Antihelminthic treatment alters cellular, but not humoral immune components in Magellanic penguin (*Spheniscus magellanicus* Forster, 1781) chicks

D'Amico V.L. [*], Palacios, M. G., Bertellotti, M.[a]

[a] *Centro para el Estudio de Sistemas Marinos (CESIMAR), CONICET, Brown 2915 (9120) Puerto Madryn, Chubut, Argentina.*

Running title: Antihelminthic treatment alters immune components in penguin chicks

*Corresponding author:

Dra. Verónica L. D'Amico

Centro para el Estudio de Sistemas Marinos (CESIMAR), CONICET, Brown 2915 (9120) Puerto Madryn, Chubut, Argentina.

damico@cenpat-conicet.gob.ar

Antihelminthic treatment alters cellular, but not humoral immune components in Magellanic penguin (*Spheniscus magellanicus* Forster, 1781) chicks

D'Amico V.L. [*], Palacios, M. G., Bertellotti, M.[a]

Abstract

We evaluate whether helminth parasites affect both cellular and humoral components of the immune system of Magellanic penguin (*Spheniscus magellanicus* Forster, 1781) chicks. We measured immune components after the administration of an antihelminthic drug to remove parasites. Cellular immune components included the complete white blood cell count (WBC) and the *in vivo* skin-swelling response to phytohemagglutinin (PHA). Humoral aspects assessed were the ability of plasma to agglutinate foreign particles and the bactericidal capacity of plasma. Antihelminthic treatment resulted in lower total WBC counts supporting the role of circulating leukocytes in fighting macroparasites. Deparasitized chicks showed a reduction in all types of leukocytes. Contrary to our expectation deparasitized Magellanic penguin chicks showed lower response to PHA injection than control chicks. The swelling response was positively correlated with body condition and with total WBC in circulation. We hypothesize that the specific helminth community naturally occurring in Magellanic penguin chicks might have an overall immunostimulatory effect on the PHA response. Antihelminthic treatment did not alter the innate humoral immune parameters measured. Our results support the prediction that, given their relatively low costs of use and maintenance, innate humoral components would not be as affected by antihelminthic treatment as more costly cellular responses.

Introduction

Birds act as hosts for a large diversity of parasites that can be important causes of morbidity or mortality (Wakelin and Apanius 1997). Infected individuals face the challenge of diverting resources, which would otherwise be allocated to critical life-history functions such as growth or reproduction, to anti-parasite defense (Lochmiller and Deerenberg 2000). Activation of anti-parasite defenses themselves, particularly immune responses, can lead to additional costs in terms of increased metabolic rate or immunopathology (Lochmiller and Deerenberg 2000; Maizel et al. 2012). Moreover, some parasites, such as gastrointestinal helminths, can impose further costs to their hosts through direct competition for nutrients in their digestive tracts (Colditz 2008; Maizel et al. 2012). Clearance of helminths by the immune system is complex and does not rely on one particular cell type or killing mechanism; instead, it depends on the interaction of multiple innate and adaptive immune components and pathways that disable, degrade, and dislocate parasites resulting in their expulsion (Maizel et al. 2012). These components include, but are not limited to, immune cells such as monocytes or macrophages, granulocytes (eosinophils, heterophils, basophils), and lymphocytes (mainly T-helper 2 and B-cells), as well as humoral components such as the complement system and neutralizing antibodies (Moreau and Chauvin 2010; Maizel et al. 2012).

Among birds, penguin species are host to a diverse array of helminth parasites (Fonteneau et al. 2011; Vidal et al. 2012; Diaz et al. 2013; Rezende et al. 2013;), which infect individuals primarily through infected food items in their diet (Diaz et al. 2013). Several studies have reported negative effects of these parasites on fitness-related parameters. For example, the presence of *Contracaecum pelagicum* (Johnston and Mawson, 1942) is associated with ulcerative gastric lesions in Humboldt penguins (*Spheniscus humboldti* Meyen, 1834) (Yáñez et al. 2012). In this regard, adult Little penguins (*Eudyptula minor* Forster, 1781) found dead at coastal areas showed renal and intestinal coccidiosis, intestinal cestodiasis, and gastric ulceration associated with

3

Contracaecum sp. (Harrigan 1991). In addition, two recent experimental studies using antihelminthic treatments provide evidence of sublethal fitness costs of helminth parasitism in two Antarctic penguin species. Chinstrap penguin (*Pygoscelis antarctica*, Forster, 1781) chicks treated with antihelminthic drugs grew more and had better body condition than untreated, control chicks (Palacios et al. 2012), an effect likely to impact fitness due to the strongly size-biased mortality of chicks soon after independence (Moreno et al. 2001). Deparasited Gentoo penguin (*Pygoscelis papua* Murphy, 1947) chicks showed altered cell-mediated immunity, suggesting that these parasites can result in an immunological burden for penguin chicks (Bertellotti et al. 2016).

Given the diversity of immune components involved in defense against helminth parasites (Maizel et al. 2012), humoral effectors, in addition to cellular ones, might be altered in parasitized individuals. Thus, the objective of this study was to evaluate whether helminth parasites affect humoral as well as cellular components of the immune system of penguin chicks, using as a model the Magellanic penguin *(Spheniscus magellanicus* Forster, 1781*)*, a species that shows greater helminth parasitism than Antarctic penguin species (D'Amico et al. 2014). Magellanic penguins, which breed along the Patagonian coast of South America between 33°-54° S (Bertellotti 2013), show high prevalence, abundance, and intensity of several species of helminths, including Nematoda, Cestoda, Digenea, and Ancantocephala, with major taxa being *Contracaecum* sp., *Cosmocephalus* sp., *Corynosoma* sp., *Tetrabothrius* sp. and *Cardiocephaloides* sp. (Diaz 2006; Diaz et al., 2010). Helminth-associated mortality has been reported in heavily parasitized individuals of this species (Diaz 2006). D'Amico et al. (2014) suggested that the relatively high helminth parasitism could be the cause of some increased cellular immune components of adult Magellanic penguins when compared to adult Antarctic penguins (*P. antarctica* and *P. Adélie* Hombron and Jacquinot, 1841), but the potential effects of these parasites on chick immunity has not been investigated.

