Polar Biol (2015) 38:1067–1074
DOI 10.1007/s00300-015-1667-2

ORIGINAL PAPER

Erythrocytic abnormalities in three Antarctic penguin species along the Antarctic Peninsula: biomonitoring of genomic damage

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Received: 4 July 2014 / Revised: 18 February 2015 / Accepted: 26 February 2015 / Published online: 11 March 2015

Abstract Pollutants and toxic contaminants produced in all parts of the world are transported to remote regions including Antarctica. Tourism, research, and fishing activities on this continent are another source of contamination. Toxic substances affect Antarctic species, and some produced genomic damage to the fauna. The genetic damage can be detected by microscopic observation of erythrocytic nuclear abnormalities (ENAs). We counted the number of ENAs in seven populations of three *Pygoscelid* penguin species, Adélie (*Pygoscelis adeliae*), Chinstrap (*Pygoscelis antarctica*), and Gentoo (*Pygoscelis papua*), and found important differences among species exposed to the same conditions. ENAs were more frequent in Adélie penguins than in the other two species. Inter-population comparisons within species showed remarkable differences in Adélie and Chinstrap penguins but not in Gentoo penguin. Frequency of ENAs in Adélie penguins was the highest in Yalour Island population, intermediate in King George Island population, and the lowest in Torgersen Island and Avian Island populations. In Chinstrap penguins, the highest number of ENAs was found on Deception Island, and significant differences were found only between Deception Island and King George Island populations. This information will provide baseline data to be used for assessing the evolution of genomic damage of penguins along the Antarctic Peninsula in the future.

Keywords Erythrocytic abnormalities · Genotoxic damage · Antarctica · Penguins · Pollutants

Introduction

Genomic damage can be produced by different factors such as some contaminants (Kleinjans and Van Schooten 2002) or radiation (Muller et al. 1996). The study of erythrocytic nuclear abnormalities (ENAs) is one of the most commonly used methods for detecting genomic damage, because it is simple and fast (Schmid 1975; Fenech 2000). ENAs are nuclear malformations that appear in erythrocytes as a result of genomic damage from genotoxic substances or radiation (Quirós et al. 2008; Muller et al. 1996) and are therefore indicators of genomic instability. Several nuclear malformation types such as kidney-shaped, lobed or tailed nuclei have been investigated. However, the most frequently studied malformation is the micronucleus (MN) (Dertinger et al. 1996), because micronucleated cells are easy to recognize. Micronuclei are found in dividing cells containing chromosome breaks and/or chromosomes unable to travel to the spindle poles during mitosis. The other nuclear abnormalities result from analogous damage (Schmid 1975; Fenech 2000). ENA detection has been used successfully to test for and report exposure to radiation (Muller et al. 1996) and genotoxic substances (i.e., PAHs, heavy metals and POPs) and evaluate their effects on organisms such as fish (Cavas and Ergene-Gozukara 2005; Matsumoto et al. 2006; Ergene et al. 2007; Van Ngan et al. 2007; Guilherme et al. 2008), birds (Quirós et al. 2008,) and amphibians (Marques et al. 2009). Moreover, some of

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these studies have used ENAs in birds as baseline data in order to do long-term comparisons of environmental deterioration and pollution in specific areas (Kursa and Bezrukov 2007).

Research on accumulation of contaminants in remote areas, such as Antarctica, shows the presence of high concentrations of toxic compounds which can arrive by transport from other areas of the planet or by local deposition (Wania and Mackay 1993). A large number of pollutants such as mercury (Dommergue et al. 2010; Marko et al. 2014) or persistent organic pollutants (POPs), such as organochlorine compounds (Wania and Mackay 1993; Van den Brink 1997) arrive by long-range atmospheric transport and other transportation pathways. In addition, research, tourism, fishing, and other human activities in recent decades have contributed to pollution on the Antarctic continent (Bargagli 2005). Substances such as polybrominated diphenyl ether (PBDE) flame retardants, petroleum hydrocarbons, polychlorinated biphenyls (PCBs), polychlorinated terphenyls (PCTs), and heavy metals are present around some Antarctic research bases (Lenihan 1992; Crockett and White 2003).

