RESEARCH/REVIEW ARTICLE

Haematological values of three Antarctic penguins: gentoo (*Pygoscelis papua*), Adélie (*P. adeliae*) and chinstrap (*P. antarcticus*)

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Abstract

It is established that haematological and biochemical parameters provide important data to assess the physiological condition and health status of wild birds. To undertake conservation physiology or ecophysiology work, it is therefore essential to establish baseline physiological parameters and how these parameters change with age and life history events. In this work, we determined and compared baseline haematology and serum biochemistry between adults and chicks of three Antarctic penguin species of the genus *Pygoscelis*: gentoo (*P. papua*), Adélie (*P. adeliae*) and chinstrap (*P. antarcticus*). Differences in adults among species were observed in haemoglobin and biochemical parameters such as total proteins, glucose and alkaline phosphatase activity. In addition, differences between adults and chicks in haematocrit, haemoglobin, total proteins and glucose concentration were determined. Moreover, we evaluated the electrophoretic protein profiles between adults and chicks of the genus *Pygoscelis,* and a conserved protein pattern was observed among species and ages in the genus. Altogether, the results suggest that biochemical and haematological differences among pygoscelids may be related to the nutritional status and energetic expenditure during breeding as well as their feeding habits and development stage.

Haematology and plasma or serum biochemical parameters can provide extremely important information about health status and autecology of free-living vertebrates (Balasch et al. 1974; Cooke et al. 2013). These analyses consider current nutritional, immune and stress status, but also different seasonal processes related with moult and breeding behaviour. Increasing evidence indicates that specific responses against environmental factors may be taxon specific (Cooke et al. 2013). The determination of these parameters provides information about the longer-term physiological and ecological processes affecting individuals or populations (Ardia 2006). In a nutritional context, body condition typically refers to vertebrates' stored fat (and protein) reserves (Schulte-Hostedde et al. 2005), which are critical in many activities and processes influencing fitness, such as long-distance migration and reproductive success (Lill et al. 2013). Therefore, establishing reference values and how these values change with age and different life history events is essential for future conservation efforts.

Migratory birds are exposed to extreme metabolic demands that, together with other factors, such as environmental pollution, stressful situations, physical activity, quality and availability of food resources or health status, can induce changes in plasma biochemistry and haematology (Sturkie & Griminger 1986; Studds & Marra 2005). Furthermore, all these factors together with human disturbance in breeding sites can limit the individual's condition, impeding its ability to migrate, breed and survive (Studds & Marra 2005; Carlini et al. 2007).

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Antarctic birds are important members of the Antarctic ecosystem, in terms of total biomass and of interaction with the environment. Of particular importance are the penguins, including the three species of the genus *Pygoscelis:* gentoo (*P. papua*), Adélie (*P. adeliae*) and chinstrap (*P. antarcticus*), whose breeding range extends south of 60°S. In penguins, haematological research has been carried out mainly on blood respiratory properties (Lenfant et al. 1969; Milsom et al. 1973; Nicol et al. 1988; Rosa et al. 1993) and on changes in plasma nutrients and metabolites during fasting in breeding season (Cherel, Leloup et al. 1988; Cherel, Robin et al. 1988; Ghebremeskel et al. 1989; Ghebremeskel et al. 1991; Ghebremeskel et al. 1992; Merino & Barbosa 1997; Vleck & Vleck 2002). Notwithstanding the great attention given to penguins because their special adaptations to environmental conditions, there are little reported data about normal haematology and plasma biochemistry of members of the *Pygoscelis* genus (Merino & Barbosa 1997; Najle et al. 2006). Moreover, comparisons of haematological reference values of adults and chicks of the genus have not been reported, although it is well established in other birds that development and life history play substantial roles in changing blood chemistry and haematology (Wolf et al. 1985; Moreira dos Santos Schmidt et al. 2007). It would be of interest to know whether patterns of haematological ageing observed in other birds are also seen in penguins, as this group is ecologically divergent from most of the world's birds (Williams 1995).

The aim of this study was to establish reference haematological and blood biochemical parameters of the three Antarctic pygoscelid penguins—gentoo, Adélie and chinstrap—using wild birds. In addition, comparisons between adults and chicks of three species were analysed.

