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ORIGINAL PAPER

Investigations of peritoneal and intestinal infections of adult hookworms (*Uncinaria* spp.) in northern fur seal (*Callorhinus ursinus*) and California sea lion (*Zalophus californianus*) pups on San Miguel Island, California (2003)

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Abstract The peritoneal cavity (PNC) and intestine of northern fur seal (*Callorhinus ursinus*) pups and California sea lion (*Zalophus californianus)* pups that died in late July and early August, 2003, on San Miguel Island, California, were examined for hookworms. Prevalence and morphometric studies were done with the hookworms in addition to molecular characterization. Based on this and previous molecular studies, hookworms from fur seals are designated as *Uncinaria lucasi* and the species from sea lions as *Uncinaria* species A. Adult hookworms were found in the PNC of 35 of 57 (61.4%) fur seal pups and of 13 of 104 (12.5%) sea lion pups. The number of hookworms located in the PNC ranged from 1 to 33 (median=3) for the infected fur seal pups and 1 to 16 (median=2) for the infected sea lion pups. In addition to the PNC, intestines of 43 fur seal and 32 sea lion pups were examined. All of these pups were positive for adult hookworms. The worms were counted from all but one of the sea lion pups. Numbers of these parasites in the intestine varied from 3 to 2,344 (median=931) for the fur seal pups and 39 to 2,766 (median=643) for the sea lion pups. Sea lion pups with peritoneal infections had higher intensity infections in the intestines than did pups without peritoneal infections, lending some support for the hypothesis that peritoneal infections result from high-intensity infections of adult worms. There was no difference in intestinal infection intensities between fur seal pups with and without peritoneal infections. Female adult hookworms in the intestines of both host species were significantly larger than males, and sea lion hookworms were larger than those in fur seals. Worms in the intestine also were larger than worms found in the PNC. Gene sequencing and (RFLP) analysis of (PCR) amplified (ITS) ribosomal DNA were used to diagnose the species of 172 hookworms recovered from the PNC and intestine of 18 *C. ursinus* and seven *Z. californianus* hosts. These molecular data revealed that *U. lucasi* (hookworm of *C. ursinus*) and *Uncinaria* species A (of *Z. californianus*) infrequently mature in the intestine of the opposite host species in California rookeries. However, there is no support from molecular data for the hypothesis that cross-infection with "the wrong" *Uncinaria* species is a contributing factor in these cases of host peritonitis. The major significance of this research is the unusual finding of adult hookworms in the PNC of so many dead pups. No obvious explanation for this occurrence could be determined. Further research, like in the present study, should help understand and monitor the apparent ever changing role of hookworm disease in the health of northern fur seal and California sea lion pups on SMI.

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Introduction

Taxonomy of hookworms (*Uncinaria* spp.) in pinnipeds is uncertain. Two species, *Uncinaria lucasi* and *Uncinaria*

hamiltoni, have been described according to morphology but intermediate types have been reported from certain hosts, including the California sea lion (CSL) (Dailey and Hill 1970). Molecular and morphometric distinctions have been demonstrated between hookworms from the intestines of northern fur seal (*Callorhinus ursinus* (NFS)) and CSL (*Zalophus californianus*) pups on San Miguel Island (SMI), CA (Nadler et al. 2000; Nadler 2002), with molecular data indicating that two different hookworm species are present, one in each host species. Recently, the first finding of adult hookworms penetrating the wall of the small intestine and entering the peritoneal cavity (PNC) of a dead CSL pup on SMI was reported (Spraker et al. 2004). This discovery precipitated interest in determining the extent of the phenomenon in NFS and CSL pups, examined at necropsy on SMI in 2003. The field study was performed in late July and early August, the months with the highest number of hookworms in the intestine of dead pups on SMI (Lyons et al. 2005). In the current research, besides examination of the PNC, the intestine also was processed for recovery of hookworms for comparison of prevalence and intensity of infection with previous studies (Lyons et al. 1997, 2001, 2005). This also permitted obtaining worm specimens to be compared morphometrically and genetically with those from the PNC.

