

RESEARCH NOTES

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Uncinaria hamiltoni (Nematoda: Ancylostomatidae) in South American Sea Lions, *Otaria flavescens*, From Northern Patagonia, Argentina

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ABSTRACT: Thirty-one South American sea lion pups (*Otaria flavescens*) found dead in Punta León, Argentina, during the summer of 2002, were examined for hookworms (*Uncinaria hamiltoni*). Parasite parameters were analyzed in 2 locations of the rookery, i.e., a traditional, well-structured breeding area and an expanding area with juveniles and a lax social structure. Prevalence of hookworms was 50% in both localities, and no difference was observed in prevalence between pup sexes ($P > 0.05$). Hookworms were concentrated in the small intestine. Transmammary transmission is assumed because only adult hookworms were found in the pups. The mean intensity of hookworms per pup was 135; the mean intensity in females (92.78) was significantly different ($P < 0.05$) from that of males (230.25). No difference ($P > 0.05$) in intensity was found between the 2 breeding areas, although prevalence was higher in the traditional breeding area than in the other area. Location was the only factor affecting hookworm prevalence (P log-linear model: 0.9552; χ^2 : 1.5629). No apparent trend between body condition and intensity of hookworms was observed.

South American sea lions, *Otaria flavescens*, occur in more than 85 rookeries and 'haul-outs' along the coast of Argentina in the southwestern Atlantic ocean, both on the midland and on islands, through the whole year (Reyes et al., 1999). The population was exploited for leather and oil during the first half of the 20th century. During this period, it was dramatically reduced, with no evident growth until 1990 when a positive increase in the number of pups was observed (Crespo and Pedraza, 1991). At present, the population in northern Patagonia is growing at an annual rate of 4–7%, although it still represents less than a quarter of the supposed original population size before the highest levels of exploitation (Dans et al., 2003). Besides population dynamics, their interactions with fisheries (Crespo et al., 1994, 1997) and the food habits of this species have been well reported (Koen-Alonso et al., 2000), although parasitological studies are scarce. However, *Uncinaria* spp. have been previously reported from the Falkland (Malvinas) Islands by Baylis (1933), from Uruguay by Botto and Mañé-Garzón (1975), and from Uruguay and Chile by George-Nascimento et al. (1992). These nematodes are found in the intestines of otariid pups (Lyons et al., 2001) and, unusually, phocid pups (George-Nascimento et al., 1992).

The taxonomic specificity of *Uncinaria* spp. remains uncertain (Lyons, De Long et al., 2000), and molecular-based analyses are underway elsewhere (Nadler et al., 2000). Originally, 2 species, *Uncinaria lucasi* Stiles, 1901, and *Uncinaria hamiltoni* Baylis, 1933, were described, although, subsequently, intermediate types were found by several authors (Lyons et al., 2001). The life cycle of *U. lucasi* is known and has been well described in northern fur seals (*Callorhinus ursinus*) from Alaska by Lyons (1963). Infections by *Uncinaria* spp. (uncinariosis) are regarded as one of the most important mortality factors in sea lion pups (Lucas, 1899; Baylis, 1933; Olsen and Lyons, 1965; Fowler, 1990).

The main objective of this study was to generate data on *U. hamiltoni* in northern Patagonia relative to the parasite's prevalence, mean intensity, range of intensity, and distribution in South American sea lion pups and to evaluate the presence of *U. hamiltoni* as a potential mortality factor.

Samples were taken in Punta León reserve (43°03'S, 64°47'W; Chubut province, Argentina), one of the largest rookeries in northern Patagonia, where approximately 2,000 pups are born every year (25% of total pup production). During the past 20 yr, together with an increase in the number of pups, there was an increase in the area occupied (from 4 to 7 km). The study was undertaken with the knowledge that the rookery now has 2 different locations, one with a traditional breeding

structure (northern area) and another developed during the past 10 yr, with a less organized breeding structure (southern area). The only other pinniped species found in Punta León is the southern elephant seal (*Mirounga leonina*).

