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HEMATOLOGY, SERUM CHEMISTRY, AND SEROLOGY OF GALÁPAGOS PENGUINS (*SPHENISCUS MENDICULUS*) IN THE GALÁPAGOS ISLANDS, ECUADOR

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ABSTRACT: The Galápagos penguin (*Spheniscus mendiculus*) is an endangered species endemic to the Galápagos Islands, Ecuador. In 2003 and 2004, 195 penguins from 13 colonies on the islands of Isabela and Fernandina in the Galápagos archipelago were examined. Genetic sexing of 157 penguins revealed 62 females and 95 males. Hematology consisted of packed cell volume ($n=134$), white blood cell differentials ($n=83$), and hemoparasite blood smear evaluation ($n=114$). Microfilariae were detected in 22% (25/114) of the blood smears. Female penguins had significantly higher eosinophil counts than males. Serum chemistry on 83 penguins revealed no significant differences between males and females. Birds were seronegative to avian paramyxovirus type 1–3, avian influenza virus, infectious bursal disease virus, Marek's disease virus (herpes), reovirus, avian encephalomyelitis virus, and avian adenovirus type 1 and 2 ($n=75$), as well as to West Nile virus ($n=87$), and Venezuelan, western and eastern equine encephalitis viruses ($n=26$). Seventy-five of 84 (89%) penguins had antibodies to *Chlamydophila psittaci* but chlamydial DNA was not detected via polymerase chain reaction in samples from 30 birds.

Key words: Chemistry, filarid, Galápagos Islands, hematology, hemoparasite, penguin, serology, *Spheniscus mendiculus*.

INTRODUCION

The Galápagos Islands straddle the equator and are 1,000 km west of continental Ecuador in the Pacific Ocean. Galápagos avifauna is comprised of 58 resident species, of which 52% are endemic (Tye et al., 2002). The Galápagos penguin (*Spheniscus mendiculus*) is an endemic species with the majority of the population residing on two western islands of the archipelago, Isabela and Fernandina; smaller populations reside on Floreana, Bartolomé, and Santiago Islands (Mills and Vargas, 1997). The Galápagos penguin is one of the world's smallest penguin species and it is the only species found in the tropics, with part of its population inhabiting the northern hemisphere (Boersma, 1977). It is considered endangered due to its small range and fluctuating population (BirdLife International, 2000). Galápagos penguin populations were dramatically reduced during the 1982–83 (Valle and Coulter, 1987) and 1997–98 El Niño events (Vargas et al., 2006). Since 1998, the adult population has been relatively stable whereas the juvenile population has been variable (Vargas and Wiedenfeld, 2004). In 2004, the population was estimated at 1505 individuals (Vargas and Wiedenfeld, 2004).

No avian species have become extinct from the Galápagos Islands (Wikelski et al., 2004), but the possibility of disease causing significant morbidity and mortality is of great concern. Avian health studies in the Galápagos Islands have been ongoing since 2001, and to date, 16 avian species on 11 islands have been sampled. The first comprehensive health assessment of the

penguin population occurred in August 2003 and March 2004. Serologic tests were determined from previous penguin disease surveys (Austin and Webster, 1993; Wallace et al., 1997; Clarke and Kerry, 2000; Gauthier-Clerc et al., 2002; Tuttle et al., 2005). Penguins also were tested for exposure to multiple poultry pathogens that could be associated with chickens in the Galápagos Islands (Gottdenker et al., 2005). Potential spillover of poultry pathogens to penguins exists via poultry waste or other infective material in freshwater runoff, aerosol transmission of viruses from inhabited coastal regions adjacent to penguins, discarded poultry products from commercial (fishing and tour) boats in areas where penguins reside, and contact with volant birds (i.e., yellow warblers or finches) that might transmit pathogens of poultry origin to penguin nesting sites. Serologic test selection was also directed by positive serologic results from other endemic Galápagos avian species (Padilla et al., 2003, 2004). In order to establish a baseline for future monitoring of the endangered Galápagos penguin, hematology and serum chemistry parameters also are reported.