Individual hookworm specimens recovered from the PNC and the intestine were diagnosed using species-specific restriction fragment length polymorphism (RFLP) profiles designed from nuclear internal transcribed spacer (ITS) sequences. This genetic analysis was done to assess whether specimens in the PNC of dead NFS and CSL pups were the same species as recovered from their own intestine or were those normally present in the intestine of the other ("opposite") host from SMI. This molecular characterization addresses the possibility that the unexpected presence of adult worms in the PNC is the result of hookworm species migrating abnormally because they were in the "wrong" host. For the molecular characterization, names used for hookworms are *U. lucasi* for individuals from northern fur seals and *Uncinaria* species A for individuals from California sea lions. The "species A" designation is based on previous molecular studies showing that hookworms from California sea lion hosts are a different species than *U. lucasi*, and that the appropriate species name remains uncertain for these hookworms (Nadler et al. 2000; Nadler 2002).

Materials and methods

The hookworm study was done on San Miguel Island, California (34°2′ N, 120°26′ W) from 29 July through 10 August, 2003, on dead NFS and CSL pups. The PNC of 57 NFS pups and 104 CSL pups was examined for hookworms at necropsy. In addition, the intestines of 43 NFS and 32 CSL of these pups were examined for hookworms. The worms were counted from all but one of these CSL pups.

Necropsy aspects

Freshly dead pups, usually in rigor mortis, were selected for necropsies; these were performed in the field. Some specific details including pup sex, blubber thickness, and rookeries of collection were recorded. Each pup was placed on its back and the ventral midline of the abdomen opened with a scalpel. Then, about 60 cc of seawater was squirted from a syringe onto the walls of the PNC and the abdominal organs. The organs were manipulated by hand to free and concentrate any hookworms into the peritoneal fluid and saltwater solution pooled in the abdominal cavity. Then, the liquid was suctioned with another syringe and discharged into a 50-mesh sieve (openings=300 μm). Saltwater from a spray bottle was expelled into the sieve to wash the material which sometimes contained particulate matter such as blood and/or fibrin. The sieve then was examined grossly for hookworms. Hookworms recovered immediately were placed in absolute EtOH for molecular study. If hookworms were found, the method for recovery of hookworms from the PNC and sieving of the fluid was repeated until no hookworms were seen after two successive washings. After the procedure was completed for recovery of hookworms from the PNC, the complete gastrointestinal tract was removed. The entire small and large intestines were processed using the "rinsing" method previously described (Lyons et al. 2005). For pups from which hookworms were found in the PNC, a few specimens were picked out of the intestinal contents and put in absolute EtOH for future molecular study. The remainder of the material from the intestine was poured into a 50-mesh sieve and reduced in volume by washing it with saltwater pumped from a carboy with a hand-operated sprayer. Residue on the sieve was washed into a funnel inserted into an eight-ounce plastic jar and preserved with isopropyl alcohol. Recovery of hookworms from the fixed intestinal contents was done in the laboratory at the University of Kentucky (Lyons et al. 1997). Hookworms recovered from the PNC and intestine were counted and sexes were recorded. For one of the 32 CSL pups examined, counts were not made of hookworms found in its intestine. The wash of the PNC from four NFS pups was examined for hookworm eggs.

Hookworm measurements

Total length and width measurements were made for all intact hookworms recovered from the PNC of NFS and

CSL pups. A sample of worms from the intestine of 35 NFS and 13 CSL pups also was preserved in EtOH. The total length (millimeters) was measured under a dissecting microscope because the length was too great to do so readily under a compound microscope. Each hookworm was straightened, using two teasing needles, onto a plastic ruler calibrated in millimeters. The width measurements were made under a compound microscope at ×63 after each specimen was placed on a glass slide with a coverslip added; absolute EtOH was flooded under the coverslip. The measurements were made at the widest area in the approximate middle of the specimen.

Molecular characterization (DNA amplification and diagnostic RFLP)

Molecular diagnostics were performed "blind" with respect to the host source (species) and site of nematode infection (intestine versus PNC). Nucleic acid templates used for polymerase chain reaction (PCR) amplifications were extracted from ~4 mm midbody pieces taken from individual ethanol-preserved adult hookworms. Hookworm tissues were digested and nucleic acids prepared using the sodium hydroxide/neutralization method (Floyd et al. 2002). One and one half microliters of DNA extract was used for each PCR reaction.