Hookworms were collected during the 2002 breeding season, between 11 January and 10 February. An additional and final survey was completed at the end of the breeding season, on 27 February. Thirty-one (13 males, 18 females) fresh, recently dead pups were collected. All animals were necropsied immediately in the field. Pups were measured and weighed, sex was determined, and thickness of subcutaneous fat (at the level of the throat, the sternum, and the belly button) was determined to assess the body condition of the pups. Intestines were removed, placed in plastic bags, and fixed in 70% ethanol. As soon as possible, i.e., within 24 and 48 hr, intestines were taken to the Marine Mammal Laboratory (LAMAMA-CENPAT/CONICET) where they were dissected according to the method of Aznar et al. (1997). Each fragment of an intestine was placed in a petri dish filled with 70% ethanol and dissected with scissors; all examinations were conducted using a stereomicroscope at $\times 6.3$ magnification. All parasites were collected, counted, preserved in 70% ethanol, and cleared in lactophenol for identification. Terminology, i.e., prevalence, mean intensity, and range of hookworm infection is from Bush et al. (1997). Parasite distribution along the intestine was determined using variance-to-mean ratios (Krebs, 1989), and significance evaluated with a chi-square test. Student's t -test (Zar, 1996) was used to assess: (1) differences in mean intensity of hookworms between female and male pups, (2) differences in mean intensity between female and male hookworms, (3) differences in mean intensity of hookworms among female pups, (4) differences in mean intensity of hookworms in male pups, and (5) differences in mean intensity of hookworms between the northern and the southern breeding areas within Punta León. Fisher's exact test (Zar, 1996) was used to examine differences in prevalence between sexes. Finally, a log-linear model (Sokal and Rohlf, 1998), with 3 classification criteria, i.e., pup sex, location within rookery, and presence-absence of hookworm, was used to evaluate which factor influenced hookworm prevalence.

Voucher specimens of *U. hamiltoni* from the South American sea lion pups were deposited (U.S. National Parasite Collection [USNPC] 94041; 94042) in the USNPC Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Maryland.

The hookworms found in the South American sea lion pups were identified as *U. hamiltoni* on the basis of comparison with described morphospecies only. Given the present uncertainty in the taxonomy of hookworms, we should say that our morphological identification is provisional and that the ongoing molecular studies may eventually result in the revision of the taxonomy of these *Uncinaria*. All the hookworms found were adults. Morphometric data corresponding to the specimens found are provided in Table I. Hookworms infected 50% of the pups examined. No difference was observed in prevalences between sexes (female pups, 56.25%; male pups, 38.46%; Fisher's exact $P = 0.4621$). Hookworms showed an overdispersed distribution in the small intestine ($I = 278.63$; $\chi^2 = 8080.25$, $df = 29$ [lower critical value = 16.05; upper critical value = 45.72]). Only 2 worms were found attached to the intestine wall; all the rest were free in the lumen and no hemorrhagic lesion was observed. The number of hookworms found in all infected pups ranged from 1 to 451: from 1 to 248 in female pups and from 17 to 451 in male pups (Table II). The mean intensity of hookworms per pup was 135 (± 146.52). The mean intensity in females (92.78 \pm 98.09) was significantly lower than in males (230.25 \pm 206.8) ($t = 2.94$; $P = 0.013$). No difference was observed in the mean intensity of female and male hookworms (72.91 \pm 69.69; 65.69 \pm 69.94, respectively; $t = 0.79$,

TABLE I. Morphometric measurements on females and males of *Uncinaria hamiltoni* found in South American sea lions (*Otaria flavescens*) from Punta León, Patagonia (mean, \pm SD, range) and from previous studies (range). All measurements are expressed in millimeters.*

