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Biological Sciences

Biomonitoring of genomic damage in shags from three Antarctic localities

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Abstract

Seabirds play an important role as top consumers in the food web and can be used as biomonitors for exposure to pollutants. Erythrocyte nuclear abnormalities (ENAs) represent one of the most important ways to detect genomic damage associated with environmental degradation and pollution. This study investigates the number of ENAs in three populations of two species of *Leucocarbo* shags. Blood samples from the Antarctic shag (*Leucocarbo bransfieldensis*) breeding on the Antarctic Peninsula and the South Shetland Islands and the South Georgia shag (*Leucocarbo georgianus*) breeding on the South Orkney Islands were analysed. The results revealed evidence of genomic damage in all individuals, with a mean number of ENAs of 26.54 and 43.51/10 000 red blood cells for Antarctic and South Georgia shags, respectively. Thus, the shags from the Orkney Islands showed a higher number of erythrocyte abnormalities, whereas no significant differences were observed among shag populations across the Antarctic Peninsula and South Shetland Islands. These results suggest that, in the northern part of the region, shags might be more exposed to pollutants. They also provide the first reference values for cytogenetic damage in this species and establish a critical baseline for future biomonitoring efforts.

Keywords: Antarctica; blue-eyed shag; environmental deterioration; erythrocytic nuclear abnormalities; *Leucocarbo*

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Introduction

Antarctica is one of the most pristine areas of the world. However, human settlements in this area and their associated activities, such as fishing, tourism and research, produce pollution through oil spills, sewage disposal, waste incineration and marine debris (Cripps 1992, Da Silva *et al.* 2023, Stark *et al.* 2016). In addition, pollutants such as persistent organic pollutants (POPs) and organochlorine pesticides (OCPs) released into environments can reach remote areas through atmospheric transport and deposition (Galban-Malagon et al. 2013, Rimondino *et al.* 2018), especially in the polar regions (Potapowicz *et al.* 2020). Consequently, various studies have detected a wide range of pollutants, including heavy metals and POPs, in the air, snow and soil of the Antarctic environment (Cipro *et al.* 2017, Na *et al.* 2020, Liu *et al.* 2021). Thus, obtaining information regarding the effects of environmental contaminants on Antarctic organisms is essential to detecting and mitigating the impacts of environmental pollution.

Seabirds provide information regarding the quality of the marine ecosystems they inhabit. Due to their role as bioaccumulators of contaminants within the food chain (Kursa & Bezrukov 2008, Skarphedinsdottir *et al.* 2010), these birds are suitable candidates as sentinels of the genotoxic agents in the surroundings of their feeding and reproductive areas. In recent years, many studies have demonstrated the presence of genotoxic substances in Antarctic seabirds, specifically in penguins (Barbosa *et al.* 2013, De Mas *et al.* 2015, Jerez *et al.* 2012), petrels (Van den Brink 1997, Colabuono *et al.* 2016) and albatrosses (Carravieri *et al.* 2014). However, there is a lack of knowledge regarding the effects of these pollutants in most Antarctic seabirds.

Antarctic birds are important members of the Antarctic ecosystem in terms of total biomass and environmental interaction (Corsolini 2011). In particular, two phalacrocoraciid species are found in Antarctica: the Antarctic shag (*Leucocarbo bransfieldensis*), which inhabits the Antarctic Peninsula and the South Shetland Islands (SSIs), and the South Georgia shag (*Leucocarbo georgianus*), which inhabits the South Orkney Islands (SOIs) and the sub-Antarctic South Sandwich Islands, South Georgia and Shag Rock (Kennedy & Spencer 2014, Orta *et al.* 2021). These shags generally reproduce in isolated areas far from anthropic activity and differ from other flying seabirds of Antarctica in their capacity to dive deeper than 100 m to feed almost exclusively on a variety of demersal fish (Casaux & Barrera-Oro 1993, Casaux *et al.* 2002, Casaux 2004), occupying in inshore-shallow waters the trophic niche of main predators of demersal fish (Casaux & Barrera Oro 2006). These characteristics make the Antarctic shags a suitable species for the study of marine contamination over wide geographical areas and at different trophic levels. Shag population data reveal a steady decline in the number of breeding pairs over the last 25 years at several colonies within their breeding range (Naveen *et al.* 2000, Casaux & Barrera-Oro 2006, Schrimp et al. 2018).