MATERIALS AND METHODS

Study area and sample collection

Galápagos penguins were studied on the coasts of Isabela (0°25′30″S, 91°7′W) and Fernandina (0°22′0″S, 91°31′20″W) Islands. All sampling procedures were in accordance with Saint Louis Zoo institutional animal care and use committee standards. Eighty-four and 112 penguins were examined in August 2003 and March 2004, respectively. On Fernandina Island, 27 penguins in 2003 and seven in 2004 were sampled, and on Isabela Island, 57 penguins in 2003 and 105 in 2004 were evaluated. Information on capture technique (Vargas et al., 2005), and morphometric measurements and aging (Boersma, 1977), has previously been described. During manual restraint, a transponder (AVID Microchip, Folsom, Louisiana, USA) was placed subcutaneously over the left dorsal mid phalangeal area and the skin defect sealed with tissue glue (3M Vetbond, St Paul, Minnesota, USA). Jugular venipuncture was performed with 20 gauge needles and syringes to collect up to 12 ml of blood per bird. Several drops of blood were immediately placed into cryogenic vials containing a lysis buffer preservative solution (Longmire et al., 1988) for genetic testing of gender, as well as preliminary hemoparasite molecular analysis. During the 2003 field season, whole blood was placed in lithium heparin (Vacutainer PST gel, Becton Dickinson, Franklin Lakes, New Jersey, USA); during the 2004 field season a small amount of whole blood was placed in ethylenediaminetetraacetic acid (EDTA) (Microtainer gel, Becton Dickinson), and the remainder placed in serum separator tubes (Vacutainer SST gel and clot activator, Becton Dickinson). Blood tubes were kept on ice packs until processing. A single sterile swab (Copan Diagnostics, Corona, California, USA) was used to collect a combined conjunctival, choanal, and cloacal swab per bird ($n=97$). The swabs were stored in cryogenic vials (Nalge Nunc International, Rochester, New York, USA). No ectoparasites were observed on feathers during either year. Fecal samples from 11 individual penguins from 2004 were collected opportunistically from fresh excreta during restraint. Feces were preserved in cryogenic vials with potassium dichromate and/or formalin and stored at room temperature for later microscopic examination.

Sample processing

The timing of full sample processing varied from less than 1 hr up to 6 hr after collection. Most samples were centrifuged within 3 hr of collection. Two blood smears were made from whole blood (lithium heparin in 2003, EDTA in 2004), and air dried and fixed with methanol in the field. Microhematocrit tubes ($n=134$) were centrifuged (Mobilespin Model 128, Vulcon Technologies, Grandview, Missouri, USA) for 20 min, and the packed cell volumes (PCV) determined. The blood in lysis buffer solution was held at ambient temperature. The lithium heparin whole blood samples ($n=74$) and serum separator samples ($n=97$) were centrifuged for 20 min, and the plasma or serum decanted and stored in cryogenic vials. The plasma, serum, and swabs were stored in liquid nitrogen in the field and in the laboratory.

Sample and data analysis

Blood smears were stained using a modified Wright–Giemsa stain (JorVet Dip-Quick, Jorgensen Laboratories, Loveland, Colorado, USA) at the Saint Louis Zoo for white blood cell (WBC) differentials based on 100 cells

counted at 1000× magnification. Blood smears were evaluated for thrombocytes and hemoparasites. Genetic testing for sex determination ($n=157$) was performed at the University of Missouri–Saint Louis (UMSL) via polymerase chain reaction (PCR) (Fridolfsson and Ellegren, 1999). Ninety of the 2004 samples were processed for serum biochemistry (AVL Veterinary Laboratory, Saint Louis, Missouri, USA), in one batch on the Ace Clinical Chemistry System (Alfa Wassermann, Inc., West Caldwell, New Jersey, USA). Seven results were not included in the analysis due to hemolysis or lipemia.