Sample size	<i>Uncinaria hamiltoni</i> (this work)		<i>Uncinaria hamiltoni</i> (in <i>Otaria flavescens</i>) (Baylis, 1933)		<i>Uncinaria hamiltoni platensis</i> (in <i>Otaria flavescens</i>) (Botto and Maté-Garzón, 1975)		<i>Uncinaria hamiltoni</i> (in <i>Eumetopias jubata</i>) (Baylis, 1933)	
	Females	Males	Females	Males	Females	Males	Females	Males
Number of hosts examined	30	29	—	—	30	30	3	1
BL	5.36–17.2 (11.37 \pm 2.94)	4.64–10.32 (7.49 \pm 1.53)	12.5–17.5	8.5–12.0†	8.3–19.4	6.4–11.4†	16.7–18.8	9.4†
BLB	0.820–1.34 (1.15 \pm 0.11)	4.88–10.64 (7.85 \pm 1.52)	1.6–1.9	1.5–1.55	1.35–1.57	1.2–1.38	1.4–1.6	1.3
OL	0.11–0.22 (0.18 \pm 0.02)	0.79–1.83 (1.09 \pm 0.19)	—	—	—	—	—	—
OW	0.21–0.34 (0.28 \pm 0.03)	0.10–0.31 (0.16 \pm 0.05)	0.32–0.38	0.28–0.3	0.24–0.29	0.21–0.26	—	—
LBC	0.2–0.31 (0.24 \pm 0.02)	0.21–0.28 (0.24 \pm 0.02)	—	—	—	—	—	—
WBC	2.13–6.62 (4.33 \pm 1.34)	0.14–0.24 (0.19 \pm 0.06)	5.1–7.0	—	3.6–5.7	—	—	—
VPE	0.1–0.14 (0.12 \pm 0.01)	—	0.13–0.14	—	0.1–0.13	—	—	—
EL	0.04–0.10 (0.07 \pm 0.01)	—	0.06–0.09	—	0.06–0.08	—	—	—
SL	—	0.57–1.05 (0.89 \pm 0.09)	—	1.0	—	0.75–0.99	—	0.67
DRL	—	0.01–0.08 (0.04 \pm 0.02)	—	—	—	—	—	—
DRB	—	0.12–0.25 (0.15 \pm 0.03)	—	—	—	—	—	—
Ratio DRB–DRL	—	1:3.65	—	1:2.4	—	1:3.0	—	—

* BL, body length; BLB, body length including bursa; OL, length of the esophagus; OW, width of the esophagus; LBC, length of buccal capsule; WBC, width of buccal capsule; VPE, distance from vulva to posterior end; EL, egg length; EW, egg width; APE, distance from anus to posterior end; SL, spicule length; DRL, dorsal ray length; DRB, dorsal ray bifurcation.

† Not specified if measured with or without the bursa.

$P = 0.43$), in the mean intensity of female and male hookworms in female pups (43.88 ± 36.88 ; 44.89 ± 44.35 , respectively; $t = 0.19$, $P = 0.85$), and in the mean intensity of female and male hookworms in male pups (112.75 ± 115.25 ; 112.5 ± 96.49 , respectively; $t = 0.02$, $P = 0.98$).

In the northern breeding area, 44.44% of the dead pups were infected, whereas 60% of the dead pups in the southern part of the rookery were infected. Among all the infected pups, 57.14% belonged to the traditional breeding areas and 42.86% to the new area. There was no interaction between the 3 factors in the log-linear model, and location was the only factor that accounted for the adjustment of the model (P of the log-linear model: 0.9552; χ^2 : 1.5629, df: 6). This suggests that the breeding area influences the prevalence of infection. In addition, no difference was found between the mean intensity of hookworms in the northern (150.5 ± 123.5) and the southern areas (110.4 ± 191) ($t = 1.025$; $P = 0.33$).

Prevalence and mean intensity of *U. hamiltoni* in the South American sea lion were moderate compared with hookworm infections in other pinnipeds studied in other areas. For example, pups had lower levels of infection than the California sea lion (*Zalophus californianus*) and the northern fur seals from California (Lyons, Spraker et al., 2000), although they were more heavily infected than the northern fur seals from Alaska (Lyons, Spraker et al., 2000) and the Juan Fernández fur seal (*Arctocephalus philippii*) from Alejandro Selkirk Island, Chile (Sepúlveda, 1998). One reason that accounts for the moderate levels of infection preliminarily found at Punta León may be the kind of substratum. As it is known, the type of soil is related to the development and transmission of hookworms. On St. Paul Island, Alaska, for example, northern fur seal pups born on sandy rookeries have higher numbers of adult hookworms than pups born on less sandy areas (Lyons, Spraker et al., 2000) or as observed for Steller sea lions (*Eumetopias jubatus*) from a rocky terrain rookery in Oregon where no hookworm is found (Lyons et al., 2003). This matter will be intensively studied at northern Patagonia in the future, with more samples from different breeding seasons.

Hookworm burden did not differ significantly between the 2 locations of Punta León rookery. Also, it has to be noticed that pup mortality was 2 times higher in the southern than in the northern area, and this was attributed to the absence of a typical nursery structure in the new breeding area (E. A. Crespo, pers. obs.) rather than to hookworm infection.

A relationship between a host's sex and the number of hookworms present has not been previously reported. California sea lions, northern fur seals (from California), and Juan Fernández fur seals did not show differences in hookworm intensities in male and female pups (Lyons et al., 1997, 2001; Sepúlveda, 1998), contrary to that observed in this work.

