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MICROFILARIAE IN GALÁPAGOS PENGUINS (*SPHENISCUS MENDICULUS*) AND FLIGHTLESS CORMORANTS (*PHALACROCORAX HARRISI*): GENETICS, MORPHOLOGY, AND PREVALENCE

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ABSTRACT: Galápagos penguins (*Spheniscus mendiculus*) and flightless cormorants (*Phalacrocorax harrisi*) live in small, isolated populations on the westernmost islands of Isabela and Fernandina in the Galápagos Islands, Ecuador. Between August 2003 and February 2005, 4 field trips, 2 in the cool, dry season (August 2003 and August 2004) and 2 in the hot, rainy season (March 2004 and February 2005), were undertaken; 298 Galápagos penguins and 380 cormorants were sampled for prevalence and intensity of hemoparasites. Microfilariae were found in both the penguins and the cormorants. Blood smears were negative for the presence of other species of hemoparasites. Overall prevalence of microfilariae across seasons was 42.0% in cormorants and 13.8% in the penguins. Intensity of infection was generally low (mean = 3.2–31.7 in 25 fields across seasons and species) with the exception of a few individuals with markedly high intensities of parasites (>300 in 25 fields in 1 cormorant). Prevalence of microfilariae increased significantly over the 4 sampling periods for cormorants, but not for penguins. Prevalences were significantly higher in cormorants than in penguins for 3 of the 4 collecting trips. Male penguins had higher prevalences than females; however, there were no gender differences in cormorants. No relation was detected between body mass and either presence or intensity of parasitism. Morphological characteristics of the microfilariae are also described and specimens from each host species were similar in all characters measured. DNA sequence data from the mitochondrial cytochrome *c* oxidase subunit I gene were consistent with the morphological evidence and together demonstrate that the penguins and cormorants are likely to be infected with the same species of microfilariae.

Galápagos penguins and flightless cormorants live in small, isolated populations endemic to the westernmost Galápagos Islands of Fernandina and Isabela. According to the most recent estimates, the population of each species is less than 2,000 individuals (Vargas et al., 2005). Penguin and cormorant populations are under pressure from both human and natural sources, including oil spills, ecotourism, fishing, El Niño events, and volcanic activity (Matamoros et al., 2006). Concern for the fate of such small populations led to incorporation of these 2 species into an ongoing biomedical survey described below.

Beginning in 2001, a team consisting of personnel from the University of Missouri–Saint Louis (UMSL), Saint Louis Zoo (SLZ), Charles Darwin Research Station (CDRS), and the Galápagos National Park (GNP) has been conducting a broad range of studies involving avifauna of the Galápagos Islands (summarized in Parker et al., 2006). One component of the project is a biomedical survey to monitor for disease and to establish baseline health parameters for Galápagos bird species (Padilla et al., 2003, 2004, 2006; Travis et al., 2006b). An additional objective of the survey is to compare disease prevalence between introduced and endemic birds to screen for the introduction of new disorders (Padilla et al., 2004; Gottdenker et al., 2005; Thiel et al., 2005). As a part of the biomedical survey, members of the team have also been collecting blood samples from Galápagos penguins and flightless cormorants.

An earlier study found microfilariae (Order Spirurida, Superfamily Filarioidea) in the Galápagos penguins and the flightless cormorants (Harmon et al., 1985). To investigate this finding more fully and determine its biological significance, we collected blood smears on all birds that were sampled. We expanded the scope of the Harmon et al. (1985) investigation to include a larger sample size, to report prevalence and intensity across seasons, and to provide preliminary genetic analyses and thorough morphological descriptions of the microfilariae.

Microfilariae have been reported in numerous species of birds, including many South American species (White et al., 1978; Campbell, 1988; Bennett et al., 1991). Unlike Nearctic locales, the Neotropics in general appear to have a higher prevalence of avian filarids (Bennett et al., 1991; Rodriquez and Matta, 2001). Consequences of filarial infection in avian hosts range from nonpathogenic cases to rare fatal infections (Campbell, 1988; Simpson et al., 1996). Historically, filarid infections in avian hosts have been generally dismissed as nonpathogenic. The pathogenicity or effects on host fitness during filarid infection are generally not well studied in birds; however, recent studies on other species of hemoparasites have demonstrated negative effects on egg laying patterns, reproductive success, parental condition, and survivorship (Earle et al., 1993; Merino et al., 2000; Votypka et al., 2003; Remple, 2004). Purple martins (*Progne subis*) had a 90% mortality when concurrently infected with the haemosporid apicomplexan, *Haemoproteus prognei*, and an unidentified filarial nematode (Davidar and Morton, 2006).

Penguins and cormorants in our study appeared healthy on physical examination; laboratory evaluations for a number of viral diseases were negative, and serum chemistries were normal (Travis et al., 2006a, 2006b). Avian skin mites, which can cause mange in wild birds, were found on louse flies (Hippoboscidae) associated with the cormorants (Whiteman et al., 2006). The only abnormal finding was an overall elevation of eosinophils in both the penguins and the cormorants, but the eosinophilia did not correlate with microfilarid detection or intensity. Eosinophil elevation is correlated with parasite burden in mammals; however, this relationship has not been established in birds (Ritchie et al., 1994).