The PCR was used to amplify a 1,400-bp region spanning from the 3′-end of the nuclear small-subunit ribosomal DNA (rDNA) to the 5′-end of the nuclear large subunit (LSU) rDNA, which included the ITS1, 5.8S, and ITS2 regions. The forward PCR primer #93 (5′-TTGAACCGGGTAAAAGTCG) and the reverse primer #608 (5′-CCTCGGTCCGTGTTTCAAGACG) were used with Taq polymerase (Promega) for PCR amplification. PCR mixes included 0.5 μM of each primer, 200 μM deoxynucleoside triphosphates, and a MgCl2 concentration of 3 mM in a total reaction volume of 25 μl. PCR cycling parameters included denaturation at 94°C for 3 min, followed by 36 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 1 min, followed by a post-amplification extension at 72°C for 7 min. One microliter of each PCR product was used to confirm amplicon size and yield by agarose gel electrophoresis (1.3% agarose in TBE buffer). When necessary, PCR products were concentrated by vacuum evaporation to approximately normalize product yields as estimated by ethidium bromide staining of DNA in agarose gels. For discrimination between *Uncinaria lucasi* (from *C. ursinus)* and *Uncinaria* species A (from *Z. californianus*), the digestion profiles of different restriction enzymes were predicted based on the rDNA sequences of these species (Nadler et al. 2000) using the program Sequencher v 3.0 (Gene Codes, Ann Arbor, Michigan). Based on predicted differences in restriction fragment sizes for different enzymatic cutters, two enzymes (NlaIII and FnU4HI) each were found to yield band profiles consistent with reliable species discrimination. The predicted restriction digest profiles for NlaIII and FnU4HI were confirmed experimentally using amplicons from DNA templates that had been sequenced for the ITS region. In addition, 15 ITS PCR amplifications from field collected hookworms (three putative *Uncinaria* species A and 12 *U. lucasi*) were directly sequenced to confirm that the RFLP profile was consistent with the diagnostic nucleotide sequence. For RFLP diagnostics of individual hookworms, 3 μl of ITS PCR product was digested overnight at 37°C using 1.5 U of restriction enzyme in the manufacturer's recommended buffer supplemented with 0.1 mg/ml BSA (New England Biolabs, Ipswich, Massachusetts). Restriction digests from *Uncinaria* individuals were resolved in 1.8% agarose gels with TBE buffer and restriction patterns determined following staining of gels with ethidium bromide.

Statistical methods

Prevalence of peritoneal and intestinal infections was computed for the complete sample of 104 sea lion and 57 fur seal pups that we examined. A subsample of those pups was used to assess infection intensities and worm morphometrics. We used non-parametric tests to avoid any distributional assumptions. For proportional data (e.g., prevalence of PNC infections across species) we used Fisher's exact test to assess whether the differences were significant. For non-paired tests of infection intensities and worm morphometrics, we used a Wilcoxon rank sum test. For paired tests of counts and worm morphometrics we used a Wilcoxon signed-rank test. The distribution of infection intensities tends to be skewed due to some animals with very large intensities. To describe the central tendency of these distributions, we have used medians and geometric means rather than arithmetic means (Lyons et al. 2005). For morphometric comparison of worms, we computed the mean length or width of the worms contained in each pup and then used those mean values for each pup as the sample statistics in the statistical summaries and tests. We did so because of the unequal numbers of worms found in each pup and the potential that the average worm length or width varied across pups.

Results

Adult *Uncinaria* spp. were recovered from the PNC of 35 (61.4%) of the NFS pups and of 13 (12.5%) of the CSL pups. Prevalence of peritoneal infection was significantly greater in NFS than CSL pups (Fisher's exact test, $p<0.001$). The number of hookworms ranged from 1 to 33 (median=3)

in the PNC for infected NFS pups (Table 1) and 1 to 16 (median=2) for infected CSL pups (Table 2). There was no difference in the prevalence of infection between male and female pups (Fisher's exact test, $p=1.0$ for NFS and $p=0.073$ for CSL pups). Identification of hookworms from the PNC according to sex revealed all were female except for three males of 190 recovered from NFS pups and two males of 44 from CSL pups. The wash of the PNC of all four NFS pups examined was positive for hookworm eggs in varying degree of embryonation to the tadpole stage.

Adult hookworms were discovered emerging from the small intestinal wall into the PNC of one NFS pup (one specimen) and two CSL pups (one specimen in one pup and two in the other). About two thirds of the body of each hookworm was free in the peritoneal cavity with the other portion within the wall of the intestine.

