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ORIGINAL ARTICLE

Assessment of persistent organic pollutants and their relationship with immunoglobulins in blood of penguin colonies from Antarctica

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ABSTRACT. Persistent organic pollutants (POPs) may affect the immune system of seabirds, and some field studies have examined this effect. There are evidences that POPs cause negative effects on the immune system, but in Antarctic penguins data are scarce. In order to assess the risks that POPs may have on wild animals, some immunological studies constitute a good alternative. Determining the levels of immunoglobulins can deliver relevant information about the potential impact of POPs in seabirds from different environments. However, there are very few records in penguins so far. The aim of the present work was to quantify the concentrations of polychlorinated biphenyls (PCBs), hexachlorobenzene (HCB), dichlorodiphenyltrichloroethane (ΣDDT), endosulfans, and immunoglobulin IgY in blood samples of colonies of chinstrap penguins (*Pygoscelis antarctica*) that inhabit in the Antarctic Peninsula area. Analysis of POPs in blood samples was carried out through extraction with QuEChERS method, followed by gas chromatography. Detection of IgY concentrations was achieved by ELISA test, using anti-chicken antibodies. There was some significant correlation between immunoglobulin and the presence of PCBs and endosulfans. Even though the levels of these pollutants are low to cause any biological effect on the birds, the results are indicating some influence on the IgY concentrations in penguin blood.

Key words: POPs, seabirds, penguins, blood, antibodies, organic pollutants, Antarctica.

INTRODUCTION

Seabirds, as free-living animals, are exposed to a variety of chemical pollutants, and their ability to fight off stress is an important component of fitness (Tizard 2012). The immune system is the main defense mechanism of an organism against aggressive agents, thus individuals with developed immunity are most able to survive in harsh conditions (French *et al* 2009). Even though the production of persistent organic pollutants (POPs) has been banned for over 35 years, their levels still remain in the environment, and because they can be transported by wind and water, their effects can be evidenced far from where they were released, even reaching cold regions (Geisz *et al* 2008). Polychlorinated biphenyls (PCBs) and chlorinated pesticides (such as DDT, hexachlorobenzene, endosulfans) are toxic xenobiotics that adversely can affect people, wildlife and ecosystems (Bright *et al* 1995, Roosens *et al* 2007, Llansola *et al* 2010).

Antarctica is the most remote continent of the planet, but despite of this it is not exempted from anthropogenic impacts (Bargagli 2008, Corsolini 2009). Ecosystems with little human presence, such as Antarctica, are of great importance to study the health status of the biota that lives there, being of great concern among scientists (Ballschmiter *et al* 2002, Corsolini *et al* 2003, Barbosa *et al* 2007). Within this biota, marine birds, such as penguins, are excellent bioindicator organisms of the health of Antarctic ecosystems, since they can clearly express the biological effects of man-made chemical pollutants (Boersma 2008, Celis *et al* 2012).

Immunoglobulin IgY is the major antibody in birds, reptiles, and lungfish (Warr *et al* 1995). In birds, the IgY is found mainly in blood and in the fluid fraction of the egg providing protection to newly hatched chick (Schade *et al* 2005). Previous studies have shown the presence of three immunoglobulin classes in birds (IgA, IgM and IgY), but IgY is found in higher concentration (5-15 mg/mL) than IgM (1-3 mg/mL) and IgA (0.3-0.5 mg/mL) in serum (Rose *et al* 1974, Kowalczyk *et al* 1985).

Certain factors can affect the immune system of birds at humoral and cellular level, and therefore the levels of immunoglobulin IgY (Tizard 2012). Some studies on fauna have shown that there is a decrease in the levels of immunoglobulin when the individual has been exposed to chemical pollutants, resulting in an increased susceptibility to infectious diseases (Grasman 2002, Bustnes *et al* 2004, Sagerup *et al* 2014). It can even lead to an increased reproductive effort and an increased parasite load (Bustnes *et al* 2004). Seabirds with high levels of POPs have decreased reproduction and survival rates, increased parasitic load, showed greater wing asymmetry and evidenced immunohaematological disorders (Sagerup *et al* 2000, Bustnes *et al* 2004). In *Pygoscelis* penguins from Antarctica, the levels of immunoglobulins indicated that certain POPs produced by humans tend to depress the immune system of birds (Jara-Carrasco *et al* 2015).

