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Environmental pollution affects genetic diversity in wild bird populations

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Abstract

Many common environmental pollutants, together with nuclear radiation, are recognized as genotoxic. There is, however, very little information on pollution-related genetic effects on free-living animal populations, especially in terrestrial ecosystems. We investigated whether genetic diversity in two small insectivorous passerines, the great tit (*Parus major*) and the pied flycatcher (*Ficedula hypoleuca*), was changed near point sources of heavy metals (two copper smelters) or radioactive isotopes (nuclear material reprocessing plant). We measured concentration of heavy metals and nucleotide diversity in mitochondrial DNA in feather samples taken from nestlings in multiple polluted areas and at control sites. In both species, heavy metal concentrations – especially of arsenic – were increased in feathers collected at smelter sites. The *P. major* population living near a smelter showed significantly higher nucleotide diversity than a control population in an unpolluted site, suggesting increased mutation rates in a polluted environment. On the contrary, *F. hypoleuca* showed reduced nucleotide diversity at both smelter sites but increased nucleotide diversity near the source of radioactivity. Our results show that heavy metal pollution and low level nuclear radiation affect the nucleotide diversity in two free-living insectivorous passerines. We suggest that the different response in these two species may be due to their different ability to handle toxic compounds in the body.

Keywords: Biomarkers; Genetic diversity; Heavy metal pollution; Insectivorous birds; Mitochondrial DNA; Nuclear radiation

1. Introduction

Many common environmental pollutants, together with nuclear radiation, are recognized as genotoxic [1]. For example, several heavy metals were found to have harmful effects on DNA [2,3]. Elevated germ-line mutations have been documented in birds and mammals in industrial areas [4,5]. Mutation rates were also increased in local bird and rodent populations after the Chernobyl accident due to high levels of radioactivity [6,7]. There is, however, very little information on pollution-related genetic effects in wild animal populations, especially in terrestrial ecosystems. The effects of low-level radiation on the genetic composition of populations are largely unknown for any free-living vertebrate. We show that heavy metal pollution and low-level nuclear radiation affect the nucleotide diversity in two free-living insectivorous passerines.

Bird feathers can be used as a non-invasive source to assess both genetic variation and pollutant levels from

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the same sample. Nestling feathers have shown to reliably indicate the body load of heavy metals [8]. We collected nestling feathers of two common insectivorous birds, the pied flycatcher (*Ficedula hypoleuca*) and the great tit (*Parus major*), at two smelter sites (Harjavalta, Finland and Revda, Russia) and at one site showing increased amount of radioactivity (Seversk Chemical Combine, Russia). Samples from multiple polluted sites were compared for nucleotide diversity in a mitochondrial control region with samples collected from the surrounding population in an unpolluted area. The same feathers were analyzed for their heavy metal content to measure the exposure to pollutants.

2. Materials and methods

2.1. Study areas and species

Nestling feathers were collected at two smelters (Harjavalta, Finland and Revda, Russia) and one nuclear material processing plant (Seversk Chemical Combine, Russia; Fig. 1). Samples of *F. hypoleuca* ($n = 98$) were taken from all three places but *P. major* feathers ($n = 40$) were collected only in Harjavalta, because population densities were too low to obtain adequate samples from the Russian sites. The Russian populations of *F. hypoleuca* belong to the subspecies *F. hypoleuca sibirica* Khakhlov, while the Finnish population belongs to the subspecies *F. hypoleuca hypoleuca* Pallas. Both subspecies of *F. hypoleuca* are trans-Saharan migrants, spending about 4 months on breeding grounds while *P. major* is a sedentary species, living in the study areas throughout the year.

All the study sites are typical point sources of pollution with relatively high levels of pollutants in the vicinity of the emission source and exponentially decreasing concentrations with increasing distance to the source. Increased levels of heavy metals in faeces of nestlings and low breeding success have been reported at both smelter sites and in both bird species [9,10].

