Assessment of DNA damage in Ardea cinerea and Ciconia ciconia: A 5-year study in Portuguese birds retrieved for rehabilitation

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\textbf{ABSTRACT}

Over the past decades, the presence of micronucleated blood cells has been used to detect genotoxic effects of xenobiotics in fish, amphibians and birds. This study assessed the frequency of micronuclei (MN) and other nuclear abnormalities in erythrocytes of individuals of Ardea cinerea and Ciconia ciconia retrieved for rehabilitation in order to evaluate the influence of age, temporal and spatial factors on the occurrence of DNA damage in Portuguese wild birds. Blood smears from 65 birds with different life-history backgrounds (e.g. geographic origin, age) were collected between 2007 and 2011 and the frequency of erythrocyte nuclear abnormalities (ENAs) was analysed. Differences in DNA damage between ages were observed to occur in C. ciconia, with chicks displaying significantly higher frequencies of ENAs (both when looking at total ENAs or only MN frequency) than juveniles and adults. Additionally, significant differences in ENAs frequencies were observed between different years and geographic origins, whereas MN frequency alone did not show significant alterations concerning spatial and temporal variations. These results suggest that the assessment of ENAs rather than MN frequency alone may be a useful and valuable tool to complement the evaluation of DNA damage in populations of birds, as prompted by individual life-history traits and environmental factors.

1. Introduction

Within a cell, the DNA molecule undergoes cyclic changes in its structure that makes it flux between a functionally stable double-stranded entity and an intermediate state, in which the double helix is partially uncoiled to allow DNA replication. During the latter state, the DNA molecule is less stable and thereby, particularly susceptible to genotoxic damage (Shugart et al., 2003). Genotoxic damage refers to damage induced in the genetic material of cells by free radicals (e.g. reactive oxygen species and reactive oxygen intermediates) generated by oxidative stress processes and/or through the (indirect) action of genotoxic substances (Cajaraville et al., 2003). This damage, triggered by the disruption of the DNA replicating processes with consequent loss of its structural integrity, may include a wide range of effects from chromosomal aberrations to the initiation of carcinogenicity in individuals, or hereditary defects that pass via germ cell mutations and teratogenicity (Baos et al., 2006; Shugart et al., 2003). Ultimately, these may alter individual fitness, reproductive success and, consequently, population dynamics (Shugart et al., 2003).

Genotoxic substances may have relevant deleterious effects upon population dynamics, thereby making it an issue of concern in conservation biology. Therefore, assessing genotoxicity is a valuable strategy in wildlife risk assessment and requires the choice of suitable and sensitive markers (Baos et al., 2006; Pastor et al., 2001). Over the years, a wide number of techniques derived from human cancer risk assessment has been employed to monitor genotoxic effects on a broad range of organisms, both in vivo and in vitro, including markers of DNA modifications (e.g. DNA adducts) and of cytogenetic effects (e.g. micronuclei and chromosomal aberrations) and mutations (Costa et al., 2011; Kleinjans and van Schooten, 2002). Two examples of cytogenetic assays are the micronucleus (MN) test and the Erythrocyte Nuclear Abnormalities (ENA) assay. The MN test is based on the detection of MN, these are small cytoplasmic bodies originated from displaced chromosomes or chromosome fragments (acentric chromosome or chromatid fragments) which are enclosed by a nuclear membrane (Fenech et al., 2011). These may be produced, for example, by un repaired or misrepaired DNA double strand breaks or impaired function of the spindle apparatus (Fenech et al., 2011). The MN test...
has been used as a tool to detect genotoxic effects induced by environmental contaminants in different animals, including birds (Baesse et al., 2015; Quirós et al., 2008). The ENA assay is based on the same principle of the MN and detects MN and other analogous abnormal nuclear structures such as kidney-shaped and lobed nuclei whose formation is as well induced by genotoxic compounds or radiation (Ayllon and Garcia-Vazquez, 2000; Gravato and Santos, 2002; Pacheco and Santos, 1997). The ENA assay has already been successfully applied to fish (Costa et al., 2008; Monteiro et al., 2011; Pacheco and Santos, 2002; Van Ngan et al., 2007) amphibians (Josende et al., 2015; Marques et al., 2009) and only more recently to birds (De Mas et al., 2015).