The determination of IgY can give valuable information about the immunological status of penguins, which can be used to design plans and programs for wildlife conservation. By using this type of biomarker is possible

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to assess the effects that certain toxic compounds of anthropogenic origin may produce on wildlife (Bustnes *et al* 2004, Barbosa *et al* 2007). In wild birds, the quantification of IgY can be made by means of enzime-linked immunosorbent assay, ELISA, without antigen administration (Martínez *et al* 2003).

The study of trace elements in penguins is valuable, because they are animals that exclusively inhabit the Southern Hemisphere and represent about 90% of the bird biomass of the Southern Ocean (Williams 1990). Three species of *Pygoscelis* penguins primarily inhabit on the Antarctic Peninsula area: gentoo penguin (*P. papua*), Adélie penguin (*P. adeliae*) and chinstrap penguin (*P. antarctica*). Currently, penguins face serious risks of survival in the future, due to larger marine conservation problems, such as pollution (Szopińska *et al* 2016). Chinstrap penguins are experiencing a declining of their population at 2.6 ± 0.7% per annum since 1979-80 (García and Boersma 2013). Although some works have reported the presence of POPs in Antarctic organisms (Corsolini *et al* 2003, Corsolini *et al* 2007, Cipro *et al* 2010, Cipro *et al* 2013, Jara-Carrasco *et al* 2015, Jara-Carrasco *et al* 2017), little is still known about their potential adverse effects on this species. In penguins, there is a lack of immunological data and scarce information with regard to their relationships with POPs. Consequently, the main goal was to assess if there is a negative relationship between POPs and IgY in chinstrap penguins that live in the Antarctic Peninsula area.

MATERIAL AND METHODS

This study was approved by the Bio-Ethical Committee for Animal Experimentation of the authors' institution.

LOCATION OF THE STUDY AND SAMPLING

The work was conducted in the summer season of 2013/2014 (21 December-7 January) through an expedition organised by the Chilean Antarctic Institute (INACH). The locations studied corresponded to sites where chinstrap penguins (*Pygoscelis antarctica*) usually nest (figure 1): Cape Shirreff (62º27'S, 60º47'W), Narębski Point (62°13'S, 58°45'W) and Kopaitic Island (63º19'S, 57º53'W). Those locations are considered as priority areas for the conservation of Antarctic fauna. Chinstrap penguins were caught using nets from their burrows, during guard phase. At each site, blood samples from adult individuals (n=15) were collected following the protocol described by Wilson (1997) in order to minimise bird stress. The birds captured in the different penguin rookeries were sampled in the same breeding phase. From each selected individual, 2 mL blood was aspirated from the medial metatarsal (caudal tibial) vein using a 25-gauge butterfly needle with a 3 mL syringe (Sergent *et al* 2004) and transferred it into 6 mL vacutainers. Blood samples were placed in a cooler with ice packs, transferred them to a freezer within 12 h of sampling and kept them frozen at -80ºC until analysis. Before freezing, blood samples were centrifuged at 12,000 rpm during 10 min to separate plasma from red blood cells.

