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# Erythrocytes nuclear abnormalities and leukocyte profile of the immune system of Adelie penguins (*Pygoscelis adeliae*) breeding at Edmonson Point, Ross Sea, Antarctica

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1 **Erythrocytes nuclear abnormalities and leukocyte profile of the**  
2 **immune system of Adélie penguins (*Pygoscelis adeliae*) breeding at**  
3 **Edmonson Point, Ross Sea, Antarctica**

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5 Silvia Olmastroni<sup>1,2</sup>, Giulia Pompeo<sup>1</sup>, Awadhesh N. Jha<sup>3</sup>, Emiliano Mori<sup>4</sup>, Maria Luisa Vannuccini<sup>1</sup>, Niccolò  
6 Fattorini<sup>4</sup>, Nicoletta Ademollo<sup>5</sup> and Iliaria Corsi<sup>1</sup>

7

8 <sup>1</sup> Dipartimento di Scienze Fisiche, della Terra e dell’Ambiente, Università di Siena, Via Mattioli 4 53100  
9 Siena, Italia

10 <sup>2</sup> Museo Nazionale dell’Antartide “Felice Ippolito” Via del Laterino 8 53100 Siena, Italia

11 <sup>3</sup> School of Biological and Marine Sciences, University of Plymouth, Plymouth, PL4 8AA, UK,

12 <sup>4</sup> Dipartimento di Scienze della Vita, Università di Siena, Via Mattioli 4, 53100 Siena, Italia

13 <sup>5</sup> Istituto di Ricerca sulle Acque, Consiglio Nazionale delle Ricerche (IRSA-CNR), Via della Mornera, 25,  
14 20047 Brugherio, Italia

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24 **Corresponding author:** [silvia.olmastroni@unisi.it](mailto:silvia.olmastroni@unisi.it); tel. +39 0577 233775; [ORCID 0000-0002-9319-9914](https://orcid.org/0000-0002-9319-9914)

25

26 **Abstract**

27 Antarctic seabirds well adapted to extreme environments often deal during their life cycle with sub-optimal  
28 conditions and occasionally with severe environmental stress. Climate changes, pollution, habitat loss,  
29 increasing human presence can all significantly affect organism's health status from molecular to individual  
30 up to population level. In the present study, erythrocytes nuclear abnormalities (ENAs) and white blood cells  
31 (WBC) differential were investigated in 19 adults of Adélie penguin (*Pygoscelis adeliae*) breeding at  
32 Edmonson Point, Antarctic Specially Protected Area (ASPA n. 165) in the Ross Sea. Micronuclei (MN)  
33 accounted for 10.50% of observed abnormalities in penguin erythrocytes while kidney-shaped nucleus  
34 (KSN) was the most abundant (20.88%). Heterophils (HE) were the most common WBC (36.93%) in  
35 agreement with the generic avian leukocytes profile while eosinophils (EO) were the lowest (7.45%). A low  
36 number of lymphocytes were detected resulting in a higher heterophils to lymphocytes ratio. ENAs and H:L  
37 ratio are confirmed as reliable indexes of penguin's health status since they reflect their individual adaptation  
38 during breeding season. These baseline data will be useful for future studies as indicators of penguin's health  
39 status mainly as response to environmental changes.

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45 **Keywords:** Adélie penguin, Antarctica, genotoxic damage, immune response, Ross Sea

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## 47 **Introduction**

48 Organisms rarely experience optimal state in their natural habitat but for the most of their life they deal with  
49 variable conditions and occasionally with severe environmental stress. A variety of intrinsic factors can  
50 influence organism's physiological stress response such as reproductive status, age, sex, developmental  
51 or/and recent experiences. Whatever the source, physiological stress is a relevant parameter to consider when  
52 assessing animal welfare in both captive and wild populations (Davis et al. 2008).

53 Blood cells counts and classification, in particular erythrocytes' nuclear abnormalities (ENAs) and white  
54 blood cells (WBC), are considered efficient tools for assessing genomic instability and immune status in  
55 wildlife (Kursa and Bezrukov 2008). Although the mechanisms of the formation of ENAs are still little  
56 investigated in birds (Clark 2015), Van Ngan et al. (2007) promote the use of ENAs count for detecting  
57 genomic damage caused by prolonged exposure to several physico-chemical stressors during organism's  
58 lifespan. According to Kursa and Bezrukov (2008), occurrence of both micronucleus (MN) and in general  
59 nuclear abnormalities (NA) should be considered useful tools for assessing genome instability also in  
60 Antarctic birds since they represent a cellular reaction to natural and environmental stressors. MN occurrence  
61 may document what happen during erythrocytes' lifetime thus reflecting possible chronic effects.  
62 Furthermore, the white blood cell count (WBC) reflects animal's immune status and response to stressful  
63 conditions. The use of blood smears for detection of ENAs and WBC have several advantages such as low  
64 amount of blood needed with consequent low impact on animal health and a quick sampling procedure  
65 which can be readily used also in extreme environmental conditions as for instance with polar birds (Dantzer  
66 et al. 2014). Heterophils/ lymphocytes ratio (H:L) is considered a suitable indicator of organism's stress  
67 associated to reproductive cycle, seasonal changes, injury and also to pathogens and parasites (Dufva and  
68 Allander 1995; Krams et al 2012). Moreover, it reflects food and water deprivation, extremes temperature,  
69 constant light, long-distance migration and social disruption too. All these stressors result in an increased  
70 level of heterophils (innate immune system), decreased number of lymphocytes (acquired immune system)  
71 and a high H:L ratio (Vleck et al, 2000).

72 Commonly, birds exhibit low level of spontaneous blood cell anomalies such MN, therefore it might be  
73 rather easy to detect any alteration due genotoxicants exposure or other environmental stressors (Zúñiga-  
74 González et al. 2000, 2001).

