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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

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A total of 330 white storks (*Ciconia ciconia*) and 138 black kites (*Milvus migrans*) were blood sampled during four consecutive years in an area heavily contaminated as a consequence of a massive spillage of toxic acid mining waste rich in heavy metals that impacted on the Doñana National Park (south western Spain), in April 1998. The alkaline single cell gel electrophoresis (Comet) assay was performed as a genotoxicity test, in order to assess whether the high level of DNA damage first detected by us 1 year after the disaster was still present in birds in each of the successive 3 years. Our results clearly show that, when compared with control individuals from non-polluted areas, white storks and black kites born in the contaminated area for a period of up to 4 years after the toxic accident have suffered an increase of at least 2- to 10-fold in the level of their genetic damage through the study period. Taken as a whole, these observations seem to indicate that the toxic spill still appears to be affecting the wildlife 4 years after the mining disaster and that attempts at cleaning up the waste have proved ineffective based on DNA damage detection.

Introduction

The spill of toxic acid mining waste from the processing of pyrite ore in Aznalcollar (south west Spain) in April 1998 has been considered as one of the worst environmental disasters to have ever happened in western Europe, posing a threat to the Doñana National Park. Many bird species, including protected ones, use this Reserve of the Biosphere (designated as such in 1981) as a breeding and wintering site, making it one of the most important wildlife sites in Europe (Grimalt *et al*., 1999).

Ecologically sensitive areas surrounding the marshlands of the park were flooded with acidic mud rich in heavy metals brought by the river Guadiamar, causing consistent decreases in pH. In addition to the presence in the contaminating sludge waste of high levels of heavy metals such as arsenic, cadmium, lead, copper and zinc, the acid conditions facilitated the solubilization of these metals, leading to water concentrations lethal to aquatic wildlife (Pain *et al*., 1998).

When monitoring for genotoxicity in the environment is needed, an issue of major importance is the selection of representative organisms as sentinels as well as the development of sensitive and reliable tests such as those designed for the evaluation of DNA damage. Regardless of the possible unique features of the contamination event, it is also very important that the assays of choice have been widely validated by different laboratories and can be used to monitor virtually any potentially endangered wild species.

As to the selection of sentinel organisms, the white stork (*Ciconia ciconia*) and the black kite (*Milvus migrans*) are appropriate bird species for evaluating the potential effects of the toxic spill on wildlife of the Doñana area, because they are long-lived species that occupy a high position in the aquatic and terrestrial trophic webs, respectively. While white storks usually prey on a variety of invertebrate and vertebrate species in marshlands (Negro *et al*., 2000), black kites do so mainly on vertebrates, such as rabbits (Veiga and Hiraldo, 1990). Diet is one of the most important factors explaining the accumulation of heavy metals readily observed in wetland birds in the area around Doñana within a few months after the spill (Hernández *et al*., 1999; Benito *et al*., 1999). As many of these heavy metals, such as As, Pb, Cd, Cu and Hg, have been reported as genotoxic, mutagenic and even carcinogenic (LeBlanc and Bain, 1997; Yager and Ostrosky-Wegman, 1997), we carried out an investigation 1 year after the toxic spill on the possible level of genotoxic damage present in white storks (Pastor *et al*., 2001a) and black kites (Pastor *et al*., 2001b) born in and around the affected area.

The alkaline single cell gel electrophoresis (SCGE) or Comet assay (Singh *et al*., 1988) was performed on peripheral blood lymphocytes from young birds sampled in the contaminated area and compared with reference animals, since this test has been demonstrated as suitable to detect genotoxic DNA damage in such different systems as onion roots (Navarrete *et al*., 1997), trout (Belpaeme *et al*., 1996), mussels (Wilson *et al*., 1998) and different tissues of mice (Miyamae *et al*., 1998). The Comet assay has a number of clear advantages that make it a test of choice in environmental studies. While classical cytogenetic techniques, such as detection of chromosomal aberrations or sister chromatid exchanges, have been useful in many instances, the large number of small chromosomes that make up the karyotype of many animals (mainly fish, reptiles and birds) has clearly made these methods useless. The main advantage of the Comet assay stems from its independence of cell division (an unavoidable prerequisite for cytogenetic techniques) as well as the possibility of measuring DNA damage in single cells, which in turn allows the assessment of inter-individual variations in a cell population.

