

**University of Veterinary Medicine
Doctoral School of Veterinary Science**

**Phylogeography and population genetics of
white-tailed eagles (*Haliaeetus albicilla*) in the
Carpathian Basin**

Brief summary of Ph.D. theses

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Introduction

The white-tailed eagle (*Haliaeetus albicilla*, Linneus 1758; hereafter referred to as WTE) is a large raptor species distributed across the Palearctic and Greenland. It belongs to the subfamily *Haliatinae* ('sea eagles') of the family *Accipitridae* in the order *Falconiformes*.

The WTE is dependent on large water bodies and predominantly feeds on fish and waterfowl. However, individuals well adapt to local prey compositions. As a top predator, it plays an important role in water-related ecosystems. WTEs are territorial, monogamous raptors; and their individual lifespan can exceed 20, or even 30 years in the wild.

Individuals become mature around their 6th calendar year. Juveniles are vagrant, and can visit populations in several hundred or thousand kilometres from their natal area. Still, tend to breed relatively close to their natal area; the species is therefore assumed as philopatric.

Sampling issues

WTEs are sensitive to human disturbance and therefore their investigations should be planned with caution: their disturbance should be avoided, especially during the incubation and hatching period. Although in several European countries co-workers of the national parks ring a number of nestlings every

year, majority of the breeding adults do not carry any individual mark.

Conventional methods of individual identification of birds on the field (ringing or wing tagging) require the capture of individuals. This can be extremely challenging and should be avoided when investigating adult individuals of large raptor species. In such cases, non-invasive methods are increasingly preferred: we can investigate individuals by molecular methods, using their traces (e.g. feathers) left behind in the wild. DNA-based methods allow researchers to investigate several questions either on individual or population level: e.g. we can follow individual movements, estimate pairwise genetic relatedness, or investigate the direction and extent of gene flow.

In territorial raptors, it is generally assumed that moulted feathers collected at occupied nest sites likely belong to the territorial pair. Nevertheless, feathers shed by nest site intruders could potentially influence investigations concentrating on the resident individuals. To our knowledge, no study aimed to assess the reliability of moulted feathers for non-invasive sampling of residents at their nest sites in WTEs so far.

Population trends of the white-tailed eagle in Europe

The WTE was a common raptor species in the Carpathian Basin and many other European areas in the 19th century. Due to landscape changes, direct persecution and intensified

agriculture, populations of this large raptor decreased dramatically in the early 20th century across Europe: in many countries only a few breeding pairs survived or the species became regionally extinct. With banning the use of DDT in agricultural practice and PCBs in industry, and strict legal protection of the species, its populations started to increase again since the 1970s. The European breeding population is currently estimated at 9,000-12,000 breeding pairs, and the Carpathian Basin maintains about 500 breeding pairs.

The Carpathian Basin has always been an important region for the WTEs: besides maintaining a breeding population, the area has provided important wintering places for WTEs coming from several European areas, even during the population decline. The Carpathian Basin population did not become extinct, but the population bottleneck was significant: about 50 breeding pairs remained in the region (10-12 breeding pairs in Hungary). Recently, extensive investigations have been published on the genetic structure of European WTE populations, but the southernmost breeding area remained poorly studied. It is unknown whether recolonization of the Carpathian Basin occurred exclusively from the local surviving population (as expected from philopatry) or individuals coming from more distant populations played a role as well (e.g. through settlement of wintering individuals).

WTEs were released in some European countries during the 20th century. In the Czech Republic, WTEs were reintroduced after a local extinction, and this reintroduction possibly has affected the genetic structure of the present population: the released individuals originated from two captive breeding pairs found injured in the wild and their population of origin remained unknown.

Behavioural ecology of the white-tailed eagle

Similarly to other large raptors, WTEs are socially monogamous. Production of extra-pair offspring is generally rare in such raptor species (i.e. they are also genetically monogamous).

Several negative effects of inbreeding on the offspring's fitness are known. Inbreeding avoidance may be more crucial in mate choice in genetically monogamous species compared to those birds which have high divorce rate or produce many extra pair offspring. Despite the general philopatry of WTEs, sex-biased or long-distance natal dispersal might decrease inbreeding in this species. However, occurrence of such strategies have been studied only in a few populations so far.

Direct inbreeding avoidance require the ability of kin recognition. Although the ability of kin recognition has been shown in some bird species, occurrence of kin avoidance in mate choice has rarely been demonstrated in wild bird

populations. To our knowledge, so far no investigations attempted to reveal whether direct kin avoidance occur in mate choice of WTEs.