75 Antarctic seabirds feed over wide geographical areas at different trophic level and therefore they are studied  
76 to monitor health conditions across large aquatic ecosystems and at different trophic levels. In turn they are  
77 able to reflect both natural and anthropogenic stressors (Mallory et al. 2010). Their health and physiological  
78 tolerance to stressors is closely influenced by their adaptation capability necessary to survive in their natural  
79 environment.

80 ENAs and immune status have been investigated in seabirds and in pygoscelid species breeding in the Sub-  
81 Antarctic and Antarctic Peninsula (Vleck et al. 2000; Kursa and Bezrukov 2008; D'Amico et al. 2014; De  
82 Mas et al. 2015; Barbosa 2013). ENA and immune status have been linked to contaminants exposure  
83 augmenting stress on penguin populations (D'Amico et al. 2014; Colominas-Ciuró et al. 2017) but also to

84 different stages of the breeding cycle, sex and individual condition and activities (Vleck et al. 2000; Moreno  
85 et al. 1998).

86 It is well known that climate changes are affecting the bioavailability of toxic contaminants in the wildlife  
87 altering the toxicokinetics due to an increase in temperature and salinity and leading to changes in organism'  
88 homeostasis and other physiological defence mechanisms (Noyes et al. 2009). Thus, during penguin's  
89 lifetime, contaminants exposure may vary according to the ecosystem changes. Different diets and foraging  
90 areas have been recognized also as major drivers for genome instability of penguin species from Antarctic  
91 Peninsula (De Mas et al. 2015).

92 In the background of above information, the present study investigates for the first time the occurrence of  
93 ENAs and WBCs in blood cells of an Adélie penguin (*Pygoscelis adeliae*, Hombron and Jacquinot 1841)  
94 population breeding at Edmonson Point, an Antarctic Specially Protected Area (ASPA n. 165) localized in  
95 the Ross Sea. The Adélie penguin is considered a keystone species of the Antarctic environment and  
96 currently most affected by environmental changes such as sea ice extent anomalies in different Antarctic  
97 regions (Ainley 2002; Olmastroni et al. 2004; Emmerson and Southwell 2008; Ropert-Coudert et al. 2013;  
98 Ducklow et al. 2013; Ballerini et al. 2009, 2015; Cimino et al. 2016).

99 In comparison with other Antarctic territories as for instance Antarctic Peninsula, the Ross Sea is still  
100 considered a pristine area (Halpern, 2008) and recently partially included in a Marine Protected Area (SC-  
101 CAMLR, 2016) to be preserved from increasing human activities. On the other hand, human pressure has  
102 increased significantly in the last twenty years mainly due to increase in fishing, tourism and number of  
103 scientific bases (De Mas et al. 2015; Tin et al. 2009). Scientific research communities strongly required  
104 protection for Antarctica from which the designation of the Ross Sea' MPA with the aim to preserve the  
105 marine ecosystem and biodiversity, as well as to limit and regulate current and future human impact. The  
106 Ross Sea is the home of 38% of the global population of Adélie penguin, therefore it is mandatory to address  
107 the current health status of population living in this territory in order to prove the efficacy of the MPA and to  
108 monitor any potential impact in the future. While ecology of Adélie penguin breeding at the Edmonson Point  
109 colony has been the focus of studies in the last 20 years (Olmastroni et al 2001, 2004; Pezzo et al 2007;  
110 Ballerini et al. 2009, 2015), genome and immune stability have not been investigated so far. This issue  
111 inspired our study on the occurrence of ENA and leukocyte profile of the immune system, with the aim to  
112 provide a baseline of health status of penguin living in the area.

113

## 114 **Materials and Methods**

### 115 **Study area and samples collection**

116 The penguin colony is located at Edmonson Point (74 ° 20' S, 165° 08' E), Ross Sea, an ice-free area of  
117 about 6 km<sup>2</sup> along the Eastern slopes of Mt. Melbourne and c. 50 Km NW from Mario Zucchelli Italian  
118 Research Station (Fig. 1). The area has been occupied by Adélie penguin (*Pygoscelis adeliae*) from almost  
119 3000 years BP (Baroni and Orombelli 1994) and breeding population size consisted of 3066 pairs in 2014/15  
120 summer season. Since 1994 Edmonson Point is a monitoring site to carry out scientific research on the

121 Adélie penguin's ecology and to collect data for the Ecosystem Monitoring Program (CEMP) lead by  
122 CCAMLR Commission for the Conservation of Antarctic Marine Living Resources.

123 All data were collected during 2014-15 austral summer, following protocols approved by SCAR (SCAR's  
124 Code of Conduct for the Use of Animals for Scientific Purposes in Antarctica, 2011) and under permission  
125 from PNRA for working in an ASPA.

126 All blood and feather samples (19) were collected from adult penguins at the beginning of the breeding  
127 season (incubation period) when mostly males occupy colony. Blood was collected from apparently healthy  
128 penguins (i.e. not showing any sign of illness or injuries). No ectoparasites, feather or skin changes or  
129 emaciation were observed.

130 In order to reduce stress induced by capturing and handling each bird was restrained for the minimum time  
131 necessary (max 5 minutes) to carry out blood and feathers sampling and to record biometrics (Vleck et al.  
132 2000). After sampling each bird was then released in front of its nest and observed until it returned to regular  
133 breeding activity. Blood samples (one drop) were collected by venipuncture of the brachial vein using a  
134 heparinized syringe with sterilized needle (22 gauge) according to Owen (2011). Up to five feathers per  
135 individual were sampled from the chest area. Feathers were conserved in sealed plastic bags at -20°C.  
136 Penguins were weighted with a Salter scale to the nearest 50 g, and bill depth and bill length measured using  
137 a calliper. Blood smears were prepared in the field immediately after collection using a drop of blood on a  
138 clean slide (15 min 10% HCl and rinsed with MilliQ water and oven-drying at 100°C). Slides were then  
139 stored at +4°C in a slides' box.

