

## THE USE OF SERUM PROTEINS AS BIOLOGICAL MARKERS OF CONTAMINATION OF GENTOO *Pygoscelis papua* AND ADELIE *P. adeliae* PENGUINS

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### ABSTRACT

Two common and widely used liquid fuels with hepatotoxic activity, and trimethyltin, a compound with neurotoxic activity which is used in several industrial processes, were tested for their toxicity in two species of *Pygoscelis* penguins. Gentoo penguins *Pygoscelis papua* were dosed with trimethyltin (TMT)(15 mg/kg) and Adelie penguins *P. adeliae* were dosed with fuel for air (JP1)(0.20 ml/kg) and land transport (Polar Diesel, PD)(0.20 ml/kg). We use the serum proteins as markers of contamination on Gentoo and Adelie penguins. The following hematological parameters were measured: hemoglobin, hematocrit, glucose, total lipids, total proteins and enzymes (aspartate transaminase and alanine transaminase). Normal hematological values obtained on all penguins are in agreement with previously published data. All tested birds showed significant toxic changes being seen clinically and by blood tests. No mortality was observed during the experiment.  $\beta$  globulin increased in Gentoo penguins treated with TMT, showing highly significant differences.  $\alpha 1$  and  $\alpha 2$  globulin was fused. There was a marked drop in total proteins in Adelie penguins dosed with JP1.  $\beta$  and  $\gamma$  globulin decreased significantly in Adelie penguins exposed to Polar Diesel. This study show that serum proteins and enzymes levels can be used as biological markers of contamination on penguins.

Palabras clave: marcadores biológicos, proteínas del suero, pingüinos, Antártida, *Pygoscelis adeliae*, *Pygoscelis papua*

### RESUMEN

Dos combustibles líquidos comunes y ampliamente usados con actividad hepatotóxica y trimetiltin, un compuesto con actividad neurotóxica, que son usados en varios procesos industriales, fueron utilizados para probar su toxicidad en dos especies de pingüinos pigoscélidos. Se administró a pingüinos papúa *Pygoscelis papua* Trimetiltin (TMT)(15 mg/kg) y a pingüinos adelia *P. adeliae* combustible de aeronaves (JP1)(0.20 ml/kg) y combustible diesel (Polar Diesel, PD)(0.20 ml/kg). Se usaron proteínas séricas como

marcadores de contaminación en pingüinos papúa y adelia. Los siguientes parámetros hematológicos fueron medidos: hemoglobina, hematocrito, glucosa, lípidos totales, proteínas totales y enzimas (AST, aspartato amino transferasa y ALT, alanina amino transferasa). Los valores hematológicos normales obtenidos en todos los pingüinos corresponden con los datos publicados previamente. Todas las aves tratadas mostraron cambios significativos clínicos y/o hematológicos. No se observó mortandad durante el experimento. Los valores de  $\beta$  globulina aumentaron en los pingüinos papúa tratados con TMT mostrando diferencias muy significativas, mientras  $\alpha 1$  y  $\alpha 2$  globulinas se fusionaron. Hubo un marcado descenso en las proteínas totales de los pingüinos Adelia tratados con JP1. En los pingüinos Adelia expuestos a PD,  $\beta$  y  $\gamma$  globulinas disminuyeron significativamente. Este estudio muestra que los niveles séricos de proteínas y enzimas pueden ser utilizados como marcadores biológicos de contaminación en pingüinos.

## INTRODUCTION

More than 100 years of human occupation of the Antarctic continent has inevitably led to anthropogenic contamination in the environment, particularly in the ice-free areas. Such contamination is concentrated around occupied and historic bases and stations, as well as field camps, where soils are often visibly contaminated by fuel residues and solid wastes, or stained by domestic waste water. Internationally agreed protocols now prohibit the discharge of any substance onto ice-free areas and soils in Antarctica. However, prior to the implementation of these protocols, there were less stringent controls on the use, storage and disposal of potential contaminants and less appreciation of the risk posed to the environment by inappropriate use and disposal of these substances. Even now, accidental spills, particularly of fuel, continue to provide a source of potential contamination and are, to some degree, an inevitable consequence of human activity (Webster *et al.* 2003).

The Antarctic Continent constitutes an exceptional environment in which communities of marine life are becoming exposed to anthropogenic disturbances, which are most serious in the areas of greatest human activity (Harris 1991). In these regions, several birds requiring rehabilitation have increased dramatically, the commonest problems being related to oil spills and starvation (Camphuysen and van Franeker 1992). Antarctic birds, particularly penguins, are the most important members of the Antarctic ecosystem, in terms of total biomass and of interaction with the environment (Woehler 1993).

