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Potential use of electrophoretic profiles in three antarctic penguin species as biological markers of contamination

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ABSTRACT

The effects of hydrocarbons on wild life include a variety of pathologic lesions resulting from external exposition and/or ingestion. Among marine birds, penguins are particularly vulnerable to hydrocarbon pollutants from oil spills; since for this species it is difficult to detect and avoid leaked petroleum as other marine birds do. Previous works reported changes in globulin levels of electrophoretic profiles of sera from penguins dosed with fuels in Antarctica. Sera of Adélie (*Pygoscelis adeliae*), Chinstrap (*P. antarcticus*) and Gentoo (*P. papua*) adult and chick penguins were analyzed by native polyacrylamide gel electrophoresis to obtain their protein profiles. The presence of immunoglobulins IgA, IgM and IgG was tested by Western blotting. All three immunoglobulins types were present. Electrophoretic profiles of the species studied differ with those of domestic fowl (*Gallus gallus*) and Olrog seagull (*Larus atlanticus*). Characterization of immunoglobulins by Western blot allows assessing the health status of individuals and can also be utilized as biomarkers of aquatic contamination.

KEYWORDS

Penguin;
Protein sera;
Globulins;
Biomarkers;
Antarctica.

INTRODUCTION

As indicator of animal health, the immunological function has proven to be a sensitive marker of exposure to pollutants[1]. The study of the immunological response is currently recognized as a valuable tool in toxicology[2], ecology[3] and in animal behavior studies of wild bird species[3-5]. Ingestion of petroleum depresses the immunological system, particularly resistance to infective diseases by reducing the number of immune cells in lymphoid organs. Oil-related polycyclic aromatic hydrocarbons (PAHs) are contaminants widely distributed throughout the environment. Oil spills provide the most visible source of contamination, producing acute stress to a localized ecosystem[4].

The level of toxicity and the multiple effects of petroleum derived products on the wild-life depend on a great variety of factors, including: variable concentration of polyaromatic hydrocarbons, metals and other additives, differences in susceptibility due to age and

species, season of the year, water and air temperatures in the location of contamination, efficiency of rescuing oil-soaked birds and time between capture and rehabilitation. Moreover, captivity can deteriorate the health status of marine birds[6,7].

Accidental and intentional releases of petroleum during oceanic transport kill many seabirds annually[8]. Although the effects of major oil spills are often dramatic, chronic pollution may be a more important cause of seabird mortality resulting from oil extraction, refining, and use. Several authors[9-11] reviewed the modes of oil toxicity to birds. Ingested oil produces significant toxicity that can lead to malnutrition and reduced chick growth. Although Briggs et al.[11] noted that humoral immunity is not greatly affected by petroleum, Newman et al.[7,12] measured increased albumin:globulin ratio (due to decreased levels of globulins) in American Coots (*Fulica americana*) rehabilitated following an oil spill. The latter authors also documented an inflammatory response, and suggested that such birds may be under stress-induced immunosuppressant. Lymphocyte development and function may be affected as a result of thymus atrophy and toxic effects of ingested oil on intestinal T lymphocytes[11].

Marine birds are a highly vulnerable to hydrocarbon contamination[8]. In this group of birds, penguins represent the most important family in the Antarctic ecosystem in terms of total biomass and interaction with the environment[13]. Penguins are colonial during their breeding season and are particularly vulnerable to oil spills while foraging at sea[14]. Oil pollution of Antarctic penguins has been reported for Magellanic penguins (*Spheniscus magellanicus*)[14], Adélie (*Pygoscelis adeliae*), Chinstrap (*Pygoscelis antarcticus*), and Gentoo penguins (*Pygoscelis papua*)[15]. In the vicinity of Antarctic stations, the environment and associated wildlife are threatened by pollution. Also, oil spills in distant areas interfere with migratory routes of some species thus representing a significant risk for some species[16].

Some physiological indices are useful in capturing subtle effects of environmental stress on birds[17]. Among the metrics frequently used as indicators of overall health in wild avian populations is the level of plasma proteins[18]. Although rarely applied in studies of wild birds, plasma or serum protein analysis of total and relative concentrations of albumins, α-, β-, and γ-globulins can provide important information on health and physiological status, including immunological and inflammatory responses[2]. Knowing the protein profile of species of conservation interest is a prerequisite to quantify them and better interpret alterations in their levels and factors behind their fluctuation. However, baseline ecological and physiological metrics, contaminant levels across species' ranges and reference intervals for bioindicators of health are lacking in most species[19].