140

#### 141 **Genome and immune analysis**

142 Slides were processed at the University of Plymouth Ecotoxicology Lab for the analysis of genome  
143 instability. The following procedure was used: slides were fixed using (100% v/v) cold methanol for 30 min,  
144 stained with 10% Giemsa stain modified solution (Giemsa buffer tablets, pH 6.4 – BDH), and DPX  
145 Mounting Media (Leica Biosystems). They were then observed under a light microscope equipped with  
146 Digital Microscope Leica DMD108 Digital Microimaging Device with 40x objective. The images were  
147 acquired, stored and processed by using LAS program (Leica Application Suite).

148 Areas with a clear distribution of erythrocytes were identified for each slide as a well-defined and separate  
149 cytoplasm. Areas which presented overlapping cells were not taken into consideration.

150 Cell counting was carried out by taking the reference coordinates x, y and progressively moving from the left  
151 to the right margin. Upon selecting the best images per slide, 1,000 erythrocytes were counted for each slide  
152 according to Clark (2015) and the number of leukocytes and thrombocytes localized between them recorded.  
153 A total amount of 19,000 cells was analysed.

154 In order to increase the identification of all known abnormalities in the nucleus of the erythrocytes of avian  
155 species, in the present study blood cells of penguins were analysed according to the established method of  
156 Kursa and Bezrukov (2008) already used for pygoscelid species by D'Amico et al (2014) and De Mas et al.  
157 (2015). Erythrocytes nuclear abnormalities were determined as follows: (a) micronucleus, (b) lobed nucleus,

158 (c) tailed nucleus, (d) two-lobed nucleus, (e) budding nucleus, (f) nucleus with cavity, (g) kidney-shaped  
159 nucleus, (h) unknown nuclear malformation. Their sum as ENAs was also calculated.

160 White blood cells were classified along the five types of leukocyte according to Samour (2006): (a)  
161 heterophils, (b) lymphocytes, (c) monocytes, (d) basophils, (e) eosinophils were identified based on  
162 morphologic and staining characteristics according to the Table 22.10 reported in the chapter by Samour  
163 (2006). Erythrocytes and WBC were counted using ImageJ 1.6.0\_24 (NIH, USA).

#### 164 **Sex determination**

165 Sex of penguins (12 males and 7 females) was determined by molecular analysis on feathers except for one  
166 individual in which blood was used. DNA was extracted using the PureLink™ DNA Mini Kit (Invitrogen, by  
167 Thermo Fisher Scientific), following the manufacturer's instructions. The reliability of DNA extraction was  
168 monitored through a negative control (no tissue added), and the DNA content determined through an  
169 Eppendorf Ultraviolet Spectrophotometer (AG Eppendorf). The chromo-helicase-DNA-binding-1 gene  
170 (*CHDI*), found on sex chromosomes, was amplified which length varies among male (sex-chromosomes:  
171 ZZ) and female (ZW) penguins (Zhang et al. 2013). The following specific primers for penguins were used:  
172 PL (5'-CCC AAG GAT GAT AAA TTG TGC-3') and PR (5'-CAC TTC CAT TAA AGC TGA TCT GG-  
173 3'). PCR was run through a 2720 Thermal Cycler (Applied Biosystems), following this profile: 3 min 94°C,  
174 30 cycles of 35" at 94°C, 45" at 55°C and 3' at 72°C, followed by 7 min at 72°C. PCR reactions were  
175 prepared with 0.5 µL of Taq Polymerase, 1 µL of each primer, 6 µL of PCR Master Mix (with PCR buffer,  
176 MgCl<sub>2</sub> and dNTPs: Genaid Biotech Ltd.) and about 20 ng of each DNA template. The electrophoresis was  
177 run for 45' on a 3% agarose gel (Zhang et al. 2013).

#### 178 **Statistical analyses**

179 Descriptive statistical analyses including average, standard error (SE), range (minimum-maximum) of blood  
180 smear parameters were carried out with R Studio software (Version 0.99.902 – © 2009-2016 RStudio, Inc).  
181 Differences between sexes were determined through the nonparametric *Monte Carlo* exact permutation test  
182 for the equality of means, which computed all the possible permutations and uses the absolute difference in  
183 means as test statistic (Anderson 2001). The *Monte Carlo* exact permutation test assumes that the two  
184 samples are equal in distribution if the null hypothesis is true (Anderson 2001). Thus, we checked that each  
185 variable, both for male and female, followed the same distribution through a *Kolmogorov-Smirnov* test for  
186 equal distributions if the null hypothesis was true. Significance level was set at  $\alpha = 0.05$ . Analyses were  
187 performed through the software *Past* (Hammer et al. 2001).

188

#### 189 **Results**

190 Erythrocytes nuclear abnormalities in the penguin's blood smears from Edmonson point colony are listed in  
191 Table 1. ENAs was found in 4.31 % over 19,000 mature erythrocytes analysed. Mean values of ENA varies  
192 from the lowest number of TLN ( $1.74 \pm 0.40$ ), which account for 4.03% of total ENAs to the highest of KSN  
193 ( $9.0 \pm 1.14$ ) accounting for 20.88% (Table 1).

194 Mean values of MN ( $4.53 \pm 0.52$ ) resulted similar to that found for lobed nucleus (LN) ( $4.79 \pm 1.64$ ) and  
195 budding nucleus (BN) ( $4.79 \pm 0.95$ ) and account for 10.5% of total ENAs (LN and BN 11.11 %  
196 respectively).

197 Therefore, the most recurring ENA are KSN, NWC and TN, followed by LN, MN and BN. Amongst those,  
198 MN is the lowest abnormality occurring with  $\leq 5$  over 1,000 mature erythrocytes while NWC, TN, LN e  
199 KSN exhibited higher variability. In addition, a small percentage showed unknown nuclear malformation  
200 (UNM) (Table 1). The figure 2 shows all ENAs detected in Adélie penguin's blood smears classified  
201 according to Kursa and Bezrukov (2008) and De Mas (2015).

