DNA damage in barn swallows (*Hirundo rustica*) from the Chernobyl region detected by use of the comet assay

Andrea Bonisoli-Alquati [a,*], Andrew Voris [b,e], Timothy A. Mousseau [c], Anders Pape Møller [d,f], Nicola Saino [a], Michael D. Wyatt [b]

[a] *Dipartimento di Biologia, Sez. Ecologia, Università degli Studi di Milano, via Celoria 26, I-20133 Milano, Italy*
[b] *Department of Pharmaceutical and Biomedical Sciences, University of South Carolina, Columbia, SC 29208, USA*
[c] *Department of Biological Sciences, University of South Carolina, Columbia, SC 29208, USA*
[d] *Laboratoire d'Ecologie, Systématique et Evolution, CNRS UMR 8079, Université Paris-Sud, Bâtiment 362, F-91405 Orsay Cedex, France*
[e] *Medical University of South Carolina, Charleston, SC 29425, USA*
[f] *Center for Advanced Study, Drammensveien 78, NO-0271 Oslo, Norway*

ABSTRACT

We investigated levels of DNA damage in blood cells of barn swallows (*Hirundo rustica*) inhabiting the Chernobyl region to evaluate whether chronic exposure to low-level radioactive contamination continues to induce genetic damage in free-living populations of animals. Blood samples were obtained from barn swallows collected at sites with different background levels of radiation, including a relatively uncontaminated area. The extent of DNA damage was evaluated using the alkaline (pH = 12.1) comet assay, a robust and sensitive electrophoresis-based technique widely employed in research ranging from biomonitoring to clinical studies. We found that levels of DNA damage, as indexed by the extent of DNA migration, were increased in barn swallows living in areas surrounding Chernobyl when compared to swallows sampled at low-level sites. The results we obtained are consistent with previous findings on this same species, which showed that swallows breeding in areas heavily contaminated with radionuclides have increased mutation rates, higher oxidative stress and incidence of morphological aberrations and tumors. Overall, these results indicate that chronic exposure to radioactive contaminants, even 20 years after the accident at the Chernobyl nuclear power plant, continues to induce DNA damage in cells of free-living animals.

© 2009 Elsevier Inc. All rights reserved.

1. Introduction

The accident at the Chernobyl nuclear power station in 1986 likely represents the worst accidental release of radionuclides (mainly [131]Iodine, [90]Strontium and [137]Caesium; Shestopalov, 1996) into the environment. Although the subsequent exposure to radiation is considered to have been at a low level (i.e. <20 mSv/year; Golikov and Balonov, 1993), harmful biological consequences spanning from the individual to the community and ecosystem level caused by the radioactive contamination have been demonstrated in a number of studies (review in Møller and Mousseau, 2006).

The barn swallow (*Hirundo rustica*), a small, long-distant migratory passerine bird, has been a model species for many of these studies, owing to its thoroughly investigated ecology and life history. Studies conducted on barn swallows have shown that individuals breeding in contaminated areas around Chernobyl experience reduced survival rate and reproductive success, as demonstrated by both a lower proportion of reproducing adults and an increase in hatching failure compared to birds breeding in uncontaminated areas (Møller et al., 2005a). In birds from the Chernobyl area, individual lymphocyte counts and immuno-globulin concentrations are reduced (Camplani et al., 1999) and antioxidants, such as carotenoids and vitamins A and E, in plasma and liver are seriously decreased (Møller et al., 2005b). Partial albinism in the plumage, together with other morphological abnormalities, is also more frequent in birds breeding in the most heavily contaminated areas and is associated with a reduction in individual survival rates (Ellegren et al., 1997; Møller et al., 2007). Consistent with this decrease in fitness, barn swallow population sizes in the Chernobyl region are declining (Ellegren et al., 1997) despite immigration from populations in uncontaminated areas (Møller et al., 2006).

At the genetic level, barn swallows from around Chernobyl have a germline mutation rate enhanced by a factor of two to ten, compared to a Ukrainian and an Italian control population, as revealed by means of a parent–offspring regression analysis at both expressed and non-coding (i.e. microsatellite) loci (Ellegren et al., 1997). Moreover, in a study where abnormal sperm were used to track individual muta-tions, the increase in the germline mutation rate was related to increased levels of background radiation (Møller et al., 2005b). In

* Corresponding author. Tel.: +39 0250314716.
E-mail address: andrea.bonisoli@unimi.it (A. Bonisoli-Alquati).

addition, this study provided support to the hypothesis of an inverse relationship in this species between the level of antioxidants in an individual bird and sensitivity to radiation (i.e. increases in mutation rate among individuals may be due to decreased levels of circulating or stored antioxidants) (Møller et al., 2005b).

Ionizing radiation can induce DNA damage either by direct effects (Iliakis, 1991) or indirectly by producing an excess of free radicals that can in turn damage DNA (Imlay and Linn, 1988; Imlay et al., 1988). Antioxidant compounds provide protection against the free radicals (i.e. reactive oxygen species, ROS) that arise as an unavoidable byproduct of mitochondrial respiration; ROS can cause significant damage to biological molecules, including DNA (Halliwell and Gutteridge, 2007). Induction of ROS by radiation would reduce the available antioxidant levels, which might increase the risk for DNA damage resulting from further exposure to ROS (Neyfakh et al., 1998a,b; Kumerova et al., 2000; see Møller et al., 2005b).

Several techniques have then been developed that allow for the quantification of the extent of DNA damage induced by genotoxic agents in vivo (Tice et al., 2000). Here, we report the first finding of an increase in DNA damage in a wild population of any animal after the Chernobyl disaster by using the alkaline (pH = 12.1) single-cell gel electrophoresis assay (or comet assay), a technique designed to detect the ability of chemical and physical agents to induce increased levels of DNA strand breaks in eukaryote cells (Tice et al., 2000). The comet assay is usually regarded as being one of the most sensitive and reliable techniques, with advantages of cost-effectiveness, speed, and the fact that observations are made at the level of the individual cell (Tice et al., 2000). Due to these advantages and to its applicability to virtually any eukaryotic cell, the use of the comet assay has recently been extended to a wide range of applications, spanning from the molecular to the epidemiological level (Collins et al., 1997; see reviews in Fairbairn et al., 1995; Collins, 2004; Tice et al., 2000; Jha, 2008).