In recent decades, many studies on toxic substances have shown significant presence of noxious products in Antarctic wildlife, specifically in south polar skuas (Tao et al. 2006; Kursa and Bezrukov 2007), penguins (Van den Brink 1997; Corsolini et al. 2007; Geisz et al. 2008; Schiavone et al. 2009; Jerez et al. 2011; Barbosa et al. 2013; Jerez et al. 2013a, b), albatrosses (Tao et al. 2006), petrels (Van den Brink 1997), seals (Tao et al. 2006; Schiavone et al. 2009), whales (Krahn et al. 2008), fishes (Van Ngan et al. 2007), krill (Corsolini et al. 2006; Jerez et al. 2013a, b), and lichens and mosses (Yogui and Sericano 2008).

Seabirds are good sentinel species of environmental contamination mainly because of their high position in the trophic web (Walker 1990; Van den Brink 1997) which can contribute to greater biomagnification of pollutants compared with animals in lower trophic levels (Van den Brink 1997). Penguins are the most abundant birds in the Antarctic region and may be then considered sentinels of the Antarctic ecosystem (Boersma 2008). Penguins are at high risk for the effects of exposure to toxic substances, and they can accumulate and biomagnify toxic chemicals in tissues (Jerez et al. 2013a, b). Moreover, penguins, like other marine vertebrates in cold regions, accumulate lipids to protect themselves from the cold temperatures, and many contaminants, such as lipophilic POPs, are accumulated in their fatty tissue (Corsolini et al. 2006, 2007; Geisz et al. 2008). The presence of heavy metals in penguins has also been shown in places with more human activity (Jerez et al. 2011). Recently, Barbosa et al. (2013) suggest a high frequency of ENAs in penguins associated with a high concentration of heavy metals such as Pb and Ni, probably due to intense human activity.

This study aims to investigate the frequency of ENAs in blood samples of several populations of three *Pygoscelid* penguins, Adélie (*Pygoscelis adeliae*), Gentoo (*Pygoscelis papua*) and Chinstrap (*Pygoscelis antarctica*) penguins, to establish baseline levels of genomic damage along the penguin populations of the west coast Antarctic Peninsula. This information will provide baseline data to be used for assessing the evolution of genomic damage of penguins in the future.

Materials and methods

Field and laboratory procedures

Ten individuals of each penguin species (Adélie, Chinstrap, and Gentoo penguins) were captured at seven localities in the South Shetland Islands and along the west coast of the Antarctic Peninsula (see Table 1, Fig. 1). Captures were made in February 2006, except for Torgersen Island (February 2003) and for Adélie and Chinstrap penguins on King George Island (February 2007). Adult penguins were captured by means of a long-handled net on the beach in order to minimize disturbance in the breeding colonies. Adults were chosen instead of chicks to avoid the probable differences in development by the time sampling was done (Barbosa et al. 2007a, b). Immediately after capture, a blood sample was taken from each individual with a heparinized capillary tube after pricking a peripheral foot vein with a sterilized needle. Blood was smeared immediately, air-dried, and fixed with ethanol 96°. Later in the laboratory, the smears were stained with Giemsa (Mallinckrodt Baker Inc., Phillipsburg NJ, Cat. 3856). ENA frequency in each blood sample was scored by scanning the smears under microscope (100× objective) per 10,000 mature erythrocytes (Schmid 1975). Nuclear abnormalities observed were micronucleated erythrocytes (MN) (Fig. 2a), lobed (Fig. 2b), tailed (Fig. 2c), two-lobed (Fig. 2d), budding (Fig. 2e), cavity (Fig. 2f), and kidney-shaped nuclei (Fig. 2g) (Kursa and Bezrukov 2007; Van Ngan et al. 2007). Erythrocytes with other nuclear malformations were classified as unknown. The total sum of ENAs was used for statistical analysis. In addition, MN was also analyzed separately because it is the most frequent abnormality studied.