Birds that are emplaced in higher trophic level of the food chain show high levels of xenobiotics and can be considered as bioindicators for monitoring the environmental pollution (Thompson *et al.* 1990), they are relatively large and easily identified. Baseline

surveys of concentration of pollutants in selected bird species have been conducted in Antarctica (Tatton and Ruzicka 1967, Szefer *et al.* 1993, van Den Brink and De Ruiter-Dijkman 1997).

Oil by-products, such as fuel for air and land transport (JP1 and Polar Diesel), as well as organometallic compounds (e.g. Trimethyltin: TMT, antifouling paint), are used in Antarctica. Liquid fuels are used in large quantities, usually stored in outdoor depots, which may leak and contaminate the nearby sea and ice. Trimethyltin has been characterized as a powerful neurotoxin that also affects other organs and tissues (feathers, muscles, liver, kidneys) (Kannan *et al.* 1998) showing a high toxic effect to aquatic life. One criterion for the persistence of organotins in the environment is their lipophile character. The accumulation of organometallic compounds by higher trophic aquatic organisms proceeds through either uptake from solution alone or of a combination with diet ingestion. Due to the extensive use in numerous areas of human activity, large amounts of organotin compounds have been introduced to various ecosystems. Thus, significant concentrations of these pollutants and their metabolites have been detected in all compartments mainly of the aquatic environment: waters, suspended matters, sediment, and biomass. Despite the high concentrations of toxic organotin compounds found in aquatic invertebrates, little is known about the accumulation and toxic effects in higher trophic vertebrate predators, which may be exposed to these pollutants via food ingestion (Hoch 2001).

The impact of a contaminating agent on an organism is reflected through changes in physiological, biochemical and cellular balances. The effects of toxic substances can be measured as disturbances at different levels of functional complexity, depending on the toxin (Larsson *et al.* 1990).

A variety of technical approaches have been ap-

plied in pollution biomonitoring programs in order to estimate the bioavailable fraction of toxic substances, which could be used as baseline levels to monitor changes in the Antarctic ecosystem.

Considering that the levels of pollutants in birds exposed to a polluted aquatic medium is the result of both its accumulation from food and from water, the aim of this study was to use the serum proteins as biological indicators of environmental contamination. For this purpose Gentoo and Adelie penguins were used.

## MATERIALS AND METHODS

Hematological parameters were measured in samples from adult Gentoo *Pygoscelis papua* and Adelie *P. adeliae* penguins. Blood samples were collected from Gentoo ( $n=6$ , mean body weight  $5215 \pm 134$  g) and Adelie ( $n=9$ , mean body weight  $4956 \pm 123$  g) penguins at Potter peninsula ( $62^{\circ}14'S$   $58^{\circ}38'W$ ) King George Island, South Shetland Islands, Antarctica, at the end of austral summer 1993, on which there is an active reproductive colony of penguins: Adelie (number of pairs NP= 14,554), Gentoo (NP= 2,325) and Chinstrap (*P. antarctica*, NP= 265) (Aguirre 1995).

Doses of potentially toxic substances, which are used and stored at Antarctic stations, were given to Gentoo and Adelie penguins to assess hematological changes. The penguins were kept in individual cages, in good weather conditions and with ad libitum availability of fresh water (snow). They were distributed in five groups of 3 penguins each: two intact control (Gentoo  $n=3$ , Adelie  $n=3$ ) and three groups of individuals of which were given pollutants (Gentoo with TMT  $n=3$ , Adelie with JP1  $n=3$ , and Adelie with Polar Diesel  $n=3$ ). The body weight of the penguins was recorded.

The experiment was conducted under ethical conditions. The protocol was approved by Instituto Antártico Argentino ethics committee. All birds were adult and were found in the beach of Potter Cove. Sample collections were done after 12 hours without food. They were handled very gently and as soon as the sampling was finished they were set free. All the penguins used in the experiments were banded and released in good condition in the same place where they were captured.

We measured proteins and enzymes activity. Electrophoresis was done on polyacrylamide 5 % native gels stained with Coomassie Blue and scanned with a densitometer to determine concentrations of

the different protein fractions. Total proteins were calculated with Rapid Lowry's technique.

Serum enzymes, aspartate transaminase (AST) and alanine transaminase (ALT) were quantified by kinetic technique. The enzymatic activity was corrected by mg of protein determined by the Rapid Lowry technique. Additionally, the following tests were undertaken: hemoglobin, hematocrit, glucose and total lipids. The hemoglobin level was determined by the cyanmethaemoglobin method and the hematocrit value by the micromethod. All determinations were done in triplicate. Results were expressed as means  $\pm$  S.D. Data were evaluated statistically by one-way analysis of variance and Tukey test to assess the differences of the means, which were accepted as significant at  $p < 0.05$ .

