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Buccal swabs : a universal alternative to sample bird DNA

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Abstract

Least intrusive sampling should be prioritized for wildlife conservation and bioethics. In birds, DNA samples are typically obtained from blood. Here we emphasized buccal swabs as an alternative source. We tested swabs in adults and nestlings representative of 17 European species (n = 189), ranging from small passerines to large Strigiformes (owls) and Larids (gulls), and characterized the extracted DNA yields. The contents were highly variable between and within species, both in terms of quantity and perhaps purity, but the amounts obtained (~100–10,000 ng) meet the requirements of standard molecular analyses. No differences were found between adults and nestlings. The DNA yields were moderately higher for larger bird species, and samples taken from experienced swabbers yielded more DNA. In line with species-specific case studies that had successfully implemented buccal swabs, our results support the universal potential of the technique, which should be more generally applied in avian research, notably when endangered species are involved.

Key words : avian research, conservation genetics, non-invasive genetic sampling.

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Résumé

Limiter l'impact des échantillonnages ADN dans le cadre de projets de recherche sur les animaux sauvages est un enjeu d'éthique et de conservation. Depuis les années 2000 et l'amélioration des techniques de laboratoire, les généticiens rivalisent d'ingéniosité pour extraire de l'ADN depuis des sources non- ou peu invasives, telles que des crottes, des régurgitas, de la salive ou encore de l'ADN environnemental. Cependant, en recherche ornithologique, la grande majorité des scientifiques continuent de prélever du sang, une procédure intrusive pour les oiseaux et rarement appréciée par les cercles naturalistes et les comités éthiques. Dans cette étude, nous avons testé l'universalité de l'écouvillonnage buccal, une technique moins invasive. Des prélèvements buccaux ont été effectués sur 189 poussins et adultes représentant 17 espèces capturées dans le cadre de projets de suivis dans le canton de Vaud, en Suisse occidentale, plus une aux îles Canaries. L'ADN a été extrait et quantifié par spectrophotomètre. Les quantités obtenues furent très variables entre individus et espèces, de l'ordre de 100-10 000 ng, avec la grande majorité (95 %) des échantillons à plus de 200 ng, et une moyenne globale à plus de 1 000 ng. Comme la plupart des analyses multilocus nécessitent rarement plus de 150 ng d'ADN, nous concluons que les écouvillons représentent une source fiable pour les applications en génétique des populations aviaires, et pourraient être également utilisables pour des protocoles de génomique nouvelle génération. De

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plus, nos statistiques ne suggéraient pas un effet de l'âge sur la quantité d'ADN récupéré, mais un effet de l'espèce et du manipulateur. L'effet espèce semble être modérément lié à la taille de l'oiseau, avec plus d'ADN obtenu pour les grandes espèces (ex: chouettes, goélands) par rapport aux plus petites (ex: mésanges, gobemouches). L'effet manipulateur illustre l'importance de l'expérience avec la technique. Ainsi, moyennant optimisations et entrainements spécifiques aux espèces cibles, nous incitons à privilégier et valoriser cette technique simple, fiable, peu onéreuse et éthiquement défendable, tant par les chercheurs eux-mêmes, que par des commissions éthiques et les organismes de financement, encore plus pour l'étude d'espèces sensibles et menacées.

Mots-clés : recherche ornithologique, génétique de la conservation, échantillonnage génétique non-invasif.

Introduction

Genetic sampling must compromise between gathering sufficient DNA and limiting the complexity and invasiveness of field protocols (Carrol *et al.* 2018). In birds, DNA is traditionally obtained from blood (> 75 % of studies reviewed by Zemanova 2019), an invasive procedure that can be stressful and risky to the animals, and which requires professional handling and specific permits issued by ethical committees. With the significant improvement of laboratory methods, ornithologists have thus been testing less intrusive DNA sources for conservation genetics, such as shed feathers (Rudnick *et al.* 2007), feces (Idaghdour *et al.* 2003), eggshells (Kjelland & Kraemer 2012), exuviae (Marrero *et al.* 2009), saliva (Handel *et al.* 2006), and more recently environmental DNA from water samples (Ushio *et al.* 2018). Nevertheless, these approaches are only useful if non-degraded DNA can be reliably retrieved for molecular markers to be amplified, sequenced and compared among the specimens of interest.