In order to evaluate whether helminth infection affects both cellular and humoral immunity in Magellanic penguin chicks, we measured cellular and humoral immune components after the administration of an antihelminthic treatment. Assessment of cellular components included the complete white blood cell (WBC) count, also known as leukocyte profile, and the *in vivo* skin-swelling response to phytohemagglutinin (PHA). Humoral aspects assessed were the ability of plasma to agglutinate foreign particles and the bactericidal capacity of plasma. The complete WBC count provides information on increases or decreases in each leukocyte type and can be diagnostic of infections and inflammatory conditions, including those mediated by parasites (Cambell 1995; Roitt et al. 2001). In particular, elevated eosinophil and monocyte counts are commonly associated with gastrointestinal parasitic infections (Roitt et al. 2001; Gebreselassie et al. 2012; Thrall et al. 2012). The PHA skin-swelling test is widely used for estimation of cell-mediated immune response *in vivo* (Martin et al. 2006; Tella et al. 2008). This inflammatory response integrates the activity of various immune cells involved in both innate (heterophils, eosinophils, basophils and monocytes) and acquired (lymphocytes) cellular responses to parasites (Goto et al. 1978; Martin et al. 2006). The ability of humoral components in plasma to agglutinate foreign particles, mediated mainly by natural antibodies, constitutes an important first line of defense against invading pathogens (Ochsenbein and Zinkeragel 2000), whereas the bactericidal capacity of plasma provides an index of complement system function (Matson et al. 2006). Both aspects of humoral immunity have been implicated in response to helminth infections (Moreau and Chauvin 2010; Maizel et al. 2012).

Medication approaches such as the one proposed in this study hypothesize that the deparasitation treatment frees energetic and nutritional resources that will influence immune function given the presumed costs of maintaining and/or using certain levels of immune defenses (e.g. Tomas et al. 2007; Bertellotti et al. 2016). Given that costs of maintenance and use vary among different immune components (Lee 2006), costly components might be more affected than less costly ones by the antihelminthic

treatment. Thus, based on the differential costs proposed by Lee (2006) we predicted that: 1) treated chicks would display lower WBC counts, particularly of the immune cells indicative of helminth infection (eosinophils and monocytes), because they should no longer carry the parasites and, therefore, they should not need to maintain an immune response to them, 2) treated chicks should be able to mount a stronger immune response to a novel challenge (PHA) than control chicks, as the local inflammatory response to PHA, which involves innate and adaptive immune cells, has a high cost of use, and 3) treated chicks should display similar (or slightly higher) values of bacterial agglutination and bactericidal capacity of plasma than controls, because the costs of maintaining and using innate humoral components is thought to be relatively low.

Materials and methods

Study site

The study was conducted at the Magellanic penguin colony of San Lorenzo (47°S, 63°W) in Peninsula Valdés, Chubut, Patagonia, Argentina, during early February 2013 of the breeding season. At this colony, chicks hatch in late November and reach their independence around early March (Bertellotti 2013). Given that the experiment involved 3 captures of each individual, we randomly selected 40 chicks (most of which were likely between 7 and 8 weeks of age) in order to guarantee an adequate sample size at the end of the experiment. Chicks were marked with a velcro ring around one of their flippers to allow individual identification during the sampling period. Rings were removed at the end of the experiment.

Experimental treatment

Following Bertellotti et al. (2016), we performed the experimental deparasitation of 20 chicks with an oral dose of 50 mg/kg of Albendazole and 5 mg/kg of Praziquantel diluted in purified water according to veterinarian deparasitation protocols (Tucker et al. 2007; Kahn 2010). Another set of 20 chicks was kept as control and received an equal

volume/kg of water as placebo. Benzimidazole and pyrazinoisoquinoline antihelminthic

drugs (as the ones used in this study) are common in public health and veterinary

medicine and are rapidly absorbed and effective producing tetanic contraction of the

parasite's musculature and rapid vacuolization of the integument within minutes to a

few hours of administration (Lacey 1990; Dayan 2003; Kahn 2010). Killing efficacies

greater than 90% for nematodes and cestodes have been reported in chickens (Tucker

et al. 2007; Saeed 2007). Because of their rapid action and efficiency, these drugs

have also been used as antihelminthic method of choice in studies in wild birds

(Grimes et al. 1989; Bustnes et al. 2007; Hanssen et al. 2003; Palacios et al. 2012;

Bertellotti et al. 2016). Helminth parasite loads of penguin chicks were not assessed in

the present study because this cannot be effectively performed by coprological

methods (Palacios et al. 2012), even when using molecular probes (Vidal 2014).

Nevertheless, we are confident that most individuals were parasitized based on

previous work reporting 100% prevalence of helminth parasites in Magellanic penguins

at our study site (Diaz 2006; Diaz et al. 2013). A blood sample was collected from all

chicks immediately before the antihelminthic treatment to assess their general health

status (i.e., body condition and hematological parameters). Individuals were then

weighed, measured (wing length, bill length), marked without distinction between

control and treatment, and then released at the site of capture.

PHA test, blood sampling, and WBC analyses

The skin-swelling response to the injection of PHA was performed 24 h after the

deparasitation treatment. Chicks (12 control and 16 deparasitized) were recaptured

and injected with 0.1 ml of a 2 mg/ml solution of phytohemagglutinin (PHA, Sigma,

L2646) in sterile phosphate buffered saline (PBS) at a marked site on the interdigital

membrane of the right foot (Moreno et al. 1998; Bertellotti et al. 2016). The thickness of

the foot web was measured with a digital thickness gauge with constant pressure

(Schwyz, model EDB-13) with an accuracy of 0.01 mm at the injection site just before

7

and 24 h after injection. The average of three thickness measures made by the same person (MGP) was considered. The swelling caused by PHA was calculated as the difference between both measurements (Smits et al. 1999). The remaining chicks (8 control and 4 deparasitized) could not be relocated at the time of the PHA test and were therefore dropped from the experiment. On the third capture (i.e., 48 h after deparasitation treatment and 24 h after PHA injection), a second blood sample was collected before measuring the swelling response. All individuals injected with PHA were recaptured the next day, thus the final sample sizes for the experiment were 12 control and 16 deparasitized chicks.