Serology testing was restricted to the 2004 samples. Testing for antibodies to *Chlamydophila psittaci* was done by the Texas Veterinary Medical Diagnostic Laboratory, College Station, Texas, USA using direct complement fixation (DCF) on serum ($n=84$); combined conjunctival, choanal and cloacal swabs from 30 of the antibody positive penguins were also tested for *C. psittaci* DNA by real-time PCR.

Serologic tests for viral antibodies, except for WNV, were conducted at the National Veterinary Services Laboratory, US Department of Agriculture, Ames, Iowa, USA. Hemagglutination inhibition (HI) testing was used to test for antibodies to avian paramyxovirus type 1–3 ($n=75$; titers ≥ 8 were considered positive) and Venezuelan (VEE), western (WEE), and eastern (EEE) equine encephalitis viruses ($n=26$; titers ≥ 10 were considered positive). Agar gel immunodiffusion (AGID) testing was used to test for antibodies to avian influenza virus, infectious bursal disease virus, Marek's disease (herpes) virus, avian adenovirus types 1 and 2, and avian encephalomyelitis virus ($n=75$). Indirect fluorescent antibody (IFA) testing was used to test for antibodies to reovirus ($n=75$). Serum was tested for antibodies to West Nile virus by plaque reduction neutralization ($n=87$) at the Animal Health Diagnostic Center, Cornell University, Ithaca, New York, USA. Fecal analysis from fecal floatation and sedimentation was conducted with microscopy at the Southeastern Cooperative Wildlife Disease Study, College of Veterinary Medicine, The University of Georgia, Athens, Georgia, USA.

A statistical software package (NCSS®, Kaysville, Utah, USA) was used for data analysis. Results were inspected for normality using a Shapiro-Wilk W test and t-tests were performed. Mann-Whitney U-tests were used on samples where normality was rejected. A statistical significance was determined as $P \leq 0.05$ except for the serum chemistry values where a Bonferroni-corrected $P \leq 0.004$ (0.05/12) was used.

RESULTS

Genetic sexing, body weight, hematology, and microscopic hemoparasite evaluations were performed on the 2003 and 2004 samples, counting each individual once because some individuals were sampled both years. Serum chemistries, serology, and fecal analysis were conducted only for the 2004 samples. All penguins examined were in good body condition, as determined both by palpation of pectoral muscles and body weight measurement. There were no external signs of disease. In 2003, 84 penguins were sampled; eight (10%) nestlings, five (6%) juveniles, and 71 (84%) adults. In 2004, 112 penguins were sampled; four (4%) nestlings, 28 (25%) juveniles, and 80 (71%) adults. Twenty-five penguins in 2004 were recaptured from the previous year. Genetic sexing of 157 individuals revealed 62 females (39.5%) and 95 males (60.5%). Body weight and hematology results are provided in Table 1. Because there was a significant difference between the sexes for body weight and PCV, separate gender means are listed. There were no differences in PCV or differentials between heparin or EDTA blood. The mean PCV of the combined male and female sampled population was 44.1% (SD 6.8%, $n=134$). Males had significantly higher body weights ($P<0.00001$) and PCV results ($P<0.006$) compared to females. There were no differences in WBC differentials between genders except that females had elevated eosinophils ($12.2 \pm 9.1\%$, $n=29$) compared to males ($5.3 \pm 0.7\%$, $n=54$) ($P<0.0002$). Microscopic evaluation of blood smears revealed microfilariae in 21.9% (25/114) of the penguins. Thrombocytes were adequate for both genders because at least two to three thrombocytes were seen on each high power field (Pierson, 2000).

Results of serum chemistries are provided in Table 2; there were no significant differences between female and male penguin biochemistry values. All penguins

TABLE 1. Weight, packed cell volume, and white blood cell differentials for free-ranging Galápagos penguins.

Parameter	Mean±SD	Range	*n*
Female weight (kg)[a]	1.9±0.3	1.4–2.5	62
Male weight (kg)[a]	2.3±0.3	1.7–3.5	103
Female packed cell volume (%)[b]	41.7±7.0	28.0–52.0	53
Male packed cell volume (%)[b]	45.3±6.8	23.0–58.0	79
Heterophils (%)	57.6±16.0	8.0–85.0	83
Lymphocytes (%)	31.5±15.5	6.0–84.0	83
Monocytes (%)	2.4±2.1	0.0–9.0	83
Eosinophils (%)	8.0±7.5	0.0–37.0	83
Basophils (%)	0.4±0.7	0.0–3.0	83

[a] Significant difference $P<0.00001$.