Although the selection of appropriate techniques and approaches are important factors when monitoring genotoxicity in the environment, an aspect as equally important is the selection of representative organisms as sentinels (Baos et al., 2006; Pastor et al., 2001). Being long-lived and representative of upper trophic levels in both aquatic and terrestrial ecosystems, birds such as storks and herons are sensitive indicators of environmental quality, as they are particularly prone to bioaccumulate organic contaminants, including genotoxins, which may compromise their fitness and survival (Barata et al., 2010; Pastor et al., 2001; Quirós et al., 2008; Shugart et al., 2003). Moreover, birds are conspicuous and abundant species, with well-established basic ecology and habitat preferences, being therefore, a relatively easy and inexpensive group to monitor (Stolen et al., 2005).

The main aim of the present study was to assess the frequency of micronuclei (MN) and other nuclear aberrations (ENA assay) in peripheral blood erythrocytes of Portuguese populations of birds, in order to evaluate the possible contribution of the individual life-history features such as age, geographic origin and sampling year to DNA damage. To accomplish that, two species were selected: the piscivorous grey heron (Ardea cinerea) and the white stork (Ciconia ciconia), which has more generalist habits, feeding opportunistically on insects, amphibians, reptiles, earthworms and fish. Blood samples were obtained from birds with different life-history features, immediately upon arrival at a wildlife rehabilitation centre.

This approach has important advantages. Firstly, using blood as a matrix to assess genotoxic damage allows to screen for recent exposure effects, as erythrocytes have a short lifespan (28–45 days) (Rodnan et al., 1957). Secondly, by taking into account individuals’ intrinsic features such as age, it is possible to infer whether or not these individual characteristics may influence the frequency of erythrocyte nuclear abnormalities detected and act as a confounding factor in genotoxicity risk assessment studies (Shepherd and Somers, 2012). To our knowledge, the present study is the first to study spatial and temporal variation of DNA damage in Portuguese birds.

2. Methods

2.1. Birds selection, geographic origin and blood collection

All birds used in this study were individuals admitted for rehabilitation between 2007 and 2011 at CERVAS (Centro de Ecologia, Recuperação e Vigilância de Animais Selvagens), a wildlife rehabilitation centre located in Gouveia (Guadã, Portugal). Briefly, 65 blood smears were analysed from a total of 65 birds retrieved from different districts within northern and central mainland Portugal (Fig. 1). The number of individuals analysed per species, district and year of retrieval are discriminated in the Supplementary Material, Table S1. Detailed information on district-specific environmental risk factors is depicted on Table 1, according to the Risk Factor classification by the Portuguese National Statistical Institute (Ine, 2009).

Blood samples, collected by the veterinary centre, were drawn from the brachial vein, immediately upon arrival at the centre and used to prepare a blood smear. Heparin was used during collection to prevent blood clotting. Considering that bird arrival at the centre cannot be predicted, the frequency and time interval between blood sampling was dependent on the arrival of new individuals, thus varying each year. Blood sampling was only performed when birds were not displaying signs of pain or stress, in order to prevent injury to the individuals analysed. Disturbance stress caused by animal handling was minimized by limiting the handling period and by covering the head. Birds’ age (e.g. chick, juvenile and adult) was scored following analysis of moult and plumage pattern in individuals received for rehabilitation, according to their species. All procedures involving handling of live birds were conducted according to the European Parliament and Council directive 2010/63/UE (22 September 2010) in Portugal represented by Decreto de Lei n°113/2013 (7 August 2013).

2.2. Slide preparation and DNA damage scoring

DNA damage was assessed in mature peripheral erythrocytes using
the ENA assay, as adapted by Pacheco and Santos (Pacheco and Santos, 1996). Briefly, one blood smear per individual was fixed in methanol, dyed using the Diff-Quik stain kit (in accordance with the guidelines recommended by the manufacturers) and air dried. Slides were then coded and scored blindly, and from each smear, 1000 erythrocytes were counted using 1000x of magnification in order to determine the frequency of the following nuclear abnormalities (Fig. 2): MN, lobed nuclei (L), kidney-shaped nuclei (K), segmented nuclei (S) and notched nuclei (N) as described by Pacheco and Santos (Pacheco and Santos, 1996). All blood smears were scored and analysed by a single operator, in order to reduce experimental bias associated with different operators.

