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Swedish University of Agricultural Sciences, SLU
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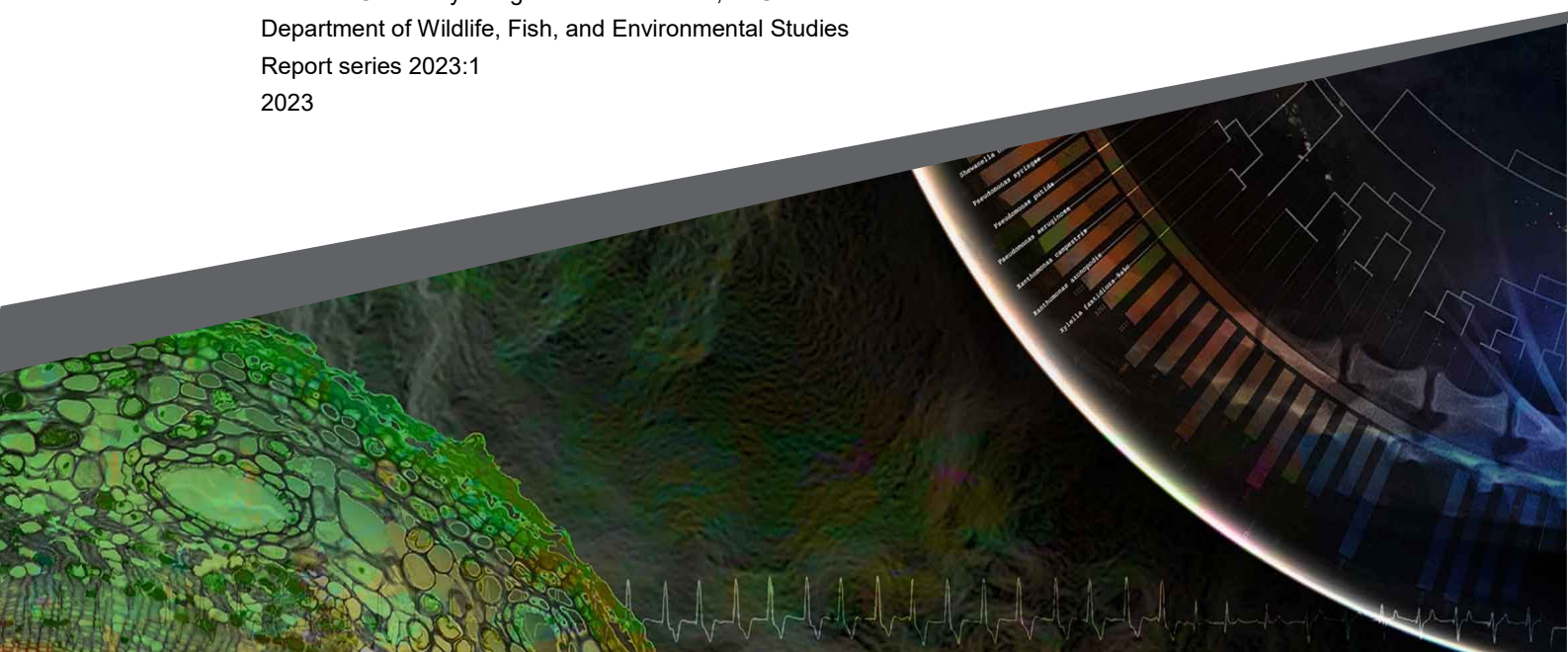

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Image on front cover: *Newly hatched Eurasian Curlew chicks in the critically endangered Iberian breeding population – an important goal for genetically sustainable conservation.* Photo: Jesús Domínguez.

Summary

Decades of decline have triggered an AEWA action plan and numerous conservation and management projects across the range of Eurasian Curlew *Numenius arquata*. Several projects include DNA studies, but more have an untapped potential to collect and analyse DNA. This document intends to promote DNA studies in Eurasian Curlew projects, in particular through pointing out the broad array of useful DNA samples that projects can collect for current and future use, often at low costs and without negatively affecting the birds. The document also provides an overview of DNA analysis methods, and advice on how to fit DNA studies sustainably into socio-economical and ethical frameworks. Additionally, the document contains numerous hands-on practical advice and an extensive reference list. The result is a long text, but we hope its structure helps you to navigate smoothly to the parts of your interest, whatever role you have or plan to play in Eurasian Curlew conservation and management projects.

Highlights:

- Solid genetic information can improve the outcome of conservation and management actions.
- We need to carefully document the genetic aspects of captive breeding and releases of eggs and chicks/fledglings.
- Everyone involved in Eurasian Curlew projects can sample DNA.
- DNA samples stored for future analyses are equally important as samples used to study contemporary objectives.
- There are many potential tools in the DNA toolbox, but several are underdeveloped for our species. More methods would lead to better and more cost-efficient studies.

Table of Contents

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Preface

Background

In November 2015, at the 6th Session of the Meeting of the Parties to the African-Eurasian Migratory Waterbird Agreement (AEWA), an *International Single Species Action Plan (ISSAP) for the Conservation of the Eurasian Curlew* (Brown 2015) was adopted. The implementation of the ISSAP is now an ongoing process, internationally and nationally, and recent years have seen an expansion of conservation, monitoring and research projects.

Based on the ISSAP, the AEWA Eurasian Curlew International Working Group (ECIWG) concretizes future actions within the framework of a work plan. The first work plan was agreed at a meeting in Aberlady, Scotland in 2018 and was updated and prolonged at a meeting in Papenburg, Germany in 2022. Work plans seek to aid implementing ISSAPs by identifying discrete projects and areas of work to take forward. Careful genetic monitoring is part of the updated work plan.

Scope and aims

The aim of this publication is to raise awareness of and provide guidance for the genetic aspects of conservation and management actions within the framework of the Eurasian Curlew ISSAP and its work plan.

Ultimately, the goal of this document is to assist efforts to move the Eurasian Curlew towards a more favourable conservation status at local, regional, national and international scale. DNA studies can help provide evidence-based knowledge of the status and biological processes in Eurasian Curlew populations and thus, direct conservation actions towards higher efficiency and sustainability. Some DNA projects are wealthy and can afford expensive, state-of-the-art methods while others must operate on a low, possibly zero budget. This does not mean low-budget projects - in particular low-budget DNA sampling projects - contribute less to Eurasian Curlew conservation and management. In terms of cost-efficiency, they are often highly competitive.

DNA-analysis methods have developed tremendously over the last decades. Currently, whole genome sequencing of high-quality DNA samples is the state-of-the-art. This concept is extensively covered in this document, but we also provide information about alternative ("older") methods (Chapter 3). We do this because these still have a great potential to deliver valuable information and fit a wider range of DNA-sources, laboratory equipment, sampling techniques, fieldworker skills and, last but not least, budgets. Some of these methods remain to be tailored for the Eurasian Curlew, though.

Good samples are vital for any DNA study. The major section of this document (Chapter 2) deals with sampling methods for a wide range of Eurasian Curlew DNA sources. The idea is to provide hands-on advice for easy, cost-effective and secure sampling in par with various objectives and thus, the analysis methods. Many valuable samples can be collected with very little costs and effort.

The content of this publication is limited to methods for genetic identification (genotyping) of Eurasian Curlew and similar species, e.g. Black-tailed Godwit (*Limosa limosa*). Neither studies of prey species nor of organisms living on or in Eurasian Curlews are considered[1].

[1] Like human bodies, the bodies of birds should be viewed as ecosystems inhabited by a huge diversity of organisms, all with their own set of genetic molecules.

Methods for studies of expressed DNA (mainly mRNA and tRNA) or resulting metabolites (proteins, hormones, etc.) are not covered either.

This document intends to be useful for anyone involved in actions to improve the conservation status of the species, from interested field assistants, via project managers to funding agencies and authorities. The "List of contents" and the "Reading guide" (Textbox 2) will help you find the information of your choice.

We intend to keep this a 'live' document and hope to improve it over time. If you have comments, questions or suggestions that could improve future versions of these guidelines, please let us know.

Textbox 1

Copyright and suggested citation

Suggested citation:

de Jong, A., Brown, D., Domínguez, J., Düttmann, H., McMahon, B.J. & Walsh, G. (2023). Genetic aspects of conservation and management of the Eurasian Curlew *Numenius arquata*. Swedish University of Agricultural Sciences. Department of Wildlife, Fish, and Environmental Studies, Umeå. Report series 2023: 1.

Textbox 2

Reading guide

This document intends to serve many different readers and many different conditions. As a result, there is probably much more information in this document than you need. Please, do not let this information-overload scare you. The editorial team has tried to facilitate easy access to the contents of this document by the use of extensive 3-level numbering of chapters. Additional information is presented in text-boxes and appendices.

If genetics and DNA are completely new things for you, we suggest you start with reading Appendix 1. After that, we advise you to approach the rest of this document along the three paths presented below. Welcome!

If you mainly work with conservation actions in the field, the list of DNA sampling methods may be your prime interest. Check the Chapter 2 entries in the "List of contents" and pick the method(s) that might suit you. For each sampling method, you will find hands-on instructions and links to further readings. Meanwhile, you might find reading the "Introduction" chapter worthwhile for putting your work into a wider context.

If you run or plan to start up a Eurasian Curlew project, we suggest you start with the "Introduction" section. Based on the information that fits your plan and resources, you can then continue to relevant parts of Chapter 3. If DNA sample collection is a feasible part of your plan, just read the information about suitable/adequate sampling methods in Chapter 2.

If you are a researcher or in charge of directing or funding conservation projects, we suggest you quickly scan Chapters 1-3 to get an overview of the possibilities (and pitfalls). For further information, you can read sections of interest more closely and/or use the referred literature for in-depth reading. Please note that the Reference list even includes items not referred to in the text and thus, can be used as a stand-alone entry to the body of literature.

In this document, you will find neither detailed information about methods of DNA extraction from samples nor about storage and pre-treatment of DNA extracts prior to final analyses (Chapter 3). This is because these methods vary tremendously between analysis methods and laboratories. You find a short overview in Chapter 3.1 and detailed descriptions in relevant references.

Textbox 3

Management vs conservation vs research

How a project/action is classified in this trinity can have a profound impact on the organizational context, funding options and, last but not least, the legal framework. In this document we use the following definitions.

Management includes all human actions intending to influence *where*, *when* and *in what numbers* organisms (here the Eurasian Curlew) occur. With this definition, conservation is a form of management, but because the term *management* is often related to hunting/culling and the promotion of harvested populations, we choose to define *conservation* separately.

Conservation includes all human actions intending to move populations towards a more favourable conservation status. This clearly implies that the action was motivated by a perceived sub-optimal conservation status of the species/population.

We use the word "intending" to mark that the outcomes of management and conservation actions are uncertain, and unfortunately, in many cases these effects are never reliably evaluated.

Research has no implicit intention to change population sizes or to exploit species. Instead, the goal is more data/knowledge/insight. Obviously, research output can be used in conservation and management, but this requires additional actions.

Eurasian Curlew projects can cover one or several of these categories, but we think it is wise to clarify their position in/across this trinity. For example:

* Management actions are expected to be underpinned by documented decisions and can become discredited if those are missing or faulty.
* Conservation actions, implicitly or explicitly, "promise" improvements for the focal species and/or their ecosystem. If these promises are not met, current and future conservation projects are likely to lose trust and support.
* A project labelled "research" is expected to follow scientific rules and to publish its results. It loses credibility if it fails to do so.

This document focuses on management and conservation actions, without denying the important role of research *per se* in providing the data, knowledge and insights needed for evidence-based management and conservation, e.g. about re-nesting frequencies, extra-pair parenthood and intra-specific nest parasitism.

1 Introduction

1.1 Why DNA studies on Eurasian Curlews?

Understanding a species' genetic properties can aid conservation and management (e.g. McMahon et al. 2014, Hohenhole et al. 2021, Theissinger et al. 2023). Conservation genetics[2] can be used to study a variety of topics, including identifying and minimising inbreeding, detecting population structure, solving taxonomic issues, defining conservation units and tracing hybridisation.

Specifically, in current conservation and management of the Eurasian Curlew, the following genetic aspects stand out: (a) the special concerns regarding the genetics of (very) small populations, (b) the population structure and migratory connectivity of (sub) populations and (c) the genetic impacts of conservation actions.

Textbox 4

Genetics vs Genomics

Genomics is the younger of these sibling terms, its name clearly inspired by the prestigious Human Genome Project of around the turn of the millennium. "Genomics" signals a focus on sequencing large portions of the genome, ultimately the entire genome (whole genome sequencing *sensu stricto*). The idea is that this massive amount of DNA sequence information will provide new knowledge/insight and better underpinning of conservation and managements actions. Currently, we are only just beginning to tap this potential (c.f. McMahon et al. 2014, Oyler-McCance et al. 2016) and most real-world conservation and management decisions are still based on other methods (c.f. Chapter 3). Several of these other methods would require species-specific development, though.

Originally, whole genome sequences were only produced for model species, e.g. *Homo sapiens*, *Gallus gallus, Escherichia coli* and *Arabidopsis thaliana*. These "reference genomes" were then used for genomic studies of these and related species. With decreasing sequencing costs, the genomes of more and more "non-model" species are sequenced[3]. Currently, no reference genome for the Eurasian Curlew is available, but several ongoing projects plan to produce one in the near future. With a reference sequence in place, advanced sequencing techniques become easier and cheaper, particularly the bioinformatics steps.

The drawback of whole genome sequencing is that per-sample costs are high, which, in most cases, results in small numbers of analysed samples. Although thoroughly described individuals can be assumed to inform us about certain genetic traits of other individuals in their population of origin, we cannot be sure without data from a sufficient amount of samples from a thorough sampling scheme. This drawback will gradually disappear when prices for sequencing come down, but for time being, this problem requires thorough consideration.

In this document, we have chosen to use the term *genetics* instead of *genomics* to indicate that all DNA methods can be useful for conservation and management (c.f. Chapter 3).

[2] In current conservation and management, the terms "genetic" and "genomic" are used as (semi-)synonyms. In this document, we will use the term "genetic", because it is more widespread and familiar among non-professional geneticists.

[3] Whole genome sequencing of non-model species is often called DE NOVO sequencing.

1.1.1 Genetic risks in small populations

As populations decrease in size, there are fewer individuals available to carry different genetic traits. This results in decreased genetic variation (Frankham 1996). At the population level, this results in a shrinking gene-pool and essential genotypes potentially being lost. For individuals, there can be an overall reduction in fitness. This is known as the Allee effect (Kramer et al. 2018) and implies that the average individual, and thus the population, produces fewer and/or lower quality offspring. Multiple drivers can cause the Allee effect including difficulty finding mates, lack of group protection and inbreeding. However, it is often difficult to sort out the relative importance of these drivers. Without thorough, long-term studies of reproductive success of individually marked birds, it is also hard to prove/quantify a possible Allee effect in the first place.

With or without an Allee effect, small populations run an increased risk of extinction through demographic stochasticity - "chance", or rather "bad luck" (e.g. Sæther et al. 2004, Courchamps et al. 2006). Environmental stochasticity, such as an outbreak of disease (Spielman et al. 2004), operates in similar ways. Reduced genetic diversity makes a species less adaptable as there is less variation for selection to act on, reducing the capacity for a species to adapt to chance events and changing environments (Purvis et al. 2000).

Small populations are also disproportionally affected by genetic drift (Kimura 1983). This is because random losses of genetic variation, more easily shifts the overall genetic pattern in small populations than in larger ones.

Finally, inbreeding increases in small and fragmented populations (Brook et al. 2002). When there are less individuals, there is a higher change of mating with a relative. Inbreeding can result in decreased fitness due to increased homozygosity of deleterious recessive alleles (Hedrick & Kalinowski 2000). This can lead to inbreeding depression. As inbreeding increases, large regions of the genome can become homozygous. These regions are known as Runs of Homozygosity (ROH) (Ceballos et al. 2018). Inbred individuals can have reduced overall fitness and lower fecundity.

Risks from stochasticity, genetic drift and inbreeding are aggravated when the population become increasingly geographically isolated and meta-population processes[4] are hampered. When populations become increasingly small, it is theorised that they may enter an 'extinction vortex' (Gilpin & Soulé 1986). Genetic rescue through translocations is often put forward to counter this. However, this is not a straightforward solution and is not suitable in all situations. Genetic information regarding population structure is required to do this effectively (c.f. Chapter 1.1.5).

It is important to note that even though (very) small populations run an increased risk of extinction, they may very well recover, either "naturally" or with the help of effective conservation actions. A common perception is that recovered populations have an inferior gene pool causing inbreeding depressions and unhealthy birds. However, some highly inbred populations do not suffer from inbreeding depression, e.g. Island Foxes *Urocyon littoralis* (Robinson et al. 2018). This shows that populations that have gone through a population bottleneck (a period of small population size) can be sustainably healthy. Consequently, the idea that small and/or recovering populations need "genetic improvement" is not necessarily true and requires genetic evidence before acceptance and interventions. One main reason for this document is the need for thorough DNA sampling and individual-level genotyping of individuals in small populations in order to inform conservation actions.

[4] Meta-population processes describe extinction and recolonization events in an "archipelago" of suitable habitats in a matrix of unsuitable habitats.

1.1.2 Population structure and phylogeography

All natural populations[5] of birds exhibit variation, morphologically, physiologically, behaviourally and genetically (Fig. 1). This variation can be completely random ("white noise"), but usually, some significant structure can be detected[6]. With modern DNA methods on adequate sample sizes, almost all populations show genetic structure (e.g. de Jong et al. 2019). For conservation and management, it is usually important to link genetic variation to other relevant traits, e.g. morphology, demography, feeding behaviour or migration patterns. In combination, these traits can characterize Evolutionary Significant Units ("ESUs", Moritz 1994) and Management Units ("MUs", Marjakangas et al. 2015).

The main mechanisms that create population structure are some form of reproductive isolation followed by genetic drift or adaptation. Typically, this isolation is the result of a geographic barrier (e.g. a mountain range, a sea or shear distance), but it can also be an effect of differences in e.g. behaviour, habitat choice or phenology. From the original common genetic diversity, separated (sub)populations will be affected differently by random genetic drift and thus, differentiate. Consequently, the overall population becomes structured.