In an early study, the analysis of individual cells (comets) from storks and kites born in the Doñana area indicated a high level of genotoxic damage, as shown by a significant increase

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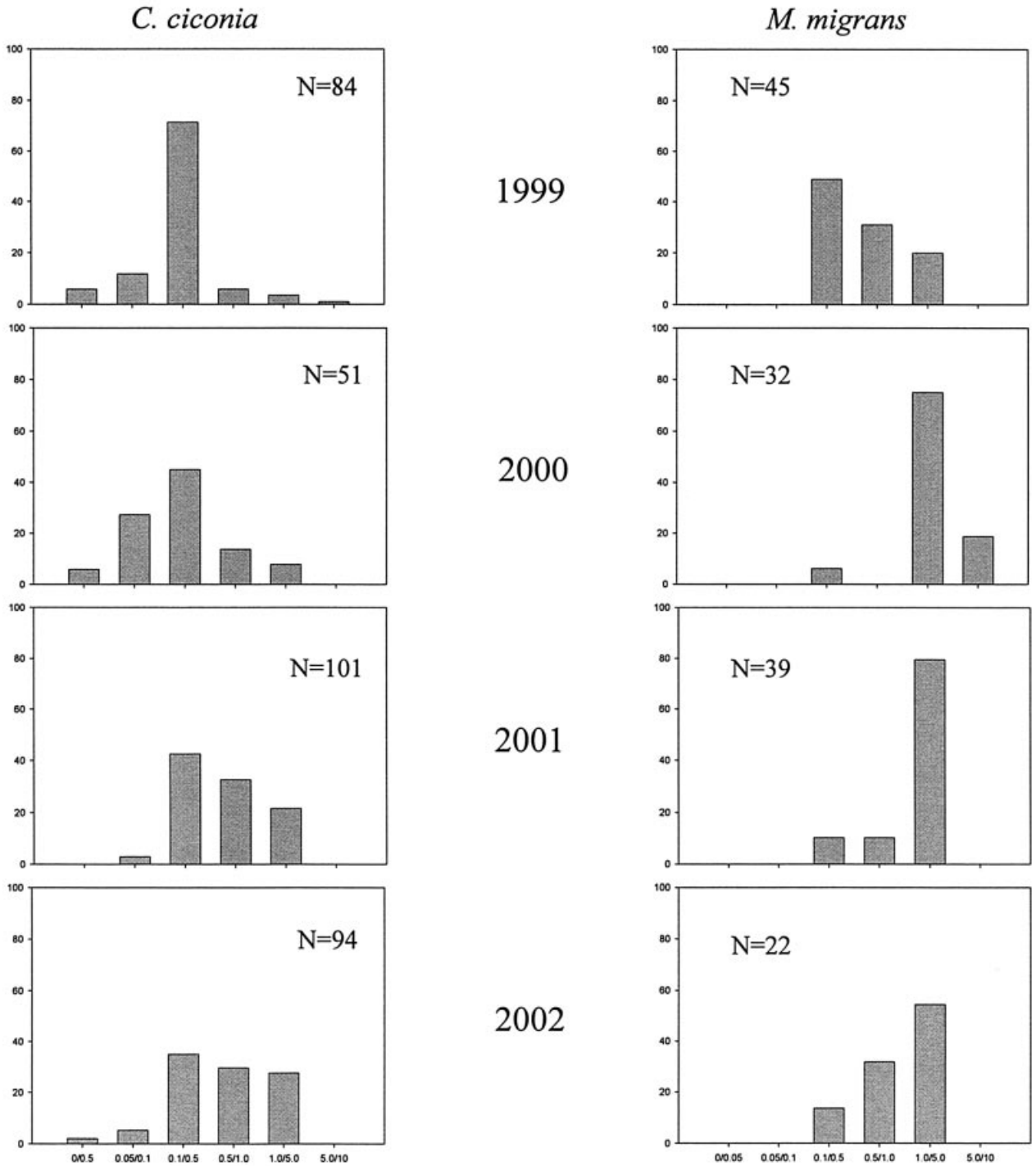

Fig. 1. Average tail moments of white stork (left column) and black kite (right column) nestlings sampled during four consecutive years after the mining waste toxic spill around Doñana National Park (south west Spain). The *y*-axis indicates the number of individuals within each category of average tail moment. Sample sizes (*n*) are indicated for each species and year. Control values were in all cases significantly lower, mainly located in the interval 0–0.05, i.e. at least ~10- to 20-fold lower than that found in birds sampled at or near the contaminated area (not shown).

in DNA migration observed in cells from exposed animals, compared with that seen in control animals sampled in non-contaminated areas (Pastor *et al*., 2001a,b). The present report describes the results from a 4 year follow-up investigation in the same areas and shows an increase in the previously reported harmful effects of genotoxicants in this threatened important wildlife reserve.