Intestines of all the pups of both host species harbored adult hookworms. The intensity of intestinal infections in CSL pups with peritoneal infections were greater than in CSL pups without peritoneal infections (Wilcoxon rank sum test $p=0.004$; Table 2). There was no significant difference in the intensity of intestinal infection between NFS pups with and without peritoneal infections ($p=0.068$; Table 1). There was also no significant difference in the intensity of intestinal infection between NFS (geometric mean=694.5) and CSL (geometric mean=574.2) hosts (Wilcoxon rank sum test, $p=0.266$). Numbers of hookworms in the intestine varied from 3 to 2,344 (geometric mean=914.3 for female and 531.2 for male hookworms) for the NFS and 39 to 2,766 (geometric mean=412.9 for female and 728.5 for male hookworms) for CSL (Table 3).

There was no significant difference in the intensity of intestinal infections of CSL pups on rocky (geometric mean=476.2) and sandy substrates (geometric mean=612.8) (Wilcoxon rank sum test, $p=0.343$). Also, there was no difference in the number of male and female hookworms in the intestines of either NFS (Wilcoxon signed-rank test, $p=0.653$) or CSL (Wilcoxon signed-rank test, $p=0.089$) pups (Table 3).

Hookworm measurements

A sample of worms from the intestine of 35 NFS and 13 CSL was measured. As expected, female hookworms were significantly larger than male hookworms in both host species (Table 4). Hookworms recovered from the PNC of NFS and CSL pups were significantly smaller in length than paired samples of worms recovered from the intestine (Table 5). The same worms were significantly smaller in width in the PNC versus the intestine of NFS but not in CSL (Table 5). The adult male hookworms from CSL were significantly larger (Wilcoxon rank sum tests) in both

Table 1 Intensity of intestinal (gut) infections in northern fur seal (*Callorhinus ursinus*) (NFS) pups with and without peritoneal (PNC) infections of adult hookworms

	Worms	
NFS	PNC	Gut
SMI 03 CU-15	1	1,917
SMI 03 CU-16	1	1,298
SMI 03 CU-21	10	387
SMI 03 CU-22	2	1,146
SMI 03 CU-23	2	443
SMI 03 CU-24	2	2,115
SMI 03 CU-26	3	861
SMI 03 CU-34	7	588
SMI 03 CU-38	3	1,155
SMI 03 CU-39	1	792
SMI 03 CU-40	9	341
SMI 03 CU-42	4	762
SMI 03 CU-43	1	315
SMI-03 CU-44	4	878
SMI 03 CU-45	33	1,035
SMI 03 CU-48	1	302
SMI 03 CU-49	2	1,676
SMI 03 CU-50	5	1,368
SMI 03 CU-51	7	887
SMI 03 CU-52	3	701
SMI 03 CU-55	17	440
SMI 03 CU-57	1	782
SMI 03 CU-59	1	1,091
SMI 03 CU-60	8	1,345
SMI 03 CU-61	2	247
SMI 03 CU-62	5	496
SMI 03 CU-63	4	797
SMI 03 CU-64	3	900
SMI 03 CU-65	20	425
SMI 03 CU-66	1	1,185
SMI 03 CU-67	1	792
	Median	886
SMI 03 CU-17	0	2,344
SMI 03 CU-18	0	1,323
SMI 03 CU-19	0	1,422
SMI 03 CU-37	0	769
SMI 03 CU-53	0	1,232
SMI 03 CU-54	0	1,219
SMI 03 CU-56	0	3
SMI 03 CU-58	0	2,003
	Median	1,278

Wilcoxon rank sum test $p=0.068$; (not significant, but trend is in wrong direction; i.e., higher intensity of infection in pups with no peritoneal infection)

Table 2 Intensity of intestinal (gut) infections in California sea lion (*Zalophus californianus*) (CSL) pups with and without peritoneal (PNC) infections of adult hookworms