This technique has traditionally been used to investigate radiation-induced DNA damage in humans and other model species (reviews in Tice and Strauss, 1995; Olive, 1999). Recently, this technique is increasingly being applied to ecogenotoxicological studies of wild organisms (Kleinjans and van Schooten, 2002), in both animal and plant species (Jha, 2004, 2008; Dhawan et al., 2009; Frenzilli et al., 2009), usually for investigating the effects of chemical pollutants and predict the genotoxic potential of chemicals. Studies on birds are relatively rare compared to other taxonomic groups (Pastor et al., 2001a,b, 2004).

The comet assay has also been used to assess levels of DNA damage in people, mainly children and clean-up workers, inhabiting contaminated areas near Chernobyl (Frenzilli et al., 1998, 2001; Garcia and Mandina, 2005). To the best of our knowledge, however, the comet assay has not previously been used to examine DNA damage in cells of any wild population exposed to the radioactive contaminants in the environment following the accident at Chernobyl in 1986.

2. Material and methods

2.1. General field procedures

During June 2005–2007, we captured barn swallows using mist nets across open doors and windows in farm buildings previously inspected for their presence. This method has been shown to be unbiased and highly efficient during the breeding season (Møller and Szép, 2002). The individuals included in this study were captured in Dytiaku (51° 07′ N, 30° 09′ E), Pysky (51° 05′ N, 30° 03′ E), Vesniane (51° 18′ N, 29° 37′ E), and Ghovtnere (50° 12′ N, 30° 49′ E) (Fig. 1). Blood samples were taken by puncturing the brachial vein, collecting the blood in heparinized capillary tubes.

Background radiation levels were measured using a hand-held dosimeter (Inspector, SE International, Summertown, TN, USA). Based on our field measurements, we classified sampling sites as low-, intermediate or high-level. Background levels of radiation were 0.02 μSv/h in the low-level site (Ghovtnere), 0.05 μSv/h in both the intermediate-level sites (Dytiaku and Pysky) and 2.9 μSv/h in the high-level site (Vesniane) (see Fig.1). Field-collected radiation measurements were highly correlated with officially published data (Shestopalov, 1996; see Møller et al., 2008a for details).

2.2. The comet assay

Blood samples were processed according to the protocol by Singh et al. (1988) with minor modifications. Samples were first frozen at −20 °C and later transferred to a −80 °C freezer. The same procedure was applied to samples from all years. Samples were quickly thawed by immersion in a 37 °C water bath, to avoid consolidation of micro-crystals of ice into larger forms, that are known to be damaging (Mazur et al., 1981). Erythrocytes were suspended in Ca^{2+}/Mg^{2+}-free PBS at 2×10^5 cells/mL. A volume of this suspension was mixed with 1% low-melting agarose (LMA) in a 1:10 ratio, and layered on two-well, precoated slides (Trevigen, Gaithersburg, MD, USA). Slides were placed at 4 °C in the dark for 30 min, then immersed in ice-cold, freshly prepared lysing solution (Lysis Solution, Trevigen, pH = 10.0, combined with 10% dimethyl sulfoxide (DMSO)), and allowed to sit in the dark for 1 h. DNA unwinding was carried out in a tray filled with freshly prepared alkaline solution (1 mM ethylenediaminetetraacetic acid disodium salt (Na_2 EDTA), 300 mM NaOH, pH = 12.1) for 45 min. DMSO, Na_2 EDTA and NaOH were all obtained from Sigma-Aldrich (St Louis, MO, USA). Electrophoresis was performed using the same solution at 250 mA and 25 V (ca. 1 V/cm) for 10 min. Unwinding and electrophoresis at pH = 12.1 were chosen to avoid the expression of alkali labile sites, which are extremely abundant in erythrocytes, owing to the high condensation of chromatin (Singh et al., 1989; Tice et al., 2000; Frenzilli et al., 2009). Our procedure thus facilitated the expression of single and double strand breaks, incomplete cross-links and excision repair sites (Miyamae et al., 1997; Tice et al., 2000). Electrophoresis buffer was rinsed by dipping the slides three times in distilled water. Slides were then immersed in 70% ethanol for 5 min, and allowed to air dry prior to staining. Slides were stained by placing 100 μL of diluted SYBR® Gold (Trevigen) onto each circle of dried agarose. Slides were scored under a Nikon Eclipse 600 microscope with a Mercury lamp ultraviolet light source, equipped with a QImaging micropublisher 3.3 camera, using the HCSA Comet Analysis System (Loats Associates, Inc., Westminster, MD, USA), which automatically provides multiple standardized measures of individual cells, including comet tail length, percent of total cellular DNA in the tail and tail moment. Each sample was run in duplicate on two separate slides and 75 cells were scored blind to the collection site. Only red blood cells were scored, while leukocytes, that are easily recognizable because of their much bigger size, were excluded from this analysis.

Cytotoxicity was checked through a neutral diffusion assay (Vasquez and Tice, 1997), which determines the frequency of cells with low molecular weight DNA. Single-cell suspensions from all tissues were processed in the comet assay except for DNA unwinding and electrophoresis, which were not performed (Vasquez and Tice, 1997). The nuclei were qualitatively examined for large or extended halos as evidence of compromised cellular integrity, according to Rider et al. (2006). There were no observable differences among the samples, which suggested that cell viability did not differ among radiation-level groups.