Materials and methods

Collection sites and birds sampled

Blood sampling fieldwork was conducted in successive years from 1999 to 2002 in the Dehesa de Abajo, where one of the largest colonies of storks from the Western Palearctic is sited (~300 breeding pairs). This colony is located less than 1 km

from the area affected by the toxic spill in April 1998 (see Pastor *et al*., 2001a for a map of the study area). Black kites were sampled in and around the Doñana National Park (Forero *et al*., 1999), in the vicinity of the contaminated area. Between 1 and 4 nestlings in the case of storks and 1 and 3 kite nestlings per nest were sampled 10–15 days prior to fledging. A total of 330 storks and 138 kites were sampled for comet analysis through the study period.

Sex is a factor that has been claimed to be related to the accumulation of some metals in birds from Doñana a few months after the spill (Benito *et al*., 1999). Both sexes of white storks and black kites look alike, so gender was determined by molecular procedures using DNA extracted from the cellular fraction of a few drops of blood (Negro *et al*., 2000). In addition, we investigated whether nestling age is a possible source of individual variation. Birds were aged according to a regression equation for age on the length of the wing for storks (see Negro *et al*., 2000). Data for the seventh primary feather length were fitted to a least squares linear regression for each individual in order to age kite nestlings (Viñuela and Bustamante, 1992).

Blood sampling and lymphocyte preparation

Blood collection from birds and lymphocyte isolation were carried out as reported elsewhere (Pastor *et al*., 2001a,b). Briefly, blood samples (1 ml) were collected from the brachial vein, placed in vials (Soria Greiner, Spain) with EDTA as an anticoagulant and transported in coolers to the laboratory on the day of collection. Isolation of lymphocytes was performed by centrifugation in a density gradient (2000 r.p.m., 30 min) using a lymphocyte separation medium (Ficoll-Paque; Seromed). Finally, cells were washed in phosphate-buffered saline (PBS) and lymphocyte concentrations were adjusted to ~10^5 cells/ml PBS.

Comet assay

The protocol of Singh *et al*. (1988) was basically followed, with some modifications to make it suitable for bird lymphocytes, as reported elsewhere (Pastor *et al*., 2001a,b). In short, the standard slides were immersed vertically in 1% normal melting point agarose (NMA) at 55°C and left vertical to allow the agarose to solidify. The slides were then kept at 4°C until they were used.

About 10 000 lymphocytes from each bird were mixed with 85 µl of low melting point agarose (LMA) (0.7% in PBS) (FMC) at 37°C and then the cell suspension was rapidly pipetted onto the first agarose layer, spread using a coverslip and kept at 4°C for 8 min for the LMA to solidify. Subsequently the coverslips were removed and a third layer of 100 µl LMA (0.7%) at 37°C was added, covered with a coverslip and again allowed to solidify at 4°C for 8 min. After the top layer of agarose had solidified, the slides were immersed in a chilled lysing solution made up of 2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris–HCl, 1% sodium sarcosinate, pH 10, with 1% Triton X-100 and 10% DMSO added just before use. They were kept at 4°C in the dark for at least 1 h to lyse the cells and to allow DNA unfolding.

After removing the slides from the lysis solution, they were subsequently drained and placed on a horizontal gel electrophoresis unit, side by side. The slides were then covered with chilled fresh alkaline solution (1 mM Na_2EDTA, 170 mM NaOH) at 4°C and pH 12.8, which allows the detection of both double- and single-strand breaks as well as alkali-labile sites (Fairbairn *et al*., 1995). Before proceeding with electrophoresis, the slides were maintained in the solution for 20 min to allow unwinding of the DNA. Finally, electrophoresis was carried out at low temperature (4°C) for 20 min at 1.6 V/cm and 300 mA. In order to prevent additional DNA damage due to photolysis, all steps described above were carried out under yellow light or in the dark.