Materials and methods

Study area

The study was conducted on Potter Peninsula on King George Island, South Shetland Islands, Antarctica (62°15′0″S, 58°40′0″W; Fig. 1). The official site name and its latitudinal and longitudinal position were obtained from the SCAR-MarBIN Portal at www.scarmarbin.be/gazetteer.php?p=details&id=13542. When this work was carried out (15 December 2010 to 15 February 2011), the three species were breeding sympatrically and a total of 14 554 pairs of Adélie penguin, 2325 pairs of gentoo penguin and 265 pairs of chinstrap penguin were present. Of note, on King George Island there are eight scientific bases with hundreds of scientists working throughout the year. Also, ongoing and continual movement of aircraft and ships in the area has the potential to contribute to the anthropogenic effects in these zones (Rakusa-Suszczewski 1998; Vodopivez & Curtosi 1998; dos Santos et al. 2005).

Sample collection

Blood samples were collected by venipuncture of the brachial vein from adults (26 gentoos, 18 Adélies and 15 chinstraps) and chicks (17 gentoos, 16 Adélies and 18 chinstraps) of similar age (15–20 days). Peripheral blood

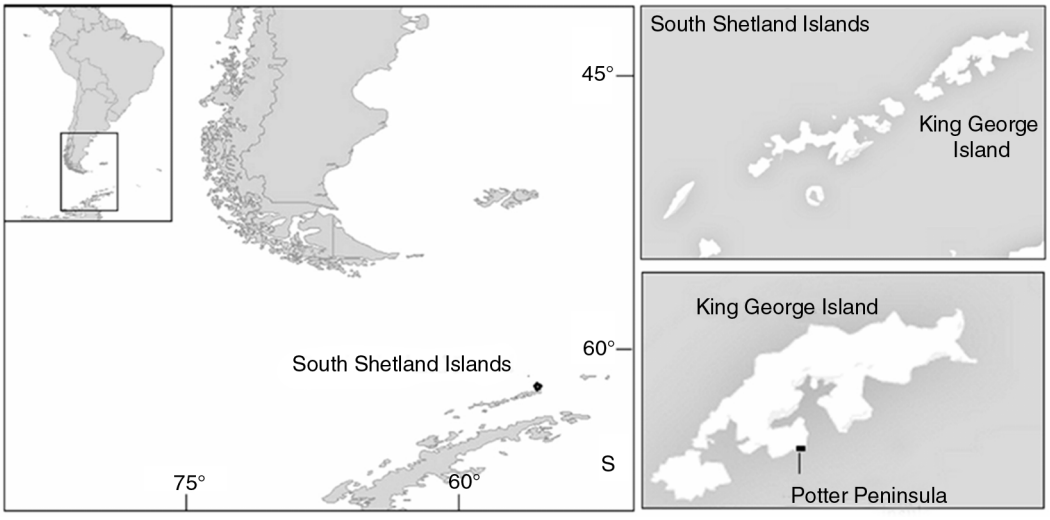

Fig. 1 Study area showing the sampling site, Potter Peninsula in King George Island, South Shetland Islands, Antarctica.

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was obtained using a single 5-ml syringe and a 23-gauge needle. Blood samples (approximately 3 ml) were taken immediately, within two minutes after bird capture, to avoid changes in biochemical and haematological parameters due to stress associated to handling (Fowler 1999). Then the sample was divided and deposited in different test tubes depending on subsequent procedures. Briefly, 1 ml of fresh blood was used to determine haematocrit, sedimentation rate, haemoglobin, glucose and electrolytes. On the other hand, 2 ml of blood were used to obtain serum. For this, blood was incubated overnight at 4°C and then clotted samples were centrifuged for 10 min at 400 × *g*. Finally, harvested serum was frozen at −20°C until determinations were performed in the laboratory. In some cases, blood volumes obtained were small and as a consequence some procedures were applied to all species, but some were only applied to gentoo penguins. Haemolysis was not detected in the sera obtained. During the sample collection, each bird was banded with a numbered band to avoid re-sampling.

Haematological and biochemical analysis

Haematological and biochemical analyses were carried out on blood samples obtained from adults and chicks of gentoo, Adélie and chinstrap penguins. Haematocrit, haemoglobin, erythrocyte sedimentation, biochemical metabolites (glucose, total lipid and proteins, urea, creatinin), alkaline phosphatase activity and ion concentration (Na^{+}, K^{+}, Cl^{-}, Ca^{+2}, P^{-3} and Mg^{+2}) were measured.

