PHYSIOLOGICAL TOOLKIT TO ASSESS THE HEALTH IMPACTS ON A MAGELLANIC PENGUIN *SPHENISCUS MAGELLANICUS* COLONY IN A HIGH-USE TOURISTIC REGION OF PATAGONIA, ARGENTINA

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ABSTRACT

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We evaluated the health status of Magellanic Penguins *Spheniscus magellanicus* inhabiting a region on the Valdés Peninsula (Patagonia, Argentina) that is subject to extensive tourism. We compared individuals nesting along tour trails with others inhabiting non-touristic zones. Hematocrits, blood cell counts, glucose, cholesterol, total proteins, and heterophil/lymphocyte were considered as stress indices. Most parameters were not affected by tourism. Adults and chicks tested positive for bacterial pathogens in both the touristic and control areas. These data complemented a long-term database of systematic physiological monitoring on penguins and will be useful tools for future comparative analyses.

Key words: bacteria test, biochemistry, hematology, Patagonia, penguins, tourism

INTRODUCTION

Physiological parameters are very useful indicators of the health status of free-living mammals and birds. Due to the functional links between the immune system, nutrition, and metabolism, hematological studies have provided accurate assessments of the overall physical condition in several vertebrate groups (Wikelski & Cooke 2006). Consequently, hematology parameters have been recently used to assess anthropogenic effects on wildlife (Geffroy *et al.* 2017, Palacios *et al.* 2018).

Ecotourism is an activity that adversely impacts wildlife by altering the behavior of wild animals (Ellenberg 2017), along with their stress condition and/or reproduction (French *et al.* 2010). For example, it has been reported that changes in behavior alter glucocorticoid levels and immunity parameters in several penguin species (Walker *et al.* 2005, 2006; Palacios *et al.* 2018).

Penguin colonies constitute one of the most popular tourism destinations, particularly during the reproductive season, in the Southern Hemisphere (Ellenberg 2017). The Magellanic Penguin *Spheniscus magellanicus* is the most important touristic resource species inhabiting the Patagonian coast in Argentina (Bertellotti *et al.* 2015). The total penguin population is estimated to be greater than 900 000 breeding pairs (Bertellotti 2013, Pozzi *et al.* 2015). One colony that is known to be growing is in San Lorenzo, which is within the Peninsula Valdés area (42°50′S, 063°49′W); it has ~135 000 breeding pairs and a growth rate of 1.21 breeding pairs per year (Pozzi *et al.* 2015). In parallel, tourist activity in San Lorenzo has also been increasing during the last 20 years (e.g., ~10 000 tourists visited the area in 2014), according to Secretaría de Turismo y Areas Protegidas (Chubut, Argentina).

We evaluated the physiological conditions of the Magellanic Penguins at San Lorenzo using hematological parameters to compare individuals occupying tourist-accessible areas with control individuals located in a portion of the colony that never receives tourist visits. Tourist visits are restricted to a small portion of the colony by way of fenced walking trails. Thus, we would expect that penguins inhabiting these touristic areas to show depressed physiological conditions when compared to individuals in the control area. Additionally, as humans could introduce novel pathogens into the system (Barbosa & Palacios 2009), we analyzed the presence of bacterial pathogens thought to be potentially harmful. The results from this study will complement a long-term database used to address the health condition of Magellanic Penguins over time. This database was formed using results from studies on cellular immunology (D'Amico *et al.* 2014), immune responses to helminth parasites (D'Amico *et al.* 2018), physiological parameters (Palacios *et al.* 2018), and oxidative stress parameters (Carabajal 2017, Colominas-Ciuró *et al.* 2017). Therefore, the final aim of this study is to add data to build a more comprehensive database that could be used as a toolkit to address the health condition of any Magellanic Penguin colony.