In the process of differentiation, separated populations are thought to adapt to their specific environmental conditions through divergent selection and thus, become locally adapted (Hereford 2009, Blanquart et al. 2013, Whitlock 2015)[7]. This, in turn could make individuals (and their genes) maladapted to foreign environments, e.g. in translocation programs. These immigrants, possibly together with the receiving population, can then suffer from outbreeding depression (Frankham et al. 2011). In highly mobile birds with complex phenotype-habitat interactions, significant levels of local adaptation are not likely to evolve (Kawecki & Ebert 2004). In Southern Dunlin *Calidris alpina schinzii*, Rönkä et al. (2021) showed genetic differentiation between isolated (relict) populations, but this differentiation does not prove that local adaption exists (Coop et al. 2010). Future genetic studies may change this, though (Savolainen et al. 2013, Tiffin & Ross-Ibarra 2014, Yeaman 2015, Hoban et al. 2016). Even without confirmed local adaptations, genetic studies of Eurasian Curlew population structure are important, e.g. for finding Evolutionary Significant Units meriting conservation effort (Moritz 1994). Although Rodrigues et al. (2019) could not demonstrate population structure; we predict that further studies - based on new techniques and more samples – will unveil significant differentiation across the range of the Eurasian Curlew.

[5] In this document we define a "population" as any assembly of specific individuals. Usually, but not necessary, populations relate to a specific geographic range. A population can include all specimen of a species, but may also refer to sub-units, e.g. populations of sub-species or locally defined groups. Populations and the taxonomic units they relate to are human constructs and thus, must be properly defined for their specific context.

[6] Detection chances tend to increase when sample size increases, analysing methods improve and more traits are taken into account.

[7] Obviously, the concept of local adaptation is relevant mainly for plants and less so for mobile organisms, particularly migratory ones.

Contemporary population structure relates to the question how this structure evolved, i.e. phylogeography. A classical model for phylogeography describes how species spread from refugia during the last (Weichselian) glaciation that ended roughly 10,000 BC, into areas freed from permanent ice cover (e.g. Weiss & Ferrand 2007). For the Eurasian Curlew, this scenario is probably not a good description of how current populations arose, because (a) the Weichselian glaciation left most of Europe and adjacent parts of Asia free from permanent ice cover, and (b) historic evidence shows that Eurasian Curlew populations have shifted their distribution repeatedly over the last centuries alone. Other processes, e.g. human induced habitat changes, are more likely to have caused current populations structures and thus, make broad-perspective studies of phylogeography highly relevant.

Figure 1. Exposé of Eurasian Curlew eggshell colouration. Probably a sign of genetic variation, possibly even population structure. Photos: Adriaan de Jong.

1.1.3 Migratory connectivity

The current use of the term "migratory connectivity" is a bit confusing. Originally, the concept referred to "the degree to which two or more periods of the annual cycle are geographically linked" (Boulet & Norris 2006). The strength of the connectivity was the proportion of the focal population that connects a starting point with one or several endpoints of a migratory event (c.f. Webster et al. 2002). For Eurasian Curlew DNA studies, this interpretation of the concepts relates to two methods. First, a genetically "unique" individual can provide evidence for the linkage (the migratory connectivity) between two (or more) sites where it was recorded. This, for example, would be the case when DNA from an egg-membrane sampled in the breeding area could be shown to come from the same individual as a Eurasian Curlew caught and sampled in a wintering area. Here, the intrinsic DNA profile functions similar to external markers, e.g. coded rings. Obviously, the resolution of the DNA analysis must be high enough to make the risk of mixing up individuals negligible. Microsatellites, SNP-panels and whole-genome sequencing match this requirement.

Secondly, the DNA profile of an individual Eurasian Curlew can demonstrate migratory connectivity if its profile matches the unique DNA profile of a known source population. For a potential source population to be convincingly distinct, the relevant variation in DNA profiles within this population *relative **to** other population's profiles* needs to be thoroughly described (e.g. Norris et al. 2006). This, in turn, requires a trustworthy sampling scheme. Assume the assignment of an individual with DNA-profile "A" builds on the fact that this profile "A" is widespread in one potential source population, but considered absent in all other populations. This assignment is only valid if the presences of "A" in all other populations is highly (significantly) unlikely based on broad sampling across the geographic range of all populations. With the use of multiple profiles (i.e. more or longer sequences), assignment accuracy can be improved, but the need for thorough genetic information about all potential source populations remains. For this class of migratory connectivity studies, microsatellites, SNP-panels and whole-genome sequencing can work.

Nowadays, the "migratory connectivity" concept is usually focused on the potential effects of individuals from various populations mixing[8] at one or several nodes in the migratory network (Somveille et al. 2021). The underlying idea is that meeting individuals can form pairs, mate and exchange information[9]. Obviously, for any of this to happen, the individuals need to come close, spatially and temporarily, and have the capacity and "will" to interact. Even here, the level of migratory connectivity is measured by the proportion of individuals going to different places (Kölzsch et al. 2019, Piironen et al. 2021). For studies of population genetics, and thus conservation and management, knowledge of genetic mixing in migratory connected systems is important (Taylor & Norris 2010). This is because if physical mixing leads to genetic mixing, this counteracts inbreeding and the formation of population structure. Some genetic effects of migratory connectivity can be estimated from observations of tagged birds, but genotyping an adequate collection of individuals at various nodes in the migratory network will provide better data. Whole-genome sequencing and SNP-panels are suitable techniques, but even microsatellite studies can answer many questions.

[8] Essentially, this mixing is an **effect** of migratory connectivity rather than a characteristic *per se*.
[9] Information-sharing is not limited to conspecifics (c.f. Madsen et al. 2023).

1.1.4 Population size estimates

Theoretically, individual-level genotyping can form the basis of population size estimates. The reliability, and thus the usefulness of such estimates, depends on adequate knowledge of the range of the population(s) and the quality of the sampling scheme. Given the current state of knowledge of EC population structure and migratory connectivity, and in combination with the high costs of a proper sampling operation, we discourage from the use of genotyping data for population size estimates except in very special situations, e.g. for small and isolated breeding populations[10].

Population sizes are often expressed by an estimate of Effective Population Size (N_e). Estimates of N_e are typically smaller than the results of censuses, partly because not all individuals actually participate in reproduction. Estimates of N_e can be made in many ways and depend on various assumptions about mating systems, genetic drift, etc. (Wang et al. (2016) and Hohenlohe et al. (2021) for details). N_e estimates can also aid in the understanding of genetic drift and inbreeding, and are an important parameter in conservation genetics.

1.1.5 Genetic effects of headstarting, re-introductions, and transfers of eggs or chicks

When Eurasian Curlew populations get alarmingly small, concrete measures to boost the population are regularly considered appropriate and urgent, regardless of whether the drivers of decline/deficiency are fully known or not. Adding new individuals to an existing small population is commonly called headstarting[11]. When the original population has gone extinct, the term re-introduction applies.

Either way, the conservation action involves the harvest of eggs from a natural population or, more seldom, from a captive breeding population. The eggs are then put in an incubator, outside the reach of predators and other threats. Finally, the eggs are placed back into the original nest[12] shortly before expected hatching **or** hatched in captivity for later release as chicks/juveniles (Colwell et al 2020, Eaton et al. 2020, Loktionov et al. 2023).

The egg-collection process as such has population-genetic consequences, because it boosts the genetic fitness of the collected eggs relative to the non-collected eggs (that are under predation risk). When eggs are repatriated to their original nest, no direct genetic transfer has occurred. Indirectly though, the genes of potentially inferior parents are artificially kept in the population instead of being weeded out by natural selection. In the longer run, this could cause genetic degradation.

More obvious effects can be expected when human-raised chicks are released into the wild. If chicks are released in the same area from where the eggs were collected, no other effects than the "missed parent-test" mentioned above will occur. If, on the other hand, chicks are released away from their area of origin, the future genetic structure of the receiving **and** the original populations are affected[13]. Based on our current knowledge, the strengths and consequences of these effects are impossible to know or estimate. The motives behind foreign releases may very well be considered to outweigh the possible genetic risks, but these risks need to be acknowledged and all released birds should be fully genetically documented. For

[10] These, on the other hand, can usually be conveniently censused with observational methods.
[11] The terms "augmentation" and "supplementary releases" are also used.
[12] The parents must be lured to incubate dummy eggs so they will accept the artificially incubated eggs later.
[13] Theoretically, the egg-harvest from the donating population could be completely random. In reality, this is a very unlikely event.

genetic documentation, immediate genotyping is not necessary, but high-quality samples from each individual need to be collected and properly stored.

Foreign releases can be a tool to improve the genetic composition of the receiving population. This is a viable argument only if evidence has shown that the genetic structure of the receiving population is truly impoverished and the released individuals really add new alleles.

In an ideal scenario, head-starting would be unnecessary because bird populations would be restored through more traditional conservation strategies such as predator control and habitat management. However, these measures take time and often involve co-operation of several stakeholder groups, including less conservation-minded bodies. In scenarios where traditional conservation measures do not work or cannot work in time, head-starting may be a necessity. If so, the population genetic consequences need to be carefully considered in the project plan and all genetic aspects thoroughly documented, i.e. all manipulated eggs and individuals should be DNA sampled.

Table 1. Overview over potential Eurasian Curlew DNA sources with related features. Source types are numbered for reference in Table 2.

Source type	Invasiveness	Sampling amount	DNA yield quantity/quality	Comment
Blood (1)	very high	< 1 ml	largest / highest	avian red blood cells contain nDNA
Tissue from live birds (2)	very high	*ca.* 1 g	largest / highest	in special cases during surgery
Mucous tissue in body cavities (3)	very high	swab	small - medium / medium - high	medical standard
Feather – pulled (4)	high	whole feather	medium - large / medium - high	more invasive than you might think
Feather – shed (5)	none	whole feather	small - medium / low - medium	lower yield than pulled feathers
Egg-shell – outside (6)	low	swab	small – medium / medium - high	maternal first, soon from both parents
Egg-shell - calcareous (7)	none	*ca.* 10 mg powder	small – medium / medium - high	maternal DNA only
Egg-shell – membrane (8)	none	< 1 ml blood or *ca.* 2 g	medium - large / medium - high	sample as soon after hatching as possible
Egg - yolk (abandoned egg) (9)	none	*ca.* 5 ml/g	small – medium / medium - high	novel method, not yet validated
Nests (10)	none	swab or > 10 g nest material	small – large / low - medium	abandoned nests preferred
Faeces (11)	none	swab or whole	small – medium / medium - high	rarely found in the field
Regurgitated pellets (12)	none	whole	small – medium / medium - high	rarely found in the field
eDNA – water (13)	none	> 0.5 l water	small – medium / low - medium	novel method, not yet validated
eDNA – soil (14)	none	100 g soil	small – medium / low - medium	novel method, not yet validated
eDNA - peat or sediment (15)	none	> 10 g per layer	small – medium / low - medium	novel method, not yet validated
Dead specimen – museum (16)	none	*ca.* 1 g	medium – large / medium - high	toe-pad standard
Dead specimen – carcass (17)	none	*ca.* 1 g	medium - large / medium - high	preferably fresh
Dead specimen – embryo (18)	none	*ca.* 1 g	small – large / medium - high	preferably fresh

Table 2. Overview over Eurasian Curlew DNA study objectives and suitable sampling and analysis methods. DNA sources and analysis methods (three at most) are presented in top-down order based on combined features of availability, ease, cost-efficiency, invasiveness and information output. Coding of potential DNA sources in Table 1. Coding of analysis methods in sub-table below.

Objective	Sources	Analysis methods
In-breeding	3, 4, 1	C, D, C
Genetic drift	3, 4, 1	D, C, B
Population structure	8, 4, 1	C, B, A
Phylogeography	1, 3, 4	D, C
Local adaptation	1, 3, 4	D
Population history (e.g. bottlenecks)	1, 3, 16	D, C
Population size estimate	8, 4, 5	C, B, D
Migratory connectivity I[1)]	8, 3, 5	C, B, D
Migratory connectivity II[1)]	3, 4, 1	C, D, A
Migratory connectivity III[1)]	1, 3, 4	C, D
Documentation of conservation actions[2)]	3, 1, 4	C, B, D (+ bio-banking)
Documentation of harvesting	2, 4	C, B, D (+ bio-banking)
Pedigree	8, 5, 3	C, B, D
Mating system	8, 5, 6	C, B, D
Parental care	6, 5, 10	C, B, D
Longevity	5, 8, 6	C, B
Hidden/unknown presence	5, 14, 13	E, A
Archeology	14, 15	C, E, A

Analysis method	code
mtDNA	A
Microsatellites	B[3)]
SNP-panel	C[3)]
Whole genome sequencing	D
DNA barcoding	E

[1)] See Chapter 1.1.3 for description of migratory connectivity study types.
[2)] Collection of eggs, production of chicks/fledglings, release of eggs, chicks/fledglings (c.f. Chapter 1.1.5).
[3)] Currently, these methods have not yet been developed for the Eurasian Curlew, but can be in a near future when interest arises.

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1.2 DNA studies *IRL*

When you plan to spend money and time on a study of Eurasian Curlew DNA, you want to make sure the results match your needs. In this chapter, we present some general information that can help you to avoid disappointing results.

1.2.1 DNA quantity and quality

DNA is a common molecule in the biosphere, because it is present in most living cells, either as single paired set of nuclear DNA (nDNA) strings or as multiple copies of circular DNA in mitochondria (mtDNA). DNA is also constantly reproduced when new cells are formed. When cells die, their DNA content is usually dispersed into the surroundings where it gradually decays (c.f. Chapter 1.2.2). The period during which this DNA can be detected varies, but under favourable conditions, meaningful DNA fragments can be found and analysed after tens of thousands of years. Consequently, it is easy to collect samples containing DNA, but the trick is to make sure it is from the desired species or even individual, and of sufficiently high quantity and quality.

Mitochondrial DNA occurs in multiple copies per cell and thus, more copies are available for sampling. The shorter and circular mtDNA molecule is also more stable than the long linear nDNA molecules. Complete or large fractions of mtDNA are thus present in many sources, including faeces, soils and surface waters. Unfortunately, mtDNA can only be used for identification at species and subspecies levels.

The cell's single set of nDNA is a rarer commodity for sampling than mtDNA, but contains much more information. For successful analyses of nDNA you need to find sources closer to its origin (ideally clusters of living or recently deceased cells) and apply advanced analysis methods. In chapters 2 and 3, you will find a full description of sampling sources and an overview of analysis methods.

In many cases, problems with low DNA quantities can be solved with the use of Polymerase Chain Reaction (PCR) techniques (Mullis et al. 1986). PCR multiplies the DNA sequences in a sample and thus, makes them easier to analyse. Neither PCR nor any other currently available method can repair degraded DNA, though. If DNA **quality** is lost, it is gone forever.

Although PCR technology can do wonders and is generally robust, it also comes with potential pitfalls. Finding the right regime of heating cycles and temperatures can be challenging, but nowadays, suitable protocols are readily available. A more serious problem arises when the PCR process amplifies foreign DNA in contaminated samples. This foreign DNA can mask target DNA or become mistakenly interpreted as target DNA. Pre-PCR sample hygiene is extremely important.

1.2.2 DNA stability

Despite its molecular complexity and high information density, the DNA molecule is surprisingly robust and persistent. DNA from tens of thousands year old sources have been used for advanced studies, e.g. the complete sequencing of Neanderthal genomes (Fu et al. 2014, Prüfer et al. 2014, Pääbo 2015).

The main threats to DNA quality are microbial[14] decay, certain chemicals (e.g. Chlorine), UV light, and undulating/high temperatures. All microbes need moisture to start decomposing organic matter, including DNA. Consequently, DNA samples are safe in truly dry conditions. In dry climates and many heated/air-conditioned buildings, ambient humidity is normally low enough to store DNA samples (Appendix 2 & 3).

Generally speaking, all chemicals that are harmless to humans are safe for DNA outside the living body. In fact, the problem is often the opposite; it is hard to find chemicals that effectively remove DNA from lab-tables, equipment, containers, etc. UV light is often used to sterilize DNA labs and equipment.

Outdoors, the UV-component of sunlight has a negative impact on DNA quality of exposed sample surfaces, e.g. of faeces and eggshells. That is why such items should be sampled soon after deposition and/or from surfaces that were not or less exposed to direct sunlight.

At the end of the day, the quality requirements depend on the objectives of your study, and thus the analyses of choice. For some types of analyses (e.g. whole genome sequencing) top quality is vital while others (e.g. SNP panels) produce excellent results from poor quality samples (Chapter 3.5 for details).

Another aspect of DNA stability concerns changes while the DNA is inside the body of a living organisms. Once inherited from the parents (nDNA) and from the mother alone (mtDNA), DNA changes ("mutates") over the lifespan of the individual. Focus tends to be on mutations in gametes (egg and sperm cells involved in reproduction), but mutations occur in all types of cells. Mutations are so frequent that one or more can be found in most cells, definitely in multiple cells in the body of a bird (containing billions of cells all stemming from the initial fertilized egg).

Most of these mutations pass unnoticed (and are usually weeded out by the organism itself), but some persist and may cause altered functionality, sometimes cancer. Mutations are caused by a range of drivers, mainly chemicals (including oxygen!), viruses and virtually haphazardous copying and sorting errors. The changes also come in many different forms, from exchanges of single nucleotides (the As, Ts, Cs and Gs), via longer sections of the DNA chain to loss or duplication of entire chromosomes. For most DNA studies, the resulting within-individual variation is irrelevant, but when analysis methods become gradually more sophisticated (e.g. moving towards true whole genome sequencing) and fine differences between individuals become important, within-individual variation becomes a factor worth considering.

[14] The main types of "bad guys" in this context are bacteria and fungi.