Blood samples were collected from the metatarsal vein with a 3-ml syringe, within 5 min of capture of the individuals to minimize capture and handling stress (Davis et al. 2008). Blood smears were immediately prepared and air-dried. Blood (~1 ml) was placed in heparinized eppendorf tubes and into microcapillary tubes. In the laboratory, smears were fixed in ethanol for 3 min and stained with Tinción 15 [®] (Biopur). Eppendorf tubes were centrifuged and plasma was separated, stored, and frozen at -20 ºC until further analysis. Blood smears were examined under a light microscope to obtain the complete WBC count. Total WBC was estimated by counting all cell types in 10 consecutive 400x monolayer fields (D'Amico et al. 2014). The proportion of each cell type was obtained from a sample of 100 WBC under 1000x (oil immersion) classified into basophils, heterophils, eosinophils, lymphocytes, and monocytes (Campbell 1995). The latter proportions were multiplied by the total WBC to obtain the corresponding total counts for each type of cell. The heterophil/lymphocyte ratio (H/L), described as a good measure of stress in birds (Davis et al. 2008), was also obtained. Ten smears were randomly selected for analysis of repeatability. Three consecutive counts were made using the described counting method for each of the 10 smears. Repeatability was calculated following Lessells and Boag (1987) for total WBC counts, heterophils, eosinophils, lymphocytes, and monocytes. Repeatability ranked

between 74 and 98%. Basophils were not included in statistical analyses because of the presence of many zeros. All white blood cell counts were made by VLD.

Humoral immune analyses

The ability of plasma components to agglutinate foreign particles, which provides an index of the constitutive levels of natural antibodies in circulation (Matson et al. 2006), was performed using a standard bacterial strain (*Escherichia coli* Migula, 1895, ATCC 8739) as foreign particles. A bacterial agglutination protocol previously developed for use in penguins was followed (D'Amico et al. 2014). Briefly, bacteria were grown in tryptic soy (TS) broth and then fixed in 1% formalin overnight at 4 ℃. Fixed bacteria were washed three times with PBS and adjusted to a concentration of approx. 1×10^9 bacteria/ml. Plasma samples (20 µl) were added to the first column of a 96-well plate and serially two-fold diluted along the rows with PBS. A negative control (PBS instead of plasma) was included in each plate. Then, 20 µl of fixed bacteria were added to all wells. Plates were vortexed, incubated at 40 ℃ for 60 minutes, and then left overnight at room temperature (~25 ℃). Agglutination titers were determined as -log2 of the highest dilution showing agglutination. Enough plasma for this assay was not available for 3 control and 2 deparasitized individuals, so sample sizes for bacterial agglutination were lower than for the remaining immune parameters.

The bactericidal capacity of plasma, which provides and index of the integrated killing ability of complement and other constitutive innate humoral factors, was performed following the original protocol by Matson et al. (2006) with modifications for use in penguins. Briefly, *Escherichia coli* (ATCC 8739) were suspended in PBS to produce a working solution containing approximately 200 colony-forming bacteria per 10 µl. All plasma samples were diluted 1:10 with sterile PBS, and sample reactions were prepared by adding 10 ml of the bacterial working solution to 90 µl of the diluted plasma samples. All sample reactions were incubated for 8 min at 40 ℃ to provide adequate time for bacterial killing to occur. Control reactions were prepared by adding

10 µl of the bacterial working solution to 90 µl PBS, and were plated before, midway, and after plating the sample reactions. All sample reactions and controls were plated using 50 µl aliquots on 4% tryptic soy agar and incubated overnight at room temperature. The number of bacterial colonies on each plate was then counted, and the percentage of colonies on each plate per the mean number of colonies in control plates was calculated. This percentage was subtracted from 100 to obtain the percentage of bacteria killed.

Statistical analyses

To test for the effects of experimental treatment on chick response variables, we used general linear models (GLM) that included treatment (deparasitized vs. control) as a fixed effect and the corresponding pre-treatment measurement as a covariate to control for initial values, with the exception of PHA response that was only measured post-treatment. Body condition (residuals of the regression of body mass on structural size) was evaluated as a potential additional covariate in all models but was kept in the final models only when significant. Structural size was estimated as the first component of a principal component analysis (PCA) that combined wing and bill lengths and explained 81% of variation in the data. Residual plots from the GLMs were visually inspected and no signs of non-normality were observed with the exception of lymphocyte counts, which were therefore $\log_{10}$-transformed before analysis. We decided to run univariate GLMs to maximize sample sizes for each dependent variable. However, given that some immune measures were intercorrelated (Table S1), we also performed a MANOVA including all immune measures to assess a multivariate anti-helminthic treatment effect. All statistical analyses were performed using JMP Pro 11.0.0 (SAS 2012). Raw data are depicted in tables and figures for visual clarity.

Ethical Considerations

Given the ethical and social responsibility that involves the scientific and technological activity, this study neither committed nor affected to human rights, nor did it cause any harm to the environment, animals and / or future generations. This study was carried out with the permits granted by the province of Chubut (Wildlife and Natural Resources and Conservation and Protected Areas offices) and considering the Nuremberg Code, the Helsinki Declaration and its amendments, and Universal Declaration on Human Genome and Human Rights adopted by the General Conference of UNESCO, 11/11/1997. This study followed the guidelines on the care and use of wildlife (Canadian Council on Animal Care, 2003 ISBN: 0-919087-39-6) and also this study did not compromise any rare, endangered or in extinction animal species.

Results

None of the variables measured before the administration of the treatment (i.e., body mass, bill length, wing length, body condition, leukocyte profile, bacterial agglutination titer, and bactericidal capacity of plasma) differed significantly between the control and the deparasitized group (ANOVA models for pre-treatment measurements, effect of treatment, all $P > 0.05$). Pre-treatment values of chicks (control and deparasitized groups pooled together) are presented in Table 1 as reference values for the species.

