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The life history strategy of a fur seal hookworm in relation to pathogenicity and host health status

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ABSTRACT

The strategies that parasites use to exploit their hosts can lead to adverse effects on human and animal populations. Here, we describe the life cycle, epidemiology, and consequences of hookworm (*Uncinaria sp.*) disease in South American fur seals (*Arctocephalus australis*), and propose that hookworm adaptation to fur seal life history traits has led to maximizing transmission at high levels of parasite-induced anemia and mortality. Fur seal pups acquire hookworms during their first days of life through their mothers' colostrum and most adult hookworms are expelled from the pups' intestine 30–65 days later. This gives hookworms little time to feed and reproduce. However, despite reaching high within-host densities, female hookworms do not decrease egg output, therefore pups with high hookworm burden contribute disproportionately to parasite egg shedding. These heavily infected pups also suffer severe anemia and high levels of hookworm-induced mortality. Alternative strategies to maximize total egg shedding and/or transmission, such as increased environmental survival of larval stages or avoidance of clearance, have not been developed by this hookworm. We propose that fur seal hookworms exploit a live fast-die young life history strategy, which translates to the highest levels of host anemia and mortality recorded among hookworms.

1. Introduction

Soil transmitted helminths are one of the most prevalent and detrimental parasitic infections in the world, with substantial resources deployed for their control and eradication (Bartsch et al., 2016; Jourdan et al., 2017). Within this parasite group, hookworms are particularly pathogenic because they establish long-lasting infections and exhibit a detrimental feeding behavior for the host (Bartsch et al., 2016). With the help of well-developed buccal plates and secretion of several anticoagulant proteins, hookworms cause bleeding wounds in the intestine in order to feed on host blood (Loukas et al., 2005). Therefore, in human and animal populations affected by these parasites, intestinal bleeding and chronic regenerative anemia are the hallmarks of infection (Bartsch et al., 2016; Jourdan et al., 2017; Seguel and Gottdenker, 2017). Additionally, because of the extraction of blood and loss of protein through the intestine, hookworm infection has been associated with retarded growth in human and wild animal populations (Bartsch et al., 2016; Seguel and Gottdenker, 2017).

Despite these adverse consequences for host health, hookworm-induced mortality is rare in humans and animals, with the exception of pinnipeds (seals, fur seals and sea lions) (Bartsch et al., 2016; Seguel and Gottdenker, 2017). In pinnipeds, and most specifically otariids (eared seals), hookworms (*Uncinaria sp.*) can cause up to 70% of the total pup mortality during a reproductive season (Spraker et al., 2007; Lyons et al., 2011; Seguel and Gottdenker, 2017). The reasons for these high levels of mortality among eared seals are unknown, but in several otariid species, parasite prevalence and burden are the most important factors driving host mortality (Castinel et al., 2007; Spraker et al., 2007; Lyons et al., 2011; Seguel and Gottdenker, 2017; Seguel et al., 2017). For instance, most necropsied South American fur seal pups (SAFS, *Arctocephalus australis*) at Guafo Island (Chilean Patagonia), are infected with an undescribed *Uncinaria sp.* but only animals with high burdens die due to a combination of hookworm lesions and secondary bacterial infection of these wounds (Seguel et al., 2017).

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For a soil transmitted nematode, pinniped hookworms (*Uncinaria sp.*) have been quite successful in a coastal environment that poses challenges to their survival. Most pinniped hosts form large aggregations along shores during the reproductive season, yet they spend most of their time in the ocean (Paves and Schlatter, 2008). Additionally, many pinniped rookeries are located in latitudes subjected to extreme temperatures and constant wash of sea and rain water (Castinel et al., 2007; Lyons et al., 2011; Seguel et al., 2013). Therefore, pinniped hookworms likely developed alternative strategies to cope with challenges to their survival and reproduction, resulting in high levels of mortality in many marine mammal populations. Some of these strategies, for instance, could be related to increased resistance of larval stages in the environment, prolonged egg shedding in the definitive host (prolonged infectious period) and/or alternative modes of transmission of infective stages. However, studies in several otariid species suggest that the life cycle of marine hookworms is similar to terrestrial carnivores, except that transmission of infective larvae is exclusively lactogenic (Olsen and Lyons, 1965; Lyons et al., 2011). This suggests that marine hookworms could have attained high prevalence among otarrids due to synchronization with the host reproductive cycles, although increased opportunities for transmission through environmental resistance of larval stages and/or prolonged egg shedding are potential contributory factors.

In this manuscript, we describe the life cycle and epidemiology of *Uncinaria sp*. infection in South American fur seals (SAFS, *Arctocephalus australis*) using field data and experiments in order to understand the dynamics of hookworm transmission in pinnipeds within the context of high levels of parasite induced mortality. Additionally, the host population dynamics were compared with that of the parasite to detect if synchronization with host reproductive cycles is feasible. Later, to better understand this hookworm life history strategy in relation to their pinniped host life history, we observed egg production in hookworm females and performed experiments to evaluate hookworm larvae environmental survival. Finally, we measured hookworm-specific mortality and anemia in fur seal pups, and examined the total contribution to hookworm egg shedding in pups that suffered the worst health consequences of parasitism compared to pups that were barely affected by hookworms. Based on this approach, we propose that pinniped hookworms exploit a live fast-die young life history strategy that results in few limitations on extraction of host resources and the highest recorded levels of anemia and mortality among hookworms.

2. Materials and methods

2.1. Study site and animal handling

The study was performed in the reproductive colony of SAFS located at Guafo Island, Northern Chilean Patagonia (43° 35′ 34.9″ S, 74° 42′ 48.53″ W) (supplementary figure 1). This rookery, of approximately 3000 individuals during the reproductive season, is located over a mix of sandy and rocky substrates. Pups captures and recaptures were performed in a rookery sector covered by a thin (3–10 cm) layer of sandy soil with moderate amount of organic matter over a rocky substrate (supplementary figure 2).

During 2014 (n = 201) and 2015 (n = 206), 1 to 7 day-old pups were captured by hand and physically restrained. Age of pups was exactly known or estimated using the peak parturition date for Guafo Island (December 15th) and by assessing the rests of placenta and umbilical cord in these young pups (Paves and Schlatter, 2008; Seguel et al., 2016). Placenta is usually loss between 24 and 48 h after birth and rest of umbilical cord are loss between day 2 and 5 (Paves and Schlatter, 2008). The exact age of a subset of pups (n = 20) was known because their birth was observed and they were marked 24 h later.

In each capture, pups were measured, weighed, and blood and fecal samples collected as previously described (Seguel et al., 2016). All pups were marked with correlative numbers using a commercial hair decolorant applied on the fur.

Each year, a randomly selected group of 30 pups, 10 of them of exact known age (1–2 days old), were treated in their first capture with 400 $\mu g^{*}kg^{-1}$ of subcutaneous Ivermectin (antiparasitic drug, control group) (Delong et al., 2009). Half of these pups (n = 30) could be monitored throughout the whole study period and were included in the final data sets.

The pups were recaptured and the sampling procedure repeated on each pup every 5–10 days during the duration of the study (10 weeks) and only pups that were captured at least 4 times were included in the study (76 in 2014 and 73 in 2015).

