COMMUNICATIONS **Communications** are short contributions (preferably ≤4 printed pages, about 3500 words), presenting biologically interesting observations within ornithology and notes on methodology and equipment. An abstract is required.

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An overlooked DNA source for non-invasive genetic analysis in birds

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Non-invasive sampling is a useful tool for genetic analyses of endangered and/or elusive species, but it is often inapplicable due to the low quality and quantity of the DNA obtained. In this study we show that the blood clot located in the superior umbilicus of the feather shaft is a better source of DNA than the previously used tip samples from moulted feathers. We found that feather clots from museum specimens provided results nearly as good as footpad and better than those from the more commonly used museum skin snips. Feather clots proved to be a good source of DNA for genetic analysis that will significantly facilitate genetic monitoring of wild bird populations.

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Improved DNA extraction from feathers

Non-invasive methods may be the only alternative for the genetic analysis of endangered and/or elusive species that are difficult to sample otherwise. Museum samples, on the other hand, provide a unique opportunity to gain insights into the past that can then be used to better understand current genetic patterns and to test hypotheses about the demographic processes that produced them. However, the use of non-invasive sampling of free ranging animals and museum samples often does not provide reliable results because of low quality and quantity of the extracted DNA that can lead to allelic drop-outs and false alleles. Two strategies have been proposed to overcome these problems: i) a multiple tubes approach, in which each sample is repeatedly genotyped (Taberlet et al. 1996), and ii) the accurate estimation of DNA concentration through quantitative PCR and the rejection of samples containing amounts of DNA lower than a reliability threshold (Morin et al. 2001). The multiple-tube approach significantly increases labour and reagent costs, rendering some projects unpractical, whereas accurate quantification of DNA by PCR requires expensive equipment and the elimination of samples with low amounts of DNA.

Some genetic studies on birds (Mundy et al. 1997b, c) have successfully used feathers plucked from living birds (i.e. non-destructive sampling according to Taberlet et al. 1999). However, only a couple of studies (Morin et al. 1994, Srikwan and Woodruff 1998, Petersen et al. 2003, Segelbacher et al. 2003) have used moulted feathers collected in the field (i.e. non-invasive sampling according to Taberlet et al. 1999). These studies reported successful mitochondrial DNA amplification (Morin et al. 1994, Srikwan and Woodruff 1998, Petersen et al. 2003), but limited success with nuclear DNA amplification (Segelbacher 2002). In several studies using museum bird specimens, skin and footpad samples have also been used, sometimes in conjunction with plucked feathers (Ellegren 1991, Mundy et al. 1997a, Gautschi 2001, Godoy et al. 2004). Although such samples can provide suitable amounts of DNA, the reported success rates are variable and the potential damage to the specimens needs to be considered.

Most previous studies using feather samples for genetic analyses, collected both invasively and non-invasively, reported that the DNA was extracted from the basal tip of the calamus (Morin et al. 1994, Mundy et al. 1997b, Srikwan and Woodruff 1998, Segelbacher 2002, Petersen et al. 2003). An alternative and potentially abundant source of DNA from feathers, a blood clot embedded in the shaft (see Fig. 1), has so far remained overlooked. Once the feather is completely developed, the mesenchymal pulp, containing a single axial artery, is completely reabsorbed from the calamus and only keratinised pulp caps remain (Hodges 1974, Fig. 1B). These pulp caps, along with feather follicle cells

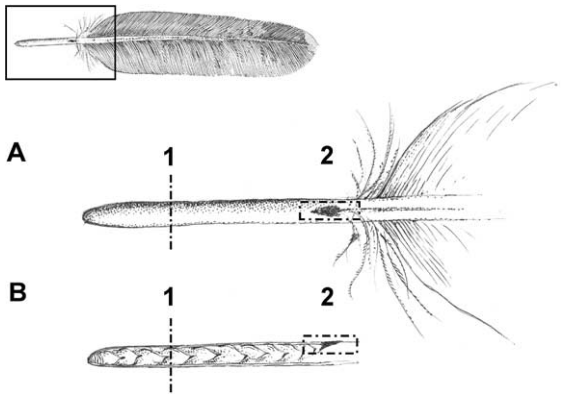

Fig. 1. General view of a typical flight feather: (A) detail of a posterior view of the base of the feather, and (B) longitudinal cross-section through the feather calamus. Two different sampling areas for feathers are shown: (1) basal tip of the calamus and (2) blood clot from the superior umbilicus.