Univariate analyses showed that after antihelminthic treatment, total WBC counts were significantly lower in deparasitized than control chicks ($F_{1,25} = 25.4$; $P < 0.0001$), resulting in a significant decrease in the total counts of all leukocyte types in response to the treatment (H: $F_{1,25} = 16.21$; $P = 0.0005$; $\log_{10}$L: $F_{1,25} = 8.03$; $P = 0.009$; E: $F_{1,25} = 16.43$; $P = 0.0004$; M: $F_{1,25} = 35.10$, $P < 0.0001$; Figure 1 a-d, respectively). H/L ratios did not show statistical differences between groups ($F_{1,25} = 0.08$, $P = 0.78$). Contrary to our prediction, antihelminthic treatment resulted in significantly smaller foot-web swelling responses to PHA injection in deparasitized than in control birds ($F_{1,25} =$

24.18, $P < 0.0001$; Figure 2a). In addition, PHA response increased significantly with chick body condition independently of treatment ($F_{1,25} = 7.25$, $P = 0.013$; Figure 2b). Furthermore, a positive relationship was found between foot-web swelling response to PHA and total WBC count after treatment administration (Linear regression: $R^2 = 0.5$, $P < 0.0001$, Figure 2c), with no evidence of significant slope differences between groups (ANCOVA, interaction term: $P > 0.05$). On the other hand, antihelminthic treatment showed no effects on the two innate humoral immune components assessed: bacterial agglutination by natural antibodies ($F_{1,20} = 2.76$, $P = 0.11$, Figure 3a) and bactericidal capacity of plasma components ($F_{1,25} = 0.13$, $P = 0.72$, Figure 3b). The MANOVA analysis showed a significant multivariate main effect for treatment ($F_{7,15} = 6.48$, $P = 0.0012$). Given the significance of the overall test, univariate tests were analyzed and supported the findings of the separate GLMs with only one exception: the effect of treatment on total eosinophil count did not reach statistical significance ($F_{1,21} = 3.70$, $P = 0.068$).

Discussion

As predicted, antihelminthic treatment resulted in lower total WBC counts in Magellanic penguin chicks. This result is consistent with other studies where experimental parasitization with helminths led to an increase in total WBC counts, such as in Red jungle fowl (*Gallus gallus*, L., 1758) (Johnsen and Zuk 1999) and two species of gulls, the Great black-backed (*Larus marinus*, L., 1758) and the Herring gull (*Larus argentatus* Pontoppidan, 1763), which showed 1.5-fold increase in total WBC counts when infected (Kuklina 2007). These results support the role of circulating leukocytes in fighting macroparasite infections (Roitt et al. 2001) and are also in line with the idea that species facing greater challenges from helminth parasites should invest in higher number of circulating leukocytes (Bordes and Morand 2009). As usually found in birds (Campbell 1995), including penguins (D'Amico et al. 2014; D'Amico et al. 2016), heterophils and lymphocytes were the most abundant leukocytes, being

basophils, eosinophils, and monocytes in lower counts. Leukocyte profiles obtained before the experimental treatment serve as baseline data for Magellanic penguin chicks (Table 1).

Gastrointestinal helminth infection is usually associated with an increase of eosinophils (eosinophilia) and monocytes (monocytemia) in peripheral blood (Campbell 1995; Thrall et al. 2012;). In accordance with this, deparasitation treatment in Gentoo penguin chicks resulted in a reduction in these two leukocyte types (Bertellotti et al. 2016). Similarly, we observed a reduction of eosinophils (only significant in the GLM) and monocytes in deparasitized Magellanic penguin chicks, however, these chicks also had a decrease in heterophil and lymphocyte counts (Figure 1). Involvement of multiple effector cells in immune defense against helminths, including the five leukocyte types, has been reported (Falcone et al. 2001; Harris and Gause 2001; Maizel et al. 2012). Different parasites, however, may activate specific immune responses (Gause et al. 2003), perhaps contributing to the diversity in results observed among studies concerning the leukocyte types most associated with infection. For instance, helminth parasitism increased eosinophil and heterophil counts in Red jungle fowl, but decreased basophil numbers and did not affect lymphocytes and monocytes (Johnsen and Zuk 1999). Likewise, it has been suggested that helminth parasites could explain the increased heterophil counts in Gentoo penguins at northern sites of Antarctica (D'Amico et al. 2016).

Given the relatively high costs of mounting a cell-mediated inflammatory response to PHA (e.g. Martin et al. 2003; Lee 2006), we had predicted that antihelminthic-treated chicks, ridden of the burden of parasites, would be able to respond more strongly than controls. In fact, this was the observed result in Gentoo penguin chicks subjected to the same experimental treatment in Antarctica (Bertellotti et al. 2016) and is in accordance with the finding of a weaker response to PHA in Red jungle fowl experimentally parasitized with helminths (Johnsen and Zuk 1999). Nevertheless, in the present study, contrary to our expectation and the latter results,

deparasitized Magellanic penguin chicks showed lower response to PHA injection than control chicks (Figure 2a). In addition, the swelling response increased with body condition independently of treatment (Figure 2b), a relationship commonly documented in avian species (e.g., Navarro et al. 2003), including adult male Magellanic penguins (Moreno et al. 2001). Interestingly, the swelling response was also positively correlated with total WBC counts in circulation after the experimental treatment (Figure 2c); that is, individuals having higher numbers of WBC in circulation (i.e., control chicks) were the ones that mounted stronger inflammatory responses to PHA. Thus, the reduction in WBC counts in deparasitized chicks could have resulted in their lower skin-swelling responses.