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The Galápagos Islands have a subtropical climate marked by 2 seasons, i.e., the hot, rainy season from December to April and the cool and dry season from May to November. Mosquitoes and other insects are likely more abundant during the hot, rainy season (Eldridge, 2005). The knowledge of seasonality is important because hemoparasite infections are predicted to be linked to 2 factors: (1) vector abundance during certain seasons, and (2) relapse of infection in animals undergoing physiological stress during the breeding season (Weatherhead and Bennett, 1991). Galápagos penguins and flightless cormorants are both year-round opportunistic breeders, with penguins experiencing an increase in breeding April–May and August–September (Boersma, 1977; Valle, 1987; Vargas et al., 2006). In view of this breeding pattern, it would seem that changes in prevalence of parasites across the seasons would be due to vector abundance and not linked to lowered immunity during the breeding season. We tested for seasonal differences in prevalence and predicted that higher prevalence of microfilarial parasites would be found during the hot, rainy season. Additional goals of the study were to compare the microfilariae found in the 2 bird species morphologically and genetically.

MATERIALS AND METHODS

Field methods

Our study was conducted in 2 mo (March 2004 and February 2005) of the hot, rainy seasons, and during 2 mo (August 2003 and August 2004) of the cool, dry seasons on the islands of Fernandina and Isabela, Galápagos Islands, Ecuador. Samples from the Galápagos penguins were also taken from the small populations of Floreana, Santiago, and Bartolome, where cormorants are absent. In total, 298 penguin and 380 cormorant individuals were sampled. A microchip was placed in the web of the foot to allow identification on subsequent trips. Blood samples were collected from the jugular vein and stored in either heparin or EDTA until prepared and stained with a modified Wright–Giemsa stain within 4 hr of blood collection. Several drops of whole blood were stored in a Longmire et al. (1988) solution for DNA extraction.

Slide analysis

Slides from 327 penguins and 448 cormorants were examined for large hemoparasites, e.g., trypanosomes and microfilariae at ×100 for 5 min. Presence or absence of hemoparasites was recorded. If slides were positive for microfilariae, 25 fields at the feathered edge were scanned at ×100, and the number of microfilariae was recorded as intensity. Additionally, 200 fields were reviewed at ×1,000 oil immersion to look for smaller hemoparasites, e.g., *Plasmodium* and *Haemoproteus*. Quantitative Parasitology 3.0 (QP) was employed to compare prevalence and intensity across seasons both within each species and between the 2 species (Rozsa et al., 2000). All individuals were grouped by species, and each hot, rainy season (February and March) was compared with each cool, dry season (August).

Body condition was assessed as the residuals from a regression of body mass against wing length. We then used a 2-sample *t*-test to compare the residuals of infected versus noninfected birds to assess for effect of infection on body condition. Specifically, body condition was analyzed among all penguins (infected vs. noninfected), and then, a regression was performed only among infected individuals to determine whether intensity, rather than presence of the parasite, was asserting a negative effect on body condition. Wing length was not measured in the cormorants. QP was also used to compare differences in prevalence and intensity between genders within each species.

Filarid genotyping and phylogenetic analyses

DNA samples were extracted from whole blood (preserved in lysis buffer) using a phenol-chloroform technique described elsewhere (Parker et al., 1995). From these, samples from 5 cormorants and 5 penguins that were positive for microfilariae from visual inspection of blood smears were diluted in sterile dH_20 (1:10) for use as template DNA in PCR, in an attempt to determine preliminarily whether the microfilariae in each host species were conspecifics. Using primers designed specifically for filarial nematodes (Casiraghi et al., 2001), we amplified a 688-bp region of the mitochondrial cytochrome *c* oxidase subunit I gene (COIintF 5′-TGATTGGTGGTTTTGGTAA-3′ and COIintR 5′-ATAA GTACGAGTATCAATATC-3′) from these samples. This locus has been shown to reliably diagnose nematode species (Blouin et al., 1998).

Each PCR tube contained 45 μl of a PCR master mix of the following components: 2.5 μl PCR buffer, 1.5 μl BIOLASE Red DNA polymerase (Midwest Scientific, St. Louis, Missouri), 3.9 μl 25 mM $MgCl_2$, 3 μl of each primer (100 μM), 1.6 μl DNTPs (100 μM), 29.5 μl sterile dH_2O; 5 μl of the diluted DNA extracted from each of the 10 bird blood samples was added in the last step, yielding a final PCR volume of 50 μl. Negative controls were included in each set of reactions. The PCR amplifications for COI were performed using a touchdown cycle (annealing temperature initially varied from 51–47 C): 94 C for 2 min, followed by 8 cycles beginning with 94 C for 45 sec and 51 C annealing, reduced each cycle by 0.5 C; 72 C extension for 1.5 min, followed by 25 cycles beginning with 94 C for 45 sec and 45 C annealing; 72 C extension for 1.5 min; and a final 72 C extension for 7 min. Amplicon size was verified on 1–2% TBE agarose gels stained with ethidium bromide and visualized under UV light. Multiple bands appeared in the lanes of each individual. Nine out of 10 samples (5 cormorants and 4 penguins) yielded bands of the expected (688 bp) size. We gel-extracted these bands using QIAQuick Gel Extraction Kit following the manufacturer's instructions (Qiagen Inc., Valencia, California), which were then verified on agarose gels. We cloned each of the 9 amplicons using the Promega (Madison, Wisconsin) pGem-T Easy Vector Cloning Kit. Positive colonies were amplified using universal M13 forward and reverse plasmid primers and visualized on agarose gels. Colonies that yielded bands of the expected size were gel-extracted using the QIAQuick Gel Extraction Kit. Direct sequencing was performed on both strands of each amplicon using the universal SP6 and T7 plasmid primers with ABI PRISM BigDye Terminator PCR cycling conditions and sequenced on an Applied Biosystems (Foster City, California) 3100l DNA Analyzer.