The haematocrit percentage value was determined by the micromethod, haemoglobin level using the cyanmethaemoglobin method (Drabkin & Austin 1935) and the standard sedimentation value of erythrocytes was calculated in heparinized blood by standing blood vertically in a Westergren pipette for 60 min. Glucose concentration was estimated using the *ortho*-toluidine method in a spectrophotometer at 505 nm (Nikkila & Hyvärinen 1962). In serum, lipid concentration was determined using the sulfo-phospho-vanillin method in a spectrophotometer at 510 nm (Frings & Dunn 1970). Total proteins were quantified by the Lowry method. Electrolyte concentrations were determined using different assays: sodium (100–200 mEq/L), chloride (50–150 mEq/L) and potassium (2–10 mEq/L) with a Konelab 60I Prime selective ion analyser (Wiener, Rosario, Argentina), calcium (0–10 mg/dl) and magnesium (0–15 mg/dl) with the colourimetric method (Konelab 60I Prime) and phosphorus (0–60 mg/dl) with the ultraviolet method (Konelab 60I prime). Urea, alkaline phosphatase and creatinine were determined by the chemiluminescence method using an Architect *i*1000SR Immunoassay Analyzer (Abbot Diagnostics, Santa Clara, CA, US). All determinations were done in triplicate.

Agarose gel electrophoresis

Protein fractions (albumin, α-, β-, γ-globulins) and A/G ratio were studied in serum samples (10 µl) from adults and chicks. Agarose gel electrophoresis was performed using a semi-automated Hydrasys device (Sebia Electrophoresis, Lisses, France). After that, gels were stained with 0.2% Amido Schwartz solution. An electrophoretic profile of each sample was obtained and analysed using PHORESIS software.

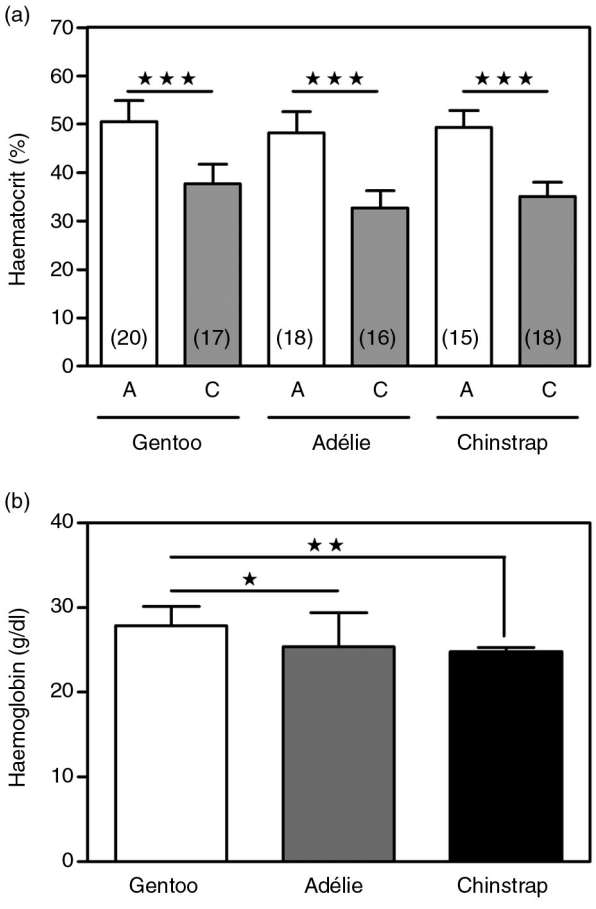

Fig. 2 Haematological values of gentoo (*Pygoscelis papua*), Adélie (*P. adeliae*) and chinstrap (*P. antarcticus*) penguins. (a) Haematocrit values are represented as mean (±SE)% of haematocrit in adults (A) and chicks (C). (b) Mean (±SE) haemoglobin concentration (g/dl) in adult penguins. Sample sizes are given in brackets. Significance values are represented by single ($p < 0.05$), double ($p < 0.01$) and triple ($p < 0.001$) asterisks.

Statistical analysis

Statistical analysis and plotting were performed using GraphPad Prism 4 software (GraphPad, San Diego, CA). Normality and homogeneity of variances were tested using Kolmogorov-Smirnov and Levene tests. Data were analysed using one-way ANOVA with Bonferroni's post-hoc test. For age comparisons in each species, data were analysed using a paired *t*-test. *F* ratio and *t* values are shown in the results, and a *p*-value less than 0.05 was considered significant. Results in tables were expressed as mean ± SD, while in bar charts as mean ± SE for each study group.