Statistical analyses

We analyzed the total number of ENAs and MN in the different species by means of generalized linear mixed models (GLMM) including locality as a random factor and species as

Table 1 Mean (±SD) number of erythrocytic nuclear abnormalities (ENA) and micronucleus (MN) per 10,000 erythrocytes in each penguin species and locality

Locality	Latitude/longitude	Species	Sample size	ENA	MN
King George I.	62°15′S 58°37′W	*Pygoscelis adeliae*	10	72.0 ± 35.3	1.9 ± 1.4
		Pygoscelis antarctica	10	11.2 ± 10.9	0
		Pygoscelis papua	10	11.9 ± 11.2	0.6 ± 0.7
Livingston I.	62°39′S 60°36′W	*Pygoscelis antarctica*	10	23.1 ± 9.3	0.1 ± 0.3
		Pygoscelis papua	10	19.3 ± 20.7	0
Deception I.	63°00′S 60°40′W	*Pygoscelis antarctica*	10	33.1 ± 31.2	1.5 ± 1.9
Rongé I.	64°40′S 62°40′W	*Pygoscelis antarctica*	10	19.0 ± 17.4	0.6 ± 0.8
		Pygoscelis papua	10	18.5 ± 25.0	1.2 ± 1.2
Torgersen I.	64°53′S 62°53′W	*Pygoscelis adeliae*	10	46.9 ± 43.5	1.3 ± 1.5
Yalour I.	65°15′S 64°11′W	*Pygoscelis adeliae*	10	109.9 ± 80.0	5.2 ± 4.1
Avian I.	67°46′S 68°64′W	*Pygoscelis adeliae*	10	41.2 ± 40.1	3.25 ± 3.7

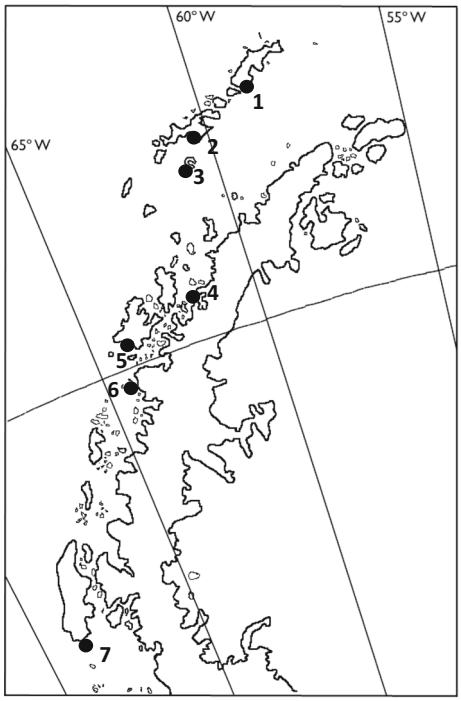

Fig. 1 Localities where blood samples were taken. *1*. King George Island (Stranger Point), *2*. Livingston Island (Hannah Point), *3*. Deception Island (Vapour Col), *4*. Rongé Island (George Point), *5*. Torgersen Island, *6*. Yalour Island, and *7*. Avian Island

a fixed factor. Case was also added as a random factor to control for over dispersion. We also analyzed the differences in ENAs and MN among the three species inhabiting on King George Island, because this was the only sampling locality where the three species live together. In these latter analyses, we used GLMs with a quasi-Poisson distribution to control for over dispersion (i.e., variance $\gg$ mean), which was presented as demonstrated by the fact that simple Poisson models showed that the residual deviance was substantially larger than the residual degrees of freedom (Crawley 2007). In order to test for differences among populations within species, we examined ENAs and MN in the three penguin species separately using GLMs, also with a quasi-Poisson distribution. In each analysis, we applied a type-III test, in which we compared a model without the independent variable of interest (e.g., species or population) against another without the dependent variable (with only the intercept fitted) by means of Wald test (Hardy and Field 1998; Agresti 2002). This comparison allows us to know whether there are differences across groups (i.e., species or populations). Finally, to compare species or populations differences, we used Tukey's tests for pairwise comparisons of group means. In order to test whether there were differences among years, we included year in a GLM in which ENA or MN number were de dependent variables. All analyses were performed using the R program (R Development Core Team 2010). For GLMM, we used the function "glmer" in package "lme4." For type-III tests, we used "Anova" within "car." For the Tukey's test, we used "glht" within "multcomp."

Results

When comparing the three species regardless of locality (i.e., locality included as a random factor), ENAs and MN were more frequent in Adélie penguins than in the other two species (Tables 1, 2). These results were confirmed in the analyses of King George Island, the only locality where all three species live together (Table 3, Fig. 3).