Raw sequence chromatograms of forward and reverse strands were assembled for each amplicon in Seqman II (DNASTAR, Madison, Wisconsin), and the entire length of each strand was evaluated by eye. Poor-quality data and primer sequences were trimmed from both strands. Seqman II was used to assemble consensus sequences resulting from the double-stranded sequences for each gene. We then returned to the original chromatograms to ensure that variable sites were unambiguously assigned. We included the 2 COI variants in a broader phylogenetic analysis of other filarid COI sequences in GenBank, although many of the available sequences are parasites of humans and other mammals. Sampling was conducted based on the phylogenetic analysis of Casiraghi et al. (2001). Filarid species used in this analysis and their GenBank accession numbers are as follows: *Brugia malayi* (AJ271610), *Brugia pahangi* (AJ271611), *Wuchereria bancrofti* (AJ271612), *Dirofilaria immitis* (AJ271613), *Dirofilaria repens* (AJ271614), *Litmosoides brasiliensis* (AJ544867), *Litomosoides sigmodontis* (AJ271615), *Onchocerca gibsoni* (AJ271616), *Onchocerca gutturosa* (AJ271617), *Onchocerca lupi* (AJ415417), *Onchocerca ochengi* (AJ271618), *Thelazia callipaeda* (AM042555), *Thelazia lacrymalis* (AJ271619), *Acanthocheilonema viteae* (AJ272117), *Dipetalonema gracile* (AJ544877), and *Loa loa* (AJ544875). These COI sequences, along with the 2 variants recovered from Galápagos penguins and flightless cormorants, were aligned in ClustalX and trimmed to 513 bp. Using this alignment, we then determined the most likely model of sequence evolution (TrN + I + G) using ModelTest 3.06 (Posada and Crandall, 1998). Using these parameters of sequence evolution, a maximum-likelihood heuristic search was performed in PAUP* (Swofford, 2002). *Thelazia lacrymalis* and *Thelazia callipaeda* were used as out-groups (Casiraghi et al., 2001). A maximum parsimony bootstrap search was also performed in PAUP* with 1,000 replications.

RESULTS

Across the 4 sampling periods, between August 2003 and March 2005, the prevalence of microfilariae in Galápagos

TABLE I. Penguin (P) and cormorant (C) metrics across seasons.

	Penguins				Cormorants			
Parameters*	n = 71 Aug 03	n = 95 Mar 04	n = 65 Aug 04	n = 67 Feb 05	n = 65 Aug 03	n = 94 Mar 04	n = 126 Aug 04	n = 95 Feb 05
Prevalence (%)	21.7	14.4	13.7	7.4	33.8	36.2	43.5	59.5
Mean abundance	0.70	1.18	1.53	2.35	2.49	2.57	5.41	16.57
Mean intensity	3.20	8.20	11.20	31.67	7.35	7.11	12.43	27.85
(95% CI)	(1.87–6.00)	(3.73–19.93)	(5.30–21.6)	(9.17–79.00)	(4.83–11.09)	(4.66–14.21)	(8.99–19.54)	(19.40–42.15)
k	0.131	0.047	0.041	0.015	0.149	0.157	0.169	0.209
Var/mean ratio	6.77	29.58	24.04	75.32	12.83	25.61	39.72	92.60

* *k*: measures eveness of distribution, with low *k* indicating clumped distribution; var/mean ratio: characterizes distribution with high values corresponding to contagion.

flightless cormorants rose from 33.8 to 59.5%, whereas prevalence in Galápagos penguins declined from 21.7 to 7.4% (Table I; Fig.1). In all but the first season, cormorants had significantly higher prevalence of microfilariae than penguins (Fisher's exact test: $P < 0.001$ last 3 seasons). Intensity of parasitism did not differ significantly between the 2 species with the exception of the first season, when intensity was higher in cormorants (2 sample *t*-test $P < 0.04$). Analysis within species across all 4 sampling periods demonstrated significant differences among periods in median intensity (MI) and prevalence in the cormorant population (intensity: Mood's median test $P < 0.001$; prevalence: Fisher's exact test $P < 0.001$). Pair-wise comparison of cormorant data for each dry season against each wet season yielded significant differences in 3 of the 4 comparisons for both mean abundance (MA) and MI (MA: 2-sample *t*-test; MI: Mood's median test; August 2003 vs. February 2005 MA: $P < 0.006$, MI: $P < 0.01$; March 2004 vs. August 2004 MA: $P < 0.03$, MI: $P < 0.02$; August 2004 vs. February 2005 MA: $P < 0.01$, MI: $P < 0.01$; August 2003 vs. March 2004 $P > 0.05$ for MA or MI). Seasonality had no effect on prevalence in the penguins when all 4 seasons were considered together; however, both species demonstrated a significant difference in prevalence between the first (August 2003) and last (February 2005) collecting trips (Fisher's exact test: penguins $P < 0.017$, cormorants $P < 0.001$), reflecting the declining incidence of filarids in penguins and increasing incidence in the cormorants. Body condition was not correlated with presence or intensity of parasitism in the penguins. Male penguins had significantly higher prevalences than females (Fisher's exact test $P < 0.009$).