attached to the outer surface of the calamus, are most likely the main source of DNA when only the tip of the feather is used. However, the superior umbilicus, where the reabsorbing mesenchymal pulp crosses the wall of the calamus in a narrow channel during feather development, typically contains a visible blood clot, which is the remnant of the axial artery (Proctor and Lynch 1993). This clot is likely to be included when the tip of a small feather is sampled, but will be totally excluded from large feathers.

In this study we evaluated the usefulness of umbilical clot samples for genetic analysis of birds and compared it to other commonly used DNA sources, from both field-collected moulted feathers and museum specimens.

Methods

Eight feathers from free-ranging Spanish imperial eagles were collected under nest or perching sites in Doñana National Park, southern Spain (37°N, 6°30′W) during August–September 2001. Feathers may have stayed on the ground for 0–7 months before being collected and stored in plastic bags at room temperature under dry and dark conditions for 7–8 months. DNA was separately extracted from: (i) the 1 cm basal tip of the calamus; (ii) the superior umbilicus containing the blood clot (Fig. 1). Five museum specimens of the Spanish imperial eagle from Doñana Biological Station were also sampled as follows: (i) one feather was cut from the back of the specimen, along with a small piece of skin (approx. 10 mm^2), around the tip (Gautschi 2001), (ii) a piece of the footpad (approx. 100 mm^3) was cut out and the outer surface removed in order to avoid contamination (Mundy et al. 1997a), and (iii) the umbilical clot of the 10th primary was collected as described above. Clots were collected without removing the feather from the specimen. DNA was extracted using the Qiagen DNeasy tissue kit following the manufacturer's instructions for animal tissues. DNA was eluted in a final volume of 120 µl. An extraction blank was always included to monitor for foreign DNA contamination. All the extractions were performed in a dedicated "clean" lab, free of modern DNA and PCR products and permanently UV irradiated when not in use. DNA concentration was measured fluorimetrically with a Hoefer-Dyna Quant 200 Fluorimeter, using Hoetsch-33258 dye. Values under 10 ng/µl were considered below the detection limit and recorded as nondetectable. Four µl of each extract were run in a 0.8% agarose gel in order to evaluate the level of DNA degradation.

Three segments of the mitochondrial genome differing in size were targeted in PCR amplifications: (i) a 210 bp fragment from the control region using primers *AID1* (Martínez-Cruz et al. 2004) and *AIR* (5′-GGGAGATTGGAGATATCTAGGC-3′), (ii) a 450 bp fragment from the control region using primers *Thr* and *Fbox* (Godoy et al. 2004), and (iii) a 1026 bp fragment, encompassing the whole cytochrome b gene using primers *MT-AB* and *MT-FB* (Seibold et al. 1996). Polymerase chain reactions (PCRs) were performed as previously described (Godoy et al. 2004). Amplification products were sized and visualised on 2% agarose gels containing 0.3 µg/ml ethidium bromide.

Molecular sexing was accomplished through amplification of *CHD1* gene fragments (Fridolfsson and Ellegren 1999). Five different PCRs per DNA extract were performed. For the Spanish imperial eagle, fragments amplified with these primers yield a product of around 700 bp in both sexes and another one of around 450 bp in females. Twelve µl of each PCR products were run in 2% agarose gel containing 0.3 µg/ml ethidium bromide and photographed under UV light.