DETERMINATION OF IgY CONCENTRATIONS

Blood samples were thawed at room temperature before any analysis. To measure circulating concentrations of total IgY, the plasma fraction was achieved by means of a direct ELISA test using a commercial peroxidase-conjugated anti-chicken IgY antibodies (Sigma, St Louis, MO, USA, A-9046). Following the procedure suggested by Martínez *et al* (2003), ELISA plates (Maxi-sorp, Nunc, Rochester, NY, USA) were coated with serial dilutions of serum (100 µL) in carbonate-bicarbonate buffer 100 mM (pH = 9.6) and incubated overnight at 4°C. Then, the plates were blocked with defatted milk diluted in PBS-Tw buffer for 1 h at 37°C (200 µL). Antichicken conjugate was added at 1/250 dilution in PBS-Tw and incubated for 2 h at 37°C (100 µL). The dilution of antichicken antibody was selected after achieving the maximum slope in the linear range. We selected the data obtained from trials using the serum dilution nearest to the center of its linear range (1/35,000). Finally, the plates were filled with a solution consisting of 2,2´-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) ABTS and concentrated hydrogen peroxide diluted to 1/1000. Following incubation for 1 h at 37°C, absorbances were measured using a plate spectrophotometer at λ = 405 nm.

DETERMINATION OF PERSISTENT ORGANIC POLLUTANT CONCENTRATIONS

The chemical pollutants analysed were hexachlorobenzene (HCB), dichlorodiphenyltrichloroethane (DDT) and its corresponding metabolites (DDE, DDD and DDT), endosulfan (α-isomer, β-isomer) and polychlorinated biphenyl (PCB 118, 138, 153 and 180). The contaminants were extracted via the QuEChERS method described by Asensio-Ramos *et al* (2010). One millilitre of sampled blood was put in a centrifuge tube (50 mL) with 10 mL of n-hexane to gas chromatography MS SupraSolv® (Merck) and stirred in a vortex mixer. Magnesium sulfate, disodium citrate salts, trisodium citrate and NaCl were added to each tube. The samples were subjected to sonication in an ultrasonic bath for 15 minutes, and subsequently all tubes were centrifuged (4°C, 20 min at 5000 rpm). Depending on the extract obtained, clean-up was performed by adding magnesium sulfate and primary-secondary amine (PSA). Then n-hexane extracts were reduced in a rotary evaporator at 40°C. The final extract (1 mL) was stored in an amber vial, brought to dryness under a stream of nitrogen and the internal standard (PCNB, 5 ppb) added, suspending it in a final volume of 1mL with n-hexane. The samples were injected into a gas chromatograph with an auto system electron capture detector (GC-ECD, Perkin

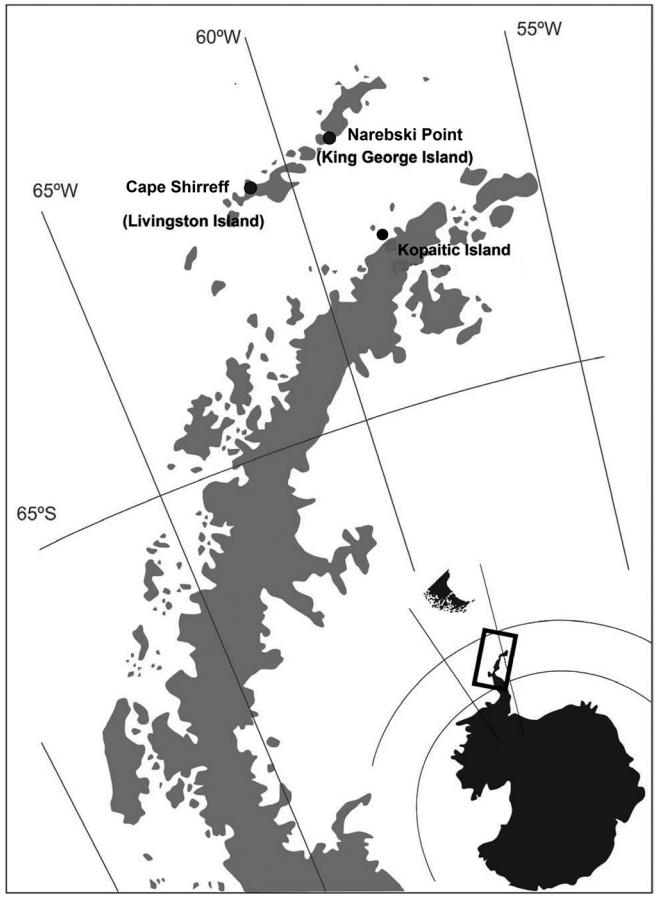

Figure 1. Location of the colonies of chinstrap penguins (*Pygoscelis antarctica*) from the Antarctic Peninsula area (King George Island and Livingston Island are part of the South Shetland Islands, while Kopaitic Island is part of the Graham Land).