2.2. Parasitological examination

Due to the lack of standardized methods to obtain a regular amount of feces from pinniped pups and calculate the number of hookworm eggs per gram (in part due to the semiliquid consistency of pup feces), a semi-quantitative protocol of hookworm egg counting was developed. A fecal swab was collected from each pup and placed in a 10 ml tube filled with Sheather's sucrose (1.275 specific gravity). In the field laboratory the swabs were removed from the tubes and additional Sheather's sucrose was added to the tube to obtain a raised meniscus. A glass cover slip was placed on top of the tube and after 1-h flotation placed on a glass slide and observed at 50X magnification with an optic microscope. The area containing more aggregates of hookworm eggs was selected and observed at 100X. Starting from this point the eggs were counted in 10 successive, random microscopic fields and the total number in the 10 fields recorded. This same coprological examination protocol was performed on 48 live captured adult female SAFS and in fecal samples (by embedding the swab in fresh feces) from 58 subadult and adult male SAFS.

To determine if the fecal egg count was a good estimate of nematode burden, the standardized fecal egg count protocol was applied to recently dead pups (n = 33). Necropsy was performed on these pups and nematodes recovered, counted, sexed and identified as previously described (Seguel et al., 2011, 2017). In addition, between 2004 and 2017 a total of 15 adult SAFS females and 26 adult SAFS males were necropsied and carefully examined for presence of metazoan parasites.

In order to determine the average number of eggs per hookworm female, randomly selected hookworm females (n = 50) and males (n = 50) from each host (necropsied pups) were measured (standard body length and width), and females body wall was macerated by placing them in sodium hypochlorite solution (0.5%) to extract all the eggs from the female's uterus. Eggs were recovered using a 38 μm sieve and suspended in a standard volume of saline solution (NaCl 0.9%). Eggs were counted 3 times using a graded chamber with 1000 μl capacity. The average of the 3 counts was recorded and the number of eggs per females calculated based on the total volume of the hookworm egg solution and the number of females macerated (Hussey and Barker, 1973). The same protocol was repeated using different numbers of females from the same sample (25, 10, 5, 1), however no significant differences were detected in the calculated number of eggs per female. Therefore, the same protocol was applied to samples with small hookworm burden using 5, 10 or 25 females per test.

In 2014, 2015 the soil from different sectors of the rookery was collected. A sector of the rookery was divided in 100^2 m quadrants and 5 collection sites were randomly selected within each quadrant (supplementary figure 3). Sampling was conducted in 5 quadrants in early December and in the same quadrants and other additional 15 quadrants through mid and late January of each year. All collection sites were located at least 10 m from the high tide line, because at Guafo Island, areas with surf wash do not contain soil but large rocks. A standard volume (50 ml) from a homogenized subsample of soil from each collection site underwent Baermann's test for detection of soil nematodes using methods described by Olsen and Lyons (1965) in Northern fur seals (*Callorhinus ursinus*). These included addition of a few drops of

concentrated hydrochloric acid to the solution obtained with Baermann's funnels to kill any potential free-living nematodes in the collected sample (Olsen and Lyons, 1965). The obtained sediments were stained with Lugol's iodine, observed under the microscope and identified according to published morphological references for *Uncinaria lucasi* (Olsen and Lyons, 1965). L3 hookworm larvae abundance was semi quantitatively recorded in each sample by a single observer using previously established criteria (0 = absent, 1 = mild, 2–4 = moderate, and 5 > severe). A particular quadrant was assigned to the same categories (absent, mild, moderate and severe) based on the larvae abundance results obtained during January in its 5 samples. The category assigned corresponded to the median of the 5 samples (*e.g.* mild, mod., sev., sev., sev. = severe). A subset of soil from samples with high larvae concentration (severe), from 2014 (n = 10) and 2015 (n = 10) were kept at 10–12 °C (average Guafo Island soil temperature) and analyzed by Baermann technique after one or two years of storage. The same Baermann's procedure and larvae identification was applied to blubber collected at necropsies of recently dead (< 12 h postmortem interval) SAFS adult females (n = 5), pups (n = 20) and adult males (n = 2), and to pieces of fresh placenta collected in the rookery with the difference that hydrochloric acid was not added to the samples. Fresh placentas were collected immediately after delivery (n = 8) and sampling included collection of at least 4 pieces (4 × 4 cm) per placenta. Similarly, blubber from 5 to 7 locations in the animal abdomen were cut into 0.5–1.0 cm^3 pieces and wrapped in filter paper to be placed in the Baermann's funnel (Olsen and Lyons, 1965; Gibbons et al., 2005). In 2015, milk samples were collected from 15 SAFS during the second week of January. These animals were captured with a net, anesthetized with isofluorane (4%) and milk collection was facilitated by a single intramuscular injection (5 IU) of oxytocine (Drag Pharma, Santiago, Chile) 5 min before collection of a single 25 ml–50 ml sample. Colostrum was opportunistically collected from 5 freshly dead adult female SAFS in December 2013 and 2014. Approximately 25 ml were collected from each animal. In order to identify hookworm larvae, Baermann tests were performed in milk and colostrum samples.

Fecal material from a subset of pups (n = 30) was left in an open plastic box containing previously sanitized (through sun exposure) rookery soil and left at room temperature at the Guafo Island field laboratory. Fecal material and soil from this box was examined every 6 (first 2 days) and 12 h (another 5 days) to assess the development of hookworm larvae.

2.3. Population end epidemiological data

Fur seal population census data was collected at Guafo Island rookeries every 3 days during the study period using previously described methods for this rookery (Paves and Schlatter, 2008; Seguel et al., 2013). The prevalence of hookworm infection was calculated throughout the study by dividing the number of infected pups by the total number of animals sampled each day. The average number of hookworm eggs per fecal smear in a given day was calculated by dividing the sum of all eggs counted in a day by the number of pups sampled the same day. The infectious period for a particular pup was defined as the number of days a pup shed hookworm eggs. This value was calculated by adding the number of days between the first positive coprological test and the first capture with a negative coprological test after the initial diagnosis of hookworm infection. To this value half the number of days between the last positive and first negative test was subtracted, according to the following formula:

Infectious Period = (N^o days between 1st positive and 1st negative test) - (N^o days between last positive test and 1st negative test/2).

2.4. Measurements of parasite induced mortality and anemia

Since hookworms are hematophagous nematodes, anemia is one of the hallmarks of infection and an indirect measurement of the extraction of resources from the host. Therefore, we used anemia (measured as low hemoglobin concentration (HG) and/or red blood cell (RBC) counts) and hookworm induced mortality as indicators of detrimental effects of hookworms on the host. RBC and HG were measured as previously described (Seguel et al., 2016), and anemia defined as HG concentrations below 10 g/dL and/or RBC bellow $3 \times 10^3/\mu L$, according to published reference values for pups in this population (Seguel et al., 2016). Additionally, we recorded the scaled body mass (total length/weight) (BM) (Banuet-Martinez et al., 2017), total serum protein concentration (TP) and white blood cell counts (WBC) as general measurements of the pups' health. These indicators were measured in each capture following previously described methods (Seguel et al., 2016), and the average for each pup was used for statistical analyses. The rookery was monitored daily to record dead and alive pups. Animals found dead during the study underwent complete necropsies and histopathology to determine cause of death as previously described (Seguel et al., 2017). Mortality rates were calculated by incorporating recapture (or re-sighting) data into survival tables, and the final cause of death recorded according to necropsy and histopathology findings in that animal.

