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Effects of antihelminthic treatment on cell-mediated immunity in Gentoo penguin chicks

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Abstract Intestinal parasites suppose a cost to hosts as they compete directly for nutritional resources. Therefore, hosts must defend themselves against intestinal parasites by mounting an immune response. Many penguin species acquire parasites through their diet and transfer these parasites to their chicks when feeding them. High parasite loads in penguin chicks could have effects on their growth and body condition, and ultimately on their survival. Here, we evaluated the effect of parasites on the cell-mediated immune system in Gentoo penguin (*Pygoscelis papua*) chicks at Stranger Point (25 de Mayo/King George Island, South Shetland Islands). To this end, 12 chicks were experimentally deparasitized with a mixture of anthelminthic drugs (albendazole and praziquantel), whereas 10 others were kept as control. We measured cutaneous cell-mediated immunity in response to immunization with phytohemagglutinin (PHA). We also analyzed the leukocyte profile in both treated and control groups before and after the treatment. After the treatment, deparasitized birds showed larger foot-web swelling in response to PHA injection than control birds. Deparasitized penguins also showed lower eosinophil and monocyte counts than controls, whereas heterophils, lymphocytes, and total white blood cell counts did not differ between groups. Our results suggest that Gentoo penguin chicks parasitized with intestinal parasites suffer a cost in terms of reduced cell-mediated immune responses that could ultimately affect their survival.

Keywords Gentoo penguins · Immunity · Leukocyte counts · Parasites · Phytohemagglutinin

Introduction

Birds can act as hosts for several kinds of parasites, which can be important causes of morbidity or mortality (Wakelin and Apanius 1997). Among them, gastrointestinal parasites are present in a large number of species (Janovy 1997) and are of particular interest because they suppose not only a direct cost to the host as they compete for nutritional resources required for growth and maintenance (Lochmiller and Deerenberg 2000; Palacios et al. 2012), but also an indirect cost through resources spent by the host on antiparasite defense (Wakelin 1978). Investment in immunity is likely to be condition-dependent as ultimately the rate of resource use determines the amount of resources available for allocation among competing body functions (Nelson et al. 2002). Gastrointestinal parasites cause characteristic immune responses by the host (Colditz 2008), activating both constitutive defenses, including macrophages, granulocytes, natural killer cells, complement, lysozyme, nonspecific antibodies, and other humoral compounds, and inducible defenses, including cytokine-mediated inflammation (Lee 2006).

Although it has been argued that parasitism has little impact in polar regions because parasites seem to be scarcer and their overall effect on hosts weaker at higher latitudes (Barbosa and Palacios 2009), some Antarctic

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penguin species show great diversity and prevalence of different parasites (Barbosa and Palacios 2009; Diaz et al. 2013). The Gentoo penguin (*Pygoscelis papua*) is infested by at least 10 species of gastrointestinal parasites, including *Ascaridia* sp., *Contracaecum heardi*, *Corynosoma bullosum*, *Corynosoma shackletoni*, *Parorchites zederi*, *Stegophorus macronectes*, *Tetrameres wetzeli*, *Stomachus* sp., *Streptocara* sp., and *Tetrabothrius pauliani* (see review by Barbosa and Palacios 2009; Diaz et al. 2013). In fact, helminth parasites incorporated by penguin chicks through food regurgitated by their parents have important effects on their growth rates, which can ultimately impact their survival (Palacios et al. 2012). However, the potential effect of these parasites on other important fitness-related components of Antarctic penguin chicks, such as immune defenses, has not been investigated.

The objective of this study was to evaluate whether some cellular components of the immune system of Gentoo penguin chicks are affected by gastrointestinal parasites. To test this, we analyzed the response to the phytohemagglutinin (PHA)-skin test and the leukocyte profile after the administration of anthelminthic drugs to remove the parasites present in the chicks. The PHA-skin-swelling test is the most widely used test available to estimate cell-mediated immune response in vivo (Smits et al. 1999; Tella et al. 2008). The PHA-skin test involves subcutaneous injection of a mitogen (PHA) and subsequent measurement of the skin-swelling response (Smits et al. 1999). This inflammatory response integrates the activity of various immune cells involved in both the innate (heterophils, eosinophils, basophils, and monocytes) and the acquired (lymphocytes) response (Goto et al. 1978; Martin et al. 2006). This response is highly condition-dependent, with individuals in better condition generally mounting stronger responses (Tella et al. 2008). Leukocyte profiles provide information on increases or decreases in each leukocyte type and can be diagnostic of infections and inflammatory conditions, including those mediated by parasites (Campbell 1995; Roitt et al. 2001). In particular, among the different leukocyte types, eosinophils are directly associated with gastrointestinal parasitic infections (Roitt et al. 2001; Thrall et al. 2012).