The Harjavalta copper smelter (61°20′N, 22°10′E) in SW Finland was built in 1944. At that time all the sulphuric oxides and heavy metals were emitted into the surroundings without filtering. Later, most of the sulphuric oxides were filtered and dust emissions have decreased considerably. However, elevated heavy metal (mainly Cu, Ni, Pb, Zn, As) concentrations still occur in the area due to current and long-term deposition [11]. For example, organic soil copper (5799 ppm, dry weight) and lead (314 ppm, d.w.) concentrations near the smelter are, respectively, 39 and 5 times higher than at background sites, 8 km from the smelter [12]. The mean surface level contamination of Cs^{137} is 0.39 Ci/km^2 and the mean radioactive exposure level in the study site is 8.3 μR/h.

The Middle Ural copper smelter (56°51′N, 59°53′E) in Revda was built in 1940. Heavy metals (mainly Cu, Pb, Zn and Cd) and sulphuric oxides are the main pollutants of this large chemical–metallurgy complex. The pollution by metallic dust combined with sulphur dioxide has increased soil acidity and metal concentrations in the upper soil layer. For example, organic soil copper (5497 ppm, d.w.) and lead (1562 ppm, d.w.) concentrations near the smelter are, respectively, 102 and 24 times higher than at background sites, 20 km from the smelter [13]. The mean surface level contamination of Cs^{137} is 0.20 Ci/km^2 and the mean radioactive exposure level in the study site is 12 μR/h.

The Seversk Chemical Combine (56°37′N, 87°47′E) was founded in 1954. This reprocessing plant currently consists of two reactors (three reactors were stopped in 1990–1992), a chemical separation plant, a reprocessing facility for uranium and plutonium, an uranium enrichment plant and storage facilities for radioactive waste. The main pollutants in the area are various radioactive isotopes (e.g. Pu^{239}, Cs^{137} and Co^{60}) and fluorine compounds. The mean surface level contamination of Cs^{137} is 0.62 Ci/km^2 and mean exposure level in the study site is 16 μR/h. During the past 40 years several accidents have been reported at Seversk, resulting in releases of radioactivity to the environment. Increased radioactivity up to 400 μR/h have been reported in this area after a severe accident in 1993 [14] and

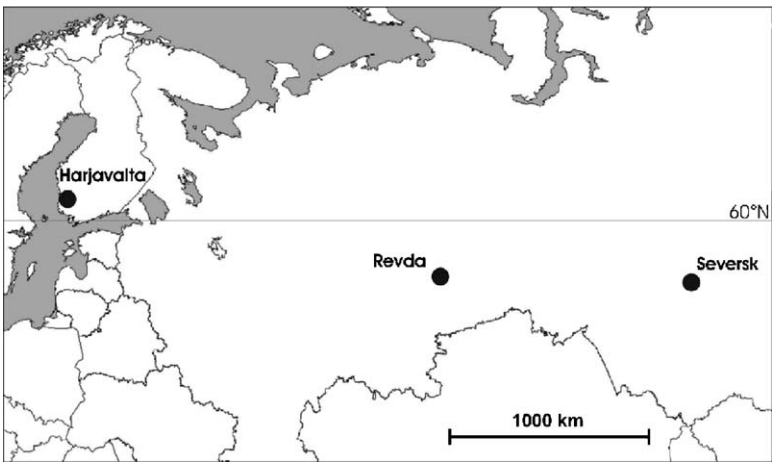

Fig. 1. The feather collection sites around two copper smelters (Harjavalta and Revda) and a nuclear material reprocessing plant (Seversk).

Table 1
Haplotype numbers of *Ficedula hypoleuca* (*F*) and *Parus major* (*P*) at study populations (boldface, in diagonal), numbers of common haplotypes between populations (below diagonal) and numbers of individuals with unique haplotypes in each population

	Harjavalta polluted, $n_F = 20$, $n_P = 18$	Harjavalta unpolluted, $n_F = 18$, $n_P = 17$	Revda polluted, $n_F = 18$	Revda unpolluted, $n_F = 18$	Seversk polluted, $n_F = 18$	Individuals with unique haplotypes
Harjavalta polluted	**F10**, **P15**					F5, P13
Harjavalta unpolluted	F3, P3	**F10**, **P11**				F7, P9
Revda polluted	F3	F3	**F8**			F3
Revda unpolluted	F2	F2	F2	**F9**		F6
Seversk polluted	F3	F3	F4	F2	**F13**	F9

Sample numbers (*n*) are shown for each population.

increased levels of chromosomal aberrations have been documented in humans living near this industrial complex [15].