2.5. Data analyses

2.5.1. Hookworms and fur seal population estimates

The within-host hookworm size variation was checked by assessing the range, standard deviation and variance of hookworm sizes in a host, and potential differences in the variances between samples (hosts) were tested with Barlett's test.

To determine if the burden of hookworm larvae in the soil was related to pup density, the soil samples were divided in groups according to their hookworm stage 3 larvae load. Pup density was calculated by counting the number of pups within each, previously divided, 100^2 m quadrant. The difference in the pup density of these groups was tested using a generalized linear model (GLM) using pup density as a continuous response and larvae concentration groups as categorical predictors.

2.5.2. Within-host density dependence of hookworm egg output

The relationship between the number of eggs in feces and number of nematodes in the pup's intestine, the number of hookworm eggs in feces and HG concentration and between number of female hookworms per host and number of eggs per female were determined by fitting several linear and polynomial regression models on the log-transformed values. The model with the lowest Akaike Information criteria (AIC) value and significant linear or polynomial interactions was selected to fit a curve with 95% confidence intervals on the graphed data points. To identify the variables that affected the hookworm female fecundity a generalized linear model with negative binomial distribution was fitted using pups BMI, male and female nematode burden, male to female ratio and hookworm female length as predictors.

2.5.3. Mortality and anemia

To determine the pup health parameters and hookworm traits that influenced pup mortality, binomial generalized linear mixed models were fitted using the number of hookworm eggs in feces, BMI, TP, WBC, RBC, HG, sex, and infectious period as predictors in the global model and including year of sampling as a random effect (R statistical software package “glmmML”). Multiple models were run by adding and subtracting variables and by including interactions between RBC and HG, HG and PT, HG and BMI, and number of hookworm eggs in feces and Infectious period in different models. The most adequate covariance structure was selected based on Akaike's information criteria (AIC) values of the same models fitted with different covariance (Barnett et al., 2010). Model selection was based on second order AIC (AICc), significance of predictors and predictability (mean absolute error). Statistical inference was done based on the output of top ranked models

(delta AIC < 2.0). Similar approaches were used to construct and select models (GLMs) with total hookworm egg shedding (burden*infectious period), number of hookworm eggs in the feces (negative binomial GLM), HG, and infectious period as response.

After the study, the non-treated pups were divided in animals with severe and mild hookworm infection based on fecal egg counts. We used the median of 6 eggs per fecal smear as the cut-off value for the severe (≥6 eggs) and mild (< 6 eggs) groups because that was the minimal value at which we observed clinical signs of hookworm infection (*e.g.* bloody feces). The mortality rates between pups with mild and severe hookworm infection and the control (ivermectin treated) group were compared using a Log-rank Mantel-Cox test. The mean RBC and HG concentrations between groups were compared using one-way ANOVAs and Tukey's multiple comparison test.

2.5.4. Contribution to parasite egg shedding

In order to test if pups suffering the worst consequences of parasitism contributed to most of the hookworm egg shedding in the environment, total egg shedding in a pup was calculated by multiplying the number of days a pup was infected with the median fecal egg count of that pup (obtained from the repeated captures of that pup). Pups were divided in animals that suffered an adverse health consequence due to parasitism and those that did not (mortality vs survival, anemic vs non-anemic) and potential differences in the total hookworm eggs shedding between the two groups assessed through a GLM with negative binomial distribution.

In all statistical tests significance was set at $\alpha = 0.05$ and for model selection a delta AIC > 2 was considered significant. All statistical analyses were performed in R 3.2.1 statistical software (R core team, Vienna, Austria, 2016).

3. Results

3.1. Hookworm and fur seal life history traits

The proposed hookworm (*Uncinaria sp.*) life cycle in SAFS is summarized in Fig. 1, and is based in several observations. Between 5 and 30 (800–1020 μm in length) stage 3 larvae (L3s) were recovered from all the colostrum samples collected (n = 5), however no hookworm larvae were found in any of the 15 milk samples collected in mid-January, when pups are on average 1-month-old. Similarly, no nematode larvae were found in fresh placentas. In the nematodes collected from 31 SAFS pups found dead, the difference between the biggest and smallest female or male hookworm within a host was never larger than 2.2 mm (less than 10% total nematode length) and the range of the standard deviations in standard length across all samples ranged between 0.14 and 0.44 mm (supplementary table 1). These intrahost differences included at least 2 cases where only immature hookworms were found in the intestine (supplementary table 1), suggesting that ingestion of larvae occurs during a short period of time. None of the 10 pups treated with ivermectin one day after birth, and none of the 20 pups treated at 2-5 days-old became re-infected. All these findings suggest that infection occurs most likely through colostrum during the pup's first days of life. The 10 pups of known age that were not treated with ivermectin released *Uncinaria sp*. eight-celled eggs 14–18 days after consumption of colostrum during their first days of life (prepatent period mean = 16.1 ± 1.6), and these eggs embryonated at Guafo Island room temperature (12–14 °C) within 24–72 h (n = 33, mean = 46.9 ± 13.2). L2 development occurred *in ovo,* and within 48–72 h (n = 33, mean = 55.2 ± 7.9), L3s were released from the eggs. Recovery of infective L3s was common in the rookery soil in January (79.5% of positive samples, 39/49) but larvae were almost not present in the same places (quadrants) sampled in early December (0.06% positive samples, 3/50), and only 1 L3 was recovered from all the soil samples left at 12 °C for one year (n = 20) and no L3s were recovered in any of the samples (n = 10) stored in the same conditions for two years, suggesting low environmental resistance of fur seal hookworm larvae. L3s were recovered from the subcutaneous tissues of pups (15/20, mean larvae number = 6.25 ± 6.64), adult females (5/5, mean larvae number = 11.2 ± 7.82) and males (1/2, 7 larvae), indicating that hookworm L3 penetrate the skin of all age/sex animals in the rookery. No hookworm eggs were detected in the feces of adult males (n = 58) and females (n = 48) and no adult hookworms were found in the intestine of necropsied SAFS adult males (n = 26) and adult females (n = 15).

Approximately ninety percent of the sampled pups in 2014 (67/77, 87%) and 2015 (66/73, 90.4%) shed hookworm eggs (Fig. 2a). The highest number of eggs were shed on January 7th (mean = 13.1 ± 14.2 eggs per fecal smear, n = 45), when pups are on average 3-weeks-old and there are large numbers of adult, reproductive, females in the rookery (Fig. 2b), which are the next host in the hookworm life cycle. Adult female density decreased in the rookery from 369 animals in December 15th to 188 individuals in January 20th, a 49% decline in a 5 weeks period. Most pups cleared hookworm infection by the second week of February (between 4 and 8 weeks after initial infection) and the infectious period ranged between 9 and 48 days (22.96 ± 8.3 days, n = 133). Infective, sheathed *Uncinaria sp.* L3s were recovered in higher numbers in areas with higher pup density (GLM, $X^2 = 1303$, df = 3, $P = 2.2 \times 10^{-16}$) (Fig. 2c), suggesting density dependent contamination of soil with hookworm eggs and L3s.

