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RESEARCH ARTICLE

Erythrocyte micronucleus cytome assay of 17 wild bird species from the central Monte desert, Argentina

Arnoldo A. M. Quero[1,2] · **Daniela M. Ferré**[1,2] · **Agustín Zarco**[2,3] · **Pablo F. Cuervo**[4] · **Nora B. M. Gorla**[1,2]

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Abstract Birds have the potential to be considered valuable bioindicators of the quality of ecosystems and the environmental impact of pollutants. The aims of this study were to determine the micronuclei frequency and other nuclear abnormalities in erythrocytes by analyzing a wild bird community from central Monte desert (Argentina) and to clarify if there were any differences among certain species. Frequencies of nuclear abnormalities were determined in 73 wild birds belonging to 17 species and two orders (Passeriformes and Columbiformes). A high proportion of individuals, 90.4 and 80.9 %, had erythrocytes with micronuclei and nuclear buds, respectively. Notched nuclei, binucleated cells, nuclear tails, and nucleoplasmic bridges were also recorded. Certain species appeared to be more informative than others with regard to the possibility of being used as bioindicators of genetic damage. *Saltator aurantiirostris* and *Columbina picui* were the only species that showed significantly different frequencies of nuclear alterations, in comparison with the other species. The frequencies here presented are the first reported for these bird species from the orders Passeriformes and Columbiformes. This research supports the notion that the use of these biomarkers could be effectively applied to evaluate spontaneous or induced genetic instability in wild birds.

Keywords Wild birds · Micronuclei · Cytome assay · Nuclear abnormalities · Passeriformes · Columbiformes · Erythrocytes

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✉ Arnoldo A. M. Quero
aamartinquero@gmail.com

Nora B. M. Gorla
noragorla@gmail.com

[1] Laboratorio de Genética, Ambiente y Reproducción (GenAR), Universidad Juan Agustín Maza, Mendoza, Argentina

[2] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Mendoza, Argentina

[3] Instituto Argentino de Investigaciones de Zonas Áridas (IADIZA), CCT Mendoza- CONICET, Mendoza, Argentina

[4] Laboratorio de Ecología de Enfermedades (LEcEN), Instituto de Ciencias Veterinarias del Litoral (ICIVET Litoral, UNL - CONICET), Santa Fe, Argentina

Introduction

Birds can be studied to provide information about the quality of the ecosystems in which they inhabit. These ecosystems can be affected by several contaminants or conditions, such as chemicals (Stončius and Lazutka 2003; Sharaf et al. 2010), radiation (Ilyinskikh et al. 1997), and urban pollutants (Skarphedinsdottir et al. 2010; Shepherd and Somers 2012). The species in this animal group could be useful as bioindicators due to their role as bioaccumulators of substances within the food chain (Kursa and Bezrukov 2007; Skarphedinsdottir et al. 2010) and are suitable candidates as sentinels of the effects of genotoxic agents. This is due to the diversity of the ecosystems that they inhabit, their abundance, and the relatively simple access to obtain samples (Stahl 1997; Bonisoli-Alquati 2014).

During the last couple of decades, biological indicators or organisms providing information about the environmental conditions of their habitat have begun to be relevant for the evaluation and management of ecosystems (Van Gestel and Van Brummelen 1996; Adams et al. 2001; Van der Oost et al. 2003). In these organisms, certain biomarkers can be analyzed

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at the cellular or molecular level as biological responses. These include biomarkers of genotoxic effect as DNA modifications and cytogenetic markers as chromosomal aberrations and micronuclei (MN) (Bonassi and Au 2002).

Most of the studies about environmental quality have been conducted using organisms from aquatic environments as bioindicators (Çavaş and Ergene-Gözükara 2005; Baršienė et al. 2013; Yu et al. 2013), while many of those that assessed the effects of the aerial and terrestrial residual power of contaminants have been developed with invertebrates and mice (Fox 2001; Bosch et al. 2011). The diversity of birds increases the likelihood of identifying appropriate model species to assess the impact of genotoxic agents and habitat degradation as a consequence of human activities (Bonisoli-Alquati 2014).