2.3. Statistical analysis

An exploratory analysis was performed using a Principal Component Analysis (PCA) in order to explore and highlight the relationships and patterns between samples, as well as to investigate which were the most important variables (among ‘age’, ‘district’, ‘year’ and ‘survival’) explaining the discrimination obtained between the different samples. Information on gender and cause of disorder of individuals was only available for some individuals, thus unsuitable to be included in statistical analysis. Categorical (qualitative) variables (e.g. survival or geographic origin) were converted to a numerical scale (survival: deceased=1, live=2; age: chick=1, juvenile=2, adult=3; geographic origin: district=1, 2, 3 and successively up for all location/species), acknowledging however, a certain degree of error associated with such a transformation.

Normality of variables was checked using the Shapiro-Wilk Test. When data deviated significantly from normality or homoscedasticity, non-parametric analysis of data were performed.

A Kruskal-Wallis analysis of variance (ANOVA on Ranks) was performed to compare differences between geographic and temporal origin of birds, as well as to discriminate age differences, when data did not meet the criteria for conducting a parametric analysis. ENAs data

<table>
<thead>
<tr>
<th>District</th>
<th>Area</th>
<th>Population density (hab/km²)</th>
<th>Mines</th>
<th>Agro-environmental indicators</th>
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<tr>
<td>Aveiro</td>
<td>2808</td>
<td>254.12</td>
<td>254.34</td>
<td>High</td>
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<td>A, B</td>
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<tr>
<td>Bragança</td>
<td>6608</td>
<td>22.53</td>
<td>20.62</td>
<td>Medium</td>
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<td>B, C</td>
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<tr>
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<td>108.97</td>
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<tr>
<td>Guarda</td>
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<td>29.17</td>
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<td>C, D</td>
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<tr>
<td>Leiria</td>
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<td>94.05</td>
<td>Medium</td>
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<tr>
<td>Portalegre</td>
<td>6065</td>
<td>20.94</td>
<td>19.54</td>
<td>Very low</td>
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<td>Viseu</td>
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<td>75.42</td>
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Fig. 2. Erythrocyte nuclear abnormalities (ENAs) observed in C. ciconia specimens: A) normal nuclei; B-C) lobed; D-E) Kidney shaped; F-G) Segmented; H) notched; I) micronuclei.
analysis between different age groups in C. ciconia met both equal variance and normality criteria and thus, one way analysis of variance (ANOVA) was performed. A Mann-Whitney rank sum test was used to compare differences in the extent of DNA damage and the survival of individuals. In all statistical analysis performed, a minimum of N=3 individuals per data group was established. This was true for all statistical analysis carried out except for PCA analysis. Multivariate analyses were performed using XLSTAT™ statistical analysis add-in (Addinsoft®). All the other analyses were conducted using SigmaPlot™ 11 software (Systat Software Inc).

3. Results

3.1. Micronucleus and total nuclear abnormalities frequencies in A. cinerea and C. ciconia

For both species studied, MN and other nuclear abnormalities were detected. MN frequencies were generally below the baseline values reported for several bird species. This was true except for 38%(C. ciconia) and 15% (A. cinerea) of the cases studied in which MN frequencies were above that value (Fig. 3). Lobed nuclei were the type of lesion most frequent in both studied species, accounting for 74.5 to 86.1% of the anomalies scored. At a lower degree, segmented nuclei was the second most scored lesion, accounting for 3.7 to 13.8% of the anomalies, followed by MN (3.4 to 9.9%), kidney-shaped nuclei (0 to 4.4%) and notched nuclei (0 to 0.4%). Further information on MN and ENAs frequencies are depicted on Table S2 of Supplementary Material.

3.2. Variability of DNA damage in Portuguese birds

Spatial and yearly variability in the frequency of MN and ENAs were observed in C. ciconia, with PC1 and PC2 generally accounting for over 73% of the overall variance in the dataset (Fig. 4). PCA showed a segregation of points into three main clusters (dashed grey lines). The year and district of retrieval of birds showed a strong influence in determining the frequency of MN and ENA 'Year' was positively correlated to PC1, explaining on its own > 47% of the information in the data, while 'District' was positively correlated to PC2, accounting for > 25% of the overall information) (Fig. 4-A). Within each cluster in Fig. 4-A, the proximity between adjacent points seems to be explained mostly by the sampling 'Year', with similar MN and ENA frequencies being predominantly observed for closer years. Geographical origin, positively correlated with PC2, contributed to discriminate three clusters corresponding to the “District” of origin, spread vertically. Moreover, the PCA also suggests a relationship between DNA damage and birds' survival, as well as between that and age (at a lower extent) (Fig. 4-B). PC1 was also strongly correlated to the variable 'Age', although to a lesser extent (compared to 'Year') (Fig. 4-B). In the same way, PC2 was also strongly correlated (positively) to 'Survival' (Fig. 4-B).