The last steps after electrophoresis consisted of gently washing the slides in neutralization buffer (0.4 M Tris–HCl, pH 7.5) to remove alkali and detergent and staining with 50 µl DAPI (5 µg/ml) in Vectashield (H-1000 mounting medium for fluorescence; Vector Laboratories).

Fifty randomly selected lymphocyte comet images were analyzed from each sampled bird. The slides were examined at 200× magnification using a 20× objective on a fluorescence microscope (Olympus Vanox AHBT3, excitation filter 550 nm and barrier filter 590 nm). Measurements were made using CASys image analysis software (Synoptics Ltd, UK) and the parameter chosen was tail moment (tail length × tail intensity or percent migrated DNA) (Olive *et al*., 1990).

Statistical analysis

Individual average tail moments were log-transformed to attain normality, thus allowing the application of parametric statistics. Since genotoxic damage could be influenced by both common parents and rearing conditions, we considered siblings as non-independent analysis units. We therefore used nest identity as a random factor in generalized linear mixed models (GLMMs) with a normal distribution and identity link function, using the GLIMMIX macro of SAS (see for example Tella *et al*., 2001). In this way, we performed a kind of ANCOVA where year and sex were introduced as fixed factors and age as covariate. Non-significant effects ($P > 0.05$) were removed in a backward procedure starting with a saturated model with all the variables fitted to average tail moment. Differences in average tail moment between species were tested for significance using Student's *t*-test.

Results

Most of the stork nestlings sampled near the contaminated area throughout the ensuing years showed tail moments in the interval 0.1–0.5 (Figure 1). However, most of the kite nestlings sampled from 2000 onwards were in the interval 1.0–5.0 (Figure 1). In both species, especially the black kite, these values are several orders of magnitude higher than that consistently observed in control birds, where, on a per year basis, the highest tail moments were located in the interval 0–0.05 (Pastor *et al*., 2001a,b).

Results of the GLMMs showed highly significant effects of year on genotoxicity for both white storks ($F_{3,101} = 17.41$, $P < 0.0001$) and black kites ($F_{3,25} = 33.73$, $P < 0.0001$) while controlling for nest of rearing, which also had a significant effect on genotoxicity for both species (*C.ciconia* $Z = 4.62$, $P < 0.0001$; *M.migrans* $Z = 1.85$, $P = 0.032$). This result indicates that genotoxic damage was more similar between siblings born in the same nest than among nestlings born in different nests, independent of the year. On the other hand, sex and age of nestlings did not affect their levels of genotoxicity (white storks, all P values >0.16; black kites, all P values >0.11) and thus these variables were not retained in the models.

Looking at inter-year differences in white storks (Figure 1), nestlings sampled in 1999 and 2000 had similar genotoxic burdens in their lymphocytes ($F_{1,101} = 0.32$, $P = 0.576$), but lower than birds sampled in 2001 ($F_{1,101} = 34.69$, $P < 0.0001$)

and 2002 ($F_{1,101} = 32.55$, $P < 0.0001$). There were no statistical differences in mean tail moments between the 2001 and 2002 sampling periods ($F_{1,101} = 0.01$, $P = 0.932$).

In black kites, an abrupt increase in genotoxicity was detected from 1999 to 2000 ($F_{1,25} = 96.27$, $P < 0.0001$), when the highest damage was recorded (Figure 1). Mean tail moments in nestlings sampled during 2001 and 2002 were similar ($F_{1,25} = 0.49$, $P = 0.493$), but lower than the mean for birds sampled in 2000 ($F_{1,25} = 19.01$, $P = 0.0002$).

On the other hand, it is worth mentioning that the level of genotoxic damage observed in black kites was higher than in white storks, regardless of the year considered ($P < 0.0001$ for all Student's *t*-test). As Figure 1 shows, kite nestlings are more severely affected, with most of the individuals showing the highest categories of damage.

