THE DRIVERS AND CONSEQUENCES OF HOOKWORM DISEASE IN SOUTH AMERICAN FUR SEALS

by

MAURICIO SEGUEL

(Under the Direction of Nicole Gottdenker and Elizabeth Howerth)

ABSTRACT

Hookworms are highly pathogenic hematophagous nematodes that parasitize more than 100 species of wild animals. Hookworms of fur seals and sea lions are particularly virulent, causing high levels of mortality, however little is known about the drivers of these effects. During 10 reproductive seasons, the dynamics of hookworm diseases was studied in the South American fur seals (SAFS, *Arctocephalus australis*, Otariidae) rookery at Guafo Island, Chilean Patagonia. Fur seal hookworms (*Uncinaria sp.*) reached 100% prevalence among pups, and in animals with high burdens (>300 nematodes) there was marked hemorrhagic enteritis, sometimes with peritoneal penetration. The life cycle of *Uncinaria sp*. in SAFS involved brief lactogenic transmission of infective larvae. This hookworm species has a live fast die young life history strategy that translates into aggressive feeding behavior and high rates of extraction of host resources. Therefore, pups with high burdens, a third of pups born each year, suffered significant levels of anemia and up to 60% hookworm-related mortality. These pups contributed disproportionally to parasite fitness, and increases in host mortality (virulence) always paid off in terms of parasite fitness, selecting for higher virulence within the parasite population. Pups that survived hookworm infection developed an efficient immune-mediated parasite clearance where

T-lymphocytes, basophils, mast cells and parasite specific IgG were key players. Pups that received higher levels of maternal attendance had better energy balance and a more reactive immune system. These pups cleared hookworm infection earlier, increasing their chances of survival. Maternal attendance decreased in years with high sea surface temperature (SST), probably reflecting less availability of prey in the environment. Therefore, in years with high SST, fur seal pups exhibited a weaker immune response and higher levels of hookworm mortality. Hookworm disease is the most significant cause of death in many otariid populations, and the present study demonstrates that the parasite is selected for higher virulence, especially when a high hookworm burden is favored. The key role of parasite immune clearance in hookworm infection dynamics and the link between environmental conditions and pups immune system present a scenario where global climate change may lead to increased hookworm virulence and mortality of fur seal pups.

INDEX WORDS: climate change, ecology, hookworm, immunology, marine mammals, pathology.

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DEDICATION

To those who walked the forests before me, to those who navigated the rivers before me, to those who named the stars. To my Mapuche people, their teachings and values.

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CHAPTER 1

IINTRODUCTION AND LITERATURE REVIEW[1]

[1]Seguel, M and N. L. Gottdenker. 2017. *International Journal for Parasitology: Parasites and Wildlife*. 6:177-192.

Abstract

Hookworms are blood-feeding nematodes that parasitize the alimentary system of mammals. Despite their high pathogenic potential, little is known about their diversity and impact in wildlife populations. We conducted a systematic review of the literature on hookworm infections of wildlife and analyzed 218 studies qualitative and quantitatively. At least 68 hookworm species have been described in 9 orders, 24 families, and 111 species of wild mammals. Black bears, red foxes, and bobcats harbored the highest diversity of hookworm species and *Ancylostoma pluridentatum*, *A. tubaeforme*, *Uncinaria stenocephala* and *Necator americanus* were the hookworm species with the highest host diversity index. Hookworm infections cause anemia, retarded growth, tissue damage, inflammation and significant mortality in several wildlife species. Anemia has been documented more commonly in canids, felids and otariids, and retarded growth only in otariids. Population- level mortality has been documented through controlled studies only in canines and eared seals although sporadic mortality has been noticed in felines, bears and elephants. The main driver of hookworm pathogenic effects was the hookworm biomass in a population, measured as prevalence, mean burden and hookworm size (length). Many studies recorded significant differences in prevalence and mean intensity among regions related to contrasts in local humidity, temperature, and host population density. These findings, plus the ability of hookworms to perpetuate in different host species, create a dynamic scenario where changes in climate and the domestic animal-human-wildlife interface will potentially affect the dynamics and consequences of hookworm infections in wildlife.