ENA frequency was observed to increase in both species from 2007 to 2010, after which it decreased slightly in 2011 (only in C. ciconia, with no samples being available for A. cinerea that year), although these differences between years were only statistically significant in C. ciconia (Fig. 5-B). Similarly, significant temporal differences were found to occur within different geographic sites, with the exception of those from Guarda, while individuals of C. ciconia retrieved at Coimbra and Portalegre followed a similar temporal pattern (Fig. 6-B). Moreover, significant geographic differences were still found in 2011, with C. ciconia individuals retrieved in Coimbra showing a significantly higher frequency of ENAs than those retrieved at Portalegre (Tukey Test, p < 0.05).

MN frequency followed a similar pattern to ENA, with MN frequency increasing from 2007 to 2011 in C. ciconia, though not statistically significant (Fig. 5-A). No defined pattern of MN variation was observed between years in A. cinerea.

No clear spatial or temporal trends were shown by PCA analysis in A. cinerea (Fig. 7-A and B), with PC1 and PC2 accounting for over 74% of the variability. PCA analysis also highlighted variability in DNA damage, influenced by survival and age of the A. cinerea individuals (Fig. 7-C and D), with PC1 and PC2 accounting for over 81% of the variability in the dataset. PCA showed a segregation of points both vertically and horizontally as an effect of individual age and survival.

Significant differences in the amount of DNA damage associated with age of individuals were observed in C. ciconia, with chicks (n=8) exhibiting a significantly higher frequency of MN than adults (n=17; Dunn's Test, p < 0.05) and a higher frequency of ENAs than juveniles (n=27) and adults (Holm-sidak Test, p < 0.05). No significant differences related to birds' age were reported in A. cinerea.

4. Discussion

Studying the occurrence of DNA-damage, which might ultimately impair the individuals' survival and reproductive success, may provide relevant information on the potential impact of environmental factors on natural populations (Baos et al., 2006). Methodologies used to monitor avian exposure to genotoxics have traditionally relied on the analysis of MN frequency or the comet assay. The present study focused on assessing the frequency of MN but also other nuclear aberrations in peripheral blood erythrocytes of A. cinerea and C. ciconia.

In all analysed individuals, MN and other nuclear abnormalities were observed. In 38% (C. ciconia) and 15% (A. cinerea) of the cases studied MN frequency was reported to be above the baseline level of MN for healthy birds (2.14 MN/1000 erythrocytes), as reviewed in the literature (Quirós et al., 2008; Zúñiga-González et al., 2000, 2001). This could suggest, in one hand, that these birds could have been exposed to abiotic (e.g. incl. chemical) stressors, which could have led to the observed DNA damage. Modifications in environmental conditions, such as changes in temperature, could have also influenced DNA stability. On another hand, all birds monitored were either injured or sick birds received for rehabilitation. For this reason, increased amount of nuclei damage as a consequence of lowered individuals' fitness is a scenario that should not be excluded as injured birds could display a lowered ability to renovate blood components. To the best of our knowledge, this study is the first to assess MN and ENA frequencies in C. ciconia and A. cinerea. Hence, the fact that values of MN observed could partly reflect normal values for the species studied should not be excluded.