The negative impact of oil spills on penguin populations is difficult to predict but potentially huge. Previous work on penguins dosed with fuels commonly used in the Antarctica showed electrophoretic profiles with altered globulin levels in but no clinical symptoms of intoxication[20,21].

Antibody response can increase γ-globulins. Likewise, inflammation can increase acute phase proteins (α-globulinas and β globulins) and decrease pre-albumin, albumin and transferrin. Three isotypes of avian immunoglobulins have been identified: IgM, homology to mammalian about 30%, 1-2mg /ml serum concentration and a high molecular weight form in serum (~900K). (Chicken IgM 823–954 kDa), IgG Molecular weight of approximately 165-200 kDa. Homology to mammalian 30-35%, 5-10 mg /ml serum concentration; and IgA homology to mammalian 32-41%, ~3mg /ml serum concentration Chicken IgA 170kDa in serum (monomeric)[22].

Analyzing wild bird sera by native gel electrophoresis, an area corresponding to the gamma-globulin fraction is obtained, which is subjective and, in some species, difficult to determine[23]. To certainly determine the gamma-globulin fraction, it is necessary to perform a Western blotting and utilize antibodies against other species such as chicken, giving that the avian immunoglobulins are coded by a limited number of genes and sequences are more preserved than in other organisms[24].

In addition, as species protection to different factors It was shown in *P. adeliae* and *P. papua* the low sensitivity to lipid peroxidation observed in several organs[25,26].

With this background, the objectives of this study were (1) to establish electrophoretic serum profiles for adults and chicks of the studied penguin species, (2) to

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establish normal physiological conditions and characterize the immunoglobulin fraction, and (3) to lay the foundation for future ecotoxiological monitoring throughout the studied species' breeding range.

MATERIALS AND METHODS

Sera

Blood samples were collected from adult and chick specimens of three penguin species (n= 8 from each species and age): Adélie (*P. adeliae*), Chinstrap (*Pygoscelis antarcticus*) and Gentoo (*P. papua*) during February 2002, in the Stranger Point breeding colony (62 º 14' S 58º 38' W), King George Island, South Shetland Islands, Antarctica. A blood sample was collected by venipuncture of the braquial vein with a syringe without anticoagulant. Sampling was conducted under special permitting and following animal welfare guidelines. All birds were released immediately after sample collection. All clotted samples were centrifuged for ten minutes at 400 x*g*. The serum was harvested and kept frozen -20ºC until assayed. Also, serum samples from chicken (*Gallus gallus*) and Olrog gull (*Larus atlanticus*) were used as references.

Polyacrylamide gel electrophoresis (PAGE)

Serum samples from adult and penguin chicks of the three species diluted 1:10 in native sample buffer, serum samples from *G. gallus* and *L. atlanticus* diluted 1:10 in sample buffer assayed as reference and a standard made of mixed proteins of known molecular weights (MW 272, 132, 45, 29 and 14 kDa), were assayed by polyacrylamide gel electrophoresis at 10% under native conditions according to Laemmli[27] and using an electrophoretic vertical plate (Hoffer, USA).

After electrophoresis, the gels were stained with 0.25 % Coomassie Brillant Blue R250 and discolored with a solution containing 10% acetic acid and 10% methanol, or transferred to a nitrocellulose membrane.

Electrophoretic profiles of each sample and the respective proportions of different protein fractions were obtained using Image Pro Plus and Origin 6.0 software. Results were analyzed using one-way ANOVA and Tukey post-hoc test. The level of significance for statistical analyses was defined as $p < 0.05$.

The relative mobility (Rf) is calculated as the ratio of the distance migrated by the molecule to that migrated by a marker dye-front. After calculation the different Rf and plotting of the molecular weight (MW) of the standards against Rf, the approximate molecular weights of each protein fraction was estimated.

Antibodies

To identify the different immunoglobulin isotypes in the sera samples analyzed, goat anti-chicken IgM-HRPeroxidase-conjugated (A30-102P), anti-chicken IgA- HRPeroxidase-conjugated (A30-103P) and anti-chicken IgG-HR HRPeroxidase-conjugated (A30-106P) conjugates (Bethyl Laboratories, INC. Montgomery, TX 77356, USA) were used in a direct immunoassay.

