Metallothionein and lipid peroxidation as markers to assess health status of chronically oiled Magellanic penguins in Argentina

Metalotioneínas y peroxidación lipídica como marcadores para determinar el estado de salud del Pingüino Magallánico crónicamente empetrolado en Argentina

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Abstract. Chronic oiling affects seabirds, with short (individual survival) and long-term (dynamics of population) effects. Magellanic penguin is the most affected species in Argentina. The aim was to evaluate the use of blood biochemical-molecular biomarkers (MT and TBARs) to assess health status of Magellanic penguin exposed to chronic environmental oiling, in addition with routine hematological analyses (differential WBC count and PCV). Oiled specimens presented significant higher RBC metallothionein and TBARs concentrations than those from shelter; while routine hematological parameters were within normal levels for penguins and did not show any difference between both groups. These results demonstrated the higher sensibility of both biomarkers, evidencing effects on health status by chronic oiling; furthermore, they are the first report in blood metallothionein and oxidative stress in *S. magellanicus*. These studies will be useful for a better assessment of the health condition in rehabilitation centers (short term); and, as early warning signals in natural colonies (long term).

Keywords: Chronic oiling; Metallothionein; Lipid peroxidation; Magellanic penguin.

Resumen. El empetrolamiento crónico afecta a las aves marinas, con efectos a corto (supervivencia del individuo) y largo plazo (dinámica de la población). El pingüino de Magallanes es la especie más afectada en Argentina. El objetivo fue evaluar el uso de biomarcadores bioquímicos-moleculares (MT y TBARS) en sangre para evaluar el estado de salud del pingüino de Magallanes expuesto a empetrolamiento crónico ambiental, además de los análisis hematológicos de rutina (recuento diferencial de leucocitos y hematocrito). Los ejemplares empetrolados presentaron concentraciones significativamente mayores de metalotioneínas en fracción celular y peroxidación lipídica que aquellos de albergue; por el contrario, los parámetros hematológicos de rutina estuvieron dentro de los niveles normales para los pingüinos y no mostraron ninguna diferencia entre los grupos. Estos resultados demuestran la mayor sensibilidad de los dos biomarcadores, evidenciando efectos del empetrolamiento crónico sobre el estado de salud; además, este es el primer reporte de concentraciones de metalotioneínas en sangre y el estrés oxidativo en *S. magellanicus*. Estos estudios son de utilidad para una mejor evaluación del estado de salud en los centros de rehabilitación (corto plazo); y, como señales de alerta temprana en las colonias naturales (largo plazo).

Palabras clave: Empetrolamiento crónico; Metalotioneínas; Peroxidación lipídica; Pingüino magallánico.

Introduction

Biomarkers are responses of organisms to environmental stress, considering it as a destabilization of the dynamic equilibrium (homeostasis). These responses are considered as "early warning signals", due to they are manifested at the slightest sign of environmental disturbance. The determination of thiobarbituric acid reactive substances (TBARs) is one of the methods to assess oxidative stress caused by different stressors. TBARS are indicative of the formation of malondialdehyde (MDA), an end-product of membrane lipid peroxidation; therefore, it is considered as biomarker of effect for this kind of stress (Janero 1990). Metallothioneins (MT) are low-molecular weight proteins rich in cysteine that bind metals and are found in all animal phyla. MT plays a primary role in the homeostasis of essential metals, such as copper (Cu) and zinc (Zn). However, nonessential metals, particularly cadmium (Cd), are also able to induce MT synthesis (Roesijadi 1996; Amiard et al. 2006). On the other hand,

hematological parameters, mainly differential white blood cells (WBC) counts and packed cell volume (PCV) are traditionally considered as indicators of health status in birds (Moreno et al. 2002; Russell et al. 2003).