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Thus, evaluating the impacts of contamination on these species is important to determine how they adapt to different environments and to understand their population dynamics. There have been few studies conducted on these species, most of which have been related to diet and ecology, and none of them have investigated the effects of pollutants.

Analysing erythrocyte nuclear abnormalities (ENAs) is a technique that can be used to assess the effects of pollutants on organisms (De Mas *et al.* 2015). ENAs are nuclear malformations that appear in erythrocytes due to genomic damage from genotoxic substances (Quirós *et al.* 2008). Thus, assessing differences in the quantification of ENAs has been considered a practical tool for evaluation and monitoring of the level of environmental contamination. The most frequently studied such malformation is in the micronucleus (MN; Schmid *et al.* 1976). MNs are cytoplasmic chromatin masses with the appearance of small nuclei that arise from chromosome fragments or intact whole chromosomes from the anaphase stage of cell division (Schmid 1976). In addition, other ENAs, such as lobed nuclei, binucleated cells, kidney-shaped nuclei and notched nuclei, have been observed in the erythrocytes of birds (Kursa & Bezrukov 2008, Clarck 2014, De Mas *et al.* 2015). Although the mechanism responsible for the formation of all ENA types has not been explained, bird research has shown that ENA analysis was effective for evaluating the environmental quality of an area (Barbosa *et al* 2013, Baesse *et al.* 2015), as well as the potential impact of environmental factors on natural populations (Baos *et al.* 2006). This study aims to investigate the frequency of ENAs in peripheral blood erythrocytes of Antarctic and South Georgia shags to obtain reference levels of genomic damage in these species.

Materials and methods

Field and laboratory procedures

Breeding Antarctic shags (*L. bransfieldensis*) were captured at Harmony Point, Nelson Island, SSIs, and at three colonies located at the Danco Coast (DC), west Antarctic Peninsula. The DC colonies were Cape Herschel (64°04′S, 61°01′W), Midas Island (within Antarctic Specially Protected Area No. 134, 64°10′S, 61°05′W) and Point Py (64°13′S, 61°00′W). Captures of South Georgia shag (*L. georgianus*) were made on Laurie Island, SOIs. Captures occurred between November 2014 and February 2015 in the SSIs and in January 2018 in DC and the SOIs. Birds were randomly chosen and captured with a handled net from the nest. Handling procedures included body weight data and blood collection. We sampled a total of 60 reproductive individuals (20 South Georgia shags and 40 Antarctic shags (20 from the SSIs and 20 from DC)). Blood samples were collected by venipuncture of the alar vein using heparinized syringes with sterilized needles (23 gauge). Blood was placed into Eppendorf tubes, which were kept cool and carried to the laboratory within 5 h of the blood draw. Once at the laboratory, blood smears were prepared with a drop of fresh blood, air-dried, fixed with 99% methanol for 10 min and stained with Tincion 15 (Biopur). For every individual captured, two slides were made. The ENA assay was performed by counting the number of MNs and other ENAs on each blood smear under a microscope (100× magnification) per 10 000 mature erythrocytes (Schmid 1975) using the zig-zag model to avoid crossing the same field more than once. ENAs observed were MN erythrocytes and lobed, tailed, two-lobed, budding, cavity and kidney-shaped nuclei (Kursa & Bezrukov 2008, De Mas *et al.* 2015). Erythrocytes with other nuclear malformations were classified as 'unknown'.

Statistical analysis

For each species a descriptive statistical analysis was performed, including average, standard deviation (SD) and range (minimum–maximum) of the total sum of ENAs and MNs of blood smears. The frequency of occurrence (F%) was calculated as the percentage of individuals with malformations out of the total number of sampled individuals.

As the number of MNs was very low, they were included in the total count of malformations, and only the ENA test was considered for the rest of the analysis. Differences in ENAs between species and localities were determined using generalized linear models (GLMs; function 'glm' in *R*). A Poisson distribution was used because the number of ENAs is a count variable (Crawley 2012). As the models showed signs of dispersion, we corrected the standard errors using a quasi-GLM (quasi-Poisson distribution) in which the variance is given by $\varphi \times \mu$, where μ is the mean and φ is the dispersion parameter (Crawley 2012).