Introduction

Hookworms (Nematoda: Strongylida: Ancylostomatoidae) are blood-feeding nematodes that parasitize the mammalian alimentary system (Popova 1964). Regardless of the large diversity within this parasitic group, all Ancylostomatoidae species share basic morphologic, physiologic and life history traits that translate into similar consequences for their host. In humans and domestic animals, the deleterious effects of hookworms are well documented at the individual and population level, being one of the most significant neglected tropical diseases of humans (Bartsch et al. 2016), and an important cause or contributory factor of anemia and neonatal mortality in domestic dogs and cats (Traversa 2012). Despite the potential deleterious impact in their hosts, there is no currently available summary on the number of hookworm species described and the significance of hookworm infection in free-ranging wild mammals.

The modification of landscapes and climate change create additional challenges for wildlife disease study, and it is predicted that these phenomena will modify the dynamics of nematode infections (Weaver et al. 2010, Weinstein and Lafferty 2015). Therefore, improved description, analysis, and understanding of hookworm infections in wildlife are necessary to direct future research efforts and understand host-parasite relationships in a regional and global scale.

In this context, the objectives of this review are to i) provide a systematic summary of the literature available on hookworm infections of wildlife, ii) evaluate the reported hookworm diversity in wildlife corrected for sampling effort, iii) identify significant pathologic features of these infections and the potential drivers of the deleterious effects of hookworms on wildlife hosts.

Materials and Methods

a) Searching methods and inclusion criteria

A systematic literature review of Ancylostomatoidae nematodes of wildlife was performed using Google scholar, Web of Science and Biosis search engines on April 27th 2016; following recommended practices for systematic reviews in the field of parasitology (Haddaway and Watson, 2016). The initial term searched was "hookworm(s)" AND "wildlife". The abstracts of the papers retrieved were reviewed and included in the study if they met the following criteria: i) The parasitic nematode found belonged to a genus within the Ancylostomatoidae family. (Studies describing the presence of eggs or nematodes as "hookworms" or "strongyles" without genus identification were excluded). ii) The host species was any free-ranging non-domesticated (wild) animal. Captive wild animals were only included if they were taken from the wild soon before the study, and therefore, transmission of parasites was assumed to occur in the wild. In the case of domestic animal-wildlife hybrids, these were included if they were free-ranging. Additional searches were performed using the preliminary list of genera identified in the initial search plus the word "wildlife" (*e.g.* "*Ancylostoma* wildlife"). The studies selected based on abstract screening (N=216) were fully reviewed and the most significant findings summarized into a master spreadsheet (supplementary material).

b) Data analyses

To identify host species with high hookworm diversity, an index penalized for sampling effort was calculated based on previously published methods (Nunn et al. 2003, Ezenwa et al. 2006). Briefly; the citations for each host species were extracted from the databases and after factor analyses condensed into one variable, citation-principal component (citation-PC). Additional sampling effort measures included the number of animals sampled in the reviewed

studies and the number of studies in our review for each host species. Negative binomial regression models were fitted for each measure of sampling effort and the residuals were used as a penalized index of hookworm diversity. To assess which hookworm species parasitized higher number of wildlife species a similar approach was used with the difference that citation-PC was not used because of "too high" penalization for highly studied hookworm species infecting humans or domestic animals (e.g. *Necator americanus*). We used instead, the total number of mammalian species screened in the studies where that hookworm species was found, since in many studies several host species were assessed. The penalized measurements of host diversity were calculated as previously described.