The micronucleus assay was initially designed to evaluate clastogenic and aneugenic effects. The test was then implemented in human oral epithelium with a cytome approach analyzing other nuclear abnormalities (Tolbert et al. 1992; Fenech et al. 2006; Thomas et al. 2009) and was later adapted for pigeons in the same cells (Shepherd and Somers 2012). In birds, however, most of the studies have been performed in erythrocytes, even the micronucleus cytome assay (Kursa et al. 2005; Sharaf et al. 2010; Hussain et al. 2012; Clark 2015; De Mas et al. 2015). MN result from factors or events that interfere with the structure or function of the mitotic apparatus, producing a failure in the incorporation of chromosomes or chromosome fragments into the principal nucleus (Tolbert et al. 1992; Fenech et al. 2006; Thomas et al. 2009). MN formation in cell lines exposed to genotoxic agents is related to changes in permeability and disruption of the nuclear envelope, affecting its continuity (Hatch et al. 2013). We have not found a dose-response study induced by genotoxic agents in Passeriformes, which would indicate the frequencies that can be reached in an experimental design.

MN frequencies, without other nuclear abnormalities, have been reported in a diverse number of bird species (Zúñiga-González et al. 2000; Skarphedinsdottir et al. 2010; Baesse et al. 2015). In particular, it was reported in broiler chicken exposed to cypermethrin (Sharaf et al. 2010), Japanese quail exposed to atrazine (Hussain et al. 2012), parrots exposed to mitomycin C (Gómez-Meda et al. 2006), and pigeons exposed to radiations (Ilyinskikh et al. 1997). A starting "baseline" (Schaumburg et al. 2012) or "spontaneous frequency" (Zúñiga-González et al. 2000; Stončius and Sinkevičius 2003) is essential to evaluate the possible effect of environmental mutagens that can alter them. Stončius and Sinkevičius (2003) highlighted that the studies showing genetic damage in wild birds are directly related to effects such as mutations in the germ lines, neoplasms, and deterioration of physiological functions of individuals, changes in typical behavior, reduced fertility, and reproductive success.

The aim of this study was to determine the frequency of MN and other nuclear alterations in erythrocytes of wild birds from the central Monte desert (Argentina), as a first approach to obtain reference values of cytogenetic damage in species not previously analyzed. Additionally, we evaluated if the levels of genetic damage differed among bird species inhabiting the same ecosystem.

Materials and methods

Study area and sampled animals

The birds were captured and sampled in an open woodland of *Prosopis flexuosa*, in the Biosphere Reserve of Ñacuñán (34° 03' S–67° 54' W), located in the central Monte desert (Mendoza, Argentina). This ecosystem stretches over 2000 km from the north to the south of Argentina and occupies approximately 46 million ha between the Andes and the Atlantic Ocean (Fig. 1). The reserve was chosen because it is one of the areas of greatest ornithological importance of the country and represents a relatively pristine ecosystem (Boshoven and Tognelli 2001, Cueto and Lopez de Casenave 2007).