2.3. Statistical analyses

For in vivo studies, the unit of comparison in statistical analyses assay is the individual animal (Lovell et al., 1999). For each individual included in our analyses, we thus reduced all the measures collected at the level of single cells to different summary statistics representing the distribution of comet values (Lovell et al., 1999). Genotoxic effects
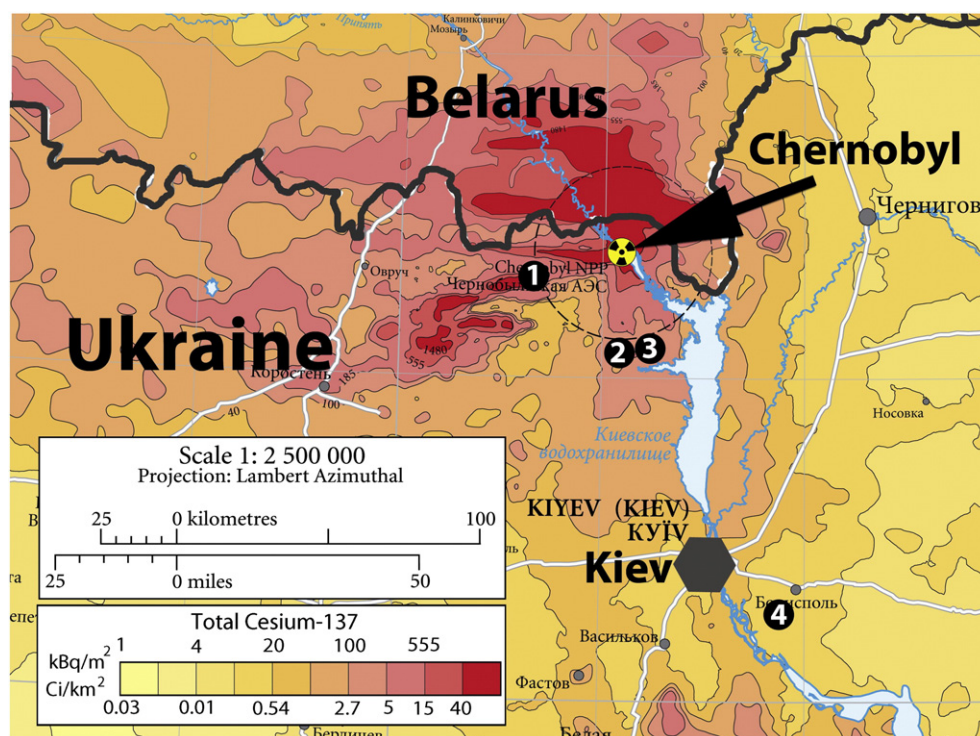
Fig. 1. Location of the study sites. 1. Vesniane (51 18′ N, 29 37′ E), 2. Pysky (51 05′ N, 30 03′ E), 3. Dytiaku (51 07′ N, 30 09′ E), 4. Ghovtnere (50 12′ N, 30 49′ E).

detected by the comet assay are usually marked by a change in both mean values and dispersion of the data (Duez et al., 2003). However, considerable uncertainty exists about which statistic is more meaningful for summarizing the data collected at the level of single cells (Lovell et al., 1999). We thus employed both the mean and median as two different measures of the central tendency of the distribution. In addition, for each individual we also computed the 75th percentile, this latter statistic being less sensitive to extreme values within the distribution (Lovell et al., 1999; Duez et al., 2003). We then tested each of these computed statistics for a relationship between radiation level and DNA damage. As measures of DNA damage, we relied on percent DNA in tail. This parameter is probably the most commonly employed in the literature, and it has been shown to be among the most reliable for detection of genotoxic effects and establishment of dose–response curves (Kumaravel and Jha, 2006).

Expectations about the damage level in each site were checked by means of ordered heterogeneity test (Rice and Gaines, 1994). This test employs a composite statistic ($r_s P_c$), where r_s is the Spearman's rank correlation between the observed and expected rankings of the groups, and P_c is the complement of the P value from the ANOVA (i.e., $P_c = 1 - P$). Thus, the P_c statistic extracts the information about magnitude of variation among the different groups and the r_s statistic extracts the information about ordering of the groups. P_c is used instead of the P value so that larger values entail more evidence against the null hypotheses of equality among the different groups (Rice and Gaines, 1994). The product $r_s P_c$ becomes increasingly large as the data increasingly refute the null hypothesis in the direction of the alternative hypothesis (Rice and Gaines, 1994). This statistical test thus allows not only to test for the existence of a statistically significant difference among groups for the variable examined, but also to check for conformity of observed rankings of the different groups to their expected rankings. We thus assigned each population in our sample an expected ranking based on measures of radiation level, and calculated the Spearman's rank correlation between their observed and expected rankings. The two intermediate-level sites were assigned the same expected ranking. The values of the composite statistic $r_s P_c$ computed for each of the three summary statistics of DNA damage (i.e. mean, median and 75th percentile DNA in tail) were

checked for statistical significance using the tables provided by Rice and Gaines (1994).

In addition, data were analyzed by means of general linear mixed models, with radiation level as a three-level fixed effect and collection site as a random effect. We also checked if the effect of radiation on genetic integrity depended upon individual sex and collection date, by including sex as a two-level factor, interaction between sex and radiation level, and sample collection date as a covariate. None of these factors reached statistical significance in any of the models, and they were thus excluded from the final models (details not shown). Post-hoc, pairwise tests were conducted when a statistically significant effect of radiation level was detected, and the Sidak correction for multiple testing was applied.

3. Results

Mean, median and 75th percentile values of percent of DNA in tail (Table 1) all followed a Gaussian distribution in each of the three

Table 1

Mean values and standard errors (SE) for the mean, median and 75th percentile of percentage DNA in tail in the three experimental groups (i.e. low-, intermediate and high-level radiation). The number of individuals in each group (N) is also indicated. Mean values represent marginal means as estimated from the model including radiation level as a fixed effect and population (i.e. collection site) as a random effect.

Parameter	Radiation	Mean	SE	N
Mean tail DNA				
	Low	49.70	3.24	33
	Intermediate	60.62	5.16	13
	High	68.41	6.21	9
Median tail DNA				
	Low	51.69	3.77	33
	Intermediate	62.47	6.02	13
	High	71.30	7.23	9
75th percentile tail DNA				
	Low	68.41	3.61	33
	Intermediate	77.51	5.75	13
	High	77.86	6.91	9

different groups (Kolmogorov–Smirnov test for normality, all P values >0.05), thus meeting the requirements for parametric statistical tests (see Fig. 2 for statistical distributions of individual values in each group). Individual- and population-level values are provided in Tables 2 and 3.

The ordered heterogeneity test (Rice and Gaines 1994; see above) disclosed a significant relationship between radiation level and DNA damage, both for mean and median percent of DNA in tail (mean percent DNA in tail: $r_s = 0.949$, $r_sP_c = 0.93$, $P < 0.01$; median percent DNA in tail: $r_s = 0.949$, $r_sP_c = 0.91$, $P < 0.01$). The increase in 75th percentile of percent DNA in tail was not significant ($r_s = -0.632$, $r_sP_c = -0.46$, $P > 0.05$).