Results

Haematocrit values from *Pygoscelis* penguins (adults and chicks) are shown in Fig. 2a. No differences were determined in haematocrit values among adult gentoo, Adélie and chinstrap penguins (gentoo versus Adélie $p = 0.320$, $F = 1.443$; gentoo versus chinstrap $p = 0.268$, $F = 1.732$; Adélie versus chinstrap $p = 0.188$, $F = 2.371$). In contrast, differences were found when comparing adults and chicks. Haematocrit values in adult gentoo, Adélie and chinstrap penguins were 1.33, 1.47 and 1.40 times higher than chicks, respectively (gentoo $p < 0.001$, $t = 8.048$; Adélie $p < 0.001$, $t = 6.233$; chinstrap $p < 0.001$, $t = 9.237$).

Differences in haemoglobin concentration were observed among species in adults. Gentoo showed higher values than the other species (gentoo versus Adélie $p = 0.038$, $F = 6.714$; gentoo versus chinstrap $p = 0.0097$, $F = 13.429$; Fig. 2b). In the case of gentoos, haemoglobin value was also determined in chicks (20.7 g/dl ± 1.6) and the results indicated significantly higher values in adults ($p < 0.001$; $t = 7.562$). In addition, the erythrocyte sedimentation rate in adult gentoo penguins was 1.45 mm/h after 60 min.

Statistically significant differences in serum glucose concentration were found among species. Chinstrap penguins showed higher values than the other species under study (versus Adélie $p = 0.0018$, $F = 28.37$; versus gentoo $p = 0.0008$, $F = 39.69$), while glucose level in Adélie was higher than in gentoo ($p = 0.0009$, $F = 35.14$). Measured total lipid concentrations showed no differences (Table 1).

Gentoo adult penguins showed higher protein concentration than Adélie and chinstrap ($p = 0.026$, $F = 8.238$). Furthermore, age variation in this parameter was found in gentoo and chinstrap penguins, being higher in adults (gentoo adults versus chicks $p < 0.01$, $t = 3.149$; chinstrap adults versus chicks $p < 0.001$, $t = 4.92$; Table 1).

Reference serum metabolites (urea, creatinin, alkaline phosphatase, sodium, potassium, chloride, calcium, phosphorus and magnesium) for the three *Pygoscelis* penguin species are shown in Table 1.

The serum protein electrophoretic profile was determined in the species studied in this work in adults and chicks. In all cases, proteins were scattered at least in four peaks corresponding to albumin (Alb), α-globulins (α_1 and α_2), β-globulin and γ-globulin. We did not observe differences in the serum globulin fraction. In addition, there was a fusion of α_1- and α_2-globulin fractions in samples of the three species, in both adults and chicks.

Table 1 Serum biochemical reference values for gentoo (*Pygoscelis papua*), Adélie (*P. adeliae*) and chinstrap (*P. antarcticus*) penguins. Mean (± SD) and sample size (*n*) of serum biochemistry values are given. Superscript letters [a] ($p < 0.05$), [b] ($p < 0.01$) and [c] ($p < 0.001$) indicate statistically significant differences between species, and single ($p < 0.05$), double ($p < 0.01$) and triple ($p < 0.001$) asterisks indicate significant differences between ages. nd: not determined.