We predicted that the effects of the deparasitation treatment would be reflected on the immune response parameters of the chicks. In particular, considering that the immune response is energetically costly (Martin et al. 2003), if the anthelminthic treatment frees resources that were previously being consumed by the parasites, then treated chicks should be able to mount a stronger immune response to a novel challenge (PHA) than control chicks. On the other hand, chicks treated with the anthelminthic drug would display lower values of the immune components indicative of infection (particularly eosinophils) because they should no longer carry live parasites and, therefore, should not need to mount an immune response to them.

Materials and methods

Study area and species

The study was conducted at Stranger Point (62.25°S; 58.67°W) in the Isla 25 de Mayo/King George Island, South Shetland Islands, during the breeding season in January 2012. The Gentoo penguin, one of the most widespread penguin species, breeds on sub-Antarctic and Antarctic Islands and on the Antarctic Peninsula, from 46° to 65°S (Lescroël et al. 2009). The modal clutch size is two eggs, and most broods consist of two chicks. After a 1-month incubation period, the eggs hatch and chicks remain in the nest for approximately another month. Thereafter, the chicks are grouped in nurseries or crèches until independence in the first weeks of February (Bertellotti 2013).

Experimental protocol

We selected 22 Gentoo penguin chicks during the crèches stage. Chick mean age was 69 days (SD = 15), as estimated from a growth model (Lescroël et al. 2009). Gastrointestinal parasite loads of these chicks were not assessed because this cannot be effectively performed by coprological methods (Palacios et al. 2012), even when using molecular probes (Vidal 2014). Nevertheless, recent work at our study site has shown that the prevalence of gastrointestinal parasites in Gentoo penguin chicks older than 20 days is 100 %, with relatively high intensities of parasitism (Diaz et al. 2013). Thus, we are confident that most chicks in the present study were parasitized. Twelve chicks were deparasitized 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), whereas other 10 chicks were kept as a control set, providing them an equal volume/kg of water as placebo. These drugs are effective within minutes of administration producing tetanic contraction of the parasite's musculature and rapid vacuolization of the integument (Kahn 2010). A blood sample was collected from each chick before treatment to test the general health status assessed by body condition and blood biochemical and hematological parameters. Immunization with PHA

was performed 24 h after the treatment, and measurement of the swelling response took place 48 h after the treatment. At this time, a second blood sample was collected. All individuals were weighed, measured, marked without distinction between control and treatment chicks, and then released at the site of capture after each manipulation.

PHA assay

Twenty-four hours after deparasitation, chicks were 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 (see Moreno et al. 1998). 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 and 24 h after injection. The average of three thickness measures made by the same person (MB) was considered. The swelling caused by PHA was calculated as the difference between both measurements (Smits et al. 1999). The maximum response to PHA is 24 h post-challenge, and the swelling disappears after 48 h (Smits et al. 1999).

Blood processing and analysis

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, S.R.L., Rosario, Argentina). Blood samples were centrifuged, and plasma was separated, stored, and frozen until further analysis for biochemical determinations. Plasma was processed on a spectrophotometer (METROLAB 1600 Plus, UV–Vis Metrolab S.A., Argentina) to determine total proteins (g/dl), cholesterol (mg/dl), triglycerides (mg/dl), and glucose (mg/dl). Microcapillary tubes were spun in a centrifuge for 12 min at 12,000*g*, and hematocrit was determined by measuring the proportion of red cells using a microhematocrit ruler calibrated in %. Blood smears were examined under a light microscope to obtain the leukocyte profile. Total leukocyte counts were estimated by counting all leukocytes in 10 consecutive 400× monolayer fields (Hale and Briskie 2007). The proportion of each leukocyte type was obtained from a sample of 100 leukocytes under 1000× (oil immersion) classified into basophils, heterophils, eosinophils, lymphocytes, and monocytes (Campbell 1995). The latter proportions were multiplied by the total leukocyte count to obtain the corresponding counts for each type of leukocyte.