CSL	Worms PNC	Gut
SMI 03 ZC-04	1	614
SMI 03 ZC-05	2	502
SMI 03 ZC-09	1	347
SMI 03 ZC-15	16	1,943
SMI 03 ZC-19	2	1,539
SMI 03 ZC-28	2	2,505
SMI 03 ZC-30	4	2,766
SMI 03 ZC-33	1	1,230
SMI 03 ZC-48	4	2,504
SMI 03 ZC-74	1	520
SMI 03 ZC-87	6	2,452
SMI 03 ZC-95	2	2,352
	Median	1,741
SMI 03 ZC-01	0	1,315
SMI 03 ZC-03	0	52
SMI 03 ZC-06	0	81
SMI 03 ZC-08	0	39
SMI 03 ZC-12	0	618
SMI 03 ZC-13	0	212
SMI 03 ZC-17	0	668
SMI 03 ZC-24	0	147
SMI 03 ZC-26	0	192
SMI 03 ZC-27	0	651
SMI 03 ZC-29	0	1,558
SMI 03 ZC-31	0	614
SMI 03 ZC-34	0	683
SMI 03 ZC-36	0	1,022
SMI 03 ZC-37	0	282
SMI 03 ZC-39	0	682
SMI 03 ZC-45	0	900
SMI 03 ZC-57	0	217
SMI 03 ZC-58	0	190
	Median	614

Wilcoxon rank sum test $p=0.004$; significantly greater intensity of infection in pups with peritoneal infections

Table 3 Geometric means of the total number of male vs. female hookworms in the intestine of California sea lion (*Zalophus californianus*) (CSL) and northern fur seal (*Callorhinus ursinus*) (NFS) pups, San Miguel Island—2003

Sex (hookworms)	Geometric mean	Minimum	Maximum
CSL pups ($n=31$)			
Male	728.5	1	1,469
Female	412.9	23	1,297
Test statistic[a] $p=0.089$			
NFS pups ($n=43$)			
Male	531.2	2	1,160
Female	914.3	1	1,184
[a]Test statistic[a] $p=0.653$			

Wilcoxon signed-rank test with continuity correction (neither relationship is significant)

length ($p<0.0001$) and width ($p<0.0001$) than were worms from NFS (Table 6). The same significant relationship exists for the width of female hookworms but not for female worm length.

Molecular characterization

Restriction digests of individual hookworm ITS rDNA, using either NlaIII or FnU4HI, provided distinctive fragment profiles that conformed to predicted band sizes of *U. lucasi* and *Uncinaria* species A, as determined from their gene sequences (results for FnU4HI, Fig. 1). One hundred seventy-two individual nematodes were used for ITS amplification and RFLP diagnostics (Fig. 1, Table 7). These 172 specimens represented 24 pinniped host individuals having hookworms in both the intestine and PNC. One group of RFLP-diagnosed nematodes included only hookworms from the intestine. Eighteen of these hosts were NFS (*C. ursinus*) and seven were CSL (*Z. californianus*); the number of nematodes RFLP-diagnosed per host individual ranged from 2 to 20. For *Z. californianus* hosts, 24 of these hookworms resided in the intestine and 18 in the PNC. In *C. ursinus* hosts, 66 of these hookworms resided in the intestine and 63 in the PNC. One hundred sixty-nine of these RFLP-typed hookworm species were recovered from their normal (expected) definitive host species irrespective of their location (intestine versus PNC) within the host. Three RFLP-diagnosed hookworms were recovered from the unexpected host species (Table 7). Two instances involved *U. lucasi* individuals occurring in the intestine of *Z. californianus* hosts, and one instance involved an *Uncinaria* species A individual in the intestine of a *C. ursinus* host. PCR products of these three individuals were sequenced to reconfirm RFLP identifications along with 12 other hookworms (Table 7). In addition, seven individual eggs were removed from an *U. lucasi* specimen for sequence-based diagnostics. Nucleic acids from these individual eggs were extracted, amplified, and sequenced using the methods described previously for adult tissues. This adult *U. lucasi* female specimen was obtained from the intestine of a *Z. californianus*, which is a species normally parasitized by a different species, here referred to as *Uncinaria* species A (all other individuals characterized from this host were *Uncinaria* species A). All seven eggs

Table 4 Morphometric measurements of both sexes of hookworms recovered from the intestine of 13 California sea lion (*Zalophus californianus*) and 35 northern fur seal (*Callorhinus ursinus*) pups, San Miguel Island—2003

Worm sex	No. of worms	Length (mm)	Standard error	Width (μm)	Standard error
California sea lion					
Male	553	7.90	0.228	308.66	5.304
Female	867	11.05	0.305	399.77	5.845
p value[a]		<0.001		<0.001	
Northern fur seal					
Male	1,013	6.65	0.228	274.48	2.756
Female	2,421	10.41	0.180	373.13	4.450
p value[a]		<0.001		<0.001	

[a] Wilcoxon signed-rank test; significance level chosen at $p<0.05$

had DNA sequences diagnostic of *U. lucasi*, with no evidence of alternative electropherogram peaks at the ITS sites used to discriminate between *U. lucasi* and *Uncinaria* species A.