Amplification of three microsatellite markers (*Aa36*, *Aa39*, *Aa53*) was performed with primers and PCR conditions as described by Martínez-Cruz et al. (2002). When using extracts from the basal tip of shed feathers, three parallel PCRs per sample were performed to evaluate the occurrence of genotyping errors. PCR products were visualised and sized in an ABI310 Genetic Analyser and alleles were assigned using Genotyper 2.5 software (Applied Biosystems).

Results

For field-collected feathers, clot samples yielded higher amounts of DNA than tip samples, as revealed by both gel electrophoresis and fluorimetric quantification. High levels of DNA degradation were observed in most cases, but less so for clot samples (data not shown).

Fluorimetric measurements detected a relatively high concentration of DNA in most of the clot samples, while extracted DNA was not detectable in any of the basal tip samples (always less than 10 ng/μl; Table 1). As expected, the observed general trend was for larger feathers to yield more DNA. The clot and footpad samples of museum specimens contained similar amounts of DNA (51.8 ± 46.6 ng/μl and 53.4 ± 33.1 ng/μl respectively), while skin samples of the same specimens provided lower DNA concentrations (26.7 ± 16.5 ng/μl) and in one case, no DNA could be detected.

In mitochondrial DNA amplifications, tip and clot samples of moulted feathers performed equally well, each producing only one failed amplification of the largest fragment. The amplification of the largest (1026 bp) mtDNA fragments from museum samples was less successful, but all three sampling methods were similarly effective (Table 1).

However, a great difference was observed between clot and basal tip samples of shed feathers in the efficiency of nuclear DNA amplification. Clot samples provided more consistent amplification of both *CHD1* fragments (Table 1) and stronger bands in all cases (data not shown). Among samples from museum specimens, the worst results were obtained from skin samples, followed by clot (one sample failing to amplify the larger fragment in all five PCRs tried) and footpad (a single failure of the 700 bp fragment). Since the product usually failing to amplify was the larger common product, this did not lead to sex misidentifications; it would, however, if the larger fragment corresponds to the female-specific product, as observed in some waterfowl (J. A. Godoy unpubl. data).

All 96 microsatellite amplifications performed from shed feathers yielded product. Two allelic dropouts and one false allele occurred, all of them from tip samples and for the same locus (*Aa39*). All museum samples yielded product except one skin sample, which did not amplify for any of the three microsatellite markers; no genotyping errors were detected.

Discussion

Our study demonstrates that the blood clot found in the superior umbilicus of moulted feathers provides more and better quality DNA than the previously used basal tip of the calamus. Despite their lower DNA yield, basal tip samples appeared to be as good a source of DNA as clot samples for mitochondrial DNA fragment amplifications (see also Morin et al. 1994, Srikwan and Woodruff 1998). Nonetheless, the lower quantity and

Table 1. Comparison of performance among five different sample types in four different DNA analyses. Moulted feathers included three remiges, four rectrices, and one covert. Museum specimens dated from 1940, 1966, 1976, 1985, and 1992.