Statistical analyses

To test for the effects of experimental treatment on chick response variables, we used general linear models that included treatment (deparasitized vs. control) as a fixed effect and the corresponding pre-treatment measurement (with the exception of PHA response, which was only measured post-treatment) as a covariate to control for initial values. Body mass, structural size (bill length and height), and body condition (residuals of the regression of body mass on structural size) were evaluated as potential additional covariates, but did not explain significant variation in any of the response variables (all $P > 0.05$) and were therefore excluded from the final models. Residual plots were visually inspected, and no signs of nonnormality were observed. However, the PHA swelling test showed unequal variance between groups and was therefore analyzed using a nonparametric (Wilcoxon) test. All statistical analyses were performed using JMP Pro 10.0.0 (SAS Institute 2012).

Results and discussion

None of the variables measured (i.e., body mass, bill length and height, blood biochemical and hematological parameters) differed significantly between control and deparasitized groups before the administration of the treatment (ANOVA models for pre-treatment measurements, effect of treatment, all $P > 0.05$). These results allow us to ensure that there were no differences between groups before administration of the antiparasitic drug.

As predicted, our results show that the deparasitation treatment affected some of the components of the immune system measured in this study (Table 1). Birds in the deparasitized group showed larger foot-web swellings in response to PHA injection than control birds (Table 1; Fig. 1). This result is in accordance with the prediction that deparasitized chicks should be able to mount a stronger PHA response, suggesting that birds without the burden of parasites would have more energetic/nutritional resources available to invest in cell-mediated immune responses to novel challenges. Palacios et al. (2012) used a similar experimental approach, treating Chinstrap penguin chicks with anthelminthic drugs and comparing them to their siblings treated with PBS as a control. These authors demonstrated that chicks treated with antiparasitic drugs increased their body mass more than control birds, supporting the prediction that gastrointestinal parasite loads affect their growth rate and deteriorate their body

Table 1 Mean values (SE between parentheses) of parameters measured in penguins after the treatment with antihelminthic drugs and in the control group

Parameter	Treatment	Control	Statistic	*P* values
Basophils	0.1 (0.1)	0.5 (0.2)	$F_{1,21} = 0.99$	0.33
Eosinophils	1.3 (0.5)	2.8 (0.3)	$F_{1,21} = 8.1$	**0.01**
Heterophils	35.6 (4.9)	30.4 (2.5)	$F_{1,21} = 0.001$	0.99
Lymphocytes	19.5 (2.4)	18.3 (1.9)	$F_{1,21} = 0.27$	0.61
Monocytes	0.9 (0.3)	2.7 (0.5)	$F_{1,21} = 16.0$	**0.0008**
Total WBC	57.3 (7.4)	54.4 (2.9)	$F_{1,21} = 0.46$	0.51
H/L ratio	1.9 (0.1)	1.5 (0.2)	$F_{1,21} = 0.05$	0.83
PHA response	1.283 (0.088)	0.167 (0.096)	$Z = -3.92$	**<0.0001**

Cell counts represent the number of each cell type counted in 10 consecutive 400× microscope fields. PHA response refers to inflammation (mm) measured 24 h after the injection of phytohemagglutinin. Significant differences between control and treatment groups are shown in bold

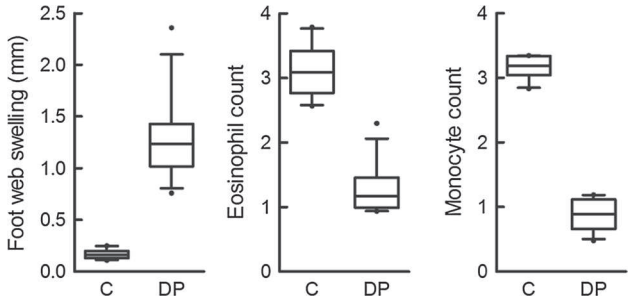

Fig. 1 Box plots of immune function variables showing significant differences between control (C) and deparasitized (DP) Gentoo penguin chicks. Plots depict medians (*horizontal lines inside boxes*), 25 and 75 percentiles (*edges of boxes*), 10 and 90 percentiles (*whiskers*), and outlying points included in the analyses (*dots*)

condition. Our results also support the notion that natural parasites do represent a burden for Antarctic penguins, extending previous findings to show that individuals freed of the cost associated with parasites are able to mount stronger cell-based immune responses. This result reinforces the idea that the immune response is energetically costly (Martin et al. 2003) and is consistent with results found by Moreno et al. (1998) showing that Chinstrap penguins in better condition are able to mount a stronger cellular immune response to PHA than individuals in poorer condition.