Discussion

As reported in recent years, the Comet assay (Fairbairn *et al*., 1995) can be considered a methodology of choice for the detection of genotoxic damage, especially due to its sensitivity in detecting DNA single-strand breaks and alkali-labile damage in individual cells (Singh *et al*., 1988; Gedik *et al*., 1992). While it is commonly necessary to perform different modifications depending upon the type of cell chosen, this methodology has been used in the detection of DNA damage after *in situ* exposure to water borne genotoxicants in erythrocytes of fish (Pandrangi *et al*., 1995; Belpaeme *et al*., 1996), as well as of amphibians (Ralph *et al*., 1996; Ralph and Petras, 1997). In our pioneer studies, the first to our knowledge to use the Comet assay in birds (Pastor *et al*., 2001a,b), we analyzed DNA damage in peripheral blood lymphocytes from storks and kites born in the Doñana area shortly after a toxic spill of mine waste (April 1998). Our present 4 year follow-up results indicate that, despite time since the ecological disaster, the exposed stork and kite populations show a consistent and elevated level of genotoxic damage through time as compared with reference birds sampled in non-contaminated locations (Pastor *et al*., 2001a,b). Moreover, levels of DNA damage have increased since our early reports (Pastor *et al*., 2001a,b) (Figure 1). To our knowledge, this is the first long-term monitoring of genotoxic damage in wild populations of birds, therefore interpretation of our results requires caution in the absence of published sources dealing with similar cases.

There is unquestionable evidence of the deleterious effects of metals, such as Fe, As, Pb, Cd, Cu, Zn, Tl and Hg (Fan *et al*., 1995; LeBlanc and Bain, 1997), some of which (i.e. Cd, Pb and As) have been reported to be genotoxic, mutagenic and even carcinogenic (LeBlanc and Bain, 1997; Yager and Ostrosky-Wegman, 1997). Accordingly, it is not unlikely that heavy metals might have played an important role in the protracted high level of genotoxic damage observed by us in storks and kites from Doñana, still present 4 years after the contamination episode. In addition to the ingestion of metals in the diet (the bioaccumulation process), soil and sediments become an important means of exposure (Beyer *et al*., 1994) for birds, because they may ingest substantial amounts of soil and sediments while feeding. In this regard, despite the immediate removal of covering sludge from the affected area and soil remediation activities carried out by the Andalusian authorities, up to February 1999 soils affected by the Aznalcollar mining spill contained significant residual contamination, especially of arsenic (Galán *et al*., 2002). Sediments from stretches of the Guadiamar river that pass the stork colony at Dehesa de Abajo had an elevated lead content, and isotopic lead identification demonstrated that the spill was the dominant lead exposure source for the white stork chicks born in this colony in 1999 (Meharg *et al*., 2002). Successive clean-up operations have been shown to be effective and levels of heavy metals and As have been reported as lower in both the biotic and abiotic compartments of the Doñana ecosystem. In general, metal concentrations in water and sediment from the Guadiamar river and Entremuros marshlands have decreased since the spill, although in July 2001 they were still higher than those measured at reference points, with some high metal peaks at certain times of sampling, indicating only a partial recovery of that zone and additional sources of metals not related to the spill (Toja *et al*., 2003).

Besides the heavy metals present in the Doñana environment as a consequence of the accidental toxic spill, other factors have traditionally threatened the wildlife in this important reserve of the biosphere (Albaiges *et al*., 1987). Intensive agriculture activity, mostly growing rice in the surrounding marshes, with the use of high levels of pesticides and fertilizers, could have contributed to the present situation, as well as in the past (Hernández *et al*., 1987; Rico *et al*., 1987).

Taken as a whole, the results reported here after gathering the data from a 4 year follow-up study give us information about the long-term consequences of the Doñana contamination episode, still apparently damaging the reserve in spite of the remedial measures taken so far. The increase in genotoxic damage observed during the study period cannot be easily explained solely on the basis of the heavy metal contamination, mainly because levels have tended to decrease in all of the compartments potentially involved (sediments, diet, etc.). In this regard, analyses of the relationship between individual genotoxic damage and metal concentrations are essential to highlight this hypothesis. Determination of aromatic compounds in bird samples for the more recent years could also help us figure out the origin of the observed damage. Moreover, more investigation is needed on the similarity in genotoxic damage between nestlings born in the same nest, which could indicate heritable genetic damage and/or a common diet, with implications for contaminant accumulation.

Taking into account the high level of genetic damage, sublethal effects such as lower reproductive success or lower survival rates could become apparent in the populations of birds living in this important wildlife area in the near future. Long-term monitoring is needed to assess whether the genotoxic damage detected in these birds might have any further effect on the population dynamics of these species.

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