3.2. Intrahost hookworm density and female fecundity

Fecal smear egg counts were a good estimator of the number of intestinal nematodes (third order polynomial regression, adj-$r^2 = 0.921$, $P = 2.2 \times 10^{-16}$) (Fig. 3a). Hemoglobin concentrations of SAFS pups were significantly and negatively correlated with the number of hookworm eggs in their feces (linear regression, adj-$r^2 = 0.43$, $P = 1.15 \times 10^{-14}$) (Fig. 3b), suggesting that the parasites deplete hemoglobin in a density dependent manner (additional models outputs in supplementary table 2). The average size of female and male hookworms was not related with the total number of female or male hookworms per host (Spearman-rho, r = −0.23, −0.15, P = 0.51–0.71). Moreover, the average number of eggs per female hookworm was not correlated with the within-host hookworm density (males, females and total) (linear regression, adj-$r^2 = -0.03$, F = 1.01, df = 36, P = 0.32) (Fig. 3c), and in multivariate analyses average hookworm female length was the only significant predictor of the number of eggs per female (GLM.NB, estimate = 0.069 ± 0.023, Z = 2.99, P = 0.003), whereas nematode burden was not significant (supplementary table 3), suggesting a lack of density dependent decline in egg output.

3.3. Parasite induced anemia and mortality

Fur seal pups with higher hookworm burdens tend to have longer infectious periods, lower hemoglobin concentrations and a higher probability of mortality (Table 1) (GLM with negative binomial distribution, $X^2 = 159.1$, df = 9, $P = 1.28 \times 10^{-65}$). Additionally, pups with higher hookworm burdens had lower numbers of RBC and WBC, lower body mass (BM) and were usually male, however the relationship between hookworm burden and these parameters was not statistically significant (RBC estimate = $6.84 \times 10^{-8} \pm 1.21 \times 10^{-7}$, P = 0.57, WBC estimate = $2.90 \times 10^{-6} \pm 1.57 \times 10^{-5}$, P = 0.85, Sex male estimate = $-3.43 \times 10^{-1} \pm 2.36 \times 10^{-1}$, P = 0.14, BM estimate = −2.77 ± 6.59, P = 0.67). According to the top ranked GLMM binomial models (Table 2), mortality likelihood increased in pups with lower RBC, longer infectious periods, higher hookworm burden and lower HG and BMI (estimates, standard errors and p-values presented in Table 3).

A third of the hookworm infected pups (53/149, 35.5%) had severe hookworm infection while the other two thirds (96/149, 65.5%) were

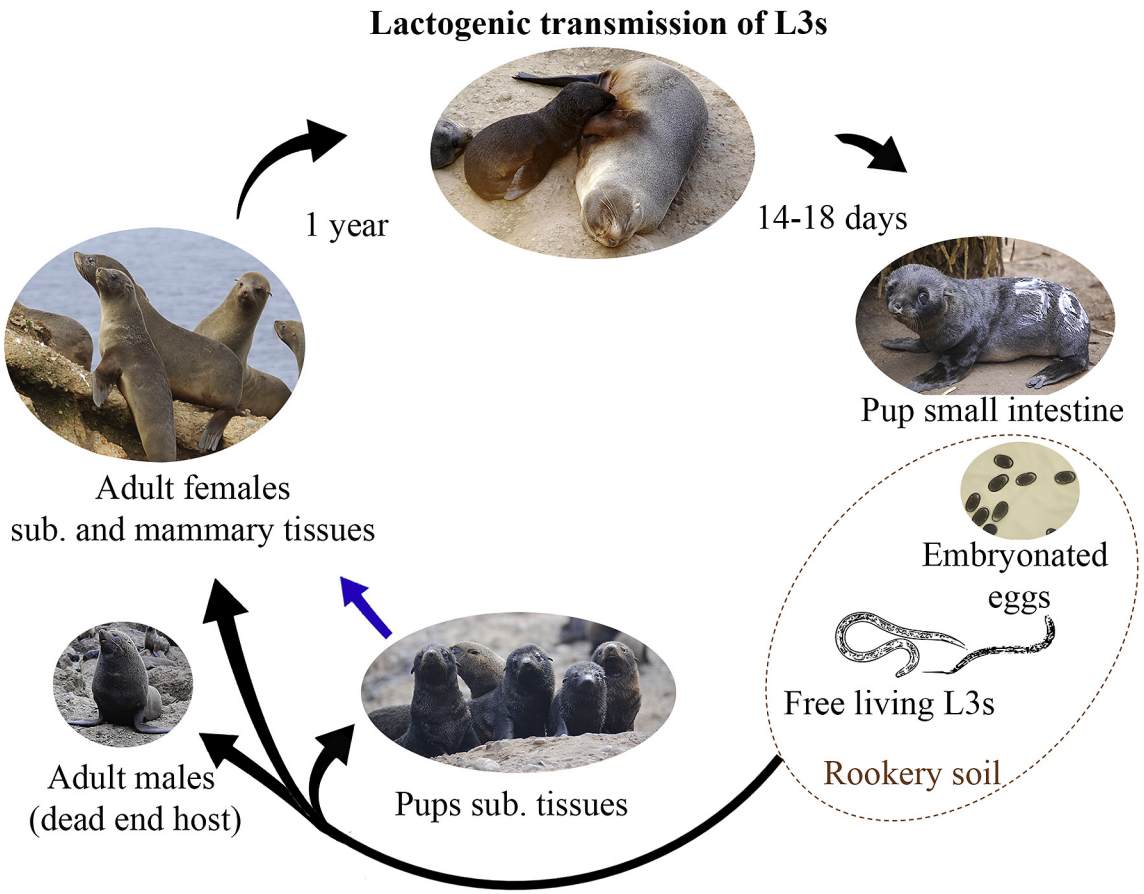

Fig. 1. Life cycle of *Uncinaria sp*. in South American fur seals (*Arctocephalus australis*). Pups get infected through ingestion of colostrum that contains infective stage 3 larvae (L3s). Within 2-weeks, hookworms reach adulthood in the small intestine and shed eight-celled eggs in the pup's feces. Eggs larvate in the rookery soil and larvae develop into sheathed infective L3s which penetrate the skin and reach the subcutaneous tissues of all animals in the rookery. However, *Uncinaria sp*. larvae only have a chance to reach the next definitive host in females, which give birth and produce colostrum once a year, repeating the cycle. It is very likely that female pups can keep larvae in their tissues until they reach maturity and pass them to their pup (blue arrow). All males are dead end hosts. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