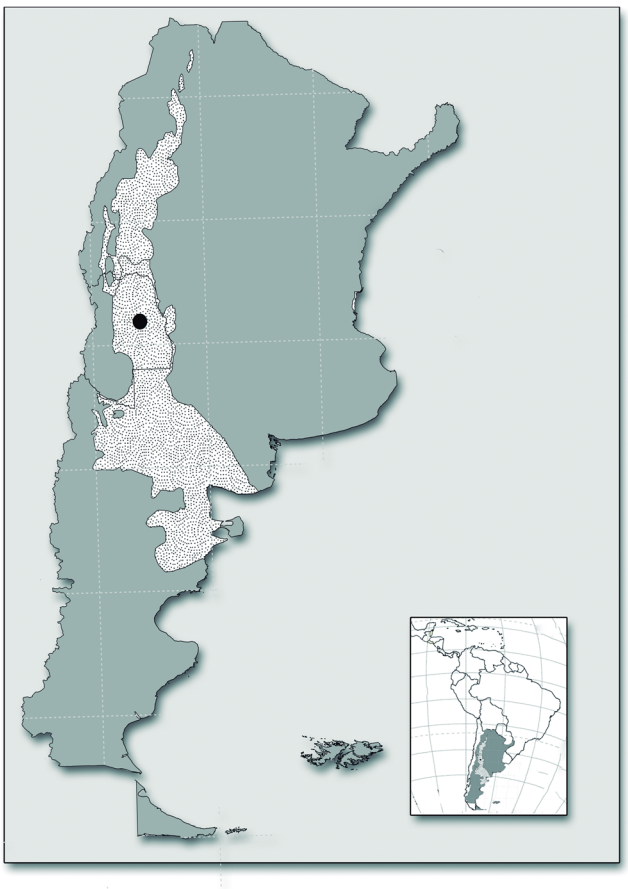

Fig. 1 Map showing the Biogeographic Province of the Monte Desert (*shaded*) in perspective from South America and Argentina. The Biosphere Reserve of Ñacuñán is indicated with a *black point*

Captures were conducted during two field sampling campaigns, between August and November 2012, during the first 4 h of dawn and were made using mist nets (12 m × 3 m, mesh size of 34 mm) separated by no more than 100 m. Each bird was removed from the nets using a systematic procedure adapted from Ralph et al. (1996). All the birds analyzed were taxonomically classified according to the South American Classification Committee (SACC) of the American Ornithologists' Union (Remsen et al. 2015) and Burns et al. (2014). Procedure protocols performed in studies involving animals were approved by the Institutional Commission for the Care and Use of Laboratory Animals (Universidad Juan A. Maza).

All the birds sampled were apparently healthy, without signs of illness. A drop of blood was obtained from the distal end of the middle finger nail. Blood smears were fixed in absolute methanol for 1 min and stained with Giemsa (Merck®) 1: 10 for 8 min (Bird and Bildstein 2007). Ten thousand mature erythrocytes were analyzed from each bird with optic microscope (×1000). Every cell having MN or other nuclear abnormality was photographed.

Criteria of analysis

The cells had to present a flat and intact cytoplasm, without overlap with other cells, absence of intracytoplasmic detritus, be intact and with bounded nucleus and homogeneous staining throughout the cell and the nucleus (Thomas et al. 2009). Identification of MN was performed according to the inclusion criteria suggested by Tolbert et al. (1992): a round or oval structure, which represents 1/3 to 1/16 the size of the nucleus, with the same focal plane, coloration and texture of the nucleus, without chromatin bridges or overlaps. According to Thomas et al. (2009), cells with nuclear buds were identified in nuclei with an apparent sharp constriction at one end, wherein the buds are between 1/4 and 1/3 the size of the principal nucleus. Following the criteria of the aforementioned authors, the binucleated cells were identified when two nuclei of the same size and staining, with or without contact between them, were found. The nuclear tails were analyzed according to the criteria set by Kursa and Bezrukov (2007), being characterized by the progressive reduction, narrowing, and elongation of one of the ends of the nucleus. According to Tolbert et al. (1992), nucleoplasmic bridges were identified whenever two nuclear structures of equal or different size, with the same color, were observed connected by a chromatin bridge. Finally, the criteria for identifying notched nucleus were adapted from fish to bird erythrocytes (Carrasco et al. 1990). These were identified as a well-defined slit of uniform width extending to an appreciable depth into a nucleus. Notches appeared to contain no nuclear material and seemed to be apparently demarcated by the nuclear envelope.

Statistical analysis

For each species, the mean ± standard error (SE) was calculated. The Kolmogorov–Smirnov test was performed to verify whether the results follow a normal distribution. For each nuclear alteration, the Kruskal–Wallis test with pairwise multiple comparison was applied to detect if the frequencies from each species with $n \geq 4$ were significantly different from the others. In order to control the familywise type I error, adjusted p values were calculated with the Bonferroni's correction and used to make the decision for each pair. The Spearman's rank–order correlation was performed to estimate whether there was a significant correlation between the frequencies of MN and each one of the remaining nuclear abnormalities. In all cases, the selected level of significance was p value <0.05. For statistical analysis, the software SPSS® Statistics version 21 was used.

Results

A total of 73 wild birds, belonging to 17 different species, were captured. Fifteen of those species belonged to five families from the Passeriformes order, while the other two species belonged to the Columbiformes order (Table 1).