METHODS

Fieldwork

Penguins were sampled in early December 2014, when adults were rearing young chicks. We randomly selected 32 adults and 22 chicks (1.5–2 months old), both in the touristic area and in the control area. The latter was comparable to the touristic area in terms of nest density, breeding phenology, and distance to the shore (Villanueva *et al.* 2014). After capture, blood samples

(0.5–1.0 mL) were extracted from the peripheral foot vein using heparinized syringes (3 mL) and heparinized capillary tubes (75 μL). Samples were kept in a cooler until laboratory processing. Thin blood smears were prepared with a drop of fresh blood, air-dried, fixed with ethanol, and stained with Tinción 15 (Biopur). Penguins were weighed with spring scales (Pesola; nearest 100 g for adults and 10 g for chicks), and bill length and depth were measured with digital calipers (Essex; nearest 0.1 mm). Adult sex was determined using a discriminant function based on morphometries (Bertellotti *et al.* 2002). Cloacal samples were obtained using swabs and placed in transport medium (Stuart's medium) for later detection of bacteria.

Laboratory analyses

Blood samples were centrifuged for 12 minutes at 12 000 *g*. Plasma was processed by colorimetric and enzymatic methods on a spectrophotometer (Metrolab 1600 Plus, UV-Vis, Argentina) to determine levels of total protein ($g \cdot dL^{-1}$), cholesterol ($mg \cdot dL^{-1}$), and glucose ($mg \cdot dL^{-1}$). These plasmatic biochemical parameters contribute to the assessment of body condition and nutritional status in birds (Brown 1996). We used a microhematocrit ruler to measure hematocrit, which is a physiological index of condition in birds when evaluated together with other hematological parameters (Fair *et al.* 2007).

TABLE 1
Physiological parameters measured for Magellanic penguins at San Lorenzo[a]

		Adult[b]				Chick			
	Area	***n***	**Mean**	**SE**	**Min–Max**	***n***	**Mean**	**SE**	**Min–Max**
Weight (g)	1	16	3 781	95.0	3 200–4 300	13	846	62.9	600–1 400
	2	16	3 813	129.7	3 000–4 800	9	694	17.6	600–750
Glucose ($mg \cdot dL^{-1}$)	1	16	142	3.5	111–164	13	175	4.8	143–206
	2	16	145	3.4	119–166	9	187	1.6	153–207
Cholesterol ($mg \cdot dL^{-1}$)	1	16	195	5.8	148–246	13	244	4.8	147–308
	2	16	204	9.3	149–298	9	241	6.1	162–314
Total proteins ($g \cdot dL^{-1}$)	1	16	7	0.1	6–8	13	5	17.9	5–6
	2	16	7	0.1	6–8	9	5	18.9	5–6
Hematocrit	1	16	37[c]	1.9	21–50	13	23	0.1	15–58
	2	16	45[c]	1.6	25–53	9	20	0.1	15–26
RBCi (%)	1	16	2	0.8	0–8	13	45	3.3	20–75
	2	16	2	0.9	0–12	9	61	6.2	20–90
tWBC	1	16	61	5.0	25–105	13	26	3.1	11–51
	2	16	58	3.9	38–95	9	28	1.3	10–72
H/L	1	16	1	0.1	1–2	13	1	4.0	1–2
	2	16	1	0.2	0–2	9	1	8.6	0–2
Basophils (%)	1	16	0	0.1	0–1	13	0	0.1	0–1
	2	16	0	0.2	0–2	9	0	0.2	0–0
Eosinophils (%)	1	16	14	1.7	6–33	13	3	0.1	0–9
	2	16	12	1.1	7–19	9	4	0.0	1–8
Heterophils (%)	1	16	43	2.0	28–55	13	43	0.6	30–64
	2	16	42	3.1	18–58	9	37	0.7	11–56
Lymphocytes (%)	1	16	36	1.5	23–45	13	49	2.8	30–64
	2	16	39	2.5	23–58	9	56	4.8	34–87
Monocytes (%)	1	16	7	1.0	0–13	13	4	3.2	1–10
	2	16	6	1.1	1–18	9	3	5.3	0–5
Thrombocytes (count)	1	16	122	10.7	30–180	13	36	0.6	5–105
	2	16	100	13.5	25–200	9	18	0.6	0–70

[a] 1 = touristic area, 2 = control area, *n* = sample size, SE = standard error, RBCi = red blood cells (immature), tWBC = total white blood cells, H/L = heterophil/lymphocyte index

[b] Adult values were from both sexes combined. Weights and hematocrits showed statistically significant differences between sexes.