2.2. Feather sampling

The advantages of sampling feathers are that they are easy to collect, sampling causes a minor stress to birds, and information on genetic structure and heavy metal concentrations can be obtained from a single feather. However, the exposure to radioactivity could not be assessed by using feather samples. The feather samples were collected from multiple heavy metal polluted sites close to the smelters (Harjavalta: six sites at 0.4–1.8 km; Revda: four sites at 1.0–2.5 km) and from relatively unpolluted (low metal levels, no effects on offspring production) control sites 4–12 km (Harjavalta: six sites) and 16–20 km (Revda: two sites) from the smelters. Due to much higher emissions in Revda compared with Harjavalta, the control sites of Revda are located farther from the pollution source. Samples from Seversk (two sites) were collected at a distance of 1.5 km from the pollution source and feathers from the Revda control area were used as a control for these samples. Sampling took place in the summer of 2000, but 10 samples were further collected in the summer of 2001 from the polluted area of Revda to increase the sample size (Table 1). We plucked one outer rectrix (*P. major* in Harjavalta and *F. hypoleuca* in Revda and Seversk) or one inner primary (*F. hypoleuca* in Harjavalta) of 14-day-old (*F. hypoleuca*) or 16-day-old (*P. major*) old nestlings. Because different feathers were taken from *F. hypoleuca* in Harjavalta and Revda, the heavy metal levels may not be directly comparable among study areas, but they are directly comparable between polluted and control areas in all cases. However, we consider it unlikely that rectrices of nestlings would show markedly different heavy metal levels than primaries, because in nestlings both types of feather are growing at the same time. Only one sample was taken from each nest.

2.3. Metal analysis

We cut the feathers into two parts near the base of a vane. The distal part was taken for metal analyses while the proximal part was preserved for DNA sequencing. Samples for metal analyses were washed vigorously in deionized water alternated with 1 mol/l acetone (Baker analyzed) to remove any external contamination, and dried at room temperature for 48 h. The samples were accurately weighed and 2 ml of Supra-pure HNO_3 and 0.5 ml of H_2O_2 were added to the samples into Teflon bombs for digestion with a microwave system (Milestone High Performance Microwave Digestion Unit mls 1200 mega). After digestion, the samples were diluted to 50 ml with deionized water (Elgastat Maxima). The determination of the elemental concentration of Al, As, Cd, Cr, Cu, Ni, Pb, Sn and Zn was done with an inductively coupled plasma mass spectrometer (ICP-MS) Elan 6100 DRC+ from PerkinElmer-Sciex. The detection limits for most elements are around the ppt (ng/l) level and below. The calibration of the instrument was done with a certified solution (Claritas PPT, Multi-element solution 2A) from Spex Certiprep. One exceptionally high value for the Al concentration (609 ppm, Revda) was omitted as an outlier from subsequent analyses.

2.4. DNA purification, PCR and sequencing

Isolation of DNA from blind feather samples was performed with a DNeasy™ tissue kit for DNA purification (Qiagen), using animal tissue protocol. The following specific primers were used in PCR to amplify a sequence close to the beginning of the mitochondrial control region: for *P. major*, H1471: 5′-AGT CAA GTT GCA CTC ATT GCT TAA T-3′ and L16700: 5′-ATC ATA AAT TCT CGC CGG GAC TCT-3′; for *F. hypoleuca*, IDH521: 5′-ATG CCC CTG AAA TAG GAA CCA GT-3′ and ND6DL2L: 5′-CAT AGT AGG GAG AAG GGT TGG AGG CG-3′. The PCR profiles were the following: for *P. major*: denaturation at 94 °C for 2 min 30 s, then 63 °C for 30 s, 72 °C for 30 s, repeated for 30 cycles and a final synthesis at 72 °C for 5 min; *F. hypoleuca*: denaturation at 94 °C for 2 min 30 s, then 55 °C for 30 s, 72 °C for 30 s, repeated for 37 cycles and a final synthesis at 72 °C for 7 min.