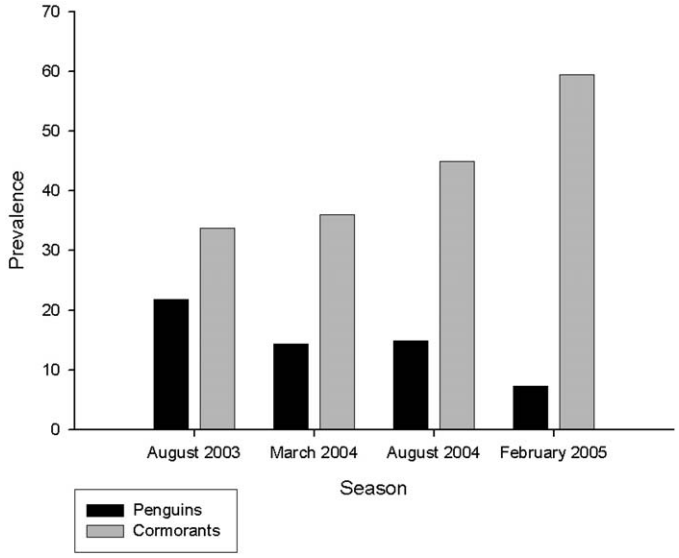

FIGURE 1. Histogram of prevalence of parasitism across seasons in cormorants (gray) and Galápagos penguins (black).

Intensity of infection in individual birds ranged from 1–300 per 25, ×100 fields in cormorants, whereas in penguins, the intensity ranged from 1–110 per 25, ×100 fields (Table I). The distribution of the intensity of parasitism for both cormorants and penguins demonstrates a negative binomial distribution of these parasites, a characteristic finding for parasites (Crofton, 1971).

Filarid genotyping and phylogenetic analyses

A 649-bp region of double-stranded filarid nematode mitochondrial COI sequence was recovered (and yielded an open reading frame) from 3 of the cormorant individuals and 2 of the penguin individuals initially screened by PCR (GenBank DQ838570–DQ838574). Filarid COI sequences from 4 of these individuals (2 cormorants and 2 penguins) were identical across the entire region. The fifth sample, from a cormorant, varied at 5 of 649 sites. To determine their identity, we subjected these putative filarid COI sequences to a BLAST search in GenBank (NCBI). The results of the BLAST search were conclusive. All of the most similar sequences in the database to the 2 submitted were filarial nematode COI sequences, although the 2 Galápagos-derived sequences were unique. To our knowledge, no other COI sequences from filarids derived from wild birds have been deposited in GenBank, although some nuclear LSU rDNA sequences from filarids derived from African rain forest birds are available (Sehgal et al., 2005). Although our sample size was very limited, nematode isolates with identical COI sequences were found in multiple flightless cormorants and Galápagos penguins. The sole variant from a flightless cormorant was <1% divergent from this sequence, suggesting that all of these sequences were derived from the same filarid species (Blouin et al., 1998). A phylogenetic analysis including these 2 sequences and several COI sequences from other filarids indicates that the filarids derived from Galápagos birds are distinct from all others in the phylogeny, forming a clade with 100% bootstrap support (Fig. 2). However, the COI gene is not as useful for understanding higher-level relationships as are other, more slowly evolving genes (Casiraghi et al., 2000), and most nodes were not highly supported in the bootstrap analysis.