[b] Significant difference $P<0.006$.

were serologically negative to all viruses. Seventy-five of 84 penguins (89.3%) were seropositive to *C. psittaci* with titers of 8 to 64. Combined swabs were submitted for *C. psittaci* PCR on 30 of the 75 seropositive penguins; all were negative. Hematology and biochemistry results did not differ between *C. psittaci* or filarial positive penguins and those that were negative. Fecal samples were negative for helminth eggs and larval nematodes, but one penguin (1/11) had three *Eimeria* oocysts detected (17×16 μm).

DISCUSSION

These are the first published hematology, serum chemistry, and large-scale serology results for the Galápagos penguin. The Humboldt penguin (*S. humboldti*) is closely related to the Galápagos penguin (Robert Fleischer, pers. comm.); therefore, blood work from a free ranging Humboldt population (Wallace et al., 1995) was used for comparison. Because the methodology of the studies was different, trends between the penguin species is discussed instead of statistical differences. The Galápagos penguins had higher chloride, calcium, uric acid, and aspartate aminotransferase values, but lower PCV, glucose, and albumin compared to the Humboldt penguins. Laboratory results can be affected by many variables such as species, gender, age, nutritional status, or geographic location (Dein, 1986), and the difference between the Galápagos and Humboldt penguins likely are not clinically significant.

A change in the Galápagos penguin

TABLE 2. Serum chemisty results for free-ranging Galápagos penguins ($n=83$).

Parameter (SI units)	Mean±SD	Range
Aspartate aminotransferase (U/l)	282.5±89.8	132.0–525.0
Total protein (g/l)	56.0± 7.7	36.0–77.0
Albumin (g/l)	12.2±1.5	9.0–17.0
Phosphorus (mmol/l)	1.6±0.8	0.8–4.2
Calcium (mmol/l)	2.5±0.2	2.2–3.7
Glucose (mmol/l)	12.1±1.9	5.3–15.7
Sodium (mmol/l)[a]	156.3±6.1	133–169.0
Potassium (mmol/l)[a]	3.9±1.3	2.2–8.1
Chloride (mmol/l)[a]	118.5±4.6	102.0–132.0
Creatine kinase (U/l)	174.4±144.5	18.0–675.0
Uric acid (mmol/l)	1.1±0.4	0.2–1.9

[a] $n=76$.

sampling protocol between years resulted in blood smears from lithium heparin anticoagulated blood in 2003 and EDTA anticoagulated blood in 2004, but no differences were appreciated microscopically between the two anticoagulants. Field conditions did not allow for hemocytometer total WBC counts. An estimated total WBC count from fixed blood smears (Fudge, 2000) was attempted months after sample collection, but the blood smear quality had diminished sufficiently to yield inaccurate results. Therefore, total WBC counts are not reported. Fortunately, the WBC differentials and hemoparasite evaluations were performed in a timely manner after sample collection while the cells were easily identifiable. The authors speculate that the marine environment was detrimental to the slides, and that the lack of cover slips further caused cell deterioration. The leukocyte differentials for the Galápagos penguin population, combining male and females ($n=83$), fell within the ranges reported for wild Humboldt penguins (Wallace et al., 1995). The clinical significance of the elevated eosinophil count in female Galápagos penguins remains unknown. Galápagos penguins were evaluated over two distinct environmental seasons, but no seasonal difference was found in eosinophil counts. Furthermore, eosinophil values between microfilarid positive and negative penguins did not differ.

