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ERYTHROCYTIC ABNORMALITIES IN THREE ANTARCTIC PENGUIN SPECIES ALONG THE ANTARCTIC PENINSULA: BIOMONITORING OF GENOMIC DAMAGE

Eva De Mas[a], Jesús Benzal[a], Santiago Merino[b], Francisco Valera[a], María José Palacios[a], José Javier Cuervo[a,b], Andrés Barbosa[a,b] *

[a] Departamento de Ecología Funcional y Evolutiva. Estación Experimental de Zonas Áridas, CSIC. Carretera de Sacramento s/n. La Cañada de San Urbano – 04120 Almería. Spain

[b] Departamento de Ecología Evolutiva. Museo Nacional de Ciencias Naturales, CSIC. C/José Gutiérrez Abascal, 2. 28006 Madrid. Spain.

* Corresponding author: Andrés Barbosa. E-mail: barbosa@mncn.csic.es. Phone number: +34914111328. Fax number: +34915645078

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-shape, 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 like fish (Cavas and Ergene-Gozucara 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 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

67 al. 2014) or persistent organic pollutants (POPs), such as organochlorine compounds
68 (Wania and Mackay 1993; Van den Brink 1997) arrive by long range atmospheric
69 transport and other transportation pathways. In addition, research, tourism, fishing and
70 other human activities in recent decades have contributed to pollution on the Antarctic
71 continent (Bargagli 2005). Substances such as polybrominated diphenyl ether (PBDE)
72 flame retardants, petroleum hydrocarbons, polychlorinated biphenyls (PCBs),
73 polychlorinated terphenyls (PCTs) and heavy metals are present around some Antarctic
74 research bases (Lenihan 1992; Crockett andWhite 2003).

75 In recent decades, many studies on toxic substances have shown significant
76 presence of noxious products in Antarctic wildlife, specifically in south polar skuas
77 (Tao et al. 2006; Kursa and Bezrukov 2007), penguins (Van den Brink 1997; Corsolini
78 et al. 2007; Geisz et al. 2008; Schiavone et al. 2009; Jerez et al. 2011; Barbosa et al.
79 2013; Jerez et al. 2013a, b), albatrosses (Tao et al. 2006), petrels (Van den Brink 1997),
80 seals (Tao et al. 2006; Schiavone et al. 2009), whales (Krahn et al. 2008), fishes (Van
81 Ngan et al. 2007), krill (Corsolini et al. 2006; Jerez et al. 2013a, b), lichens and mosses
82 (Yogui and Sericano 2008).

83 Seabirds are good sentinel species of environmental contamination mainly
84 because of their high position in the trophic web (Walker 1990; Van den Brink 1997)
85 which can contribute to greater biomagnification of pollutants compared to animals in
86 lower trophic levels (Van den Brink 1997; Burger and Gochfeldt 2004). Penguins are
87 the most abundant birds in the Antarctic region and may be then considered sentinels of
88 the Antarctic ecosystem (Boersma 2008). Penguins are at high risk for the effects of
89 exposure to toxic substances and they can accumulate and biomagnify toxic chemicals
90 in tissues (Jerez et al. 2013a, b). Moreover, penguins, like other marine vertebrates in
91 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; Corsolini et al. 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 like 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 lab 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 and 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 96º. 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 (100x objective) per 10000 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 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>>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 tests for pairwise comparisons of group means. In order to test if 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 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 and 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 ($z = 1.416$, $p = 0.486$). 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).

192 # Discussion

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

198 Information about ENAs in wild birds is scarce. In Antarctica, three studies
199 have reported erythrocytic malformations in birds, one in south polar skuas (0.7 MN per
200 10000 erythrocytes (Kursa and Bezrukov 2007)) and two in Gentoo penguins (3.0 MN
201 per 10000 erythrocytes (Afanasieva et al. 2006), and 19.10 and 5.3 ENAs per 10000
202 erythrocytes (Barbosa et al. 2013)). Our results show that mean number of MN in
203 *Pygoscelid* penguins of the Antarctic Peninsula ranged from 0 to 5.2 per 10000
204 erythrocytes.