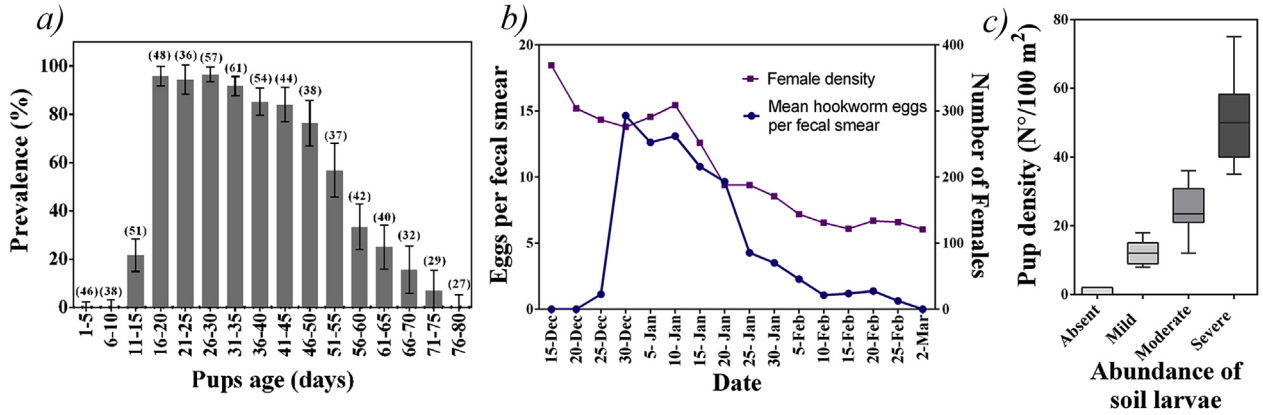

Fig. 2. Hookworm prevalence, egg shedding and abundance of larvae in the soil are correlated with adult females and pup density. (a) Prevalence reach over 90% when pups are between 20 and 30 days old, then substantially decline and by 75 days-old on average, all pups have cleared hookworm infection. Numbers in parenthesis indicate sample size. Bars represent binomial confidence intervals (b) Mean fecal hookworm egg count follow a similar curve with the highest number of eggs being shed when the number of adult fur seal females in the rookery is still high, between December 30 and January 15th, when pups are on average 15 to 30 days-old. (c) The soil from areas of the rookery with higher pup density had larger numbers of hookworm larvae (GLM, $X^2 = 1303$, df = 3, $P = 2.2 \times 10^{-16}$). Whiskers represent 95% confidence intervals.

categorized as mildly infected based on the fecal egg count cutoff value. There were marked differences in the survival rates of pups with severe hookworm infection compared to the “mild infection” and “hookworm free (ivermectin-treated, control)” groups (Log-rank Mantel-Cox test, $X^2 = 44.43$, df = 2, $P = 5.41 \times 10^{-10}$) (Fig. 4a). In the severe infection group, 30 pups died (30/53, 56.6% mortality), and in at least 26 cases they were confirmed to die due to hookworm infection by necropsy and histopathology (26/53, 49.1%). In the mild infection group (n = 96), 9 animals died (9.4% mortality) due to trauma (n = 4), starvation (n = 2) or unknown causes (n = 3). In the ivermectin-

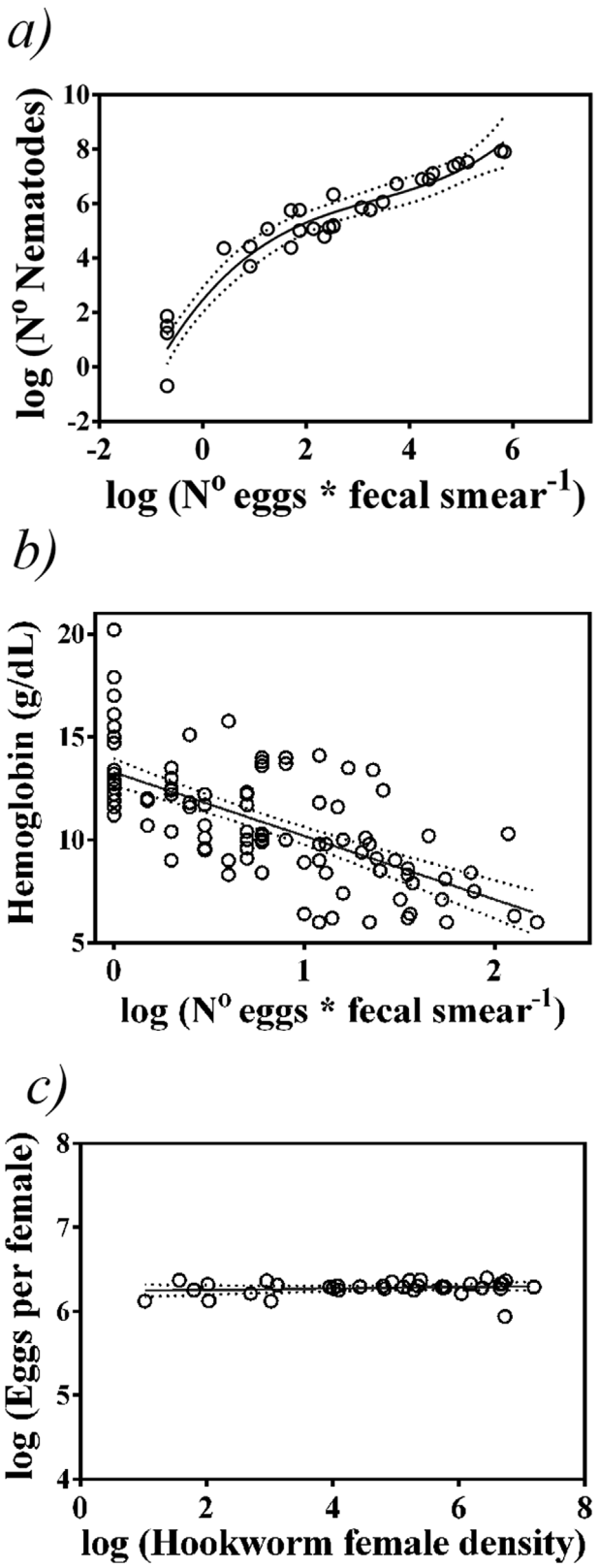

Fig. 3. Correlations between hookworm burden, egg shedding and extraction of host resources. (a) Hookworm burden is highly correlated with egg shedding in pup's feces (third order polynomial regression, adj-$r^2 = 0.921$, $P = 2.2 \times 10^{-16}$). (b) Hemoglobin concentration decreases as the number of hookworm eggs in pup's feces increase (second order polynomial regression, adj-$r^2 = 0.401$, $P = 2.09 \times 10^{-14}$), suggesting that extraction of host resources depends on parasitic burden. (c) Female hookworms harbor similar number of eggs in their uterus regardless of parasitic burden (linear regression, adj-$r^2 = -0.03$, $F = 1.01$, $df = 36$, $P = 0.321$), suggesting that there is no decline in egg output even at high hookworm densities. The solid lines represent the best fit model with 95% confidence intervals (dashed lines).

treated group (n = 30), 2 animals (6.6%) died due to trauma. The difference in survival between the mild infection and treated groups was not significant (Log-rank Mantel-Cox test, $X^2 = 0.3728$, $df = 1$, $P = 0.542$).

The hemoglobin concentration in the pups was markedly influenced by the hookworm burden (GLM estimate $= -4.56 \times 10^{-2} \pm 1.21 \times 10^{-2}$, $P = 2.49 \times 10^{-3}$). Hemoglobin concentrations were similar in hookworm free pups (treated with ivermectin) and those with mild hookworm infection, however pups with severe infection had considerably lower values compared to the other two groups (ANOVA, $F = 31.47$, $df = 2$, $P = 2.12 \times 10^{-12}$) (Fig. 4b).