To assess which factors influenced the likelihood of finding a mammalian-hookworm species relationship that had detrimental effects for the host, the paired mammalian-hookworm species were categorized as 1 if there was at least one study describing adverse health effects at the individual or population level and as 0 if there were no studies registering such effect. Evidence of adverse health effect included mortality, anemia, retarded growth, and significant degrees of macroscopic or histologic tissue damage (*e.g*. fibrosis, inflammatory infiltrate). Generalized linear models were fitted using these two categories (no pathologic effect vs. pathologic effect) as binomial response and the number of studies for each mammalian-hookworm species relationship, number of animals sampled, Citation-PC for the animals and hookworm species, hookworm host diversity index, average prevalence in the studies reporting pathology or no effect, mean infection intensity in studies reporting pathology or no effect, average host weight (in kilograms), average hookworm length (in millimeters), host weight-hookworm size ratio, geographic location (continent) and study methodology were used as predictors in the general model. We used a stepwise algorithm and calculated Akaike's

information criteria (AIC) to determine which set of independent variables (including potential interactions between covariates) provided the best fit to the data.

Results

1. Characterization of studies describing hookworm infections in wild animals

The initial search yielded 3710 abstracts in Google scholar, 38 in Web of Science and 42 in Biosis. Out of these, 180 papers met the inclusion criteria and later searches by hookworm genera produced additional 38 papers, totaling 218 studies. Most of the studies were conducted in North America (n=66) followed by Eastern Asia (n=35) and Europe (n=35) (Figure 1.1a), and the most commonly used methodology of data collection was through necropsies of culled or incidentally found dead animals (n=153). Studies using experimental or molecular approaches for data collection corresponded to a minority of the papers (n=10 and n=8 respectively) (Figure 1.1b). Most of the studies described hookworm infections in the order Carnivora (n=125), particularly within the Canidae (n=73) Felidae (n=28) and Otariidae (n=25) families. Regarding host species, most studies were performed in red fox (n=26), coyote (n=16), raccoons (n=11) and Northern fur seals (n=10). After penalizing by sampling effort (number of studies and number of animals), the host species harboring the larger number of hookworm species were black bear (*Ursus americanus*), bob cat (*Lynx rufus*), and red fox (*Vulpes vulpes*) (Figures 1.2a and 1.2b).

At least 68 hookworm species have been described in 9 orders, 22 families, and 108 species of wild mammals. Before penalizing for sampling effort the hookworm species with the largest host range were *Uncinaria stenocephala* (n=11), *Ancylostoma caninum* (n=10), *Ancylostoma tubaeforme* (n=10), *Ancylostoma pluridentatum* (n=6) and *Necator americanus* (n=6); however, after penalizing for the number of sampled animals (Figure 1.3a) or the number

of species assessed in the studies of a particular hookworm species (Figure 1.3b), *U. stenocephala* and *A. caninum* became less generalists, but the feline hookworms *A. tubaeforme*, *A. pluridentatum*, *A. braziliencis* and the human nematode *N. americanus* remained as more generalist parasites, affecting several host species.

Thirty-five studies reported detrimental effects of hookworms at the individual or population level. All these studies were conducted on carnivores with the exception of a few reports on elephants and giraffes (n=5). The final model to assess the likelihood of finding a detrimental host-hookworm relationship included the level of study of the particular hookworm species (citation-PC), the mean prevalence and infection intensity in the studies and the average hookworm length as predictors (binomial GLM, p value= 0.0003, df=79); however, only the mean prevalence was statistically significant (p-value=0.0008).

2. Mammalian orders and families infected with hookworms

2.1 Carnivora

2.1.1 Canidae

Nine hookworm species have been described in canids, all of them members of the *Ancylostoma*, *Uncinaria* and *Arthrostoma* genera (Table 1.1). Among canids, the best studied species are the red fox (*Vulpes vulpes*) and coyotes (*Canis latrans*), probably related to their widespread distribution and because they are commonly hunted/culled.