Discussion

Prior to the current study, the only information available on the presence of adult hookworms (*Uncinaria* spp.) in the PNC of pinnipeds was in a CSL pup on SMI (Spraker et al. 2004). The present study describes the discovery of adult hookworms penetrating the intestinal wall and their recovery from the PNC of NFS and CSL pups on SMI. Prevalence and intensity of PNC and intestinal infections of hookworms were evaluated and specimens from both anatomical locations were diagnosed to species using molecular methods.

In the present study, the 61% prevalence of adult *Uncinaria* spp. in the PNC of NFS pups was approximately five times greater than that of CSL pups, which was also statistically significant. The high prevalence of peritoneal infections found in both host species was not anticipated because the normal location of adult hookworms is the intestine. Extra-intestinal location of these parasites is not only remarkable but also extremely detrimental. Hookworms can produce injury to the intestinal wall resulting in bacteremia and also peritonitis with migration through the gut wall into the PNC (Spraker et al. 2007). Either one of these conditions can lead to death of the pups. Female hookworms predominate over males in the body cavity, but the reason for this bias is unclear. Males are smaller than females, which makes their detection and recovery more difficult, but it seems unlikely to be responsible for the dramatic prevalence of female worms recovered from the PNC.

Morphometric data from measurement of a large number of specimens from the intestines of NFS and CSL in the present study indicate a significant difference in size of the hookworms in the two hosts. This was reported by Nadler et al. (2000) but that study was based upon few hookworms from a few hosts. The difference in measurements and molecular characteristics indicate that hookworms in NFS and CSL are separate species (Nadler et al. 2000; Nadler 2002). However, the number of different *Uncinaria* species in pinniped hosts is currently unknown, and resolution of this question requires additional studies profiling morphometric and molecular make-up of hookworms from other host species, preferably including in-depth study of additional morphological structures of these hookworms. Such studies may clarify reports of pinniped hookworms that do not fit the morphological descriptions of *U. lucasi* and *U. hamiltoni* (Dailey and Hill 1970; Castinel 2006; Castinel et al. 2007).

It is of interest that the hookworms in the PNC overall are smaller than those in the intestines of each of the two hosts. We assume this is evidence that worms in the peritoneal cavity do not find adequate nutrition to achieve

Table 5 Morphometric measurements of female hookworms recovered from the peritoneal cavity and intestine (gut) of nine California sea lion (*Zalophus californianus*) (CSL) and 34 northern fur seal (*Callorhinus ursinus*) (NFS) pups, San Miguel Island—2003

	Length (mm)			Width (μm)		
Host species	Gut	Peritoneal cavity	*p* value[a]	Gut	Peritoneal cavity	*p* value[a]
CSL	11.06	9.91	0.009	399.77	386.05	0.065
NFS	10.41	9.78	0.019	373.13	357.57	0.004

[a] Wilcoxon signed-rank test; significance level chosen at $p<0.05$

Table 6 Morphometric comparative measurements of male and female intestinal hookworms of 13 California sea lion (*Zalophus californianus*) (CSL) and 35 northern fur seal (*Callorhinus ursinus*) (NFS) pups, San Miguel Island—2003

Host species	No. of worms	Length (mm)	Standard error	Width (μm)	Standard error
Female hookworms					
CSL	867	11.05	0.305	399.77	5.845
NFS	2,421	10.41	0.180	373.13	4.450
p value[a]		0.121		0.002	
Male hookworms					
CSL	553	7.90	0.228	308.66	5.304
NFS	1,013	6.65	0.228	274.48	2.756
p value[a]		<0.0001		≤0.0001	

[a] Wilcoxon rank sum test; significance level chosen at $p<0.05$

growth comparable to those in the intestine of the same animal. Although we do not know the stage or size of hookworms when they enter the PNC, it is known that at least some of them enter the PNC as adults. This is based on necropsy finding of some adult specimens in the act of penetrating the intestinal wall with the head end in the PNC and the tail in the lumen of the intestine. Possibly, some younger ones entered the PNC and were somewhat stunted in growth when located there.