3.4. Hookworm egg shedding and pup's health status

Based on the estimated total hookworm egg shedding parameter (median burden multiplied by the number of days infected), fur seal pups with anemia (hemoglobin concentration < 10 g*dL^{-1}) shed on average more hookworm eggs compared to pups that were not anemic (GLM with negative binomial distribution, Anemic pups $= 1.36 \pm 0.23$, $Z = 5.92$, $P = 3.09 \times 10^{-9}$) (Fig. 5a). When the total number of hookworm eggs shed was calculated for all the studied pups, animals that were anemic (n = 36) contributed to 61.1% of the total egg shedding while non-anemic pup contributed to only 38.9% of the egg shedding despite being the most numerous group (n = 50). Regarding mortality, fur seal pups that died due to hookworm disease shed on average more hookworm eggs when compared to pups that survived hookworm infection (GLM with negative binomial distribution, Dead pups $= 1.40 \pm 0.22$, $Z = 6.16$, $P = 7.24 \times 10^{-10}$) (Fig. 5b). Pups that died due to hookworm disease (n = 26) contributed to 60.7% of the total egg shedding in the environment whereas pups that survived hookworm infection (n = 70) contributed to 39.2% of the egg shedding.

4. Discussion

The marine lifestyle of pinnipeds creates a major challenge for a parasite that depends on the development of larval stages in soil to complete its life cycle. We suggest that fur seal hookworms have overcome this problem through tight synchronization with the host reproductive cycles. Our epidemiological data and evidence from similar studies in other pinniped species suggest that transmission of hookworm infective larvae to pups occurs only through fur seal's milk (Castinel et al., 2007; Lyons et al., 2011; Marcus et al., 2014). Since many pinniped species have highly synchronized birth periods (Paves et al., 2016), transmission of hookworms occurs with initiation of lactation in a short period of time for most of the pup population. This fact plus the early clearance of adult hookworms from the pups' intestine gives pinniped hookworms little time for growth, reproduction, eggs shedding, and development of larvae in the soil. Thus, an r-selected life history strategy has been favored in this hookworm species, which translates into a lack of restriction in the extraction of host resources, despite reaching high within-host density. This could explain in part why a third of the pups born each year suffer significant levels of anemia and mortality as consequence of hookworm infection, yet these pups provide more than 60% of the total number of eggs shed in the environment.

Biological rhythms have been proposed as a significant player in host-parasite relationships (Martinez-Bakker and Helm, 2015). In the case of fur seal hookworms, their particular life cycle could be the result

Table 1
Coefficients and statistical significance for predictors in several statistical models for hookworm burden as response in South American fur seal (*Arctocephalus australis*) pups (negative binomial Generalized Linear Models). Models are ranked based on Akaike's information criteria.

Model	Predictors									log-lr	X^2	df	p-value	AIC	delta AIC
	HG	WBC	PT	RBC	HG[a]RBC	Mortality (yes)	BMI	Sex (Male)	Infectious Period						
1	−0.35[a]		0.22[a]	7.30E-08		1.23[a]	−2.97	−0.36	0.03[a]	−321.833	159.167	9	1.28E-65	661.67	0.00
2	−0.35[a]	2.91E-06	0.22[a]	6.84E-08		1.22[a]	−2.77	−0.34	0.03[a]	−321.821	159.18	10	1.26E-65	663.64	1.97
3	−0.34[a]		0.17	5.52E-08		1.23[a]		−0.41	0.02[a]	−327.566	153.435	8	3.60E-63	671.13	9.46
4	−0.65[a]			−9.36E-07[a]	9.40E-08[a]	1.26[a]	−3.78	−0.34	0.03[a]	−328.344	152.656	9	7.75E-63	674.00	12.33
5	−0.32[a]		0.16			1.20[a]		−0.42	0.02	−332.583	148.418	7	5.01E-61	679.17	17.50
6	−0.72[a]			−1.07E-06[a]	1.10E-07[a]	1.25[a]			0.03[a]	−334.758	146.243	7	4.26E-60	683.52	21.85
7	−0.28[a]					1.25[a]		−0.52[a]		−363.2	117.8	5	4.65E-48	736.00	74.33
8	−0.28[a]					1.23[a]		−0.48[a]	0.01	−361.081	119.92	6	2.69E-48	736.89	75.22

HG = Hemoglobin, WBC = White blood cell count, PT = Total serum proteins, BMI = body mass index.
[a] Predictors are significant at α = 0.05.

of synchronization with the host reproductive rhythms. In SAFS, as well as in other studied pinniped species, pups are infected by L3s ingested through the milk (Olsen and Lyons, 1965; Castinel et al., 2007; Marcus et al., 2014). In northern fur seals, subcutaneous L3s do not migrate to the small intestine and experimental infection is only successful if attempted with L3s obtained from pregnant females (Olsen and Lyons, 1965). L3s extracted from the subcutaneous tissue of non-pregnant females, pups, adult males or from the soil do not mature in the fur seal pup's intestine (Olsen and Lyons, 1965). Additionally, northern fur seal pups born by cesarean section, and not exposed to colostrum, do not develop hookworm infection (Olsen and Lyons, 1965), suggesting lack of transplacental infection and exclusive lactogenic transmission, as shown in other hookworm species (Burke and Robenson, 1985). In SAFS, something similar could happen since our epidemiological and pathological data (Seguel et al. 2011, 2017), hookworm size variation, and analyses in fur seals milk, tissues and colostrum suggest that successful hookworm colonization of the intestine only occurs during the first week of pup's life. Although the presence of L3s in placenta and milk during the 2nd or 3rd week of lactation cannot be completely ruled out, these potential routes of transmission apparently do not result in patent infections. The opposite occurs with terrestrial mammal hookworm species L3s, which can infect the host and reach maturity in the intestine, regardless of their source or the infection route (skin and ingestion) (Loukas et al., 2005; Seguel and Gottdenker, 2017). This suggests that pinniped hookworms have developed efficient mechanisms to detect host reproductive status, probably through sensitivity to mammalian hormones. From the evolutionary point of view, to favor this trait, there must be a strong selective pressure to favor synchronized transmission in pinnipeds.

Table 3
Coefficients, standard errors and P values of predictors of mortality in South American fur seal pups (*Arctocephalus australis*) infected with hookworms (*Uncinaria sp.*). Values correspond to the top ranked model (GLMM) based on second order Akaike's information criteria (AICc) (Table 2).

Predictor	Estimate coefficient	Standard error	Z value	P value
Intercept	1.078	1.55	0.69	0.488
Red blood cell count	−8.047e-07	3.374e-07	−2.385	0.01710
Hookworm burden	0.032	0.015	2.063	0.03920
Infectious period	0.124	0.041	3.004	0.00267
Hemoglobin: BMI	−2.623	1.112	−2.359	0.01830

BMI = Body mass index.
GLMM = Generalized linear mixed model.

In the fur seal hookworm life history, there is remarkable timing in egg shedding, which is probably associated with the synchronization of reproductive cycles of SAFS at Guafo Island, where up to 90% of births occur in a two-week span (Paves and Schlatter, 2008; Paves et al., 2016). Therefore, most pups in the rookery are born and infected through their mother's colostrum during a short period of time, which translates in hookworm egg shedding starting in about 80% of the pups the first week of January, which is approximately 2 weeks (the prepatent period) after the peak births at Guafo Island (December 15, Paves and Schlatter, 2008). The lactogenic transmission during first

Table 2
Top ranked binomial generalized linear mixed models (GLMM) for hookworm induced mortality in South American fur seal pups (*Arctocephalus australis*). Models are ranked based on second order Akaike's information criteria (AICc).