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

213 Erythrocytic malformation reflects exposure to factors generating genomic
214 damage during erythrocyte formation. Although we do not know the timing of this
215 process in the studied species, erythrocyte formation in birds usually takes around one
216 week. Therefore, our data may reflect genomic damage that occurred very shortly

217 before sampling. However, considering that many pollutants are bioaccumulative, it is
218 also possible that erythrocytic malformations were caused by exposure to pollution
219 throughout the lifetime of the individual. If this is the case, the differences found in
220 genotoxic effects between Adélie penguins and the other two species might be due to
221 either a different diet or to different wintering areas.

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

230 Our study also found significant inter-population differences in ENAs in the
231 Adélie and Chinstrap penguins, but not in Gentoo penguins. In the Adélie penguin
232 population on Yalour Island, the number of ENAs or MN was higher than on King
233 George, Torgersen and Avian Islands. Unfortunately, little information is available
234 about the levels of contaminants in Yalour Island. Jerez et al. (2011) studied the
235 presence of trace elements in the feathers of Adélie penguins breeding on this island and
236 found Ni, Cu, Zn and Se concentrations higher than or similar to those on King George
237 Island, where human activity is intense (Tin et al. 2009) and contaminant levels are
238 considered to be high (e.g. in aerosols (Artaxo et al. 1992) and penguins (Cipro et al.
239 2010, Jerez et al. 2011; 2013a, b)). High concentration of trace elements in Yalour
240 Island could be attributed to human activity, because this island is close to the Ukranian
241 Antarctic Research Base Vernadski as well as to natural sources. Interestingly, our study

242 did not show a large number of ENAs in Adélie penguins inhabiting Torgersen Island,
243 which is very close to where a major oil spill (600000 l of diesel fuel) occurred in 1989
244 when the ship Bahía Paraíso ran aground. Although the oil spill had a dramatic impact
245 on seabirds living in Palmer Archipelago (Eppley and Rubega 1989; Eppley 1992) and
246 hydrocarbon pollution was detected in fish and invertebrates (Kennicutt et al. 1992a, b)
247 up to two years after the accident (Kennicutt and Sweet 1992), genomic damage in
248 Adélie penguins 14 years later was low. Our results of low frequency of ENAs in the
249 Adélie penguins of Avian Island are in agreement with the low level of heavy metals
250 found in this population in comparison with the populations of Yalour and King George
251 islands (Jerez et al. 2011).

252 Our study also found significant differences in the number of ENAs among
253 Chinstrap penguin populations. Chinstrap penguins from Deception had more ENAs
254 than Chinstrap penguins on Livingston, Ronge and King George Islands. Deception
255 Island show high levels of contaminants and trace elements due to human activity and
256 volcanism (Deheyn et al. 2005; Guerra et al. 2011) In addition, higher concentrations of
257 trace elements, such as Al, Mn and Fe, were found in Chinstrap penguin feathers from
258 this island in comparison with penguins living in the other islands (Jerez et al. 2011).
259 The number of ENAs did not differ significantly among the three Gentoo penguin
260 populations despite the differences in heavy metals found in feathers of this species
261 (Jerez et al. 2011). Afanasieva et al. (2006) reported in the heavily visited penguin
262 rookery on Petermann Island (65º10'S 64º10'W) similar levels of ENAs (20.0 per
263 10000 erythrocytes) to those reported here for Gentoo penguins. The Gentoo penguin
264 rookery on Petermann Island is presumably highly polluted by human activity. All these
265 results suggest that ENAs in Gentoo penguins might increase significantly only when a
266 certain pollution threshold is reached, and small variations below that threshold would

267 not affect the number of ENAs significantly. Alternatively, the pollution level of the
268 sampled localities could be similar which produces similar level of erythrocytic
269 abnormalities. This could be consistent with differences found in this species when
270 localities with low pollution level are compared with localities with higher pollution
271 levels (Barbosa et al. 2013).