Similarly to other territorial raptors, conspecific territorial intrusions occur, even around the breeding season. Several authors suggested that intruders could gain opportunity to breed or acquire a territory. It has also been observed several times, that the vagrant juveniles can visit their natal area from time to time; and potentially, they can become territorial intruders as well during these visits. Nevertheless, our knowledge on the background of this behaviour is scarce.

Aims of the dissertation

1. We tested the **reliability of a non-invasive DNA sampling method** for breeding WTEs: We assessed whether moulted feathers collected at occupied nests sites belong to the breeding pairs, or feathers lost by intruders can potentially bias studies using such samples.

2. With a comprehensive sampling of WTEs across Europe we studied two main topics on population level:

- We investigated the **genetic structure of several European breeding populations** (from the northern to the southernmost areas) to reveal the history of the population recovery in the Carpathian Basin: did it occur exclusively through local expansion or did gene flow from other populations substantially contribute to it?
- We inferred the **origin of the captive birds released** between 1978 and 1989 in the Czech Republic to compare this population and its history with the naturally recovered neighbouring populations.

3. Occurrence of two strategies related to **inbreeding avoidance** was tested in the Carpathian Basin population:

- We tested the hypothesis that WTEs consider relatedness when **choosing a mate**, by comparing

mean pairwise genetic relatedness of actual breeding pairs to mean values predicted under random mating.

- We addressed whether **natal dispersal** is sex-biased in the Carpathian Basin, inferring from genetic and spatial data on male and female WTEs breeding in the area.

4. Using moulted feathers collected at occupied WTE nest sites, we investigated whether **nest site intrusions** can be explained by three non-exclusive hypotheses. Accordingly, intruders may visit their natal area, seek opportunity to breed, or to occupy a suitable territory.

Materials and methods

We investigated DNA samples extracted from a total of 247 shed WTE feathers, and 167 nestling feathers (pulled during the ringing process) from the Carpathian Basin, and an additional 118 DNA samples extracted from several tissues collected across Europe.

All investigations below were largely based on 11 or 12 loci microsatellite genotypes.

We also estimated the age of the sampled individuals, if colour pattern of their shed feathers allowed age estimation.

Reliability of moulted feathers for sampling residents

As WTEs are monogamous, we assumed that an individual is resident at the territory where it was sampled, if its genotype matched with the nestling genotypes (i.e. it could be the father or mother of the nestlings) from the same territory in the year of sampling.

Fisher's exact test was used to compare the ratio of residents to non-residents among the individuals identified from at least 3 feathers to the ratio of residents to non-residents among the individuals identified from less than 3 feathers in a sampling event. We similarly tested whether the odds of finding the resident females differed from the odds of finding the resident

males: we recorded whether the resident females and males were sampled or not at each nest site in each year.

Current genetic structure across Europe

Based on microsatellite genotypes of breeding individuals and nestlings sampled across Europe, genetic structure was investigated using two Bayesian clustering methods: we inferred the most probable number of genetic clusters with, or without information on geographical distances between the individuals.

We compared the Carpathian Basin with all previously investigated populations using a 499 bp fragment of the mitochondrial DNA. We sequenced this DNA region in 59 nestling samples collected across the Carpathian Basin.

To identify source populations for the recolonized areas of the Carpathian Basin and the Czech Republic, we carried out assignment tests on our microsatellite dataset, using both frequency-based and Bayesian approaches.

Mate choice and territorial intrusions

Residents and territorial intruders were investigated in the Boronka Landscape Protection Area (hereafter Boronka forest) and the associated contiguous forest, using shed feathers collected at occupied nest sites between 2013 and 2015. Shed feathers from resident pairs collected at other Carpathian Basin

areas were used as well, to investigate kin avoidance in mate choice.

Using individual microsatellite genotypes of WTEs, we estimated their pairwise relatedness. We used randomization tests to investigate 1) whether pairwise relatedness of actual breeding pairs differs from that expected under random mating, and 2) whether pairwise relatedness of intruders and opposite-sex residents differs from that expected if intrusions occur randomly.

The ratio of the odds of intrusion in old territories to the odds of intrusion in new ones was calculated using Fisher's exact test.

Analyses of parentage, maternity and paternity were performed to assess whether intruders sampled at the Boronka forest could originate from this area.

Genetic signs of a sex-biased natal dispersal

Sex-bias in natal dispersal was investigated by comparison of pairwise genetic relatedness and breeding distance of WTEs breeding across the Carpathian Basin.

As WTEs are long-term faithful to their territories, we assumed that the pairwise breeding distance of close relatives was generally longer if their natal dispersal distance was longer. Wilcoxon rank sum test was calculated to assess whether the

distribution of geographic breeding distances of close relative females was generally longer than that of close relative males.