	Gentoo		Adélie		Chinstrap	
	Adults	Chicks	Adults	Chicks	Adults	Chicks
(*n*)	22	17	21	16	18	18
Serum biochemistry	Mean ± SD		Mean ± SD		Mean ± SD	
Proteins (g/dl)	3.71 ± 0.42[a,**]	2.47 ± 0.74	4.39 ± 0.6	4.20 ± 0.43	3.46 ± 1.1[a,***]	2.20 ± 0.87
Lipids (g/l)	8.3 ± 1.7	nd	7.9 ± 0.8	nd	8.3 ± 0.9	nd
Glucose (mg/dl)	201.2 ± 21[c,*]	193.0 ± 20.71	285.6 ± 46[b]	nd	321.3 ± 15.3[b,c,***]	227 ± 12.86
Urea (mg/dl)	10.5 ± 2.12[a]	11.25 ± 2.5	15.8 ± 0.07[a]	18.67 ± 1.15	13 ± 5.65	13.67 ± 5.03
Creatinine (mg/dl)	0.43 ± 0.05	0.47 ± 0.01	0.33 ± 0.30	0.55 ± 0.09	0.34 ± 0.28	0.38 ± 0.27
Alk. phosphatase (mg/dl)	108 ± 15.56	108.7 ± 30.35	120 ± 53.55	87.67 ± 23.59	71 ± 7.07*	138 ± 11.31*
Sodium (mEq/l)	142.5 ± 6.36	152 ± 9.055	130 ± 20.22	141.7 ± 5.68	164.7 ± 30.6	151.7 ± 1.52
Potassium (mEq/l)	2.5 ± 0.42[b]	3.57 ± 0.25	19.05 ± 1.76[b]	15.87 ± 3.33	6.23 ± 2.09[b]	2.7 ± 0.34
Chloride (mEq/l)	98.5 ± 3.53	114.3 ± 12.09	125.3 ± 4.04	117 ± 0	131.3 ± 38.7	111 ± 4.35
Calcium (mg/dl)	3.2 ± 1.41	3.42 ± 0.80	5.2 ± 1.31	3.53 ± 1.01	3.23 ± 2.04	3.76 ± 1.00
Phosphorus (mg/dl)	5.35 ± 0.21[b]	4.85 ± 1.34	13.04 ± 2.67[b]	16.8 ± 3.1	5.23 ± 1.66	4.53 ± 0.32
Magnesium (mg/dl)	2.2 ± 0.28	1.45 ± 0.62	2.26 ± 0.30	2.2 ± 0.34	1.4 ± 0.98	1.56 ± 0.58

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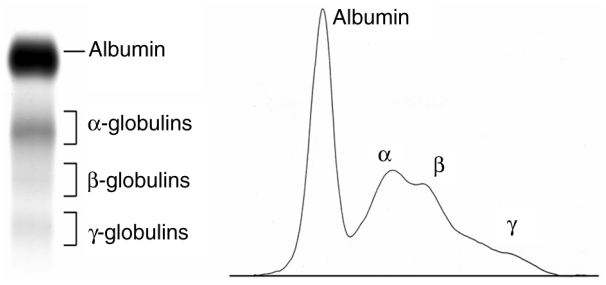

Fig. 3 Determination of *Pygoscelis* serum protein fraction by native agarose gel electrophoresis. Serum samples from gentoo (*Pygoscelis papua*), Adélie (*P. adeliae*) and chinstrap (*P. antarcticus*) adults and chicks were assessed in native agarose gel electrophoresis. Using PHORESIS software, by densitometric analyses four peaks were determined in each sample corresponding to albumin, α (α_1 and α_2), β and γ fractions.

Finally, the A/G ratio was similar among pygoscelid adults and lower than chicks (Fig. 3, Table 2).

Discussion

Haematocrit values determined for the three *Pygoscelis* species were similar to those previously described for these penguins and other migratory birds (Milsom et al. 1973; Myrcha & Kostelecka Myrcha 1980). No differences were observed in haematocrit among adults, but significant age-related differences were identified. This agrees with the observation that haematocrit value is different for each stage of development, and is associated with physiological processes such as haematopoiesis, which occurs during late embryonic growth and the early post-hatching period. As young animals grow, there is a trend to raise the production of red blood cells, which increases haematocrit value (Fair et al. 2007). Haemoglobin values were similar to those previously reported for pygoscelids (Milsom et al. 1973). Gentoo penguins showed higher haemoglobin levels in comparison with Adélie and chinstrap, potentially related to gentoo penguins being able to dive deeper distances for longer periods than the other species (Williams 1995). The adult–chick differences observed in haematological values (haematocrit and haemoglobin-only in gentoo) are in line with expectations. Adults should have a higher oxygen affinity to support adult behaviours such as long continuous diving and swimming (Culik et al. 1994).

Gentoo penguins showed lower glucose concentration than Adélie and chinstrap penguins. The glucogenic mechanisms in gentoo penguin are likely to be important because of its feeding habits (Aguilera et al. 1993). Gentoo penguins feed inshore and are deep divers, and have both greater average prey size and body mass than the other two species. However, values reported here for the penguin species were lower than those described previously (Aguilera et al. 1993).