The different nuclear abnormalities observed are shown in Fig. 2a–p. These nuclear alterations, from the highest to the lowest frequency found, were MN, nuclear buds (bud), notched nuclei (notch), binucleated cells (bin), nuclear tails (tail), and nucleoplasmic bridges (bridge) (Table 1). In seven species, each nuclear alteration was contrasted against the others. From the pairwise comparisons analyzed (a total of 126, corresponding to 21 pairwise comparisons per nuclear alteration), 17 were statistically different (Bonferroni's corrected p values <0.0024) specifically in MN, buds, notch, tail, and bridge (Table 1) and always involving *Saltator aurantiirostris* or *Columbina picui* (additional figures are available in Online Resources 1–6).

A positive correlation was found between the frequencies of MN and notched nuclei (rho = 0.35, p value = 0.0025). Furthermore, these abnormalities were found simultaneously affecting the same nucleus in three different cells (Fig. 2m–o).

Discussion

Those species with no apparent exposure to a contaminant and that present a high frequency of MN are useful for the analysis of this biomarker and could be used as bioindicators (Zúñiga-González et al. 2001, Poletta et al. 2008). The amount and variety of nuclear abnormalities found in the captured birds, mostly Passeriformes, lead us to think that some of the species from this group could be useful and informative to assess

Table 1 Frequency of nuclear alterations in wild bird species from central Monte desert, Argentina

Species	*N*	Type of Nuclear alterations/ 1000 erythrocytes/ animal- mean ± SE					
		MN	Bud	Notch	Bin	Tail	Bridge
Passeriformes							
Emberizidae							
Zonotrichia capensis—rufous-collared sparrow	16	0.44 ± 0.07	0.21 ± 0.04	0.14 ± 0.05	0.11 ± 0.02	0.05 ± 0.05	0.01 ± 0.01
Furnariidae							
Cranioleuca pyrrhophia—stripe-crowned spinetail	2	0.45 ± 0.15	0.10 ± 0.10	0.20 ± 0.10	0.05 ± 0.05	–	–
Lepidocolaptes angustirostris—narrow-billed woodcreeper	2	0.65 ± 0.25	0.15 ± 0.05	0.55 ± 0.15	0.05 ± 0.05	–	–
Pseudoseisura lophotes—brown lophotes	2	0.40 ± 0.10	0.20 ± 0.20	0.50 ± 0.10	0.35 ± 0.25	–	–
Icteridae							
Molothrus bonariensis—shiny cowbird	2	0.10 ± 0.00	0.65 ± 0.25	0.45 ± 0.05	0.25 ± 0.15	0.10 ± 0.10	0.05 ± 0.05
Thraupidae							
Lophospingus pusillus—black-crested finch	1	0.30	0.60	1.90	0.20	0.20	–
Phrygilus carbonarius—carbonated sierra-finch	3	0.26 ± 0.12	0.13 ± 0.08	0.33 ± 0.24	0.23 ± 0.06	0.03 ± 0.03	–
Poospiza ornata—cinnamon warbling-finch	4	0.25 ± 0.06	0.27 ± 0.14	0.40 ± 0.17	0.12 ± 0.09	–	–
Poospiza torquata—ringed warbling- finch	6	0.21 ± 0.04	0.21 ± 0.09	0.20 ± 0.08	0.18 ± 0.08	0.01 ± 0.01	0.01 ± 0.01
Saltator aurantiirostris—golden-billed saltator	4	1.12 ± 0.37	0.95 ± 0.14[a]	6.65 ± 1.76[b, c]	0.17 ± 0.07	0.22 ± 0.06[d–e]	0.20 ± 0.08[f, g]
Saltatricula multicolor—nany-colored chaco-finch	6	0.15 ± 0.11	0.30 ± 0.18	0.51 ± 0.45	0.06 ± 0.03	–	0.01 ± 0.01
Tyrannidae							
Elaenia albiceps—white-crested elaenia	8	0.38 ± 0.09	0.37 ± 0.07	0.10 ± 0.05	0.15 ± 0.03	0.02 ± 0.01	–
Pyrocephalus rubinus—vermilion flycatcher	2	0.10 ± 0.00	0.10 ± 0.01	0.30 ± 0.20	0.05 ± 0.05	–	–
Sublegatus modestus—southern scrub-flycatcher	3	0.20 ± 0.11	0.70 ± 0.32	0.36 ± 0.13	0.43 ± 0.20	–	–
Xolmis coronatus—black-crowned monjita	3	0.23 ± 0.06	0.60 ± 0.60	0.06 ± 0.03	0.30 ± 0.17	–	–
Columbiformes							
Columbidae							
Columbina picui—picui ground dove	8	0.87 ± 0.19[h]	0.62 ± 0.28	1.77 ± 0.84[i, j]	0.35 ± 0.18	0.01 ± 0.01	0.01 ± 0.01
Zenaida auriculata—eared dove	1	0.40	0.30	2.50	0.10	–	0.10
Percentage of individuals that presented at least one nuclear abnormality		90.4 %	80.9 %	74.0 %	71.2 %	21.9 %	13.7 %