Results

Reliability of moulted feathers for sampling residents

Reliability of moulted feathers (N=152) for sampling resident individuals was investigated in overall 25 WTE territories. On average 79% of the moulted feathers belonged to resident females and 18% to resident males, while 2% were shed by intruders and 1% by nestlings.

Fisher's exact test showed that the odds that the sampled individual was a resident was significantly higher if it had been identified from at least 3 moulted feathers than if it had been identified from less than 3 feathers ($p < 0.005$).

The odds for finding a resident female was significantly greater than the odds for finding a resident male ($p < 0.005$).

Current genetic structure across Europe

Based on 218 individuals, we found three genetic clusters being present with different frequencies along a north-south line: accordingly, we will refer to them as the northern, the central and the southern clusters. The southern cluster was exclusively present in the Carpathian Basin, while the northern and central clusters occurred in each studied region.

Based on genetic and geographic data together, the studied populations could be divided to five clusters: (1) Carpathian Basin, (2) Germany and Poland (together with north-western Czech Republic), (3) Czech cluster, (4) Finnish Lapland and Lithuania and (5) Finnish Baltic coast.

We found overall 4 mitochondrial haplotypes in our newly sequenced samples, out of which one (B12) is exclusively known from the Carpathian Basin, and 17% percent of the WTEs sampled in the area carried this haplotype.

According to our results, majority of the individuals with accepted origin in the re-colonized area of the Carpathian Basin originated from the local population (at least 33% of all sampled individuals), but some of them likely originated from the northern, and central European populations. The proportion of WTEs with northern origin was remarkably high (41%).

Mate choice and territorial intrusions

Microsatellite genotypes of a total of 24 residents (12 males, 12 females) and 16 intruders (5 males, 10 females, 1 of uncertain sex) were used from the Boronka forest and additional 24 residents from other Hungarian areas.

The observed mean pairwise relatedness of breeding pairs (0.013, $sd=0.03$, 12 pairs) in the Boronka forest was significantly lower than expected under random mating ($p<0.03$). The results

were similar when we extended the study area to southwestern Hungary (using 16 pairs).

Relatedness of observed dyads of intruders (males and females) and opposite-sex residents of the visited territories was generally low (median=0 for each sex). Still, the observed mean pairwise relatedness did not significantly differ from that expected under random intrusions neither for males nor for females.

Fisher's exact test showed that the odds of choosing an old territory was 5 times greater for male than for female intruders, however not significant (possibly due to our limited sample size).

Results of the parentage, paternity and maternity tests suggested that the intruders likely originated from outside the Boronka forest, and none of the sampled intruders were offspring of the current breeding pairs.

Three recorded events were especially interesting: 1) one female intruder that became resident next year, 2) replacement of the resident male in a good quality territory frequently visited by intruders and 3) a resident female with successful brood visited the nest site of another successfully breeding pair.

Genetic signs of a sex-biased natal dispersal

Pairwise relatedness and breeding distance of a total of 73 residents (24 males, 49 females; sampled by overall 214 moulted feathers) was calculated.

The spatial genetic autocorrelation analyses failed to find any genetic structure either among males or females breeding across the Carpathian Basin.

Overall 43 female-female and 13 male-male dyads were assumed to consist of closely related individuals, based on their pairwise maximum likelihood relatedness being at least 0.4. The median breeding distance was 136 km among related females (median=378 km) and 38 km among related males (median=247 km). The Wilcoxon rank sum test revealed that distribution of pairwise geographic distances was significantly shifted towards greater values for close relative females than for close relative males.

Conclusions

We showed that moulted feathers collected at occupied nest sites of WTEs are reliable DNA sources for studies concentrating on resident individuals of the sampled territories. As a proportion of these feathers can belong to nest site intruders, we suggest that genetic analyses of a higher number of feathers is necessary from each territory where the lack of nestling DNA samples makes impossible to unambiguously address the breeding pair.

Analyses of 11 loci microsatellite genotypes across Europe found three genetic clusters and their geographic distribution suggest a division for three major WTE populations: southern (Carpathian Basin countries: Hungary, Croatia, Serbia and Slovakia, south-eastern Czech Republic and north-eastern Austria), central (Poland, Germany, northern Austria and probably autochthonous Czech birds), and northern (Finland, Lithuania and probably Estonia). The northern population could be further divided to a coastal and an inland population.

