

# Using naturally shed feathers for individual identification, genetic parentage analyses, and population monitoring in an endangered Eastern imperial eagle (*Aquila heliaca*) population from Kazakhstan

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## Abstract

Genetic analyses on noninvasively collected samples have revolutionized how populations are monitored. Most noninvasive monitoring studies have used hair or scat for individual identification of elusive mammals, but here we utilize naturally shed feathers. The Eastern imperial eagle (EIE) is a species of conservation concern throughout Central Asia and, like most raptors, EIEs are inherently challenging to study because adults are difficult to capture and band using conventional techniques. Over 6 years, we noninvasively collected hundreds of adult feathers and directly sampled EIE chicks at a national nature reserve in Kazakhstan. All samples were genetically sexed and genotyped at a suite of microsatellite loci. Genetically profiled adult feathers identified and monitored the presence of individual eagles over time, enabling us to address a variety of issues related to the biology, demography, and conservation of EIEs. Specifically, we characterized (i) the genetic mating system, (ii) relatedness among mated pairs, (iii) chick sex ratios, and (iv) annual turnover in an adult breeding population. We show that EIEs are genetically monogamous and furthermore, there is no apparent relatedness-based system of mate choice (e.g. inbreeding avoidance). Results indicate that annual adult EIE survivorship (84%) is lower than expected for a long-lived raptor, but initial analyses suggest the current reproductive rate at our study site is sufficient to maintain a stable breeding population. The pristine habitat at our study site supports an EIE population that is probably the most demographically robust in the world; thus, our results caution that populations in marginal habitats may not be self-sustaining.

*Keywords:* *Aquila*, conservation genetics, genetic tag, noninvasive sampling, parentage, population monitoring

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## Introduction

Over the last several years, genetic analyses on noninvasively collected samples (e.g. hair, scat, and feathers) have become established methods for providing information on both rare and elusive wildlife species (Taberlet *et al.* 1997; Sloane *et al.* 2000). The ability to genetically profile

noninvasively collected samples has proven particularly important for conservation, allowing researchers to genetically 'tag' individuals of threatened or endangered species without capture. Unlike conventional tags, genetic tags are permanent and noninvasive assessment does not impact survival or recapture rates. While hair and scat have been used extensively for genetic tagging, large-scale studies utilizing feathers for individual identification remain rare. Herein, we use the Eastern imperial eagle (*Aquila heliaca*) to demonstrate how noninvasive genetic tagging can be of particular use when studying raptors.

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The Eastern imperial eagle (EIE) is a mid-sized raptor found throughout Central Asia. While once common in many parts of its range, the species experienced rapid population declines during the middle of the 20th century (del Hoyo *et al.* 1994). Because EIEs are currently very rare or extinct in many parts of their historic range (IUCN 2003; UNEP-WCMC 2003), their biology is of considerable conservation interest. Like many raptors, EIEs are difficult to study because (i) males and females cannot be easily distinguished visually (they are sexually dimorphic only in size, not plumage), (ii) adults are exceedingly difficult to capture and mark, and (iii) individuals migrate long distances. We used genetically profiled adult feathers to identify and monitor the presence of individual eagles over time, which enabled us to address a variety of issues related to the biology, demography, and conservation of EIEs. We investigated (i) the genetic mating system, (ii) relatedness among mated pairs, (iii) chick sex ratios, and (iv) annual turnover in an adult breeding population.

Genetic mating systems and the relatedness of mated individuals are two central aspects of mating systems theory. Conventional theory regarding avian mating systems was that most bird species are monogamous (Lack 1968), but a paradigm shift occurred when molecular techniques revealed the presence of extra-pair fertilizations (EPFs) in broods produced by supposedly monogamously mated individuals (Avisé 1996). Because substantial variation in EPF rates has been observed among avian species, a continuing characterization of these rates remains important for understanding their evolutionary underpinnings. While recent attention has focused on genetic mating systems (Avisé 2001; Griffith *et al.* 2002), other aspects of avian mating systems also are important. For example, the criteria by which individuals choose mates contributes to their own reproductive success and, indirectly, to that of their progeny (particularly for species that form long-term pair bonds). A potential mate's quality is often associated with its level of relatedness and consequently, relatedness forms the basis of choice in many mate-choice models (e.g. Bateson 1983; Tregenza & Wedell 2000).

Annual turnover is a basic parameter of population biology, and estimates of turnover are important for understanding the stability of EIE populations. In general, eagles are long-lived (often > 20 years), socially monogamous birds that exhibit high nesting site fidelity (del Hoyo *et al.* 1994). This implies that breeding populations should be relatively stable, with few individuals entering or leaving these populations on a yearly basis. However, to determine the true stability of a breeding population, it is necessary to monitor the presence of specific individuals over time. To circumvent conventional (invasive) marking techniques like wing bands, we have used noninvasively collected feathers to genetically identify and monitor the presence of breeding EIEs at our study site.