DNA analysis	Sampling methods/Number (%) of cases				
	Clot-shed feather	Tip-shed feather	Clot-museum specimen	Footpad-museum specimen	Skin-museum specimen
1. DNA quantity	n = 8	n = 8	n = 5	n = 5	n = 5
DNA visible on gel	8 (100%)	1 (11.1%)	5 (100%)	5 (100%)	4 (80%)
DNA detectable with fluorimeter (>10 ng/μl)	7 (87.5%)	0 (0%)	5 (100%)	5 (100%)	4 (80%)
Mean DNA concentration ± SD (ng/μl)	92.1 ± 76.8*	–	51.8 ± 46.6	53.4 ± 33.1	26.7 ± 16.5*
2. mtDNA amplification	n = 8	n = 8	n = 5	n = 5	n = 5
210 bp fragment amplification	8 (100%)	8 (100%)	5 (100%)	5 (100%)	5 (100%)
450 bp fragment amplification	8 (100%)	8 (100%)	4 (80%)	5 (100%)	5 (100%)
1026 bp fragment amplification	7 (87.5%)	7 (87.5%)	3 (60%)	4 (80%)	3 (60%)
3. Molecular sexing	n = 8 × 5	n = 8 × 5	n = 5 × 5	n = 5 × 5	n = 5 × 5
450 bp fragment amplification (in females only)**	25 (100%)	22 (88%)	20 (100%)	20 (100%)	15 (75%)
700 bp fragment amplification	40 (100%)	12 (30%)	20 (80%)	24 (96%)	15 (60%)
Correct sexing after one PCR per sample	40 (100%)	25 (55.6%)	25 (100%)	25 (100%)	20 (80%)
Correct sexing after five PCRs per sample	8 (100%)	7 (87.5%)	5 (100%)	5 (100%)	4 (80%)
4. Microsatellite genotyping	n = 8	n = 8 × 3	n = 5	n = 5	n = 5
Aa36 (109–119 bp) amplification	8 (100%)	24 (100%)	5 (100%)	5 (100%)	4 (80%)
correct genotype	8 (100%)	24 (100%)	5 (100%)	5 (100%)	4 (80%)
Aa39 (191–223 bp) amplification	8 (100%)	24 (100%)	5 (100%)	5 (100%)	4 (80%)
correct genotype	8 (100%)	21***(87.5%)	5 (100%)	5 (100%)	4 (80%)
Aa53 (123–133 bp) amplification	8 (100%)	24 (100%)	5 (100%)	5 (100%)	4 (80%)
correct genotype	8 (100%)	24 (100%)	5 (100%)	5 (100%)	4 (80%)

*: Mean for the seven and four samples that could be quantified respectively.
**: n = 5 × 5 for shed feathers from females and n = 4 × 5 for female museum specimens.
***: Two allelic dropouts and one false allele occurred.

quality of DNA obtained from basal tip samples sometimes resulted in failed amplifications of nuclear DNA and genotyping errors. Segelbacher (2002) reported similar problems when using basal tip samples from shed feathers of capercaillie *Tetrao urogallus* and concluded that only approximately 50% of all feathers could be used for reliable genotyping. In contrast, we found that the higher DNA concentration of clot samples consistently resulted in efficient molecular sexing and microsatellite genotyping.

The improved yield and quality of this new feather DNA source might render the expensive procedures of the multiple-tube approach (Taberlet et al. 1996), or quantitative PCR (Morin et al. 2001), unnecessary in some cases. However, since species particularities, feather types and environmental conditions might influence DNA yield and quality, a careful evaluation of performance should be carried out before deciding to rely solely on umbilical clot sampling. As flight feathers (i.e. remiges and rectrices) of all 67 bird species belonging to 30 different families examined contained visible superior umbilical blood clots (M. Horváth et al. unpubl. data), clots may be a universal source of suitable DNA for birds.

Of the three type of tissues sampled from museum specimens, umbilical clot and footpad provided similarly good results, whereas skin samples performance was slightly inferior. Clot samples may be preferred by some curators as the sampled area remains hidden and multiple samples (one clot per large feather) are usually available from a single specimen. Although our results were obtained with specimens less than 60 years old, and therefore the performance of the new source needs to be evaluated in older specimens, we have obtained reliable genotypes from imperial eagle specimens up to 115 years old using clots (B. Martínez-Cruz et al. unpubl. data).

The early promises of non-invasive sampling for field studies of vertebrates have not yet been fulfilled, as reflected by the relative scarcity of published studies (but see Morin et al. 1994, Srikwan and Woodruff 1998, Petersen et al. 2003 and Segelbacher et al. 2003, for examples with birds). Extensive application of non-invasive methods has clearly been hampered by the limitations imposed by the low-quality and low-quantity of DNA obtained from most non-invasively collected remains. The discovery of a new suitable source of DNA in shed feathers significantly alleviates these limitations and will hopefully facilitate new exciting studies on bird behaviour, population genetics and conservation based on non-invasively collected feathers.

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