We found a unique genetic cluster in the Carpathian Basin based on microsatellite genotypes and found that the mitochondrial haplotype B12 is not only unique, but frequent in this population. Our results both confirmed a mainly local recolonization in the Carpathian Basin after a population bottleneck in the 1970s, and suggest that some WTEs coming

from more northern populations contributed to its current genetic structure as well. Our results furthermore suggest that WTEs released in the Czech Republic had a significant impact on the current genetic structure of this population.

Despite its high migration capacity, the WTE is known as philopatric, suggesting that some level of genetic structure should be present among (or within) breeding populations. Our results highlighted that the Carpathian Basin maintains a WTE population with unique genetic composition, but no within-population genetic structure was found on a fine spatial scale. We suggest that the lack of fine-scale genetic structure within the Carpathian Basin is at least partly caused by female-biased long-distance natal dispersal events.

Understanding individual movements of floaters (future members of the breeding population) is important for species conservation. Here we provided some information on this topic, by investigating natal dispersal and nest-site intrusions in the Carpathian Basin WTE population. Our results confirmed that natal dispersal is sex-biased and suggest a sex-bias in territorial intrusions as well. We assume that sex-biased natal dispersal contributes to a generally low intersexual relatedness among potential mates in southwestern Hungary, and nest-site intrusions may be related to territory choice and mate choice in WTEs.

Based on pairwise genetic relatedness data, we gave the first report on kin avoidance in mate choice of a raptor species. We suspect that the long-term genetic monogamy could have promoted some direct mechanism for kin avoidance in WTEs.

Relevance for practical conservation biology

Our findings suggest that the Carpathian Basin is important for the species' conservation not only because it maintains a significant abundance of breeding WTEs, but it is also important for the preservation of genetic diversity of the species. Our findings on some level of gene flow from the northern populations suggest that the protection of the major wintering places in the Carpathian Basin can significantly contribute to the long-term preservation of the local breeding population.

We showed that using conservative criteria, a breeding WTE population can be non-invasively studied by using moulted feathers collected at occupied nest sites. This can be an effective method for long-term studies related to the species' conservation.

Relevance for veterinary science

Besides being apex predators, as facultative scavengers, WTEs may also have a significant role in clearing out carcasses from the wild. Consequently, they can provide an important sanitary service for wild animal populations, including game animals, by

preventing diseases from spreading from rotting carcasses left in the wild.

The main distribution range of a feather abnormality disease (the pinching off syndrome) of WTEs concurs with the WTE population of Germany, Poland and the Czech Republic. With the recovery of the European WTE populations, individuals may disperse from this central European population to the neighbouring ones, and occurrence of this so far locally distributed disease may become increasingly wider in the future, and may appear in the Carpathian Basin population as well.

Results new to science

1. Collection of moulted feathers at occupied nest sites during the breeding season is a reliable non-invasive method for sampling resident WTEs. However, due to potential influence of feathers lost by intruders conservative criteria are needed for addressing the sampled individuals as residents.

2. The studied European countries can basically be divided into three major WTE populations: southern (Carpathian Basin countries: Hungary, Croatia, Serbia and Slovakia, south-eastern Czech Republic and north-eastern Austria), central (Poland, Germany, northern Austria and probably autochthonous Czech birds), and northern (Finland, Lithuania and probably Estonia).

3. The previously described haplotype B12 at the mt-hvr1 is not only unique, but frequent in the Carpathian Basin WTE population. Our results on both the microsatellite genetic clusters and the mitochondrial haplotypes suggest that recovery in the Carpathian Basin occurred predominantly from a small surviving local population after the bottleneck period in the 1970s.

4. Assignment analyses on the individuals sampled in the recolonized areas suggest that immigration from the central and northern European populations also affected the genetic composition of the Carpathian Basin WTE population.

5. Genetic composition of the Czech WTE population suggests that although natural recolonization of the area could have been mainly local, it was largely influenced by the reintroduction of birds of presumably northern origin.

6. Our results based on pairwise genetic relatedness suggest that WTEs can avoid kin in mate choice. To our knowledge, no studies found evidence for similar strategy for inbreeding avoidance in raptor species so far, however it should be of outstanding importance for species with long-term genetic monogamy.

7. Spatial distribution of genetically related breeding individuals suggest that long-distance natal dispersal occurs within the otherwise philopatric WTE population of the Carpathian Basin, and these events are female-biased.