Another potential explanation for the lower swelling response in antihelminthic-treated chicks could be linked to the particular helminth fauna of Magellanic penguins, as the specific composition of the helminth community can influence the response to PHA. For instance, swelling was not related to total helminth intensity in shrews (*Crocidura russula* Hermann, 1780), but instead it was negatively related to cestode intensity and positively related to nematode intensity, suggesting a potential immunosuppressive effect by the former and an immunostimulatory effect by the latter worm type (Goüy de Bellocq et al. 2007). On the other hand, experimental parasitization of Red jungle fowl with an intestinal nematode (*Ascaridia galli* Schrank, 1788) had a suppressive effect on the PHA response (Johnsen and Zuk 1999), highlighting that a suppressive or stimulatory effect might depend on the specific parasite species, host, or host-parasite combination. Thus, we hypothesize that the natural helminth community in Magellanic penguin chicks has an overall immunostimulatory effect on the PHA response, such that antihelminthic treatment results in reduced swelling with respect to controls. Nematodes, cestodes, and digeneans are found in high prevalence and intensity in Magellanic penguins (Diaz et al. 2010), thus further studies are warranted to evaluate the relationship between the specific helminth community and host immunity.

Contrary to the changes observed in cellular immunity, antihelminthic treatment did not alter the innate humoral immune parameters measured in Magellanic penguin chicks (Figure 3). The selected assays, despite using bacteria as foreign particles, integrate the activities of several innate humoral factors known to participate in defense against helminths, including neutralizing natural antibodies and components of the complement cascade. Regardless, our result supports the prediction that, given their relatively low costs of maintenance and use (Lee 2006), innate humoral components would not be as affected by antihelminthic treatment as more costly cellular responses. This finding is in accordance and complements those by other researchers regarding acquired humoral immune components, which are also thought to have relatively low cost of use (Lee 2006). For instance, experimental parasitization with helminths had no effect on immunoglobulin G (IgG) levels in Red jungle fowl (Johnsen and Zuk 1999) and antihelminthic treatment did not affect specific antibody production against sheep red blood cells (SRBC, primary and secondary responses) by village chickens showing natural helminth infections (Sassa et al. 2014). Further support for the differential costs of mounting cellular versus humoral responses comes from our finding of condition-dependence of the PHA swelling response (Figure 2b) but not of humoral components, as has also been reported for other bird species (e.g. Møller and Petrie 2002; Palacios et al. 2009).

Our results suggest that treatment to remove helminth parasites has an impact on cellular but not on innate humoral components of the immune system of Magellanic penguin chicks. This differential response follows predictions based on the relative costs of use and maintenance of these immune components, with more costly cellular responses being more affected than less costly humoral ones. Our study contributes to the knowledge on the diversity of responses of immune defenses to antihelminthic treatment in birds, showing contrasting results even within relatively closely related taxa such as different penguin species. The possibility of an immunostimulatory effect

on the swelling response to PHA by the specific helminth community in Magellanic

penguins deserves further study.

Acknowledgments

Literature cited

Bertellotti, M., D'Amico, V.L., Palacios, M.G., Barbosa, A., and Coria, N. 2016. Effects
of antihelminthic treatment on cell-mediated immunity in Gentoo penguin chicks.
Polar Biol. **39**: 1207-12012.

Bertellotti, M. 2013. Pingüino de Magallanes: Embajador de la Patagonia. 1st edition
Vázquez-Mazzini, Buenos Aires.

Bordes, F., and Morand, S. 2009. Coevolution between multiple helminth infestations
and basal immune investment in mammals: cumulative effects of polyparasitism?
Parasitol. Res. **106**: 33-37.

Bustnes, J.O., Erikstad, K.E., Hanssen, S.A., Tveraa, T., Folstad, I., and Skaare, J.U.
2006. Anti-parasite treatment removes negative effects of environmental

pollutants on reproduction in an Arctic seabird. Proc. R. Soc. Biol. Sci. **273**: 3117-3122.

Campbell ,T.W. 1995. Avian Hematology and Cytology, 2nd edition. University Press, Iowa State, Iowa.

Colditz, G. 2008. Six costs of immunity to gastrointestinal nematode infections. Parasite Immunol. **30**: 63–70.

D'Amico, V.L., Bertellotti, M., Diaz, J., Coria, N., Vidal, V., and Barbosa, A. 2014. Leukocyte levels in some Antarctic and non-Antarctic penguins. Ardeola, **61**: 145-152.

D'Amico, V.L., Coria, N., Palacios, M.G., Barbosa, A., and Bertellotti, M. 2016. Physiological differences between two overlapped breeding Antarctic penguins in a global change perspective. Polar Biol. **39**: 57-64.

Davis, A.K., Maney, D.L., and Maerz, J.C. 2008. The use of leukocyte profiles to measure stress in vertebrates: a review for ecologists. Funct. Ecol. **22**: 760-77.

Dayan A.D. 2003. Albendazole, mebendazole and praziquantel. Review of non-clinical toxicity and pharmacokinetics. Acta Tropica, **86**: 141-159.

Diaz, J.I., Cremonte, F., and Navone, G. 2010. Helminths of the Magellanic Penguin, *Spheniscus magellanicus* (Sphenisciformes), during the breeding season in Patagonian coast, Chubut, Argentina. Comp. Parasitol. **77**: 172–177.

Diaz, J.I., Fusaro, B., Longarzo, L., Coria, N.R., Vidal, V., Jerez, S., Ortiz, J., and Barbosa, A. 2013. Gastrointestinal helminths of Gentoo penguins (*Pygoscelis papua*) from Stranger point, 25 de Mayo/King George Island, Antarctica. Parasitol. Res. **112**: 1877-1881.

Diaz, J.I. 2006. Las comunidades parasitarias como expresión de distinto comportamiento trófico en aves del Mar Argentino. Doctoral Thesis, Universidad Nacional de La Plata, Argentina.

Falcone, F.H., Pritchard, D.I., and Gibbs, B.F. 2001. Do basophils play a role in immunity against parasites? Trends Parasitol. **17**: 126–129.

Fonteneau, F., Geiger, S., Marion, L., Le Maho, Y., Robin, J.P., and Kinsella, J.M. 2011. Gastrointestinal helminths of King penguins (*Aptenodytes patagonicus*) at Crozet Archipelago. Polar Biol. **34**: 1249–1252.