In this respect, the strictly non-invasive sampling techniques *i. e.* when birds are not seen and disturbed, have been attempted for taxonomic and even individual identification in diversity surveys and population monitoring, respectively, with variable success (e. g. Segelbacher 2002, Regnaut *et al.* 2006, Rudnick *et al.* 2007, Marrero *et al.* 2009). Alternatively, nondestructive, minimally invasive samples, as obtained from buccal swabs and plucked feathers, may provide a more reliable solution to limit DNA degradation, and when specimen capture is a prerequisite (e. g. Harvey *et al.* 2006, Yannic *et al.* 2011). Buccal swabs have been successfully applied for various molecular applications in several other vertebrate groups, such as reptiles (Beebee 2007) and amphibians (Broquet *et al.* 2007). Swabs are cheap, easy to use and store during fieldwork expeditions, often do not require additional handling authorization, and cause little impact on animal welfare, hence representing a tool of choice for genetic surveys on endangered wildlife. Nevertheless, swabs still remain unpopular among scientists, who refrain to change traditional practices, and often assume that nondestructive DNA samples would be improper for downstream analyses (Zemanova 2019, 2020).

While previous studies have targeted specific species (e. g. Handel *et al.* 2006, Yannic *et al.* 2011), here, we compare and demonstrate the potential of buccal swabs to retrieve DNA from a wide array of bird taxa. We sampled, extracted and quantified DNA from specimens representative of 17 species. The substantial amounts retrieved suggest that swabs represent a universal resource for avian genetics.

Methods

We tested buccal swabs obtained from 189 individuals (17 species, 13 families, 6 orders). All but one species were captured in Western Switzerland during the year 2013, as part of monitoring/ringing projects approved by the Swiss Ornithological Institute; nine at the ringing station of the Jaman Pass (Canton of Vaud) during the autumnal migration, and seven at breeding sites. Samples from the last species (*Falco eleonorae*) were taken at the breeding colony of Alegranza (Canary Islands) during a field survey in 2011. Nestlings being smaller than adults, the use of swabs may be less proficient in the former, so when possible, both age classes were swabbed for comparisons. Table 1 lists species and sample sizes.

Buccal sampling was performed by rolling sterile rayon swabs (model 155C, Copan) under the birds' tongues, counting approximately 15 clockwise and anticlockwise movements. For repeatability, the procedure was preliminary agreed between the four samplers involved (GL, CD and two anonymous). Swabs were stored at -20 °C within a few hours post sampling. DNA was extracted using the Qiagen DNEasy blood & tissue extraction kit, with the following modifications from the manufacturer's protocol: proteinase K and ATL buffer volumes were doubled (total volume: 400 µl) in order to fully immerse the swabs, and the digestion was performed overnight in 2 ml Eppendorf tubes; Qiagen Qiashredder columns were used to recover all the digestion product absorbed by the cotton; elution was performed at 37 °C for 20' in 50 µl. DNA was then immediately quantified with a Nanodrop ND-1000 spectrophotometer (Thermofisher). We recorded the DNA concentration as well as the A260/A280 and A260/A230 absorbance ratios provided by the instrument. The A260/A280 ratio is sensitive to the DNA/RNA/protein content, with ideal values around 1.8 (pure DNA solutions), while lower values indicate protein or phenol contamination. The A260/A230 ratio is also used to detect contaminants, notably organic compounds, with ideal values around 1.8–2.2. Note that these ratios were shown to be more reliable when measured from high-concentration samples (ideally >50 ng/µl; Koetsier & Cantor 2019). Finally, the total amount of DNA retrieved per sample was calculated from the concentration measured.