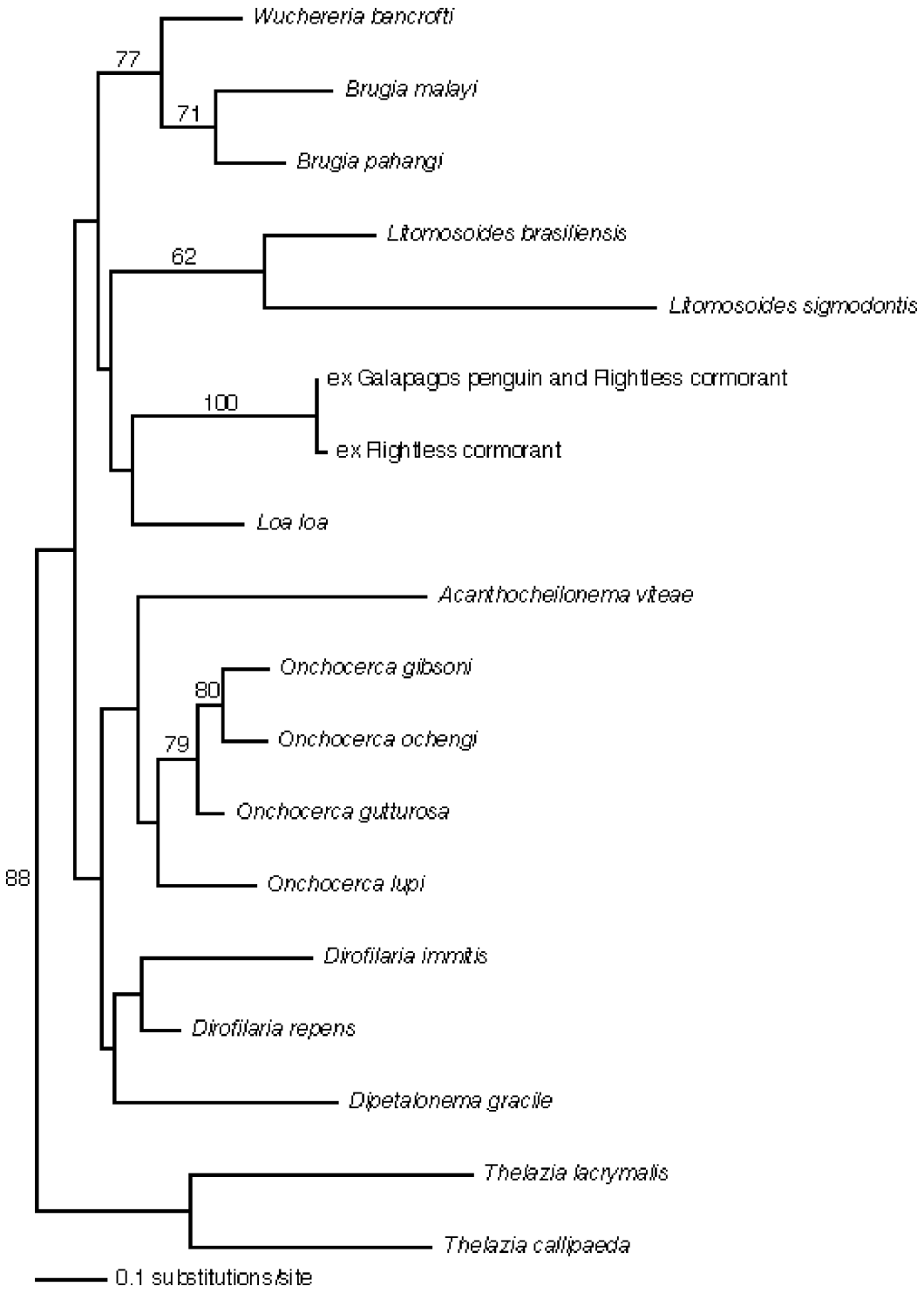

FIGURE 2. Single maximum-likelihood phylogenetic tree of 513 bp from the mitochondrial cytochrome *c* oxidase subunit I gene of filarid nematodes, including sequences obtained from microfilariae infecting Galápagos penguins and flightless cormorants. The TrN + I + G model of sequence evolution was chosen as the most likely (−ln = 1,807.56) and the score of the best tree was 3,177.10116. Values above branches indicate bootstrap support for nodes from a bootstrap analysis of the sequences in a heuristic search in maximum parsimony (1,000 replications). Only values >50% are reported; thus, support for nodes without values is equivocal. Note that the accessions from penguins and cormorants are the only avian-derived COI sequences available from GenBank.

Morphology of the microfilariae from flightless cormorant *Phalacrocorax harrisi*

Description (Fig. 3; Table II): Relatively long microfilariae, unsheathed, usually disposed extended or undulating. Maximum width in anterior quarter of worm, around level of nerve ring, thereafter tapering gradually to the posterior end; tail blunt, with nuclei extending almost to tip. Long cephalic space, nerve ring at about one-quarter of distance from anterior end, excretory pore about one-third and G^1 cell about three-quarters from anterior end, well defined. Inner body often stained bright pink; large, G^1 cell dark, immediately posterior to inner body.

Microfilariae from penguins *Spheniscus mendiculus* were similar in appearance and in all measurements to those from cormorants (Table II). Although this filarid may represent a novel species, adults are required for species descriptions.

DISCUSSION

In this study, we found high prevalence of nematode microfilariae in Galápagos penguins and flightless cormorants. Over the 2-yr study period, prevalence declined in penguins and increased in cormorants. Preliminary genotyping and morphological analyses suggest that these 2 hosts are infected with the same species of filarid nematode (Fig. 4).