In Asia, the native hookworms *Arthrostoma miyazakiense* and *Ancylostoma kusimaense* are the most common gastrointestinal nematodes of native (raccoon dogs, *Nyctereutes procyonoides*) and introduced canids (red foxes) (Sato et al. 2006, Shin et al. 2007). Interestingly, introduced raccoon dogs in Denmark lack their native Asian hookworms but are

infected with *U. stenocephala* (48.5% prevalence), a very common parasite of red foxes in that region (Al-Sabi et al. 2013), highlighting the potential of canine hookworms to infect multiple species (see table 1.1).

Many common canine hookworms such *U. stenocephala*, *A. caninum* and *A. ceylanicum* are important zoonotic pathogens, especially in Australia and Southeast Asia (Smout et al. 2013).

2.1.2 Felidae

Twelve species of hookworms within the *Ancylostoma*, *Uncinaria*, *Galoncus* and *Arthrostoma* genera have been described in wild felids (table 1.2). Despite the significant diversity of hookworm species within felines, and the vulnerable conservation status of many of them, considerably less research has been performed on hookworm parasites in felids compared to canids, and many aspects of their hookworms' biology are unknown.

The dog hookworm *A. caninum* infects felids in many areas where wild cats are sympatric with domestic or wild canids, such as the southeastern United States (Miller and Harkema 1968, Little et al., 1971, Mitchel and Beasom 1974). In other areas, however, the domestic cat hookworms, *A. tubaeforme* and *A. braziliense*, are the predominant species in wild felids (Waid and Pence 1988, Pence et al. 2003, Smith and Kok 2006), and in some occasions these wildlife infections are most likely because of spillover from feral domestic cats (Millan and Basco-Costa 2012).

In Asia, *Uncinaria felidis* and *Uncinaria maya* are the most common hookworms of native felids such as the leopard (*Prionailurus bengalensis*) and Iriomote cats (*Prionailurus iriomotensis*) (Hasegawa 1989, Yasuda et al. 1993, Shimono et al. 2012). Although apparently rare, the nematode *Arthrostoma hunanensis* infects the bile duct of leopard cats in some areas (Yasuda et al. 1993).

2.1.3 Otariidae

Four *Uncinaria* species have been described in eared seals (otariids); however, molecular analyses suggest that there are at least 5 additional undescribed *Uncinaria* species (Nadler et al. 2013 Seguel et al., unpublished data) (Table 1.3).

2.1.4 Procyonidae

Six species of hookworms within the *Necator*, *Arthrocephalus*, *Ancylostoma*, *Arthrostoma* and *Uncinaria* genera have been described in procyonids (Table 1.4). Most studies in procyonids have been conducted in raccoons (*Procyon lotor*), which in their native North America are infected with *Arthrocephalus lotoris*; however, in Japan, where they have been introduced, raccoons are infected with the native raccoon dog hookworms *Ancylostoma kusimaense* and *Arthrostoma miyazakiense* (Matoba et al. 2006, Sato and Zuzuki 2006).

2.1.5 Mustelidae

Four species of hookworms within the *Uncinaria* and *Tetragomphius* genera and at least one unknown species within the *Ancylostoma* genus have been described in mustelids (table 1.5). The most studied host species is the European badger (*Meles meles*), which is usually infected with *Uncinaria criniformis,* and in Korea *Tetragomphius procyonis* has been described in the Asian badger (*Meles leucurus*) (Son et al. 2009).

In Europe, pine martens (*Martes martes*) are infected with *Uncinaria sp*., *U. criniformis* and an *Ancylostoma* sp. (Segovia et al. 2007, Borecka et al. 2013).

2.1.6 Ursidae

Six species of hookworms within the *Ancylostoma*, *Arthrocephalus* and *Uncinaria* genera have been described in bears (table 1.6). The most important hookworm species in black bears is *Uncinaria rauschi,* which can reach up to 72% prevalence in some areas of Canada (Catalano et

al. 2015). *Uncinaria yukonenesis* is more common in brown bears in North America, while in Japan brown bears are infected with *Ancylostoma malayanum* (Catalano et al. 2015, Asakawa et al. 2006). The canine hookworm, *A. caninum*, and the raccoon hookworm, *A. lotoris*, infect black bears in the southeastern United States; however, the mean intensities are usually low (< 15 nematodes per animal) (Crum et al. 1978, Foster et al. 2011).