All analyses and figures were generated in R v3.4.2 (R Core Team 2020). We first tested for an effect of age (adult *vs* nestling), sampler, and species on log-transformed DNA yields using ANOVAs. We further tested whether the interspecies differences in DNA yields could be explained by body size, using the average weight for each species as a proxy (taken from Maumary *et al.* 2007 and https//www.oiseaux.net), by fitting a linear regression on log-transformed species average DNA yields.

Results

We obtained DNA from the 17 bird species sampled with buccal swabs, which yielded variable amounts within and between species (table 1, figure 1). Over all samples, the average yield was 1304 ng (95 % distribution = 223–4240 ng), with a minimum of 75 ng (an adult of *Turdus philomelos*) and a maximum of 13,424 ng (a nestling of *Larus michahellis*).

There was a significant effect of the species ($P = 3\times10^{-11}$, $F = 6.5$, $df = 16$), of the sampler ($P = 0.003$, $F = 4.8$, $df = 3$), but not of the age ($P = 0.13$, $F = 2.3$, $df = 1$) on the amount of DNA retrieved. Specifically, more DNA was obtained from the largest species, e. g. the nut-

Table 1. DNA yield and absorbance ratios measured from extraction eluates in 17 European bird species. DNA yield are shown in nanogram (ng). Averages and standard deviations are given. Total sample size (*n*) is given for each, with the amount of nestling in brackets. The samplers of each species (noted 1 to 4) are listed.

Tableau 1. Quantités d'ADN et ratios d'absorbance mesurés sur les produits d'extraction de frottis buccaux pour 17 espèces européennes d'oiseaux. Les quantités d'ADN sont données en nanogrammes (ng). Les valeurs montrent les moyennes et écart-types. Le nombre total d'échantillons (*n*) est donné pour chaque espèce, avec le nombre de jeunes en parenthèses. Les échantillonneurs (sampler) sont listés (numérotés de 1 à 4).

Scientific name	English name	French name	Order	Family	Sampler	n	DNA yield	A260/A230	A260/A280