Gause, W.C., Joseph, F., Urban. Jr. J.F., and Stadecker, M.J. 2003. The immune response to parasitic helminths: insights from murine models. Trends Immunol. **24**: 275.

Gebreselassie, N.G., Moohead, A.R., Fabre, V., Gagliardo, L.F., Lee, N.A., Lee, J.J., and Appleton, J.A. 2012. Eosinophils preserve parasitic nematode larvae by regulating local immunity. J. Immunol. **188**: 417-425.

Goto, N., Kodama, H., Okada, K., and Fujimoto, Y. 1978. Suppression of phytohemagglutinin skin response in thymectomized chickens. Poult. Sci. **57**: 246–250.

Goüy de Bellocq, J., Ribas, A., Casanova, J.C., and Morand, S. 2007.
Immunocompetence and helminth community of the white-toothed shrew,
Crocidura russula from the Montseny Natural Park, Spain. Eur. J. Wildl. Res. **53**:
315–320.

Grimes, J., Suto, B, Greve, J.H., and Albers H.F. 1989. Effect of Selected Anthelmintics
on Three Common Helminths in the Brown Pelican (*Pelecanus occidentalis*). J.
Wildl. Dis. **25**: 139-142.

Hanssen, S.A., Folstad, I., Erikstad, K.E., and Oksanen, A. 2003. Costs of parasites in
common eiders: effect of antiparasite treatment. Oikos, **100**: 105–111.

Harrigan, K.E. 1991.Causes of Mortality of Little Penguins *Eudyptula minor* in Victoria.
Emu, **91**: 273-277.

Harris, N., and Gause, W.C. 2011. To B or not to B: B cells and the Th2-type immune
response to helminths. Trends Immunol. **32**: 80-88.

Johnsen, T.S., and Zuk, M. 1999. Parasites and tradeoffs in the immune response of
female red jungle fowl. Oikos, **86**: 487–492.

Johnston, T.H. and Mawson, P.M. 1942. Nematodes from Australian albatrosses and
petrels. Trans. R. Soc. S. Aust. **66**: 66-70.

Kahn, C.M. 2010. The Merck Veterinary Manual. 10th edition. Merck and Co., Inc.
Whitehouse Station, New York.

Kulklina, M.M. 2007. Specific features of metabolism in male Herring Gulls (*Larus argentatus*, Pontop.) on the Murman Coast. Dokl. Biol. Sci. **416**: 278-281.

Lee, K. 2006. Linking immune defenses and life history at the levels of the individual and the species. Integ. Comp. Biol. **46**: 1000-1015.

Lessells, C.M., and Boag, P.T. 1987. Unrepeatable repeatabilities: a common mistake. The Auk, **104**: 116-121.

Lochmiller, R.L., and Deerenberg, C. 2000. Trade-offs in evolutionary immunology: just what is the cost of immunity? Oikos, **88**: 87–98.

Maizel, R.M., Hewitson, J.P., and Smith, K.A. 2012. Susceptibility and immunity to helminth parasites. Curr. Opin. Immunol. **24**: 459–466.

Martin, L.B., Han, P., Lewittes, J., Kuhlman, J.R., Klasing, K.C., and Wikelski, M. 2006. Phytohemagglutinin-induced skin swelling in birds: histological support for a classic immunoecological technique. Funct. Ecol. **20**: 290–299.

Martin, L.B., Scheuerlein, A., and Wikelski, M. 2003. Immune activity elevates energy expenditure of house sparrows: a link between direct and indirect costs? Proc. R. Soc. Lond. Biol. Sci. **270**: 153–158.

Matson, K.D., Tieleman, B.I., and Klasing, K.C. 2006. Capture stress and the bactericidal competence of blood and plasma in five species of tropical birds. Physiol. Biochem. Zool. **79**: 556-564.

Møller, A.P., and Petrie, M. 2002. Condition dependence, multiple sexual signals, and immunocompetence in peacocks. Behav. Ecol. **13**: 248–253.

Moreau, E., and Chauvin, A. 2010. Immunity against helminths: interactions with the host and the intercurrent infections. J. Biomed. Biotechnol. Article ID 428593. http://dx.doi.org/10.1155/2010/428593

Moreno, J., de León, A., Fargallo, J.A., and Moreno, E. 1998. Breeding time, health and immune response in the chinstrap penguin *Pygoscelis antarctica*. Oecologia, **115**: 312-329.

Moreno, J., Potti, J., Yorio, P., and Borboroglu, P.G. 2001. Sex differences in cell-mediated immunity in the Magellanic penguin *Spheniscus magellanicus*. Ann. Zool. Fenn. **38**: 111-116.

Navarro, C., Marzal, A., de Lope F., and Møller, A.P. 2003. Dynamics of an immune response in house sparrows *Passer domesticus* in relation to time of day, body condition and blood parasite infection. Oikos, **101**: 291–298.

Ochsenbein, A.F., and Zinkernagel, R.M. 2000. Natural antibodies and complement link innate and acquired immunity. Immunol. Today, **21**: 624–630.

Palacios, M.G., Cunnick, J.E., Vleck, D., Vleck, C.M. 2009. Ontogeny of innate and adaptive immune defense components in free-living tree swallows, *Tachycineta bicolor*. Dev. Comp. Immunol. **33**: 456-463.

Palacios, M.J., Valera, F., and Barbosa, A. 2012. Experimental assessment of the effects of gastrointestinal parasites on offspring quality in chinstrap penguins (*Pygoscelis antarctica*). Parasitology, **139**: 19-824.

Rezende, G.C., Baldassin, P., Gallo, H., and Silva, R.J. 2013. Ecological aspects of helminth fauna of Magellanic penguins *Spheniscus magellanicus* (aves: Spheniscidae) from the Northern Coast of the State of Sao Paulo, Brazil. Braz. J. Biol. **73**: 61-66.

Roitt, I., Brostoff, J., and Male, D. 2001. Immunology. 6th edition. Mosby International Ltd, London.

Saeed, A.M.A. 2007. Efficacy of albendazole against experimental *Raillietina tetraqona* infection in chickens. Res. J. Pharmacol.**1**: 5-8.