Two species of filarial nematode have been previously described from a cormorant species, i.e., *Parornithofilaria shaldybini* (Sonin, 1963), subsequently placed in *Chandlerella* (Splendidofilariinae) by Anderson and Freeman (1969), was described from *Phalacrocorax urile* (red-faced cormorant) from the Kurile Islands in the Northwest Pacific Ocean. Adult worms were found in the host kidneys, and the microfilariae were not described. *Serratospiculum helicina* (Molin, 1858), subsequently placed in *Paronchocerca* (Splendidofilariinae), was described from the brain of *Anhinga anhinga* (anhinga), a group closely related to cormorants. Despite the limited information on adult filarial worms in this order of birds, reports of microfilariae from several species around the world indicate that infections are widespread. Takos (1947) found microfilariae in a single *A. anhinga* specimen examined in Panama, and Ashford et al. (1976) reported microfilariae in 1 of 7 *Anhinga rufa* (African darter) specimens examined in Ethiopia. Microfilariae were also reported by Mackerras (1962) from the cosmopolitan *Phalacrocorax carbo* (great cormorant) from Townsville (N. Queensland, Australia). In none of these 3 reports was a description given of the microfilariae. However, Cleland and Johnston (1911) briefly describe and illustrate microfilariae recovered from little cormorants (*Phalacrocorax melanoleucus*) in New South Wales, Australia. These appear similar in form (Plate XXXII) to those collected from the Galápagos cormorants, with a large cephalic space and a blunt tail, but are slightly smaller (116–140 μm long). Subsequently, Cleland (1915) described 2 forms of microfilariae, one sheathed, collected from a mixed colony of *Plotus* (=*Anhinga*) *novae-hollandiae* and *Phalacrocorax sulcirostris* in SE Queensland, Australia. It is not clear, however, whether both forms occurred in both species of bird (Johnston, 1912). None of the original material can be traced to allow direct comparisons, but the illustration in Cleland and Johnston (1911) is similar in form to the specimens collected from flightless cormorants. Another 9 species in the Phalacrocoracidae have been examined (*Halietor africanus*, *Halietor niger*, *Halietor pygmaeus*, *Phalacrocorax atriceps*, *Phalacrocorax auritus*, *Phalacrocorax fuscicollis*, *Phalacrocorax olivaceus*, *Phalacrocorax pelagicus*, and *Phalacrocorax penicillatus*), and no microfilariae were reported (references in Bennett et al., 1982; Bishop and Bennett, 1992).

Additional tissue-dwelling nematodes occurring in large water birds, including cormorants, are *Desmidocercella* spp. in the Aproctoidea (Superfamily). Adults are found in the birds' air sacs; eggs contain fully formed first-stage larvae (Anderson, 1959). Swans and geese are known to be infected with a filarial heartworm, *Sarconema eurycerca* (Seegar et al., 1976).

Adult *Paronchocerca straeleni* have been recovered from the heart of a Galápagos penguin kept in captivity (Chabaud and Ball, 1964). That was a postmortem finding, and there is no record that a filarial infection was noted before death. This was a previously undescribed species, thus increasing the likelihood that it was an exotic infection, acquired before the host's capture. Microfilariae recovered from the uteri of these worms and cleared in lactophenol were of a similar length to those described herein, but possessed sheaths. However, differences in provenance, preparation, and staining in that study and in the present one preclude making comparisons between the microfilariae. Nematodes from the heart of a little penguin (*Eudyptula minor*), collected on Kangaroo Island, South Australia, in 1945, and labeled as Filarioidea (Mawson et al., 1986 p. 225), have been examined and found to be Ascaridoidea.

Many studies have been conducted on blood collected from penguins in the wild and in captivity; no hematozoa have been recorded from either Antarctic or sub-Antarctic species in the wild (summary in Jones and Shellam, 1999), though *Plasmodium* spp. have been reported from 9 penguins species in captivity (references in Bennett et al., 1982), often with high morbidity or mortality. However, several blood-inhabiting protozoa have been found in wild penguins in temperate regions, including *Leucocytozoon tawaki* in *Eudyptes pachyrhynchus* in New Zealand (Fallis et al., 1976) and in *Spheniscus demersus* in South Africa (Earle et al., 1992); *Plasmodium relictum* from *Spheniscus demersus* and 3 other species of penguin in South Africa (Fantham and Porter, 1944); *Babesia peircei* from *Spheniscus demersus* in South Africa (Earle et al., 1993); and *Trypanosoma eudyptulae* in *Eudyptula minor* in Tasmania (Jones and Woehler, 1989).

Without knowledge of the adult worms, it cannot be ascertained whether the microfilarial forms from *P. melanoleucus* (little cormorants) are of the same species as those from the Galápagos cormorants. Host specificity of filarial worms varies, but some species exhibit low host-specificity (Anderson and Bain, 1976), e.g., *Cardiofilaria inornata* (Anderson and Freeman, 1969), and therefore, the phylogenetic distance between Spheniscidae and Phalacrocoracidae need not preclude this possibility. Furthermore, as noted above, *Paronchocerca* sp. has been reported from both anhingas (*P. helicina*) and the Galápagos penguin (*P. straeleni*). As both the flightless cormorant and the penguins breed in close proximity on the shores of Isabela and Fernandina, it is possible that they share the same species of hematophagous arthropod, which may act as intermediate hosts. Possible vectors of these microfilariae include *Ochlerotatus taeniorhynchus*, a mosquito species known to breed in brackish water, and *Culex quinquefasciatus*, an introduced mosquito species (Barnett, 1985; Whiteman et al., 2005).