The amplification product was purified from agarose gel and sequenced by using the following primers: for *P. major*, H636: 5′-GAG ATG AGG AGT ATT CAA CCG AC-3′ and H737: 5′-GCA CTG GAA GGG TTT ATT GAA G-3′; for *F. hypoleuca*, IDH409: 5′-GGT TCT CGT GAG AAA CAC GAT-3′. Sequencing was performed using dye terminator chemistry of Applied Biosystems and the ABI Prism 377 automatic

sequencer. All samples were sequenced twice to reduce a possibility of genotyping errors. Five samples of *P. major* and six samples of *F. hypoleuca* were discarded from the subsequent analyses due to low-quality sequences.

2.5. Sequence divergence analysis

Two overlapping sequences were aligned with a sequence merger of the European Molecular Biology Open Software Suite (EMBOSS) by using Needleman & Wunsch alignment algorithm to create a merged sequence. Merged sequences were further aligned to the same length (*P. major*: 557 bp; *F. hypoleuca*: 358 bp) by using BioEdit Sequence Alignment Editor version 5.09 [16]. The estimates for the nucleotide diversity (π = proportion of sites different in two random copies of a sequence) were calculated with Jukes and Cantor correction [17] for each population using DnaSP version 3.51 [18]. The differences in nucleotide diversity among populations (within species) were tested with pairwise *t*-tests. Among the five *F. hypoleuca* populations we calculated genetic distances on the basis of average number of nucleotide substitutions per population (with Jukes and Cantor correction). These distances were then used to construct a population dendrogram by the UPGMA method in MEGA 3.1 [19].

3. Results

We found 23 different haplotypes for *P. major* ($n = 35$ individuals) and 36 haplotypes for *F. hypoleuca* ($n = 92$ individuals). For *P. major* there were 15 haplotypes in the polluted and 11 haplotypes in the unpolluted site, of which only three were common to both sites (Table 1). The frequency of individuals with unique haplotypes (Table 1) did not differ between sites ($\chi^2 = 1.39$, d.f. = 1, $P = 0.24$). The most common haplotype (eight individuals) is identical to one in GenBank (accession number AF059662). Despite the distant geographic location of sampling sites and the fact that two subspecies were sampled, two most common haplotypes of *F. hypoleuca* were found in all the sampled populations, and geographically proximate populations did not show more common haplotypes than geographically distant populations (Table 1). Neither in this species the frequency of individuals with unique haplotypes differed among sites ($\chi^2 = 5.4$, d.f. = 4, $P = 0.25$). The two most common haplotypes (21 individuals both) are identical to two sequences in GenBank (accession numbers AJ275164 and AJ275134).

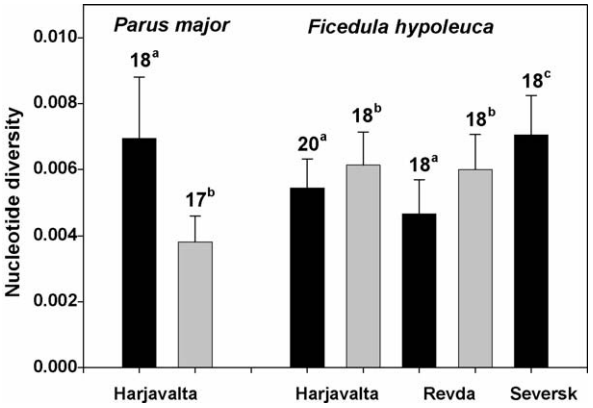

Fig. 3. The nucleotide diversity ($\pi \pm$ S.D.) in the mitochondrial control region for populations of *Parus major* and *F. hypoleuca* at polluted (black bars) and unpolluted (grey bars) sites. Within species, means with the same letter are not significantly different (*t*-test).