Significance *p values* deduced using Kruskal–Wallis test with pairwise multiple comparison. Adjusted *p* values were calculated with the Bonferroni's correction: *Saltator aurantiirostris* was significantly different than (a) *Z. capensis* ($p = 0.002$), (b) *E. albiceps* and *Z. capensis* ($p \leq 0.001$), (c) *S. multicolor* ($p = 0.002$), (d) *P. torquata*, *E. albiceps*, and *Z. capensis* ($p = 0.001$), (e) *P. ornata*, *S. multicolor* and *C. picui* ($p \leq 0.001$), (f) *E. albiceps* and *Z. capensis* ($p \leq 0.001$), (g) *P. ornata* and *C. picui* ($p = 0.001$). *Columbina picui* was significantly different than (h) *S. multicolor* ($p = 0.001$), (i) *E. albiceps* ($p = 0.001$) and (j) *Z. capensis* ($p \leq 0.001$)

N number of individuals/species, *SE* standard error, *MN* micronuclei, *bud* nuclear buds, *notch* notched nuclei, *bin* binucleated cells, *bridge* nucleoplasmic bridges, *tail* nuclear tails

possible genetic damage caused by environmental pollutants. A mean MN frequency ≥ 0.35 is suggested as a cut-off for a species to be useful as a bioindicator of genotoxic risk (Zúñiga-González et al. 2001). According to this proposal and analyzing only the species with $n \geq 4$ individuals, the highest MN frequencies were found in *S. aurantiirostris* and *C. picui,* (Table 1 and Online Resource 1). In contrast, the lowest frequencies observed were detected in *Saltatricula multicolor.*

The MN frequencies described in this study are consistent with rates reported in previous studies by Zúñiga-González et al. (2000, 2001), Kursa and Bezrukov (2007), Skarphedinsdottir et al. (2010) and De Mas et al. (2015) for other families and orders of wild birds. A study with numerous species of Passeriformes (Baesse et al. 2015), which included the genus *Saltator*, reported the following MN mean frequencies: *S. maximus* 0.45 ($n = 10$) and *S. similis* 0.85 ($n = 5$). Our study shows a MN mean frequency of 1.12 for *S. aurantiirostris* ($n = 4$).

The Biosphere Reserve of Ñacuñán is a protected area where anthropogenic pollutants are considered to be absent or at least scarce, and then, frequencies obtained could be considered as a reference for adult birds of *Zonotrichia capensis, Poospiza ornata, Poospiza torquata, S.*