Regarding the leukocyte profile, our results show that deparasitized birds displayed a significant decrease in eosinophil and monocyte counts compared to controls (Table 1; Fig. 1). Both types of leukocytes are related to helminth infections (Campbell 1995; Davis et al. 2008). The increase in both eosinophils and monocytes is characteristic of gastrointestinal helminth infestations, particularly those by nematodes (Thrall et al. 2012), which interestingly constitute one of the main gastrointestinal parasite groups found in Gentoo penguins in Stranger Point (Diaz et al. 2013). Therefore, our results reinforce the role of these two leukocyte types in the defense against gastrointestinal parasites. Lymphocytes and heterophils were the most abundant cells in the peripheral blood of both treated and control Gentoo chicks (Table 1), which is usual in birds (Campbell 1995), including penguins (D'Amico et al. 2014). Basophils, heterophils, lymphocytes, total white blood cell counts, and the heterophil to lymphocyte ratio, an index of stress in birds (Davis et al. 2008), showed no significant differences between groups (Table 1).

It is interesting to note that our results are also in accordance with the Th1–Th2 paradigm of regulation of immune responses. To resolve infections, mammals and also birds have evolved immune reactions regulated by antigen-specific T helper (Th) cells that are reciprocally downregulated (Degen et al. 2005). In general, Th1 cells orchestrate inflammatory responses that are effective against intracellular parasites, whereas Th2 cells orchestrate an optimal reaction against metazoan parasites via antibody production (including IgE responses) and promotion of mast cell and eosinophil proliferation and function (Muraille and Leo 1998). Our results support the idea that chicks parasitized with gastrointestinal parasites are mounting a Th2 immune response and therefore show reduced ability to mount a cell-mediated Th1 inflammatory response when challenged with PHA.

In summary, deparasitation increased cell-mediated immunity through a stronger PHA inflammatory response and reduced the number of immune cells specialized in fighting gastrointestinal parasites (eosinophils and monocytes). This suggests that gastrointestinal parasites constitute a burden to Gentoo penguin chicks, having effects on specific components of their immune systems, which could in turn reduce their survival (Møller and Saino 2004). Thus, together with the results by Palacios et al. (2012) showing enhanced growth of deparasitized penguin chicks, our results on the immune response provide further evidence that parasites can have important negative effects on

these organisms. These results have great importance in a scenario of climate change in the Antarctic Peninsula, considering that penguins can become exposed to novel parasites either caused by changes in their diet due to decreased krill stocks (Atkinson et al. 2004) or in the distribution of parasites themselves (Harvell et al. 2002). Some of these changes could produce a cascade of physiological effects on penguins, including those on immune function (Xavier et al. 2013), making them more susceptible to emerging diseases in the face of increasing human activities in Antarctica (Grimaldi et al. 2014).

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References

Atkinson A, Siegel V, Pakhomov E, Rothery P (2004) Long-term decline in krill stock and increase in salps within the Southern Ocean. Nature 432:100–103. doi:10.1038/nature02996

Barbosa A, Palacios MJ (2009) Health of Antarctic birds: a review of their parasites, pathogens and diseases. Polar Biol 32:1095–1115. doi:10.1007/s00300-009-0640-3

Bertellotti M (2013) Pingüino de Magallanes: Embajador de la Patagonia. Vázquez Mazzini, Buenos Aires **60**

Campbell TW (1995) Avian hematology and cytology, 2nd edn. Iowa State University Press, Ames **105**

Colditz G (2008) Six costs of immunity to gastrointestinal nematode infections. Parasite Immunol 30:63–70. doi:10.1111/j.1365-3024.2007.00964.x

D'Amico V, Bertellotti M, Díaz J, Coria N, Vidal V, Barbosa A (2014) Leukocyte levels in some Antarctic and non-Antarctic penguins. Ardeola 61:145–152. doi:10.13157/arla.61.1.2014.145

Davis AK, Maney DL, Maerz JC (2008) The use of leukocyte profiles to measure stress in vertebrates: a review for ecologists. Funct Ecol 22:760–777. doi:10.1111/j.1365-2435.2008.01467.x

Degen WGJ, van Daal N, Rothwell L, Kaiser P, Virgil EJC, Schijns VEJC (2005) Th1/Th2 polarization by viral and helminth infection in birds. Vet Microbiol 105:163–167. doi:10.1016/j.vetmic.2004.12.001

Diaz JI, Fusaro B, Longarzo L, Coria NR, Vidal V, Jerez S, Ortiz J, 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. doi:10.1007/s00436-013-3341-3

Goto N, Kodama H, Okada K, Fujimoto Y (1978) Suppression of phytohemagglutinin skin response in thymectomized chickens. Poult Sci 57:246–250. doi:10.3382/ps.0570246