The analysis of genetic distances revealed that the Seversk population was most distant among the five *F. hypoleuca* populations (Fig. 2). However, the exposed populations of Harjavalta and Revda were most similar and it seems that geographic distances do not explain the genetic distances among the sampled populations (Fig. 2).

The *P. major* population living near a smelter showed significantly higher nucleotide diversity than the control population in an unpolluted site (Fig. 3). On the contrary, the *F. hypoleuca* population showed reduced nucleotide diversity at both smelter sites (Fig. 3). However, the nucleotide diversity of *F. hypoleuca* was increased in Seversk, near the source of radioactivity (Fig. 3).

The concentrations of heavy metals in feathers are shown in Table 2. Element profiles differ markedly

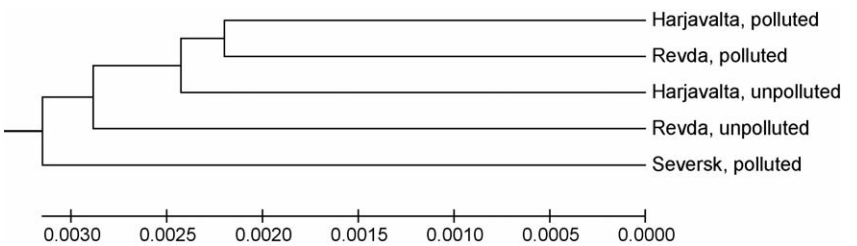

Fig. 2. A dendrogram showing the genetic distances among five populations of pied flycatchers (*Ficedula hypoleuca*). The tree was calculated from mtDNA control region sequences by using distance matrix of average number of nucleotide substitutions per population.

Table 2
Metal concentrations (μg/g, dry weight) in feathers of *F. hypoleuca* and *P. major* nestlings from three polluted areas

Population	Metal	Polluted			Unpolluted			*F*	*P*
		n	$\bar{x}$	S.E.	*n*	$\bar{x}$	S.E.		
F. hypoleuca									
Harjavalta	Al	20	39.1	3.08	20	39.9	2.74	0.17	0.69
	As	20	2.15	0.39	20	0.38	0.03	62.0	<0.0001
	Cd	20	0.09	0.01	20	0.10	0.01	1.86	0.18
	Cr	20	1.73	0.13	20	1.64	0.10	0.12	0.73
	Cu	20	14.0	1.28	20	12.6	0.86	0.69	0.41
	Ni	20	12.9	1.02	20	9.57	0.36	8.88	0.0050
	Pb	20	3.09	0.26	20	3.09	0.32	0.02	0.90
	Sn	20	1.28	0.24	20	2.19	0.14	24.6	<0.0001
	Zn	20	146.3	7.10	20	130.5	3.86	3.63	0.064
Revda	Al	17	45.7	6.10	20	27.36	1.75	11.0	0.0021
	As	18	8.41	1.64	20	0.63	0.07	149.6	<0.0001
	Cd	18	0.17	0.03	20	0.14	0.03	0.77	0.39
	Cr	18	2.96	2.01	20	0.45	0.04	16.7	0.0002
	Cu	18	15.3	1.60	20	8.79	0.96	19.9	<0.0001
	Ni	18	8.00	0.88	20	3.80	0.40	25.0	<0.0001
	Pb	18	23.6	4.02	20	2.13	0.15	217.0	<0.0001
	Sn	18	1.04	0.53	20	0.06	0.02	28.99	<0.0001
	Zn	18	185.4	8.17	20	145.31	2.68	26.1	<0.0001
Seversk	Al	20	53.74	9.90	Unpolluted area of Revda (above)			10.0	0.0031
	As	20	0.24	0.03	was used as a control of Seversk			38.7	<0.0001
	Cd	20	0.04	0.01				30.2	<0.0001
	Cr	20	0.90	0.07				29.9	<0.0001
	Cu	20	11.6	1.28				5.28	0.027
	Ni	20	3.82	0.23				0.31	0.58
	Pb	20	1.02	0.10				44.2	<0.0001
	Sn	20	0.72	0.38				7.06	0.012
	Zn	20	134.4	3.02				8.05	0.0073
P. major									
Harjavalta	Al	20	45.5	3.92	20	29.7	2.60	13.0	0.0009
	As	20	1.10	0.10	20	0.26	0.02	137.0	<0.0001
	Cd	20	0.03	0.00	20	0.01	0.00	7.09	0.011
	Cr	20	0.82	0.06	20	0.46	0.04	29.9	<0.0001
	Cu	20	15.9	1.15	20	8.32	0.58	43.5	<0.0001
	Ni	20	9.18	1.06	20	3.84	0.85	34.7	<0.0001
	Pb	20	2.00	0.64	20	1.67	0.41	0.94	0.34
	Sn	20	3.44	0.20	20	1.74	0.26	21.5	<0.0001
	Zn	20	132.4	2.56	20	126.5	2.65	2.90	0.097