202 Table 2 summarizes WBC identified in Adélie penguin's blood samples. White blood cells were 658 over  
203 19,000 cells scored in penguin's blood smears. Heterophils (HE) were the most common WBC, followed by  
204 lymphocytes (LY), basophils (BA), monocytes (MO) and eosinophils EO (Table 2 and shown in Fig. 2).  
205 Although not significant, toxic HE (THE) resulted higher than normal HE (NHE) in the total HE found (243  
206 over 19,000 erythrocytes scored). LY resulted lower than total HE (23.70% compared to 36.90%) while BA  
207 were 19.45% of the total WBC (Table 2 and Fig. 3).

208 Total MO resulted 12.46% of the total leukocytes over 19,000 cells scored. Mean TMO numbers resulted  
209 higher than NMO even though not significantly different. The lowest WBC (Fig. 3) detected were EO  
210 (7.45%). Heterophil: Lymphocyte ratio (H:L) was calculated and the mean value was  $3.08 \pm 0.87$ .

211 The number of HE (*Monte Carlo* exact permutation test:  $p = 0.016$ ) and the number of NHE (*Monte Carlo*  
212 exact permutation test:  $p = 0.012$ ) were approximately four times greater in males ( $n = 12$ ) than in females ( $n$   
213 = 7) (Fig. 4), the rest of parameters analysed showed not gender differences.

214



215 Discussion

216 The present study investigates for the first time the occurrence of ENAs and WBCs in blood cells of Adélie  
217 penguin (*Pygoscelis adeliae*, Hombron and Jacquinot 1841) breeding at Edmonson Point, an Antarctic  
218 Specially Protected Area (ASPA n. 165) localized in the Ross Sea.

219 The most frequent ENAs described for bird populations including penguins have been observed in blood  
220 smears of Adélie from Edmonson Point (Kursa and Bezrukov 2008 and De Mas 2015); in particular  
221 peculiar nuclear anomalies as TN, KSN and TL were observed (Lucas and Jamroz, 1961) as well as BN and  
222 LN which are considered in interphase as precursors of MN formation and associated to cell death, genomic  
223 instability, or cancer development (Webster et al. 2009).

224 MN frequency is also in the range of natural values reported for birds (from 0.40 to 4.30 over 1000  
225 erythrocytes scored) (Zúñiga-González et al. 2001), thus suggesting a low genome instability of individual  
226 nesting at the Edmonson Point colony.

227 The analysis of immune parameters also reveals that total number of WBC are within the normal range  
228 reported for birds (Kursa and Bezrukov 2008). By comparing ENAs and H:L ratio observed in Adélie from  
229 Edmonson Point with those documented in penguins breeding in Antarctic Peninsula (i.e. more  
230 anthropogenically impacted: Tin et al. 2009; SCAR 2010), some considerations can be made.

231 ENA values result similar to those reported by De Mas et al. (2015) in Adélie penguin from Torgensen and  
232 Avian Islands ( $43.11 \pm 27.59$ ;  $46.90 \pm 46.50$ ;  $41.20 \pm 40.10$  respectively) whereas those of penguins from  
233 Yalour and King George Island result far higher ( $109.90 \pm 80$  and  $72 \pm 35.3$ ). Lower values are on the  
234 contrary reported by D'Amico et al. (2014) in penguins from Potter Peninsula at Stranger Point ( $26.20 \pm$   
235  $3.20$ ) scoring 40,000 mature erythrocytes out of 20 individuals.

236 MN values ( $4.53 \pm 0.52$ ) are similar to the range reported in penguins breeding in colonies located in the  
237 Antarctic Peninsula (Yalour Island,  $5.2 \pm 4.1$ ; Avian Island,  $3.25 \pm 3.7$ ) but higher than those recorded in  
238 penguins from Torgensen Island ( $1.3 \pm 1.5$ ) and King George ( $1.9 \pm 1.4$ ) (De Mas et al. 2015).

239 Interspecific comparison among *Pygoscelis* genus, shows lower MN values in individuals of *Pygoscelis*  
240 *papua* (Gentoo penguin) and *Pygoscelis antarcticus* (Chinstrap penguin) (De Mas et al. 2015 and reference  
241 within) compared to Adélie from Edmonson Point (this study).

242 Several ecological and environmental factors, such as species-specific sensitivity, diet, wintering areas and  
243 exposure to toxic pollutants, could affect penguin's genome and immune stability (Bargagli 2005; Barbosa et  
244 al. 2013; De Mas et al. 2015). According to De Mas et al. (2015), a different sensitivity to environmental  
245 disturbance of Gentoo and Chinstrap penguins compared to the strictly sea ice dependent Adélie penguin,  
246 might have resulted in the development of a physiological defence mechanism able to cope better with  
247 genotoxic agents. In addition, it has been hypothesized that some of the observed differences among species  
248 could be related to the diet spectrum, which is wider in Gentoo penguins compared to Adélie (D'Amico et  
249 al. 2016). D'Amico et al. (2016) address also anthropic sources as responsible of observed ENAs recorded in  
250 Adélie penguins from Stranger Point where high levels of heavy metals (Ni, Cu, Zn and Se) have been  
251 detected in their feathers. Ancora et al. (2002) reported heavy metals (Cd, Pb and Hg) in stomach contents,

252 excreta, and feathers of Adélie penguins breeding at Edmonson Point. At that time a natural occurrence has  
253 been hypothesized for Cd and, to a lesser extent, for Hg, but not a direct anthropogenic impact of local  
254 sources. In fact the nearest scientific stations are far (*c.* 50 Km) from the Edmonson Point colony.