For Eurasian Curlew DNA studies, the following stability aspects are of special interest:

- Mitochondrial DNA is much more stable (mutates less often) than nuclear DNA. Consequently, large portions of mtDNA are usually identical across whole populations at species or subspecies level. This means that selected parts of mtDNA are very suitable for telling apart specific (sub)species from other (sub)species. On the other hand, mtDNA is not suitable for identifying individuals or groups within populations. In short, mtDNA analysis is a blunt, but robust tool.
- Some parts of the nuclear DNA are expressed (used by the cell to produce molecules) and others are not. Expressed DNA can be under pressure of (natural) selection, which in this context means, has a stabilizing effect (= reduces genetic variation). Genotyping expressed DNA has a larger discriminative power than mtDNA, but if high discriminative power is important (e.g. for individual identification or pedigree studies), genotyping non-coding (not expressed) DNA is preferred. Microsatellites are non-coding DNA sections "suffering" from frequent mutations and thus, microsatellite analyses (Chapter 3.4) are very useful for the identification of individuals and small groups (e.g. in studies of population structure). SNP-panels (Chapter 3.5) exploit the fact that even minor mutations in expressed DNA can escape from selection pressure (at least temporarily) and are allowed to vary within a population. Variability for each SNP is low, but by combining many SNPs on an analysis panel, high discriminative power can be reached.

1.2.3 From DNA source to scientific evidence

In DNA studies, there is a process chain between the sampled material and all the way to the dissemination of the acquired knowledge. This chain needs to be fully transparent and reliable. Any link that can be questioned or falsified spoils the credibility of your study and thus, your case in conservation and/or science. If you cannot convincingly answer questions like: *Were the samples kept frozen uninterruptedly?* or *How did you avoid contamination?*, your conclusions are likely to be challenged. Maybe you should not even trust them yourself.

Ensuring high quality standards is mainly about experience, planning and careful work, but also about awareness and attitude. The stakes are high, but it is fully doable, even for semi-professionals and amateurs. Throughout this document, you will find a wealth of advice and suggestions that can help you strengthen the quality of your DNA project.

1.2.4 Access to analysis techniques

Although you could extract DNA in your kitchen, making proper DNA analyses requires specialized skills and fairly advanced equipment. Consequently, we advise EC conservation and management projects to affiliate with specialized laboratories for their analyses of choice. Some projects may have the economic muscles to buy services from commercial laboratories, but for most projects, collaboration with scientific institutions is the only viable option. Many laboratories/institutions have interest in genetic studies for conservation purposes and are willing to do analyses at reduced prizes, or even *pro bono*. Being able to offer shared authorship of scientific publications is often an effective door-opener, but this requires a sound scientific design of the whole study. The DNA sampling design is a core aspect of scientific quality. It is always wise to involve expertise in genetic analyses **and** biological sampling in the project planning process as early as possible (e.g. Zinger et al. 2019).

1.2.5 Cost-efficiency and future developments

Almost all projects have limited resources (i.e. money and manpower) for the DNA studies they wish to perform and thus, high cost-efficiency is a desirable goal. All types of costs should be included in study design decisions, including the "costs" of suffering "paid" by the individual birds in the project (c.f. Chapter 1.3). Due to the wide range of objectives, conditions and techniques, it is virtually impossible to provide precise guidance for how optimal cost-efficiency should be reached. It is a matter of awareness and careful consideration. Overall, neither overdoing nor "too cheap" will lead to acceptable levels of cost-efficiency.

It is often tempting to choose a fancy new method rather than an "old" one. Especially when you plan to publish your results in high-ranking scientific journals. Sometimes, new genetic methods are better **and** cheaper, but if they are more expensive, you need to make sure the extra costs are really motivated. Given a fixed budget, it may be better to have **more** samples analysed with a cheap method rather than **fewer** samples with an expensive one. But the opposite may also be true.

If samples are collected for future use (Chapters 2.1.5 and 2.1.6); it's wise to recognize that analysis methods tend to become better and cheaper over time. This means that collected samples can render more information and/or more samples can be analysed at the same price in the future. Without much exaggeration, the best strategy is to collect as many DNA samples as possible within given limits of time, packaging and storage. *A non-existing sample can never be analysed, but an existing (well preserved and fully documented!) sample may provide valuable information in times ahead.*

1.3 DNA projects in their human-societal context

1.3.1 Legal aspects

Your Eurasian Curlew DNA project needs to comply with a range of rules and regulations. You find an overview of the important ones in Appendix 5. A major problem is that these rules and regulations vary between societies and over time. Another one is that awareness and enforcement varies. Offences that you may consider futile can be seen as severe violations by others and *vice versa*. If your opponent has strong means to exert power, you may be in deep trouble. This trouble may even have an adverse impact on the goals of your project and in the longer run, jeopardize the status and future success of biodiversity conservation at large. In conclusion, obedience to laws and regulations may take some extra effort initially, but tends to pay off in the end through better results, less problems and more appreciation.

1.3.2 Ethics[15]

Probably even more important than the legal aspects are the ethical/moral[16] aspects of your actions. These apply on a personal and a societal level. Personally, you have to carefully decide what actions are right and appropriate in your own opinion. And skip any action that is not explicitly *right*. In a social context, you need to relate to the norms and regulations held by societies around you, from local to international. If your action upsets a passing neighbour, brakes the national law for ethical permissions, spoils international collaborations or makes your results unpublishable in serious journals, you jeopardize the goals of your own work. The problem is that norms and ethical regulations vary widely among societies and countries. In addition, levels of implementation and law-enforcement vary. Simply put, there exist very different ethical "cultures". You need to find out exactly what applies for your planned actions.

Harm-benefit analyses[17] are the cornerstone of ethical decision-making. They imply a careful evaluation of all the positive and negative effects. If the benefits clearly outweigh the harm, the action can be taken. If not, the planned action must be abandoned, but after modification, and a new harm-benefit analysis, the revised action may be accepted. A general problem with harm-benefit analyses is finding a common "currency" to measure harm and benefit (i.e. avoiding "comparing pears with apples"). Also, the analyses usually require estimates of uncertainty. Last but not least, the harms and benefits often involve multiple stakeholders, i.e. the subjected individual animal vs their species/population or human society.

The EU regulations for the use of animals in research and education[18] apply in all member-states, and many countries outside the EU have similar legislation. Most Eurasian Curlew range states have formal systems for ethical approval of actions that involve (wild) animals. The problem is that the regulations differ between countries regarding (a) the definition of what research is and what is not, (b) which species are covered, and (c) the minimum level of harm that calls for formal approval. The EU regulations apply a traditional definition of "research" (i.e. an institutional project with a plan, organization and a scientific goal) and a

[15] Please note that a number of ethical considerations are embedded in the legal aspects of conservation, management and research (c.f. Chapter 1.3.1 and Appendix 5).
[16] Simply put: we use "ethics" for the underpinning of norms and "moral" for how one follows these norms (c.f. Baard 2021)
[17] These are basically the same as cost-benefit analyses in economics and "cost"-benefit analyses in ecology. In the latter, "costs" can refer to e.g. lost foraging-time or minor injuries while benefits are ultimately expressed in higher survival and more offspring.
[18] https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:32010L0063

threshold level expressed as “the stick of a needle”. The EU-regulations prescribe firm standards for organisations, the competence of staff, the facilities and the methods used. Some countries, e.g. Sweden, have extended the animal welfare issue much further than the EU minimum standard, and there is an international trend to include more species and more contexts (i.e. not only research *per se* but also conservation actions) and to lower the threshold of acceptable harm.

This variation may seem alarming and confusing, but a pro-active approach for sound ethics in your Eurasian Curlew project is to embrace the “Principles of the 3Rs” and the “Culture of Care” concepts (see Textbox 5). Even when this is not (yet) strictly mandatory! In the context of this document, choosing a non-invasive instead of an invasive sampling method whenever possible is an obvious conclusion of recognizing the welfare of the Eurasian Curlews we aim to protect and manage sustainably.

Textbox 5

The Principles of the 3Rs and the Culture of Care

The Principles of the 3R’s were originally postulated by William Russell and Rex Burch (Russell & Burch 1959) for research using animals in laboratories. Nowadays these principles are applied in research on domestic animals and wildlife as well. A key feature is that the implementation of the 3Rs not only safeguards minimum harm to the animals, but also leads to improved scientific results. In the EU and many other countries, the recognition of the Principles of the 3Rs in research is mandatory[19].

The 3Rs stand for *Replace*, *Reduce* and *Refine*. Increasingly, animals used in research can be **replaced** by cell cultures, computer models, etc. In pharmaceutical and chemical research, these alternative methods are now commonplace and their importance is rapidly growing. At first glance, the *Replace* principle may seem inappropriate in wildlife research, conservation and management, but can often be reached by the use of non-invasive methods, e.g. the use of shed feathers instead of blood samples.

Through thorough planning, pilot studies and statistical (power) analyses, the numbers of individuals used in a study can be **reduced** to the lowest level needed for significant results. Taking samples from more live animals than strictly needed for the purpose of the study violates this principle. Meanwhile, the use of too few individuals is also condemned under the *Reduce* principle, because this leads to unreliable results and thus, the suffering of the animals will have been in vain.

Finally, the lives of individuals really needed for the study must be **refined** as much as possible. Refinement includes all measures that reduce the harm anticipated by the individual animals, including reduced handling and storages times. Research on wild birds still has a lot to learn about how this refinement can be implemented. The core concept of the implementation of *Refine* is the **Culture of Care**, the overall will and attitude to improve the well-being of the animals used in a project (c.f. https://norecopa.no/more-resources/culture-of-care).

The Principles of the 3Rs do not provide a fixed set of rules and regulations. Instead, they prescribe a process of continuous improvements leading to less harm to the individual animals and more benefit for the species/population and their environment (including human society).

[19] https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:32010L0063

1.3.3 "FAIR play"

The main goals of conservation and management actions are their effects on populations (for hunting even the reward of harvesting). The actions also generate fulfilment, and possibly income, for the people involved. Last but not least, all these actions can, potentially, generate valuable data, knowledge and insight. These "bi-products" are worth sharing with others.

In science, the acronym FAIR stands for Findability, Accessibility, Interoperability and Reuse of scientific assets (Wilkinson et al. 2016). Generally, this is about digital assets, but the concept could also be extended to include physical samples and extracts (see Chapter 2.1.6 about bio-banking). In short, it is about letting others built upon your experiences to make their future conservation actions more successful.

Obviously, thorough documentation and publication of *What was done* and *What the results were* are key in letting others (now and in the future) benefit from your experiences (Mills et al. 2015). If preparing a manuscript for a truly scientific journal is beyond your ambition/capacity, just make a simple report. Posting such documents to the Eurasian Curlew International Working Group and its newsletter is an effective way to disseminate your findings.

For measuring the effects of your actions, we strongly advise you to use a Before-After Control-Impact (BACI) study design (e.g. Lengyel et al 2023). In contrast to just a documented trajectory after a conservation measure has been implemented, a BACI study provides true evidence of the effectiveness of your project's actions. May sound like an over-the-top enterprise, but probably requires just a minor extension of your overall scheme (see de Jong et al. 2021 for hints on feasible monitoring methods).

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2 DNA sampling methods

In this chapter, we start with some thoughts and advice about DNA sampling in general (Chapter 2.1), and continue with an overview of Eurasian Curlew DNA sources and how to sample those (Chapters 2.2-2.9).

Figure 2. Proud producer and source of Eurasian Curlew DNA. Male walking on an airfield next to the breeding area in Spain. Photo: Jesús Domínguez.

2.1 Before you start sampling DNA

Whether you have a distinct goal for your DNA sampling or just want to be able to make use of any sampling opportunity, the following points can help you to become a well-prepared and efficient DNA collector.

2.1.1 DNA sources and sampling strategies

This document describes a wide variety of Eurasian Curlew DNA sources (Chapters 2.2 - 2.9), and anyone involved in a conservation or management project has the opportunity to collect useful DNA samples. If you decide to collect DNA samples, you may want to develop a "sampling strategy". A sampling strategy stands on two legs. The one is about *where* and *when* to collect DNA samples (the sampling design) and the other about all the practical issues that ensure collected samples can produce useful information (the sampling technique). This document contains ample information about sampling techniques in Chapter 2 and Appendix 2, 3 & 4.

Obviously, your sampling design depends on your (project's) objectives, resources and study environment, but it may also be wise to consider the statistical and scientific aspects of sampling. At the one end, you can choose to apply an opportunistic sampling design, i.e. collect whatever becomes available. At the other end, you can choose to apply a strictly randomized scheme based on a pilot study and power analysis. Most likely, your choice will lay somewhere in between. The essential question is whether your samples (and thus results) represent a wider population or not. A limited number of samples can very well describe a large number of individuals (a population), but for a generalization claim to be trustworthy, you need to make sure the collected samples are a representative subset of all possible samples. For example, a study of, say, Finnish DNA samples cannot claim to represent all Eurasian Curlews across their entire range. For trustworthy generalisations, samples from across the whole relevant range should be included. Sampling design is a well-established field in (bio)statistics (e.g. Quinn & Keough 2002). If you are not familiar with sampling design, we suggest you affiliate with a partner who does.

2.1.2 Where to find DNA

In the media, forensic "DNA hunters" are commonplace. In reality, DNA is almost anywhere and the trick is to find the *right* DNA for the purpose of your study. The word *right* concerns both quantity and quality of Eurasian Curlew DNA (c.f. Chapter 1.2.1). The good news is that developments in sampling and analysis techniques gradually make smaller quantities of lower qualities suitable for successful analyses, and that the species identification is readily built in the DNA itself. Soon, it may be possible to "sniff" Eurasia Curlew DNA from the air. The bad news is that widely spread detectable DNA may become a "contamination blanket" covering the target DNA you want to sample for your study.

2.1.3 How to pick it up

You cannot just pick up DNA as such, it is always embedded into or attached to something else. Chapters 2.2 – 2.9 explain how to collect DNA from various sources. The key issue is to avoid harmful contamination. Contamination with dirt and chemicals is undesirable, but is seldom a major problem (but see Chapter 3.1.3). Contamination with DNA from species other than Eurasian Curlews (including your own) should be avoided, but in most cases, foreign DNA does not interfere with analyses of Eurasian Curlew DNA. In conservation genetics, the main issue is cross-contamination = contamination of another Eurasian Curlew's DNA into the focal sample. When DNA from multiple individuals are mixed in a sample, the foundation of most important analysis methods collapse. For the analyses presented in Chapter 3, only DNA barcoding (Chapter 3.7) and some mtDNA methods (Chapter 3.3) can handle multi-individual samples.

2.1.4 Where to put it

After collection, the DNA sample needs to be kept in a way that facilitates storage, transport and, most importantly, preservation. How this should be done depends on the DNA source (Chapters 2.2 - 2.9) and future handling and analyses (Chapter 3 and Appendix 3).

Generally speaking, the simpler and cheaper, the better. In the cost-evaluation, handling times (e.g. for screwing off lids) and potential losses of valuable samples should be included. The whole work-cycle counts. It is wise to follow the standards developed for professional projects, but a cheaper solution may be a good alternative when you are on a tight budget.

2.1.5 Multi-purpose sampling

Whatever samples you decide to collect for your DNA study, it may be worth considering if the samples could be used for other studies as well, either now or in the future. These studies can be repeat or alternative DNA studies (Chapter 3), but also studies of e.g. stable isotopes, eggshell thickness, stress hormones and residues of pharmaceuticals and pesticides. More and more options keep emerging when new technologies become available at reasonable prices. An open attitude and some extra planning can create huge potential benefit for science and conservation at very low costs.

2.1.6 Bio-banking

Bio-banking means the storage of samples for future use[20], linked to proper documentation and the dissemination of information about available samples. These samples can be either complete samples or leftovers from the samples you already used. Keeping leftovers can even prove to be a lifesaver in case the original samples were compromised, or your results are challenged scientifically.

Professional bio-banking systems are costly because they focus on (deep)freezer storage and thus, may not be a viable option for *good-to-have* samples for uncertain future uses (c.f. www.isber.org). Instead, you can consider truly down-to-earth solutions, e.g. a box with well-documented feathers or swabs. Making your collection known and available is paramount.

[20] Museum collections are a traditional form of bio-banking, c.f. Chapter 2.9.1.

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2.2 Blood and tissue

Living cells keep their DNA in the best possible condition and thus, are the prime DNA source for any DNA study based on any analysis method. The downside is that sampling living cells is highly invasive and harmful for the birds.

2.2.1 Blood

Blood is the ultimate source of DNA, in particular because red blood cells in birds contain nuclear DNA[21] . Very small volumes (< 1 ml) of blood are sufficient for all the DNA analyses methods covered by this document. Unfortunately, blood sampling is highly invasive to the birds (c.f. Textbox 5) and, in most countries; blood sampling is strictly regulated and requires permission(s) (Chapter 1.3). Blood sampling also requires training, sometimes even a license. Make sure you and your project comply with all the rules before beginning to take blood samples.

At population level, blood sampling (*sensu* taking blood from veins) seems to have no or insignificant effect on survival, reproduction or behaviour (Sheldon et al. 2008, Voss et al. 2010, but see also Brown & Brown 2009, Orzechowski et al 2019). At the individual level, these statistically derived effect levels are not applicable, and effects like pain, stress, disrupted time schedules and reduced wellbeing must be considered. The problem is that these qualities are hard to measure/observe (c.f. Maho et al. 1992). The fact that procedures are common practice and included in contemporary guideline documents (e.g. Morton et al. 1993, Gaunt & Oring 2010) does not prove they are harmless (c.f. Textbox 6). Responsible conservation and management projects should give animal welfare the benefit of doubt whenever possible and thus, avoid invasive blood sampling unless strictly necessary.

Blood samples can be taken from various places and in different ways. Blood can sometimes become available unintentionally from minor injuries during catching, storage or handling. Well-prepared projects are ready to collect this blood in (micro-)capillary tubes or on FTA cards (Fig. 3 and description below). Clearly, there are contamination issues attached to this “method”, but missing the chance of a “free meal” is always a pity.

In all other instances, blood must be collected from punctures in the skin, and often also the wall of a vein. These punctures open up the body’s protective shield and create risks for infections and unintentional after-bleeding. To minimize infection risks you must sterilize the place where the puncture will be made with an antiseptic solution (e.g. chlorhexidine) and minimize the size of the puncture[22].

[21] In mammals, red blood cells do not contain nuclear DNA.
[22] Repeated punctures are unnecessary and usually result from lack of experience or poor working conditions.