ANOVA for differences between polluted and unpolluted sites. Values were log-transformed before ANOVA.

among areas. In the polluted area of Harjavalta, feathers from *F. hypoleuca* contained relatively (enrichment factors in brackets: polluted/unpolluted) more As (5.7) and Ni (1.3), whereas the concentration of Sn (0.6) was higher at the control site. Compared with the background level, the feathers from *P. major* from the polluted area of Harjavalta contained more of all the metals, except Pb and Zn. In *P. major* the highest relative differences were found for As (4.2), Cd (3.0), Ni (2.4) and Sn (2.0). In the polluted area of Revda, *F. hypoleuca* feathers contained relatively more of all the metals, except Cd. The highest relative differences were found for Sn (17.3), As (13.3), Pb (11.1) and Cr (6.6). In Seversk, *F. hypoleuca* feathers contained relatively more Sn (12.0), Cr (2.0), Al (2.0) and Cu (1.3) than in the control area. In Seversk there were less Cd (0.3), As (0.4) and Pb (0.5) than in the control area. In general, As was typically strongly associated with the distance to the copper smelter in both species and at both sites. The clearest difference between the smelters sites of Harjavalta and Revda is the very high concentration of Pb in Revda.

4. Discussion

A common molecular mechanism in the toxicities of many xenobiotics may be a production of reactive oxygen species, i.e. free radicals, which may result in a condition known as oxidative stress. Free radicals are produced in cells by ionizing radiation, by a variety of chemicals (including heavy metals) and by normal metabolic processes [20]. For example, Cu is considered to be an important factor in the generation of free radicals and the formation of DNA damage in target cells [21]. The intensity of peroxide oxidation (MDA; mmol of malonic dialdehyde/g) of lipids (a measure of oxidative stress) was 1.5 times higher in 17-day-old nestlings of *P. major* living close to the Revda smelter compared with the control population [22]. Furthermore, this value correlated strongly ($r = 0.60$, $n = 28$, $P = 0.00074$) with the Cu concentration in the nestling skeleton [22]. Our data suggest that higher oxidative stress at polluted sites may cause increased mutation rates in *P. major* nestlings. Heavy metals may also inhibit repair of DNA damage and form adducts in nucleotide bases of DNA [1].

The nucleotide diversity in *P. major* population of Harjavalta also seems to be high when compared with that in several other European populations. Kvist et al. [23] compared mtDNA among eight European great tit populations from Spain to northern Finland. Their sample also included the background population of Harjavalta, in which the average nucleotide diversity was highest ($\pi = 0.00330$) among the studied populations and comparable with the value in our study ($\pi = 0.00381$). Since the focal *P. major* populations are not isolated, they are not geographically distant and there are no major differences in breeding densities [24], we believe that the most likely explanation for higher nucleotide diversity near the pollution source is mutations caused by toxic substances like heavy metals. Increased nucleotide diversity even in the background area suggest further that genetic effects extend much farther from the pollution source than, e.g., the detrimental effects on reproduction that have been documented in the vicinity of the smelter [10].