Three Gentoo penguins were given a single dose of 15 mg/kg body mass of trimethyltin (TMT). The toxic substances were given orally via an sterile plastic tube. Samples of blood were taken at 0, 17, 24 and 48 h from the commencement of the experiment, and birds were examined every three hours. Kerosene-based aviation fuel (JP1) was given orally to three Adelie penguins (0.20 ml/kg), and Polar Diesel (PD) (0.20 ml/kg) to the other three via an sterile plastic tube. Blood samples were taken at 0, 24 and 48 h (Szubartowska and Gromysz-Kalkowska 1992).

## RESULTS

The normal hematological values of two species of penguins were calculated to determine the good health of birds (**Table I**). Values obtained on all penguins are in agreement with previously published data (Milsom et al. 1973, Kostelecka-Myrcha and Myrcha 1980, Rosa et al. 1993). No mortality was observed during the experiment. The toxic substances used did not affect the penguins body weight.

Gentoo Penguins dosed with TMT showed symptoms within 24 hours, mainly neurological ones (difficulty with balance and walking). Comparison between the intoxicated animals and control birds 48 h after the intoxication, revealed differences in several estimates. In our experimental conditions serum proteins values obtained on the 0 time are in agreement with unpublished data from adults birds taken in the same season (D. Montalti, unpublished data). There was a significant increase in total protein from time 17 h to 48 h, due mainly to a rise in betaglobulin; also the  $\alpha 1$  globulin and 2 fractions coalesced, due to that reason, none of them were focus separated on the next three samples. Prealbumin, albumin and gamma

**TABLE I.** NORMAL VALUES OF HEMATOLOGICAL PARAMETERS OF GENTOO *Pygoscelis papua* AND ADELIE *P. adeliae* PENGUINS FROM KING GEORGE ISLAND, ANTARCTICA

	Hemoglobin (g/100 mL)	Hematocrit %	Glucose (g/L)	Total lipids (g/L)	Total proteins (g/L)	AST (UI/L)	ALT (UI/L)
Gentoo Penguin (n=6)	17.9 ± 1.2	49.7 ± 5.3	2.7 ± 0.2	8.3 ± 1.1	59.3 ± 5.2	2.3 ± 0.4	4.1 ± 1.1
Adelie Penguin (n=9)	15.4 ± 1.8	45.9 ± 7.7	1.8 ± 0.1	6.9 ± 1.3	57.9 ± 3.2	2.0 ± 0.2	2.9 ± 0.4

Values expressed as mean ± S.D.

globulin didn't show significant differences after the intoxication.  $\beta$  globulin increased during the experiment showing highly significant differences.  $\alpha_1$  and  $\alpha_2$  globulin was amalgamated, there was no variation in their percentage from the sample taking at the 17 h, until the end of the experiment (**Table II**).

Thus, on comparing control specimens with intoxicated Gentoo penguins with TMT, highly significant differences were found in the serum enzymes levels after 48 h (**Table II**).

Adelie penguins treated with JP1 remained in apparently good health with no discernible signs or symptoms. There was a marked drop in total proteins, showing highly significant differences after the experiment. Prealbumin-albumin and alfa globulin increased showing highly significant differences at 24 h while there was no difference at 48 h, showing the same concentration. On the other hand,  $\beta$  and  $\gamma$  globulin, fell to half their normal values in the samples taken at 24 h, showing highly significant difference and keeping their normal values in the samples taken at 48 h (**Table III**).

No symptoms were observed in Adelie penguins exposed to Polar Diesel. The biochemical changes were similar to those seen with JP1, but were not so marked. Total proteins had a significant decreased at 24 h as well as 48 h. Prealbumin and albumin showed no significant differences after 48 h from the intoxication. Alfa globulin increased and highly significant differences were found.  $\beta$  and  $\gamma$  globulin decreased significantly in the two sampled periods (**Table IV**).

## DISCUSSION

The normal values quoted in this study are mostly within the ranges published for several penguin species (Allison and Feeney 1968, Ghebremeskel *et al.* 1991, Aguilera *et al.* 1993, Rosa *et al.* 1993, Ferrer *et al.* 1994). Between Gentoo and Adelie penguins no significant differences were found in the normal values of hematological parameters, except in some of them (e.g. concentration of glucose in Adelie

**TABLE II.** EFFECTS OF DOSING WITH TMT ON SERUM PROTEINS AND ENZYMES OF GENTOO PENGUIN *Pygoscelis papua*

	0 hours	17 hours	24 hours	48 hours
Total proteins (g/L)	59.3 ± 5.2	66.1* ± 3.3	68.8* ± 4.1	68.9* ± 2.7
Prealbumin	7.6 ± 0.2	7.8 ± 0.3	7.8 ± 0.4	7.9 ± 0.3
Albumin	46.9 ± 1.3	46.8 ± 1.3	45.4 ± 1.4	46.7 ± 2.1
$\alpha_1$ globulin	18.8 ± 0.7			
$\alpha_2$ globulin	14.7 ± 0.4			
$\alpha_1\alpha_2$ globulin		29.9 ± 1.3	28.4 ± 0.9	28.7 ± 1.6
$\beta$ globulin	12.0 ± 0.5	14.6* ± 1.2	16.4* ± 0.8	16.7* ± 1.3
AST (UI/L)	2.3 ± 0.4			27.4** ± 2.7
ALT (UI/L)	4.3 ± 0.5			7.9** ± 0.3