Blood under the skin occurs either in fine capillaries in soft tissues or in veins[23]. You can access capillary blood by making a small incision through the skin with a medical lancet (old style or "clicker-type", Fig. 3)[24]. You need to make the incision away from bones and major veins (and arteries!), typically in the exterior parts of the wing (e.g. near the base of primaries or secondaries). From the blood emerging from this incision, you collect your sample in one or a few micro capillary tubes (Fig. 3). Next, you transfer the blood in the capillary tube to either an FTA card (Fig. 3) or in a storage solution (typically >70% ethanol)[25]. You do so by connecting the sample-end of the vertically held capillary tube with the surface of the FTA card or the storage solution. The blood will simply leak out of the capillary tube. Blood on the FTA card or in solution can be stored at room temperature or frozen at -20° C[26].

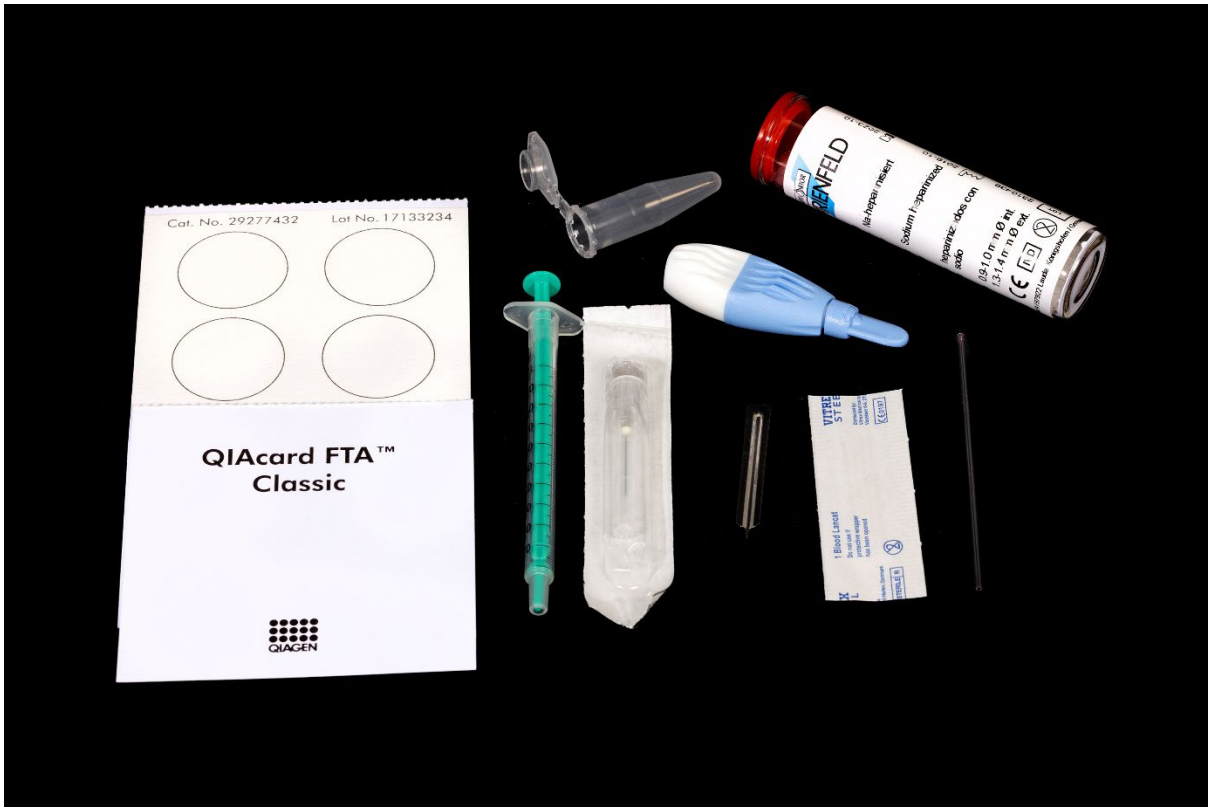

Figure 3. Blood sampling and storage equipment.
Left: FTA card for four separate or quadruple samples. The card comes in a readymade envelope for marking, storage and sending. The absorbing surfaces contain chemicals that effectively preserve DNA and many other features (e.g. antibodies).
Lower centre: 1 ml syringe with "25" gauge needle[27].
Lower right: Medical blood sampling lancet (open and sealed) and single-use skin puncturer ("clicker") for blood sampling from tiny skin punctures.
Right: Micro-capillary tubes for collecting blood from incisions or wounds.
Upper: Eppendorf tube for storage of blood samples in solutions (e.g. ethanol).

[23] Here we ignore the unlikely situation that your study prescribes that blood is taken from an artery or directly from the heart. We also ignore "exotic" methods like collecting blood from the growth-pad of toes through invasive toe-clipping (c.f. Owen 2011)
[24] You could also use the tip of a syringe to make a similar incision, but this takes a steady hand and good working conditions.
[25] Some labs require the blood is stored in EDTA vacuettes/vacutainers, not ethanol. Check with your laboratory.
[26] For storage in liquid nitrogen, pure blood can be stored in any suitable container.
[27] The higher the gauge number (code), the finer the needle.

Textbox 6

Blood extraction - How much? If at all.

Assuming your project aims to work for the good of Eurasian Curlew individuals and populations, ethical questions about invasive blood sampling are highly relevant. In line with common sense and the Principles of the 3Rs (Textbox 5) the overall strategy should be: **Try to avoid invasive sampling altogether, but if really needed, make the procedure as *gentle* as possible.**

The "gentle" notion has two aspects. First, the level of penetration. Making a puncture in regular tissue or a small capillary is preferred over a puncture in a major vein. Also, a narrow syringe or lancet is preferred over a thicker one. Secondly, the amount of blood (intentionally) extracted must be kept at a minimum. Eurasian Curlews are fairly large birds and applying the 1% of body weight rule-of-thumb would "allow" for the extraction of 5 ml or more. Current DNA analysis technologies do not require blood volumes at that level, though, and future techniques will certainly require even less (e.g. Voss et al. 2021). This means there is no reason to consider taking samples of more than 250 – 500 µl (≈ 0.25 – 0.5 ml) for DNA identification studies[28]. These volumes even allow for sample sharing and compensation for possible losses or instrumental failures.

The potential of non-invasive sampling methods is rapidly growing. Even for advanced techniques like whole genome sequencing, the requirements of sample sources are easing up (e.g. Schweizer et al. 2021). In fact, these requirements are much more about quality than quantity. Many non-invasive sources can provide high quality samples.

To sum up: *Blood taken from under the skin is nice, but often unnecessary and thus ethically inappropriate.*

Mild after-bleeding from the puncture after blood extraction can occur. You can reduce and finally stop after-bleeding by gently pressing dry cotton wool over the wound, just as a nurse at your medical station does. A small amount of ironhydroxisulphate powder applied over the wound can help[29]. Keep this "band aid" in place for *ca.* one minute and check if the bleeding stopped (Owen 2011). If the bleeding did not stop, the puncture was poorly applied and a veterinarian should be consulted.

Historically, syringe sampling from a vein was mandatory to provide the blood volumes required for earlier analysis techniques. Nowadays, sampling from a vein with a small syringe is still a viable option when your DNA analysis method of choice requires relatively large volumes of blood (c.f. Chapter 3)[30]. Veins are the low-pressure blood vessels that transport blood back to the heart. For blood sampling under outdoors conditions, three types of veins are available (https://lafeber.com/vet/venipuncture/). The jugular veins in the neck allow for quick extraction of large amounts of blood, but are technically difficult to sample, and sampling from them comes at considerable risks for the birds (e.g. Hoysak & Weatherhead 1991). Consequently, we strongly discourage blood sampling from the neck.

[28] Even for many other studies, e.g. studies of anti-bodies or stress hormones, sub-1 ml volumes are sufficient.
[29] Ironhydroxisulphate is sold in pet-shops for stopping bleeding after claw-clipping.
[30] **Never** re-use syringes for blood sampling. Used syringes can cause serious infections in the birds and come at a high risk of cross-contamination between samples (Chapters 1.2.1 & 2.1.2).

The brachial veins in the wings and the femoral veins in the legs are adequate alternatives for blood-sampling Eurasian Curlews (e.g. Kelly & Alworth 2013). In field ornithology, veins in the wings are most frequently used, mainly because veins in the thin legs of passerines are difficult to sample (Owen 2011). Even in Eurasian Curlew, the brachial vein is a good choice for blood sampling. The brachial veins are clearly visible under the thin skin of the underside of the radius ("arm"). The disadvantage of brachial veins over femoral veins is that they are shorter and winding and lay embedded in complex/flexible tissue. The femoral vein in the metatarsus of the leg is long, straight and embedded in a simple structure. Also, the leg-development is advanced over wing-development and thus, the femoral vein is more easily sampled than the brachial vein in small (< *ca.* 10 days old) chicks. Unfortunately, the femoral vein is covered by non-transparent, tough skin. Owen (2011) reports that sampling from the femoral vein has a lower risk for hematomas than sampling from other veins, but on the other hand, a wound on the leg is more exposed for infection risks from the environment than a wound on the wing. Overall, the choice between sampling the brachial and the femoral vein seems to be more about tradition and former experience than science.

For metatarsal and brachial vein sampling, you can use a 1 ml or 3ml syringe with a 25-gauge needle[31]. For extra safety, we suggest you use syringes with luer lock function (check with your lab or supplier). Move the syringe plunger up and down several time to prime it and ensure the plunger is completely down before inserting into the vein. This method requires a handler to hold the bird and for someone else to extract the blood. Insert the syringe needle in a shallow angle with the bevel side facing up (= the pointed side down) and in the direction of the blood-flow (= pointing towards the body/heart). Make sure the tip of the needle is inside the vein when you start to draw blood into the syringe. Once the desired amount of blood is withdrawn (Textbox 6), you slowly remove the syringe. Prior to extraction, you can apply a non-adhesive bandage pad or cotton ball to lessen bleeding. To encourage clotting and thus, prevent excessive after-bleeding, you should keep gentle pressure on the pad for *ca.* one minute after the needle was released, e.g. Owen (2011) for further details. *Again, it is important to highlight that blood extraction should be undertaken by trained individuals* (Fair et al. 2010).

From the syringe, you transfer the blood sample to an FTA card or a container (Fig. 3). There is no need to remove the needle to do so. FTA cards can be stored dry at room temperature (Textbox 7). For storage in containers (e.g. Eppendorf tubes or vacutainers), you should mix the blood with a storage medium, i.e. ethanol or lysis buffer[32]. Depending on planned duration and further processing, you can store these "wet samples" at room temperature or frozen (c.f. Appendix 2). Di Lecce et al. (2022) provides a useful overview over preservation methods for blood and DNA samples.

[31] A thicker 22-gauge needle is also an option.

[32] You could quick-freeze the blood sample alone, but this will probably lead to clotting and make future processing difficult.

Textbox 7

FTA cards for blood sample storage

FTA cards provide a secure and convenient way to take care of blood samples. The absorbing "paper" contains chemicals that preserve the sample and protect it from degradation by bacteria and fungi. The wrapping adds physical protection and allows for marking and labelling (Fig. 3 and Appendix 4). Some designs can be safely sent by snail-mail, but others need an additional envelope.

You transfer the blood from a syringe or a capillary tube by connecting the open end gently to the active surface of the card. The blood is automatically drawn from the syringe/tube into the papery substance. Stop adding blood as soon as the blood is no longer quickly absorbed. The card should be surface-dry directly after loading. If more blood is available, transfer it to a new card (or well on the same card). Overloading FTA cards significantly increases the risk of cross-contamination between samples and poor DNA quality of the surplus volume on the surface of the card (where it is out of reach from the protective chemicals).

FTA cards with DNA can be kept dry at room temperature for months and years without significant loss of DNA quality, but can also be stored frozen (Appendix 2). For analysis methods with very high quality demands, i.e. current whole-genome methods (Chapter 3.6), the use of FTA cards can be questioned. For most other analysis methods, FTA cards are truly appealing.

Standard kits are available for the extraction of DNA from FTA cards. In most cases, only a small fraction of the card is needed, leaving the rest for back-up or bio-banking.

2.2.2 Body tissues

Fresh tissue is an equally good source of avian DNA as blood (Chapter 2.2.1). Tissue sampling from live birds is even more invasive and formally restricted than blood sampling, but may be feasible under certain conditions, e.g. when surgery is made for therapeutic reasons. Full compliance to rules and regulations is paramount (Chapter 1.3 and Appendix 5).

All internal body tissues contain high concentrations of mitochondrial and nuclear DNA (Wong et al. 2012) and thus, you only need small quantities (ca. 1 g ≈ 1 ml ≈ "little finger tip halfway up the nail") of tissue for DNA analyses[33]. You can use a DNA-free scalpel or scissors (Fig. 4) to remove the tissue sample from a non-contaminated part of the body, and DNA-free tweezers to transfer the sample to the storage container. You will probably need to open up the body under anti-sceptic conditions to reach a non-contaminated surface[34] from which to sample. You can preserve tissue samples at -20° C, preferably in ethanol, but some special (non-DNA) analysis methods may require ultra-freezing (c.f. Appendix 2).

[33] Obviously, larger samples can be taken when supplies and storage capacity permits. If you plan to use 1.5 ml Eppendorf tubes for storage, your tissue sample needs to be significantly less than 1 ml to ensure the protective solution (probably ethanol) will effectively cover and conserve the sample.

[34] Probably, a moderately contaminated tissue surface will do when your analysis method is not extremely demanding. Most likely, the sheer amount of target DNA in the sample will override any significant effect of the contamination.

Skin can be viewed as a special case in this context. Dead and dying cells with good quality DNA are constantly released from the surface of animal skin. These cells can be collected by swabbing the skin surface without harming the bird (Textbox 8 and Chapter 2.4.3). Swabs can either be stored dry in paper bags at room temperature or under >70% ethanol.

You can even collect useful DNA samples from tissues in carcasses and museum specimen; see Chapters 2.9.1 and 2.9.2, respectively.

Figure 4. Equipment for sampling tissues and solid objects (e.g. eggshells).
Top: Disposable gloves. For Eurasian Curlew DNA sampling, the use of expensive medical grade gloves is overkill. Chances that fresh gloves contain wader DNA is practically null.
Bottom from left to right: 1) Needlework-type scissors are very suitable for extraction of tissue samples. 2) Metal tweezer for multiple use. They allow efficient disinfection/DNA removal. 3) Scalpel with removable blade. With intermittent DNA cleaning, you can use blades multiple times if cost is an issue. 4) Individually wrapped tweezers. Even those can be DNA-cleaned and thus re-used multiple times

2.3 Mucous tissues and external body fluids

Cells on the surface of mucous tissues are loosely attached and can be easily collected by mild abrasive force of swabbing. Living cells are also present in fluids produced on these surfaces or in embedded glands and can be sampled directly (in combination with mucous tissue sampling) or later from places in which the fluids were deposited.

2.3.1 Mucous tissues

You can sample mucous tissue surfaces in the mouth and the cloaca[35] with buccal swabs. A wide range of dedicated swab types are commercially available, but simple and cheap "tops" work well in Eurasian Curlew studies (c.f. Textbox 8 and Fig. 5). You can store swabs dry in a paper bag or in >70% ethanol at room temperature (c.f. Appendix 2). The swab will contain small to moderate quantities of high-quality DNA.

2.3.2 External body fluids

Compared with mammals, birds do excrete less fluid, e.g. saliva and tears, but Monge et al. (2020) demonstrated successful genotyping of DNA from saliva left on fruits partly eaten by parrots. If you happen to come across a sampling opportunity in your Eurasian Curlew project, you can sample the body fluid with a swab, similar to sampling mucous tissue surfaces (Chapter 2.3.1) or eggshells (Chapter 2.5.1). Like other swabs, you can store these samples at room temperature, either dry or in >70% ethanol (c.f. Textbox 8, Appendix 2).

[35] Theoretically even the ear- and nose-openings, but in birds, these are inferior alternatives.

Textbox 8

Swabbing

Most of us have seen crime-scene investigators in movies take swabs, and since the Corona virus pandemic outbreak, many of us have been "swabbed" for Covid-19 tests ourselves. Basically, swabbing is the collection of DNA from surfaces, including mucous tissues in body cavities, e.g. cloaca, mouth or nose (Handel et al. 2006, Vilstrup et al. 2018, Fig. 5). Be aware of the fact that swabbing mucous tissues is painful for the bird and must be performed with care.

When the surface is dry, re-wetting it with a gentle spray of clean water or ethanol improves sampling efficiency. Avoiding cross-contamination is important because DNA sampled by swabbing may be of medium to low quality and comes in small quantities. Wear disposable gloves!

After air-drying, swab-samples can be stored dry in room temperature. Alternatively, the sample material is transferred into a preservation fluid in a test tube (Fig. 3) and stored in accordance to the requirements of downstream treatment (Table 2). Make sure to properly mark all samples (Appendix 4).

Figure 5. Swabbing tools. Top left: professional forensic swab kit in dry storage tube. The bottom of the tube is permeable so you can store the sample dry at room temperature, if needed, in an outer container with desiccant (silica gel).
Left: Regular cleaning tops. A viable alternative for many DNA studies, suitable for swabbing tissues and surfaces (e.g. eggshells).
Centre and right: Barbed toothpicks are suitable for swabbing soft surfaces, e.g. faeces, but also mucous tissues.

2.4 Feathers

Birds produce large numbers of feathers and these are an excellent source of good to medium quality DNA. Most of the DNA is found at the base of the shaft (rachis). This DNA is easily extracted with standard extraction kits. DNA is also present inside the keratin matrix of the vane, but this fraction occurs in very low concentrations and is much harder to extract (Campos and Gilbert 2019). In the following, we ignore the DNA inside the keratin matrix.

2.4.1 Plucked feathers[36]

When pulling feathers from live birds (often harder than you may expect!), a small clump of tissue will come out with the feather. This clump is a rich source of high-quality DNA and can be viewed and treated as a tissue sample (Chapter 2.2.2). When (very) high-quality DNA is needed for your study, we suggest you separate the basal tip (+ clump) from the rest of the feather as soon as possible, and treat it as a high-quality DNA resource. For all other purposes, pulled feathers can be air-dried and stored dry at room temperature (Appendix 2 & 3).