Our research focuses on an EIE population found at the Naurzum Zapovednik in north-central Kazakhstan. Given the inconsistent environmental record of the former Soviet Union, the zapovedniks (national nature reserves) of this region are essential resources for studying the biology of Central Asian fauna and flora. Today, several different types of protected areas are found throughout the former Soviet Union, but the zapovedniks are unique in that they were the first protected areas in the world to be established primarily for scientific research and conservation (Weiner 1988). The Naurzum Zapovednik, established in 1931, currently encompasses approximately 87 700 ha and represents an exceptional ecosystem in which southern fragments of Siberian forest meet Central Asian steppe. Approximately 25 raptor species breed within the reserve (Bragin 1989), including four eagle species: the EIE, the steppe eagle (*Aquila nipalensis*), the golden eagle (*Aquila chrysaetos*), and the white-tailed sea eagle (*Haliaeetus albicilla*). The population of EIEs residing in the zapovednik is likely the largest and most dense in the world, rendering this site particularly valuable from a conservation perspective.

## Materials and methods

### Sample collection

EIE samples were collected at the Naurzum Zapovednik in the Kostanay Oblast of north-central Kazakhstan (Fig. 1) over a 6-year period (1998–2003). Roughly 1200 naturally molted adult feathers were noninvasively collected from nesting sites and developing blood feathers were directly plucked from 227 chicks. These samples represent > 90% of all nesting territories occupied in the zapovednik during each year of collection. Adult feathers were stored dry at room temperature, while developing chick feathers were placed in a lysis buffer (100 mM Tris-HCl pH 8.0, 100 mM EDTA, 10 mM NaCl, 2% SDS) immediately upon collection. Chick samples were stored at room temperature for several months before being ultimately stored at  $-80^{\circ}\text{C}$ . The genetic



**Fig. 1** The breeding range of Eastern imperial eagles in Central Asia. The location ( $51^{\circ}\text{N}$ ,  $64^{\circ}\text{E}$ ) of the Naurzum Zapovednik in north-central Kazakhstan is indicated by an asterisk.

data presented herein were gathered from a subset of the total number of samples collected, those from the 1999–2002 breeding seasons.

#### DNA methods

Even though developing chick feathers were placed in a lysis buffer, the feathers never digested completely. Consequently, chick DNA was isolated from a small piece of tissue roughly 2–3 mm in diameter. To isolate DNA from single adult feathers, *c.* 5 mm was cut from the tip each shaft. Adult feather tips and chick tissues were diced, and DNA was isolated from these samples by the following methods. First, samples were incubated overnight at 55 °C in 700 µL of extraction buffer [86 mM NaCl, 43 mM Tris base, 21 mM EDTA, 0.08 M Tris Cl (pH 8.0), 0.01 mg Protease K, 3% sodium dodecyl sulphate, and 0.007 M dithiothreitol]. Next, 233 µL 7.5 M ammonium acetate was added for protein precipitation, extractions were centrifuged, and the supernatant was recovered. Finally, an isopropanol precipitation was used to recover DNA from the supernatant (Sambrook & Russell 2001). A negative control containing no eagle tissue was included in each group of DNA extractions.

The sex of each sample was genetically determined by polymerase chain reaction (PCR), using Fridolfsson & Ellegren (1999) 2550F and 2718R primers. PCRs were performed in a final volume of 25 µL and contained 0.5 unit *Taq* DNA polymerase (New England BioLabs), 1× Thermopol PCR Buffer, 0.2 mM each dNTP, and 0.1 µM forward and reverse primers. The thermal profile included an initial denaturation step of 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 52 °C for 1 min, and 72 °C for 2 min. A final extension step of 72 °C for 5 min concluded the profile. PCR sexing products were electrophoresed in 3% agarose gels and stained with ethidium bromide for visualization. The 2550F and 2718R primers are advantageous in that both males and females display PCR product when visualized in an agarose gel; males exhibit a single band, while females exhibit two distinct bands.

DNA samples were genotyped at the following nine microsatellite loci: *IEAAAG-4*, *IEAAAG-15*, *Aa02*, *Aa35*,

*Aa36*, *Aa43*, *Aa49*, *Aa39*, and *Aa56* (Martinez-Cruz *et al.* 2002; Busch *et al.* 2005). PCRs for *IEAAAG-4* and *IEAAAG-15* were performed in a final volume of 20 µL and contained 0.5 unit *Taq*, 1× Thermopol Buffer, 0.2 mM each dNTP, and 0.25 µM forward and reverse primers. The thermal profile included an initial denaturation step of 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. PCRs for each *Aa* locus were performed in a final volume of 20 µL and contained 0.5 unit *Taq*, 1× Thermopol Buffer, 0.25 mM each dNTP, and 0.25 µM forward and reverse primers. Thermal profiles for *Aa* loci are described by Martinez-Cruz *et al.* (2002).

For a small proportion (<10%) of adult feathers that failed to amplify with traditional PCRs, microsatellites were genotyped by modifying a ‘multiplex preamplification method’ for use with rare DNA templates (i.e. low-quantity DNA; Piggott *et al.* 2004). The preamplification method includes two steps of PCR. The first step was performed in a final volume of 50 µL and contained 1.5 units *Taq*, 1× Thermopol Buffer, 0.25 mM each dNTP, and 0.01 µM forward and reverse primers for either (i) both *IEAAAG* loci or (ii) all *Aa* loci. Thermal profiles were those corresponding to the *IEAAAG* and *Aa* loci described earlier. The second step in the preamplification method utilized the product from the first step as a template, with all other conditions being the same as those detailed for the traditional PCRs.

PCR products were electrophoresed in 4.75% denaturing polyacrylamide gels using an ABI377. Alleles were sized with respect to the electrophoretic mobility of an internal size standard using the GENESCAN and GENOTYPER (ABI) software. Microsatellite genotypes were scored blind, such that the scorer had no knowledge of specific nesting sites from which samples were collected.