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

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

The authors declare that there are not conflicts of interest.

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455 Table 1. Mean (±SD) number of erythrocytic nuclear abnormalities (ENA) and
456 micronucleus (MN) per 10000 erythrocytes in each penguin species and locality.

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Locality	Latitude/Longitude	Species	Sample size	ENA	MN
King George I.	62º15'S 58º37'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.	62º39'S 60º36'W	*Pygoscelis antarctica*	10	23.1 ± 9.3	0.1 ± 0.3
		Pygoscelis papua	10	19.3 ± 20.7	0
Deception I.	63º00'S 60º40'W	*Pygoscelis antarctica*	10	33.1 ± 31.2	1.5 ± 1.9
Rongé I.	64º40'S 62º40'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.	64º53´S 62º53´W	*Pygoscelis adeliae*	10	46.9 ± 43.5	1.3 ± 1.5
Yalour I.	65º15'S 64º11'W	*Pygoscelis adeliae*	10	109.9 ± 80.0	5.2 ± 4.1
Avian I.	67º46´S 68º64´W	*Pygoscelis adeliae*	10	41.2 ± 40.1	3.25 ± 3.7

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463 Table 2. Inter-specific comparisons of erythrocytic nuclear abnormalities and
464 micronucleated erythrocytes using Generalized Linear Mixed Models (see the text for
465 details). Sample size n = 10 in all cases.

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	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

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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). Sample size n = 10 in all cases.

	Erythrocytic nuclear abnormalities				Micronucleated erythrocytes			
	Estimate	SE	t-value	p	Estimate	Std.Error	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

Figure legends

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, 7. Avian Island.

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.

Fig. 3. Boxplot of the number of erythrocytic nuclear abnormalities per 10000 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.

Fig. 4. Boxplots of the number of erythrocytic nuclear abnormalities per 10000 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, RO = Rongé Island. The box contains the 50% of values. Median, minimum and maximum values are indicated.