To explore sex differences, we examined the ENAs of the two shag species separately using GLMs with sex as a fixed factor. In the case of the Antarctic shag, by including two populations, the fixed effect of locality was incorporated. In this case, the interaction term between these main effects was also tested in the models to determine whether locality differences depended on the sex considered. In all cases, the best model was selected using a manual stepwise backwards deletion of non-significant terms from the full global models.

To compare models with different levels of complexity, we used the 'Anova' function with *F*-tests. Then, we used Tukey's honestly significant difference (HSD) *post hoc* test to compare locality or sex differences. All analyses were performed using *RStudio* version 4.1.2 (R Development Core Team 2021).

Results

We determined the ENAs and MN frequency for Antarctic shags and South Georgia shags. A small percentage of Antarctic shags showed MNs, whereas no MNs were found in the smears of the South Georgia shags (Table I). South Georgia shags showed a significantly higher proportion of ENAs than Antarctic shags ($F = 9.92$, $P = 0.002$; Table I). In addition, the breeding site of

Table I. Number of erythrocytic nuclear abnormalities (ENAs) and micronuclei (MNs) per 10 000 erythrocytes in each shag species.

Species	Sample size	ENAs			MNs		
		Mean ± SD	Range	F%	Mean ± SD	Range	F%
Antarctic shag (*Leucocarbo bransfieldensis*)	40	26.54 ± 14.61	5–58	100	1.29 ± 0.49	1–2	12.5
South Georgia shag (*Leucocarbo georgianus*)	20	43.41 ± 20.74	7–90	100	0	0	0

F% = frequency of occurrence percentage; SD = standard deviation.

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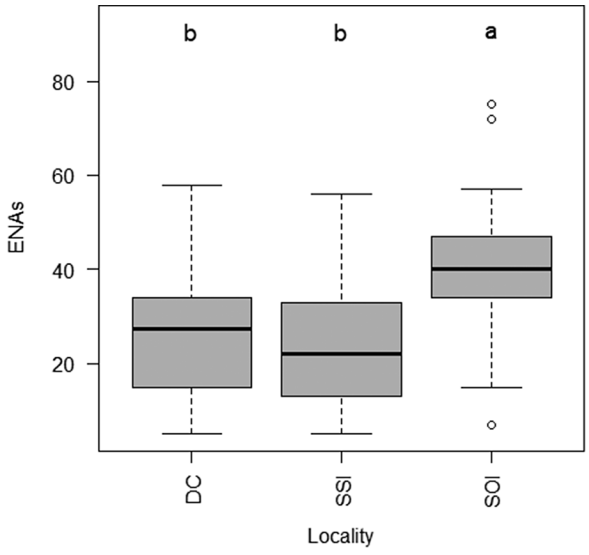

Figure 1. Boxplot of the number of erythrocytic nuclear abnormalities (ENAs) per 10 000 erythrocytes in three breeding localities of the two shag species. The boxes contain 50% of the values. Median, minimum and maximum values are indicated. Different letters indicate significant differences in ENA frequencies. DC = Danco Coast; SOI = South Orkney Islands; SSI = South Shetland Islands.

the shags had a strong influence on determining the frequency of ENAs (F = 4.95, P = 0.01). ENAs were significantly more frequent in shags from SOIs than in those from the other two localities (Fig. 1).

Regarding sexual differences, South Georgia shags did not show significant differences in the frequency of ENAs between females and males (F = 2.45, P = 0.13; Table II). In Antarctic shags, the number of ENAs varied between the sexes according to the locality studied (sex × locality; F = 1.77, P = 0.01). The number of ENAs was higher in males than in females from DC, Antarctic Peninsula (W = 71.5, P < 0.01), whereas non-significant differences were found between the sexes from Harmony Point, SSIs (W = 70.5, P = 0.28). Intra-specific comparisons within the species did not show differences between Antarctic shags from the SSIs and DC (F = 0.13, P = 0.71; Table II).