Western blotting

Serum samples of seagull, chicken and of the three adults penguin species were run in polyacrylamide gels under non-denaturing conditions according to the technique described by Laemmli[27]. Following electrophoresis, the separated proteins were transferred to nitrocellulose membranes by electroelution as described by Towbin et al.[28], using a horizontal electrophoretic transfer apparatus (Nova Blot Pharmacia). Transferring conditions were set at 0.8mA/cm^2 during two hours in transfer buffer (Tris 25 mM, glycine 192 mM and methanol 20% (v/v), pH 8.3). Then, the membrane was withdrawn and transfer efficiency was evaluated by Ponceau S stain (Ponceau Red 0.2% in triclhoroacetic acid 3%). After the transfer of the proteins from the gel, the membrane was blocked for one hour with PBS plus 3% of non-fat dry milk (PBS-L). Membranes were then incubated for 8 hours at 4ºC or two hours at room temperature with the different immunochemical reagents (all of them were diluted 1:1000 in PBS). After each incubation, the membrane was washed twice with PBS plus Tween-20 (PBS-T) (NaCl 0.9%, 10 mM, Tween-20 0.05%) and once with PBS alone. Immunocomplexes were visualized as brown precipitate within 6 min of incubation, by the addition of 0.05% (w/v) diaminobenzidine (DAB)(Sigma) as cromogen and 0.01% (v/v) H_2O_2 (100 Vol-30%). The reaction was stopped placing the membranes in tap H_2O and rinsing the membrane twice with distilled water. The presence of IgM, IgG and IgA was determined by comparing

their respective position to the run of standards. Positive reactions in the nitrocellulose support were recorded with a Video Camera Module CCD SONY and digitalized with a frame grabber (Pc Plus, Imaging Technology Inc., 521x512 pixels).

RESULTS AND DISCUSSION

Electrophoresis

Six similar protein bands were observed in samples of the three penguin species, both in adults and chicks (Figure 1). The electrophoretic patterns in penguin sera differed with that of the control species (*G. gallus* and *L. atlanticus*, Figure 1). Protein fractions of both adults and chicks were not different among penguin species (ANOVA, $P > 0.05$) (Figure 2 a, b and c).

The mean electrophoretic profiles of the three penguin species showed a characteristic pattern with respect to other species studied (*G. gallus*, *L. atlanticus*).

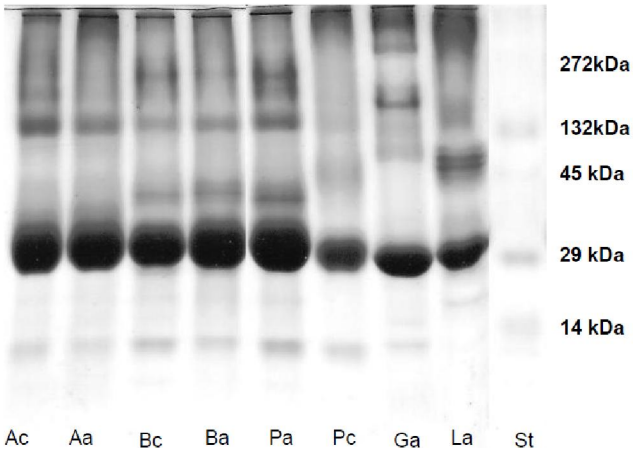

Figure 1 : Native polyacrylamide gel electrophoresis 10%. Ap: *P. adeliae* chick; Aa: *P. adeliae* adult; Bp: *P. antarcticus* chick; Ba: *P. antarcticus* adult; Pa: *P. papua* adult; Pp: *P. papua* chick; Ga: *G. gallus*; La: *L. atlanticus*; St: standard protein mixture of known molecular weights.

Western blotting analysis

All analyzed sera showed positive results for the presence of the three immunoglobulin isotypes. The three polyclonal antibodies against the Ig isotypes of chicken presented a specific interspecies cross-reaction with the penguins immunoglobulins (TABLE 1).

Demonstrated a light contamination of the IgM fraction with IgA and IgG. IgM is found in the first bands, being the protein with the highest molecular weight, while IgA is the smaller, being its band closest to the dye front. On the other hand, IgG ranges an area located between the IgM and IgA bands. In western blotting, it appears that IgM, IgA and IgG, are contained in the gamma fraction of serum protein, IgG can also find in the beta region or beta-gamma (Figure 3).