#### Genetically tagging adults

The microsatellite genotypes attributed to a given sample collectively represented a genetic profile for that sample (Table 1). The program MATCH-MAKER (Rudnick *et al.* submitted) was used to assign genetically profiled feathers to individual adults using an unbiased estimator of the

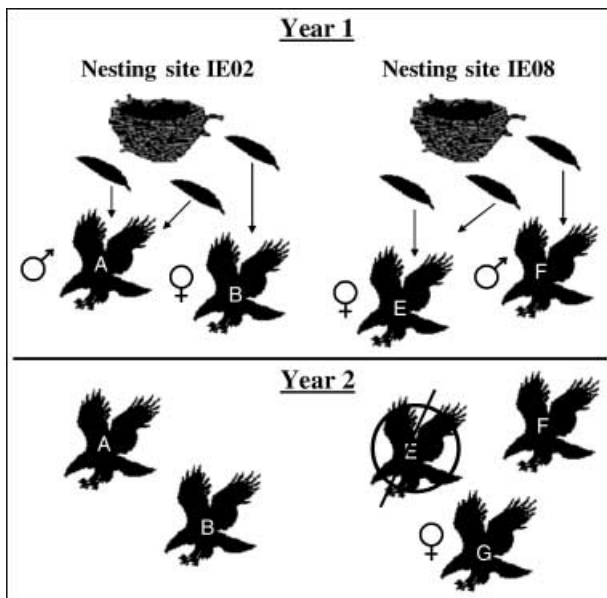
**Table 1** Genetic data generated from noninvasively collected eagle feathers. Dashes represent missing data. Four feathers collected from nesting site 31 were grouped by genetic profile to represent a single adult. Genotyping errors were identified by comparing the genetic profiles of feathers assigned to a given individual. The bold genotype for locus *Aa02* represents a genotyping error

Feather	Sex	Microsatellite Loci																	
		<i>IEAAAG-4</i>	<i>IEAAAG-15</i>	<i>Aa02</i>	<i>Aa35</i>	<i>Aa36</i>	<i>Aa39</i>	<i>Aa43</i>	<i>Aa49</i>	<i>Aa56</i>									
31-01	F	238	246	112	112	<b>149</b>	<b>151</b>	253	259	113	127	—	—	115	119	—	—	262	262
31-05	—	238	246	112	112	149	157	253	259	113	127	—	—	—	—	149	151	262	262
31-06	F	—	—	—	—	149	157	253	259	—	—	198	198	115	119	149	151	262	262
31-10	F	238	246	112	112	149	157	253	259	113	127	198	198	115	119	149	151	—	—

probability of identity ( $P_{ID}$ ; Paetkau *et al.* 1998). The  $P_{ID}$  threshold was set to 0.001, meaning all feathers assigned to a given individual exhibited a multilocus  $P_{ID}$  of less than 0.001 (i.e. the probability of two individuals having identical genetic profiles by chance is 1 in 1000). In our view, a threshold of 0.001 is conservative given that the number of breeding adults in the zapovednik is consistently less than 100. Genetic 'tags' were created for individual adults by combining the genetic profiles of the feathers assigned to each individual.

### Measuring turnover

Because eagles are socially monogamous and highly territorial, the adult feathers collected from a given nesting site during a single breeding season were assumed to represent one or both members of the social pair occupying that site. Turnover was directly assessed by using the noninvasively collected feathers to monitor the presence of genetically tagged adults over time (Fig. 2). In some cases, the feathers collected at a nesting site represented only a single adult. Turnover was assessed indirectly in these cases by utilizing parentage analyses. Consider a situation



**Fig. 2** A method for measuring turnover in an eagle population by utilizing noninvasively collected feathers. Adult feathers were collected at nesting sites, microsatellite genotyping and genetic sexing were combined to generate a genetic profile for each feather collected, and feathers were subsequently assigned to individuals based on their genetic profiles. The feathers collected from a given territory (i.e. nesting site) were assumed to represent the social pair occupying that site. Male A and female B were present at nesting site IE02 in both year 1 and year 2; no turnover occurred at that site between those years. At nesting site IE08, female E and male F were present in year 1 but female E was replaced by female G in year 2. This scenario represents one turnover event.

in which the feathers collected from a particular nesting site in a given year represented both members of the social pair occupying that site, but the feathers collected at the same nesting site in the following year only represented the social male. If the female that was present in the first year could be excluded as a parent of the chicks present at the nesting site in the second year, one turnover event was recorded. If the female could not be excluded, that individual was assumed to still be present and breeding in the population.

### Parentage analyses

The genetic mating system of EIEs was investigated by searching for EPFs among chicks. The previously described turnover analyses were used to identify the social pair of adults occupying a given nesting site each breeding season. EPFs were identified if one of the social adults present at a nesting site could be genetically excluded as a parent of one or more of that site's chicks. Population-level allele frequencies for the nine microsatellite loci utilized in this study were used to calculate the combined neither-parent-known exclusion probability (Selvin 1980).

### Relatedness

We used Queller & Goodnight's (1989) unbiased estimator of true relatedness ( $r$ ) to calculate relatedness values for mated pairs of individuals. To evaluate the average relatedness observed between mated pairs ( $\bar{r}_{obs}$ ) in the context of mate choice, we compared the likelihood of  $\bar{r}_{obs}$  to the theoretical expectation assuming a random union of gametes (Landry *et al.* 2001). To generate a distribution of  $\bar{r}$  values under the conditions of random mating, groups of randomly generated mated pairs were pulled 10 000 times from the pool of all genetically tagged adults. Ultimately,  $\bar{r}_{obs}$  was compared to the simulated random mating distribution.