Larus michahellis	Yellow-legged gull	Goéland leucophée	Charadriiformes	Laridae	1	7 (7)	6628 ± 4254	1.85 ± 0.34	2.06 ± 0.04
Strix aluco	Tawny owl	Chouette hulotte	Strigiformes	Strigidae	1	14 (8)	1945 ± 1223	2.04 ± 1.02	1.85 ± 0.14
Tyto alba	Barn owl	Effraie des clochers	Strigiformes	Tytonidae	1	10 (8)	871 ± 244	0.76 ± 0.43	1.60 ± 0.14
Falco tinnunculus	Common kestrel	Faucon crécerelle	Falconiformes	Falconidae	1	8 (8)	1875 ± 1500	1.30 ± 0.39	1.82 ± 0.12
Falco eleonorae	Eleonora's falcon	Faucon d'Eleonore	Falconiformes	Falconidae	1	12 (12)	508 ± 260	1.54 ± 1.23	1.47 ± 0.21
Coturnix coturnix	Common quail	Caille des blés	Galliformes	Phasianidae	1, 2	10	1402 ± 1531	0.97 ± 0.64	2.14 ± 0.38
Tachymarptis melba	Alpine swift	Martinet à ventre blanc	Apodiformes	Apodidae	1, 3	22 (11)	1088 ± 703	0.60 ± 0.28	1.63 ± 0.23
Cyanistes caeruleus	Eurasian blue tit	Mésange bleu	Passeriformes	Paridae	1	8 (7)	845 ± 248	0.74 ± 0.24	1.73 ± 0.15
Parus major	Great tit	Mésange charbonnière	Passeriformes	Paridae	1	18 (17)	965 ± 873	0.90 ± 0.44	1.74 ± 0.23
Anthus trivialis	Tree pipit	Pipit des arbres	Passeriformes	Motacillidae	1, 2	16	837 ± 769	1.16 ± 0.53	1.65 ± 0.13
Ficedula hypoleuca	European pied flycatcher	Gobemouche noir	Passeriformes	Muscicapidae	1, 2	10	477 ± 234	1.12 ± 0.70	1.46 ± 0.28
Erithacus rubecula	European robin	Rougegorge familier	Passeriformes	Muscicapidae	1, 2, 4	12	1090 ± 901	1.28 ± 0.84	2.18 ± 0.66
Nucifraga caryocatactes	Spotted nutcracker	Cassenoix moucheté	Passeriformes	Corvidae	1, 2, 4	10	2352 ± 2340	1.00 ± 0.38	1.63 ± 0.27
Locustella naevia	Common grasshopper warbler	Locustelle tachetée	Passeriformes	Locustellidae	1, 2	11	1171 ± 931	0.87 ± 0.76	1.80 ± 0.23
Fringilla coelebs	Common chaffinch	Pinson des arbres	Passeriformes	Fringillidae	4	10	772 ± 1040	0.55 ± 0.42	2.00 ± 0.62
Spinus spinus	Eurasian Siskin	Tarin des aulnes	Passeriformes	Fringillidae	4	2	414 ± 192	0.80 ± 0.80	2.00 ± 0.05
Turdus philomelos	Song Thrush	Grive musicienne	Passeriformes	Turdidae	1, 4	9	534 ± 413	1.98 ± 1.57	1.51 ± 0.19