Elmer, series 9000). The injector and GC-ECD were at 240°C and 360°C, respectively. The analytes of interest were separated using a PTE-5 capillary column (30 m × 0.25 mm inner diameter and 0.25 µm thick stationary phases) and helium as the make-up gas. The temperature program of the column was: 100°C for 10 min using a gradient of 5°C/min to reach 280°C for 12 min. The total run time was 58 min. Analysis of POPs in blood samples was accompanied by a rigorous quality control program, which consisted of repetitive analysis of blanks and blood samples doped with known concentrations of the compounds. The detection limits were 0.002 µg/L.

STATISTICAL ANALYSIS

The values obtained were first log (x+1) transformed and tested for any evidence of a normally distributed population (Shapiro-Wilk test). Then, multivariate analyses were employed to handle data. The one-way multivariate analysis of variance (one-way MANOVA) was applied to assess differences in pollutant and immunoglobulin concentrations. Posthoc test was used if significant differences ($P<0.05$) appeared between data (Tukey HSD test). A principal component analysis (PCA) was applied (SPAD v5.5) in order to allow the projection of data onto a multidimensional space according to their concentrations. Statistical analysis was conducted with SPSS 12.0.1 (SPSS Inc., Chicago, IL, USA).

RESULTS

Distributions of POPs (HCB, ΣDDT, α-endosulfan, β-endosulfan, PCB-118, PCB-138, PCB-153, and PCB-180) and immunoglobulin (IgY) measured in the blood of chinstrap penguins are listed in table 1. At Kopaitic Island, the concentrations of IgY were the highest whereas the levels α-endosulfan and β-endosulfan were the lowest. On the contrary, the concentrations of IgY were the lowest at Cape Shirreff, where were found the highest levels of β-endosulfan, PCB-138 and PCB-180.

The analysis of multivariable correlation (table 2) revealed that of all contaminants detected in penguin blood, α-endosulfan ($P=0.0042$), β-endosulfan ($P=0.0009$), PCB-138 ($P=0.0018$) and PCB-153 ($P=0.0205$) showed a significant correlation when compared with the respective concentrations of IgY.

Table 1. Persistent organic pollutants (ng/g, wet weight) and immunoglobulin (μg/mL) levels in blood of chinstrap penguins (*Pygoscelis antarctica*) from three locations of the Antarctic Peninsula area.

	Cape Shirreff	Kopaitic Island	Narebski Point
HCB (n = 15)			
Mean ± SD	0.79 ± 0.17	0.85 ± 0.20	0.90 ± 0.17
Min–max	0.55-1.10	0.60-1.24	0.63 ± 1.22
∑DDT[1] (n = 15)			
Mean ± SD	6.90 ± 0.51	7.34 ± 0.77	3.19 ± 0.31
Min–max	6.20-7.90	6.20-9.02	2.67-3.65
α-endosulfan (n = 15)			
Mean ± SD	2.50 ± 0.34	1.38 ± 0.20	2.71 ± 0.46
Min–max	1.80-3.10	1.05-1.78	2.00-3.12
β-endosulfan (n =15)			
Mean ± SD	5.22 ± 0.59	2.60 ± 0.35	2.79 ± 0.41
Min–max	4.40-6.20	1.97-3.05	2.01-3.46
PCB 118 (n = 15)			
Mean ± SD	2.18 ± 0.40	2.24 ± 0.42	2.90 ± 0.37
Min–max	1.50-2.80	1.65-2.88	2.00-3.55
PCB 138 (n =15)			
Mean ± SD	2.40 ± 0.46	1.14 ± 0.25	1.10 ± 0.27
Min–max	1.30-3.10	0.71-1.55	0.75-1.40
PCB 153 (n = 15)			
Mean ± SD	1.20 ± 0.35	2.19 ± 0.60	2.18 ± 0.31
Min–max	0.70-1.80	1.03-2.79	1.45-2.68
PCB 180 (n = 15)			
Mean ± SD	2.20 ± 0.36	1.99 ± 0.31	1.09 ± 0.22
Min–max	1.60-2.70	1.45-2.56	0.70-1.47
IgY (n =15)			
Mean ± SD	155.48 ± 20.89	181.48 ± 3.35	166.98 ± 5.29
Min–max	122.44-189.01	178.00-187.12	159.44-177.21