A significant effect of radiation level was found using generalized linear mixed models for analyses of mean percent of DNA in tail ($F_{2,52} = 4.26$, $P = 0.019$; Table 1). After the Sidak correction for multiple comparisons, this difference was found to be explained by a significant increase in damage from the low-dose group to the high-dose group ($P = 0.030$), while no significant difference emerged between the low-dose group and the intermediate-dose group, or between the intermediate- and the high-dose group ($P = 0.219$ and $P = 0.711$, respectively).

Qualitatively similar results were obtained if individual median values were used, instead of mean values, as summary statistics ($F_{2,52} = 3.34$, $P = 0.043$; Table 1). Percentage of DNA in tail increased from the low- to the high-dose group, although the difference between the two groups was marginally non-significant after the Sidak correction was applied ($P = 0.058$). The difference between the low-dose and the intermediate-dose group, and between the intermediate- and the high-dose group was not statistically significant ($P = 0.353$ and $P = 0.729$, respectively).

No significant variation among radiation-level groups was found if the 75th percentile was used for summarizing values at the level of individual cells (all P values >0.05; Table 1).

In addition, no among-populations difference existed in any of the analyses (all P values >0.05; Table 3).

4. Discussion

After the Chernobyl accident in 1986, various studies aimed at evaluating the outcomes of the disaster have reported an increase in mutation rate in organisms as diverse as wheat, Scots pine, oligochaetes,
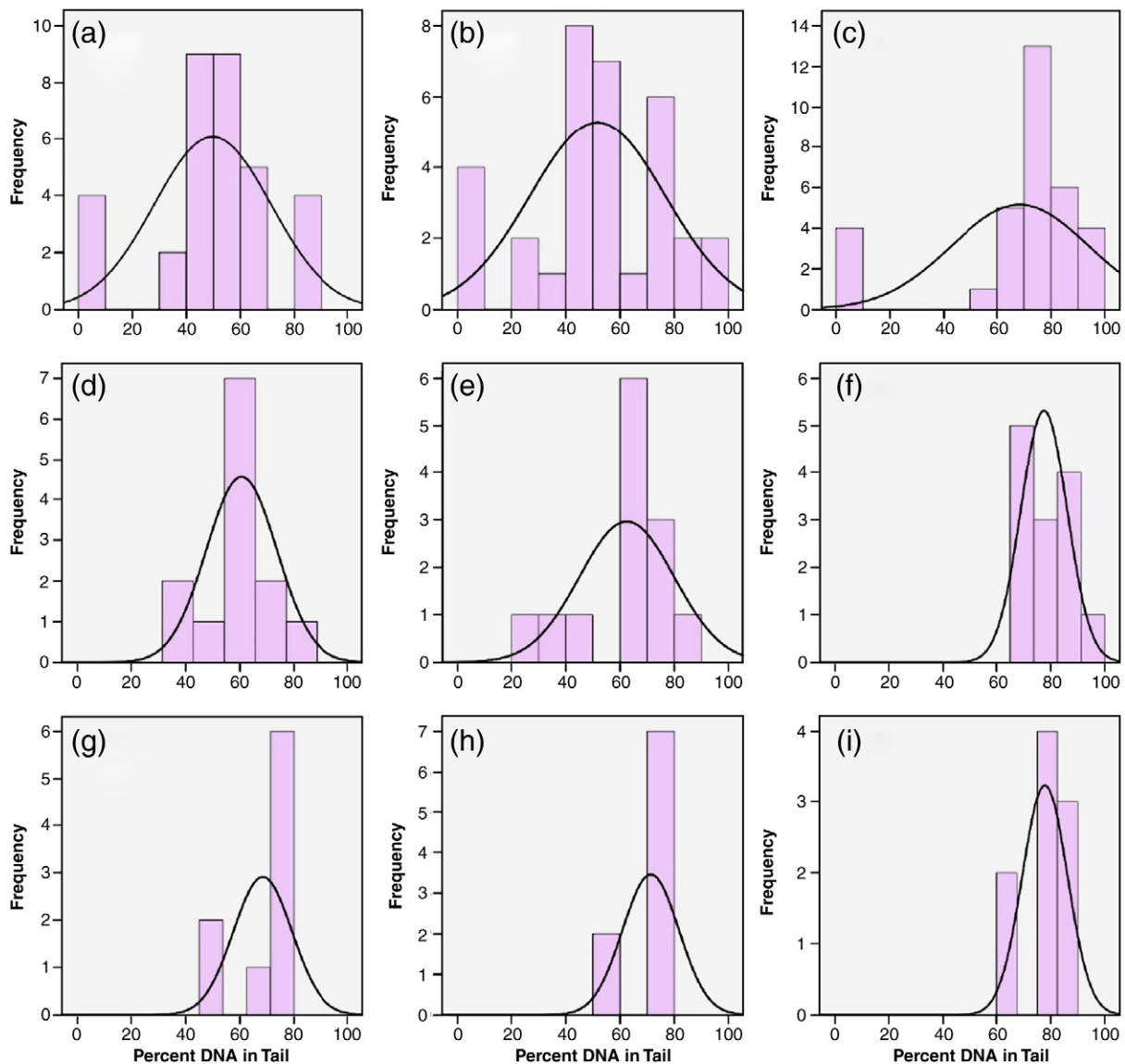

Fig. 2. Frequency distribution of the mean values for percent DNA in tail in the three experimental groups. (a)–(d)–(g): frequency distribution of mean percent DNA in tail in the low-, intermediate and high-dose group; (b)–(e)–(h): frequency distribution of median percent DNA in tail in the low-, intermediate and high-dose group; (c)–(f)–(i): frequency distribution of 75th percentile of percent DNA in tail in the low-, intermediate and high-dose group.

Table 2
Mean percentage of DNA in tail for each individual bird included in the analyses. Population (i.e. collection site), radiation level (low (L), intermediate (I) or high (H)), and individual sex (male (M) or female (F)) are also indicated.