Figure 1

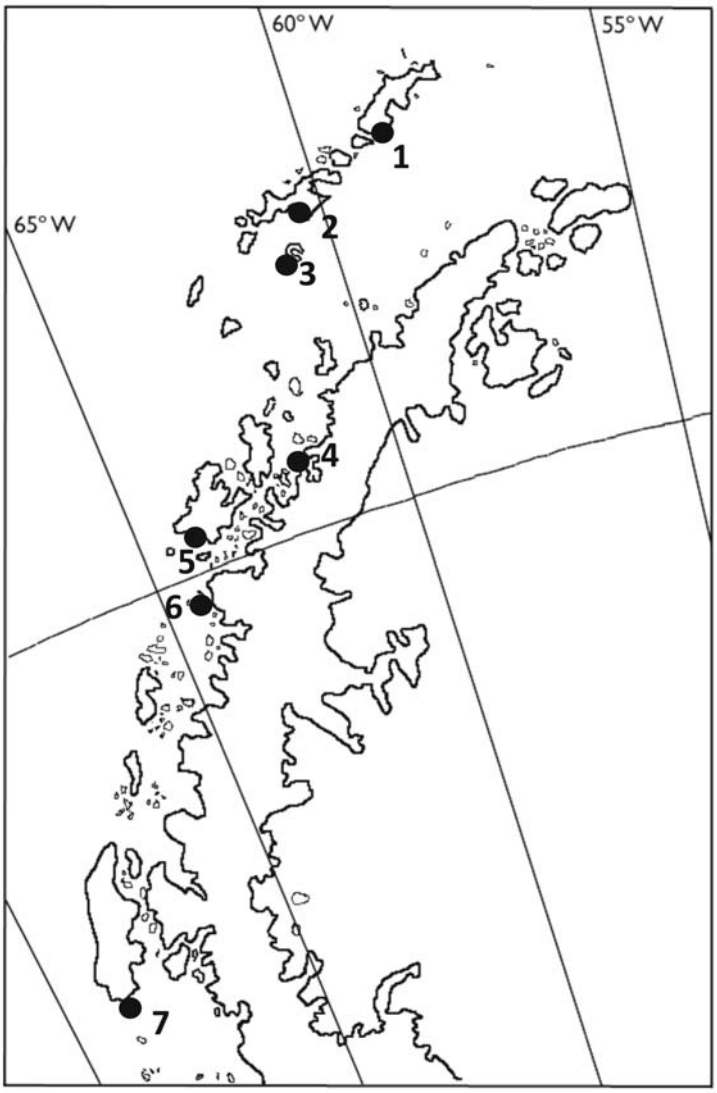


Figure 2

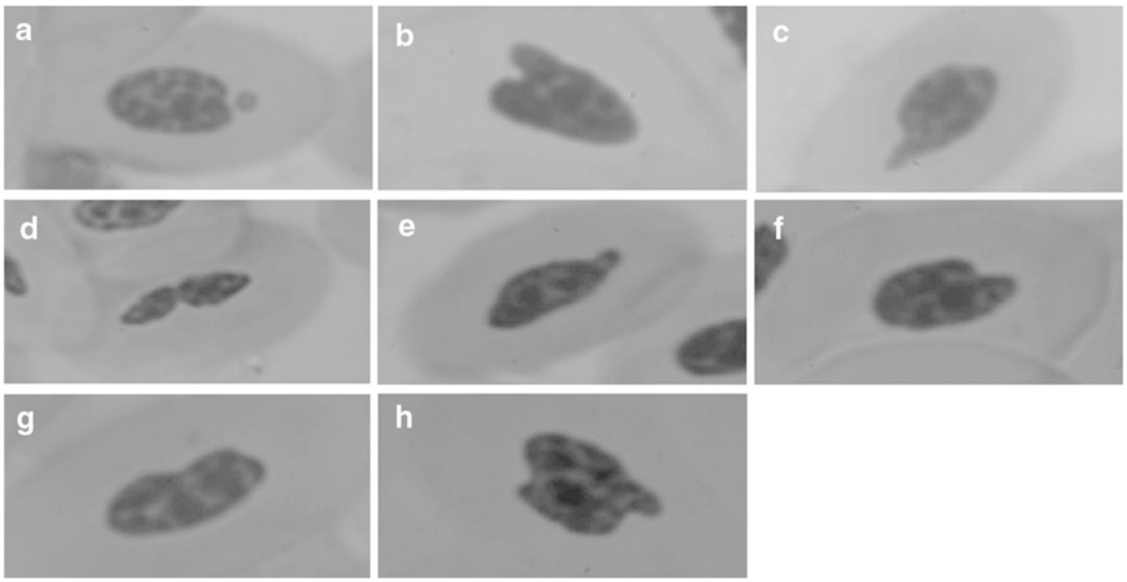
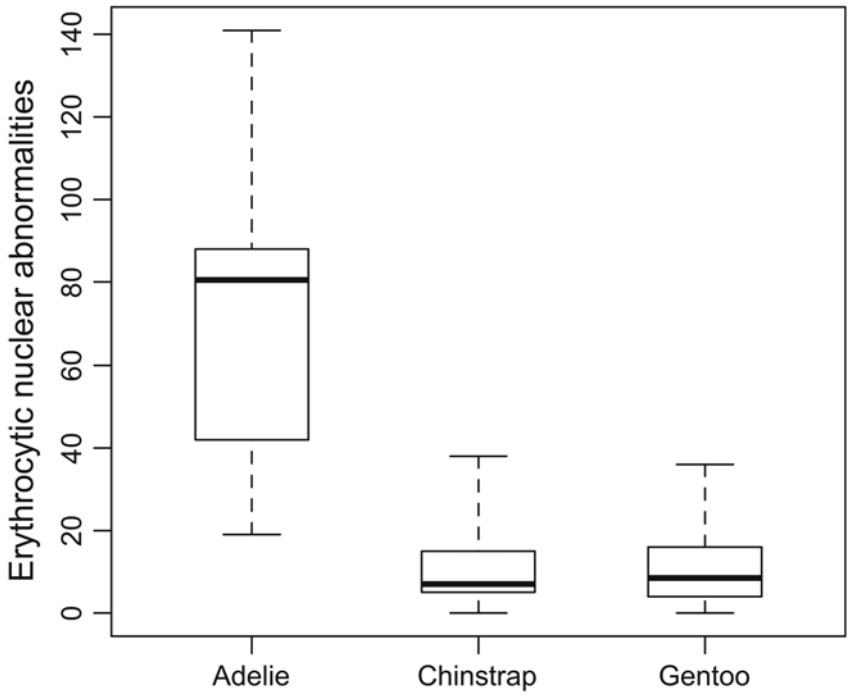