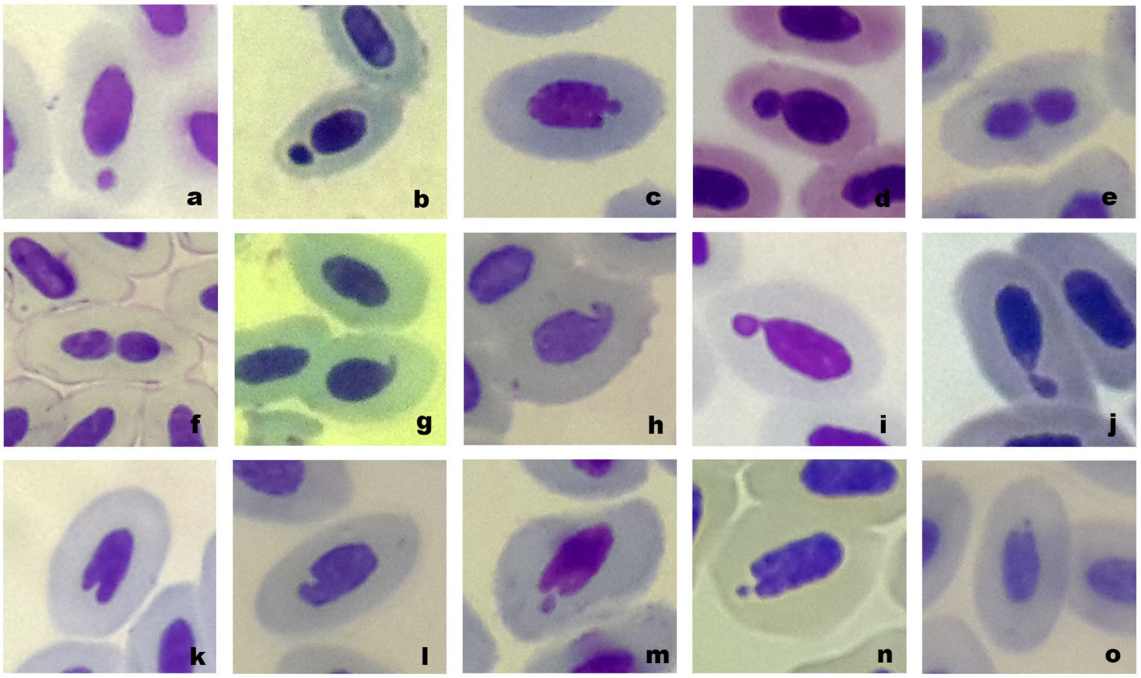

Fig. 2 Nuclear alterations with Giemsa stain in bird erythrocytes (×1000). **a**, **b** Micronucleus. **c**, **d** Nuclear buds. **e**, **f** Binucleated cells. **g**, **h** Nuclear tails. **i**, **j** Nucleoplasmic bridges. **k**, **l** Notched nuclei. **m**, **n**, **o** Micronucleus and notched nuclei in the same cell

aurantiirostris, S. multicolor, Elaenia albiceps and *C. picui*. It is desirable to increase the number of individuals per species to validate this suggestion. Having a baseline of genetic instability from different species of birds is necessary when planning to perform dose-response trials or to evaluate the effect of environmental disturbances.

Studies in birds have reported information considering both MN presence and the "cytome" approach. Nuclear buds could be a measure of gene amplification or acentric fragments (Fenech et al. 2011). In our research, the 80.9 % of the individuals from different species has presented at least one nuclear bud, from 0.10 to 0.95 ± 0.14/1000 erythrocytes. In a controlled assay in a *Psittacidae* species (*Aratinga canicularis*) exposed to mitomycin C, the reported frequency of nuclear buds was 1.28/1000 cells in the negative controls administered with water (Gómez-Meda et al. 2006), while the value reported for the only passerine species previously studied (*Parus major*) was 0.08 ± 0.02 nuclear buds/1000 erythrocytes (Kursa et al. 2005). We argue that the variation in the frequency of this biomarker among birds exposed to different gradients of anthropogenic impact (including absence of impact) could be due to the influence of intrinsic variables (specie, sex, age, diet, status, and migratory behavior) or extrinsic ones (habitat, altitude, season, type of food available), other than obvious environmental contaminants. In spite of this, nuclear buds have been proposed to be as useful as MN in its role as biomarker of genetic damage (Gómez-Meda et al. 2006).

Regarding binucleated cells in birds, reported mean frequencies were between 0.09 and 0.18 for bird species (Kursa et al. 2005). The results here are similar to that report, with mean values ranging between 0.05 and 0.40. Binucleated cells are likely to be the result of failures in cytokinesis during mitosis, and its relevance as a biomarker of genetic instability is still poorly understood (Thomas et al. 2009).

The presence of nucleoplasmic bridges was only recorded in six out of the 17 species of birds studied. We did not find previous reports describing the presence of such nuclear abnormality in peripheral blood erythrocytes of birds. However, nucleoplasmic bridges are found in human buccal mucosa cells and are indicative of DNA repair errors, chromosomal rearrangements, or telomeric fusions (Fenech et al. 2011).