255 Concerning other source of anthropic pollution, contaminants stored in pack ice during years (via global  
256 distillation process), could be released as a result of the seasonal melting also amplified by increasing  
257 temperatures of surface waters as a consequence of climate changes (SCAR 2010). For instance, Persistent  
258 Organic Pollutants (POPs) have been documented to cause alteration on immune system (Jara et al. 2018)  
259 and to correlate with alterations in ENAs and WBC in penguin's species (Jara-Carrasco et al. 2015). In  
260 Adélie penguin population breeding at Edmonson Point, legacy POPs have been reported in stomach  
261 contents, blood samples and unhatched eggs by Corsolini et al. (2003, 2011, 2017), but overall toxicity was  
262 estimated to be low compared to other Antarctic areas. Emerging contaminants like PBDEs (Corsolini et al.  
263 2017) and PFAS (Ademollo, unpublished data) were also detected in Adélie penguin blood samples and eggs  
264 from Edmonson Point. Therefore exposure to contaminant in penguins breeding at Edmonson Point cannot  
265 be considered negligible; Antarctic penguin's colonies are also considered a secondary source of POPs  
266 (Roosens et al. 2007). Nonetheless the impact of human activities that determines local inputs need further  
267 investigations (Wang et al. 2017). A small seasonal field camp (average of 2 personnel unit) located 600 m  
268 far from the breeding groups represents so far the only local source of contamination at Edmonson Point  
269 (Olmastroli 2002).

270 As far as immune status parameters, mean H:L value results higher compared to those reported by D'Amico  
271 et al. (2014; 2016) in Adélie penguin from Stranger Point ( $1.10 \pm 0.20$  and  $1.07 \pm 0.11$  respectively). In  
272 particular, the percentages of LY, HE and EO result lower than those reported by D'Amico et al. (2014)  
273 while MO and BA are 37% higher. MO and EO can be used to make a distinction among factors that alter  
274 the leukocyte profile: stress, disease and infection. In fact, MO number increases in case of infections and  
275 diseases since their main role is to phagocyte foreign particles. On the opposite, a reduction in EO number is  
276 commonly a measure of stress reaction and rarely a response to disease. Early studies in human and  
277 mammals confirmed that glucocorticoids induced by stress often carry out a reduction on EO numbers  
278 (Davis et al. 2008 and references within). THE were also detected and described by Jara-Carrasco et al.  
279 (2015) as a cytological alteration consequent to exposure to various stress agents. THE in Adélie penguins  
280 accounted to 53.50% of total HE and may suggest a bird's response to stress. The presence of THE  
281 associated with the abnormal high number of BA identified in Adélie penguin's blood smears may indicate  
282 some disease occurring in the population under study. Mild lymphocytosis and moderate basophilia have  
283 been associated with feather loss in penguins population from the Ross Sea (Grimaldi et al., 2014) which has  
284 been lately observed also in individuals from Edmonson Point in a similar percentage of occurrence  
285 (Olmastroli personal. observation, 2018-19 Antarctic expedition).

286 Concerning WBC, higher values are reported by D'Amico et al. (2016) in Adélie penguin from different  
287 Islands around Antarctic Peninsula. However, among them, similar values as those measured in our study

288 were reported in Adélie penguins from Stranger Point in which in a comparable number of individuals was  
289 analysed (n = 20). HE shows the highest percentage and this type of WBC are phagocytic cells that increase  
290 when the organism needs to cope with infections causing an increase in the level of H:L ratio. For instance,  
291 HE are the first line of defence that an organism uses as immune response against gastrointestinal parasites  
292 incorporated through the diet (D'Amico et al. 2016). An organism affected by heterophilia and lymphopenia  
293 presents the same leukocyte profiles as one who is experiencing infection and/or diseases. In addition,  
294 despite anthropogenic pressure may have a strong influence on H:L ratio, this factor might have had less  
295 influence in penguins monitored in the present study since penguins from Edmonson Point colony seem less  
296 affected by organic pollutants compared to other colonies (Schiavone et al. 2009).

297 Although difficult at this stage to connect to any contamination or stress sources, this information will be  
298 helpful for future investigation for comparison with different seasons, colonies and breeding stages. In  
299 addition, some aspects of the breeding ecology need to be considered for assessing the health status of the  
300 penguin population. During the breeding stage, females usually arrive later at the breeding colonies (Ainley  
301 2002), and fasting period and intraspecific competition are reduced if compared to mates. At the time of  
302 sampling adults were incubating eggs or attempting to breed, according to Edmonson Point breeding  
303 chronology (Olmastroni et al. 2000; Pezzo et al. 2007). Consequently males were fasting from their arrival at  
304 the breeding colony (late October) and underwent competition with conspecifics for territory occupation,  
305 nest building and mating. Thus, reproductive cycle, seasonal changes, fasting, long-distance migration,  
306 competition for resources and injuries can all affect health status e.g. H:L ratio (Moreno et al. 1998; Vleck et  
307 al. 2000; Minias 2019). Seasonal changes may influence organism's stress levels forcing individuals to use  
308 more energy for thermoregulation. Vleck et al. (2000) reported that injured birds during fights for defending  
309 their territory and/or nest, exhibit higher H:L ratio level than healthy birds. In addition, pathogens, ecto and  
310 endoparasites are known to affect immune status. Individuals sampled in the present study were healthy  
311 penguins, as their weights ranged 3100-5650 g, no sign of illness or injuries, and no ectoparasites, feather or  
312 skin changes or emaciation were observed. There are no studies available on pathogens or parasites on  
313 Edmonson Point population. We cannot exclude potential influence of disease or parasites hampering health  
314 status in the studied population, but no evidence of blood pathogens was detected in the current study. In  
315 addition, studies on pygoscelids suggested absence of blood parasites and a low richness of ecto and  
316 endoparasites for wild sub-Antarctic and Antarctic species (Jones and Shellam 1999; Diaz et al. 2016;  
317 Vanstreels et al. 2014, 2016).

318 Environmental natural stressors and increasing anthropogenic impact on wildlife are expected to grow in  
319 Antarctica in the near future, potentially by altering individual's level of stress and immune status. The  
320 present results depict a preliminary overall assessment of the health status of Adélie penguin's colony at  
321 Edmonson Point since it reflects the different components of an organism's response to its environment.  
322 ENAs and H:L ratio parameters represent a first baseline for future monitoring and assessment of genome  
323 and immune stability of Adélie penguin population in the mid Victoria Land area. Because high H:L ratio  
324 may represent a corticosterone-mediate response of organism to various exogenous stressors and an adaptive

325 evolutionary trait (Minias 2019) future investigation and sampling will be carried out in the framework of the  
326 ongoing research program PNRA2016 AZ1.11 (PenguinERA). Blood parameters such as estimations of  
327 ENAs, WBC and H:L could be useful physiological and ecological indicators in monitoring and conservation  
328 studies to assess population and ecosystem health in a changing environments.