Lipid concentration is related to nutritional status in free-living birds (Jenni-Eiermann et al. 2002). Serum lipid values were similar to those previously described for these species. In addition, among adult penguins lipids were similar, and this may be because these species have the similar diets which are constituted of krill and fish with high fat content (Reinhardt & Van Vleet 1986; Williams 1995).

Gentoo penguins showed higher protein concentration than Adélie and chinstrap penguins, and also age-related differences were identified in gentoo. In addition, some studies have found chick birds to have higher protein levels than adults, whereas others have found the opposite (Dawson & Bortolotti 1997). This could provide an avenue for future research as it would be interesting to know what proteins make up these differences (e.g., immunological, homeostatic regulation, growth regulation) and what the ecological and fitness roles are for changing protein concentrations across ages. Studies of grown birds have been unable to detect age differences.

Protein electrophoresis is an invaluable diagnostic tool to assess the avian physiological status by determining the relative and total amounts of albumin, α-, β-, and γ-globulin fractions. Variations in these fractions are related to inflammatory processes, age and nutritional status

Table 2 Blood serum proteins in gentoo (*Pygoscelis papua*), Adélie (*P. adeliae*) and chinstrap (*P. antarcticus*). Values of albumin, $\alpha_1\alpha_2$-globulins, β-globulins, γ-globulins and A/G ratio are represented as mean (±SD) protein fraction (%) in adult and chick penguins. Samples sizes are given in parentheses.

	Gentoo		Adélie		Chinstrap	
	Adults (3)	Chicks (4)	Adults (3)	Chicks (3)	Adults (3)	Chicks (3)
Fraction (%)	Mean ± SD		Mean ± SD		Mean ± SD	
Albumin	48.65 ± 3.18	52.15 ± 8.11	48.73 ± 1.55	51.17 ± 1.92	46.43 ± 4.70	54.45 ± 2.19
$\alpha_1\alpha_2$-Globulins	29.4 ± 2.82	27.15 ± 4.93	31.53 ± 2.67	30.9 ± 3.20	33.17 ± 7.55	28.35 ± 3.18
β-Globulins	12.55 ± 1.20	10.98 ± 4.37	12.23 ± 1.57	10.43 ± 2.21	14.23 ± 1.46	11.2 ± 2.54
γ-Globulins	9.4 ± 0.84	9.72 ± 1.67	7.5 ± 0.0	7.5 ± 1.65	6.167 ± 3.6	6 ± 2.82
A/G ratio	0.95 ± 0.12	1.13 ± 0.35	0.95 ± 0.05	1.047 ± 0.08	0.87 ± 0.16	1.19 ± 0.10

(Grasman et al. 2000). We observed a conserved protein pattern among *Pygoscelis* species and ages, which reflects the close taxonomic relationship of these species. Adults showed lower A/G ratio values than chicks. This observation in adults could be a consequence of the energetic costs during breeding, as well because of chronic infection or acute disease, as was reported by others (Ots et al. 1998). Moreover, the fusion observed in α_1- and α_2-globulin fractions was previously described in gentoo and Adélie, as a physiologic response to infectious agents and organic compounds in the environment (Najle et al. 2006).

Interestingly, the baseline biochemical values observed in this work differ and were lower in comparison with those previously reported (Aguilera et al. 1993), in particular concentrations of protein, glucose, urea, creatinine, alkaline phosphatase, electrolytes and protein fraction patterns. A possible explanation for this is that serum metabolites or proteins may vary in response to specific nutritional, pathological or environmental conditions. Supporting our thoughts on this, previous works reported that in different Antarctic regions where human activities were significative, modifications in baseline plasma biochemical parameters and immunoglobulin levels were induced in *Pygoscelis* species (Barbosa et al. 2007; Carlini et al. 2007). Sample collection was performed from breeding colonies on King George Island, a site with high anthropogenic pressure (Harris 1991; Carlini et al. 2007). Therefore, we believe that the environment could be exerting pressure, modifying baseline serum biochemistry as a physiological adaptation, although further work comparing King George Island populations with other less disturbed colonies is needed to confirm this.

In this work, we established haematological and serum biochemical reference values for wild Antarctic *Pygoscelis* adult and chick penguins. While we acknowledge that sample sizes for some species are small, we consider that the reference values reported here represent basic physiological information from which future comparisons and interpretations can be made. Altogether, this information contributes to our knowledge of physiology and their overall health status of these species. Once reference values are established, clinical evaluations, ecological physiology and veterinary care will be improved, and this will substantially aid in future conservation efforts.

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