SAS 2012. Institute. Using JMP. JMP, A Business Unit of SAS. SAS Campus Drive Cary.

Sassa, N., Siwila, J., M'kandawire, E., and Nalubamba, K.S. 2014. Antibody response of non dewormed and dewormed village chickens to sheep red blood cells. Poult. Sci. **13**: 353-356.

Smits, J.E., Bortolotti, G.R., and Tella, J.L. 1999. Simplifying the phytohemagglutinin skin testing technique in studies of avian immunocompetence. Funct. Ecol. **13**: 567-572.

Tella, J.L., Lemus, J.A., Carrete, M., and Blanco, G. 2008. The PHA test reflects
    acquired T-cell mediated immunocompetence in birds. PLoS One, **3**: e3295,
    doi:10.1371/journal.pone.0003295.

Thrall, M.A., Weiser, G., Allison, R., and Campbell, T.W. 2012. Veterinary Hematology
    and Clinical Chemistry. 2nd ed. Wiley-Blackwell New, York.

Tomas, G., Merino, S., Moreno, J., Morales, J., and Martínez de la Puente, J. 2007.
    Impact of blood parasites on immunoglobulin level and parental effort: a
    medication Weld experiment on a wild passerine. Funct. Ecol. **21**: 125–133.

Tucker, C.A., Yazwinski, T.A., Reynolds, L., Johnson, Z., and Keating, M. 2007.
    Determination of the anthelmintic efficacy of albendazole in the treatment of
    chickens naturally infected with gastrointestinal helminths. J. Appl. Poult. Res.
    16: 392-396.

Vidal, V., Ortiz, J., Diaz, J.I., Ruiz de Ybañez, M.R., Amat, M., Palacios, M.J., Benzal,
    J., Valera, F., de la Cruz, C., Motas, M., and Barbosa, A. 2012. Gastrointestinal
    parasites in Chinstrap Penguins from Deception Island, South Shetlands,
    Antarctica. Parasitol. Res. **111**: 723-727.

Vidal, V. 2014. Parasitofauna de pingüinos Pigoscélidos: morfología, infección,
    caracterización molecular y aplicaciones (Parasite fauna of Pygoscelid penguins:
    morphology, infection, molecular characterization and applications). Doctoral
    Thesis. University of Murcia, España.

Wakelin, D., and Apanius, V. 1997. Immune defence: genetic control. *In*: Host-Parasite
    Evolution, General Principles and Avian Models. Edited by D.H. Clayton and J.
    Moore. University Press, Oxford. pp 30–58.

Yáñez, F., Fernández, I., Campos, V., Mansilla, M., Valenzuela, A., González, H.,
    Rodríguez, C., Rivas, M., Alveal, K., and Oyarzún, C. 2012. First pathological
    report of parasitic gastric ulceration in Humboldt penguin (*Spheniscus humboldti*)
    along the coast of south-central Chile. Lat. Am. J. Aquat. Res. **40**: 448-452.

Table 1. Morphometric, hematological, and immune parameters of 7-8 week-old Magellanic penguin (*Spheniscus magellanicus* Forster, 1781) chicks at the reproductive colony of San Lorenzo. All values correspond to pre-treatment measurements pooling control and deparasitized chicks (*n* = 40). Leukocyte counts represent the number of each type of leukocyte in 10 consecutive microscope fields at 400x. WBC= white blood cells; H/L ratio= heterophil/lymphocyte ratio; BC= bactericidal capacity of plasma; AGG= bacterial agglutination.

Parameters	Mean ± Standard Deviation	Median	Range Minimum-Maximum
Mass (g)	2446.3 ± 426	2475	1600 - 3500
Bill length (mm)	45.2 ± 3	45.8	40 - 51
Wing length (mm)	184.0 ± 8	184	170 - 198
Total WBC	74.4 ± 23	69	41 - 137
Total Basophils	0.1 ± 0.03	0	0 - 1
Total Eosinophils	7.5 ± 5.7	6.3	0 - 29
Total Heterophils	35.5 ± 15.8	31.8	13 - 78
Total Lymphocytes	23 ± 7.5	23.2	9 - 43
Total Monocytes	8.2 ± 4	7.3	3 - 20
Basophils (%)	0.2 ± 0.4	0	0 - 2
Eosinophils (%)	9.9 ± 6	9.1	0 - 25
Heterophils (%)	46.7 ± 11	46.9	27 - 70
Lymphocytes (%)	31.9 ± 9	32.8	16 - 51
Monocytes (%)	11.3 ± 4	11.3	5 - 24
H/L ratio	1.7 ± 1	1.3	1 - 4
BC (%)	68.5 ± 31	82	10 - 100
AGG (titer)	6.6 ± 1	6.5	6 - 8

Figure captions

Figure 1. Leukocyte profiles of control (C) and deparasitized (DP) Magellanic penguin (*Spheniscus magellanicus* Forster, 1781) chicks after antihelminthic treatment. a) heterophil counts, b) lymphocyte counts, c) eosinophil counts, d) monocyte counts. Leukocyte counts represent the number of each cell type in 10 consecutive fields at 400x. Box plots depict medians (horizontal lines inside boxes), 25 and 75 percentiles (edges of boxes), and extreme values (whiskers). Asterisks indicate significant differences between treatment groups based on the GLMs.

Figure 2. Phytohemagglutinin response (foot-web swelling) of control (C) and deparasitized (DP) Magellanic penguin (*Spheniscus magellanicus* Forster, 1781) chicks after antihelminthic treatment. a) Box plots depict medians (horizontal lines inside boxes), 25 and 75 percentiles (edges of boxes), and extreme values (whiskers). Asterisk indicates significant differences between treatment groups based on the GLMs., b) residual foot-web swelling (controlling for treatment group) as a function of chick body condition, c) foot-web swelling as a function of total white blood cell (WBC) counts of chicks.