Transmammary transmission of hookworms in South American sea lions has not been demonstrated, although it is assumed. Lyons (1994) and Lyons and De Long et al. (2000) found that adult hookworms in California sea lion and Northern fur seal pups were derived only from parasitic third stage larvae passed through mothers' milk, for a short time postpartum, and that there may be no other way of transmission (Lyons, 1994). The same pattern of transmission is presumed in the present case because only adult hookworms were found throughout the period studied, supporting the idea that pups would only be infected with colostrum. In addition, if the number of hookworms is directly related to the quantity of milk consumed by the pup, and assuming only transmammary transmission, it might be supposed that male South American sea lion pups acquired more milk than female pups. Our preliminary findings would support this prediction even though there is no evidence that female nursing differs according to the sex of the offspring (Cappozzo et al., 1991).

Hookworms appeared to be concentrated in the small intestine. This site is the same as that selected by hookworms in Juan Fernández fur seals (Sepúlveda, 1998) and California sea lions (Dailey and Hill, 1970; Lyons, De Long et al., 2000). However, it is different from the northern fur seal pups, where they appear concentrated in the cecum and proximal large intestine (Lyons et al., 1997).

Higher prevalences and mean intensities should be expected in the traditional nursing area than in a new expanding area. In the first site, reproductive females rather than juveniles (nonreproductive) are concentrated, facilitating hookworm transmission. Consistent with the expected pattern, differences in prevalence were observed in South Amer-

TABLE II. Mean intensity \pm SD and range of intensity of *Uncinaria hamiltoni* found in South American sea lions (*Otaria flavescens*) from Punta León, Patagonia, Argentina.

Host	Total <i>Uncinaria hamiltoni</i> (n = 1,656)	Female <i>Uncinaria hamiltoni</i> (n = 800)	Male <i>Uncinaria hamiltoni</i> (n = 856)
All pups	135 \pm 46.52 (1–451)	72.91 \pm 69.69 (7–243)	65.69 \pm 69.94 (1–208)
Female pups	92.78 \pm 98.09 (1–248)	43.88 \pm 36.88 (12–101)	44.89 \pm 44.35 (1–136)
Male pups	230.25 \pm 206.8 (17–451)	112.75 \pm 115.25 (7–243)	112.50 \pm 96.49 (9–208)

ican sea lions. However, contrary to the expected pattern, they exhibited no difference in the number of hookworms present in pups. The total number of individuals of the southern area of the rookery is now one-quarter that of the northern area. Therefore, density-dependent mechanisms could be still relaxed compared with a population size close to its carrying capacity k . This may be the more plausible explanation for the lack of differences observed between the 2 areas.

Longevity of hookworms in intestines of pinnipeds from North America is well known (Lyons, De Long et al., 2000, and references therein). For *O. flavescens* pups, this phenomenon is yet to be examined. Long-term examinations of pups dying during the nursing period should give an indication when hookworms are eliminated, assuming that they resemble California sea lion and northern fur seal pups (Lyons, De Long et al., 2000).

A clear trend between the body condition of the pup and the number of hookworms present was described in other pinnipeds (cf. Lyons et al., 2001, and references therein), although a similar relationship could not be observed in South American sea lion pups. Critical biological features (related to thickness of the subcutaneous fat or pup weight and health status) not yet known for the species are required to establish any possible pattern or trend (if there were any).

One additional fact to consider in the future is the presence of southern elephant seals in the same area as another potential host species for *U. hamiltoni*, although hookworm infection is believed to be unusual in phocids (George-Nascimento et al., 1992).

Population dynamics of pinniped hosts and hookworms are correlated (Lyons, Spraker et al., 2000). The *O. flavescens* population is increasing and, hence, a corresponding increase in its hookworm population may exist. Within the present conditions, a study during the future breeding seasons should reveal such trends.

Finally, uncinariosis is considered one of the most important mortality factors among pinniped pups in the Northern Hemisphere (e.g., Lucas, 1899; Baylis, 1933; Olsen and Lyons, 1965). However, it may not be an important cause of mortality for the species studied in the Southern Hemisphere based on the low intensity of infection and the absence of lesions in the small intestine (Sepúlveda, 1998). Consequently, the moderate infection levels found in South American sea lion pups may show that uncinariosis could become a potential mortality factor as the population increases toward its original size.