Values express as mean ± S.D. of percentages of serum fractions obtained by electrophoresis at different times post-dose. Values in the 0 h column are those obtained at 0 h of the experimental group (n= 3) and those of the control group (n= 3) (each sample run was triplicated). \* Significantly different from control (p < 0.05). \*\*Highly significant differences (p < 0.001).

**TABLE III.** EFFECTS OF DOSING WITH JP1 ON SERUM PROTEINS OF THE ADÉLIE PENGUIN *Pygoscelis adeliae*

Time post-dose (hours)	Total proteins (g/L)	Prealbumin albumin %	$\alpha$ globulin %	$\beta$ and $\gamma$ globulin %
0	59.2 $\pm$ 4.1	36.9 $\pm$ 1.2	7.4 $\pm$ 0.4	55.7 $\pm$ 3.8
24	40.9* $\pm$ 2.0	59.9* $\pm$ 2.7	13.7* $\pm$ 0.8	26.4* $\pm$ 2.4
48	37.5* $\pm$ 1.3	60.6* $\pm$ 3.7	13.4* $\pm$ 0.5	26.0* $\pm$ 2.1

Values expressed as mean  $\pm$  S.D. of percentages of serum fractions obtained by electrophoresis at 0 time (n= 3) and times post-dose (n= 3) (each sample run was triplicated).

\* Significantly different from control (p < 0.05).

penguins was significantly lower than in Gentoo penguin). The glycogenic mechanisms are likely to be important, because of differences in their feeding habits (Aguilera *et al.* 1993). Gentoo penguins feed inshore and are deep divers, and average body mass is greater than that of the Adélie penguin (Trivelpiece *et al.* 1987). According to Smith and Bush (1978) total protein estimation is a valuable indicator of the general nutritional state, with low protein suggesting either malnutrition, bacterial or chemical toxemia.

In our experiment, the protein levels increased in Gentoo penguins treated with TMT (**Table II**), while in the Adélie penguins dosed with JP1 and PD, the serum proteins decreased showing highly significant differences (**Tables III** and **IV**).

Gentoo penguins dosed with TMT showed a significant increase in protein levels in their blood, due mainly to an increase in betaglobulin. One of the functions of this fraction is excretion of toxins.

The fusion of  $\alpha$  1 and  $\alpha$  2 globulins suggests hepatic shock as a result of ingestion of a potent hepatotoxin. AST increase more than one order of magnitude, while ALT rise around two times. This variation, would be due to the hepatic alterations produced by the dosed TMT.

The electrophoretic pattern of serum samples

from JP1-dosed penguins showed low levels of beta and gamma globulin. In spite of this, the penguins did not show clinical evidence of intoxication. However, these low globulin levels may lead to greater susceptibility to concomitant diseases. Variations in the normal pattern serum proteins, are indicative of toxic provoked alterations.

The observed serum proteins and enzymes changes would be indicative of hepatotoxic substances. In our study, the hematological values changed due to the pollutants administrated to the penguins.

A further possible explanation for the lack of correlation between the degree of contamination and hematological values could be the bird's capacity to carry out a complete or partial regulation of pollutants.

Our findings show that serum proteins and enzymes levels can be used as biological markers of contamination on several penguin species.

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**TABLE IV.** EFFECTS OF DOSING WITH POLAR DIESEL (PD) ON SERUM PROTEINS OF THE ADÉLIE PENGUIN *Pygoscelis adeliae*

Time post-dose (hours)	Total proteins (g/L)	Prealbumin albumin %	$\alpha$ globulin %	$\beta$ and $\gamma$ globulin %
0	58.3 $\pm$ 2.4	40.3 $\pm$ 2.7	9.4 $\pm$ 1.8	50.3 $\pm$ 0.8
24	53.3* $\pm$ 1.9	41.2 $\pm$ 4.8	13.4* $\pm$ 1.3	45.4* $\pm$ 2.1
48	47.8* $\pm$ 0.7	43.6 $\pm$ 3.1	18.1* $\pm$ 1.0	38.3* $\pm$ 1.5

Values expressed as mean  $\pm$  S.D. of percentages of serum fractions obtained by electrophoresis at 0 time (n= 3) and times post-dose (n= 3) (each sample run was triplicated). \* Significantly different from control (p < 0.05)

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