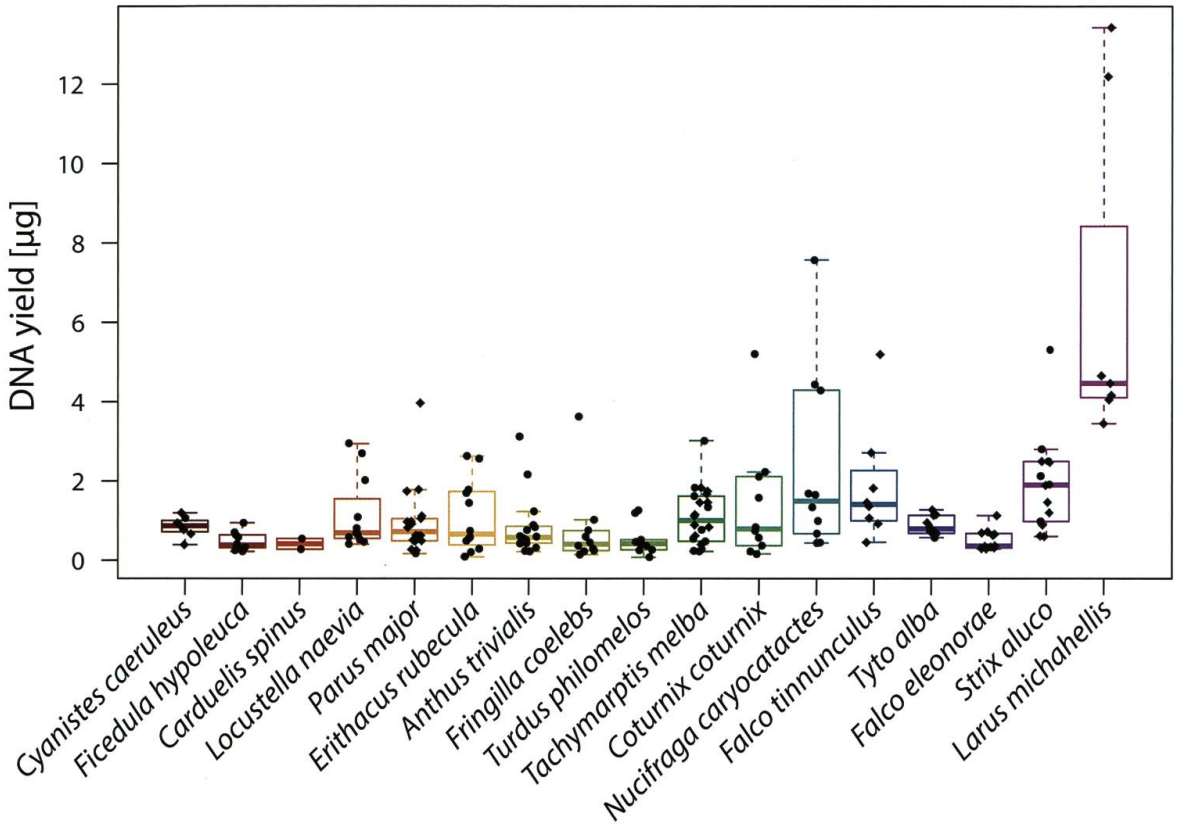

Figure 1. DNA yields obtained from buccal swabs in 17 European bird species. These are arranged by the average body size of species. Black symbols show the observations, distinguishing adults (dots) from nestlings (diamonds).

Figure 1. Quantités d'ADN obtenues à partir des frottis buccaux de 17 espèces d'oiseaux européens. Les espèces sont arrangées par leur taille moyenne. Les symboles noirs montrent les observations, en distinguant les adultes (points) des jeunes (losanges).

cracker *Nutrifraga caryocatactes*, the owl *Strix aluco*, the gull *Larus michahellis*, compared to the smaller passerines (table 1, figure 1). Accordingly, there was a positive correlation between average DNA yields and species body size ($P = 0.01$, $R^2 = 0.36$, $df = 15$; figure 2A), which however was no longer significant when excluding *Larus michahellis* ($P = 0.11$, $R^2 = 0.17$, $df = 14$; figure 2B); this single species appears a clear outlier, with disproportionate leverage in the regression (> 0.08, while others species are all < 0.02), thus strongly influencing the R^2.

The absorbance ratios were also highly variable (figure 3). Most A260/A230 ratios fell below ideal values (1.8–2.2), while the A280/A260 ratios showed a large variance around the 1.8 optimum. However more than half of our samples ($n = 117$) were measured from low-concentration elution templates (< 20 ng/µL of DNA), which affects the reliability of assessing sample purity from absorbance ratios quantified by microvolume fluorometers (Koetsier & Cantor 2019). Therefore, these values must be taken with caution and we did not perform statistical analyses on the absorbance dataset.

Discussion

In the wake of taxon-specific studies that experimented the use of swabs in bird conservation genetics (e. g. Handel *et al.* 2006, Yannic *et al.* 2011), our analyses confirm that significant amounts of DNA can be retrieved from a vast array of adults and nestlings from many differ-