Within-species analyses of ENAs showed significant differences in Adélie and Chinstrap penguins but not in Gentoo penguins (Fig. 4). Frequency of ENAs in Adélie penguins was the highest in Yalour Island population, intermediate in King George Island population, and the lowest in Torgersen Island and Avian Island populations (Table 1). Frequency of ENAs in Yalour Island population was significantly different from that of Avian Island ($z = 2.906$, $p = 0.018$) populations, but was not significantly different from those of Torgersen Island ($z = 2.478$, $p = 0.063$) and King George Island population

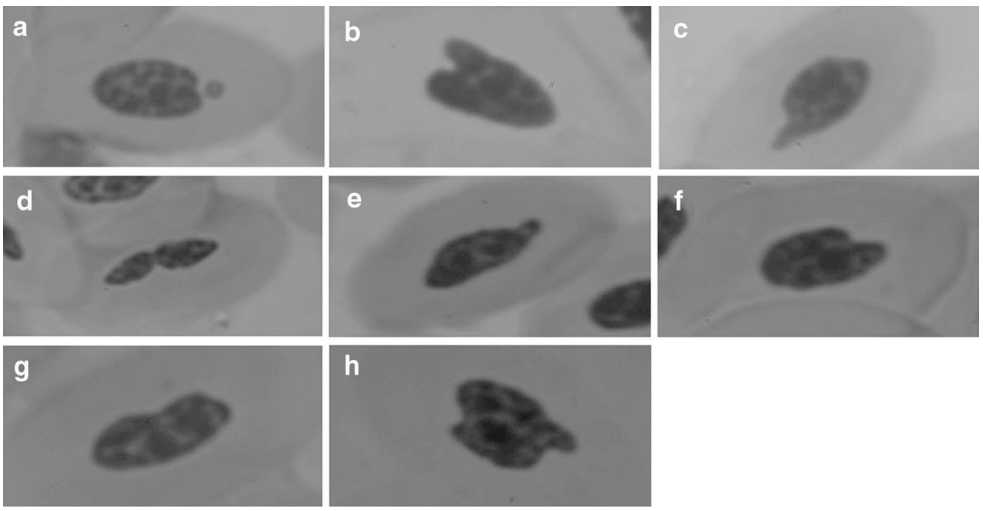

Fig. 2 Erythrocytic nuclear abnormalities observed in *Pygoscelid* penguins: **a** micronucleus, **b** lobed nucleus, **c** tailed nucleus, **d** two-lobed nucleus, **e** budding nucleus, **f** nucleus with cavity, **g** kidney-shaped nucleus, **h** unknown nuclear malformation

Table 2 Inter-specific comparisons of erythrocytic nuclear abnormalities and micronucleated erythrocytes using generalized linear mixed models (see the text for details)

	Erythrocytic nuclear abnormalities				Micronucleated erythrocytes			
	Estimate	SE	z value	p	Estimate	SE	z value	p
Chinstrap–Adélie	−1.232	0.256	−4.807	<0.0001	−2.213	0.528	−4.177	<0.001
Gentoo–Adélie	−1.535	0.276	−5.560	<0.0001	−1.514	0.493	−3.070	0.006
Chinstrap–Gentoo	−0.301	0.254	−1.190	0.459	0.698	0.473	1.477	0.301

Sample size $n = 10$ in all cases

Table 3 Inter-specific comparisons of erythrocytic nuclear abnormalities and micronucleated erythrocytes in King George Island using generalized linear models (see the text for details)

	Erythrocytic nuclear abnormalities				Micronucleated erythrocytes			
	Estimate	SE	t value	p	Estimate	SE	t value	p
Chinstrap–Adélie	−1.860	0.365	−5.098	<0.0001	−1.064	0.215	−4.956	<0.0001
Gentoo–Adélie	−1.800	0.355	−5.062	<0.0001	−0.595	0.182	−3.260	0.003
Chinstrap–Gentoo	0.060	0.473	0.128	0.990	0.470	0.236	1.990	0.113

Sample size $n = 10$ in all cases

($z = 1.416$, $p = 0.486$). Torgersen Island, Avian Island, and King George Island populations did not differ significantly in number of ENAs ($-1.159 \leq z \leq 1.531$, $p \geq 0.415$ in the three tests). In Chinstrap penguins, the highest number of ENAs was found on Deception Island (Table 1), and significant differences were found only between Deception Island and King George Island populations ($z = -2.517$, $p = 0.056$; in all other five comparisons, $-1.548 \leq z \leq 1.126$, $p \geq 0.376$). In Gentoo penguins, differences in the number of ENAs among populations were not statistically significant ($-0.878 \leq z \leq 0.795$, $p \geq 0.653$ in the three tests).