[1]Levels including the metabolites p,p-DDD, p,p´-DDE, and p´p-DDT.

Table 2. Multivariable correlation coefficients between the levels of persistent organic pollutants and immunoglobulin detected in blood of chinstrap penguins (*Pygoscelis antarctica*) from the Antarctic Peninsula area.

	HCB	∑DDT	α-End	β-endosulfan	PCB118	PCB138	PCB153	PCB180	IgY
HCB	1								
∑DDT	–0.23	1							
α-endosulfan	0.004	–0.56	1						
β-endosulfan	–0.22	0.31	0.38	1					
PCB118	0.09	–0.58	0.34	–0.34	1				
PCB138	–0.18	0.39	0.24	0.86	–0.28	1			
PCB153	0.08	–0.35	–0.31	–0.69	0.12	–0.63	1		
PCB180	–0.02	0.83	–0.34	0.46	–0.49	0.52	–0.51	1	
IgY	0.12	0.08	–0.43	–0.49	–0.05	–0.46	0.29	–0.07	1

PCA was applied to identify trends in the distribution of POPs and IgY (figure 2). Projection of the data, as described by the two main components, enabled graphic visualisation of the trends among the variables considered. It is noted that IgY levels presented negative correlations with α-endosulfan, β-endosulfan and PCB-138. No significant correlation was observed between HCB, ΣDDT, PCB-118 and PCB-180 with IgY concentrations.

DISCUSSION

In general, there is a lack of published data on POPs concentrations in penguin blood that makes it difficult to discuss these results. Moreover, immunoglobulin IgY concentrations in seabirds available in the literature are not always expressed in comparable units. In Artic seabirds, Sagerup *et al* (2014) expressed IgY levels as percentage of a pre-defined positive control, while Bourgeon *et al* (2012) reported IgY levels in units of absorbance. Those few studies in penguin blood performed by Barbosa *et al* (2007) and Bourgeon *et al* (2007), reported levels of IgY in absorbance units instead of mass/volume (mg/L, g/L, or μg/mL), making almost impossible any comparison with our results.

Haematological studies in penguins are valuable because they enable assessment of overall health, thus providing information on physiological, immunological and reproduction responses (Vleck *et al* 2000, Vanstreels *et al* 2014). Some components of the reproductive endocrine system (such as prolactin and growth hormone) are vital for growth, development, osmoregulation and reproduction, and also are promoters of phagocytosis, which all depend upon immunological status of seabirds (Poulsen and Escher 2012).

Some evidence has shown that polar seabirds present an inverse relationship between chemical pollutants and immunohaematological function, thus they are more exposed to acquire parasitic infections at higher pollutant