Model	Predictors	df	logLik	AICc	Delta AICc	Weight
1	Infectious period + HW Burden + RBC + HG: BMI	6	−37.03	86.88	0.00	0.32
2	Infectious period + HW Burden + RBC + HG + BMI	7	−36.49	88.08	1.20	0.18
3	Infectious period + HW Burden + RBC + HG + BMI + WBC	8	−35.85	89.12	2.24	0.10
4	Infectious period + HW Burden + RBC + HG	6	−38.21	89.22	2.35	0.10
5	Infectious period + RBC + HG: BMI	5	−39.73	90.04	3.16	0.07
6	Infectious period + HW Burden + RBC + HG + BMI + HG: BMI	8	−36.43	90.29	3.42	0.06
7	Infectious period + HW Burden + RBC + HG: TP	6	−38.81	90.42	3.54	0.05
8	Infectious period + HW Burden + RBC + HG + BMI + WBC + Sex (male)	9	−35.74	91.27	4.40	0.04
9	Infectious period + HW Burden + RBC + HG + Infectious period: HW Burden	7	−38.21	91.50	4.62	0.03
10	Infectious period + HG: RBC	4	−41.85	92.08	5.20	0.02

HW Burden = Hookworm burden.
RBC = Red blood cell count.
HG = Hemoglobin.
BMI = Body mass index.
WBC = White blood cell count.
TP = Total serum protein.

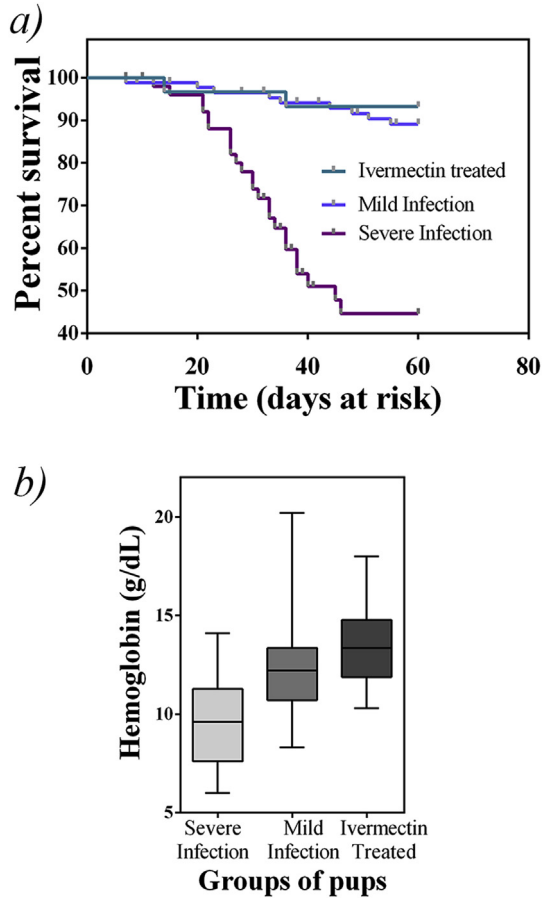

Fig. 4. Anemia and mortality are driven by parasite burden. (a) Survival rates of pups with severe hookworm infection was 44.4%, compared to 90.6% survival of pups with mild hookworm infection and 93.4% survival of pups treated with the antiparasitic ivermectin (Log-rank Mantel-Cox test, $X^2 = 44.43$, df = 2, $P = 5.41 \times 10^{-10}$). (b) Hemoglobin concentrations were markedly lower in the group with high parasitic burden (severe infection) (ANOVA, $F = 31.47$, df = 2, $P = 2.12 \times 10^{-12}$). Whiskers represent 95% confidence intervals.

days of pup life means that eggs released by these pups, once they become larvae in the rookery soil, could have higher chances to find a new host (females), in order to continue the parasite life cycle. This could occur because as shown in this study, the population density of fur seal rookeries decreases as the reproductive season advances, in part due to pup mortality, departure of adult males, juveniles, and some females without pups (Paves and Schlatter, 2008; Paves et al., 2016). Many of these animals will only return to the rookery the next reproductive season (Paves and Schlatter, 2008; Paves et al., 2016), and, given the fact that hookworm larvae do not survive in Guafo Island soil from one season to the next, the only chances for these animals to come in contact with hookworm larvae in the soil is during the first weeks of the pupping season (Late December early January at Guafo Island) before they leave the rookery.

Terrestrial hookworms live in their hosts' intestine several months to years thanks to the successful immunomodulation that the parasites elicit in the host to avoid clearance (Loukas et al., 2005; Periago and Bethony, 2012; Seguel and Gottdenker, 2017). The short period that pinniped hookworms have to maximize their transmission due to host density constraints could explain why this parasite has not evolved successful mechanisms to avoid clearance. In fur seals, 100% of the adult hookworms are dead within 4–8 weeks, due to host mortality or clearance. This short adult life span leaves these nematodes with little time to feed, growth, reproduce and release eggs, which probably explains the aggressive feeding behavior of this parasite. Pinniped hookworms are the only members of the *Ancylostomatidae* family that dig deep into the intestine, sometimes even penetrating the intestinal wall, causing peritonitis and death (Spraker et al., 2007; Seguel et al., 2017). Additionally, contrary to terrestrial hookworms (Anderson and Schad, 1985), there is lack of density dependent depletion in marine hookworm female egg output, further supporting our observations that pinniped hookworms are voracious eaters- and rapid extraction of host resources is probably more important for hookworm fitness than avoiding host anemia and mortality.

This is one of the few studies that has measured hookworm anemia and mortality under natural conditions using recapture-resighting methods (Delong et al., 2009; Seguel and Gottdenker, 2017). At Guafo Island, about a third of the pups born each year experience anemia due to hookworms and approximately 17% (26/149) of all pups born die due to hookworm disease. In other pinniped rookeries, mortalities