Grimaldi W, Seddon PJ, Lyver POB, Nakagawa S, Tompkins DM (2014) Infectious diseases of Antarctic penguins: current status and future threats. Polar Biol. doi:10.1007/s00300-014-1632-5

Hale KA, Briskie JV (2007) Decreased immunocompetence in severely bottlenecked population of an endemic New Zealand bird. Anim Conserv 10:2–10. doi:10.1111/j.1469-1795.2006.00059.x

Harvell CD, Mitchell CE, Ward JR, Altizer S, Dobson AP, Ostfeld RS, Samuel MD (2002) Climate warming and disease risks for terrestrial and marine biota. Science 296:2158–2163

Janovy J (1997) Protozoa, helminths, and arthropods of birds. In: Clayton DH, Moore J (eds) Host-parasite evolution. General principles and avian models. Oxford University Press, Oxford, pp 303–337

Kahn CM (ed) (2010) The Merck veterinary manual, 10th edn. Merck & Co., Inc., Whitehouse Station

Lee K (2006) Linking immune defenses and life history at the levels of the individual and the species. Integr Comp Biol 46:1000–1015. http://www.jstor.org/stable/3884887

Lescroël A, Bajzak C, Bost CA (2009) Breeding ecology of the gentoo penguin *Pygoscelis papua* at Kerguelen Archipelago. Polar Biol 32:1495–1505. doi:10.1007/s00300-009-0647-9

Lochmiller RL, Deerenberg C (2000) Trade-offs in evolutionary immunology: just what is the cost of immunity? Oikos 88:87–98. doi:10.1034/j.1600-0706.2000.880110.x

Martin LB, Scheuerlein A, Wikelski M (2003) Increased immune activity elevates energy expenditure of house sparrows: a link between proximate and ultimate costs of immune activity? P Roy Soc Lond B Bio 270:153–158. doi:10.1098/rspb.2002.2185

Martin LB, Han P, Lewittes J, Kuhlman JR, Klasing KC, Wikelski M (2006) Phytohemagglutinin-induced skin swelling in birds: histological support for a classic immunoecological technique. Funct Ecol 20:290–299. doi:10.1111/j.1365-2435.2006.01094.x

Møller AP, Saino N (2004) Immune response and survival. Oikos 104:299–304. doi:10.1111/j.0030-1299.2004.12844.x

Moreno J, de León A, Fargallo JA, Moreno E (1998) Breeding time, health and immune response in the chinstrap penguin *Pygoscelis antarctica*. Oecologia 115:312–329. doi:10.1007/s004420050522

Muraille E, Leo O (1998) Revisiting the Th1/Th2 paradigm. Scand J Immunol 47:1–9. doi:10.1111/j.1365-3083.1998-47-1.00383.x

Nelson RJ, Demas GE, Klein SL, Kriegsfeld LK (2002) Seasonal patterns of stress, immune function and disease. Cambridge University Press, Cambridge **308**

Palacios MJ, Valera F, Barbosa A (2012) Experimental assessment of the effects of gastrointestinal parasites on offspring quality in chinstrap penguins (*Pygoscelis antarctica*). Parasitology 139:819–824. doi:10.1017/S0031182011002381

Roitt I, Brostoff J, Male D (eds) (2001) Immunology, 6th edn. Mosby International Ltd, London

SAS Institute (2012) Using JMP. JMP, a business unit of SAS. SAS Campus Drive, Cary **462**

Smits JE, Bortolotti GR, Tella JL (1999) Simplifying the phytohemagglutinin skin testing technique in studies of avian immunocompetence. Funct Ecol 13:567–572. doi:10.1046/j.1365-2435.1999.00338.x

Tella JL, Lemus JA, Carrete M, 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 MA, Weiser G, Allison R, Campbell TW (eds) (2012) Veterinary hematology and clinical chemistry, 2nd edn. Wiley-Blackwell, New York

Tucker CA, Yazwinski TA, Reynolds L, Johnson Z, 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. doi:10.1093/japr/16.3.392

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 of Faculty of Veterinary Medicine, University of Murcia, 184

Wakelin D (1978) Immunity to intestinal parasites. Nature 273:617–620

Wakelin D, Apanius V (1997) Immune defence: genetic control. In: Clayton DH, Moore J (eds) Host-parasite evolution, general principles and avian models. Oxford University Press, Oxford, pp 30–58

Xavier JC, Barbosa A, Agusti S et al (2013) Polar marine science in Portugal and Spain: recent advances and future perspectives. J Sea Res 83:9–29