of buffer is a good target, and if more blood is available, placing it in duplicate tubes is preferable. Some of the compromises regarding blood storage may be less harmful if DNA is extracted as soon as possible, as some respondents reported DNA quality declining with time for some storage methods (lysis buffer or filter paper).

Other promising techniques

Blood sampling has been the preferred source of DNA from wild birds because of the presence of nucleated erythrocytes, which provide high yields of good quality DNA suitable for a wide array of molecular applications (McDonald and Griffith, 2011). Recent evidence suggests that a growing number of less invasive techniques might yield good enough DNA to address a wide variety of questions in the fields of ecology and evolution (Beja-Pereira et al., 2009). We did not survey respondents about these techniques, but a brief review of the literature is instructive as there are pros and cons to these alternatives. For instance, feathers are an alternative source of DNA (Harvey et al., 2006; Vilstrup et al., 2018). However, the presence of potential PCR inhibitors such as the proteins melanin and keratin (McDonald and Griffith, 2011) result in a lower quality of genetic data

compared to blood (Harvey et al., 2006; Maurer et al., 2010; Sacchi et al., 2004). Also, considerable variation occurs in DNA quantity and quality between plucked and shed feathers (Yannic et al., 2011) and feather sampling is not possible for very young nestlings with no feathers (Seki, 2003; Wellbrock et al., 2012). Another alternative to blood sampling is buccal swabs, which are proving to be a reliable source of DNA (Brubaker et al., 2011; Bush et al., 2005; Handel et al., 2006; Wellbrock et al., 2012). Buccal swabs provide several advantages over blood sampling: they are less invasive, they require minimal training of personnel and the chance of injuring birds is very low (Vilstrup et al., 2018). Also, handling time for buccal swabs is shorter and minimizing disturbance from the experimenter is important, for instance when studying breeding success, behaviour and survival and when birds might be more susceptible from stress, such as during cold weather or molt (Handel et al., 2006). Furthermore, studies of paternity and sex ratios might benefit from using buccal swabs because of the ease of sampling very young nestlings thanks to their gaping behaviour (Handel et al., 2006). Nevertheless, not all assays or study questions allow switching from drawing blood (for example to assess immune components, hormones in blood or molecular markers related to this tissue type having a particular cellular turnover rate, e.g. for telomere length measurements). Future investigations will determine whether buccal sampling is able to yield suitable amount and quality of genomic DNA for high-throughput technologies or the latest molecular technological advancements and result in comparable quality of genetic data as blood sampling.

Conclusions

There is a clear consensus on the need of preserving the biological material that has been collected from wild animals as successfully as possible. The experiences of respondents show that choosing storage method and temperature has the potential to generate considerable variation in DNA quality and/or quantity, with possible non-trivial consequences for research outcomes. We found no perfect method, and the collective wisdom of the avian researchers' community indicates that multiple factors must be considered when choosing storage conditions. Depending on research aims, an optimal preservation method should be able to guarantee adequate quality and enough DNA required by the planned assay, but also be flexible enough to offer suitable material for future

possible technological developments, as in the case of telomere length measurements. We recommend that researchers setting up or planning to introduce changes in long-term biological archives carefully take into consideration the effectiveness of currently available preservation methods, together with funding opportunities and logistic limitations.

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Data Accessibility

Supporting Information and data are available via Figshare at [10.6084/m9.figshare.17281793](https://doi.org/10.6084/m9.figshare.17281793).

Competing interests

The authors declare no conflict of interests.

Author contributions

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Acknowledgements

Funding: Polish National Science Centre grant no. 2016/21/B/NZ8/03082 awarded to Marta Szulkin.