2.1.7 Mephtididae, Herpestidae, Phocidae, Hyenidae and Viverridae.

There are few studies reporting hookworm infections in the Mephitidae (skunks), Herpestidae (mongoose), Phocidae (true seals), Hyenidae (hyenas) and Viverridae (civets) families (table 1.7). Skunks can be infected with the raccoon hookworm *A. lotoris* in North America (Dikmans and Goldberg 1949), while in South America the native skunk, *Conepatus chinga*, harbor its own hookworm, *Ancylostoma conepati* (Ibanez 1968). In Taiwan a few individuals of *Arthrostoma vampire* were found in the Palawan stink badger (*Mydaus marchei*) (Schmidtz and Kuntz 1968). *Arthrocephalus gambiensi* has been described in herpestids inhabiting Gambia and Taiwan (Ortlepp 1925, Myers and Kuntz 1964). Compared to their otariid relatives, hookworms have been rarely described in phocids; however, the description of *Uncinaria sp*. in Southern elephant seals (*Mirounga leonina*) from Antarctica (Ramos et al. 2013) highlights the extreme adaptability of some hookworm species. The human hookworm, *Ancylostoma duodenale*, has been found in low numbers (n=7) in spotted hyenas (*Crocuta crocuta*) in Ethiopia (Graber and Blanc 1979), and in other study in Kenya up to 90% of hyenas harbored an *Ancylostoma sp.* (Engh et al. 2003). The Malay civet (*Viverra tangalunga*) in Borneo harbors the zoonotic hookworm, *Ancylostoma ceylanicum*, in low prevalence (3%); however, they are more commonly infected (33% prevalence) with a different *Ancylostoma sp*. (Colon and Patton 2012).

2.2 Arctiodactyla

2.2.1 Bovidae

Hookworm infections of bovines are dominated by the genera *Agriostomum*, *Bunostomum* and *Gaigeria* (table 1.8). All *Agriostomum* species have been described in South African bovids such as blue wildebeest (*Connochaetes taurinus*) and kudu (*Tragelaphus strepsiceros*) (Van Wyk and Boomker 2011). The cattle hookworm *Bunostomum phlebotomum* has been described in endangered European bison (*Bison bonasus*) in Poland (Karbowiak et al. 2014); however, numerous African bovines, including the African buffalo (*Syncerus caffer*) harbor hookworms of the *Bunostomum* genus although the definitive species have not been fully described (Ocaido et al. 2004, Phiri et al. 2011). Sheep hookworms also affect wild ruminants; *Bunostomum trigonocephalum* infects European wild bovids (Perez et al. 1996, Karbowiak et al. 2014) and *Gaigeria pachyscelis* infects the cecum and colon of several South African bovines (Anderson 1978, Horak et al. 1983).

2.2.2 Suidae and Tayassuidae

The domestic pig hookworm, *Globocephalus urosubulatus*, has been described in wild boars, feral domestic pigs-wild boar hybrids (*Sus scrofa*) and the central America wild pig, pecari (*Pecari tajacu*) (Table 1.9) (Coombs and Springer 1974, Romero-Castanon et al. 2008, Senlik et al. 2011). *G. urosubulatus* dominates in the Americas and Europe, and has been sporadically reported in eastern Asia; however, in Japan and Korea wild boars are usually infected with *G. samoensis* and *G. longimucronatus* (Kagei et al. 1984, Sato et al. 2008). In Africa, the bushpig (*Potamochoerus porcus*) harbors a different species of hookworm, *G. versteri* (Van Wyk and Boomker 2011).