Pulling feathers from live birds is highly invasive and strictly regulated in many countries (Chapter 1.3.1, Appendix 5). Pulling flight or tail feathers is particularly disturbing, both at intervention and by affecting flight efficiency and manoeuvrability during the re-growth period. It is not at all obvious that sampling large feathers is less harmful to the bird than blood sampling (from a vein). Despite this, there are several benefits to plucking feathers over blood (or tissue) sampling. These include less waste, easier storage, shorter handling times and less training.

The yield of DNA from feathers varies between species and seems to vary with size (Johansson et al. 2012). The type of feather also has an effect, with wing and tail feathers superior to body contour feathers (Villi et al. 2013). For most DNA-studies, there is no real need to sample large feathers, though. With modern extraction, PCR and analysis techniques, DNA from medium sized (>50 mm) body feathers or tail/wing covers will yield good results.

2.4.2 Shed feathers

Johansson et al. (2012) showed that plucked feathers yielded better DNA than shed feathers, but the latter can be collected without negatively affecting the birds. Shed feathers are available in many places throughout the year, but most feathers are shed during specific periods and often in specific moulting areas (e.g. Miño & Del Lama 2009). Based on basic knowledge about moult in Eurasian Curlew, large numbers of shed feathers can be found and collected with modest effort. Flight and tail feathers are shed after the breeding season, i.e. usually in staging areas. Body feathers are shed throughout most of the non-breeding period, but can also be found near nests (Fig. 6). Small downy feathers regularly occur inside the nest cup.

It is important to collect shed feathers as soon as possible to limit degradation (c.f. Chapter 1.2.2). Shed feathers can be conveniently short- and long-term stored dry in paper bags at room temperature. Although shed feathers usually lack the tissue-clump at the base of pulled feathers, the interior part of the shaft is a good source of DNA, particularly the blood clot after the growth-supporting vein in the superior umbilicus (Horváth et al. 2005, Vili et al. 2013). Wing and tail yield more DNA than smaller contour feathers, but feather condition has also shown to be important factor (Hogan et al. 2008, Vili et al. 2013).

[36] Obviously, pulling feathers requires that the birds is caught and handled. Unless this procedure is motivated for other reasons, its effects should be recognized in the harm-benefit analysis of the study (c.f. Chapter 1.3). In many countries, plucking feathers from live birds requires an ethical permission.

Figure 6. Shed feathers near a nest. Photo: Adriaan de Jong.

2.4.3 Growing feathers

Growing feathers are initially enclosed in a sheath that gradually erodes when the feather emerges. Flakes of sheath material can be sampled with swabs or small pliers from chicks or moulting adults. This material contains low-to-medium quality DNA, but it is available in significant quantities, and can be collected (usually without extra permissions) from birds that are caught and handled for other purposes (c.f. Chapter 1.3). Sampled sheath material can be stored dry at room temperature in paper bags or in a -18-20^{o} C freezer in any type of suitable container.

Growing feathers can also be pulled, although this is even more invasive than pulling fully developed feathers. Pulled growing feathers come with significant amounts of tissue cells and blood. Basically, pulled growing feathers can be viewed as fresh tissue samples and treated accordingly (Chapter 2.2.2).

2.5 Eggs and eggshells

You can sample DNA from eggs and eggshells, often non-invasively and/or non-destructively (Pearce et al. 1997, Strausberger & Ashley 2001, Trimbos et al. 2009). Sources of DNA include egg content, eggshell membranes, the outside of the eggshell and the hard, calcareous eggshell itself. The first two of those sources represent the offspring (the chick), but the latter two the parents. DNA inside the shell matrix originate from the mother alone, but DNA on the outside of the egg is likely to come from both parents.

Figure 7. Eggs are a good source of DNA. Here incubated eggs in an active nest. Photo: Adriaan de Jong.

2.5.1 DNA on the outside of eggshells

The outside of the egg is a viable source of good to medium quality DNA, albeit in small quantities (Schmaltz et al. 2006, Martín-Gálvez et al. 2011, Dai et al. 2015). Conditions differ between newly laid eggs and ones that have been in the nest and incubated for some time (Fig. 8). Either way, swabbing is the appropriate sampling technique (c.f. Textbox 8). Swabs can either be stored dry at room temperature or transferred to tubes with storage liquid (e.g. > 70% ethanol) and frozen.

During egg laying, cells from the female's oviduct and cloaca are deposited on the outside of the egg. If eggs can be sampled directly (within an hour or so) after laying, the outside of the egg is an excellent source of the mother's DNA. In the field, this means that only one egg can be sampled at a time and that sampling multiple eggs comes at the cost of repeated disturbances[37]. Under the assumption that the same female is the mother of all eggs, there is no reason to sample more than a single newly laid egg, but this assumption may very well be invalid due to polygyny and intra-specific nest parasitism (c.f. de Jong et al. 2021). Occasionally, newly laid eggs have small stains of blood on their surface. These stains are a particularly good sources of DNA and sampling should focus on those (Schmaltz et al. 2006).

In Eurasian Curlew, both parents incubate and thus, eggs are covered with DNA from both parents soon after laying, either from direct contact with the parent's bodies or mediated by the nest material. Consequently, swabs from eggs will contain DNA from two individuals (possibly more). For many studies (e.g. species or population identification), this may not be a problem, but if the identification of individual birds is necessary, you need to find alternative or supplementary DNA sources to identify parents (probably feathers, c.f. Chapter 2.4). The swabbing technique and sample storage is the very same as for newly laid eggs (c.f. Textbox 8).

Figure 8. Just waiting to be swabbed? Photo: Jesús Domínguez.

[37] If you plan to sample successive eggs, you need to mark the one you already sampled with e.g. a permanent marker pen.

2.5.2 DNA in the calcareous shell

During the final steps of egg formation, the shell (including the cuticle) is formed in the uterus, and in this process, some maternal cells and their DNA become embedded in the eggshell matrix. DNA concentrations in eggshells are very low, but the quality often remains well-preserved (Grealy et al. 2019, Oskam et al. 2010, Grealy et al. 2021). Useful DNA has even been extracted from museum and fossil eggshells (Grealy et al., 2019, Oskam & Bunce 2012). This DNA is solely of maternal origin[38].

For DNA extraction from the calcareous material, you need to turn the shell material into powder (Egloff et al. 2009). For empty shells (Fig. 9), this is a pure technical question (c.f. Oskam et al. 2010, Oskam & Bunce 2012), but for live eggs, the security of the embryo needs special attention. Although Eurasian Curlew eggs are large and robust compared with most passerine eggs (Martín-Gálvez et al. 2011), the removal of material **will** affect the functionality of the eggshell, with possible negative consequences for hatchability. From this, in combination with low yields and laborious extraction, we conclude that DNA from the calcareous eggshell is not a DNA method of choice for Eurasian Curlew conservation/management projects. In case, though, knowledge of the mother's DNA is vital, and no other means to collect it are available, then eggshells may be the only possible source.

Figure 9. For weeks or even months after hatching or predation, fragments of the calcareous eggshell are available in the nest. If genotyping of the female parent is important, these fragments can be successfully analysed.
Photo: Jesús Domínguez.

[38] This statement is true only if the extraction process efficiently excludes contamination from DNA on the eggshell surface and possibly, even from the pores.

2.5.3 DNA in the cell membranes

Eggshell membranes are a very useful DNA source (Pearce et al. 1997, Trimbos et al. 2009, Maia et al. 2017), particularly in Eurasian Curlew, where eggshells frequently remain in the nest after hatching or predation (Bellebaum & Boschert 2003, Rodrigues et al. 2019). It is advisable to sample nests "immediately" after hatching (Trimbos et al. 2009). Ideally, you should use separate disposable gloves for each membrane, but because membranes in freshly hatched/predated eggs lay inside its eggshell (Fig. 10), you can usually prevent cross-contamination by touching only the outside of the eggshell. If this is possible, one pair of gloves per nest is OK.

Membranes in newly hatched eggshells are an equally good source of DNA as fresh body tissue (Chapter 2.2). For transportation and short-term storage, you can put the individual eggshells with attached membranes either in individual paper bags/cardboard boxes or in commercial egg-containers, one per nest (c.f. Appendix 3). If individual-level genotyping is your goal, you should **not** immerse eggshells with membranes in ethanol, because this may mobilize parental/sibling DNA from the outside of the eggshell and contaminate the membrane sample.

At the inside of newly hatched eggs, you can often see blood vessels containing liquid blood. This blood is an excellent DNA source and you can collect it easily with a micro-capillary tube after puncturing the vessel (c.f. Chapter 2.2.1). With the right equipment, you can do this in the field.

Figure 10. Newly hatched egg with blood-filled vessels. The other eggs are still in the process of hatching, which often lasts several days. Please note the parental body feather, another viable DNA source. Photo: Adriaan de Jong.

Chicks and adults can turn hatched eggs into a “pancake” of membranes and eggshell fragments on the bottom of the nest (Fig. 11). The DNA content of this mess is still useful for many analysis methods. You can either collect the whole mass for treatment in the lab or try to separate individual membranes in the field (disposable gloves and DNA-free tools!). In individual-level genotyping studies, you need to carefully separate individual membranes and surface-clean them prior to DNA extraction, to avoid cross-contamination with DNA from parents and/or siblings.

When the membranes have become seriously fragmented after some time in the abandoned nest, the DNA content may still be useful. Under these conditions, you may prefer to collect the whole nest (Chapter 2.6) and to extract and treat membrane fragments in the lab. Obviously, DNA extracted from degraded membranes will be of low quality, and you will have to adjust analysis methods to this.

You can long-term store eggshell membranes individually in bags/containers with desiccant, at room temperature (Bush et al. 2005) or frozen (Trimbos et al. 2009, Appendix 2), alternatively in >70% ethanol at room temperature (Rodrigues et al. 2019).

Figure 11. After hatching, eggshell remains are gradually fragmented. Even small fragments are useful DNA sources. Photo: Adriaan de Jong

2.5.4 DNA in the egg content

From the outside alone, you cannot tell whether an intact egg is a) fresh, incubated or abandoned, b) living or dead, c) fertile or unfertilized and d) containing an embryo or not. The egg's temperature and a flotation test (de Jong 2021) can help to sort out fresh eggs from old ones. For further categorization, you need more information, probably through multiple nest visits or remote observation. When you decide it is safe to collect the egg (collecting living eggs is a No-Go!), we suggest you take the whole egg, stuff it in a suitable container (Appendix 3) and store it at -20 °C until it can be opened, sampled and DNA-extracted by a competent lab. Expect eggs to crack open during freezing and thus, make sure the packaging and handling prevents contamination and fouling.

The DNA content of an intact egg depends on its history. Unfertilized, fresh eggs contain a single set of female DNA (basically, the egg is a single cell). This female DNA is in the "germination point" at the side of the yolk and can be retrieved by skilled laboratory staff. It will be high in quality but very low in quantity. This is probably not your DNA source of choice. Under field conditions, unfertilized eggs will soon start to rot and its DNA to decompose. Overall, this makes unfertilized eggs a poor source of DNA.

A fertilized egg also contains paternal DNA, probably from several sperm cells, possibly from multiple males. Before the egg is laid, cell division has started and even newly/un-incubated eggs contain a minute embryo that can be sampled and forms a good DNA source. See Chapter 2.2.2 for sampling and storage of tissues.

Abandoned eggs contain embryos in various stages of development (depending on how long the egg was incubated before it was abandoned/died). If the embryonic tissues are fresh, they form an excellent source of DNA, equivalent to fresh body tissue (c.f. Chapter 2.2.2). More likely, the embryo has started to decompose and DNA quality decreased. Decaying embryos resemble carcasses found in the wild and can be used and handled similarly (c.f. Chapter 2.9.1).

Final remark. Dead eggs can be used for many studies, e.g. of senescence in very small, low reproducing populations (c.f. Textbox 9). Other information that can be gained from dead eggs concern: eggshell thickness, toxins, microbes, etc. No dead eggs should be left in the field.

Textbox 9

Senescence and unhatched eggs

In peripheral and isolated Eurasian Curlew populations, as on the Iberian Peninsula, senescence problems are growing and unhatched eggs are increasingly found in nests (Fig. 12 & 13, Domínguez & Vidal 2021). Senescence-driven reproductive failure can speed up population declines, and ultimately, contribute to local extinctions. Increasing numbers of unhatched eggs are also found in less extreme populations, e.g. in Germany (Salewski et al. 2020). Genetic studies may clarify the causes.

Figure 12. Hatched chicks together with an unhatched egg. Photo: Jesús Domínguez.

Figure 13. Unhatched eggs in a fledged nest. Photo: Jesús Domínguez.

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2.6 Nests

In addition to possible eggshells and feathers (c.f. Chapters 2.5 and 2.4, respectively), the nest material and the soil underneath the nest are potential sources of DNA. This DNA originates from both parents and, after hatching/predation, the chicks[39].

If the nest is still active, you can swab the inner parts of the nest-cup (c.f. Textbox 8) or collect some of the nest material/soil, but these is probably an inferior alternative to swabbing the eggs (Chapter 2.5.1). After predation or hatching, the whole nest-cup is available (Fig. 14). Use disposable gloves and transfer the nest material into a labelled paper bag (Appendix 4 and Fig. 24). If you see feathers or fragments of eggs, we suggest that you collect those separately (c.f. Pearce et al. 1997). The sample can be stored dry at room temperature (c.f. Appendix 2), but needs to be protected from invertebrates (c.f. Appendix 3 and Fig. 22). Although the quantity and quality of DNA in nest material is often fully acceptable for many analysis methods, extraction may be difficult and fine plant material may contain PCR-inhibitors. This makes nest material a sub-optimal source of DNA, but because sampling is easy and does not harm the birds, there is little reason **not** to collect it.

Occasionally, remains of dead adults or chicks can be found in or near a nest (Fig. 16 & 17). These carcasses are useful sources of DNA and well worth collecting (c.f. Chapter 2.9.1).

Figure 14. Predated nest, but still a potential source of Eurasian Curlew DNA (and predator DNA). Photo: Jesús Domínguez.

[39] DNA in nest material has also been used to identify predators (e.g. Hopken et al. 2016).

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2.7 Faeces and regurgitated pellets

2.7.1 Faeces

Faeces contain DNA of the depositor and DNA of food items (e.g. Waits & Paetkau 2005). Eurasian Curlew faeces are a source of medium-quality DNA, but unfortunately, they are rarely found in the field (Fig. 15). Decent quantities can be collected from curlew roosting sites, though (Lovas-Kiss et al. 2019). When sampling roosting sites, you are recommended to select places where monospecific flocks[40] stayed and to sample fresh faeces after the birds have flown away, paying attention to leave at least 1 m between the samples to minimize the risk of repeated sampling of the same individual (Lovas-Kiss et al. 2019).

During handling, Eurasian Curlew often defecate and thus, deliver sampling opportunities. Under such conditions, sampling faeces can be a less-invasive alternative to cloacal swabbing (Chapter 2.3.1). Finally, when birds are kept in captivity (e.g. in head-starting projects), faeces and pellets can be easily available, but so will be other DNA sampling options (Ramón-Laca et al. 2018).

Complete faeces found in the field usually show the distinct structure of a dark bulk (the poop) with a whitish smear on top (the urine). Depending on the down-stream handling and analysis, you can choose to pick up the whole unit or surface-swab it (c.f. Textbox 8). If the surface is too dry for swabbing, you can re-wet it with water or 70-90% laboratory ethanol. Tap water works fine, but deionized/distilled water is marginally safer.

To avoid cross-contamination, you should pick up each individual faeces with a new DNA-free tool or by hand protected by a new disposable glove (Fig. 4, Thalinger et al. 2022). If you plan to store your samples at -20° C or colder, almost any durable container would do, e.g. individual plastic bags, plastic tubes, etc. (see also Appendix 3). For dry storage at room temperature, we suggest you use plastic tubes with drying function, in combination with desiccation agent (silica gel) and vacuum-sealing (c.f. Fig. 5 & 22).

Figure 15. Faeces of Fieldfare *Turdus pilaris* on agricultural land in spring. Eurasian Curlew droppings are larger but have a similar structure. Photo: Adriaan de Jong.

[40] Faeces and pellets of unknown origin can be identified to species in retrospect with DNA methods, but, depending on your analysis strategy, this may require extra steps and thus, costs.

2.7.2 Regurgitated pellets

Eurasian Curlews, like many other birds, regularly regurgitate indigestible items, but these pellets are even scarcer than faeces in the field. However, they can be found in roosting sites and where Eurasian Curlews are kept in captivity, e.g. in head-starting projects.[41]

Preferably, pellets should be collected from roosts of monospecific flocks and shortly after the birds have flown away (Lovas-Kiss et al. 2019). The procedure for sampling and storing pellets is similar to protocols described for faeces (Chapter 2.7.1).

[41] In rare cases, regurgitated pellets of raptors and owls, as well as droppings of mammalian predators, will contain remains of Eurasian Curlew. Finding useful ones is so unlikely that we do not dwell further upon this potential DNA source in this document.

2.8 Environmental DNA

The fact that DNA from higher organisms persists in the environment, where it can be sampled, extracted and analyzed, has been a major technological and scientific breakthrough during the last decade (Piggott & Taylor 2003, Thomsen & Willerslev 2015, Beng & Corlett 2020). Significant amounts of an individual bird's DNA end up in the environment, but most of this environmental DNA (eDNA) rapidly degrades, and finally disappears. Compared to the quality of the DNA retrieved from blood, tissue and other non-invasive samples, environmental samples contain less template DNA, show lower amplification rates for genetic markers and are likely to be contaminated with non-target DNA (Emami-Khoyi et al. 2021). However, under favourable conditions, eDNA persists for sufficiently long time and in reasonably high concentrations to form a relevant source for DNA studies. Moreover, the sample collection is standardisable, is truly non-invasive, is often more cost-efficient than many traditional methods and in addition, surveys can be conducted independent of many environmental conditions, e.g. weather and season (Thomsen & Willerslev 2015).

eDNA-sampling is a growing field and studies based on avian eDNA are gradually emerging (Ushio et al. 2018, Day et al. 2019, Lewis 2019, Monge et al. 2020, Neice & McRae 2021), with recent contributions relating to wader species (Schütz et al. 2020).