Extraerythrocytic microfilariae were detected in the Galápagos penguins. Similar microfilariae have been detected in the flightless cormorant (*Phalacrocorax harrisi*) which shares the same geographic distribution as the penguins (Travis et al., 2006). Filarids are usually incidental findings (Greiner and Ritchie, 1994), and are rarely reported in penguins. There is only one known case of filariasis in Galápagos penguins (Chabaud and Ball, 1964). A detailed investigation is currently underway to further classify and characterize the microfilaria seen in 22% of the Galápagos penguins. The *Eimeria* oocysts found in one penguin fecal sample are not considered significant in a healthy bird. Coccidial infections have been reported as incidental findings in little penguins (*Eudyptula minor*) (Obendorf and McColl, 1980; Harrigan, 1992). Evaluating the impact of these various parasites in the Galápagos penguins necessitates a long-term study.

The Galápagos penguins were seronegative for exposure to all tested viruses. The seronegative Marek's disease (herpesvirus) results concurred with a previous study (Miller et al., 2001). Because most tests have been validated for domestic chickens, serologic results should be interpreted with caution. The Galápagos penguins were seropositive for *C. psittaci* (89%, 75/84). *Chlamydophila psittaci* is found worldwide in asymptomatic birds (Gerlach, 1994) and antibodies have been detected in multiple penguin species (Sieburth, 1958; Moore and Cameron, 1969; Wallace et al., 1997; Karesh et al., 1999). An outbreak at a zoo in the USA in a *Spheniscus* penguin species revealed that *C. psittaci* can be an important penguin pathogen (Freeland Dunker, pers. comm.). The significance of seropositive Galápagos penguins in the absence of DNA from PCR is unclear. A false positive antibody result or a latent or inactive infection cannot be ruled out. *Chlamydophila psittaci* has been extensively studied in the Galápagos Islands and antibodies have been detected in flightless cormorants (Travis et al., 2006) and domestic chickens (Gottdenker et al., 2005), and chlamydial DNA has been detected in Galápagos doves (*Zenaida galapagoensis*; Padilla et al., 2004) and cormorants (Travis et al., 2006).

Chickens in the Galápagos Islands have tested positive for parasites and diseases for which penguins might be susceptible, including Newcastle disease (Gottdenker et al., 2005). Although this study suggests that Galápagos penguins have not been exposed to poultry pathogens, the threat of pathogen transmission is real. Chickens

range freely in Puerto Villamil on Southern Isabela and they have been observed on beachfront properties adjacent to where a small penguin population resides (Vargas and Wiedenfeld, 2004). Recent surveys suggest that penguin numbers are slowly increasing at this site through migration (Vargas, unpubl. data); therefore, measures should be implemented to reduce or prevent contact between penguins and poultry.

We report the most extensive West Nile virus (WNV) monitoring in the archipelago to date with negative results for the Galápagos penguins, flightless cormorants (Travis et al., 2006), and domestic chickens (Gottdenker et al., 2005). WNV is an emerging threat to naïve avian populations especially where known mosquito vectors reside (Peck et al., 1998; Turell et al., 2001). Because penguins are susceptible to WNV and mortality has been confirmed in black-footed (*Spheniscus demersus*) and Humboldt penguins (Centers for Disease Control, 2005), monitoring and prevention programs for WNV are in place or being devised for the Galápagos Islands.

A Population and Habitat Viability Analysis (PHVA) workshop was conducted on the Galápagos penguin facilitated by the Conservation Breeding Specialist Group (CBSG) of the International Union for Conservation of Nature (Matamoros et al., 2006). This workshop prioritized oil spills, strong El Niño events, and diseases among the top threats to the Galápagos penguin. Anthropogenic (fishing, tourism, oil spills) and climatic factors can stress fauna which can precipitate disease. The Galápagos National Park monitors and controls activities in the marine reserve to lessen negative impacts on the fragile ecosystem, and to assist in this monitoring, we provide the first comprehensive health information for the Galápagos penguin. Because the penguins are immunologically naïve to all viruses tested, a viral agent, especially one of high virulence, could be devastating. Even though the penguin population is slowly recovering after the crashes caused by strong El Niño events, disease could jeopardize the survival of this species. Disease surveillance should continue, as well as the development of contingency plans in order to respond rapidly to new pathogens.

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