Ring number	Sex	Population	Level	Mean
1403	F	Ghovtnere	L	6.25
1406	F	Ghovtnere	L	84.32
1408	F	Ghovtnere	L	3.16
1408	F	Ghovtnere	L	50.96
1422	F	Ghovtnere	L	44.36
1423	F	Ghovtnere	L	57.89
1424	F	Ghovtnere	L	47.34
1425	F	Ghovtnere	L	43.01
1426	F	Ghovtnere	L	44.97
1430	F	Ghovtnere	L	52.39
1431	F	Ghovtnere	L	64.43
1436	F	Ghovtnere	L	60.32
1438	F	Ghovtnere	L	45.41
1441	F	Ghovtnere	L	45.03
A053382	F	Ghovtnere	L	54.08
A053388	F	Ghovtnere	L	53.36
A053393	F	Ghovtnere	L	47.13
1404	M	Ghovtnere	L	83.31
1407	M	Ghovtnere	L	4.16
1411	M	Ghovtnere	L	57.98
1413	M	Ghovtnere	L	63.59
1414	M	Ghovtnere	L	2.85
1415	M	Ghovtnere	L	86.77
1417	M	Ghovtnere	L	86.19
1421	M	Ghovtnere	L	52.64
1427	M	Ghovtnere	L	57.04
1428	M	Ghovtnere	L	60.85
1432	M	Ghovtnere	L	39.12
1433	M	Ghovtnere	L	42.94
1434	M	Ghovtnere	L	34.58
1442	M	Ghovtnere	L	55.48
A053368	M	Ghovtnere	L	43.85
A058110	M	Ghovtnere	L	64.43
A050570	M	Dytiaku	I	70.65
A053296	M	Dytiaku	I	58.79
A053321	M	Dytiaku	I	59.02
A050675	F	Pisky	I	40.39
A050675	F	Pisky	I	85.55
A058984	F	Pisky	I	74.72
A059029	F	Pisky	I	55.77
A059041	F	Pisky	I	61.92
A079027	F	Pisky	I	48.38
A058985	M	Pisky	I	64.45
A058988	M	Pisky	I	64.50
A058990	M	Pisky	I	64.96
A059032	M	Pisky	I	38.95
B033785	F	Vesniane	H	73.89
B033785	F	Vesniane	H	72.09
B033796	F	Vesniane	H	69.41
A050653	M	Vesniane	H	51.01
A053307	M	Vesniane	H	48.77
B033789	M	Vesniane	H	75.87
B033793	M	Vesniane	H	78.15
B033797	M	Vesniane	H	74.07
B033798	M	Vesniane	H	72.46

Table 3
Mean and standard error (SE) of mean, median and 75th percentile of percentage of DNA for each population (i.e collection site). Number of birds from each population (N) and radiation level (low (L), intermediate (I) or high (H)) are also indicated.

Statistic	Population	N	Level	Mean	SE
Mean percent DNA in tail					
	Ghovtnere	33	L	49.70	3.78
	Dytiaku	3	I	62.82	3.91
	Pisky	10	I	59.96	4.62
	Vesniane	9	H	68.41	3.60
Median percent DNA in tail					
	Ghovtnere	33	L	51.69	4.34
	Dytiaku	3	I	67.70	4.33
	Pisky	10	I	60.91	6.20
	Vesniane	9	H	71.30	3.46
75th percentile DNA in tail					
	Ghovtnere	33	L	68.41	4.45
	Dytiaku	3	I	79.98	3.87
	Pisky	10	I	76.77	2.90
	Vesniane	9	H	77.85	2.77

the area around Chernobyl heavily contaminated with radionuclides. We suggest that this finding may be due to (i) the direct effect of ionizing radiation on the genetic material; or (ii) through genomic damage caused by elevated oxidative stress. Note these causative pathways are not mutually exclusive. In the latter case, the presence of antioxidants as free-radical scavengers can influence the genomic damage induced by chronic exposure to ionizing radiation.

Indeed, strand breaks (i.e. one of the genetic lesions typically detected by the comet assay; Tice et al., 2000) are usually regarded as being a characteristic lesion induced by ionizing radiation (Fairbairn et al., 1995), and it has been shown that incomplete or incorrect repair of these lesions may ultimately lead to chromosome damage and cell death (Iliakis, 1991). On the other hand, ionizing radiation has been shown to induce intracellular oxidative stress (Clutton et al., 1996), leading to reactive oxygen species (ROS) formation (Halliwell and Gutteridge, 2007). Active oxygen radicals can in turn efficiently produce allelic deletion and permanent chromosomal instability and aberrations (Moody and Hassan, 1982; Fairbairn et al., 1995), with possible trans-generational effects due to the transfer of damaged genetic material to the progeny. For example, hydrogen peroxide (H_2O_2), a common product of a variety of oxidative stresses, has been demonstrated to induce genetic damage leading to mutagenesis (Imlay and Linn, 1988; Imlay et al., 1988). As a result, ionizing radiation has been proposed to induce an increase in mutation rate through a depletion of available antioxidants, which can be utilized for scavenging free radicals that arise from radiolysis of water and other biomolecules (Neyfakh et al., 1998a,b; Kumerova et al., 2000). Indeed, high levels of radiation in people heavily exposed to the Chernobyl fallout have been found to negatively predict individual antioxidant defense because of their use for elimination of active oxygen species (Neyfakh et al., 1998a,b; Kumerova et al., 2000). In a comet assay study, a significant, negative correlation has been observed between plasma level of carotenoids, a major class of antioxidant compounds, and oxidative DNA damage measured as oxidized bases in lymphocytes (Collins and Horváthová, 2001).

Previous findings in our model species are also consistent with this framework. Antioxidant levels (carotenoids, Vitamins A and E) in barn swallow blood, liver and eggs were found to be reduced in Chernobyl compared to uncontaminated control areas (Møller et al., 2005b) and these reductions were the best predictor of the observed increased frequency of sperm aberrations in males from the Chernobyl region (Møller et al., 2005a). Moreover, in a recent study of this same species where we evaluated total antioxidant capacity and the level of metabolites of reactive oxygen species (reactive oxygen metabolites or ROMs), we found that ROMs were highest in the most contaminated sites (Bonisoli-Alquati et al., in press). The ratio of the plasma level of

mice, birds and humans (Dubrova et al., 1996; Kovalchuk et al., 2000; Kal'chenko and Fedotov, 2001; Weinberg et al., 2001; Tsytsugina and Polikarpov, 2003; Tsyusko et al., 2006; review in Møller and Mousseau, 2006). Moreover, the germline mutation rate was found to be elevated in each of the four studies in which it was measured, compared to control populations (Ellegren et al., 1997) or to 'internal' controls (e.g. sibling conceived before the accident; Weinberg et al., 2001). However, other studies have failed to find an increase in somatic mutations in other species (e.g. voles of the genera Microtus and Clethrionomys, and a point mutation mouse model, the Big Blue® Transgenic Rodent Mutation Assay System) (Wickliffe et al., 2002, 2003; Meeks et al., 2007; see also Chesser and Baker, 2006, and references therein).