2.8.1 DNA in water

DNA from birds can be found in natural waters, either in cell (fragment)s or colloidal-bound to particles. The DNA needs to be filtered out from the water body, either in the field or in the laboratory. When the water is rich in particles, a pre-filtering step may be necessary. Filters with DNA can be stored at -20^{o} C or dry at room temperature. DNA is extracted from the filter mass using standard extraction kits (Day et al. 2019, Ushio et al. 2018).

eDNA sampling for target species is readily available and fairly cheap, but concentrations are low which makes the technique sensitive to contamination risks and false-negatives. Moreover, the volume of water is also likely to influence the probability of DNA detection (Day et al. 2019). The decay of eDNA in freshwater beyond the threshold of detectability has been demonstrated to happen at a scale of days or weeks, enabling rivers and streams to transport eDNA over distances of hundreds of meters or even several kilometers (Thomsen & Willerslev 2015). eDNA fragments in seawater degrade beyond detectability within days and there appears to be a low probability of long-distance dispersal (Thomsen & Willerslev 2015).

2.8.2 DNA in soils

DNA from birds also leaks into soil environments. Local concentrations may be significantly higher than in water, although detection success in water vs. soil is likely to be site- and season-dependent (Feist et al. 2022). Prior to extraction, soil samples can be stored at -20º C or in a buffer solution at room temperature. Regardless of method, and to prevent cross-contamination, bleach-sterilized equipment and/or single-use consumables should be used between locations and sites (Feist et al. 2022). Specific soil DNA extractions kits are available (Feist et al. 2022, Neice & McRae 2021) and improved versions are gradually emerging. Multi-species analyses of soil samples are becoming commonplace (Chapter 3.7), but species-specific analysis techniques are still in their infancy. When soil DNA is your option of choice, we suggest you collaborate with a laboratory well experienced in this field.

2.8.3 DNA in peat and layered sediments

Peat and layered lake sediments are suitable environments for long-term preservation of DNA (Thomsen & Willerslev, 2015). They also have the advantage of providing a fixed time-line allowing for age-determination of samples. You need a core sampler to bring up peat and sediment samples (e.g. Garcés-Pastor et al. 2019). To preserve the vertical structure (the layers), you will often need to freeze the material around the probe and make special arrangements to keep things in place after surfacing the core. These advanced techniques were mainly developed in the field of paleo-botany and we recommend you to collaborate with people in this field if you want to gain access to this special DNA source.

Once surfaced, you can take a time-line of subsamples along the length of the core; taking regular precautions against contamination of presumably low-concentration DNA, i.e. DNA-free tools and disposable gloves. Freezing (or rather keeping them frozen) is usually the best way to store eDNA samples from peat and sediments.

2.9 Dead birds

Dead Eurasian Curlews can be found in the field, along roads and near nests (Fig. 16 & 17). Sometimes, Eurasian Curlews die during catching or handling in conservation projects. These and specimen in museums around the world are an important source of DNA for genotyping studies.

Figure 16. Remains after a dead chick found dead in the field. Photo: Jesús Domínguez.

Figure 17. Dead chick and unhatched egg at the nest. Photo: Jesús Domínguez.

2.9.1 Carcasses

Carcasses of dead Eurasian Curlews can be found under many circumstances, e.g. as road-kill or remains of meals of raptors (Fig. 18 & 19). Be aware of the fact that carcasses may fall under legal restrictions, c.f. Chapter 1.3 & Appendix 5. Handling carcasses also comes with human health risks and thus, protective gloves and careful hygiene are mandatory. Decaying processes start immediately after death and thus, you need to protect collected items from further decay as quickly as possible, either by freezing or under >70% ethanol. We recommend that you sample one or a few feathers from each carcass in parallel with sampling body parts (c.f. Chapter 2.4).

In most cases, you probably want to take the whole carcass to the lab for necropsy (Fig. 19), but with the right equipment (disposable gloves, DNA-free tools and containers); you can do this in the field.

When the carcass is reasonably fresh, you can take high-quality DNA samples from any soft tissue[42], e.g. muscle, heart or liver. You can then treat these samples like other tissue samples (Chapter 2.2.2). When the carcass has started to decompose just recently, you should try to sample tissue from the inner parts of large muscle groups (e.g. the breast muscles), because these are fairly well protected from initial microbial decay. From heavily decayed carcasses, you can still extract useful DNA samples, e.g. in tendons (sinews) or skin (c.f. Michaud & Foran 2011). You can store tissue samples from carcasses either in >70% ethanol at room temperature or at -20° C.

Bones (like eggshells and feather keratin) contain low quantities of well-conserved DNA (Tsai et al. 2019, Hong et al. 2020) and thus, you may want to save bone samples from carcasses, even from highly degraded ones. Bones can be stored dry at room temperature in paper bags or cardboard boxes (c.f. Appendices 2 & 3).

Depending on the state of the carcass, consider bringing it to a natural history museum for specimen voucher preparation (study skin, skeleton, spread wing, etc.). Associated meta-data are now commonly made available through various networks (e.g. VertNet[43], GBIF[44]), and could be used by other researchers on topics the initial collector did not think about.

[42] If fresh blood is available, this is an excellent source of DNA.
[43] http://vertnet.org
[44] https://www.gbif.org

Figure 18. Eurasian Curlew chick that died young. Cold and wet weather conditions may have contributed to its death. Photo: Jesús Domínguez.

Figure 19. Carcass of a radio-tagged Eurasian Curlew in the Mittelradde-Niederung (Lower Saxony, Germany). Necropsy showed typical signs of predation by a bird of prey like almost devoured pectoral muscles and typical marks at the sternum (right). Photos: Jennifer Düttmann.

2.9.2 Museum specimen

Museum specimen (Fig. 20) are also a potential source of (e)DNA (e.g. Mundy et al. 1997, Jackson et al. 2012, Russell et al. 2013)[45]. Museums are well aware of the extra value of having their specimen DNA sequenced and consequently, often willing to donate small pieces of skin or a footpad. The samples have been used for various questions from phylogenies to phylogeographics, and now almost routinely for whole genome resequencing (Segelbacher et al. 2014, Rodrigues et al. 2019, Tsai et al. 2019, Andrews et al. 2021, Iresdedt et al. 2022).

You can treat these old samples the same way as fresh tissue samples (Chapter 2.2.2), but the extracts will probably contain shorter fragments due to degradation. For example, a modern sample can yield extracted DNA sequences of 100,000 bp. In contrast, most museum skins will produce DNA sequences of 250 to 500 bp in length, sometimes even shorter. Time since collection is an important explanatory factor, but other factors that may have had an effect are preserving chemicals, humidity and temperature in the storage room, etc. Although DNA from museum specimen is not 'ancient DNA' *sensu stricto*, it is usually advised to use a separate room to handle historical DNA, to avoid contamination with modern samples, as well as the use of different sets of reagents. You will also need to adjust/complement the bioinformatics analysis to ensure accurate results.

Figure 20. Stuffed Eurasian Curlew used in a visibility experiment (Umeå, northern Sweden, 27th of April 2022). Photo: Adriaan de Jong.

[45] Please be aware of the risk of unreliable information about *where* and *when* the specimen was collected, particularly in old specimen from remote areas.

3 DNA analysis methods

3.1 Preparatory steps

Before the original DNA sample can be analysed, it must be treated in several steps, e.g. cleaned from unwanted fractions. How this is done depends on sample type, analysis method, objective, etc. Unless you are a DNA lab technician yourself, you will need help from an experienced lab to choose and perform the right steps. Similarly, the analyses will generate data that require expertize in bioinformatics and statistics to be turned into knowledge and insight. You probably need the help of partners in these steps, too. Although individual people can easily do DNA sampling, a full DNA project usually requires the collaboration of multiple partners.

3.1.1 The sampling-transport-storage-analysis-conclusion chain

The final quality and thus, the conservation and management impact, of your Eurasian Curlew DNA project is determined by the weakest link in the sampling-transport-storage-analysis-conclusion chain. If any step along the way fails or can be challenged, your results may be ignored and your goal may not be reached. Careful planning and documentation is paramount, together with doing things *right*.

3.1.2 Extraction and clipping (primers, enzymes)

You will need to extract the DNA in your original sample before it can be analysed further. You will also have to decide whether to do the extraction before or after long-term storage. DNA extracts usually require less storage space, but may be less durable (c.f. Di Lecce et al. 2022).

There are numerous techniques to extract DNA and their descriptions fall outside the scope of this document. You can find information about extraction techniques in most scientific publications and your lab probably has access to, or knows how to acquire, these techniques. This is a rapidly growing field and new "kits" become available on the market frequently, especially for "problematic" samples, e.g. DNA in soils or faeces. Many of those kits are expensive, and you need to keep an eye on their cost-efficiency (c.f. Chapter1.2.5).

Different extraction methods require different amounts of hands-on action. Unless you do this laboratory work yourself, labour costs tend to be a heavy burden on DNA projects. Modern labs often have highly automated equipment (robots) for DNA extraction. These are expensive, but may very well improve the cost-benefit equation of your project. Usually, these robots require special consumables (e.g. tubes). If these are already used in the sampling/storage process, extraction and further processes will be quicker and cheaper. Check with your analysis lab for advice about smooth throughput.

All analysis methods use advanced chemicals to clip (fragment) DNA chains and to label/mark the resulting fragments. Like DNA extraction, these techniques fall outside the scope of this document. If you are not a DNA lab technician yourself, you need to align with suitable competences at a lab.

3.1.3 PCR

The Polymerase Chain Reaction ("PCR") method has revolutionized DNA technology since the 1980s and is now a standard component in most analyses (Mullis et al. 1986, Martín-Gálvez et al. 2011). Simply put, PCR produces multiple copies of DNA sequences and thus, enhances the **quantity** of DNA but not the **quality** (fragment lengths). PCR is embedded in several of the analysis protocols covered in the following chapters and can also be used as a (semi) standalone method (e.g. Sorenson et al. 1999).

Tweaking the PCR parameters (i.e. changing temperatures and cycling features) can dramatically improve overall analysis results and thus, the delivery of the analysis method in relation to the objectives. For your Eurasian Curlew conservation or management project, it is important to find or acquire the necessary PCR competence.

Some substances can inhibit PCR amplification, e.g. plant fibres. You need to make sure to avoid including such substances in your original sample and/or to remove them during DNA-extraction. PCR-protocols that can handle inhibitors are emerging. We suggest you check-up with your DNA lab or search the web for recent developments.

3.2 Bioinformatics

The data produced by DNA analysis methods need further numerical analyses to make sense. This field of bioinformatics (including biostatistics) is rapidly growing and your Eurasian Curlew DNA project probably needs to consult experts for help. In particular, new (next-generation) sequencing methods require massive data processing to select informative SNPs (for SNP panels) or to stitch together information from DNA fragments (in whole genome sequencing). Oyler-McCance et al. (2016) provides a useful introduction to bioinformatics for ornithologists, but this field is rapidly changing and much has changed since the onset of their publication (Brandies & Hogg 2021). Check for the latest developments and team up with experienced partners before making crucial decisions.

Table 3

Cost for DNA analyses
(rough estimates of rapidly changing values)

Pre-analysis[46]:

DNA extraction and preparatory steps:	15 - 25 € / sample[47]
PCR:	5 € / sample

Development and analysis:

Method	cost (€)	
mtDNA analysis	15 – 30 / sample	
Microsatellite development	*ca.* 10,000	
Microsatellite analysis	*ca.* 20 / sample	
SNP-panel development	20-50,000	
SNP-panel analysis	*ca.* 30 / sample	
Whole genome seq., short reads	100 – 600 / sample	(+ bioinformatics)
Whole genome seq., long reads	*ca.* 2,000 / sample	(+ bioinformatics)
Reference genome development	*ca.* 5,000	
DNA fingerprinting analysis	*ca.* 25 / sample	

*Please note that the per-sample cost for DNA analysis depends on how many samples are analysed in a combined session. Usually, a complete preparation - analysis - post-processing cycle for a **single** sample is prohibitively expensive. However, through smart matching of batch sizes and laboratory capacity, you can probably bring down the per-sample costs to an acceptable level.*

[46] Costs for sample collection, documentation and storage are not covered here. These can be substantial.
[47] Costs vary slightly with analysis method. Modern technologies, e.g. extraction robots, save labour costs but add costs for kits, equipment, etc. The overall costs have been surprisingly stable.

3.3 Mitochondrial DNA methods

Studies based on mtDNA can benefit from the high number of copies per cell, its limited length (*ca.* 17,000 base pairs in curlews) and low mutation rate. The circular mtDNA molecule is also well-structured and thoroughly studied. mtDNA is inherited strictly along the maternal line (= does not contain information about the male that sired the individual). Genetic mtDNA variability is lower in birds than in mammals (Berlin et al 2007, Hickey 2008, Lane 2008).

mtDNA methods cannot distinguish between individuals on the same maternal line (e.g. full siblings) and are usually restricted to identification to the species or sub-species level (c.f. Chapter 3.7), and for low-tech, cost-efficient studies of population structure (e.g. Honka et al. 2017).

In ornithology, certain sectors of mtDNA have proven particularly useful (e.g. the "cytochrome C oxidase subunit") and matching chemicals (primers) and protocols are readily available (e.g. Sorenson et al. 1999, Ruokonen et al. 2000). After extraction and clipping, the focal fragments are sequenced (Sanger or NextGen). Specific sequences are diagnosed against a database of known taxa. Alternatively, the pattern of mutations (numbers and locations on the DNA string) for a number of individuals can be plotted and quantified to hint on clustering and evolutionary processes (c.f. Ruokonen et al. 2008).

Currently, there is no complete description of the mitochondrial genome of the Eurasian Curlew, but there is one for the Whimbrel (*Numenius phaeopus*) which can be used to select primers and for sequence alignment (Ding et al. 2016).

mtDNA based analyses are simple, quick and can be run on variable numbers of samples (no fixed batches). After method development and training, analysis cost per sample are in the range of 10-20 € plus labour costs.

3.4 Microsatellites

Microsatellites are regions with highly repetitive fragments of non-coding nDNA (Primmer et al. 1996). The number of repeats influences the migration speed during electrophoresis, with long fragments moving more slowly than short ones (Ellegren 1992). After staining, a pattern of bands becomes visible on the agar-gel. Microsatellite fragment lengths are calculated from measurements of migration distances relative to a mixture of standard fragments applied in the same run (Goldstein & Schlotterer 1999).

By cutting the original DNA with several primers (all relating to different places [loci] on the DNA string), multiple microsatellite-measurements can be made for each sample. By combining information from multiple loci (preferably >15), microsatellite analyses can distinguish individual birds at high levels of probability (1 per million or better).

Microsatellite-primers are species-specific[48] and rather difficult/costly to develop, but once developed, these primers can be used in labs with basic equipment (e.g. Kleven et al. 2016). Microsatellite analyses are labour-intensive, but fairly cheap (ca. 15 € per sample, virtually independent of the number of samples). They can be used for medium-to-good quality DNA samples (e.g. shed feathers). Currently, there are no primers for Eurasian Curlew, but with the help of new whole genome sequencing data, these could be selected and evaluated relatively easy.

[48] Primers developed for closely related species can sometimes be used (e.g. Honka et al. 2017)

3.5 SNP-panels

Single Nucleotide Polymorphs (SNPs) are short DNA sequences with a base pair that varies within the population of interest (typically a species). Due to frequent mutations (past and recent), millions of SNPs occur across the full DNA sequence of each species. SNP-panels use a smart selection of these SNPs to make a powerful diagnostic tool (e.g. Jonker et al. 2012, Spitzer et al. 2016, von Thaden et al. 2017, Giangregorio et al. 2019, Wilson et al. 2019).

The concept of SNP-panels is based on a three-step process (e.g. Norman et al. 2013). First, a carefully selected set of high-quality samples are RAD-sequenced[49]. Second, with bioinformatics, a number of highly informative Single Nucleotide Polymorphs (SNPs) are selected and validated for their discriminating power for the focal species. Finally, new samples are "matched" against all the SNPs in the panel. For the second step, a suitable reference genome is useful, but by no means necessary.

Currently, the numbers of SNPs per standard panel is *ca.* 90 (96 minus a few that are used for other purposes, e.g. sexing), each with its unique short DNA sequence. When the DNA of a sample matches a SNP (= binds to the DNA-fragment), this is indicated by fluorescence. The pattern of fluorescence points across the panel characterizes the genotype of the sample/individual. Because many samples can be run simultaneously (each with its own fluorescence-wavelength), high-volume, low-cost, highly-automated analyses are possible. SNP-panels can be used for samples of very low-quality[50] and better.

Compared with whole-genome sequencing, SNP-panels are sometimes blamed for wasting large amounts of genetic information. This may be true in some cases, but by focusing on highly informative sequences, SNP-panels provide low-cost analyses of virtually all sorts of DNA sources without repeated tedious bioinformatics steps.

The development of a 96 well SNP-panel in an experienced lab falls in the range of 20 to 50 thousand €. Once operational, the SNP-panel can analyse at a cost of *ca.* 20 € per extracted sample (given a full batch of *ca.* 90 simultaneously run samples). Currently, there is no SNP-panel for the Eurasian Curlew, but with the help of recent whole genome sequencing data, a SNP-panel could be developed in the near future.

Currently in Sweden, the vast majority of decisions made in conservation and management of mammal and fish populations are based on large-scale sampling and SNP-panel analyses.

[49] RAD-Seq stands for Restriction site-Associated DNA sequencing.
[50] Here meaning highly fragmented samples.

3.6 Whole genome sequencing

This class of DNA analyses aims at describing as much of an individual's DNA as possible (e.g. McMahon et al. 2014, Lou et al. 2021). This technique requires very high quality samples (typically fresh blood or tissue). After sequencing, DNA fragments are aligned and fitted together into very large segments of the entire DNA constellation in a massive bioinformatics process. Each sample is treated separately to produce individual DNA profiles. These can then be mapped against a high quality reference genome, if available. Currently, complete descriptions of the **whole** genome is a goal that has not yet been reached[51], but improved results are gradually delivered (Peona et al. 2018).