### Results

The nine microsatellites used in this study averaged 7.9 alleles per locus among adults. The mean observed heterozygosity was 0.68, and no significant deviations from Hardy-Weinberg expectations were detected. The estimated frequency of null alleles was low, ranging from -0.012 to 0.019 across loci (CERVUS 2.0; Marshall *et al.* 1998), and gametic phase disequilibria as assessed in GENEPOP (Raymond & Rousset 1995) were nonsignificant after performing a sequential Bonferroni correction for multiple comparisons (Rice 1989). The mean probability of misassigning a feather sample to an individual (i.e. the multilocus  $P_{ID}$ ) was  $8.9 \times 10^{-10}$ . Of the 551 adult feathers we attempted to genotype, 388 amplified at a sufficient number of loci to be assigned to adults with a  $P_{ID}$  of less than 0.001. The remaining 163

feathers were excluded from all analyses. We genetically tagged 82 adults, with an average of 3.7 feathers being genotyped per adult and an average of 3.3 identical genotypes per locus contributing to each adult's genetic tag.

#### Quality control

Microsatellite genotypes generated from noninvasively collected samples can be prone to allelic dropout and allelic misprinting (Taberlet *et al.* 1999). Because the adult feathers collected from a given nesting site during a single breeding season were assumed to represent the social pair occupying that site, most feathers were likely to belong to one of only two possible individuals. Consequently, the method by which samples were collected facilitated the identification of genotyping errors (Table 1). A genotyping error rate was calculated for the adult feathers collected during the 1999 field season. A total of 138 feathers were genetically profiled, representing 919 genotypes across all loci. Three of the 919 genotypes were identified as being incorrect, giving a genotyping error rate of 0.33%.

Two additional error rates were calculated for the genotypes that were generated by the multiplex preamplification method. This method employs sequential PCRs, such that the product of the first PCR is utilized as the template for the second PCR. To investigate the error rate associated with genotypes generated by this method, 19 samples were independently genotyped twice (i.e. two DNA aliquots from the same sample independently underwent both PCR steps) and genotyping errors were identified if the replicate genotypes were not identical. A total of 112 genotypes could be compared between replicates, and no errors were detected (error rate 0.0%). To test just the repeatability of the second PCR, 14 samples underwent the first step of PCR, then the product from each of the 14 reactions was independently genotyped two times. Sixty-six genotypes could be compared between replicates, and only one was identified as a genotyping error. Thus, when only the second PCR was considered, the error rate for genotypes generated from the multiplex preamplification method was 1.5%.

#### Turnover in the adult breeding population

Table 2 summarizes the demographic data from 1999 to 2002. Losses from the breeding population can be split into two categories: those individuals that were replaced (i.e. turnover events) and those individuals that were not replaced (i.e. missing individuals that left behind unoccupied nesting sites). The mean annual rate of loss from the population, including both turnovers and missing individuals, was 16% (7 individuals per year). A total of 20 turnover events were inferred between 1999 and 2002; these represented 5 male turnovers and 15 female turnovers. Only 3 individuals (1 male, 2 females) were lost without replacement.

**Table 2** Losses from the Naurzum Zapovednik's EIE population between 1999 and 2002

Year 1–Year 2	Adults in year 1	Turnover between years	Missing after year 1	Average total loss per year
1999–2000	51	4	1	
2000–2001	50	13*	0	7 (16%)
2001–2002	30	3	2	

\*No samples were available for a number of the nesting sites occupied in 2001. Consequently, 6 of the 13 turnover events listed for 2000–2001 may have occurred between either 2000–2001 or 2001–2002; 'turnover between years' refers to individuals that were present at the zapovednik in one year but absent the following year, with new individuals of the same sex filling the vacated positions in the population; 'missing after year 1' refers to those individuals that were present at the zapovednik in one year, but absent without replacement in following years (i.e. nesting sites occupied in one year were empty in all subsequent years of the study); 'ave. total loss per year' encompasses both of the two previously described categories of loss.

#### The genetic mating system

The combined neither-parent-known exclusion probability was calculated to be 0.999 for the markers used in this study. One hundred sixty-six chicks from 86 broods were examined for EPFs and, in all but three cases, neither social adult occupying a given nesting site could be excluded as a parent of one or more of that site's chicks. In the anomalous three cases, a social adult could be excluded as the parent of at least one chick by only a single microsatellite locus. These cases most likely represent one instance of mutation and two instances of null alleles, as opposed to EPFs or genotyping errors (Rudnick, unpublished data). Our conclusion is that genetic monogamy is by far the predominant mating system in this population of EIEs.