When the number of MN was examined in the three penguin species separately, the results were similar to those found for ENAs. In Adélie penguins, the number of MN was significantly higher in Yalour Island population than in Torgersen Island ($z = 2.677$, $p = 0.035$) populations, but was not significantly different from Avian Island population ($z = 1.329$, $p = 0.536$) and King George Island ($z = 2.249$, $p = 0.106$). Torgersen Island, Avian Island, and King George Island populations did not differ significantly in number of MN ($-1.714 \leq z \leq 1.329$, $p \geq 0.309$ in the three tests). In Chinstrap penguins, differences in the frequency of MN were only significant between Livingston Island and Deception Island populations ($z = -2.409$, $p = 0.053$; in all other five comparisons, $-1.742 \leq z \leq 1.524$, $p \geq 0.244$), whereas in Gentoo penguins, there were no significant differences in frequency of MN among the populations studied ($0.008 \leq z \leq 1.667$, $p \geq 0.182$ in the three tests). There was no effect of year either on ENA or on MN ($t = -0.439$, $p = 0.662$ for ENA and $t = -0.210$, $p = 0.834$ for MN).

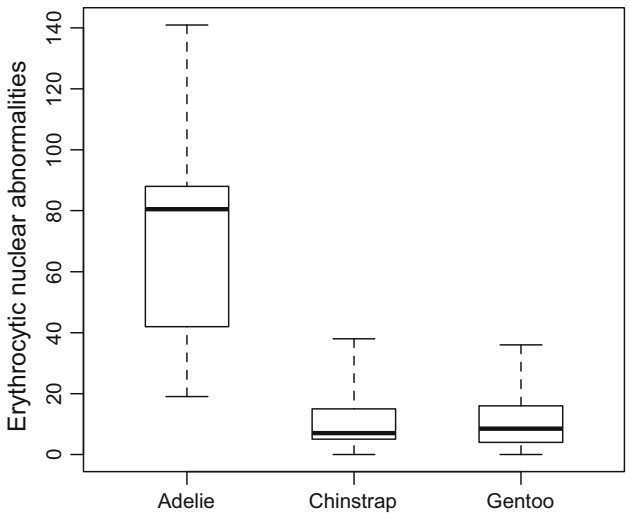

Fig. 3 *Box plot* of the number of erythrocytic nuclear abnormalities per 10,000 erythrocytes in the three *Pygoscelid* penguin species in King George Island ($n = 10$ for the three species). The *box* contains the 50 % of values. Median, minimum, and maximum values are indicated

Discussion

In this study, we found evidence of genomic damage in three species of *Pygoscelid* penguins in the same colonies where high levels of heavy metals such as Pb, Cr, Cu, or Ni (Jerez et al. 2011; Barbosa et al. 2013; Jerez et al. 2013a, b) and also persistent organic pollutants such as PCBs, PFCs, or phthalates (Jerez 2012) have been found.

Information about ENAs in wild birds is scarce. In Antarctica, three studies have reported erythrocytic malformations in birds, one in south polar skuas [0.7 MN per 10,000 erythrocytes (Kursa and Bezrukov 2007)] and two in Gentoo penguins [3.0 MN per 10,000 erythrocytes (Afanasieva et al. 2006), and 19.10 and 5.3 ENAs per 10,000 erythrocytes (Barbosa et al. 2013)]. Our results show that mean number of MN in *Pygoscelid* penguins of the Antarctic Peninsula ranged from 0 to 5.2 per 10,000 erythrocytes.

Our study found important differences in the frequency of erythrocytic abnormalities among the three penguin species studied, suggesting that factors contributing to them do not affect all species alike. The most robust results supporting different species sensitivity were found in King George Island, where the three species live and form mixed Adélie and Gentoo penguin colonies. In this locality, Adélie penguins showed the highest genetic instability, while the other two species had fewer ENAs. Therefore, the Adélie penguin seems to be the *Pygoscelid* species most affected by factors driving the appearance of ENAs.