Prices for whole genome sequencing range depend on the requested output. For a short-read whole genome sequencing, analysis prices are 100 – 600 €, and for longer linked-reads analyses *ca.* 2,000 €. These figures refer to the cost of sequencing an individual sample in a batch analysis ("lane"). Batch sizes, and thus total costs, vary with instrumental setting. If your samples do not fill a complete batch, it is often possible to combine similar types of samples in the same batch analysis. There is a trade-off between number of samples in a batch and output quality (e.g. sequence lengths). In addition to the costs for sequencing *per se*, you need to add costs for sample preparation and bioinformatics (c.f. Table 3).

Regarding reference genome development, analysis costs are in the region of 5,000 € per sample (Theissinger et al. 2023).

Last but not least: Prices for NexGen sequencing continue to steadily fall, and with reference genomes for Eurasian Curlew well in the making, whole genome sequencing studies will become increasingly competitive.

3.7 DNA barcoding

DNA barcoding is an mtDNA-based[52] technique for the identification of species in a sample (Hebert et al. 2004, Kress & Erickson 2012). After extraction, cleaning, clipping and amplification, short fragments (typically 400-800 bp[53]) of the cytochrome C oxidase subunit are sequenced with Sanger or NextGen methods. The post-processed ("edited") nucleotide strings are then compared with taxonomically assigned strings in a database, e.g. the Barcode of Life Data (BOLD) system (Ratnasingham & Hebert 2013). A match is assumed to confirm the presence of the species in the sampled environment. If no match is reached, the DNA sequence is assumed to originate from an unknown, possibly new species[54]. Non-matching sequences can become the starting point of a haunt for the missing species, or just stored for future matching attempts. The power of the barcoding concept grows with the number of reference sequences in the database.

Being an mtDNA-based method, DNA barcoding builds on the advantages of mtDNA (i.e. multiple copies per cell, low mutation rates and well-studied genomes, c.f. Appendix 1 and Chapter 3.3), but lacks the power to efficiently detect within-species variation. DNA barcoding of a wide variety of sample sources can be used for presence/absence studies in Eurasian Curlew.

[51] Not even for the galGAL5 reference chicken genome, a model species.
[52] Theoretically, nDNA sequences could also be used.
[53] For eDNA samples, even shorter fragments can be used (e.g. ca. 200 bp).
[54] Special care must be taken to assure the results do not represent "false positives" or "false negatives".

With the use of longer or multiple sequences and more advanced databases, DNA barcoding evolves into DNA fingerprinting, commonly used in human forensic studies (Michaud & Foran 2011, Hopken et al. 2016). In this context, we refrain from describing this hybrid-technique in further detail. Instead, we suggest the use of microsatellites, SNP-panels or whole-genome sequencing for Eurasian Curlew studies where individual or group identification is important (c.f. Chapters 3.4 – 3.6).

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Basic information about the distribution, ecology and behaviour of the Eurasian Curlew is available in handbooks, e.g. Handbook of the birds of Europe, the Middle East and North Africa: Vol. 3 Waders to Gulls (Cramp & Simmons 1983), in national breeding bird atlases and on national and international websites (e.g. https://www.iucnredlist.org). Please note that some of the knowledge presented in these sources may be neither fully up-to-date nor scientifically evaluated.

*This reference list contains all texts mentioned in the text plus a selection of additional genetics-related entries that can be useful for Eurasian Curlew conservation and management projects. These latter are marked with * signs.*

DOIs are provided if available. Underlined DOIs in the electronic version of this document hold an external link to the source document. Accessibility to these documents depends on your/your organization's access rights. You can also copy the DOI to the search-field in your browser.

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Appendix 1

Avian DNA basics

Where DNA sits

Two types of DNA occur in animal cells: nuclear DNA (nDNA) and mitochondrial DNA (mtDNA). Nuclear DNA resides in the nucleus of each cell and is controlled, used and copied by the organism itself, i.e. the individual Eurasian Curlew. Cells contain one double set of nDNA, packed in chromosome pairs, one from each parent. The exceptions are the reproductive cells (gametes) which contain a single set of nDNA, because the chromosome pairs are split between gametes during meiosis.

Mitochondria are organelles inside cells and are inherited strictly along the maternal line (from mother to offspring). Originally, mitochondria were separate microorganisms that became incorporated and now live and reproduce, mutually integrated, in cells of most higher organisms. They are sometimes called "alien hitch-hikers". Mitochondria play a vital role in metabolic processes (e.g. energy conversions) and have their own set of DNA to control their work. Because cells can contain many mitochondria each, the number of mtDNA copies per cell, and thus per sample, are larger than for nDNA.

Although DNA is formed and used inside metabolically active cells, significant amounts can also be found in metabolic inactive body parts like feathers, nails, beaks and bones. From the outside of bird bodies, DNA can be sampled from the skin and the mucous tissues inside body cavities (cloaca, mouth, nose, eye and ear), as well as from the fluids excreted at their surfaces. Well outside their bodies, bird leave DNA traces inside and on the surface of eggs and faeces[55]. After death, DNA in carcasses gradually decomposes, but carcasses can remain a good source of DNA for a long period of time.

A growing field of genetic research deals with DNA away from living birds. This environmental DNA (eDNA[56]) originated from living birds, but has "escaped" from their bodies. If this DNA is retrieved a long time after it was released in the environment, it is called ancient DNA (aDNA). Well-known sources of aDNA are peatlands and lake sediments.

Avian vs mammalian DNA

Chromosomes are individually "packaged" nuclear DNA sequences that occur in pairs, one from each parent. Birds have more chromosomes than the average mammal (2n = *ca.* 80 in birds vs 2n = 40-60 in mammals), but many avian chromosomes are very short.

Mammals, and thus humans, have two equivalent (homomorphic) sex chromosomes (XX) in females and two different (heteromorphic) ones in males (XY). In birds, this condition is reversed. Male birds have two equivalent chromosomes (ZZ) while females have different sex chromosomes (ZW). Genetic sexing works equally well in birds as in mammals.

[55] Avian faeces contain both poop and urine, which are deposited separately in mammals.
[56] The term ancient DNA is used for DNA inside or outside the bodies of long dead animals. Because all eDNA is retrieved after it left the bodies of birds

The length of avian nuclear DNA is *ca.* one billion ($1*10^9$) base pairs (bp), while mammalian DNA has *ca.* seven times as many. In contrast, the length of the mDNA molecule is roughly the same in mammals and birds, *ca.* 17,000 bp.

Last but not least, an important difference between DNA in birds and in mammals is that red blood cells of birds contain nuclear DNA while those of mammals do not. This means that avian blood samples are very rich in nuclear DNA, while mammal blood are not. This has a major impact on the amount of blood that needs to be collected (Chapter 2.2.1).

What DNA does

DNA is the biochemical template for protein production, but large parts of the DNA molecule have no known function. This non-coding DNA just sits there and thus, is not under selective pressure. Some of this non-coding DNA consists of chains of repeated, identical short fragments, microsatellites. The length of these microsatellite chains varies and thus, can be used for individual-level genotyping (Chapter 3.4).

For the production of proteins, a section of the DNA string (a "gene") is copied single-sided to an mRNA string. This mRNA is released from the nucleus into the cytoplasm where it is used by ribosomes for protein synthesis from amino-acids. The order in which nucleotides occur in the DNA string, and thus the mRNA string, determine the order of amino-acids in the protein. This order, in turn, determines the physical and biochemical properties of the protein. Proteins form the building blocks of many biological structures and are the agents of many biochemical processes.

Last but not least, DNA needs to be copied in the process of cell division. If this division aims at producing two sister cells (mitosis), than all nDNA is duplicated and split between the nuclei of the new cells, chromosome by chromosome. For the production of gametes (eggs and sperms) during meiosis, all DNA is duplicated followed by the distribution of the chromosomes (now two pairs) among four new cells. These cells then contain only one set of chromosomes each.

The DNA-molecule

Sixty years after James Watson, Francis Crick and Maurice Wilkins received the 1962 Nobel Prize for the discovery of its double helix structure[57], DNA is a global celebrity in science. You probably know that DNA consists of chains of paired nucleotides (Adenine+Thymine and Cytosine+Guanine), fixated between strings of sugar-phosphates. We also know that the order of the nucleotides in the DNA orchestrates life processes and that DNA is copied to sister cells in growth and reproduction. Although the basic concept may seem simple, there are lots of bells and whistles, but those fall outside the scope of this document (c.f. e.g. Hedrick 2011).

For our understanding of the potential of DNA studies, the following properties of DNA are particularly important.

- In sampling and analyses, the amount **and** the quality of DNA matters. The amount in your sample will depend on how much DNA was available in the source (Chapter 2) and how much of it you were able to collect. Fully intact DNA molecules in living cells represent the top quality level (accessible through careful sampling from blood or living tissue), but for many studies, partly degraded DNA is fully useful, even in

[57] Shamefully ignoring the role of Rosalind Franklin.

small quantities. The DNA sequence fragments will be shorter, but much of the essential information is still there.

- DNA is are fairly stable molecule, but is broken down by e.g. microbes, UV-light and high temperatures (c.f. Chapter 1.2.1 and 1.2.2). Under proper conditions (typically dry or frozen), DNA samples can be stored for decades without significant loss of quality.
- Inside living organisms, DNA occasionally changes = mutates. Only if these mutations occur in egg- or sperm-cells, these mutations are transferred to offspring (are inherited), but most mutations occur in other cells and vanish from the population when the organism dies. Inherited mutations contribute to genetic variation in populations and may be selected for under natural (or artificial) selection. They form the basis of the evolutionary process.

For a genetics/genomics glossary: see Oyler-McCance et al. 2016 or a standard Genetics textbook.

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Appendix 2

Storage temperatures

Low temperatures slow down chemical and biological processes and thus, the transformation and decay of DNA and other genetically relevant molecules. Theoretically, the lower storage temperature, the better. The question is: How low a temperature is feasible for the storage of a specific sample?

DNA is a fairly stable molecule and can be safely stored at room temperature for months and years. That is why museum specimen are good sources of DNA. Other genetic molecules, i.e. RNA and the metabolites they assist forming, are much less stable and require acute low temperature storage or chemical fixation. Although studies of these molecules fall outside the range of the document, in your contact with professional biochemists, you should be aware of their field of experience and their protocols. These often stem from work on human DNA and the advanced molecular techniques (and research environment!) they have access too. Field ornithology still in *kindergarten* by comparison, but quickly progressing. When laboratory DNA people start suggesting storage on Carbon dioxide ice or in liquid Nitrogen, keep listening, but draw your own conclusions.

From normal storage of food items, we know that non-freezing temperatures below 8ºC keep biological materials in good conditions for days. Consequently, for *temporal* storage of DNA samples, refrigerator temperatures work fine **IF** your planned analysis can handle medium to low quality DNA (Chapter 3). In the lab, fairly quick transfer to a refrigerator suffices, and in the field and during short (< 10 h) transports, a cooling bag is all you need. In samples that have started to decay (rot), bacteria are already in full swing and will no longer be stopped by simple cooling (they also produce their own heat!). Such samples need more drastic measures. If submerging the sample in ethanol is a viable option[58], this is a good choice. When the ethanol really reaches all the bacteria, this will effectively stop any microbial decay and your sample is OK to be stored in a refrigerator or at room temperature.

For mid- to long-term storage, minus 18-20ºC is the temperature of choice, be it a freezer cabinet/box or a freezer-room. Stable temperatures and disruption control[59] are paramount for valuable samples, especially when ambient temperatures are high (i.e. summer and [sub-]tropical conditions). The control function must be linked to service staff that can check-up the situation 24/7, and an alternative storage facility in case the reset or repair takes time.

Thawing, in particular repeated thawing, is really harmful to DNA quality, regardless the nominal storage temperature. Thawing tends to work its way in from the surface of the sample. This means that even when most of the sample is unaffected, the outer parts may be. In reality, it's impossible to determine where the borderline goes.

Very special samples may require ultra-freezing. Ultra-freezers are very expensive cabinets that typically operate at minus 50 to minus 90 degrees centigrade. These temperatures can guarantee multiple-year storage without significant degradation. Ultra-freezers are commonly used for studies of RNA and metabolic products (e.g. anti-bodies). Unless you can get access to an ultra-freezer at negligible costs, using an ultra-freezer for DNA studies is overkill and a

[58] Ethanol potentially affects DNA analyses and needs to be handled in the pre-processing of your sample (Chapter 3.1). Make sure the labelling and documentation informs about the presence of ethanol (or other conservation/buffer solution) in your sample.

[59] "Disruption control" is any 24/7 system that automatically alarms when temperatures drift out of the pre-set range **and** a service team that cures the problem before samples are spoiled.

waste of resources. Storage in liquid nitrogen is a potential alternative to ultra-freezing, but is costly, labour-intensive and potentially dangerous. For all ultra-freezer systems, proper documentation and in-out logistics are important.

The cons of refrigerators and freezers are costs, energy consumption, storage space and transportation. Many combinations of DNA source - sampling method - analysis technique make cooling/freezing obsolete. Dry storage is often the most convenient way to go (Chapter 1.2.2).

Last but not least, the packaging and labelling of DNA samples needs to match storage temperatures and duration (c.f. Appendices 3 & 4). Dehydration through un-tight wrapping, lost markers, broken containers etc. can easily spoil valuable samples. To make things worse, in reality, many samples will be stored much longer than originally anticipated. Give storage, not only storage temperature, a proper though, and check-up with the experts.

Appendix 3

Packaging of DNA samples

The quality of your samples and analysis results depend on how you pack your samples during transport and storage, and thus, packaging decisions should be carefully made[60]. Factors to consider are e.g.:

- Type of DNA sample.
- Planned and possible future analyses.
- Expected duration of storage.
- Risk for physical damage, including wetting.
- Easy and quick handling.
- Availability and price.

See Appendix 4 for information about the relationship between packaging and documentation/marking/labelling.

Dry samples

Dry samples (including FTA-cards for blood samples) are preferably kept in paper bags/wrapping, not plastic. Plastic has the disadvantage of water-film formation on the inside when outside temperatures are (temporarily) lower than inside. This water-film facilitates microbial growth that can be potentially harmful to the DNA in the sample. The paper should be as free from artificial chemicals (print dyes, glue, etc.) as possible, because these chemicals can potentially interfere with downstream analyses (e.g. PCR). Plain bags for food items are generally safe. Paper also has the advantage of providing a surface for writing information about the sample (Appendix 4). The old-fashioned black pencil is still the option of choice, because it does not contain solvents and other potentially harmful chemicals, and has an unprecedented life-length.

The downside of paper is that it does not protect the sample from moist/water and harmful insects. If these risks are real, proper plastic wrapping can come to a rescue. One or several paper bags can then be stored in sealed plastic together with Silica Gel (a commercial drying agent). The plastic shield can either be a box (Fig. 21) or a vacuum-sealed bag (Fig. 22). Equipment for vacuum-sealing is readily available in stores for household equipment at reasonably low prices (ca. 100 €). Rolls of sheeting fall into the range of ca. 10 €/10 m, off the shelf (less when bought at larger quantities). Please make sure the samples are air-dry before sealing to avoid the need for unnecessary large amounts of Silica Gel.

Wet samples

Wet samples (usually blood or fresh tissue) require plastic[61] containers. Without a protective fluid (ethanol or some dedicated buffer-solution), wet samples must be put in a cold environment as quickly as possible (Appendix 2). With a protective fluid, this is not an urgent need. DNA-samples in ethanol can be stored at room temperature for considerable time, but

[60] Any packaging should be able to protect the sample from collection to analysis, because re-packaging comes with the risk of contamination and losses.
[61] Glass containers will do but are no longer a viable option except in special occasions.

> 35ºC temperatures should be avoided. Make sure the protective fluid is carefully described, preferably on the container, and that it will not interfere with downstream preparation and analyses. In the long run, almost all containers will leak vapours of the protective fluid. This may harm the sample, and thus, needs to be monitored and cured.

For small samples, standard 1.5 ml Eppendorf tubes (Fig. 21) are an attractive choice. Eppendorf tubes are cheap, small, robust and widely available. Unfortunately, they are not suitable for automated sample processing (e.g. extraction robots) used in many modern DNA-labs. For overall cost-efficiency, it may be wiser to use other (more expensive) tubes that fit downstream processing (Fig. 21). Check-up with the staff of the lab you intend to engage.

Figure 21. Selection of commercially available containers for DNA samples.
Top: Lunch-box type container suitable for large or several, individually wrapped, small samples. This type has built-in freezer gel in the lid and thus, is suitable for cool transportation of samples from the field to the lab.
Bottom from left to right: 1) 1.5 ml Eppendorf tube open. 2) Eppendorf tube closed. 3) Straight container with lid for use in many modern laboratory equipment, including extraction robots. 4) 15 ml and 5) 50 ml Falcon tubes respectively. Falcon tubes are suitable for e.g. eggshells, faeces, nest material and large tissue samples.
In all these containers, samples can be store under liquid (e.g. ethanol). In the large ones, samples can be stored dry together with desiccation agent (silica gel).