Chronic oil pollution (small but frequent discharges) accounts the most petroleum pollution in the ocean (USNRC 2003). Seabirds are one of the most vulnerable groups of marine animals affected by oil pollution (Clark 1984; Dunnet 1987; Piatt et al. 1990). Oil destroys the water-proofing quality of the feathers and cause loss of buoyancy and insulation (Ambrose 1990). Their feeding is reduced or absent, further weakening their body condition and fat reserves. If close to shore, birds may choose to go on land to escape or reduce the effects of hypothermia (Camphuysen 2006). It was also reported that oiling can cause skin irritation, ulceration in eyes, skin, mouth and nose, poisoning and intoxication in birds (Leighton 1986, 1993; Balseiro et al. 2005; AMSA 2007). Furthermore, it can depress avian immune system (Briggs et al. 1997) resulting in increased parasitic diseases (Gandini et al. 1994). Oiling has also been linked to oxidative stress (Reish et al. 1999; Pérez et al. 2010). It has been proposed that oxidative damage during development may potentially constrain organisms' fitness in adulthood (Mangel and Munch 2005) being a key element of the population dynamics (Noguera et al. 2011).

Penguins are most vulnerable and sensitive to oil pollution than other seabirds, since they don´t fly and are less able to detect and avoid petroleum than other seabirds (Gandini et al. 1994). Along the coast of Argentina, chronic oil pollution is the main problem that penguins have to face (Gandini et al. 1994); and the Magellanic penguin (*Spheniscus magellanicus*) is the most abundant species in this area. They have a non-breeding season between April and September, where they migrate from colonies in the south of Argentina to Buenos Aires province, Uruguay and Brazil, in the north. These migratory routes in the Atlantic Ocean overlap with heavy maritime traffic and petroleum development (Stokes and Boersma 1998; Pütz et al. 2000). Magellanic penguin conservation status is Near Threatened (UICN 2014). On the other hand, oil pollution is likely contributing to the long-term decline at the largest Magellanic penguin colony at Punta Tombo, Argentina, where active nests have declined over 20% since 1987 (Boersma 1997; 2008). It has even been reported that there may be a reduction of circulating hormones affecting reproduction of *Spheniscus magellanicus* (Fowler et al. 1995). Therefore, due to the stress affects development and reproduction in animals and that their effects involve the conservation of species, the aim of this study was to evaluate the use of blood biochemical-molecular biomarkers (MT and TBARs) to assess health status of Magellanic penguin exposed to chronic environmental oiling, in addition with routine hematological analyses (differential WBC count and PCV).

Materials and methods

Sampling

The animals were cared for in accordance with guidelines of the Institutional Committee for Care and Use of Laboratory Animals of Mar del Plata University (CICUAL) based on the "Guide for the Care and Use of laboratory Animals" (2010, 8th Edition, National Research Council, The National Academies Press, Washington DC) and Directive 2010/63/UE of the European Parliament and of the Council on the protection of animals used for scientific purposes.

Peripheral blood samples of adults oiled, recovering and shelter penguins from Fundación Mundo Marino (FMM, San Clemente del Tuyú, Argentina) were taken during May-July 2011. The average height of all penguins was 45 cm, and the average weight of oiled penguins was between 2 and 3 kg, while penguins in recovery and shelter were between 3 and 5 kg.

Oiled penguins (OLP; N=20) were rescued by the FMM in the beaches of Buenos Aires province (36 ° 22 'S / 56 ° 44' W), Argentina; and corresponded to individuals in non-breeding season, during their trophic migration from reproductive colonies of southern Argentina (*Figure 1*). OLP came ashore as results of their disability for thermoregulate. They were partially covered with oil and without compromise of the digestive system. Sampling was performed before washing them. Recovering penguins (RECP; N=27), which had been previously oiled, were treated in the rehabilitation center to be successfully reintegrated into the environment; they were sampled before reintegration. And finally, 64 penguins, which lived in FMM shelter (SHEP), were also sampled. They lived in a stable colony and they reproduced successfully.