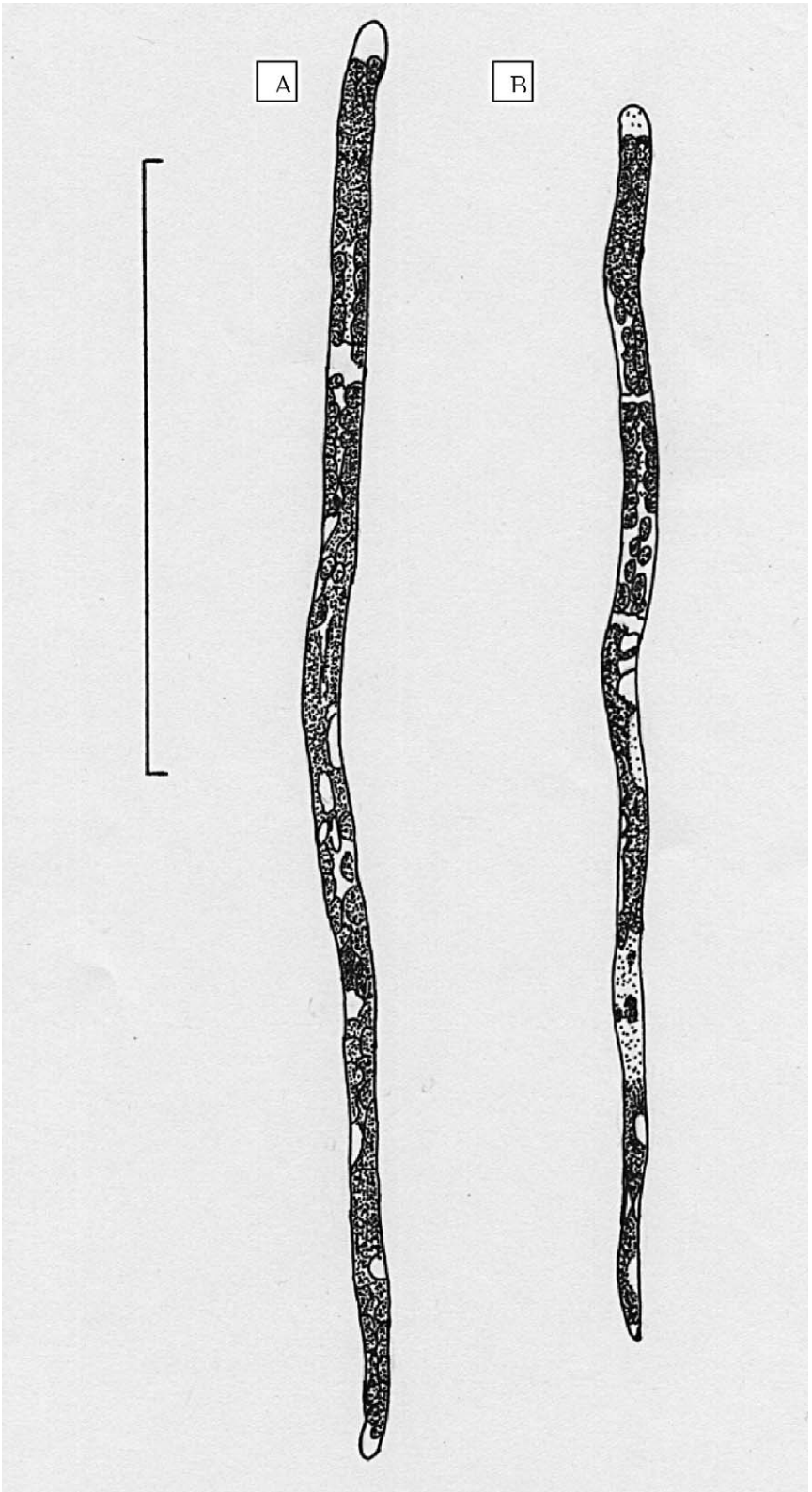

FIGURE 3. Microfilariae from peripheral blood of the Flightless Cormorant *Phalacrocorax harrisi* (**A**) and from the Galápagos penguin *Spheniscus mendiculus* (**B**). Bar = 50 μm.

TABLE II. Measurements (μm) of microfilariae from peripheral blood of flightless cormorants (*Phalacrocorax harrisi*) (n = 3 birds) and Galápagos penguins (*Spheniscus mendiculus*) (n = 3 birds).

Measurements	Flightless cormorants (n = 30) (mean and range)	Galápagos penguins (n = 21) (mean and range)
Length	164 (128–184)	164 (136–200)
Maximum width	53 (40–68)	53 (48–60)
Cephalic space	64 (40–88)	52 (40–80)
Nerve ring (%)*	25 (18–31)	24 (20–31)
Excretory pore (%)*	38 (32–47)	37.6 (32–46)
Inner body (%)*	64 (54–73)	63 (52–72)
G 1 cell (%)*	74 (63–90)	75 (62–84)
Tail	181 (120-224)	197 (128-296)

* Proportion of distance from anterior end of microfilariae.

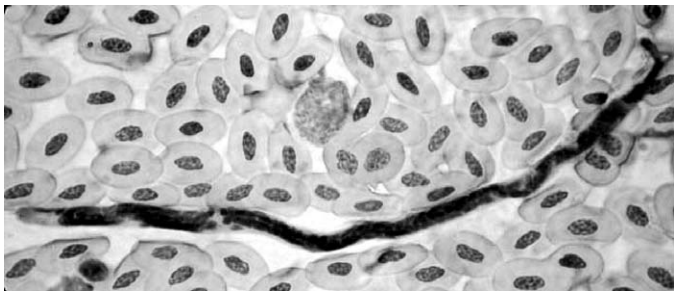

FIGURE 4. Photomicrograph of microfilariae from a flightless cormorant.