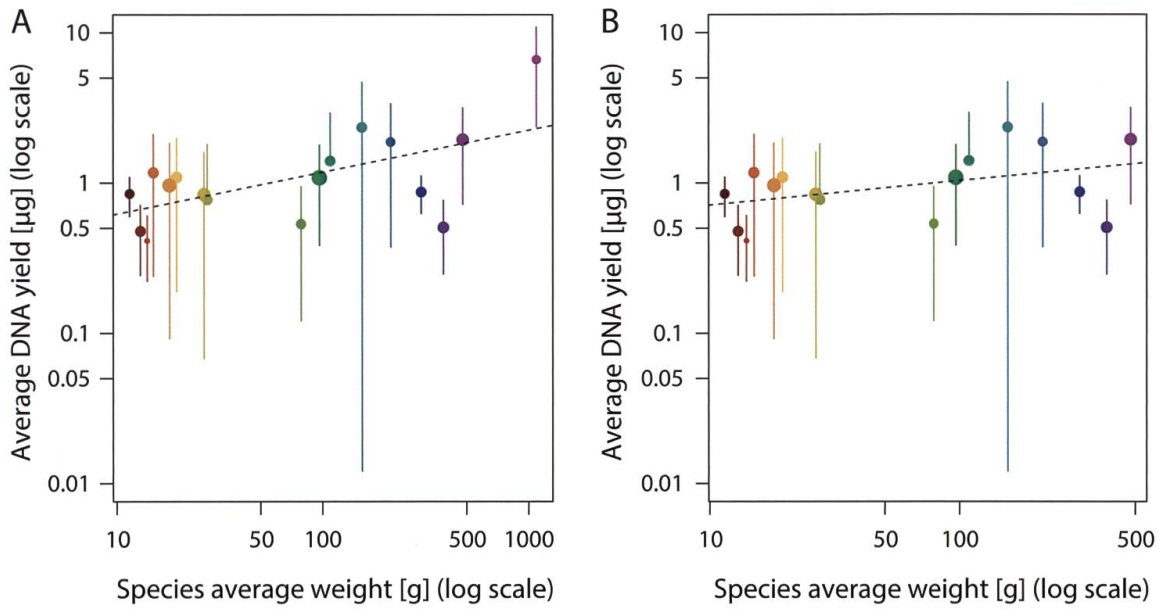

Figure 2. Correlations between average DNA yields and species average body weight in 17 European bird species. The linear regression trend lines are shown. **A)** Positive correlation among all sampled species. **B)** Weaker (non-significant) correlation when the outlier *Larus michahellis* is excluded. Colors as in figure 1.

Figure 2. Corrélations entre la quantité moyenne d'ADN obtenue et le poids moyen chez 17 espèces d'oiseaux européens. Les droites de régression sont indiquées. **A)** Corrélation positive en considérant toutes les espèces échantillonnées. **B)** La corrélation est plus faible (et non significative) quand *Larus michahellis* est exclu. Couleurs comme pour la figure 1.

ent bird species, thus demonstrating the universal utility of this technique for avian research. Typically, the standard volume of DNA extract required for a single locus PCR (e. g. barcoding of a mitochondrial marker) is about 1 µl, with concentrations about 10 ng/µl, thus representing only 10 ng of DNA. Microsatellite multiplexing necessitates larger volumes (~3 µl of DNA per reaction), totaling about 120–150 ng of DNA in order to amplify ~20 loci from 4–5 multiplexes (e. g. Yannic *et al.* 2011). Even considering replicate PCRs to account for allele dropout, the DNA yields obtained from our swabs (> 220 ng in 95 % of samples) thus appear sufficient for applications such as mitochondrial barcoding and microsatellite genotyping.

These yields are in principle compatible with high-throughput sequencing library preparation. Genomic approaches such as RAD-sequencing, now routinely used in population genomics and phylogeography (Lexer *et al.* 2013, Pante *et al.* 2015), also requires relatively small amounts of DNA (e. g. 6 µL of 20 ng/µl DNA, *i. e.* 120 ng in the protocol developed by Brelsford *et al.* 2016). While here the variable absorbance ratios gave little indication on DNA purity, the spectrometer profiles were comparable to those obtained from swabs of many amphibian species successfully analyzed with RAD-sequencing (Dufresnes *et al.* 2020 and references therein). Hence, we can assume that the DNA obtained from our bird swabs would be similarly suitable for high-density multilocus analyses.

Care must yet be taken with non-specific genotyping methods. For instance, RAD-sequencing is based on the amplification of any DNA fragment located near restriction sites, which consist of a few base pairs only. Swabs, like any nondestructive samples, can be contaminated by foreign DNA, e. g. buccal microbiota and ingurgitated food. Our quantification method based on spectrophotometry does not allow to distinguish DNA from the focal bird