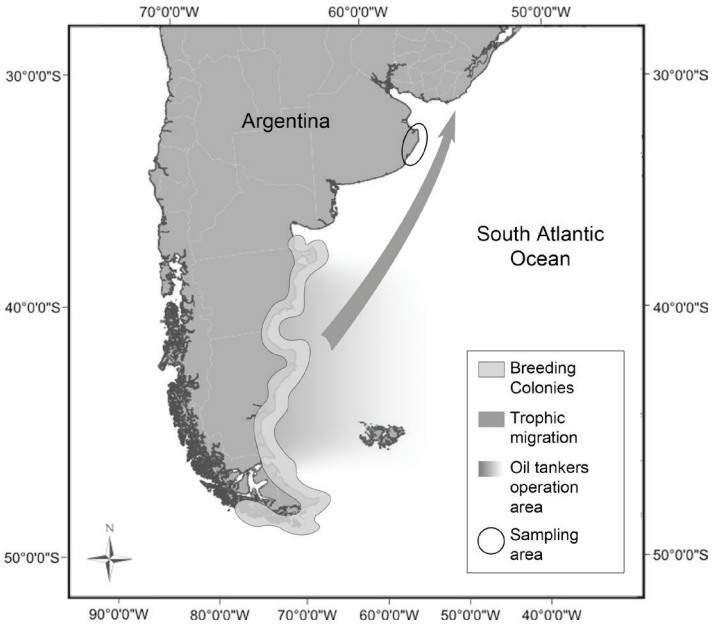

Figure 1. Distribution of breeding colonies of *S. magellanicus* in the southern coast of Argentina, showing trophic migration and oil activity area in Argentina shelf.

Blood was obtained by drawn from the metatarsal vein of the hind leg. One (1) ml of blood was collected in clean microcentrifuge tubes to obtain serum samples and another ml of blood was collected in tubes containing sodic heparin for red blood cell (RBC) fraction. All tubes were immediately centrifugated for 10 min at 3000 rpm for blood separation. After this, samples were frozen with liquid nitrogen and storage at -80°C until processing.
At the same time, a thin layer of blood was smeared on individual slides, air-dried, fixed *in situ* with methanol and, once in the laboratory, they were stained with Giemsa for 45 min. Slides were observed at microscope (1,000x with oiled immersion) to perform differential WBC count and quantify the relative percentage of the most abundant cells in avian blood: heterophils (H) and lymphocytes (L) (Clark et al. 2009). PCV was quantified by determining the microhematocrit technique; results provided by the Rehabilitation Centre of FMM.

Lipid peroxidation (LPO)
MDA, as final product of LPO, was measured by the TBARS assay, according to the method described by Oakes and Van Der Kraak (2003) with modifications to avoid pyridine. Briefly, 100 µl of serum were homogenized in 1.15% KCl containing butlylated hydroxytoluene (BHT) as antioxidant to prevent formation of TBARS in sample manipulation. After that, butanol was added and the homogenate was centrifugated, the immiscible organic layer was removed and the formed chromogen was measured by UV-Vis Spectrometry at 532 nm. The concentration was expressed as nmol of TBARS per µl of serum, which was calculated using the $\varepsilon = 155\ mM^{-1}cm^{-1}$ (Kosugi et al. 1991).

Metallothionein assay
The MT assay was performed according to the spectrometric method described by Viarengo et al. (1997). The absorbance was read at 412 nm, and MT concentration was quantified using reduced glutathione (GSH) as a reference standard. The amount of MT was calculated based on cysteine content in rabbit (18 cysteines/mol), assuming a similar SH group content in Magellanic penguin MT. Concentration was reported as nmol MT per µl of RBC.

Metals determination
Cu and Zn concentration was determined by Atomic Absorption Spectrometry (Perkin–Elmer Analyst 300, Massachusetts, USA). Samples were digested with perchloric and nitric acid (1:3) according to the FAO/SIDA method (1983). A Certified Reference Material LUTS-1 (Lobster hepatopancreas from the National Research Council of Canada) was used to validate results. A blank, treated under the same conditions of samples and Certified Reference Material, was also performed. Results were expressed in µg per ml RCB.