Why was the nucleotide diversity of *F. hypoleuca* lower at smelter sites than at unpolluted control sites? Several effects at the population level are known to affect genetic variance [25,26]. First, the population size is known to be related to genetic diversity [27]. All our sampling sites represent a continuous and practically infinite population of *F. hypoleuca*, but the density of the breeding populations, which is sometimes used as a measure of local population size, varies among study sites. In Harjavalta, breeding density was similar in both areas (polluted 108 pairs/km^2, unpolluted 121 pairs/km^2) [22], but in Revda it was about 10 times higher in the control area (in 1996–2000; 161 pairs/km^2) than in the polluted area (14 pairs/km^2). No dramatic temporal changes have been observed in breeding densities at smelter sites during the past 15 years. Breeding density was very high in Seversk (in 1996–2000; 927 pairs/km^2) where also the nucleotide diversity was highest. We are not aware of any studies on the relationship between population density and genetic diversity in birds. In plants, however, population density was found to be unrelated to genetic diversity [28]. Second, spatial distribution of sampling sites around a point source of pollutants might affect the result [see 29]. Sampling sites near the pollution source tend to be closer to each other than those in the control area. However, in our data there were not more shared haplotypes between proximate than distant populations and geographic distances seemed not to explain the genetic distances among populations, suggesting that distance between sampling sites had little effect on variance, even at the large scale. Third, environmental stress like pollution might indirectly decrease genetic variance in a population, e.g., via adaptation or assortative settling of breeding birds to polluted and often unfavourable environments. In the case of *F. hypoleuca* we consider adaptations to relatively small areas around point sources of pollutants unlikely, because natal and breeding dispersal of this species is very intensive [30]. Furthermore, the control region of mtDNA is considered to be a neutral marker and insensitive to the effects of selection. The Tajima's D assessing neutrality of the mtDNA control region was negative (-1.77) and did not deviate significantly from zero ($P > 0.05$) for the combined population of *F. hypoleuca*. We have no evidence of assortative settling of breeding birds to polluted environments for several morphological parameters [31] but for other variables this possibility cannot be excluded.

Why did two species respond differently to heavy metal pollution? The natal and breeding dispersal of *F. hypoleuca* is much more extensive than that of *P. major* [32]. It is thus likely that *F. hypoleuca* populations mix up every year to a greater extent than *P. major* populations, which would inhibit the accumulation of mutations in the exposed population of *F. hypoleuca*. This explanation does not, however, fit to increased diversity of *F. hypoleuca* in the radioactive area. Alternatively, the difference might be connected to detoxication capacity. Since our earlier studies have shown that *F. hypoleuca* nestlings get the same or greater amounts of heavy metals in their food than *P. major* [33,34], one explanation might be a different detoxication ability of these species.

The detoxication capacity of *F. hypoleuca* seems to be considerably better than that of *P. major* when measured with the activity of EROD enzyme, one of the detoxifying enzymes in the liver. EROD activity of *F. hypoleuca* nestlings and adults are inherently about 3.7 and 5.5 times higher than in *P. major* nestlings and adults, respectively [35]. Thus, *F. hypoleuca* may be more capable to handle toxic substances and less prone to suffer from oxidative stress than *P. major*. This mechanism cannot protect birds from ionizing radiation and, accordingly, our data showed increased nucleotide diversity in *F. hypoleuca* population exposed to radioactivity. Ilyinskikh et al. [14] showed that the frequency of abnormal erythrocytes increased in feral pigeons (*Columba livia*) after a severe accident and radioactive fallout at Seversk in 1993. We could not make a distinction between partial (heteroplasmic) and full mutations, but some of the observed mutations in our data are likely to be heteroplasmic since Forster et al. [36] have shown that most of the observed radiation induced mtDNA point mutations are heteroplasmic.

To conclude, our study shows that environmental pollution affects genetic variation in free-living bird populations and the effect depends on species and type of emission. The exact mechanism whereby genetic differences have arisen cannot be assessed by our study, but we consider increased mutation rates at polluted sites as the most likely explanation. We also suggest that the different response of the two species may be due their different ability to handle toxic compounds in the body.

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