2.2.3 Cervidae and Giraffidae

The cattle and sheep hookworms *B. phlebotomum, B. trigonocephalum* are the most common Ancylostomids of deer in Europe and Asia (table 10). In North America, however, *Monodontus lousianensis* has been found in the intestines of white-tailed deer (*Odocoileus virginianus*) (Chitwood and Jordan, 1965). *Monodontella giraffae* has been found in the bile duct of a captive giraffe (Giraffa camelopardalis) (Ming et al. 2010) and in all (n=7) necropsied giraffes in one study in Namibia (Bertelsen et al. 2009).

2.3 Primates

Primates within the Cercopithecidae, Hominidae and Loricidae families are affected by hookworms, and, as in people, *Necator* and *Ancylostoma* are the most important genera in non-human primates (Table 1.11). *Necator gorillae* has been described in western mountain gorillas (*Gorilla gorilla*) in the democratic Republic of Congo (Noda and Yamada 1964), and in humans in close contact with gorillas in the Central African Republic (Kalousova et al. 2016). Additionally, the human hookworm *Necator americanus,* has been found in gorillas and chimpanzees in areas where this parasite is common among people (Hasegawa et al. 2014). In Cameroon, *Ancylostoma sp.* nematodes were common in endemic cercopithecids sold as bushmeat in a local market (Pourrut et al. 2011). In the endangered lion-tailed macaque (*Macaca silenus*), groups close to human populations have 40 to 70% prevalence of *Ancylostoma sp* while groups in areas with no human settlements had 0% prevalence (Hussain et al. 2013).

Occasional gray literature (technical reports), describe hookworms as common in non-human primates; however, they do not report the genus or species, and therefore could not be incorporated into this review.

2.4 Rodentia

Hookworms have been sporadically described in rodents. Most studies have focused in the nematode species description and little is known about the prevalence and patterns of hookworm infection in this animal group. *Monodontus* is one of the most common hookworm genera in rodents in the Americas (table 1.12). In Australia, the native water rat (*Hydromys chrysogaster*) harbors *Uncinaria hydromydis* (Smales and Cribb 1997), and in Malaysia *Cyclodontostomum purvisi* infects several native murid species (Balasingam 1963). In Africa, the greater cane rat (*Thryonomys swinderianus*) is infected by two species of the *Acheilostoma* genus, of which *A. simpsoni* is found in the gallbladder and bile ducts of 60% of animals (Kankam et al. 2009).

2.5 Perissodactyla

The knowledge of hookworm infections in this order is limited to parasite descriptions usually based on a few nematodes recovered from a single animal (Khalil 1922b). Within the perissodactyla, hookworms have been found in tapirs (*Tapitus sp.*) (Travassos 1937), and the black Rhinoceros (*Rhinoceros bicornis*) (table 1.13) (Neveu-lemaire 1924).

2.6 Proboscidea

Elephants are the only living family within the proboscidea order. In this group, despite the low number of studies conducted, at least 3 species of hookworms have been described in the African elephant (Loxodonta Africana) and another 3 in the Asian elephant (*Elephas maximus*) (table 13). In these animals, hookworms in the *Grammocephalus* genus inhabit the bile duct

while those in the *Bunostomum* and *Bathmostomum* genera inhabit the small and large intestines (Monning 1925, Debbie and Clausen 1975, Setasuban 1976).

2.7 Pholidota

In Asia, pangolins (*Manis sp*) are infected with low intensities (less than 16 nematodes per host) of the human hookworm *N. americanus* (table 1.13) (Cameron and Myers 1960, Mohapatra et al. 2015).

2.8 Afrosoricida and Scandentia

In the Afrosoricida order the greater hedgehog tenrec (*Setifer setosus*) is the only species in which hookworms have been described (*Uncinaria bauchoti*) (table 1.13) (Chabaud et al. 1964). In the Scandentia order the hookworm *Uncinaria olseni* was described in a treeshrew (*Tupaia sp*) (table 1.13) (Chabaud et al. 1974).