Statistical analyses
The statistical analyses were performed using the software Statistica® 6.0 (Statsoft, Inc.). The differences between states were checked by the parametric ANOVA test, being previously ascertain the variance homogeneity by Levene's test. The significance level was $p<0.05$. The Pearson correlation test was performed between Zn and Cu concentration vs. MT levels.

Results and Discussion

Metallothioneins and essential metals
MT blood levels reported here for *S. magellanicus* constitute the first record for penguin species. OLP presented higher ($p<0.0001$) RBC MT levels (48.1±15.8 $nmol.ml^{-1}$) than RECP (23.0±15.0 $nmol.ml^{-1}$) and SHP (17.8±11.1 $nmol.ml^{-1}$) (*Figure 2a*); there were no statistical differences between RECP y SHP.

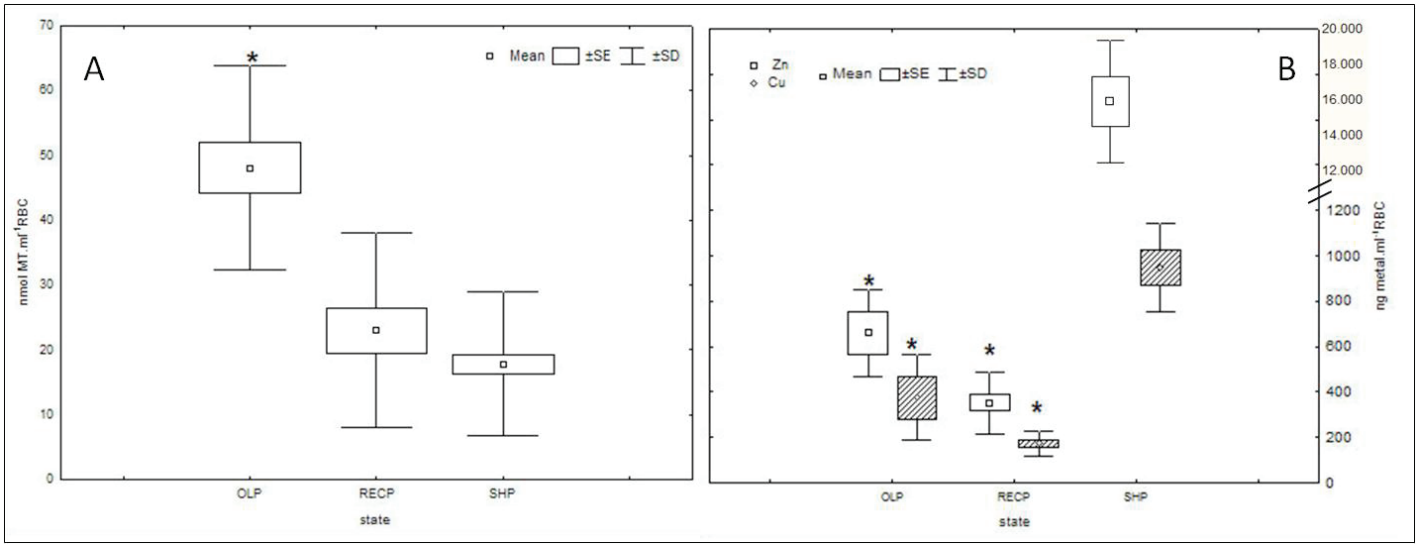

Figure 2. MT (nmol.ml^{-1}; A) and Cu and Zn (ng.ml^{-1}; B) concentrations in RBC according to states in *S. magellanicus*. *indicated significant difference with SHP.

MT are cytosolic proteins characterized by a high content of thiol groups (-SH) due to the high percentage of cysteine residues, binding up to seven atoms of divalent metals. They are involved in the homeostasis of essential metals such as Cu and Zn, and their detoxification when they are above physiological levels, and those non-essential highly toxic metals (Vallee 1995).