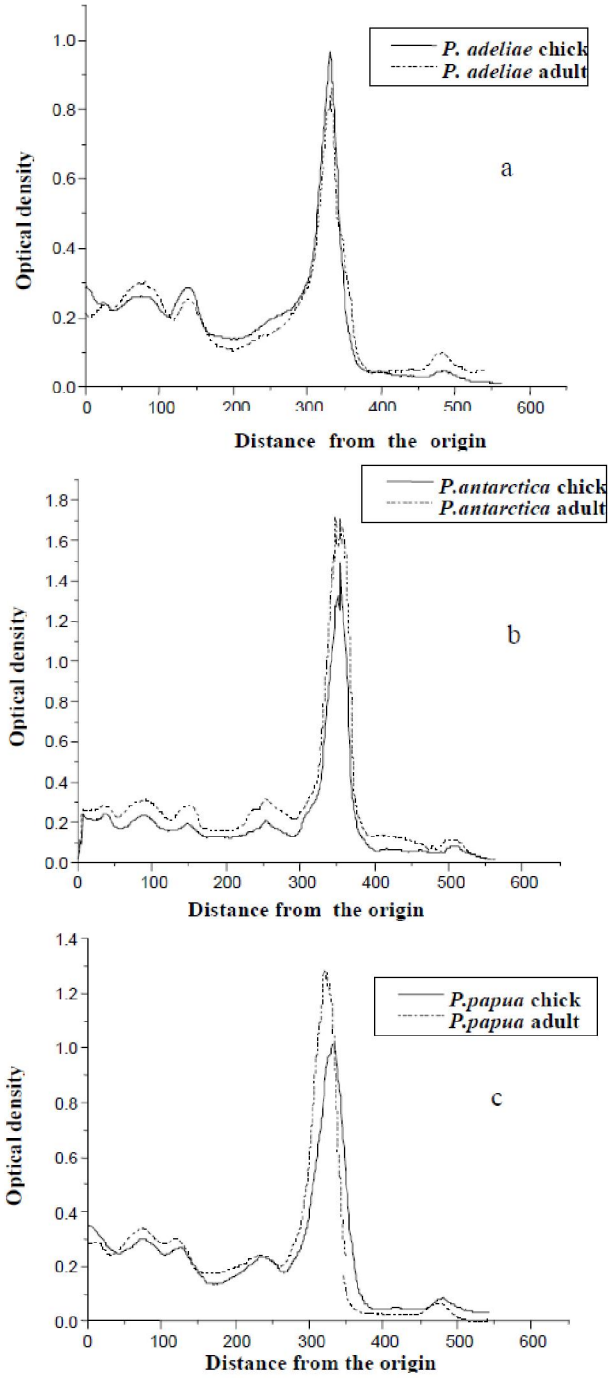

Figure 2 : (A) mean electrophoretic profile of adults and chicks of *P. adeliae*; (B) mean electrophoretic profile of adults and chicks of *P. antarcticus*; (C) mean electrophoretic profile of adults and chicks of *P. papua*.

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This work is the first comprehensive study of plasma electrophoretic profiles in the penguins *P. antarcticus*, *P. papua*, and *P. adeliae*, and in the Olrog gull *L. atlanticus* in the peer reviewed literature. All three penguin species studied had six serum protein fractions; *i.e.*

TABLE 1 : Approximate molecular weights of individual protein bands (peaks) and likely proteins in the serum.

Proteic band	Molecular weight	Probable proteic fraction
First	281 kDa	γ globulins
Second	177 kDa	β globulins
Third	125 kDa	α_1 globulins
Fourth	70 kDa	α_2 globulins
Fifth	40 kDa	Albumin
Sixth	15 kDa	Pre-albumin

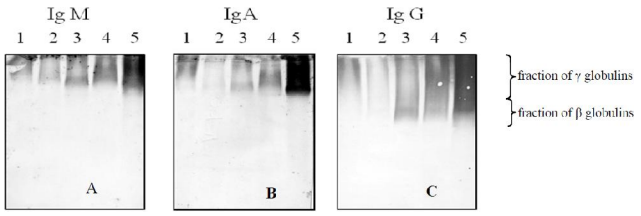

Figure 3 : Western blot of serum samples of adults of the three penguin species, seagull and chicken. Lane 1: *P. adeliae* adult; lane 2: *P. antarcticus* adult; lane 3: *P. papua* adult; lane 4: *L. atlanticus*; lane 5: *G. gallus*. (A) with goat anti-chicken -IgM antibody (B) with goat anti-chicken -IgA antibody and (C) with goat anti-chicken -IgG antibody.