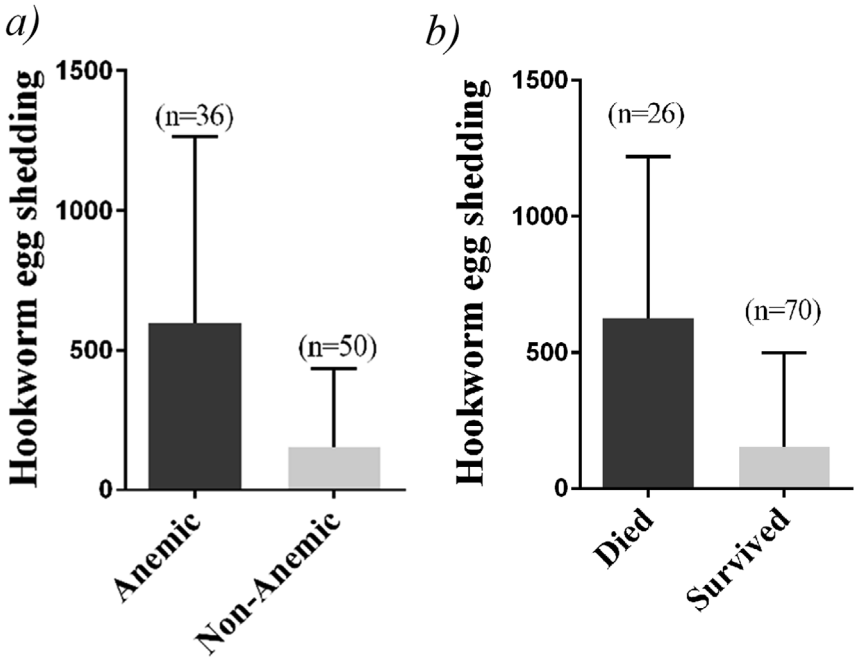

Fig. 5. South American fur seal (*Arctocephalus australis*) pups that suffer the worst consequences of hookworm (*Uncinaria sp.*) infection contribute to most of the egg shedding in the environment. Hookworm egg shedding represents the product of the median number of eggs per fecal smear and the number of days a pup was infected with hookworms (Burden * infectious period). (a) Anemic pups shed on average more hookworm eggs compared to non-anemic pups (GLM with negative binomial distribution, Anemic pups = 1.36 ± 0.23, $Z = 5.92$, $P = 3.09 \times 10^{-9}$). (b) Fur seal pups that died due to hookworm disease shed on average more hookworm eggs when compared to pups that survived (GLM with negative binomial distribution, Pups died = 1.40 ± 0.22, $Z = 6.16$, $P = 7.24 \times 10^{-10}$).

between 40% and 70% have been reported (Spraker et al., 2007, Marcus et al., 2014), however those percentages correspond to a fraction of the total pup mortality, which usually fluctuates between 10% and 30% of all pups born in a year (Seguel et al., 2013). Therefore, our reported mortality rates could be comparable with what has been observed during hookworm epidemics in California sea lions (*Zalophus californianus*) (hookworm mortality; 70% of total mortality, Spraker et al., 2007) and in regular years in Northern fur seals at San Miguel Island, California (hookworm mortality; 26% of all pups born, Delong et al., 2009). Mortality due to hookworm infection is rare in animals other than pinnipeds and it has only sporadically been reported in canids, felids and ursids (Seguel and Gottdenker, 2017), however the results of controlled experiments to measure hookworm induced mortality in these species have not been published.

The reasons for the enhanced pathogenicity of pinniped hookworms is unknown, however, based on the assumptions of the transmission-virulence trade off hypothesis, parasites should reach virulence levels that maximize their fitness, or transmission within a population (Alizon et al., 2009, Alizon and Michalakis, 2015; Schmidt-Hempel, 2011; Hatcher et al., 2012; Cressler et al., 2015). Although this hypothesis posits that parasites can evolve different levels of virulence, most host-parasite systems evolve towards intermediate virulence (Alizon et al., 2009, Alizon and Michalakis, 2015; Schmidt-Hempel, 2011; Hatcher et al., 2012; Cressler et al., 2015). In the case of human and animal pathogens that cause high levels of mortality (*e.g.* Ebola, avian influenza), these are usually considered novel host-pathogen relationships where the parasite is not yet adapted to the host population. The strongest evidence supporting this hypothesis arises from the studies on the evolution of HIV and myxoma virus in human and rabbit populations respectively (Fraser et al., 2007; Kerr et al., 2012). In both cases, a pathogen introduced into a naïve population caused significant mortality initially. However, over time, evolution favored the selection of viral strains with intermediate levels of virulence (Fraser et al., 2007; Kerr et al., 2012). In the case of hookworm infection in pinnipeds, there is most likely a long-standing host-parasite relationship (Lyons et al., 2011; Seguel et al., 2017; Seguel and Gottdenker, 2017), which has resulted in remarkable adaptation of the parasite to the marine lifestyle of the host, favoring, for instance, exclusive lactogenic transmission of infective larvae to the pups (Lyons et al., 2011; Seguel and Gottdenker, 2017).

Given the lack of density dependent decline in egg production by hookworm females, it was expected that animals with higher burden caused most of the egg shedding and suffered the adverse consequences of parasitism in terms of anemia and mortality. However, animals that die decrease their infectious period, an important component of the total egg shedding in the environment (Vanderwaal and Ezenwa, 2016). Therefore, it could also have been expected that these animals did not contribute too much to the total hookworm egg shedding. Given the fact that all pups clear infection, the difference in the days infected with hookworms between pups that die and those that clear the infection is probably not large enough to compensate for the higher burden in animals suffering mortality. In other host-parasite systems, animals with high burdens that die become “bad infection spreaders” because they decrease their contact rate and infectious period (Vanderwaal and Ezenwa, 2016). To compensate for this fact, and to allow the host to tolerate higher loads and become a better infection spreader, several parasitic nematodes experience a decline in feeding activity and egg output (Anderson and Schad, 1985; Irvine et al., 2001). Although this adaptation decreases the short term individual reproductive output of the nematode, it allows a better tolerance by the host and prolongs the infectious period, potentially avoiding sickness behavior, allowing the infected host to spread the infection more efficiently (Anderson and May 1985; Anderson and Schad, 1985; Vanderwaal and Ezenwa, 2016). Therefore, at the end of the nematode life history, it can be beneficial for the nematode fitness to produce eggs in a slower rate and feed less intensively in the host (Anderson and May 1985; Anderson and Schad, 1985; Vanderwaal and Ezenwa, 2016). Fur seal hookworms have apparently developed a different strategy, where there is little restriction in extraction of host resources, and a disproportionate contribution to the total egg shedding by animals experiencing the worst consequences of parasitism.

Why pinniped hookworms have not exploited other alternatives to increase their chances of transmission is not clear, but it could be related to the reproductive ecology and habitat of fur seals and sea lions. For a parasite with indirect transmission, increasing the environmental survival of larval stages in the soil would increase its chances of transmission, however, as shown in this and similar studies (Lyons et al., 2011), fur seal hookworm larvae do not survive from one reproductive season to the next. Pinnipeds live on shores with ocean runoff and in places with extreme winter temperatures (*e.g.* Alaska, Antarctica), therefore, lengthened hookworm survival has probably not been possible, since larvae would have had to survive extreme conditions for at least one year until the next reproductive group arrives to the coast. However, reaching a fur seal subcutaneous tissues as soon as possible and remain there until the animal become reproductively active seems as a safer and better strategy for a parasite facing harsh environmental conditions.

Another significant component of parasite transmission is the infectious period. The universal and early clearance of hookworms by fur seals significantly reduces the parasite time to release eggs. However, it is possible that extending the infectious period does not payoff as much in terms of fitness for the parasite as producing more eggs over a short period of time, due to the marked seasonal density changes in pinniped populations (Paves and Schlatter, 2008; Paves et al., 2016). Additionally, avoidance of clearance usually involves host immunomodulation, which is energetically costly for parasites (Loukas et al., 2005; Mulvenna et al., 2009), therefore a significant advantage for the parasite is probably necessary to favor this trade-off.

This study found that adult pinniped hookworms ‘live fast ‘compared to other members of their nematode family. However, they die young due to host death due to hookworm disease or the universal clearance that pups experience after 3–8 weeks of patent infection. This particular life history strategy could have resulted from the many particularities and challenges that the marine lifestyle of the host imposes in a soil-transmitted nematode, suggesting that marine hookworms are highly adapted to their hosts. However, this strategy has also resulted in a lack of restriction in the extraction of host resources and the highest levels of anemia and mortality recorded in animal populations due to hookworms, suggesting that “parasite adaptation” is probably better explained in terms of parasite transmission and fitness and not necessarily linked to host health status.

Conflicts of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ijppaw.2018.07.003.

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