MT synthesis could be induced by physic and physiologic stress conditions such as fasting and starving, like a mechanism for conservation of metals in the body (Richards et al. 1987; Debacker et al. 2001; Kondoh et al. 2003) but MT were not seem to be induced by a normal dietary intake of essential metals (Coyle et al. 2002). It has been reported that oiled specimens come ashore, and as a result, there is a reduction in the feed rate causing starvation and decreased body weight (Culik et al. 1991). This situation could lead to the MT levels found in the different states in Magellanic penguins.

Zn concentrations were significantly higher than those from Cu for all studied states ($p<0.05$) (*Figure 2b*). Cu and Zn had an important role in biochemical functions (Ma and Betts 2000). In the blood of birds, the essentiality of Zn is directly related to the activity of carbonic anhydrase (Holm et al. 2001), meanwhile, Cu is linked to the maintenance of blood vessel resistance and synthesis of hemoglobin (Harris 2001). While both metals are essential, Zn participates in the activation of a significantly higher number of enzymes compared to Cu (over 300 enzymes) (Sandstead 1978). The mentioned situation could be responsible for the presence of higher Zn than Cu concentrations in Magellanic penguin.

Significant differences between penguin groups were found for each metal. Zn and Cu levels were higher ($p<0.05$) in SHP (14,700±3,122 ng.ml^{-1}; 948±197 ng.ml^{-1} respectively) than OLP (Zn=661±190; Cu=377±188 ng.ml-1) and RECP (Zn=357±137; Cu=172±57 ng.ml-1) (*Figure 2b*). Richards et al. (1986) reported that Zn and Cu blood concentration in turkey decline in 58% and 63%, respectively, after a period of starvation. Therefore, a re-feeding after this period did not show a return of metals concentration to control levels. On the other hand, it has been reported that excretion of Zn and Cu increase drastically during starvation (Jackson et al 2006). Therefore, it is not surprising to find lower concentration of these essential metals in OLP and RECP compared to SHP.

Zn and Cu levels were significant higher ($p<0.05$) in OLP compared to RECP. The main effect of oiling in birds is the change in feathers' structure, losing impermeability with the consistent disability of thermal isolation and producing hypothermia (Jenssen 1994). There is an increase in metabolic rate to counteract the decrease of body temperature affecting buoyancy (Culik et al. 1991). In fact, on land,

increased heat loss results also in increased metabolic rates and fat consumption. It is known that essential metals, such as Zn and Cu, are involved in maintenance of metabolic rate (Klaasen 2001). For example Zn participates in the synthesis and degradation of carbohydrates, lipids, proteins and nucleic acids (Parkin 2004); and Cu enzymes are involved in a variety of metabolic reactions such as utilization of oxygen during cell respiration (Valko et al. 2005). Furthermore, both metals are also required for synthesis and activation of an important enzyme, superoxide dismutase (SOD) (Mozo et al. 2005; Sahin et al. 2009), which is relevant in antioxidant systems. Therefore, higher Zn and Cu concentration in OLP than RECP could be due to the increased metabolic rate for heat production.

MT pattern in RBC is correlated with Zn ($p<0.05$; $r=0.54$) and Cu ($p<0.05$; $r=0.72$) concentration only in OLP and RECP. This result evidenced that MT in RCB is related to the requirements of these metals and they could be good biomarkers of the condition index in oiled and recovering penguins.

Lipid peroxidation

The information presented in this study is the first report about biomarkers of oxidative stress in blood of *S. magellanicus*, from Argentina. TBARs levels found in OLP (1.6±0.3 nmol.µl^{-1}) and RECP (1.8±0.4 nmol.µl^{-1}) were significantly higher ($p<0.0001$) than those from SHP (0.3±0.1 nmol.µl^{-1}) (*Figure 3*).