Molecular systematic studies of *Uncinaria* spp. hookworms from California sea lions *(Z. californianus*) and northern fur seals (*C. ursinus*) have confirmed the existence of separate hookworm species from these hosts (Nadler et al. 2000; Nadler 2002). Similarly, statistically significant morphometric differences between hookworms from these two host species also have been reported (Nadler et al. 2000 and the present study), and were consistent with the molecular delimitation of separate species. Molecular characters delimiting these host-associated hookworms as separate species include nucleotide sequences derived from nuclear (LSU) and ITS rDNA, and mitochondrial 12S rDNA (Nadler et al. 2000; Nadler 2002). Hookworms from *C. ursinus* and *Z. californianus* often have been referred to as *U. lucasi* Stiles and *U. hamiltoni* Baylis, respectively. However, there is considerable uncertainty concerning the accuracy of *U. hamiltoni* as the proper name for hookworms from CSL, given that the morphology of these nematodes does not match the original description (Dailey and Hill 1970) and the type host of *U. hamiltoni* is a South American pinniped. Demonstrating that hookworm individuals from the topotype locality of *U. lucasi* are characterized by their diagnostic sequences remains necessary to fully corroborate *U. lucasi* as the proper name for these NFS hookworms (for detailed discussion, see Nadler et al. 2000).

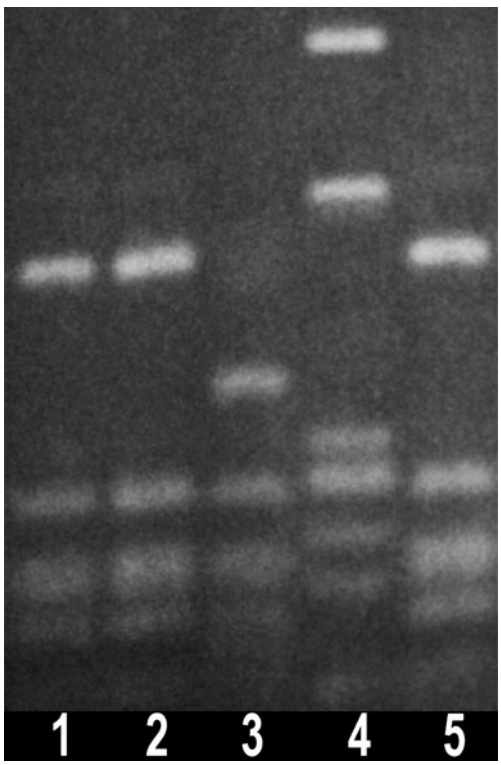

Fig. 1 Ethidium bromide-stained agarose gel (1.8%) showing diagnostic FnU4HI RFLP digests of nuclear ITS rDNA from *Uncinaria* of northern fur seals versus California sea lions. *Lanes 1*, *2*, and *5*, *U. lucasi* from *Callorhinus ursinus*. *Lane 3 Uncinaria* species A from *Zalophus californianus*. *Lane 4* molecular size marker (in bp: 872, 603, 310, 281, 271, and 234)

Previous molecular diagnostics of these hookworm species have failed to reveal evidence of cross-transmission (i.e., *U. lucasi* maturing in *Z. californianus* or *Uncinaria* species A maturing in *C. ursinus)*; however, these studies were based on a relatively small sample (~125) of *Uncinaria* individuals. Nucleotide sequence and RFLP data in this study confirm that both *U. lucasi* and *Uncinaria* species A can occur in the intestines of the "opposite" host species but at low frequencies in this study (<2% of specimens characterized). This opportunity for cross-infection occurs in circumstances where rookeries of these two hosts are adjacent, allowing host exposure to infective L_3 of both species. From previous research, it is clear that the differences in pathogenesis, hookworm location, and nematode longevity between NFS pups and CSL pups (Lyons et al. 1997, 2000) are not the result of the differential consequences of one hookworm species in two