In general, frequency of ENAs was shown to be more sensible to individual (e.g. age, geographical origin) and seasonal (e.g. year) factors than MN. To what extent, however, can this increased frequency be related to a higher sensitivity of ENA assay comparatively to MN
frequency is arguable. MN formation has been used as an indicator of chromosome breakage and/or loss, which has been directly associated with an increase in cancer risk and/or aging (Fenech, 2000). Contrarily to MN formation, and although other nuclear abnormalities (e.g. lobed or kidney-shaped nuclei) have been interpreted as analogous to MN, the mechanisms underlying their development are not yet fully understood (Oliveira et al., 2010). The ENA assays has been reliably applied to different vertebrate species (De Mas et al., 2015; Guilherme et al., 2008). Therefore, without both an extensive knowledge of the mechanisms involved in the occurrence of these type of nuclear lesions and a
clear link between such an occurrence and genotoxic exposure, it is currently challenging to ascertain whether or not such lesions might directly imply loss of genes’ transcription/expression and constrain tangible individual survival/fitness costs. Considering the present knowledge gap, ENAs frequency should only be considered as a complementary tool to MN assessment in evaluating DNA damage in bird species. In this speciﬁc point, results of this study suggest that in order to assess DNA damage in bird populations, the ENA assay may be a useful contribution to increase the sensitivity of genotoxic assessment. Data interpretation, however, should always take into account that an increase in ENAs might not be directly linked to an increase in genetic modiﬁcations susceptive to alter individual fitness, in order to prevent overestimation of the effect of genotoxic damage at the individual level.

Generally, ENA and MN frequencies in *C. ciconia* and *A. cinerea* tended to increase yearly, being this variation statistically signiﬁcant for the ENAs frequency in *C. ciconia*. This could suggest an increased exposure of individuals to environmental stressors, with a peak in 2010. This pattern of variation was consistently repeated when focusing the analysis at the district level, with individuals of *C. ciconia* from the districts of Coimbra and Portalegre evidencing also a signiﬁcant yearly increase in ENAs frequencies on 2010 and 2011, respectively.

Spatial differences were also reported in *C. ciconia* with Coimbra’s population exhibiting ENA values signiﬁcantly higher than the population of Portalegre in 2011. Additionally, Coimbra was also the district that registered the steadiest increase in ENAs and MN frequencies, not declining in 2011 as observed in Guarda and Portalegre, which could indicate that storks’ populations inhabiting this area could be more exposed to environmental stressors. Differences in human activities, land use and population density of the geographical areas where the birds were retrieved could also be a factor contributing to this pattern. For example, the basin of river Mondego (Coimbra) is historically one most important areas of rice production in Portugal, being therefore a target area of contamination by pesticides (Lima, 1997; Marques et al., 2011), which has been reported in the literature to increase genotoxic damage (Bajpayee et al., 2006; Quirós et al., 2008). The spatial differences observed could, as well, be connected to population density. Amongst the districts studied, Coimbra has one of the highest population densities. High human population densities are likely to increase the disposal of contaminants into the environment, thus increasing the risk of exposure of white stork local populations. Even though *C. ciconia* is a migratory species and it is expected that the movement of individuals between districts may occur after the breeding season, it must be highlighted that the stork populations have become increasingly sedentary in the Iberia Peninsula (Gilbert et al., 2016; Sanz-Aguilar et al., 2015) and thus movement between districts are less likely to occur.

DNA damage (MN and ENAs) was also observed to be higher in chicks of *C. ciconia* than in juveniles or adults. In two consecutive reports of baseline levels of MN in vertebrates, a similar trend was detected in a wide number of mammal species such as the dog (*Canis familiaris*), the grey squirrel (*Sciurus aureogaster*) and the white-tailed deer (*Odocoileus virginianus*) (Zúñiga-González et al., 2000, 2001). Similarly, DNA damage was observed to decrease signiﬁcantly with age, with higher MN frequencies in younger individuals than in adults. As hypothesized by the authors, these differences could be due to the inability of cellular mechanisms of chicks to cope with DNA
damage as efficiently as adults or juveniles (Zúñiga-González et al., 2000, 2001). More specifically, these differences could be related to the fact that the reticuloendothelial system, which is involved in the removal of old and damaged erythrocytes from the blood, matures with age (Zúñiga-González et al., 2000). Thus, younger individuals of C. ciconia could exhibit higher rates of DNA damage due to their reduced ability to eliminate damaged erythrocytes, which to our knowledge, is the first time that has been reported in birds.

In summary, yearly and geographic differences were detected in wild birds retrieved from the North and Centre of Portugal. ENAs frequencies of C. ciconia individuals retrieved for rehabilitation were observed to increase significantly with year and, in 2011, to differ spatially. Age was also found to be an important factor influencing both ENAs and MN frequency, which increased with decreasing age of individuals in C. ciconia. Overall, the results of the present study suggest that the assessment of ENAs, rather than MN frequencies alone, may be a useful complementary tool for the study of induced DNA damage in birds, as it showed to respond sensitively to different life history traits that can influence DNA damage in birds.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2016.10.039.

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