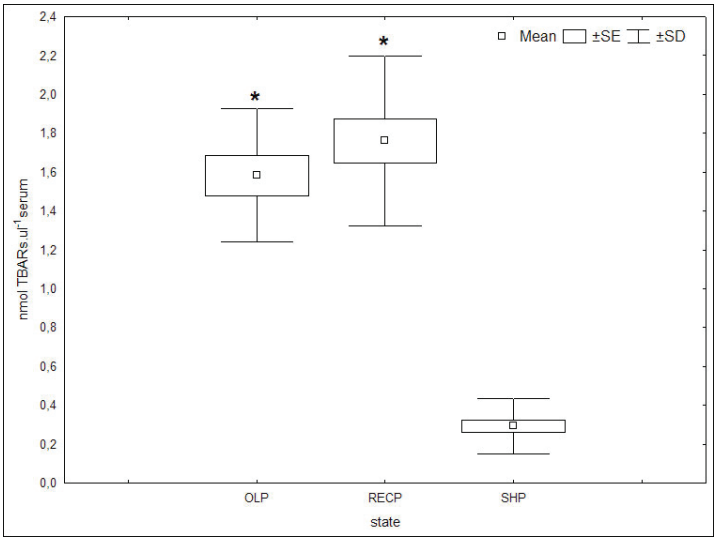

Figure 3. TBARS concentrations (µmol.µl-^{1}) according to states in *S. magellanicus*.
*indicated significant difference with SHP.

Oxidative stress results from a mismatch between the production of damaging reactive oxygen species (ROS) and the organism's capacity to mitigate their damaging effects (Monaghan et al. 2009); and oxidants are generated from normal intracellular metabolism in cells (Finkel and Holbrook 2000). It is a potentially important physiological cost implicated in life-history trade-offs (Monaghan et al. 2009) and senescence (Finkel and Holbrook 2000). While chronic exposure to oil is not lethal, even brief exposure of migrating shore birds to repeatedly cleaned but re-oiled shorelines, could lead to reduced survival during migration or decreased reproductive success at nesting grounds (Burger and Tsipoura 1998).

The relation between oiling and oxidative damage is documented. Petroleum products containing high concentrations of polyaromatic hydrocarbons which likely cause oxidative damage to cellular organelles, membranes and proteins (Newman et al. 1999; Gonzalez 2005). Pérez et al. (2010) observed an increase in lipid peroxidation levels in gulls fed with a supplementary diet containing heavy fuel oil from the Prestige oil spill compared to control; although they were not statistically different, suggesting a protective role of vitamin E as antioxidant. Despite the direct effect of oil in the production of oxidative damage, the situations mentioned in section 3.1 (increased metabolic rate; starvation and disability of thermoregulation) produce an increase in the production of ROS and this could be reflected in higher levels of LPO (Lindgard et al. 1992; Finkel and Holbrook 2000; Mozo et al. 2005).

It had been reported that MT could act as part of the antioxidant system (Prior and Cao 1999; Viarengo et al. 2000; Sato and Kondoh 2002). The pattern of MT and LPO observed in Magellanic penguin, suggested that MT had no role in decrease oxidative damage levels, and they were only involved with essential metals.

Based on the results, the oxidative damage caused by chronic oiling could not be remediated with rehabilitation treatment, despite of the recovery of homeostasis; suggesting that other actions should be taken to reduce levels of LPO in penguins, such as administration of antioxidants in food (e.g. vitamin E).

Hematological parameters

OLP and RECP showed similar patterns in percentages of H and L ($p>0.05$) (*Figure 4*).