Discussion

The present study investigates for the first time the occurrence of ENAs in the blood cells of the two species of shags from three Antarctic localities along a longitudinal gradient. We found evidence of genotoxic damage, measured as the number of ENAs, in all breeding individuals from the three localities studied. All individuals presented at least one type of erythrocyte abnormality, whereas MNs appeared in low proportions or were absent from Antarctic and South Georgia shags, respectively. Other studies have reported a lack of sensitivity when using the MN test alone, suggesting that the ENA test represents a more effective alternative for assessing genotoxic damage (Guilherme *et al.* 2008, Monteiro *et al.* 2011).

Previous studies have evaluated the frequency of erythrocyte abnormalities in several bird taxa from different environments. For instance, Martinez-Haro et al. (2017) registered 18.6 and 40 ENAs/10 000 red blood cells (RBCs) for burrowing owls (*Athene cunicularia*) from pristine and urbanized areas, respectively. Frixione et al. (2020) found 71.5 ENAs/10 000 RBCs in American kestrels (*Falco sparverius*) from an agricultural area. In contrast, a heterogeneous sample of 25 bird species in southern Brazil presented an overall mean frequency of ENAs of 16.68/10 000 RBCs (Tomazelli *et al.* 2022). However, information regarding ENAs in seabirds is scarce, and such research has mainly been conducted in gulls and penguins. In black-headed gull (*Chroicocephalus ridibundus*) embryos, the means of investigations of two natural populations in Lithuania ranged from 0.057‰ to 4.7‰ (Stoncius *et al.* 2003). In Audouin's gulls (*Ichthyaetus audouinii*) captured in Italy, the mean ENA value was 33/10 000 RBCs (Borghesi 2016). In Antarctica, a few studies have reported on ENAs in seabirds: one in south polar skuas (*Stercorarius maccormicki*), with 0.71 ENAs/10 000 RBCs (Kursa & Bezrukov 2008), and several in pygoscelids (Barbosa *et al.* 2013, De Mas *et al.* 2015, Olmastroni *et al.* 2019). ENA averages of 20 and 19/10 000 RBCs have been registered for gentoo penguins (*Pygoscelis papua*) in Antarctic localities with high visitor rates (Afanasieva *et al.* 2006, Barbosa *et al.* 2013), in comparison with 5.3/10 000 RBCs for a rarely visited gentoo penguin rookery (Barbosa *et al.* 2013).

Our results show that the mean numbers of ENAs in shags from Antarctica were 26.54 and 43.51/10 000 RBCs for *L. bransfieldensis* and *L. georgianus*, respectively. Considering the frequencies observed in other seabirds from polluted environments, it is probable that the observed frequencies of ENAs in shags from Antarctica have been caused by exposure to genotoxic agents. ENA counts represent one of the main methods for detecting genomic damage related to environmental deterioration and pollution (Stoncius & Lazutka 2003, Van Ngan et al. 2007). In this sense, several studies have shown the presence of heavy metals such as mercury (Seco *et al.* 2019) and POPs such as polycyclic aromatic hydrocarbons and OCPs in several localities of the SSIs, the SOIs and the Antarctic Peninsula (Cao *et al.* 2018, Vergara *et al.* 2019), as well as in seabirds' faeces and feathers (Metcheva *et al.* 2011, Brasso *et al.* 2012). Alternatively, it is possible that exposure to genotoxic agents might have occurred outside of the breeding season. Unfortunately, there is no information available regarding wintering areas for these species to assess the level of exposure to pollutants during that season.

ENAs were similar between the sexes in shags from SOIs and SSIs. However, males from DC showed greater ENAs than females. The Antarctic shag is known for its inter-sexual variation in foraging strategies during the breeding season, including in relation

Table II. Number of erythrocytic nuclear abnormalities (ENAs) per 10 000 erythrocytes in each shag species by sex and locality. Sample size n = 10 in all cases.

	Antarctic shag (*Leucocarbo bransfieldensis*)				South Georgia shag (*Leucocarbo georgianus*)	
	Danco Coast		Harmony Point		South Orkney Islands	
	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range
Male	36.89 ± 10.98	25–58	28.73 ± 13.78	8–56	38.33 ± 20.16	7–90
Female	18.11 ± 9.83	5–34	22.40 ± 12.15	5–53	55.60 ± 18.42	34–75

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to diving depth, distance from the colony and diet composition (Casaux *et al.* 1998, 2001). Thus, the observed sexual differences in the number of ENAs in DC shags might be related to local conditions and foraging habitats in terms of exposure to pollutants. However, assumptions regarding differences in foraging patterns in these areas and exposure levels between the sexes need to be further investigated.