#### Sex ratios among chicks

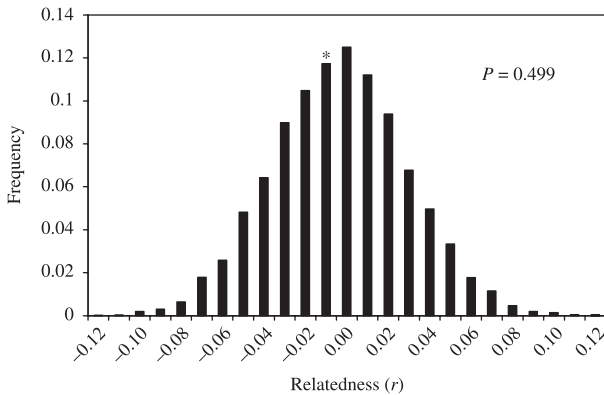
Of the 166 chicks sampled during our study period, we genetically determined the sexes of 164. Table 3 summarizes the sex ratio data from 1999 to 2002. Only one of the five sex ratios presented in Table 3 is statistically different from a ratio of 1:1 (2001:  $\chi^2 = 7.26$ , d.f. = 1,  $P = 0.007$ ). The significant departure from a 1:1 sex ratio in that year remains unclear because, by most ecological measures, 2001 was unremarkable.

#### Relatedness

Relatedness values were calculated for mated pairs when both members of a pair were genetically tagged. A total of 31 mated pairs met this criterion from 1999 to 2002, and  $\bar{r}_{\text{obs}}$

**Table 3** Hatching sex ratios

Year	No. of males	No. of females	M : F Ratio	<i>P</i> value
1999	17	18	1:1.1	0.866
2000	33	28	1:0.85	0.522
2001	23	8	1:0.35	0.007*
2002	16	21	1:1.3	0.411
Total	89	75	1:0.84	0.274

\* $\alpha < 0.05$ .**Fig. 3** Frequency distribution of  $\bar{r}$  values expected under random mating, created from 10 000 randomly generated groups of 31 mated pairs (see Materials and methods). The position of  $\bar{r}_{\text{obs}}$  in the simulated distribution is marked, and the corresponding *P* value is indicated by an asterisk.

was calculated to be  $-0.0027 \pm 0.0367$ . Because 31 mated pairs were used to calculate  $\bar{r}_{\text{obs}}$ , 31 randomly generated mated pairs were included in each simulation iteration used to generate the distribution of  $\bar{r}$  values expected under random mating. When  $\bar{r}_{\text{obs}}$  was compared to the simulated distribution (Fig. 3), the observed value was not significantly different from that expected under random mating ( $P = 0.499$ ). These results are consistent with a random union of gametes model and thus, provide no support for a relatedness-based system of mate choice.

## Discussion

### *Avian mating systems theory — monogamy*

A dramatic shift in the avian monogamy paradigm occurred when molecular techniques revealed that less than 25% of socially monogamous bird species are genetically monogamous (Griffith *et al.* 2002). Substantial variation in EPF rates has subsequently been observed among avian species; the percentage of extra-pair offspring ranges from 0 to 72% and the percentage of broods containing extra-pair young

ranges from 0 to 95% (Griffith *et al.* 2002). The detection of EPFs has been so influential, genetic promiscuity has now effectively replaced the historical standard of monogamy in avian mating systems theory.

Despite the near-pervasiveness of promiscuity in bird species studied to date, none of the 99 socially monogamous or polygynous species reviewed by Griffith *et al.* (2002) were members of the family Accipitridae (eagles, hawks, Old World vultures). The family Accipitridae is one of the largest avian families (237 species distributed among 64 genera), and most of these species are known to be socially monogamous (del Hoyo *et al.* 1994). The omission of this family from investigations of genetic parentage is particularly significant given that over 50% of the interspecific variation observed in EPF rates occurs between avian families or orders (Griffith *et al.* 2002).

Current evidence suggests all eagles are socially monogamous, but no genetic information on EPFs in eagles has been published. In fact, molecular data on EPFs in the family Accipitridae are sparse. To our knowledge, only two EPF rates have been published for socially monogamous members of the family to date; 5% of sparrowhawk young (*Accipiter nisus*; Møller & Birkhead 1993) and 1.3% of Northern goshawk young (*Accipiter gentiles*; Gavin *et al.* 1998) are reported to be extra-pair. While those data suggest socially monogamous members of the Accipitridae exhibit low levels of EPFs, our results suggest that the Naurzum Zapovednik's population of EIEs is truly genetically monogamous.

Many of the biological characteristics exhibited by EIEs are consistent with hypothesized correlates of genetic monogamy. For example, raptors generally are long-lived species that display social monogamy both within and between breeding seasons. Furthermore, male raptors also bear high levels of parental investment in reproduction; they often provide food to their young, as well as to their females during the prelaying and egg-laying periods (Watson 1997). Under both the longevity and paternal care hypotheses (Neudorf 2004), raptors are expected to tolerate only very low or zero rates of EPFs. Our data on the EIE are consistent with this expectation.

### *Avian mating systems theory — relatedness*

Another crucial aspect of mating systems theory is mate choice (DeWoody 2005). Studies in a wide variety of taxa show that the genetic similarity between a pair of mates can affect reproductive success (Amos *et al.* 2001), offspring survival (Bean *et al.* 2004), and parasite load (Coltman *et al.* 1999). Studies also have asserted that some species employ a relatedness-based system of mate choice (Keane 1990; Peacock & Smith 1997), presumably as a mechanism to reduce inbreeding and/or outbreeding depression. To our knowledge, the only published data on

relatedness-based mate choice in raptors is for the American kestrel (*Falco sparverius*; Duncan & Bird 1989). In their seminatural setting, female kestrels chose mates randomly with respect to relatedness when presented with the choice of a male sibling or an unrelated male of the same age and breeding experience. Our results for wild EIEs corroborate the American kestrel data in that there is no evidence for a relatedness-based mate choice system (e.g. inbreeding avoidance).