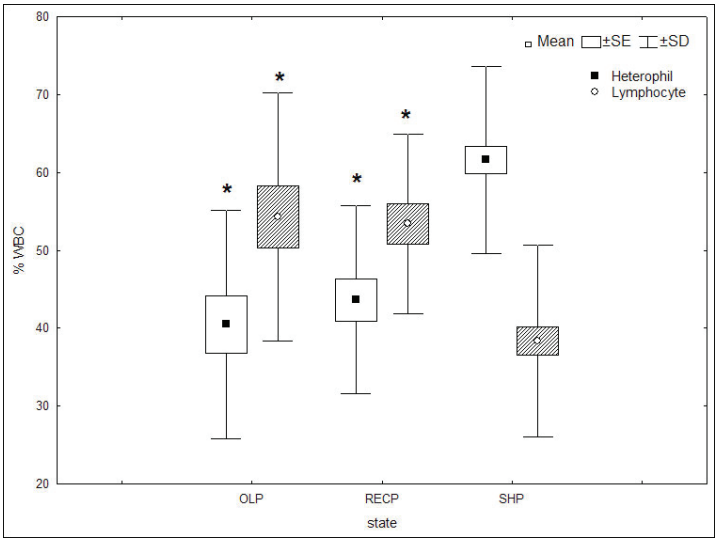

Figure 4. Relative percentages of heterophils and lymphocytes according to states in *S. magellanicus*. *indicated significant difference with SHP.

Percentages of lymphocytes (OLP: 54.3±16.0%; RECP: 53.4±11.5%) were higher than heterophils (OLP: 40.5±14.7; RECP: 43.6±12.1) in both groups. These percentages were within the parameters previously reported for wild Magellanic penguins by Hawkey et al. (1989), and other penguin species (Gentoo -*Pygoscelis papua*- and Rockhopper penguin -*Eudyptes crestatus*-; Hawkey et al. 1989).
Percentages of heterophils in SHP (61.6±12.0%) were higher than those for lymphocytes (38.3±12.4%) ($p<0.05$) (*Figure 4*). This situation was previously reported by Hawkey et al. (1989) for captive rockhopper and gentoo penguins, where H was the most numerous white cell present in captive specimens, in comparison with wild penguins, where the most abundant WBC was lymphocyte. Stoskopf et al. (1983) and Villouta et al. (1997) also reported higher H than L in captive African Blackfoot penguin (*Spheniscus demersus*) and Humboldt penguin (*Spheniscus humboldti*), respectively.
Clinical hematology is one of the most informative procedures to assess health status in birds (Bowerman et al. 2000; Moreno et al. 2002; Russell et al. 2003; Quillfeldt et al. 2004). Among them, PCV and leukocyte count are the most frequently measured variables in wild bird health assessment, due to they reflect the efficiency of oxygen carrying capacity and the immune system status, respectively (Artacho et al., 2007). Lymphocytes and heterophils are the most abundant types of leucocytes of avian blood, and changes in these parameters can reflect responses of an organism to stressful agents (Campbell 1995). Therefore, differential cell count indicated that no petroleum-induced inflammatory response occurred in studied penguins, and H and L percentages did not exceed reference intervals for the species. Although avian species can develop leukocytosis, heterophilia and lymphopenia in association with stress (Campbell 1995); these changes could not be observed in this study.
There were not significant differences ($p>0.05$) between states for PCV values (*Table 1*).

Table 1. Pack Cell Volume percentages according to states in S. magellanicus.

State	PCV (%)
OLP	42.9±5.7
RECP	41.8±4.7
SHP	44.0±3.2

These percentages were according with the proportion reported for wild specimens of the species (Hawkey et al. 1989), and in another penguin species (Hawkey et al. 1989; Merino and Barbosa 1997; Nicol et al. 1988).
Newman et al. (1999) also found no significant differences between oil-exposed marine birds and control for PCV and cell counts of heterophils and lymphocytes, among other blood parameters. These findings supported the results of this study, indicating that traditional hematologic parameters were not good biomarkers for chronic oiling affection in Magellanic penguin as it was proposed by international protocols (Russell et al. 2003).

Conclusion

MT y TBARS presented more sensibility to chronic oiling in comparison with hematological parameters, constituting good biomarkers of this problematic and an important tool to implement in rehabilitation centers. These types of analyses allowed a better understanding of the overall health status of penguins. Therefore this information will allow to improve the treatment (e.g., dietary supplements rich in antioxidants such as vitamin E), and to control in the evolution of penguins to

be returned to the environment. These parameters could also be used as early warning signals of potential problems in natural colonies, to prevent effects on reproduction, survival and fitness.

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