Although only rarely implicated as vectors of microfilariae, hippoboscid flies (*Olfersia sordida*) are abundant in cormorant nesting areas (Olsen, 1974). Prevalence of *O. sordida* is high on these cormorants; however, hippoboscid flies have not been observed on the adult penguins by the researchers (J. Merkel, H. Vargas, pers. obs.). Absence of hippoboscid flies on adult penguins does not preclude the possibility that penguin chicks, with more easily penetrable feathers, can be bitten.

Recently, *Dirofilaria immitis* was recovered from the heart of a captive Humboldt penguin (*Spheniscus humboldti*) and is believed to have been the reason for death (Sano et al., 2005). *Dirofilaria immitis* is known to occur on the inhabited island, Isabela, on which some of the penguins and cormorants reside (Barnett, 1985). *Dirofilaria immitis* infects feral dogs and sea lions and many of the human inhabitants have circulating antibodies to this filarid. However, morphologically, *D. immitis* is dissimilar to the microfilariae in the penguins and cormorants, and the phylogenetic analysis presented here suggests that *D. immitis* and the form infecting the 2 bird species in Galápagos are not especially close relatives. Often, filarid infections in wild avian hosts are dismissed as an incidental finding; however, in the absence of long-term mark–recapture studies that monitor return rates, it is difficult to know what effect filarids have. Filarid infections may predispose the birds to other illnesses by lowering immunological barriers. Negative life-history consequences resulting from filarial infection cannot be ruled out either, i.e., delayed egg laying, fewer chicks per brood, or decreased immunity.

Seasonality

Our most interesting finding over the course of the 4 seasons was the increased prevalence of the microfilariae in the cormorants with a concurrent decreased prevalence in the penguins. Long-term studies are needed to reveal why the prevalences are changing in the manner here described because many factors could be responsible. Additionally, during the last season (February 2005), the range in intensity increased for both species. Our original hypothesis that the hot, rainy season would result in higher prevalence and intensity because of the higher inferred presence of vectors was not supported. Although the cormorants did show significant differences between seasons, that was because of a rising prevalence across the 4 seasons and not a dichotomy between hot, wet seasons and cool, dry seasons. Prepatency in this species of parasite may be too long to allow circulating microfilariae to be seen within the same season.

Cormorants always exhibited a higher prevalence of microfilariae than penguins in each season, which leads to speculation that the cormorants may be the preferred host for this species of microfilariae. However, behavioral differences between the 2 species may account for differences in prevalence. Penguins are known to spend more time in the sea than cormorants (du Toit et al., 2004), and nesting behaviors vary, in that penguins often nest in holes in the lava, whereas cormorants nest in open areas, presumably allowing vectors easier access. Locating the adult parasite to allow positive identification would help clarify with which host species this parasite is most commonly associated.

Male penguins had significantly higher prevalence than females. There are at least 2 explanations for the higher prevalence in males: (1) immunosuppressive effects of testosterone, or (2) differences in life history traits, e.g., visiting habitats that expose them to more vectors than females. Recently, an endogenous increase in testosterone was shown to increase abundance of *Leucocytozoon fringillinarum* in dark-eyed juncos (*Junco hyemalis*) (Deviche and Parris, 2006). Klein (2004) found that in 84.5% of cases of parasitism (58 parasite species), male hosts had either higher prevalence or intensity of parasitism than females. Of the filarid parasites examined in the previous study, all demonstrated that males had either higher prevalence or intensity of parasites. In our investigation, we found that, although penguin males had significantly higher prevalence than females, the differences in intensities were not statistically significant between genders.

Filarid genotyping

The successful preliminary mitochondrial genotyping of microfilariae isolated from peripheral blood of flightless cormorants and Galápagos penguins suggests that the same species of filarid infected those 5 birds and is consistent with the morphological data set. Further genotyping, with primers designed specially for this filarid lineage, may allow development of a reliable PCR assay for presence of microfilariae in the peripheral blood of each host species. The phylogenetic analysis, although not exhaustive, shows that the Galápagos penguin and flightless cormorant–derived filarids are distinct from all others included in the analysis, which represented most major genera known to infect humans and other mammals. However, this marker (COI)

is not ideal for a higher-level phylogenetic analysis because of the high rate of nucleotide substitution (Casaghiri et al., 2001).

Although microfilariae are often nonpathogenic, there are reports of negative health impacts, and the rare death, of some affected birds caused by filarid infection (Campbell, 1988; Simpson et al., 1996; Davidar and Morton, 2006). Flightless cormorants and Galápagos penguins are both endangered species (BirdLife International, 2004), so monitoring for the potential adverse effects of microfilariae is essential. The small size of these populations renders them vulnerable to new parasites (Whiteman et al., 2006). Hemoparasites can contribute to extinctions as evidenced by Hawaii's loss of much of its endemic avifauna (Warner, 1968; Van Riper et al., 1986).

Our future work will focus on the spatial epidemiology of this microfilarial parasite, as well as a concerted effort to capture potential vectors of the microfilariae. We will also continue to attempt to find an adult filarid nematode from both the penguins and cormorants. In view of findings of microfilariae in Phalacrocoracidae from several parts of the world, it would be of particular interest to study the taxonomy, arthropod vectors, and geographical range of filarial worms in this cosmopolitan family of marine and aquatic birds.

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