Figure 3


Erythrocytic nuclear abnormalities
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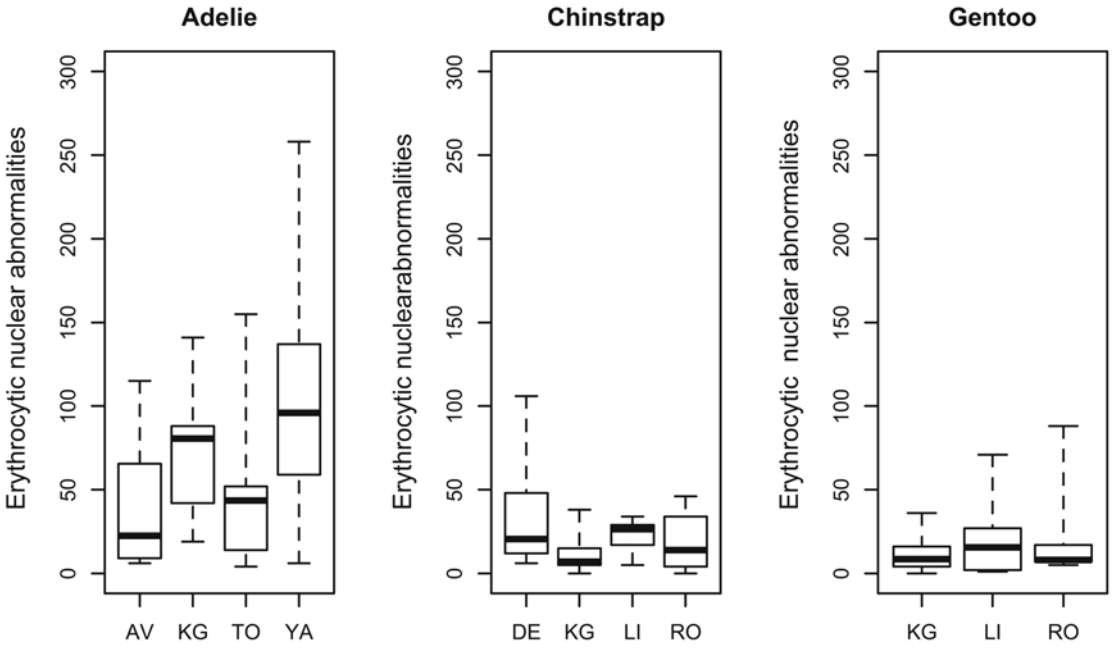
Adelie
Chinstrap
Gentoo

Figure 4


Adelie
Chinstrap
Gentoo
Erythrocytic nuclear abnormalities
Erythrocytic nuclearabnormalities
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