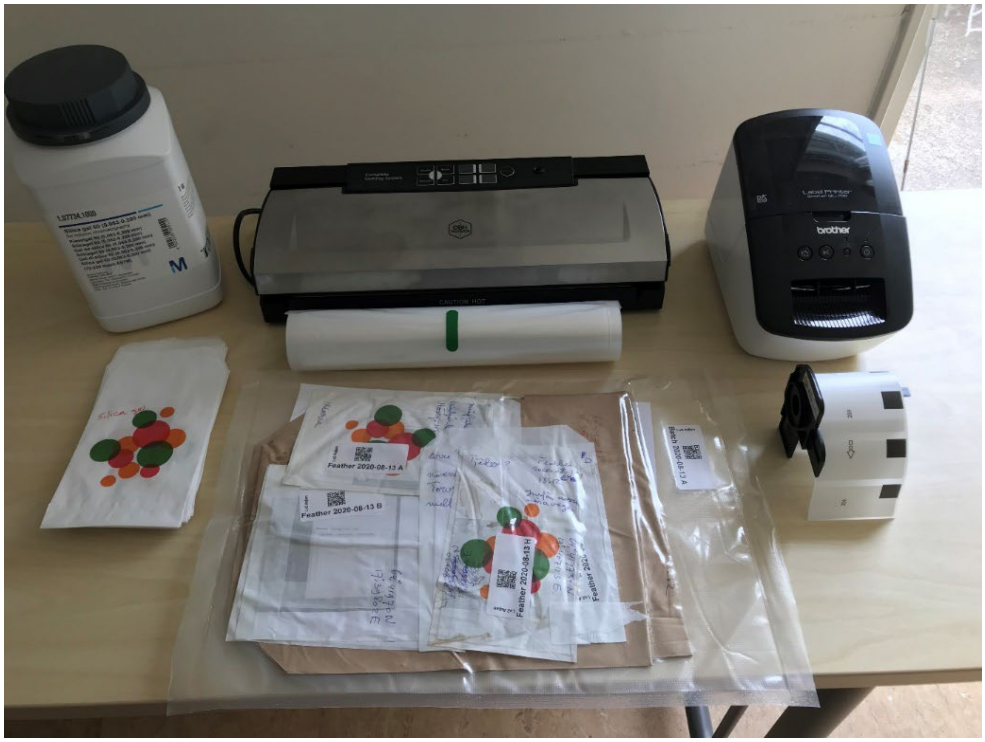

Figure 22. Complete system for packing solid samples (here feathers) for dry storage at room temperature. Samples are stored in paper bags (example left), and then vacuum-sealed and labelled (front and back). Dry conditions inside the plastic wrapping is guaranteed by adding a small amount of silica gel (top left) inside the plastic wrapping. An off-the-shelf label printer is convenient for labelling samples, batches and storage boxes. Packed this way, samples are safe from moisture, mold, bacteria and insects.

Miscellaneous

Larger samples (e.g. parts of carcasses) may call for special solutions. These are likely to be plastic wrapping and freezing. Generally speaking, large samples take longer time to decay/spoil than small ones, which gives you some extra flexibility, time wise.

Eggs and eggshells make a special case because there are easily broken. For remains of hatched eggs, regular egg-boxes are a good choice (Fig. 23), but special cases for large eggs are commercially available (yet expensive). If the samples are to be used for analyses that are sensitive to cross-species contamination risk, used egg-boxes will not be suitable (they are full of “foreign” avian DNA). In “normal” Eurasian Curlew DNA-studies, though, DNA from hen or duck will not be a problem.

Figure 23. Eggshell remains can be stored in regular hen's egg containers and semi-vacuum sealed in plastic foil together with silica gel. Photo: Adriaan de Jong.

Appendix 4

Documentation, marking and labelling

Documentation

Samples without proper documentation are useless and should be discarded. “Proper” = vital documentation includes *where*, *when* and *by whom* the sample was collected, **at minimum!** Depending on the objectives of your project, “proper” information could also include data about the birds (e.g. sex and age), their biological status (e.g. breeding or migrating), etc. This vital information should be in direct, permanent connection to every single physical sample **and also** in a notebook or a computer file. Redundancy is good, often critically important.

We suggest you use international coordinates (preferably decimal latitudes and longitudes in WGS84) for position data (the *where* part). Coordinates in national grids or just site names probably make perfect sense to you, but not for others, neither elsewhere nor in the future. Dates are probably just fine for the *when* description, but adding time of day comes at almost no cost at all, so why not add it *just in case*? In hind-sight, it can prove valuable in future analyses or publications. The reasons for documenting *who* did the sampling may not be obvious (you know it yourself!), but is utterly important. First and foremost, it gives you credit, but even responsibility. It also gives others a chance to get in touch for confirmation or additional information. Last but not least, future readers appreciate to have a “face” attached to artefacts and data. Obviously, the *by whom* information should include all people involved, and their contact information and affiliation.

In many cases, additional information about the sampling event can be important. This information may concern information about sampling method/protocol, weather conditions, sampling environment, and any abnormities or interesting observations. This additional information does not necessarily be stored together with the samples themselves, but in ways that are sustainably connected to them.

Last but not least, photos and films are powerful documentation tools. With a smartphone, you can easily produce high-quality testimonies, complete with time-stamp and location data (Fig. 24). Just make sure to include a reliable and permanent link between the photo/film and each sample. The time-stamp or the position data can sometimes work as the link with the sample[62], but often it is wise to include a visible clue, e.g. a representation of the marker or label on the container (Fig. 25).

[62] Please note that the time-stamp and position data may be changed or lost when files are transferred to another digital medium. Make sure to save the original files.

Figure 24. Photo documentation of vole nest sampling in spring. The sample label, the coordinates, the sampling environment and the sampled material are all captured in the same photograph. Photo: Adriaan de Jong.

Figure 25. QR-code label equipment.
QR-code labels on the lid of sample tubes. Combined alphanumeric and QR-code on larger label. Hand-held scanner for USB-port connection of a computer. Example of suitable label printer in Fig. 22. Photo: Jörgen Wiklund.

Coding systems

In many situations, the use of a coding system is smart in documentation/marking/labelling processes. Two main risks are attached to the use of codes. First and foremost, codes need to be unique within their operational environment. For your private cabinet or freezer, this is unlikely to be a problem, but for international collaboration and long-term storage, the operational environment of codes should be considered from an *ad infinitum*, global perspective. It is possible to expand a coding system to remedy conflicts, but that is often a challenging process. With some extra care in choosing coding systems, many future conflicts can be avoided.

Another major risk with the use of coding systems is the breakage of links between parts of the documentation chain. Laboratories often use internal codes for their Laboratory Information Management System (LIMS). Commonly, the only link between their codes and yours is a "translation table" (comparable with the Rosetta stone that provided the link between hieroglyphs and "modern" languages). If this translation table gets lost, the analysis results and the samples (with all their information) are no longer connected, and most likely, your DNA study spoiled.

Obviously, there is a trade-off between complex, unique and informative codes and coding efficiency (= short codes are easier to use/write). In addition, much effort must be put into safeguarding the links between codes in various sub-systems. Redundancy (multiple copies) and systematic backups are the prime cures. Non-informative and ambiguous codes are a disaster.

For coding samples that are stored in batches (e.g. Eppendorf tubes in a cardboard box), you can apply a hierarchical coding system by combining the code on the individual container (the Eppendorf tube) with the code on the cardboard box. You can extend this over multiple levels. Hierarchical coding can help mitigate the efficiency-uniqueness conflict, but does not prevent the broken-link problem.

Marking

Marking things can be seen as "child-play" – just putting text or codes on stuff. In reality, things are not always that easy. The pens you use need to fit the surface of the sample unit and must remain visible over the full storage period. Permanent marker pens fit many surfaces and can last for years. Be aware, though, of the risks of bleaching/discoloration (especially when exposed to sunlight or UV-radiation) and washing-off by ethanol or other organic solvents. Test any pen type and brand properly before use in real-world applications. An "old-fashioned" (lead) pencil may be a reliable alternative to modern marker pens. Surprisingly persistent.

Samples can also be marked in many other ways, either planned or as an emergency solution. Carvings in the surface of the container can serve for identification and so can artefacts added inside the container. The latter may consist of notes with text or codes, or solid objects, e.g. coloured beads. A fancier type of physical markers are tiny PIT-tags (Fig. 26). These are biologically and chemically inert and can be identified with a RFID-reader without opening the container. PIT-tags are relatively expensive (down to 2 € apiece when bought in larger quantities), but hand-held scanners are now available for under 20 €. Many RFID projects discard used tags ("death tags") which then can be used in unrelated projects. Please be aware, though, that PIT-tags come in different versions in terms of response frequency. Make sure your tags and scanner match.

It is important to make sure that things stored together with the sample do not affect the DNA itself, chemically or biologically. Foreign objects also increase the risk of cross-contamination (Chapter 2.1.2).

Figure 26. PIT tag and hand-held scanner. Smaller tags are available, but may require shorter reading distance. Photo: Jörgen Wiklund.

Labelling

Labels can replace or complement markings. Nowadays, cheap and user-friendly label printers with integrated label-design software are readily available. Labels come in many types and sizes. Most importantly, they need to stick permanently to your container and the text must remain readable for, at least, the full storage time. High-quality, durable labels cost more, but may be worth the extra money[63].

Label glues are chemically advanced and may pose a threat to DNA quality. This is not a problem when labels are applied on the outside of plastic/metal/wooden containers, but may be so when applied on paper/cardboard containers (i.e. bags and boxes). Although the chances for DNA degradation are probably minute, it is worth checking up before use on precious/rare samples.

Information on labels can contain alpha-numeric text and/or computer readable codes (and even images). "Normal" text and numbers can be read (but not necessarily understood) by humans, but take up a lot of space (= are low in information-density). Barcodes, QR codes, etc. can be read and input into a computer with a scanner (or scanning app in a smartphone). For labelling samples, QR codes are often preferred over barcodes because they can host more information per area unit. For example, a 4-5 digit QR code label readable with an off-the-shelf scanner fits nicely on the lid of an Eppendorf tube (Fig. 25). When label size is not a major problem, you can use labels with alphanumerical text **and** QR-codes (Fig. 24 & 25). Baseline labels cost <0.01 € apiece and a combined bar & QR-code scanner < 200 €.

Final note. *Usually, individual sample containers are stored in higher-level containers (e.g. Eppendorf tubes in a collection box). All aspects of documentation, marking and labelling apply to these higher-level containers/packaging as well.*

[63] Extreme labels can survive an airplane crash, but those are probably overkill for your project.

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Appendix 5

Legal aspects of Eurasian Curlew DNA projects

Legal status of the species

The Eurasian Curlew is classified *Near Threatened* by the IUCN (per 26th of October 2022). This means the species is **not** considered threatened, neither globally nor in Europe[64]. Even if it had been classified as threatened (VU or higher), this would have no direct legal implications.

Similarly, the species is **not** included in the CITES system (Appendices I, II and III)[65] and thus, no restrictions for cross-border **trade** apply within this framework.

Being a migratory species, the Eurasian Curlew falls under CMS/UNEP's Bonn Convention[66] and its European counterpart the Bern Convention (Treaty 104 of the Council of Europe)[67]. These conventions basically state that the species is protected across these conventions' legislative ranges.

The EU Birds Directive (2009/147/EC)[68] relates to all species of naturally occurring birds in the wild state in the European territory of the Member States to which the Treaty applies. It covers the protection, management and control of these species and lays down rules for their exploitation. With regard to DNA sampling it is important to know that protection of naturally occurring bird species in the EU member states shall be applied to birds, their eggs, nests and habitats. Member States shall take the requisite measures to maintain the population of the species at a level which corresponds in particular to ecological, scientific and cultural requirements, while taking account of economic and recreational requirements, or to adapt the population of these species to that level.

The Eurasian Curlew is referred to in Annex II, Part B, of the Directive: Owing to their population level, geographical distribution and reproductive rate throughout the Community, the species listed in Annex II may be hunted under national legislation. Member States shall ensure that the hunting of these species does not jeopardise conservation efforts in their distribution area. Being listed on part B means that the species may be hunted only in the Member States in respect of which they are indicated (in the case of Eurasian Curlew: Denmark, Ireland and France).

Trans-border transfer of DNA samples

Bringing or sending Eurasian Curlew DNA samples across national borders is likely to fall under legal restrictions. These vary between countries, but can be sorted into three categories.

a) The Nagoya protocol (a supplementary agreement to the Convention on Biological Diversity)[69] aims to protect the genetic resources and traditional knowledge about natural resources from unfair exploitation by foreign agents (mainly industries, but also scientists). The goal is the sharing of benefits, known and potential. In reality, you will

[64] https://www.iucnredlist.org/en
[65] https://cites.org/eng/app/index.php
[66] https://www.cms.int/sites/default/files/instrument/CMS-text.en_.PDF
[67] https://rm.coe.int/1680078aff
[68] https://ec.europa.eu/environment/nature/legislation/birdsdirective/index_en.htm
[69] https://www.cbd.int/abs/

need an *a priori* **export** permission for any type of sample put on the list by the country that has ratified the protocol. The Access and Benefit-sharing Clearing-house platform (https://absch.cbd.int/en/) will help you find your way to legally sound solutions.
PS. For the unlikely event you plan to bring live Eurasian Curlews across national borders make sure to check the Cartagena Protocol on Biosafety (https://bch.cbd.int/protocol).

b) Transportation of biological materials always comes with human, veterinary and ecosystem health security risks. These risks do not emerge from living organisms alone (c.f. Cartagena Protocol on Biosafety[70]), but from any material that is not completely biologically inert. Materials from birds are particularly risky (think avian flu), and the Covid19 pandemic has put many countries/authorities at high alert. The threat may very well arise from "hitch-hiking" agents like viruses, fungi, prions, etc.
For the necessary **import** permission(s), you need to consult the rules of the receiving country (probably your own). The EU has special rules for transfer between member states (and some other countries). Be aware of the fact that some countries apply regulations even for the transfer of biomaterials within national borders.
Usually, import permissions require a specially licenced recipient institution. Universities with international research collaboration in the life sciences usually have this kind of licenses. Check and possibly affiliate with those.

c) Some nations have their very own national, or even regional, **export** regulations. It is important to make sure you know and comply with these special regulations. If you collaborate with foreign partners, ask them to check up their national legislation. If not, find your way to the appropriate authorities and ask them to clarify your obligations.
Violation of these import/export rules can cause you great trouble and may also jeopardize the status and future success of biodiversity conservation. Playing unknowingly seldom helps.

Legal aspects of DNA sampling

The process of **DNA sample collection** itself is also subject to many rules and regulations. The details depend on the type of sample and where you plan to collect it. In general, sampling from live birds is much more regulated than non-invasive sampling from faeces, feathers, the environment, etc. Although international regulations exist (e.g. AEWA, EU Birds Directive), national legislations are the most important ones to keep an eye on. Here we present the main categories in four functional clusters.

For **legal access to lands and waters** from which to sample, you need to conform to:

- ownership, e.g. state, private or corporate
- land-use rights, e.g. husbandry, farming, hunting rights, aquaculture
- land-use, e.g. crops, installations, homes and gardens
- special restrictions, e.g. nature protection, military, infrastructure, hazard risk

Showing consideration for these various interests is common sense, but because DNA sampling is an unusual activity, you should be prepared to be met with questions, possibly suspicion. As long as you comply with their rules (including applying for permissions) and behave nicely and transparently, the risk for conflicts can usually be kept to a minimum.

For **legal access to living Eurasian Curlews** things tend to be a bit more complicated. Firstly, the Eurasian Curlew is a fully protected species in all European range states and thus, killing them is not allowed without special permissions. Moreover, even catching birds on the

[70] https://bch.cbd.int/protocol/text/

nest (e.g. for ringing) requires permissions in the EU member states. Secondly, all European countries have animal protection/welfare acts. Although these acts primarily aim to protect production and pet animals from harm and suffering in research, most country apply the same rules on wild animals kept in temporary custody (i.e. during handling by scientists or conservationists between catch and release). These are strong legal bodies and can result in severe penalties. Unfortunately, their enforcement is weak when it comes to wild birds.

In many countries, catching wild birds or taking their eggs is classified as "**non-lethal hunting**" and thus, these activities fall under the national hunting legislation. Nations differ in their views and legislation concerning hunting, but in general you will need an official authorization that overrides the normal restrictions for *where*, *when* (most of our DNA-sampling tends to take place outside the hunting season) and *how* to take Eurasian Curlews or their eggs. The *where* issue becomes extra important when you plan to catch/collect in protected areas. For all collection sites, you need a landowner agreement, which implies that the landowner accepts the infringement, possibly with compensation for potential damages. The *how* issue relates to the catching technique. Within the EU, only licensed equipment (traps, nets, etc.) is allowed for non-lethal hunting.

Please note that a ringing license does not automatically allows you to take DNA samples from the birds in your ringing project (c.f. Chapter 1.3).

If you plan to **sample DNA from other sources than living Eurasian Curlews**, life is much easier. Although carcasses of dead birds (e.g. roadkill or prey remains) have an uncertain legal status, they are usually safe to sample (except in France?). For shed feathers, faeces, eDNA etc. there should be no doubt that these sources can be legally sampled without any further ado. None of these sampling methods does any harm to the birds, because they are completely non-invasive. In the odd case a landowner (or anyone else) claims the collection of these items intrudes his/her material rights, you can probably talk your way out of the situation by explaining/demonstrating what you do. If that does not solve the problem, then probably, the ultimate causes of their complaint have little to do with your DNA-sampling activity and should be met accordingly.

Legal aspects relating to project stakeholders

A final legal aspect of DNA projects concerns financing and partnerships. Many DNA project can be run at small scale on a non-profit basis and without external funding. In such projects, conflicts about money and external obligations seldom occur. As soon as external funding comes in, things may change. Without written agreements, conflicts can easily arise and escalate over issues like "Who is responsible for task X?" or "How much financial compensation should Y get?" Also, there are legally binding obligations attached to funding contracts that need to be fulfilled. But by whom? And who takes the blame when obligations remain unfulfilled? If the project is run by an established institution (public, academic or NGO), existing organizational mechanisms will moderate most emerging conflicts. Without such mechanisms, things can get nasty and the goals of the project become wasted.

Ownership of samples and scientific results is another potential source of conflicts, especially in non-professional projects and in international collaborations. Here, too, written agreements can be useful[71]. At the start of the project, it is wise to realize that the samples and/or the findings can prove very valuable. Probably not, but possibly. From a Eurasian Curlew conservation point of view, the main issue is not that friends/colleagues turn into enemies or some get rich and others do not. The main problem is that such conflicts tend to hamper progress in science and conservation for the species (c.f. Chapters 1.3.2 & 1.3.3).

Overall, obedience to laws and regulations may take some extra effort initially, but pays of in the end through better results, less problems and more recognition, possibly even support, from local society.

[71] Most universities and authorities have offices of legal affairs that you can consult.