[c] Statistical differences of hematocrit values in adults between areas ($P = 0.005$).

Blood smears were scanned under a light microscope to analyze erythrocytes and leukocytes. Immature erythrocytes were registered as a percentage in the total erythrocyte counts (Martinho 2012). The leukocyte profile provides valuable information on the cellular components of the immune system, and it was assessed by estimating the total white blood cell count (number of leukocytes per 10000 erythrocytes, tWBC), the five leukocyte types (heterophils, eosinophils, basophils, lymphocytes, and monocytes), and thrombocytes (Campbell 1995). The tWBC was estimated under 400× magnification, the percentage of each leukocyte type was obtained from a sample of 100 leukocytes under 1000×, and thrombocytes were estimated as a total count (Campbell 1995). The heterophil/lymphocyte (H/L) index, which is considered a reliable measure of stress in birds (Davis *et al.* 2008), was calculated from the corresponding leukocyte counts.

Bacteria with zoonotic potential were scanned through specific techniques of selective and differential culture (Ryan & Ray 2004). To test for *Salmonella* spp. and *Shigella* spp., samples were incubated for 18–24 h in selenite broth and then cultured on *Salmonella-Shigella* agar (agar SS). Tryptone-soya agar (TSA) was used to test for *Corynebacterium* spp. To test for enteropathogenic *Escherichia coli* bacteria, a differential medium agar that was cysteine-lactose deficient in electrolytes (agar BD-CLDE) was used. To test for *Staphylococcus* spp., a blood agar–enriched medium was used.

All variables were normally distributed (Kolmogorov-Smirnov test > 0.05). Statistical analyses were done using one-way ANOVA. All P values < 0.05 were termed significant. The relationships between the physiological parameters and age and sex were evaluated using a Pearson test. All statistical analyses were done using Statistica package 7.0 (StatSoft, Inc. 2007).

RESULTS AND DISCUSSION

Biochemical and hematological parameters

Values of physiological parameters obtained during the reproductive season are presented in the Table 1. Male penguins are heavier than females (Bertellotti 2013), with male mass ranging between 3500 g and 4800 g (mean = 4047 g, SD = 321 g) and female mass ranging between 3000 g and 3500 g (mean = 3318 g, SD = 172 g). No significant differences in adult mass were observed between touristic and control areas. Similar results were observed in chicks, though chicks inhabiting touristic trails tended to be heavier (Table 1). The physiological parameter results of adult females and males were pooled because no significant differences in these parameters were observed between sexes (adult females $n = 11$, adult males $n = 21$).

Plasmatic metabolites reflect the nutritional status, as they are the main source of energy during prey digestion (Brown 1996). Values obtained in this study were in the range previously reported for healthy birds (Campbell 1995), including the Magellanic Penguin (Palacios *et al.* 2018). Glucose levels in chicks in both touristic and control areas were higher than the values reported by Palacios *et al.* (2018) (175 mg·dL^{-1} and 187 mg·dL^{-1} here, respectively, and 137 mg·dL^{-1} and 134 mg·dL^{-1}, respectively, in Palacios *et al.* 2018). However, no significant differences in glucose, total proteins, and cholesterol values were observed between penguins, either adults or chicks, sourced from the touristic and control areas. These parameters are directly related to diet; the main prey species for adults is the Argentine anchovy *Engraulis anchoita*, which represents at least 90 % of their diet and is, in turn, transferred to their chicks (Wilson *et al.* 2005). Therefore, although in different proportions, both adults and chicks feed on the same prey. Other studies reported higher values of glucose and cholesterol in chicks compared to adults, suggesting that adults maximize the food quality to feed their chicks by choosing prey with higher energy content (Forero *et al.* 2002).