Publications related to the dissertation

1. Full text publications in peer-reviewed journals with an impact factor assigned

Nemesházi E., Kövér Sz., Zachos F.E., Horváth Z., Tihanyi G., Mórocz A., Mikuska T., Hám I., Literák I., Ponnikas S., Mizera T., Szabó K.: **Natural and anthropogenic influences on the population structure of white-tailed eagles in the Carpathian Basin and central Europe**, J. Avian Biol., 47. 795-805, 2016. (IF₂₀₁₆: 2.228)

Nemesházi E., Szabó K., Horváth Z., Kövér Sz.: **The effects of genetic relatedness on mate choice and territorial intrusions in a monogamous raptor**, J. Ornithol., 'online first', 2017. (IF₂₀₁₆: 1.468)

2. Full text publications in peer-reviewed journals with no impact factor assigned

Nemesházi E., Horváth Z., Mórocz A., Mikuska T., Tihanyi G., Szabó K.: **A Kárpát-medence rétisas-populációjának (*Haliaeetus albicilla*) filogeográfiai és populációgenetikai vizsgálata**, Állattani Közlemények, 98. 65-79, 2013.

3. Manuscript submitted to a peer-reviewed journal

Nemesházi E., Szabó K., Horváth z., Kövér Sz.: **Genetic structure confirms female-biased natal dispersal in the White-tailed Eagle population of the Carpathian Basin.**

Beküldve az Acta Zool. Acad. Sci. Hungaricae folyóirathoz
2017-ben. (IF₂₀₁₆: 0.52)

4. Oral presentations at international and Hungarian
conferences

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populációjának filogeográfiai és populáció-genetikai
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Szakosztályának 1007. előadójelése, Budapest, 2013.

**Nemesházi E.: A Kárpát-medence rétisas-populációjának
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genetikai vizsgálata.** XXXI. Országos Tudományos
Diákköri Konferencia, Szeged, 2013.

**Nemesházi E., Szabó K., Kövér Sz.: Filogeográfiai és
populáció-genetikai vizsgálatok a Kárpát-medencei
rétsisas-populációban (*Haliaeetus albicilla*).** Annual
meeting of the Committee for Veterinary Science of the
Hungarian Academy of Science and the Postgraduate
School of Veterinary Science, Budapest, 2014.

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Nemesházi E., Kövér Sz., Szabó K.: A rétisas (*Haliaeetus albicilla*) európai állományainak genetikai struktúrája, különös tekintettel a Kárpát-medencére. Akadémiai beszámoló (MTA Állatorvos-tudományi Bizottsága és SZIE Állatorvos-tudományi Doktori Iskola), Budapest, 2015.

5. Poster presentations at international and Hungarian conferences

Nemesházi E., Kövér Sz., Szabó K.: Population structure of European populations of the white-tailed eagle (*Haliaeetus albicilla*), paying particular attention to the Carpathian Basin. XVI. Student Conference on Conservation Science. Cambridge, 2015.

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Nemesházi E., Szabó K., Horváth Z., Kövér Sz.: Rétisasok rokonsági viszonyainak jelentősége a

párválasztásban és a betolakodásban. XVIII. Magyar
Etológus Konferencia, Debrecen, 2016.

Publications not related to the dissertation

1. Full text publications in peer-reviewed journals

Vili N., **Nemesházi E.**, Kovács Sz., Horváth M., Kalmár L.,
Szabó K.: **Factors affecting DNA quality in feathers
used for non-invasive sampling**, J. Ornithol., 154. 587-
595, 2013. (IF₂₀₁₃: 1.927)

Bókony V., Kövér Sz., **Nemesházi E.**, Liker A., Székely T.:
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reversals: the type of sex determination matters**,
Philos. Trans. R. Soc. Lond. B Biol. Sci., 372. 20160325,
2017. (IF₂₀₁₆: 5.846)

2. Manuscript submitted to a peer-reviewed journal

Ágh N., Kovács Sz., **Nemesházi E.**, Szabó K.: **Feasibility of
universal CHD1 sexing markers in various bird orders**.
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3. Oral presentations at international and Hungarian conferences

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Bókony V., Kövér Sz., **Nemesházi E.**, Liker A., Székely T.:

Climate-driven shifts in adult sex ratios via sex reversals. “Adult sex ratios and reproductive decisions: integrating data and theory across the biological and social sciences” workshop, Berlin, 2017.

Nemesházi E., Kövér Sz., Liker A., Székely T., Bókony V.:

Climate-driven shifts in adult sex ratios via sex reversals: the type of sex determination matters. XVI. Congress of the European Society for Evolutionary Biology (ESEB), Groningen, 2017.

4. Poster presentations at international and Hungarian conferences

Vili N., **Nemesházi E.**, Kovács Sz., Horváth M., Szabó K.:

Factors affecting DNA quality in feathers used for non-invasive and non-destructive sampling. 3rd European Congress of Conservation Biology, Glasgow, 2012.