Erythrocytic malformation reflects exposure to factors generating genomic damage during erythrocyte formation. Although we do not know the timing of this process in the studied species, erythrocyte formation in birds usually takes around 1 week. Therefore, our data may reflect genomic damage that occurred very shortly before sampling. However, considering that many pollutants are bioaccumulative, it is also possible that erythrocytic malformations were caused by exposure to pollution throughout the

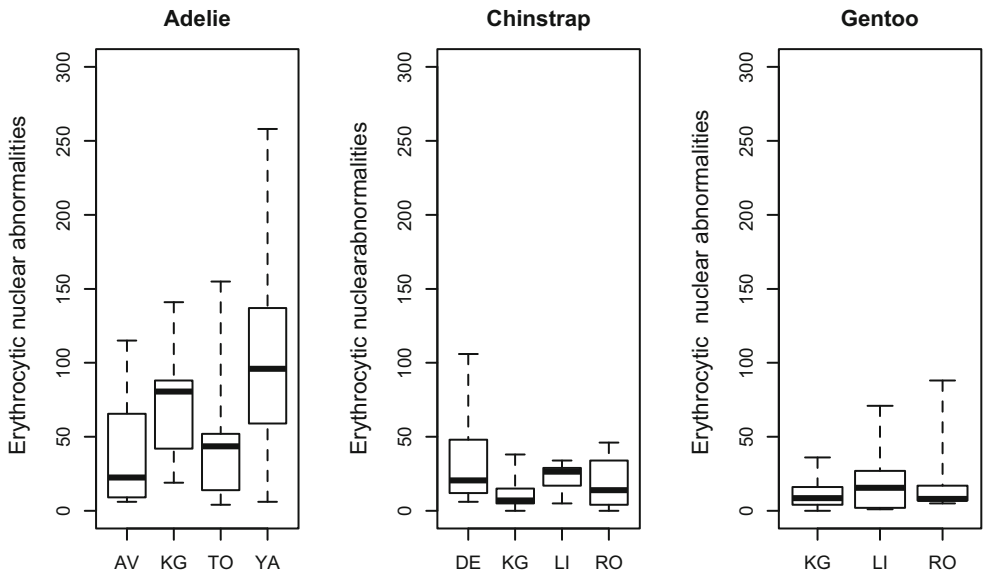

Fig. 4 *Box plots* of the number of erythrocytic nuclear abnormalities per 10,000 erythrocytes in each penguin species in different localities. *AV* Avian Island, *KG* King George Island, *TO* Torgersen Island, *YA* Yalour Island, *DE* Deception Island, *LI* Livingston Island, and *RO* Rongé Island. The *box* contains the 50 % of values. Median, minimum and maximum values are indicated

lifetime of the individual. If this is the case, the differences found in genotoxic effects between Adélie penguins and the other two species might be due to either a different diet or to different wintering areas.

As mentioned above, the other possible explanation for differences in number of ENAs among species is a species-specific sensitivity to genomic damage. Long-term differences in geographical distribution among the three penguin species could help understand the hypothetical differences in such sensitivity. Contrary to Adélie penguins, which are strictly Antarctic birds, the Gentoo and Chinstrap penguins have a predominantly sub-Antarctic distribution. Isolation of the Adélie penguin in one of the areas with the lowest human disturbance could have prevented the development of physiological defense mechanisms against environmental disturbances.