Parental relatedness has been shown to influence pup survival in grey seals (*Halichoerus grypus*; Bean *et al.* 2004), birth weight and juvenile survival in red deer (*Cervus elephus*; Coulson *et al.* 1998, 1999) and harbour seals (*Phoca vitulina*; Coltman *et al.* 1998), as well as adult reproductive success in grey seals, wandering albatrosses (*Diomedea exulans*), pilot whales (*Globicephala melas*), and red deer (Slate *et al.* 2000; Amos *et al.* 2001). Still, when different types of fitness components are considered, patterns of association between parental relatedness and reproductive success are not always consistent within a species. For example, Hansson (2004) reported a negative association between egg-hatching success and parental relatedness in great reed warblers (*Acrocephalus arundinaceus*), but no relationship between parental relatedness and clutch size, proportion of fledglings, or proportion of recruits was detected. These results suggest that for a given species, some fitness components may be more suitable than others for detecting associations between reproductive success and parental relatedness.

We tested for a correlation between parental relatedness and clutch size in EIEs. For each breeding eagle pair, clutch size was defined as the average number of chicks produced by that pair during the 1999–2002 breeding seasons; no association between clutch size and parental relatedness was detected ( $F_{1,29} = 0.11$ ,  $P = 0.074$ ). Furthermore, these data also complement the lack of support for a relatedness-based system of mate choice (Fig. 3). If a negative correlation were to exist between clutch size and parental relatedness, we would expect the average relatedness among mated pairs to deviate from a model of random mating.

#### *Are breeding populations stable?*

Annual mortality rates for large raptors typically range from 3% to 10% (Ricklefs 2000; Saether & Bakke 2000). For the Naurzum Zapovednik's EIE population, the average yearly rate of loss was 16% between 1999 and 2002. Several possible explanations exist for this elevated rate of loss. For example, annual survivorship in EIEs may be lower than in other raptor species, perhaps as a legacy of habitat degradation, human over-exploitation, or some combination thereof. Alternatively, nesting site fidelity may be relatively low among adults. This latter explanation is unlikely for at least two reasons: (i) all birds present at the zapovednik over

multiple breeding seasons *were* strongly philopatric, and (ii) eagles are highly territorial, so an adult failing to return to its own territory would have to acquire another; we detected no such movements within the zapovednik. Given the information available on EIE biology, we believe it is reasonable to use the average yearly rate of loss we observed at the zapovednik as a proxy for the annual adult mortality rate.

The average yearly rate of loss we observed at the zapovednik may be an indication that the current number of breeding adults occupying the reserve cannot be maintained. The population of EIEs in the zapovednik may be in jeopardy of extinction if its annual rate of reproduction is insufficient to compensate for an annual adult mortality rate of 16%. To gain a general understanding of the breeding population's stability, we developed the following model:  $N_{t+1} = [\phi_A + h_{(ave)}\phi_A^r]N_{t(ave)}$ , where  $N_{t(ave)}$  is the average number of adults in the breeding population,  $h_{(ave)}$  is the average number of chicks annually produced per adult,  $\phi_A$  is the annual rate of survival, and  $r$  is the minimum age of first reproduction. If  $\phi_A + h_{(ave)}\phi_A^r$  equals or exceeds 1, the rate of reproduction in the zapovednik's EIE population is sufficient to compensate for adult mortality. The zapovednik's breeding population currently averages *c.* 60 individuals and annually produces *c.* 0.7 chicks per adult (Rudnick, Katzner, and Bragin, unpublished data; averages are based on nesting sites at which breeding was attempted). EIEs begin breeding at approximately 5 years of age, but no information on juvenile survival rates is currently available. If juvenile and adult survival rates are assumed to be equivalent (a conservative estimate for juveniles),  $\phi_A + h_{(ave)}\phi_A^r$  equals 1 if the annual rate of survival is 79%. With an annual adult mortality rate of 16%, the EIE population at the Naurzum Zapovednik exhibits an estimated annual adult survival rate of 84%. This suggests that the number of breeding adults present at the reserve can be maintained by recruitment, but this simple model produces only a preliminary estimate of the population's viability. More complex population modelling (e.g. a model incorporating age structure) and accurate estimates of juvenile survival are required to firmly establish the stability of this breeding population.

Our data suggest there may be a sex bias in the mortality rates of EIEs. Among the 20 turnover events observed during our study period, five represent male turnover events and 15 represent female turnover events, a statistically significant difference between the sexes ( $\chi^2 = 5.0$ , d.f. = 1,  $P = 0.025$ ). However, the biological significance of these data is unclear. Because EIEs are socially monogamous, signatures of a sex-biased adult mortality rate are expected to be present in other biological parameters exhibited by the species. For example, either (i) an excess of male 'floaters' (i.e. nonmated individuals) might be present or (ii) fledgling sex ratios could be skewed to produce an excess

of females. The presence of floaters is difficult to evaluate because of the sexual monomorphism in EIEs, but the parentage data clearly indicate that, if present, floaters do not breed. We can more directly evaluate chick sex ratios: hatchling sex ratios are 1:1 in the Naurzum Zapovednik, and preliminary analyses indicate the sex ratio continues to be 1:1 among the subadults present at our study site (unpublished data). This suggests that the EIE population in the Naurzum Zapovednik does not produce a sexually skewed group of potential recruits. While these results provide no additional evidence for a sex-biased adult mortality rate, information on the sex ratio of floaters in our study population and, more importantly, additional turnover data are needed to fully evaluate the biological significance of our data.