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First Isolation of *Neospora caninum* From an Aborted Bovine Fetus in Spain

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ABSTRACT: *Neospora caninum* was isolated from the brain of a 6-month-old aborted bovine fetus from Galicia, Spain. The fetal brain homogenate was inoculated intraperitoneally into cortisonized mice. The peritoneal exudate from the infected mice, along with mouse sarcoma cells (Tg180), was inoculated into a second group of mice, and parasites were harvested from the peritoneal exudate. The parasites were adapted to in vitro growth in Vero monolayers. The tachyzoites from the peritoneal exudate reacted positively with anti-*N. caninum* antibodies and not with anti-*Toxoplasma gondii* antibodies on indirect fluorescent antibody test. The tachyzoites were lethal to interferon gamma gene knock out (KO) mice and could be identified immunohistochemically in the tissues. The identity of the parasite was also confirmed by polymerase chain reaction amplification of *N. caninum*-specific fragments. The sequences of the amplified gene 5 fragments (GenBank AY494944) were found to be identical to that of an Austrian isolate of *N. caninum* but not to that of NC-1. This is the first isolation of viable *N. caninum* from Spain.

Neospora caninum is a major cause of abortion in cattle worldwide (Dubey, 2003). In Spain, antibodies to *N. caninum* have been detected in bovine sera, and *N. caninum* antigen has been detected in aborted bovine fetuses (Fondevila et al., 1998; González et al., 1999; Mainar-Jaime et al., 1999; Quintanilla-Gozalo et al., 1999; Pereira-Bueno et al., 2003). We report the first isolation of viable *N. caninum* from an aborted bovine fetus in Spain.

A 6-month-old aborted bovine fetus and a blood sample of the dam were collected from a farm located in La Coruña district, Galicia, Spain. The cow was Holstein-Friesian, barn housed, milked twice a day, and was fed total mixed rations. The entire fetus and serum from the dam were refrigerated and sent by overnight mail to the Center of Parasite Immunology and Biology Laboratory, Porto, Portugal.

The cow was found to have an antibody titer of 1:160 or more in the *Neospora* agglutination test as described by Romand et al. (1998). The fetal brain was used for isolation of *N. caninum* according to the procedure of Canada, Meireles, Rocha, Sousa et al. (2002). Briefly, the brain was trypsinized and the washed homogenate was inoculated in-

traperitoneally into cortisonized mice. The peritoneal exudate from these mice, along with mouse sarcoma cells (Tg180), was inoculated into a second group of mice, and parasites were harvested from the peritoneal exudate of this group. For in vitro propagation of the parasite, the final pellet obtained from the peritoneal wash was inoculated onto confluent monolayers of Vero cells as described (Canada, Meireles, Roch, Sousa et al., 2002).

Two months after the initiation of the cell culture, a flask infected with this isolate of *N. caninum* was sent to the Animal Parasitic Diseases Laboratory (APDL), U.S. Department of Agriculture, Beltsville, Maryland for further characterization. Tachyzoites (1×10^6) from culture were inoculated subcutaneously into 2 interferon gamma gene knock out (KO) mice, 2 Swiss Webster mice, and 2 gerbils (*Meriones unguiculatus*) as described (Dubey et al., 1998; Dubey and Lindsay, 2000), and observed for infection. The surviving animals were killed after 3 mo, their sera tested for *Toxoplasma gondii* antibodies by modified agglutination test (MAT) (Dubey and Desmonts, 1987), and brain smears examined for protozoan tissue cysts. The tissues were examined microscopically after immunohistochemical staining using anti-*N. caninum* antibodies (Lindsay and Dubey, 1989).

The tachyzoites from the culture supernatant were used to extract DNA using DNazol (MRC, Cincinnati, Ohio) according to the manufacturer's instructions. Polymerase chain reaction (PCR) was performed with the *N. caninum*-specific primers Np6–Np21, as described (Yamage et al., 1996), including positive (NC-1) and negative controls. The PCR products were resolved on a 1.5% agarose gel and observed for the presence of the specific target fragment. The specific PCR product was extracted from the gel using MinElute gel extraction kit (Qiagen, Valencia, California) and directly sequenced in both directions using the Big Dye terminator system, version 3.1 (Applied Biosystems, Foster City, California) using an ABI 377 sequencer. The sequence chromatograms were edited using Sequencher 4.1 software (Genecodes Corp., Ann Arbor, Michigan). Searches were performed in BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>) to determine whether the sequences were similar to any of the previously published sequences of *N. caninum* available in the public database.

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