α_1, α_2, β, γ globulins, albumins and pre-albumins with their respective molecular weights, which is in agreement with previous research[20,21,29,30].

The electrophoretic profiles and molecular weight of the different protein fractions of penguins differ markedly from those of other bird species such as *G. gallus* and *L. atlanticus* (Figures 1 and 3) resulting in an additional tool to determine the health status of the species of the genus *Pygoscelis*.

In river otters (*Lutra canadensis*) exposed to Exxon Valdez oil spill, alterations in albumins, haptoglobulin, and β_2-globulins were observed, possibly as direct toxic effect of the oil and (or) a dietary effect of altered food supply[31].

Variations in the normal serum protein patterns are indicative of alterations caused by toxic substances[21]. The biological impact that oil spillages could have on the penguin colonies is very difficult to determine due to the lack of information about biochemical and physiological parameters of the species exposed to contamination. Hence, the normal electrophoretic serum protein profile from Antarctic penguins (Adélie, Chinstrap and Gentoo) would be useful biomarker to detect early alterations caused by stressing factors or contaminant agents.

In the present work, the presence of IgM, IgG and IgA in all the studied species could be corroborated by immunolocalization in all the sera analyzed (chicken, seagull and adult penguins). We show that the different immunoglobulin isotypes of penguins can be detected by specific cross-reacting polyclonal antibodies raised against the Ig isotypes of chicken. The three polyclonal antibodies, anti-chicken/IgA (o-chain specific, affinity purified), anti-chicken/IgG (Fc-fragment specific) and antichicken/IgM (p-chain specific), showed an interspecies cross-reactivity with the corresponding Ig isotypes of penguins. This is in agreement with a report by Castrelos et al.[29] for *P. adeliae*. Localization of the respective bands of immunoglobulins agrees with reports in other vertebrate species *ie*. in turkeys[31].

Previous research in the pygoscelid penguins *P. papua*, *P. antarcticus*, and *P. adeliae* showed that changes in plasmatic immunoglobulin levels are associated with fuel pollution and spatial location[20,21,33].

This serum protein profile determined by native PAGE constitutes another tool to determine the health status of this species. As was pointed out, the determination of the normal serum protein profile can be used to detect the effect of fuel contamination on exposed animals in petroleum spillage zones[21]. Although plasma protein fractions and specific protein analyses are not routinely performed in immunotoxicological studies of wildlife species, these techniques are promising, since they can determine the immunological status and inflammation in wild animals. Long-term comprehensive research reaching across relevant disciplines such as toxicology and immunology, and spanning larger latitudinal ranges, is needed to evaluate the effects of pollution on physiological parameters and to select reliable indicators of health for Antarctic bird populations.

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REFERENCES

[1] J.E.G.Smits, G.R.Bortolotti; Comparative Biochemistry and Physiology A, **151**, 711-716 **(2008)**.

[2] K.A.Grasman; Integrative and Comparative Biology, **42**, 34-42 **(2002)**.

[3] J.L.Tella, M.G.Forero, M.Bertellotti, J.A.Donázar, G.Blanco, O.Ceballos; Proceedings Royal Society London B, **268**, 1455-1461 **(2001)**.

[4] A.Fairbrother, J.E.Smits, K.Grasman; Journal of Toxicology and Environmental Health Part B: Critical Reviews, **7**, 105-137 **(2004)**.

[5] K.A.Hale, J.V.Briskie; Animal Conservation, **10**, 2-10 **(2007)**.

[6] A.M.Hoffman, F.A.Leighton; Journal of the American Veterinary Medical Association, **187**, 1125-1128 **(1985)**.

[7] S.H.Newman, J.K.Mazet, M.H.Ziccardi, C.L.Lieske, D.A.Fauquier, J.G.Gardner, J.G.Zinkl, M.M.Christopher; Comparative Haematology International, **9**, 60-67 **(1999)**.

[8] J.F.Piatt, C.K.Lensink, S.W.Butler, M.Kendziorek, D.K.Nysewander; Auk, **107**, 614-622 **(1990)**.

[9] A.E.Burger, D.M.Fry; Effects of oil pollution on seabirds in the northeast Pacific, in: The status, ecology and conservation of marine birds in the North Pacific, Canadian Wildlife Service Special Publication, Ottawa, 254–263 **(1993)**.