### Conservation implications

International concern for the EIE is heightened, in part, due to the status of its critically endangered conspecific: the Spanish imperial eagle (*Aquila adalberti*). Like its sister species, the Spanish imperial eagle also experienced population declines during the last century. Today, the species is considered to be one of the most endangered birds of prey in the world (Collar & Andrews 1988). Genetic comparisons between Spanish and Eastern imperial eagles have proven to be both valuable and enlightening. For example, by comparing the species' current genetic diversities, Martinez-Cruz *et al.* (2004) ascertained that the demographic bottleneck suffered by Spanish imperial eagles did not impact the species' nuclear genetic variation. Thus, in addition to being informative in their own right, the genetic and biological parameters described by our research will be useful in future investigations of eagle biology and conservation.

Our results suggest EIEs exhibit a higher annual rate of mortality than other raptors. However, we also suggest that the current reproductive rate of our study population is sufficient to maintain the number of breeding adults present. Two additional lines of research are needed: (i) similar estimates of the annual rate of loss are needed from additional populations to firmly establish the range of survival rates in adult EIEs, and (ii) additional population modelling is needed to better establish the stability of this breeding population, as well as to assess the stability of other breeding populations. The Naurzum Zapovednik provides protected habitat that is particularly well suited to the EIEs and thus this population may enjoy increased reproductive success relative to other EIE populations, allowing it to compensate for high adult mortality. The same may not be true for populations in marginal habitats and, given recent population declines, comparisons with other EIE populations are critical for assessing the true conservation status of the species.

Our research utilized noninvasive feather sampling in investigating both theoretical and practical aspects of eagle biology. We have demonstrated that single, noninvasively collected eagle feathers yield sufficient quantities of DNA for both genetic sexing and microsatellite genotyping. Furthermore, we have demonstrated that our genotyping error rate was very low and that this type of sample is appropriate for individual identification. Because raptors are inherently difficult to capture and band using conventional techniques, we suggest our noninvasive method of population monitoring can be a valuable asset to future raptor research.

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### References

- Amos W, Wilmer JW, Fullard K *et al.* (2001) The influence of parental relatedness on reproductive success. *Proceedings of the Royal Society of London. Series B, Biological Sciences*, **268**, 2021–2027.
- Avisé JC (1996) Three fundamental contributions of molecular genetics to avian ecology and evolution. *Ibis*, **138**, 16–25.
- Avisé JC, ed. (2001) DNA-based profiling of mating systems and reproductive behaviors in poikilothermic vertebrates. *Journal of Heredity*, **92**, 99–211.
- Bateson P (1983) Optimal outbreeding. In: *Mate Choice* (ed. Bateson P), pp. 257–277. Cambridge University Press, Cambridge.
- Bean K, Amos W, Pomeroy PP *et al.* (2004) Patterns of parental relatedness and pup survival in the grey seal (*Halichoerus grypus*). *Molecular Ecology*, **13**, 2365–2370.
- Bragin EA (1989) *Biology of birds of prey of pine forests of the Kustanay Steppe*. PhD Thesis, Kazakhstan Academy of Sciences, Almaty, Kazakhstan.
- Busch JD, Katzner TE, Bragin E, Keim P (2005) Tetranucleotide microsatellites for *Aquila* and *Haliaeetus* eagles. *Molecular Ecology Notes*, **5**, 39–41.
- Collar NJ, Andrews P (1988) *The ICBP World Checklist of Threatened Birds*, ICBP Technical Publication 8. ICBP, Cambridge, UK.
- Coltman DW, Bowen WD, Wright JM (1998) Birth weight and neonatal survival of harbour seal pups are positively correlated with genetic variation measured by microsatellites. *Proceedings of the Royal Society of London. Series B, Biological Sciences*, **265**, 803–809.
- Coltman DW, Pilkington JG, Smith JA, Pemberton JM (1999) Parasite-mediated selection against inbred Soay sheep in a free-living, island population. *Evolution*, **53**, 1259–1267.