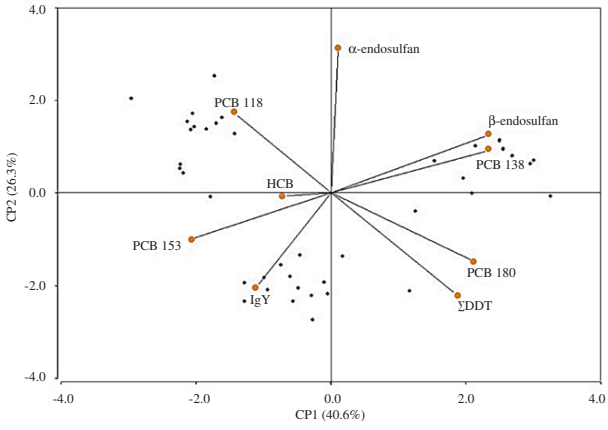

Figure 2. Multidimensional principal component analysis (PCA) based on the POPs and IgY concentrations in blood of *Pygoscelis antarctica*.

concentrations (Sagerup *et al* 2000, Bustnes *et al* 2004). Previous evidence has shown that IgY levels in blood tend to decrease when an organism has been exposed to high concentrations of organic pollutants, implying a greater susceptibility to infectious diseases (Sagerup *et al* 2014). A study performed in Artic glaucous gulls (*Larus hyperboreus*) showed a negative correlation between chick body condition and the parent´s blood levels of HCB and PCBs (Gabrielsen 2007). Another work showed a negative response between POPs and IgY in Atlantic puffins (*Fratercula arctica*) (Sagerup *et al* 2014). Similarly, our results showed that the chinstrap penguin colonies at Cape Shirreff exhibited the highest concentrations of PCBs with the lowest IgY response, although with low exposure levels. A previous study on chinstrap penguins showed that the presence of ∑DDT and PCBs had significant correlations with eosinophils, lymphocytes and heterophyls, although those levels are within established physiological ranges for wild birds (Jara-Carrasco *et al* 2015).

The presence of POPs can be a serious threat to Antarctic ecosystems and their endemic fauna, because POPs bioaccumulate and biomagnify in aquatic food webs, resulting in high concentrations in higher predatory wildlife species, like penguins (Szopińska *et al* 2016). Antarctic trophic chains are relatively simple and short, where chinstrap penguins feed almost exclusively on krill, although they also can prey on fish (Espejo *et al* 2014). Biomagnification is the main route of contamination at the higher levels, where the feeding habits of penguins play a crucial role in POPs intake (Corsolini 2009). Concentrations (ng/g, ww) of ∑DDT (0.41), PCB-153 (0.1) and HCB (0.06) have been reported in Antarctic krill (Cipro *et al* 2010). Our ∑DDT, PCB-153 and HCB concentrations in chinstrap penguin blood are about 14, 19, and 14 times higher than those found in krill, respectively. Also, our concentrations of HCB and ∑DDT are 1.3 and 1.9 higher than those of HCB (0.66) and ∑DDT (3.04) found in Antarctic fish, respectively (Cipro et *al* 2013), thus evidencing those POPs effectively biomagnify into the Antarctic food web.

In seabirds, PCBs comprise the majority of POPs, followed by DDT, chlordanes, HCB and HCHs (Savinova *et al* 1995, Borgå *et al* 2001). Similarly, our results showed concentrations of POPs as follows: PCBs > ∑DDT > endosulfans > HCB. In penguins, differences across POPs could be explained by diet and geographical location (Jara-Carrasco *et al* 2015). The presence of PCBs in blood is highly related to penguin diet, as the congeners 138 (hexa-chlorinated) and 180 (hepta-chlorinated) constitute most of the residues of PCBs from food ingested by penguins (Focardi *et al* 1995). A study showed that twelve PCB congeners (including 153, 138 and 180) are considered to be environmentally important (Oliver and Niimi 1988). The congener PCB-138 is classified in a group more potent than PCB-180 in inducing hepatic function alterations and immunotoxicity in animals, including humans (Llansola *et al* 2010). Endosulfans are of more recent appearance in

polar environments, being first detected in eggs of chinstrap penguins from Antarctica at 1.03 ng/g, ww (Cipro *et al* 2013). The presence of endosulfans in Antarctica can be due to an intensive use of this insecticide by South American farmers, especially in Argentine, where it was manufactured until its prohibition by July 2013 (Pérez *et al* 2013). Although many of the POPs are decreasing, new compounds are entering ecosystems thus creating a sort of cocktail effect of different environmental toxicants may be severe in seabirds, and an additional stress to the breeding populations (Bustnes *et al* 2015).