Here, we report the first finding of an increase in DNA damage using the comet assay in any wild population of animals inhabiting

these compounds to the plasma antioxidant capacity was also increased, thus indicating that the overall balance between the antioxidant defenses and the oxidative threat they have to face was also affected (Bonisoli-Alquati et al., in press). Moreover, an inter-specific survey of species abundance in the region found that species-specific behavioral correlates of antioxidant use (in the context of maternal allocation to the eggs, for quenching ROS generated by migratory flight, and for sexual signaling) were predictors of decline in abundance in highly contaminated sites (Møller and Mousseau, 2007b). As a whole, these findings were thus consistent with the hypothesis of a link between radiation and individual level of antioxidants, suggesting that mutation rates may differ within natural populations of animals owing to individual differences in the abundance of antioxidants (Møller et al., 2005a, 2008a). We consider the present finding of a positive relationship between background radiation level and genetic damage to be comprised within this previously defined framework, for which it represents an important missing link.

For results of the comet tests to be comparable among different laboratories, it has been advised that percent of DNA in the tail in control samples should be within 10–20% (Tice et al., 2000). Although the higher level of damage detected in our study is outside of these recommendations, there are a number of possible explanations, including elevated ROS levels in migrating birds (Costantini et al., 2007). However, we are confident that the levels of DNA damage observed in our study do not confound our overall interpretations, because all samples were handled and treated according to the same protocol. Future studies will be designed to better understand endogenous and environmental sources of DNA damage in this species.

The biological consequences of the disaster are still debated, though the popular description of the Chernobyl region as a thriving ecosystem appears to be based more on narrative, anecdotal evidence than on quantitative, rigorous assessment of field procedures and data (see Møller et al., 2008b). Indeed, some of the studies cited by Møller and Mousseau (2006) are not included in the definitive (sic) survey of the UN Chernobyl Forum (2005). Therefore, the optimistic view summarized in this report seems not rooted in state-of-the-art scientific knowledge about the possible harmful consequences of the Chernobyl disaster.

The recent claim for a conjunct effort of the scientific community in exploring the ecological consequences of this disaster, as well as for setting standards for radioecological inquiries, remains largely unanswered, despite the fact that this claim has been raised by both sides of the controversy (e.g. Chesser and Baker, 2006; Møller and Mousseau, 2006).

Moreover, little is known about the life history and evolutionary consequences of exposure to radioactive contaminants, with some remarkable exceptions for barn swallow and other passerine species. In the barn swallow, a decline has been shown in reproductive performance and output and reduced adult survival rate (Møller et al., 2005a), as well as in population sizes in the most contaminated areas (Ellegren et al., 1997; Møller et al., 2006). These findings were recently extended to the inter-specific level, with large-scale censuses showing a decline in both abundance and richness of bird and invertebrate species likely caused by susceptibility to the effects of exposure to radiation in Chernobyl (Møller and Mousseau, 2007a,b, 2009a,b).

We suggest that future studies should improve study design, by utilizing fresh blood, and extending the tests to a higher number of individuals and a wider geographic area, to include European populations outside Ukraine as reference populations. The test for a relationship between DNA damage and behavioral and physiological traits will also allow the assessment of fitness consequences of DNA damage in natural populations. Inference about consequences of contaminants on survival and reproductive success should be the primary aim of ecotoxicological studies (Jha, 2008). In our study species, physiological analyses of oxidative stress levels and enzyme-modified versions of the comet test could both be employed in the future for detecting specific damage categories (e.g. oxidative damage) and investigating mechanistic processes linking life-history and population-level phenomena to the underlying biochemical processes.

References

Bonisoli-Alquati, A, Mousseau, T.A., Møller, A.P., Caprioli, M., Saino, N., in press. Increased oxidative stress in barn swallows from the Chernobyl region. Comp. Biochem. Physiol. A. doi:10.1016/j.cbpa.2009.10.041.

Camplani, C., Saino, N., Møller, A.P., 1999. Carotenoids, sexual signals and immune function in barn swallows from Chernobyl. Proc. R. Soc. Lond. B 266, 1111–1116.

Chesser, R.K., Baker, R.J., 2006. Growing up with Chernobyl. Am. Scient. 94, 542–549.

Clutton, S.M., Townsend, K.M.S., Walker, C., Ansell, J.D., Wright, E.G., 1996. Radiation-induced genomic instability and persisting oxidative stress in primary bone marrow cultures. Carcinogenesis 17, 1633–1639.

Collins, A.R., 2004. The comet assay for DNA damage and repair: principles, applications, and limitations. Mol. Biotech. 26, 249–262.

Collins, A.R., Horváthová, E., 2001. Oxidative DNA damage, antioxidants and DNA repair: applications of the comet assay. Biochem. Soc. Trans. 29, 337–341.

Collins, A., Dusinská, M., Franklin, M., Somorovská, M., Petrovská, H., Duthie, S., Fillion, L., Panayiotidis, M., Raslová, K., Vaughan, N., 1997. Comet assay in human biomonitoring studies: reliability, validation, and applications. Environ. Mol. Mutagen. 30, 139–146.

Costantini, D., Cardinale, M., Carere, C., 2007. Oxidative damage and anti-oxidant capacity in two migratory bird species at a stop-over site. Comp. Biochem. Physiol. C 144, 363–371.

Dhawan, A., Bajpayee, M., Parmar, D., 2009. Comet assay: a reliable tool for the assessment of DNA damage in different models. Cell Biol. Toxicol. 25, 5–32.