Table 7 Summary of ITS RFLP results for species of *Uncinaria* hookworms sampled from California sea lion (*Zalophus californianus*) (CSL) and northern fur seal (*Callorhinus ursinus*) (NFS) pups

Host species	No. hosts	No. hookworms	No. intestinal *U. lucasi*	No. peritoneal *U. lucasi*	No. intestinal *Uncinaria species A*	No. peritoneal *Uncinaria* species A
CSL	7	42	2 (2)[a]	0	22 (2)[a]	18
NFS	18	130	66 (8)[a]	63 (2)	1 (1)[a]	0

[a] Numbers in parentheses indicate the number of hookworms that had RFLP results confirmed by direct sequencing of ITS PCR products

different host species, but a result of two distinct species of *Uncinaria* (Nadler et al. 2000). However, a related question is whether frequent cross-infection with the "wrong *Uncinaria* species" leads to an increased frequency of peritoneal infections in pups due to aberrant migrations of adults of the "wrong species" through the intestine. In this study, no evidence for such a result was found. For the 24 hosts examined with hookworms recovered from both the intestine and the PNC, none had hookworms representing the "wrong species" in the PNC. It appears from these findings that factors other than cross-infection are responsible for peritoneal infections of adult hookworms in these pups.

The presence of adult hookworms in the PNC is cause for posing the question: Why would a parasite, which as an adult is "supposed" to locate in the lumen of the intestine, migrate to the PNC? Adult hookworms in the NFS and CSL pups on SMI typically pierce the intestinal mucosa and submucosa while feeding. So, could some specimens be stimulated by an unknown internal or external factor cause them to feed deeper than normal and penetrate through the intestinal walls? It is of interest that the hookworm eggs in the wash of the PNC of the NFS pups were fertile, indicating that the females had been inseminated. Whether the mating occurred before or after the females penetrated the intestinal wall is unknown.

It would be advantageous to know if penetration of the intestinal wall by adult hookworms only occurs in NFS and CSL pups on SMI. The fur seals and sea lions on some rookeries on SMI have a sympatric relationship. Further studies are needed to be done to ascertain whether there are adult hookworms located in the PNC of pinniped pups in an allopatric relationship in other geographical areas. There is one report of hookworms not being found in the PNC of New Zealand sea lion pups (A. Castinel, personal communication).

Periodic studies like the one reported here allow determination of any changes in hookworm prevalence, behavior, differences in species (based on molecular and morphometric characteristics) present, and effect on the health of NFS and CSL pups born on SMI. This type of study also shows the value of doing field research by performing necropsies of dead pups. By this method, it is possible to detect infections in areas other than the intestines and recover all hookworms in the PNC and intestines. These data are more beneficial than just examining feces for hookworm eggs. Uniqueness of hookworms (*Uncinaria* spp.) in pinnipeds becomes more evident as research data accumulate. The most extensive studies have been done in NFS with less in CSL, followed by other pinniped hosts.

Some features of pinniped hookworms are:

1. *U. lucasi* in NFS was the first helminth in any mammal found to pass as a larval form through the mammary system of the mother and then mature in her nursing offspring (Lyons 1963; Lyons and Olsen 1962; Olsen and Lyons 1962, 1965).
2. Parasitic third-stage larvae that pass through the mammary system are the only stage of hookworm that matures in NFS pups and probably other species of infected pinnipeds (Lyons 1963; Lyons and Olsen 1962; Olsen and Lyons 1962, 1965).
3. Transmammary transmission also has been reported for at least three other pinniped hosts including CSL (Lyons et al. 2003) (summarized by Castinel et al. 2007).
4. Free-living infective third-stage larvae, instead of maturing in the intestine, locate in tissues, especially in the ventral abdominal area of NFS and probably other hookworm-infected pinniped hosts
5. Species status in the genus *Uncinaria* in pinnipeds is unresolved (Nadler et al. 2000).
6. Penetrating the intestine and entering the PNC, like in NFS and CSL on SMI, are an atypical trait for adult nematodes normally living in the intestine (Spraker et al. 2004, 2007; current research).
7. Pathogenicity due to PNC hookworms can be at a high level because of the tendency to cause bacteremia, enteritis, and peritonitis in addition to the typical anemia reported for hookworms (Spraker et al. 2007).

The discovery and unexplained presence of hookworms in the PNC of such a large number of dead pinniped pups were the highlight of the research reported here.

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