- Coulson TN, Pemberton JM, Albon SD *et al.* (1998) Microsatellites reveal heterosis in red deer. *Proceedings of the Royal Society of London. Series B, Biological Sciences*, **265**, 489–495.
- Coulson TN, Albon S, Slate J, Pemberton J (1999) Microsatellite loci reveal sex-dependent responses to inbreeding and outbreeding in red deer calves. *Evolution*, **53**, 1951–1960.
- DeWoody JA (2005) Molecular approaches to the study of relatedness and parentage: extensions of pedigree analyses. *Journal of Wildlife Management*, in press.
- Duncan JR, Bird DM (1989) The influence of relatedness and display effort on the mate choice of captive female American Kestrels. *Animal Behaviour*, **37**, 112–117.
- Fridolfsson A, Ellegren H (1999) A simple and universal method for molecular sexing of non-ratite birds. *Journal of Avian Biology*, **30**, 116–121.
- Gavin TA, Reynolds RT, Joy SM, Leslie D (1998) Genetic evidence for low frequency of extra-pair fertilizations in Northern Goshawks. *Condor*, **100**, 556–560.
- Griffith SC, Owens IPF, Thuman KA (2002) Extra pair paternity in birds: a review of interspecific variation and adaptive function. *Molecular Ecology*, **11**, 2195–2212.
- Hansson B (2004) Marker-based relatedness predicts egg-hatching failure in great reed warblers. *Conservation Genetics*, **5**, 339–348.
- del Hoyo J, Elliott A, Sargatal J, eds (1994) *Handbook of the Birds of the World*, vol. 2, *New World Vultures to Guinea-fowl*. Lynx Edicions, Barcelona.
- IUCN (2003) *IUCN Red List of Threatened Species*. Available at <http://www.redlist.org>. Downloaded on 3 December 2003.
- Keane B (1990) The effect of relatedness on reproductive success and mate choice in the white-footed mouse, *Peromyscus leucopus*. *Animal Behaviour*, **39**, 264–273.
- Lack D (1968) *Ecological Adaptations for Breeding in Birds*. Methuen Ltd, London.
- Landry C, Garant D, Duchesne P, Bernatchez L (2001) 'Good genes as heterozygosity': the major histocompatibility complex and mate choice in Atlantic salmon (*Salmo salar*). *Proceedings of the Royal Society of London. Series B, Biological Sciences*, **268**, 1279–1285.
- Marshall TC, Slate J, Kruuk L, Pemberton JM (1998) Statistical confidence for likelihood-based paternity inference in natural populations. *Molecular Ecology*, **7**, 639–655.
- Martinez-Cruz B, David VA, Godoy JA *et al.* (2002) Eighteen polymorphic microsatellite markers for the highly endangered Spanish imperial eagle (*Aquila adalberti*) and related species. *Molecular Ecology Notes*, **2**, 323–326.
- Martinez-Cruz B, Godoy JA, Negro JJ (2004) Population genetics after fragmentation: the case of the endangered Spanish imperial eagle (*Aquila adalberti*). *Molecular Ecology*, **13**, 2243–2225.
- Møller AP, Birkhead TR (1993) Cuckoldry and sociality: a comparative study of birds. *American Naturalist*, **142**, 118–140.
- Neudorf DLH (2004) Extrapair paternity in birds: understanding variation among species. *Auk*, **121**, 302–307.
- Paetkau D, Waits LP, Clarkson PL *et al.* (1998) Variation in genetic diversity across the range of North American brown bears. *Conservation Biology*, **12**, 418–429.
- Peacock MM, Smith AT (1997) Nonrandom mating in pikas *Ochotona princeps*: evidence for inbreeding between individuals of intermediate relatedness. *Molecular Ecology*, **6**, 801–811.
- Piggott MP, Bellemain E, Taberlet P, Taylor AC (2004) A multiplex pre-amplification method that significantly improves microsatellite amplification and error rates for faecal DNA in limiting conditions. *Conservation Genetics*, **5**, 417–420.
- Queller DC, Goodnight KF (1989) Estimating relatedness using genetic markers. *Evolution*, **43**, 258–275.
- Raymond M, Rousset F (1995) GENEPOP (version 1.2): population genetic software for exact tests and ecumenicism. *Journal of Heredity*, **86**, 248–249.
- Rice WR (1989) Analyzing tables of statistical tests. *Evolution*, **43**, 223–225.
- Ricklefs RE (2000) Density-dependence, evolutionary optimism, and the diversification of avian life histories. *Condor*, **102**, 9–22.
- Rudnick JA, Ivy C, DeWoody JA (submitted) MATCH-MAKER 1.0: a computer program that uses the probability of identity to detect genetically identical samples within large datasets generated through noninvasive sampling. *Conservation Genetics*.
- Saether BE, Bakke O (2000) Avian life history variation and contribution of demographic traits to the population growth rate. *Ecology*, **81**, 642–653.
- Sambrook J, Russell DW (2001) *Molecular Cloning: A Laboratory Manual*, 3rd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
- Selvin S (1980) Probability of nonpaternity determined by multiple allele codominant systems. *American Journal of Human Genetics*, **32**, 276–278.
- Slate J, Kruuk LEB, Marshall TC, Pemberton JM, Clutton-Brock TH (2000) Inbreeding depression influences lifelong breeding success in a wild population of red deer (*Cervus elaphus*). *Proceedings of the Royal Society of London. Series B, Biological Sciences*, **267**, 1657–1662.
- Sloane MA, Sunnucks P, Alpers D, Beheregaray LB, Taylor AC (2000) Highly reliable genetic identification of individual northern hairy-nosed wombats from single remotely collected hairs: a feasible censusing method. *Molecular Ecology*, **9**, 1233–1240.
- Taberlet P, Camarra JJ, Griffin S *et al.* (1997) Noninvasive genetic tracking of the endangered Pyrenean brown bear population. *Molecular Ecology*, **6**, 869–876.
- Taberlet P, Waits LP, Luikart G (1999) Noninvasive genetic sampling: look before you leap. *Trends in Ecology & Evolution*, **14**, 323–327.
- Tregenza T, Wedell N (2000) Genetic compatibility, mate choice and patterns of parentage: invited review. *Molecular Ecology*, **9**, 1013–1027.
- UNEP-WCMC (2003) *UNEP-WCMC Species Database: CITES-Listed Species*. Available at <http://www.cites.org/eng/resources/species.html>.
- Watson J (1997) *The Golden Eagle*. T&AD Poyser, London.
- Weiner DR (1988) *Models of Nature Ecology, Conservation, and Cultural Revolution in Soviet Russia*. Indiana University Press, Bloomington.

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