As expected, hematocrit values were higher in adults than chicks (ANOVA $P = 0.003$; Table 1); hematocrit values typically increase with age in birds (Fair *et al.* 2007, Smith & Barber 2012). Hematocrit values were higher in adults sourced from the control area (ANOVA $P = 0.005$), but no significant differences were observed in hematocrit values for chicks between the different areas. Considering that exposure to environmental stressors affects hematocrit values (Fair *et al.* 2007), the higher values observed in adults from the touristic area could be related to human presence during breeding. However, as birds have natural variation in hematocrit caused by age, reproductive status, geographical elevation, season, parasitism, and nutritional status (Fair *et al.* 2007), this issue needs further analysis.

The percentage of immature erythrocytes was higher in chicks than adults (Table 1). Nearly half of the erythrocytes in chicks were immature, with no differences between the touristic and the control sites. Similarly, levels of immature erythrocyte were similar in adults regardless of whether sourced from the touristic or control areas. Leukocytes constitute the primary line of defense against pathogens (Roitt *et al.* 1998). In this study, adults and chicks from touristic and control areas showed similar tWBC values (ANOVA $P > 0.05$). Lymphocytes and heterophils were the most abundant cells displayed for adults and chicks in both areas. Total counts and percentages of basophils, eosinophils, and monocytes remained low, which is expected in healthy birds (Campbell 1995; Table 1). Adults and chicks showed no statistical differences in leukocyte types between areas (ANOVA $P > 0.05$). Mean values of tWBC, H/L, and leukocyte types observed in adult penguins were similar to those previously found in the same colony five years earlier (D'Amico *et al.* 2014). When compared with values from the previous season (December 2013) at the same site, adults maintained similar values. Chicks, however, except for eosinophils and monocytes, exhibited lower values among the remaining leukocyte types, tWBC, H/L index, and thrombocytes (see Palacios *et al.* 2018). Although, leukocyte counts are influenced by several factors, such as food deprivation, parasites, severe weather changes, contamination, habitat modification, and human impacts (Davis *et al.* 2008), the main cause of leukocyte production and activation would be exposure to several pathogens (Campbell 1995).

Bacterial tests

The growing touristic activities around the penguin colony could contribute to the dissemination of diseases by incidental transport of pathogenic agents. Therefore, as already found in other colonies, penguins could be exposed to new pathogens carried by visitors. A direct relationship between human presence and an increase in the range distributions, abundance, and/or virulence of parasites and pathogens was already reported for Antarctic penguins (Barbosa & Palacios 2009). Here, 38 samples were tested for bacteria (20 adults, 18 chicks; Table 2). All samples showed positive results for all bacteria tested. *E. coli* was the most prevalent bacterium in both adults and chicks (60 % and 56 %, respectively). Adults in touristic areas carried higher concentrations of *E. coli* than control adults (Table 2), but chicks showed equal percentage in both areas (55.5 %).

Although our study did not go deeply into the analysis of bacterial strains, there are several reported cases of transmission of *E. coli* and other bacteria from humans to animals, a process known as a reverse zoonosis. Many of these have been associated with tourism among Antarctic penguin colonies (Barbosa & Palacios 2009, Griekspoor *et al.* 2009, Hernández *et al.* 2012). In addition, thrombocytes exhibit phagocytic activity in several bird species. For instance, it has been documented in ducks as a response to *Staphylococcus aureus* (see more examples in Claver 2005). Furthermore, thrombocytes can phagocytose 1.7 times more bacteria in the blood, making it three times faster than heterophils and monocytes together (Chang & Hamilton 1979).