In penguins, high POPs concentrations for a prolonged time may reduce the number of B cells, which implies a reduction of immunoglobulin synthesis (Vleck *et al* 2000). Our results showed that the highest PCB-138, PCB-180 and β-endosulfan concentrations tend to reduce the IgY levels, as particularly noted in penguin colonies at Cape Shirreff. Nevertheless, our findings revealed that PCBs (7.35-8.04 ng/g) are far below the range of no-observed effect levels (1,300-11,000 ng/g) related to reproduction success in birds, and in blood (600 ng/g) from healthy Artic glaucous gulls of normal body condition (Gabrielsen 2007).

It is well known that the production of POPs was stopped some decades ago (Geisz *et al* 2008), and for that reason there has been a reduction in the levels of some POPs in Antarctica (Van den Brink *et al* 2011). In concordance with that, our levels of HCB were lower than those reported by Corsolini *et al* (2007) in blood of *Pygoscelis* penguins. Yet, our results are indicating that PCBs, DDT, HCB and endosulfan are present somehow in the Antarctic food web. On the other hand, the Antarctic wildlife is more sensitive to chemical contaminants at lower concentrations than similar species from temperate latitudes (Poland *et al* 2003). Polar animals have a high content of lipids and a slow metabolism to save energy, and therefore they have a very slow process of pollutants detoxification (Chapman and Riddle 2005), which implies that Antarctic organisms might be more vulnerable to the adverse effects of POPs than animals from other regions (Bargagli 2008).

Deleterious biological effects reported in several species of vertebrates have been associated to induction by PCBs, as for example through interference with the thyroid hormone T4 transport proteins (Sandau 2000). Levels of PCBs and organochlorine pesticides have been correlated negatively with circulating levels of thyroid hormones in male glaucous gulls (Verreault *et al* 2004).

Although our study provided evidence of the presence of POPs in blood of chinstrap penguins from Antarctica, our POP levels are lower than those found in blood of great skuas (*Stercorarius skua*) from the Northern Hemisphere (Bourgeon *et al* 2012). Also, our HCB, PCB-118, PCB-138, PCB-153 and PCB-180 concentrations are lower than those levels reported by Sagerup *et al* (2014) in blood of black-legged kittiwakes (*Rissa tridactyla*) from the Artic (3.80, 4.87, 18.39, 12.43 and 2.27 ng/g, respectively), and they are not likely to be a threat for this population. However, even if concentrations of these pollutants are low, they may have a combined effect with some others chemicals, such as heavy metals (Sebastiano *et al* 2016). Commonly, wildlife is exposed to a wide array of contaminants (POPs, heavy metals, etc.) which makes it difficult to set correlations with the effects on fauna (Bustness *et al* 2003). For that reason, it is complicate to state that PCBs and other POPs could be the sole cause of the physiological alterations in chinstrap penguins. Even so, it is possible to say that these pollutants are distinctly and could be indirectly affecting the immune systems of the penguin colonies studied here.

In conclusion, some POPs may affect the immune system of seabirds. There are evidences that POPs cause negative effects on the immune system of birds, but in Antarctic penguins data are scarce. Some relationship was observed for the IgY response of some POP concentrations in chinstrap penguins, but with low explanatory values. Data indicate that POP concentrations were lower than the threshold of the effect level for the proposed biomarker. However, it is necessary to complement this study with the combined effects with others pollutants, as well as include other immunological parameters, such as cytokines, lysozymes, metallothioneins, among others. Also, it is necessary to supplement the information obtained in this study with other locations, in order to have a wider scenario about some potential effects that certain organic pollutants may have on other colonies of penguins that inhabit in the Antarctic Peninsula area.

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