[10] F.A.Leighton; Environmental Review, **1**, 92-103 **(1993)**.

[11] K.T.Briggs, M.E.Gershwin, D.W.Anderson; ICES Journal of Marine Science, **54**, 718–725 **(1997)**.

[12] S.H.Newman, D.W.Anderson, M.H.Ziccardi, J.G.Trupkiewicz, F.S.Tseng, M.M.Christopher, J.G.Zinkl; Environmental Pollution, **107**, 295-304 **(2000)**.

[13] E.J.Woehler, J.P.Croxall; Marine Ornithology, **25**, 43-66 **(1997)**.

[14] P.García-Borboroglu, P.D.Boersma, V.Ruoppolo, L.Reyes, G.A.Rebstock, K.Griot, S.Rodríguez Heredia, C.A.Adornes, R.Pinho da Silva; Marine Pollution Bulletin, **52**, 193-198 **(2006)**.

[15] B.M.Culik, R.P.Wilson, A.T.Woakes, F.W.Sanudo; Marine Pollution Bulletin, **22**, 388-391 **(1991)**.

[16] P.Gandini, P.D.Boersma, E.Frere, M.Gandini, T.Holik, V.Lichtschein; Auk, **111**, 20-27 **(1994)**.

[17] G.A.Fox, D.A.Jeffrey, K.S.Williams, S.W.Kennedy, K.A.Grasman; Journal of Toxicology and Environmental Health A, **70,** 1443-1470 **(2007)**.

[18] R.D.Dawson, G.R.Bortolotti; Comparative Biochemistry and Physiology A, **117**, 383-390 **(1997)**.

[19] M.L.Mallory, S.A.Robinson, C.E.Hebert, M.R.Forbes; Marine Pollution Bulletin, **60**, 7-12 **(2010)**.

[20] A.S.Fernández, R.Najle, A.Curtosi, I.Galufa, C.Vodopidez, H.Solana; Contribución Científica del Instituto Antártico Argentino, **441**, 1-8 **(1994)**.

[21] R.Najle, H.Solana, D.Bottino, M.Juáres, M.Mauad, D.Montalti; Revista Internacional de Contaminación Ambiental, **22**, 107-112 **(2006)**.

[22] F.Davison, K.E.Magor, B.Kaspers; Structure and evolution of avian immunoglobulins, in: Avian Immunology, F.Davison, B.Kaspers, K.Schat (Eds); Elsevier, 107-127 **(2008)**.

[23] J.Martínez, G.Tomás, S.Merino, E.Arriero, J.Moreno; Functional Ecology, **17**, 700-706 **(2003)**.

[24] I.Roitt, J.Brostoff, D.Male; Immunology, Mosby-Year Book Europe Limited, **(1993)**.

[25] M.Gavazza, M.Marmunti, D.Montalti, A.M.Gutiérrez; Acta Biologica Hungarica, **59**, 185-194 **(2008)**.

[26] M.Marmunti, A.M.Gutiérrez, D.Montalti, M.Gavazza, A.Palacio; Biochemistry An Indian Journal, **6**, 162-168 **(2012)**.

[27] U.K.Laemmli; Nature, **227**, 680-685 **(1970)**.

[28] H.Towbin, T.Stahelin, J.Gordon; Proceedings of the National Academy of Sciences, **76**, 4350-4354 **(1979)**.

[29] O.Castrelos, M.Romero Mercado, C.Abatángelo, R.A.Margni; Biochimica et Biophysica Acta, 400, 137-142 **(1975)**.

[30] C.D.Rosa, R.Rosa, E.Rodrigues, M.Bacila; Comparative Biochemistry and Physiology A, **104**, 117-123 **(1993)**.

[31] L.K.Duffy, R.T.Bowyer, J.W.Testa, J.B.Faro; Journal of Wildlife Diseases, **29**, 353-359 **(1993)**.

[32] A.Van Nerom, R.Ducatelle, F.Haesebrouck, S.Arnouts, B.Goddeeris, T.F.Davison, B.Kaspers; Veterinary Immunology and Immunopathology, **57**, 305-314 **(1997)**.

[33] A.Barbosa, S.Merino, J.Benzal, J.Martínez, S.García-Fraile; Polar Biology, **30**, 219-225 **(2007)**.