In our study, *Corynebacterium* spp. were present solely in adults (20 %), regardless of area. One adult tested positive for *Salmonella* spp. in the control area (5 %), as did one chick in the touristic area (5.5 %). *Staphylococcus* spp. had a prevalence of 30 % in adults and 44 % in chicks, with a similar percentage in both areas (Table 2). *E. coli* and *Staphylococcus* spp. were positively correlated to the thrombocyte counts of chicks in both touristic and control areas (Pearson P = 0.021). The presence of *Salmonella* spp. in the touristic area was correlated with the H/L stress index (Pearson P = 0.022). For adults, *E. coli* in both areas was correlated with the production of L (Pearson P = 0.0032), H/L index (Pearson P = 0.024), and E (Pearson P = 0.047), while *Corynebacterium* spp. was correlated to the production of tWBC (Pearson P = 0.03), H (Pearson P = 0.0139), L (Pearson P = 0.02), and, consequently, induced a higher H/L index (Pearson P = 0.0028) in both areas.

Using the physiological parameters obtained in this study (except for hematocrits in adults), we suggest that growing tourism does not impact the health condition of adults and chicks inhabiting the San Lorenzo colony. These results agree with previous work showing that penguins at San Lorenzo colony did not show alterations on their physiological parameters compared to their congeners at Punta Tombo (Chubut, Argentina), which showed physiological indicators of chronic stress, altered immunity, and poor general health (Palacios *et al.* 2018).

One shortcoming of our work was the low sample size. However, these data complement the systematic physiological monitoring that has been carried out on penguins at San Lorenzo since 2007. Villanueva *et al.* (2012) and Walker *et al.* (2006) suggested that a continuous monitoring of touristic activity would be important, as a history of visitation disturbance seemed to have behavioral and physiological effects on how birds responded to tourists. Likewise, Villanueva *et al.* (2014) found that penguins displayed different behavioral responses depending of the proximity to touristic areas: penguins inhabiting touristic zones were more tolerant to human approach than those inhabiting non-touristic areas. Palacios *et al.* (2018) showed an integrated set of immune-state and health-state indices that could be used to evaluate the effects of ecotourism on Magellanic Penguins, and they concluded that high exposure to humans resulted in physiological stress and poor health despite a long history of exposure and behavioral habituation to human visitation. Likewise, oxidative stress is a chemical imbalance occurring when there is an excessive production of reactive oxygen species or an inefficient elimination of them. Oxidative stress is considered to be a physiological indicator of environmental stressors, including human activities such as tourism, recreation, fishing activities, burning fuels, and dumping or spilling solid and liquid human waste. Measurements in penguins at San Lorenzo showed that individuals nesting around touristic trails exhibited higher oxidative stress compared with control areas (Carabajal 2017).

Taken together, our hematological and health condition parameters can provide useful information to better understand the physiological responses of penguins to global environmental change.

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TABLE 2
Bacterial testing of Magellanic penguins at San Lorenzo colony[a]

		Adults[b]			Chicks		
		n	Prevalence (%)		*n*	Prevalence (%)	
	Area		per site	Total		per site	Total
Escherichia coli	Touristic area	9	81.8	70	5	55.6	56
	Control	5	62.5		5	55.6	
Salmonella spp.	Touristic area	0	0	5	1	11.1	6
	Control	1	12.5		0	0	
Corynebacterium spp.	Touristic area	2	18.2	20	0	0	0
	Control	2	25		0	0	
Staphylococcus spp.	Touristic area	3	27.3	30	4	44.4	44
	Control	3	37.5		4	44.4	

[a] Adults: Touristic area = 11 samples analyzed; Adult control = 8 samples analyzed. Chicks: Touristic area = 9 samples analyzed; Chick control = 9 samples analyzed.

[b] Adult values were from both sexes combined because they did not show statistically significant differences.

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