329

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339

### 340 **Author's contribution**

341 SO and IC conceived of this study and wrote up the manuscript, IC and ANJ planned genome and immune  
342 lab analyses, SO and NA collected data, GP performed genome and immune lab analyses, EM and MLV  
343 performed molecular lab analyses, NF performed statistical analyses. All authors helped to draft the  
344 manuscript, read and approved the final manuscript.

345

### 346 **Ethical approval**

347 All applicable international, national and /or institutional guidelines for the care and use of animals were  
348 followed. All procedures performed in studies involving animals were in accordance with the ethical  
349 standards of SCAR's Code of Conduct for the Use of Animals for Scientific Purposes in Antarctica.

350

### 351 **Conflict of interest**

352 We declare that we have no conflict of interest with the data presented in this scientific contribution.

353

### 354 **Figure and table captions**

355 **Fig. 1** Adélie penguin colony at Edmonson Point (74°20' S, 165°08' E), Victoria Land, Ross Sea.

356 **Fig. 2** Erythrocytes nuclear abnormalities (ENAs) in Adélie penguin blood samples according to Kursa and  
357 Bezrukov (2008): (a) micronucleus (MN), (b) lobed nucleus (LN), (c) tailed nucleus (TN), (d) two-lobed  
358 nucleus (TLN), (e) budding nucleus (BN), (f) nucleus with cavity (NWC), (g) kidney-shaped nucleus (KSN),  
359 (h) unknown nuclear malformation (UNM).

360

361 **Fig. 3** Differential white blood cells (WBC): (a) Heterophil (HE), (b) Toxic Heterophil (THE), (c)  
362 Lymphocyte (LY), (d) Monocyte (MO), (e) Toxic Monocyte (TMO), (f) Basophil (BA), (g) Eosinophil (EO).

363 **Fig. 4** Mean  $\pm$  standard error of n. of heterophils and n. of normal heterophils counted in males and females  
364 Adélie penguin (females: n = 7; males: n = 12)

365 **Table 1** Number of micronucleus (MN) and other erythrocytes nuclear anomalies (ENA) analysed per  
366 19,000 mature erythrocytes of Adélie penguin's blood smears according to Kursa and Bezrukov (2008), and  
367 to De Mas (2015)

368 **Table 2** White blood cells (WBC) per 19,000 mature erythrocytes in Adélie penguin's blood samples and H:  
369 L ratio

370

371 **References**

- 372  
373 Ainley D (2002) *The Adélie Penguin: Bellwether of Climate Change*. Columbia University Press  
374  
375 Ancora S, Volpi V, Olmastroni S, Focardi S, Leonzio C (2002) Assumption and elimination of trace  
376 elements in Adélie penguins from Antarctica: a preliminary study. *Mar Environ Res* 54: 341-344  
377  
378 Anderson M J (2001) Permutation tests for univariate or multivariate analysis of variance and regression.  
379 *Can J Fish Aquat Sci* 58:626-639  
380  
381 Ballerini T, Tavecchia G, Olmastroni S, Pezzo F, Focardi S (2009) Nonlinear effects of winter sea ice on the  
382 survival probabilities of Adélie penguins. *Oecologia* 161:253-265  
383  
384 Ballerini T, Tavecchia G, Pezzo F, Jenouvrier S, Olmastroni S (2015) Predicting responses of the Adélie  
385 penguin population of Edmonson Point to future sea ice changes in the Ross Sea. *Front Ecol Evol* 3:1-11  
386  
387 Barbosa A, De Mas E, Benzal J, Diaz JI, Motas M, Jerez S, Pertierra L, Benayas J, Justel A,  
388 Lauzurica P, Garcia-Peña FJ, Serrano T (2013) Pollution and physiological variability in gentoo penguins at  
389 two rookeries with different levels of human visitation. *Antarct Sc* 25:329-338  
390  
391 Bargagli R (2005) Antarctic ecosystems: environmental contamination, climate change and human impact.  
392 In *Ecological Studies* 175 Springer Czeschlik D. Ed Heidelberg, Germany  
393  
394 Baroni C and Orombelli G (1994) Abandoned penguin rookeries as Holocene paleoclimatic indicators in  
395 Antarctica. *Geology* 22:23-26  
396  
397 Beyersmann D, Hartwig A (2008) Carcinogenic metal compounds: recent insight into molecular and cellular  
398 mechanisms. *Arch of Toxicol* 82: 493-512  
399  
400 Cimino MA, Lynch HJ, Saba VS, Oliver MJ (2016) Projected asymmetric response of Adélie penguins to  
401 Antarctic climate change. *Sci Rep Uk* 6:28785  
402  
403 Clark P (2015) Assessment of avian erythrocytes that exhibit variant nuclear morphology. *Comp Clin Pathol*  
404 24:485-490  
405  
406 Colominas-Ciuró R, Santos M, Coria N, Barbosa A (2017) Reproductive effort affects oxidative status and  
407 stress in an Antarctic penguin species: an experimental study. *PLoS ONE* 12:e0177124  
408  
409 Corsolini S, Ademollo N, Romeo T, Olmastroni S, Focardi S (2003) Persistent organic pollutants in some  
410 species of a Ross Sea pelagic trophic web. *Antarct Sci* 15:95-104  
411  
412 Corsolini S, Ademollo N, Martellini T, Randazzo D, Vacchi M, Cincinelli A (2017) Legacy persistent  
413 organic pollutants including PBDEs in the trophic web of the Ross Sea (Antarctica). *Chemosphere* 185: 699-  
414 708  
415  
416 Corsolini S, Borghesi N, Ademollo N, Focardi S (2011) Chlorinated biphenyls and pesticides in migrating  
417 and resident seabirds from East and West Antarctica. *Environ Int* 37:1329-1335  
418  
419 D'Amico VL, Coria N, Palacios MG, Barbosa A, Bertellotti M (2014) Physiological differences between  
420 two overlapped breeding Antarctic penguins in a global change perspective. *Polar Biol* 39:57-64  
421  
422 D'Amico VL, Marcelo B, Benzal J, Coria N, Vidal, V, Diaz JI, Barbosa A (2016) Leukocyte counts in  
423 different populations of Antarctic Pygoscelid penguins along the Antarctic Peninsula. *Polar Biol* 39:199-206  
424  
425 Davis AK, Maney DL, Maerz JC (2008) The use of leukocyte profiles to measure stress in vertebrates: a  
426 review for ecologists. *Funct Ecol* 22:760-772