Our study revealed notable variation in the frequency of ENAs between the two shags species studied, which could be due to species-specific sensitivity. For example, Barbosa *et al.* (2013) found that on Isla 25 de Mayo/King George Island, where three penguin species live in mixed colonies, Adélie penguins had the highest genetic instability and the highest number of ENAs compared with gentoo and chinstrap penguins. The species-specific differences could reflect genetic variations in the capacity to produce such ENAs or a lower physiological ability to remove these altered cells (Zúñiga-González *et al.* 2001). However, species-specific ecological characteristics, such as diet and trophic level during the breeding season, might contribute to the observed differences in the frequencies of abnormalities between the species, as they play a role in both contaminant exposure and the biomagnification processes that may have adverse impacts on DNA. However, Antarctic shags and South Georgia shags are closely related, both genetically and in terms of feeding strategies and trophic position (Casaux *et al.* 2016). Thus, more probable explanations for these observed differences might be associated with spatial variation. In this sense, *L. georgianus* breeds on the SOIs and South Georgia Island, much further north than *L. bransfieldensis*, which is found in the Antarctic Peninsula and the SSIs. Thus, the higher ENA concentration found in shags from the SOIs could be attributed to a geographical gradient in the bioavailability of trace elements (Cossa *et al.* 2011), partly influenced by the proximity to the American continent. Clear latitudinal gradients in persistent contaminants, such as mercury, were previously described across the Southern Ocean (Mills *et al.* 2022). For example, Seco *et al.* (2019) observed that krill from the SOIs have total mercury concentrations five- to seven-times higher than Antarctic krill from the Antarctic Polar Front, reflecting differential contaminant bioavailability in the Southern Ocean. In this sense, Corsolini *et al.* (2011) also detected higher contaminant concentrations in migrating seabirds (*S. maccormicki* and brown skua, *Stercorarius antarcticus*) in comparison with sub-Antarctic species (snow petrel, *Pagodroma nivea*) and Antarctic species (*Pygoscelis* spp.) from the same sampling sites, suggesting higher contamination events at lower latitudes. Similarly, the differences in ENA concentrations between individuals of similar species but from different sampling areas suggest geographical variations in the concentration of bioavailable contaminants in seabirds. We would need to measure the level of pollutants in birds in each locality to validate this hypothesis.

In summary, we have established the baseline data on ENAs as biomarkers of genomic damage in shag populations from Antarctica. The results and conclusions of our study are subject to certain limitations. These include the difficulty of achieving a large sample size, the lack of additional information on the concentration of pollutants at the survey sites and the limited knowledge regarding the foraging areas and movements of these species during winter. Therefore, although it is difficult to identify the source of pollution, this study is the first attempt to establish baseline values for cyto-genetic damage in these species, and such data may be useful for long-term comparisons regarding the health of shag populations. Future studies should include assessments of contaminant levels in individuals in order to investigate potential relationships between such contaminants and genotoxic damage.

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Competing interests. The authors declare none.

Ethical approval. The protocol for capturing and sampling birds was ethically reviewed and approved by the Departamento de Gestion Ambiental de la Dirección Nacional del Antártico. This study was carried out in accordance with the Nuremberg Code, the Helsinki Declaration and its amendments. This study followed the guidelines established in the Code of Conduct of the Scientific Committee on Antarctic Research (SCAR) for the Use of Animals for Scientific Purposes in Antarctica.

Author contributions. All authors contributed to the conception and design of the study. Material preparation, data collection and analysis were performed by Marianela Beltran. The draft of the manuscript was written by Marianela Beltran, and all authors collaborated on the various versions of the manuscript. Marianela Beltran and Verónica D'Amico read and approved the final manuscript.

Data availability statement. The datasets generated and analysed during the current study are not publicly available because some of the data are still under analysis as part of a doctoral thesis that is being carried out by a researcher from the Argentine Antarctic Institute. However, some of these data are available from the corresponding author on reasonable request.

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