Dubrova, Y.E., Nesterov, V.N., Krouchinsky, N.G., Ostapenko, V.A., Neumann, R., Neil, D.L., Jeffreys, A.J., 1996. Human minisatellite mutation rate after the Chernobyl accident. Nature 380, 683–686.

Duez, P., Dehon, G., Kumps, A., Dubois, J., 2003. Statistics of the comet assay: a key to discriminate between genotoxic effects. Mutagenesis 18, 159–166.

Ellegren, H., Lindgren, G., Primmer, C.R., Møller, A.P., 1997. Fitness loss and germline mutations in barn swallows breeding in Chernobyl. Nature 389, 593–596.

Fairbairn, D.W., Olive, P.L., O'Neill, K.L., 1995. The Comet assay: a comprehensive review. Mutat. Res. 339, 37–59.

Frenzilli, G., Lori, A., Panasiuk, G., Ferdeghini, M., Barale, R., 1998. Comet assay on children's leukocytes 8 years after the Chernobyl disaster. Mutat. Res. 415, 151–158.

Frenzilli, G., Bosco, E., Antonelli, A., Panasiuk, G., Barale, R., 2001. DNA damage evaluated by alkaline single cell gel electrophoresis (SCGE) in children of Chernobyl 10 years after the disaster. Mutat. Res. 491, 139–149.

Frenzilli, G., Nigro, M., Lyons, B.P., 2009. The Comet assay for the evaluation of genotoxic impact in aquatic environments. Mutat. Res., Rev. Mutat. Res. 681, 80–92.

Garcia, O., Mandina, T., 2005. DNA damage evaluated by the comet assay in lymphocytes of children with ^{137}Cs internal contamination caused by the Chernobyl accident. Mutat. Res. 565, 191–197.

Golikov, Y.Y., Balonov, M.I., 1993. Estimation of external gamma radiation doses to the population after the Chernobyl accident. In: Merwin, S.E., Balonov, M.I. (Eds.), The Chernobyl papers. Richland, WA.

Halliwell, B., Gutteridge, J.M.C., 2007. Free Radicals in Biology and Medicine. Clarendon Press, Oxford.

Iliakis, G., 1991. The role of DNA double-strand breaks in ionizing radiation-induced killing of eukaryotic cells. BioEssays 13, 641–648.

Imlay, J.A., Linn, S., 1988. DNA damage and oxygen radical toxicity. Science 240, 1302–1309.

Imlay, J.A., Chin, S.M., Linn, S., 1988. Toxic DNA damage by hydrogen peroxide through the Fenton reaction in vivo and in vitro. Science 240, 640–642.

Jha, A.N., 2004. Genotoxicological studies in aquatic organisms: an overview. Mutat. Res. 552, 1–17.

Jha, A.N., 2008. Ecotoxicological applications and significance of the comet assay. Mutagenesis 23, 207–221.

Kal'chenko, V.A., Fedotov, I.S., 2001. Genetic effects of acute and chronic ionizing irradiation on Pinus sylvestris L. inhabiting the Chernobyl meltdown area. Russ. J. Genet. 37, 341–350.

Kleinjans, J.C.S., van Schooten, F.J., 2002. Ecogenotoxicology: the evolving field. Environ. Toxicol. Pharmacol. 11, 173–179.

Kovalchuk, O., Dubrova, Y.E., Arkhipov, A., Hohn, B., Kovalchuk, I., 2000. Wheat mutation rate after Chernobyl. Nature 407, 583–584.

Kumaravel, T.S., Jha, A.N., 2006. Reliable Comet assay measurements for detecting DNA damage induced by ionising radiation and chemicals. Mutat. Res. 605, 7–16.

Kumerova, A.O., Lece, A.G., Skesters, A.P., Orlikov, G.A., Seleznev, J.V., Rainsford, K.D., 2000. Antioxidant defence and trace element imbalance in patients with postradiation syndrome: first report on phase I studies. Biol. Trace Elem. Res. 77, 1–12.

Lovell, D.P., Thomas, G., Dunbow, R., 1999. Issues related to the experimental design and subsequent statistical analysis of *in vivo* and *in vitro* comet studies. Teratog. Carcinog. Mutagen. 19, 109–119.

Mazur, P., Rall, W.F., Rigopoulos, N., 1981. Relative contribution of the fraction of unfrozen water and of salt concentration to the survival of slowly frozen human erythrocytes. Biophys. J. 36, 653–675.

Meeks, H.N., Wickliffe, J.K., Hoofer, S.R., Chesser, R.K., Rodgers, B.E., Baker, R.J., 2007. Mitochondrial control region variation in bank voles (*Clethrionomys glareolus*) is not related to Chernobyl radiation exposure. Environ. Toxicol. Chem. 26, 361–369.

Miyamae, Y., Iwasaki, K., Kinae, N., Tsuda, S., Murakami, M., Tanaka, M., Sasaki, Y.F., 1997. Detection of DNA lesions induced by chemical mutagens using the single-cell gel electrophoresis (Comet) assay. 2. Relationship between DNA migration and alkaline condition. Mutat. Res. 393, 107–113.

Møller, A.P., Mousseau, T.A., 2006. Biological consequences of Chernobyl: 20 years after the disaster. Trends Ecol. Evol. 21, 200–207.

Møller, A.P., Mousseau, T.A., 2007a. Species richness and abundance of birds in relation to radiation at Chernobyl. Biol. Lett. 3, 483–486.

Møller, A.P., Mousseau, T.A., 2007b. Determinants of interspecific variation in population declines of birds from exposure to radiation at Chernobyl. J. Appl. Ecol. 44, 909–919.

Møller, A.P., Mousseau, T.A., 2009a. Reduced abundance of raptors in radioactively contaminated areas near Chernobyl. J. Ornithol. 150, 239–246.

Møller, A.P., Mousseau, T.A., 2009b. Reduced abundance of insects and spiders linked to radiation at Chernobyl 20 years after the accident. Biol. Lett. 5, 356–359.

Møller, A.P., Szép, T., 2002. Survival rate of adult barn swallows *Hirundo rustica* in relation to sexual selection and reproduction. Ecology 83, 2220–2228.

Møller, A.P., Mousseau, T.A., Milinevsky, G., Peklo, A., Pysanets, E., Szép, T., 2005a. Condition, reproduction and survival of barn swallows from Chernobyl. J. Anim. Ecol. 74, 1102–1111.