Nucleoplasmic bridges and nuclear tails might be produced by the same mechanism (Anbumani and Mohankumar 2015). The first one is formed when a dicentric chromosome is divided into opposite poles during mitosis, but somehow the chromatids would give rise to a nucleoplasmic bridge that could break and end up forming a MN (Fenech et al. 2011). Additionally, a cytoplasmic constriction of the nucleoplasmic bridge could result in a nuclear tail through breakage-fusion-bridge cycle (Anbumani and Mohankumar 2015). In bird erythrocytes, this abnormality has been initially described by Kursa et al. (2005) and Kursa and Bezrukov (2007). These authors described mean values for nuclear tails per 1000 erythrocytes in five different species (mean = 0.46), including one passerine. De Mas et al. (2015) described this abnormality in penguins, while Alimba and Bakare (2015) described it in *Coturnix coturnix japonica*. We recorded this abnormality in 21.9 % of the individuals studied, with a mean range between

0.05 and 0.22. There are no reports in any of the aforementioned literature about frequencies of nuclear tails in species belonging to these orders to compare with our observations.

The species *S. aurantiirostris* presented the highest frequency of cells with notched nuclei (Table 1 and Online Resource 3), abnormality named in this way by Carrasco et al. (1990), but as indentations by Clark (2015) and De Mas et al. (2015). This abnormality was present in every species studied and it was observed in 74 % of the individuals analyzed. The high proportion of individuals presenting this nuclear abnormality and its high frequency in several species may suggest that notched nuclei could be as informative as MN and nuclear buds as biomarkers in a bird community. The causes and mechanisms of formation of notched nuclei are still unknown.

A positive correlation between the MN and notched nuclei frequencies was detected in the population studied, and three cells were seen with both types of nuclear abnormalities in the same nucleus. This might suggest that the mechanism of MN formation could be related with the presence of other nuclear alterations. Harabawy and Mosleh (2014) have postulated that diverse nuclear alterations detected in fish erythrocytes can act as precursors of MN and binucleate cell formation. Other experimental studies (Da Silva Souza and Fontanelli 2006; Jindal and Verma 2015) have reported a related increase between the MN frequency and different nuclear abnormalities observed, including notched nuclei. A strong cross-correlation between MN, nuclear buds, and nucleoplasmic bridges has been also reported (Fenech et al. 2011). However, as far as we know, the connection between the mechanisms of formation of notched nuclei and MN remains unknown.

Some other authors have reported that bird age and gender could be factors affecting background MN frequencies, but results have been inconclusive (Shepherd and Somers 2012). In our study, all birds tested were adults. With regard to gender, a high proportion of the species analyzed had no external sexual dimorphism, and thus, we suggest that any future investigation should consider a molecular sex determination procedure to determine the gender of the birds under study.

S. aurantiirostris and *C. picui* were the species that evidenced higher frequencies of the nuclear abnormalities analyzed (Table 1 and Online Resources 1–6). These results would suggest that the two species aforementioned could be taken into consideration as possible candidates for genotoxicity assessment. A higher frequency of micronucleated erythrocytes could reflect a greater capacity to produce them or a lower physiological ability to remove the altered cells (Zúñiga-González et al. 2001). It should be considered that birds are capable to exchange all their erythrocytes in 25–45 days (Jones 2015); therefore, the low capacity to remove altered red blood cells is questionable. This could mean that *S. aurantiirostris* and *C. picui* can generate these biomarkers because they have a differential susceptibility, one of the features that should have a sentinel (Rabinowitz et al. 2010).

MN, nuclear buds, nuclear tails, binucleated cells, nucleoplasmic bridges, and notched nuclei frequencies were not previously reported for the species studied. This represents the first approach to determine reference values from certain species (*Z. capensis* or *C. picui*) also present in human-impacted areas, such as contaminated areas by landfill leachates, agricultural pollution, and others. The application of micronucleus test and cytome approach in peripheral blood has proved to be feasible in wild birds. Within the Passeriformes, it was detected that some species could be more informative than others to be used as bioindicators of genetic damage. Furthermore, these results are valuable in future assessments of experimental or field exposure to genotoxic agents.

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Compliance with ethical standards Procedure protocols performed in studies involving animals were approved by the Institutional Commission for the Care and Use of Laboratory Animals (Universidad Juan Agustín Maza).

Conflict of interest The authors declare that there they have no conflict of interest.

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