427  
428 Diaz JI, Fusaro B, Longarzo L, Coria NR, Vidal V, D'Amico V, Barbosa A (2016) Gastrointestinal  
429 helminths of Adélie penguins (*Pygoscelis adeliae*) from Antarctica. Polar Res 35, 28516  
430  
431 De Mas E, Benzal J, Merino S, Valera F, Palacios MJ, Cuervo JJ, Barbosa A (2015) Erythrocytic  
432 abnormalities in three Antarctic penguin species along the Antarctic Peninsula: biomonitoring of genomic  
433 damage. Polar Biol 38:1067-1074  
434  
435 Ducklow HW, Fraser WR, Meredith MP, Stammerjohn SE, Doney SC, Martinson DG, Sailley SF, Schofield  
436 OM, Steinberg DK, Venables HJ, Amsler CD (2013) West Antarctic Peninsula: an ice-dependent coastal  
437 marine ecosystem in transition. Oceanography 26:190-203  
438  
439 Dufva R and Allander K (1995) Intraspecific variation in plumage coloration reflects immune response in  
440 Great Tit (*Parus major*) males. Funct Ecol 9:785-789  
441  
442 Emmerson L, Southwell C (2008) Sea ice cover and its influence on Adélie Penguin reproductive  
443 performance. Ecology 89:2096-102  
444  
445 Grimaldi WW, Hall RJ, White DD, Wang J, Massaro M & DM Tompkins (2015) First report of a feather loss  
446 condition in Adélie penguins (*Pygoscelis adeliae*) on Ross Island, Antarctica, and a preliminary investigation  
447 of its cause. Emu - Austral Ornithology 115:185-189  
  
448 Halpern BS, Walbridge S, Selkoe KA, Kappel CV, Micheli F, D'Agrosa C, Bruno JF, Casey KS, Ebert C,  
449 Fox HE, Fujita R, Heinemann D, Lenihan HS, Madin EMP, Perry MT, Selig ER, Spalding M, Steneck R,  
450 Watson R (2008) A Global Map of Human Impact on Marine Ecosystems. Science 319:948-952  
451  
452 Hammer Ø, Harper DAT, Ryan PD (2001) PAST: Paleontological statistics software package for education  
453 and data analysis. Palaeontol Electron 4:1-9  
454  
455 Jara S, Celis JE, Araneda A, González M, Espejo W, Barra R (2018) Assessment of persistent organic  
456 pollutants and their relationship with immunoglobulins in blood of penguin colonies from Antarctica. Aust J  
457 Vet Sci 50:43-49  
458  
459 Jara-Carrasco S, González, González-Acuña D, Chiang G, Celis J, Espejo W, Mattatall P and Barra R.  
460 (2015). Potential immunohaematological effects of persistent organic pollutants on chinstrap penguin.  
461 Antarct Sci 27:373-381  
462  
463 Jones H.I and Shellam GR (1999) Blood parasites in penguins, and their potential impact on conservation.  
464 Mar Ornith 27:181-184  
465  
466 Krams I, Vrublevska J, Cirule D, Kivleniece I Krama T, Rantala MJ, Sild E, Hõrak P (2012)  
467 Heterophil/lymphocyte ratios predict the magnitude of humoral immune response to a novel antigen in great  
468 tits (*Parus major*). Comp Biochem Physio A 161:422-428  
469  
470 Kursa M and Bezrukov V (2008) Health status in an Antarctic top predator: micronuclei frequency and white  
471 blood cell differentials in the South Polar Skua (*Catharacta maccormicki*). Polarforschung 77:1-5  
472  
473 Lucas, AM, & Jamroz C (1961) Atlas of avian hematology. Agriculture monograph n. 25 U.S. Dept. of  
474 Agriculture Washington, DC  
475  
476 Mallory ML, Robinson SA, Hebert CE, and Forbes MR (2010) Seabirds as indicators of aquatic ecosystem  
477 conditions: a case for gathering multiple proxies of seabird health. Mar Poll Bull 60:7-12  
478  
479 Minias P (2019) Evolution of heterophil/lymphocyte ratios in response to ecological and life-history traits: A  
480 comparative analysis across the avian tree of life. J An Ecol <https://doi.org/10.1111/1365-2656.12941>  
481