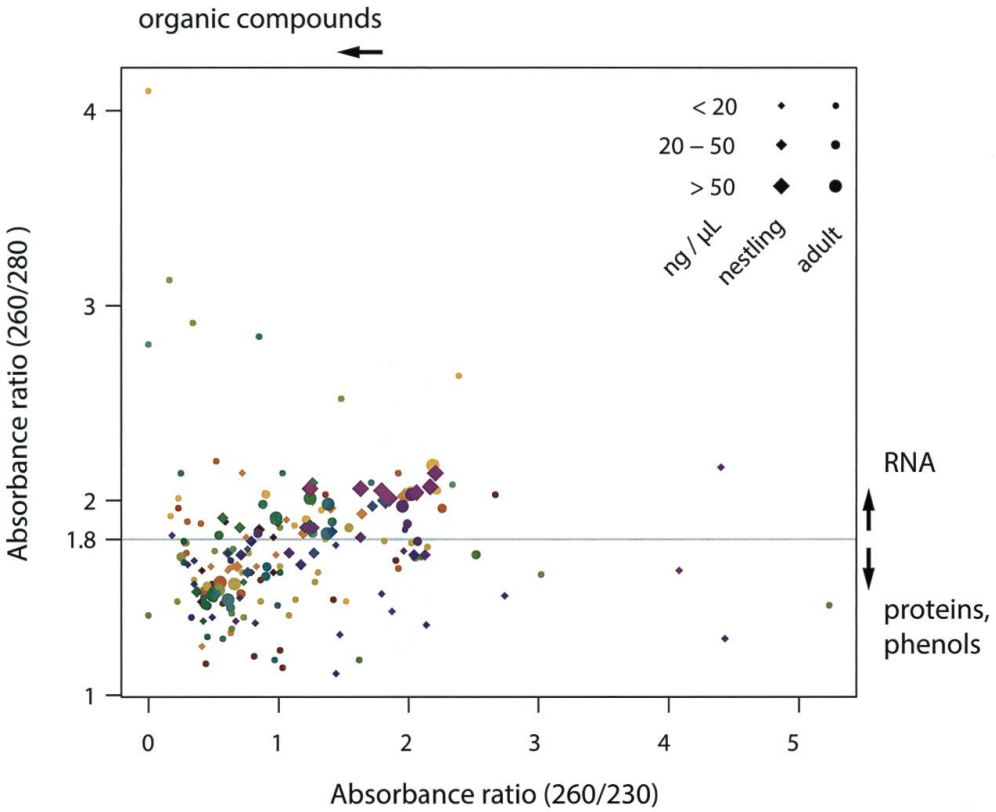

Figure 3. Absorbance ratios measured in 189 DNA extracts from buccal swabs, representative of 17 bird species. Ideal ratios *i. e.* supposedly reflecting uncontaminated (pure) DNA, are shown by blue lines. Symbols distinguish species (colors, as in figure 1), age (shape), and eluate concentrations (size). Note that most concentrations were too low (< 20 ng/µl) to accurately assess purity based on these ratios (see Results).

Figure 3. Ratios d'absorbance mesurés pour 189 produits d'extraction d'ADN obtenus à partir de frottis buccaux de 17 espèces d'oiseaux. Les lignes bleues indiquent les ratios « idéaux », supposés refléter de l'ADN pur (c. à. d. sans contaminants). Les symboles illustrent les espèces (couleurs, comme pour la figure 1), l'âge (forme) et la concentration des éluas (taille). À noter que les concentrations étaient pour la grande majorité trop faibles (< 20 ng/µl) pour une bonne appréciation de leur pureté sur la base de ces ratios (voir Résultats).

species *vs* other DNA contaminants, which would require specific protocols like qPCR using bird primers. The reliability of genomic libraries prepared from swabs is thus sensitive to the ratio of focal / foreign DNA (and the genome size of the latter), so the issue should be negligible if bird DNA is in excess. In any case, a reference genome can be useful to retain sequence reads that exclusively belong to the species of interest.

As our results suggest, the use of swabs may be less efficient for small birds, from which epithelial cells are less easily grasped from the tongue and palate. The protocol could thus probably be optimized, for instance by using narrower swabs and/or by rolling them for longer. Training to the method is also key. The sampler effect we detected stemmed from greater DNA quantities obtained by the two of us (GL and CD), who both had a long-term experience of swabbing, compared to the other samplers who were applying the technique for the first time.

Despite their underusage in vertebrates (Zemanova 2019, 2020), buccal swabbing thus holds great promises for bird research. Given their reliability, simplicity of use, lesser animal constraints and greater acceptance by the amateur naturalist community compared to blood sampling, we encourage ornithologists, ethical committees and funding bodies to promote

and implement least intrusive DNA sampling protocols such as buccal swabs in avian genetic research, particularly when endangered taxa are involved.

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