Møller, A.P., Surai, P.F., Mousseau, T.A., 2005b. Antioxidants, radiation and mutation in barn swallows from Chernobyl. Proc. R. Soc. Lond. B 272, 247–253.

Møller, A.P., Hobson, K.A., Mousseau, T.A., Peklo, A.M., 2006. Chernobyl as a population sink for barn swallows: tracking dispersal using stable isotope profiles. Ecol. Appl. 16, 1696–1705.

Møller, A.P., Mousseau, T.A., de Lope, F., Saino, N., 2007. Elevated frequency of abnormalities in barn swallows from Chernobyl. Biol. Lett. 3, 414–417.

Møller, A.P., Mousseau, T.A., Lynn, C., Ostermiller, S., Rudolfsen, G., 2008a. Impaired swimming behaviour of sperm from barn swallows *Hirundo rustica* in Chernobyl. Mutat. Res. 650, 210–216.

Møller, A.P., Mousseau, T.A., de Lope, F., Saino, N., 2008b. Anecdotes and empirical research in Chernobyl. Biol. Lett. 4, 65–66.

Moody, C.S., Hassan, H.M., 1982. Mutagenicity of oxygen free radicals. Proc. Natl. Acad. Sci. U. S. A. 79, 2855–2859.

Neyfakh, E.A., Alimbekova, A.I., Ivanenko, G.F., 1998a. Vitamin E and A deficiencies in children correlate with Chernobyl radiation loads of their mothers. Biochemistry (Moscow) 63, 1138–1143.

Neyfakh, E.A., Alimbekova, A.I., Ivanenko, G.F., 1998b. Radiation-induced lipoperoxidative stress in children coupled with deficit of essential antioxidants. Biochemistry (Moscow) 63, 977–987.

Olive, P.L., 1999. DNA damage and repair in individual cells: applications of the comet assay in radiobiology. Int. J. Radiat. Biol. 75, 395–405.

Pastor, N., López-Lázaro, M., Tella, J.L., Baos, R., Forrero, M.G., Hiraldo, F., Cortés, F., 2001a. Assessment of genotoxic damage by the comet assay in white storks (*Ciconia ciconia*) after the Doñana ecological disaster. Mutagenesis 16, 219–223.

Pastor, N., López-Lázaro, M., Tella, J.L., Baos, R., Forrero, M.G., Hiraldo, F., Cortés, F., 2001b. DNA damage in birds alter the mining waste spill in southwestern Spain: a comet assay evaluation. J. Environ. Pathol. Toxicol. Oncol. 20, 317–324.

Pastor, N., Baos, R., López-Lázaro, M., Jovani, R., Tella, J.L., Hajji, N., Hiraldo, F., Cortés, F., 2004. A 4 year follow-up analysis of genotoxic damage in birds of the Doñana area (south west Spain) in the wake of the 1998 mining waste spill. Mutagenesis 19, 61–65.

Rice, W.R., Gaines, S.D., 1994. Extending non-directional heterogeneity tests to evaluate simply ordered alternative hypotheses. Proc. Natl. Acad. Sci. USA 91, 225–226.

Rider, C.F., Smith, C.C., O'Donovan, M.R., Martin, E.A., 2006. The neutral diffusion assay as an estimate of cytotoxicity in the comet assay. Toxicology 226, 58–59.

Shestopalov, V.M., 1996. Atlas of Chernobyl Exclusion Zone. Ukrainian Academy of Science, Kiev.

Singh, N.P., McCoy, M.T., Tice, R.R., Schneider, E.L., 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp. Cell Res. 175, 184–191.

Singh, N.P., Danner, D.B., Tice, R.R., McCoy, M.T., Collins, G.D., Schneider, E.L., 1989. Abundant alkali-sensitive sites in DNA of human and mouse sperm. Exp. Cell Res. 184, 461–470.

Tice, R.R., Strauss, G.H., 1995. The single cell gel electrophoresis/comet assay: a potential tool for detecting radiation-induced DNA damage in humans. Stem Cells 13, 207–214 (Suppl.).

Tice, R.R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., Miyamae, Y., Rojas, E., Ryu, J.C., Sasaki, Y.F., 2000. Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. Environ. Mol. Mutagen. 35, 206–221.

Tsytsugina, V.G., Polikarpov, G.G., 2003. Radiological effects on populations of Oligochaeta in the Chernobyl contaminated zone. J. Environ. Radioact. 66, 141–154.

Tsyusko, O.V., Smith, M.H., Oleksyk, T.K., Goryanaya, J., Glenn, T.C., 2006. Genetics of cattails in radioactively contaminated areas around Chornobyl. Mol. Ecol. 15, 2611–2625.

UN Chernobyl Forum Expert Group "Environment", 2005. Environmental consequences of the Chernobyl accident and their remediation: twenty years of experience. IAEA, WHO, UNDP, New York.

Vasquez, M., Tice, R.R., 1997. Detecting genotoxic activity against high molecular weight DNA using the alkaline single cell gel (SCG) assay. Environ. Mol. Mutagen. 29, 53.

Weinberg, H.S., Korol, A.B., Kirzhner, V.M., Avivi, A., Fahima, T., Nevo, E., Shapiro, S., Rennert, G., Piatak, O., Stepanova, E.I., Skvarskaja, E., 2001. Very high mutation rate in offspring of Chernobyl accident liquidators. Proc. R. Soc. Lond. B 268, 1001–1005.

Wickliffe, J.K., Chesser, R.K., Rodgers, B.E., Baker, R.J., 2002. Assessing the genotoxicity of chronic environmental irradiation using mitochondrial DNA heteroplasmy in the bank vole (*Clethrionomys glareolus*) at Chernobyl, Ukraine. Environ. Toxicol. Chem. 21, 1249–1254.

Wickliffe, J.K., Bickham, A.M., Rodgers, B.E., Chesser, R.K., Phillips, C.J., Gaschak, S.P., Goryanaya, J.A., Chizhevsky, I., Baker, R.J., 2003. Exposure to chronic, low-dose rate gamma radiation at Chernobyl does not induce point mutations in Big Blue® mice. Environ. Mol. Mutagen. 42, 11–18.