482 Moreno J, de León A, Fargallo JA, Moreno E (1998) Breeding time, health and immune response in the  
483 Chinstrap penguin *Pygoscelis antarctica*. *Oecologia* 115:312-319  
484  
485 Noyes PD, McElwee MK, Miller HD, Clark BW, Van Tiem LA, Walcott K.C, Erwin KN, and Levin ED  
486 (2009) The toxicology of climate change: environmental contaminants in a warming world. *Environ Int*  
487 35:971-986  
488  
489 Olmastroni S (2002) Factors affecting the foraging strategies of Adélie penguin (*Pygoscelis adeliae*) at  
490 Edmonson Point, Ross Sea, Antarctica. Dissertation, University of Siena  
491  
492 Olmastroni S, Corsolini S, Pezzo F, Focardi S, Kerry K (2000) The first five years of Italian-Australian joint  
493 programme on the Adélie penguin: an overview. *Ita J Zool* 67: 141-145  
494  
495 Olmastroni S, Pezzo F, Volpi V, Focardi S (2004) Effects of weather and sea-ice on the reproductive  
496 performance of the Adélie penguin at Edmonson Point, Ross Sea. *CCAMLR Sci* 11:99-109  
497  
498 Owen JC (2011) Collecting, processing, and storing avian blood: a review. *J Field Ornith* 82:339-354  
499  
500 Pezzo F, Olmastroni S, Volpi V, Focardi S (2007) Annual variation in reproductive parameters of Adélie  
501 penguins at Edmonson Point, Victoria Land, Antarctica. *Polar Biol* 31:39-45  
502  
503 Roosens L, Van Den Brink N, Riddle M, Blust R, Neelsa H and Covaci A (2007) Penguin colonies as  
504 secondary sources of contamination with persistent organic pollutants. *J Environ Monit* 9:822  
505  
506 Ropert-Coudert Y, Kato A, Meyer X, Pellé M, MacIntosh AJJ, Angelier F, Chastel O, Widmann M, Arthur  
507 B, Raymond B, Raclot T (2015) A complete breeding failure in an Adélie penguin colony correlates with  
508 unusual and extreme environmental events. *Ecography* 38:111-113  
509  
510 Samour J (2006) Diagnostic value of hematology. In Harrison and Lightwood Eds *Clinical Avian Medicine*  
511 pp 587-609  
512  
513 SC-CAMLR (2016) SC-CAMLR-XXXV Report of the thirty-fifth meeting of the Scientific Committee.  
514 Hobart, Australia, 17-21 October 2016  
515  
516 SCAR (2011) IP 53: Code of Conduct for the use of Animals for Scientific Purposes in Antarctica (May  
517 2011). ATCM XXXIV and CEP XIV 2011, Buenos Aires, Argentina.  
518 <https://www.scar.org/library/policy/antarctic-treaty/atcm-xxxiv-and-cep-xiv-2011/2847-atcm34-ip053/>  
519  
520 SCAR (2010) Antarctic climate change and the environment. Published by the Scientific Committee on  
521 Antarctic Research, Cambridge. ISBN 978-0-948277-22-1  
522  
523 Schiavone A, Corsolini S, Borghesi N, Focardi S (2009) Contamination profiles of selected PCB congeners,  
524 chlorinated pesticides, PCDD/Fs in Antarctic fur seal pups and penguin eggs. *Chemosphere* 76:264-269  
525  
526 Tin T, Fleming Z, Hughes K, Ainley D, Convey P, Moreno CA, Pfeiffer S, Scott J and Snape I (2009)  
527 Impacts of local human activities on the Antarctic environment. *Antarct Sci* 21:3-33  
528  
529 Van Ngan P, Gomes V, Passos MJA, Ussami KA, Campos DY, da Silva Rocha AJ, Pereira BA (2007)  
530 Biomonitoring of the genotoxic potential (micronucleus and erythrocyte nuclear abnormalities assay) of the  
531 Admiralty Bay water surrounding the Brazilian Antarctic Research Station “Comandante Ferraz,” King  
532 George Island. *Polar Biol*, 30:209-217  
533  
534 Vanstreels RET, Flavia RM, Ruoppolo V, de Almeida Reis AO, Schneider Costa E, Rodrigues de Lira  
535 Pessôa A, Machado Torres JP, Schmauder Teixeira da Cunha L, da Cruz Piuco R, Valiati VH, González-  
536 Acuña D, Labruna MB, Petry MV, Epiphanyo S, Catão-Dias JL (2014) Investigation of blood parasites of



537 pygoscelid penguins at the King George and Elephant Islands, South Shetlands Archipelago, Antarctica.  
538 *Polar Biol* 37:135-139  
539

540 Vanstreels SR, Braga É, and Catão-Dias J. (2016) Blood parasites of penguins: A critical review.  
541 *Parasitology* 143:931-956  
542

543 Vleck CM, Verticalino N, Vleck D, Bucher TL (2000) Stress, corticosterone, and heterophil to lymphocyte  
544 ratios in free-living Adélie penguins. *The Condor* 102:392-400  
545

546 Wang P, Li Y, Zhang Q, Yang Q, Zhang L, Liu F, Fu J, Meng W, Wang D, Sun H, Zheng S, Hao Y, Liang  
547 Y, Jiang G (2017) Three-year monitoring of atmospheric PCBs and PBDEs at the Chinese Great Wall  
548 Station, West Antarctica: Levels, chiral signature, environmental behaviors and source implication. *Atmos*  
549 *Environ* 150:407-416  
550

551 Webster M, Witkin KL, and Cohen-Fix O (2009) Sizing up the nucleus: nuclear shape, size and nuclear-  
552 envelope assembly. *J Cell Sci* 122:1477-1486  
553

554 Zhang P, Han J, Liu Q, Zhang J, and Zhang X (2013) Sex Identification of Four Penguin Species Using  
555 Locus-Specific PCR. *Zoo Biol* 32:257-261  
556

557 Zúñiga-González G, Torres-Bugarín O, Luna-Aguirre J, González-Rodríguez A, Zamora-Perez A, Gómez-  
558 Meda BC, Ventura-Aguilar AJ, Ramos-Ibarra ML, Ramos-Mora A, Ortiz GG, and Gallegos-Arreola MP  
559 (2000) Spontaneous micronuclei in peripheral blood erythrocytes from 54 animal species (mammals, reptiles  
560 and birds): Part two. *Mutat Res-Gen Tox En* 467:99-103  
561

562 Zúñiga-González G, Torres-Bugarín O, Zamora-Perez A, Gómez-Meda BC, Ibarra MR, Martínez-González  
563 S, and Gallegos-Arreola MP (2001) Differences in the number of micronucleated erythrocytes among young  
564 and adult animals including humans: Spontaneous micronuclei in 43 species. *Mutat Res-Gen Tox En*  
565 494:161-167