Our study also found significant inter-population differences in ENAs in the Adélie and Chinstrap penguins, but not in Gentoo penguins. In the Adélie penguin population on Yalour Island, the number of ENAs or MN was higher than on King George, Torgersen, and Avian Islands. Unfortunately, little information is available about the levels of contaminants in Yalour Island. Jerez et al. (2011) studied the presence of trace elements in the feathers of Adélie penguins breeding on this island and found Ni, Cu, Zn, and Se concentrations higher than or similar to those on penguins on King George Island, where human activity is intense (Tin et al. 2009) and contaminant levels are considered to be high [e.g., in aerosols (Artaxo et al. 1992) and penguins (Cipro et al. 2010; Jerez et al. 2011, 2013a, b)]. High concentration of trace elements in Yalour Island could be attributed to human activity, because this island is close to the Ukranian Antarctic Research Base Vernadski as well as to natural sources. Interestingly, our study did not show a large number of ENAs in Adélie penguins inhabiting Torgersen Island, which is very close to where a major oil spill (600,000 l of diesel fuel) occurred in 1989 when the ship Bahía Paraíso ran aground. Although the oil spill had a dramatic impact on seabirds living in Palmer Archipelago (Eppley and Rubega 1989; Eppley 1992) and hydrocarbon pollution was detected in fish and invertebrates (Kennicutt et al. 1992a, b) up to 2 years after the accident (Kennicutt and Sweet 1992), genomic damage in Adélie penguins 14 years later was low. Our results of low frequency of ENAs in the Adélie penguins of Avian Island are in agreement with the low level of heavy metals found in this population in comparison with the populations of Yalour and King George islands (Jerez et al. 2011).

Our study also found significant differences in the number of ENAs among Chinstrap penguin populations. Chinstrap penguins from Deception had more ENAs than Chinstrap penguins on Livingston, Ronge, and King George Islands. Deception Island show high levels of contaminants and trace elements due to human activity and volcanism (Deheyn et al. 2005; Guerra et al. 2011) In addition, higher concentrations of trace elements, such as Al, Mn and Fe, were found in Chinstrap penguin feathers from this island in comparison with penguins living in the other islands (Jerez et al. 2011). The number of ENAs did not differ significantly among the three Gentoo penguin populations despite the differences in heavy metals found in feathers of this species (Jerez et al. 2011). Afanasieva et al. (2006) reported in the heavily visited penguin rookery on Petermann Island (65°10′S 64°10′W) similar levels of ENAs (20.0 per 10,000 erythrocytes) to those reported here for Gentoo penguins. The Gentoo penguin rookery on Petermann Island is presumably highly polluted by human activity. All these results suggest that ENAs in Gentoo penguins might increase significantly only when a certain pollution threshold is reached, and small variations below that threshold would not affect the number of ENAs significantly. Alternatively, the pollution level of the sampled localities could be similar which produces similar level of erythrocytic abnormalities. This could be consistent with differences found in this species when localities with low pollution level are compared with localities with higher pollution levels (Barbosa et al. 2013).

Finally, ultraviolet (UV) radiation can also induce erythrocytic malformations (Muller et al. 1996), and consequently, our results might be influenced by this factor. Unfortunately, nothing is known about the direct effects of UV radiation on penguins (Muller et al. 1996). However, UV radiation does seem to increase from north to south in Antarctica (Barbosa et al. 2007b), and our results did not show any latitudinal trend in erythrocytic malformations. Therefore, it does not seem probable that UV radiation can explain ENA variation in the studied penguin populations.

As a summary, we have established the baseline data on ENAs as biomarkers of genomic damage in order to make long-term comparisons to assess the health of penguin populations. Considering the potential of penguins as environmental sentinels, these data could be used for monitoring the health of the Antarctic ecosystem. Future directions would include an assessment of contaminant levels and ENAs in individual penguins to examine potential relationships between contaminants and genotoxic damage.

Acknowledgments M.J.P. was supported by a Ph.D. grant from the Spanish Ministry of Science and Education (BES2005-8465). We thank the Spanish Antarctic base "Gabriel de Castilla," the Argentinean Antarctic base "Carlini" (ex Jubany), the UTM (Maritime Technology Unit, CSIC), the Spanish Polar ship Las Palmas and the Argentinean Antarctic Institute for their logistic support. Permission to work in the study area and for penguin handling was granted by the Spanish Polar Committee. This is a contribution to the International Polar Year project 172 BIRDHEALTH and to PINGUCLIM project. Deborah Fuldauer revised the language of the manuscript. We greatly

appreciate for the comments of three anonymous reviewers which improved an early version of the manuscript. This study was funded by the Spanish Ministry of Science and Innovation and the European Regional Development Fund under the Projects REN2001-5004/ANT, CGL2004-03148, POL2006-05175, CGL2007-60369 and CTM2011-24427 during writing.

Conflict of interest The authors declare that there are not conflicts of interest.

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