Figure 3. Innate humoral immune parameters of control (C) and deparasitized (DP) Magellanic penguin (*Spheniscus magellanicus* Forster, 1781) chicks after antihelminthic treatment. a) bacterial agglutination titer, b) bactericidal capacity of plasma (% bacterial killed). Box plots depict medians (horizontal lines inside boxes), 25 and 75 percentiles (edges of boxes), and extreme values (whiskers).
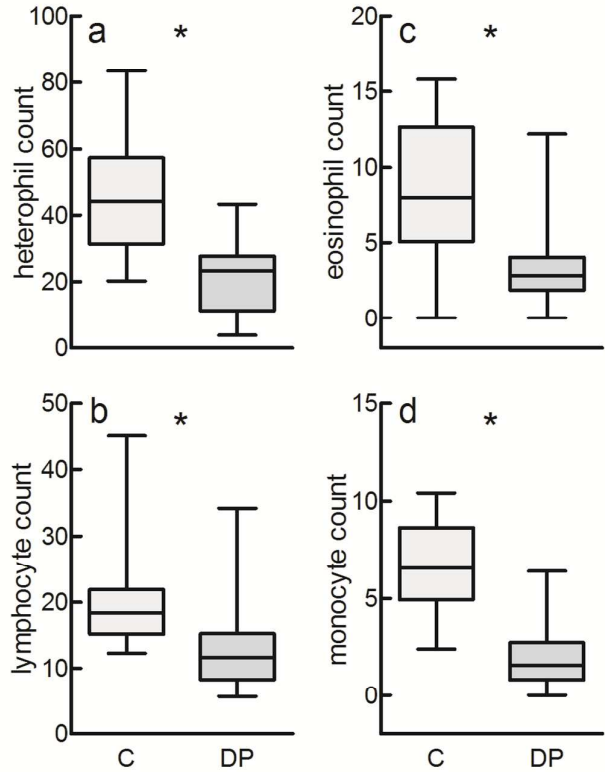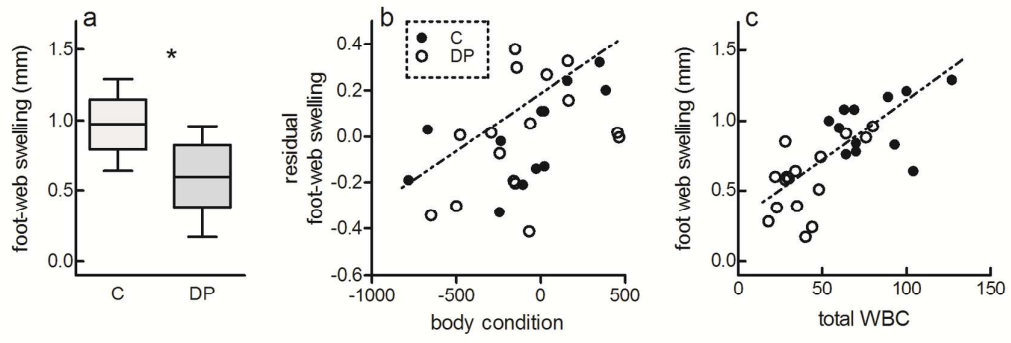
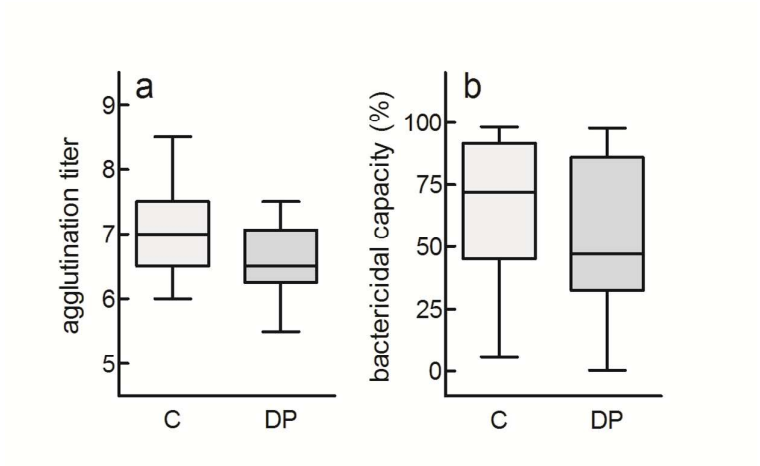**Figure 1**

Figure 2

Figure 3

Table 1. Morphometric, hematological, and immune parameters of 7-8 week-old Magellanic penguin (*Spheniscus magellanicus* Forster, 1781) chicks at the reproductive colony of San Lorenzo. All values correspond to pre-treatment measurements pooling control and deparasitized chicks (n = 40). Leukocyte counts represent the number of each type of leukocyte in 10 consecutive microscope fields at 400x. WBC= white blood cells; H/L ratio= heterophil/lymphocyte ratio; BC= bactericidal capacity of plasma; AGG= bacterial agglutination.

Parameters	Mean ± Standard Deviation	Median	Range Minimum-Maximum
Mass (g)	2446.3 ± 426	2475	1600 - 3500
Bill length (mm)	45.2 ± 3	45.8	40 - 51
Wing length (mm)	184.0 ± 8	184	170 - 198
Total WBC	74.4 ± 23	69	41 - 137
Total Basophils	0.1 ± 0.03	0	0 - 1
Total Eosinophils	7.5 ± 5.7	6.3	0 - 29
Total Heterophils	35.5 ± 15.8	31.8	13 - 78
Total Lymphocytes	23 ± 7.5	23.2	9 - 43
Total Monocytes	8.2 ± 4	7.3	3 - 20
Basophils (%)	0.2 ± 0.4	0	0 - 2
Eosinophils (%)	9.9 ± 6	9.1	0 - 25
Heterophils (%)	46.7 ± 11	46.9	27 - 70
Lymphocytes (%)	31.9 ± 9	32.8	16 - 51
Monocytes (%)	11.3 ± 4	11.3	5 - 24
H/L ratio	1.7 ± 1	1.3	1 - 4
BC (%)	68.5 ± 31	82	10 - 100
AGG (titer)	6.6 ± 1	6.5	6 - 8

1