3. The impact of hookworm infections on wildlife.

All members of the Ancylostomatoidae family are hematophagous and have developed efficient systems to extract and digest their host blood (Hotez et al. 2016). Most hookworm species use their well-developed buccal capsules to attach to mucosal surfaces and cut-out pieces of the tissue to produce "wounds" that bleed, in part because of the secretion of several anticoagulant proteins (Periago and Bethony 2012, Hotez et al. 2016). Since most hookworms live in the small intestine, this process creates a perfect environment for chronic blood loss, secondary bacterial infections and significant inflammation in the mucosae, impairing digestion and absorption (Seguel et al. in press). Therefore, the main adverse effects of hookworms

recorded in humans, domestic animals and wildlife species are anemia, retarded growth, secondary bacteremia and mortality (Traversa 2012, Hotez et al. 2016, Seguel et al. in press). The following section summarizes available evidence of such effects on wildlife hosts and explores potential drivers of those effects on wildlife populations.

3.1 Pathologic effects

3.1.1 Anemia

Anemia is rarely documented in wildlife species infected with hookworms, because few studies include assessment of blood values. In wolves, *A. caninum* infection has been associated with iron deficiency anemia in pups (Kazacos and Dougherty 1979). In Florida, USA, a young cougar was found markedly anemic due to *A. pluridentatum* infection (Dunmbar et al. 1994). In Michigan, USA, the reintroduced American martens (*Martes americana*) infected with hookworms (not species specified), were more likely to have anemia (Spriggs et al. 2016). The pups of Northern fur seals, California sea lions (Acevedo-Whitehouse et al. 2006), New Zealand sea lions (Acevedo-Whitehouse et al. 2009), Australian sea lions (Marcus et al. 2015b) and South American fur seals (Seguel et al. in press), present with mild to severe anemia related to *Uncinaria sp.* infection. Hookworm-induced anemia is markedly regenerative in Australian sea lions (Marcus et al. 2015b). In New Zealand and California sea lions, a single nuclear polymorphism (SNP) is linked with the degree of hookworm-associated anemia (Acevedo-Whitehouse et al. 2006, 2009).

3.1.2 Retarded growth

The only hookworm-infected wildlife species in which retarded growth has been accurately measured are Northern fur seals (DeLong et al. 2009), New Zealand sea lions

(Chilvers et al. 2009) and South American fur seals (Seguel et al. unpublished data). In Australian sea lions, although pups from rookeries with lower hookworm prevalence had higher body mass index (Marcus et al. 2014), controlled deworming experiments did not find significant difference between treated and hookworm-infected animals (Marcus et al. 2015a). The scarce literature on the effect of hookworms on wildlife host growth rates is probably related to logistic limitations to perform experimental studies in most free-ranging populations, an approach that facilitates measurement and comparison of growth rates in infected and uninfected animals. Additionally, hookworm infection is a primarily neonatal disease in pinnipeds, because infective stage 3 larvae only develop into adults when ingested by a pup with its mother's milk (Lyons et al. 2011, Seguel et al. submitted), therefore, retarded growth is a significant component of hookworm disease in these mammalian species (Chilvers et al. 2009, DeLong et al. 2009, Seguel et al., submitted).

3.1.3Tissue damage and inflammation

Probably most hookworm species cause some level of tissue damage as an unavoidable consequence of parasite feeding. This effect, however, along with inflammation, has been rarely documented in wildlife species, probably because most studies only assess these changes through gross examination of carcasses, where subtle lesions can be easily missed (Seguel et al., in press).

When hookworms feed on the intestinal mucosa they leave small 1-2 mm erosions on the mucosal surface that sometimes can be observed grossly, as is the case of Coyote pups infected with low numbers of *A. caninum* (Pence et al. 1988), California sea lions, South American fur seals, New Zealand sea lions, and Northern fur seals infected with *Uncinaria sp* hookworms (Spraker et al. 2007, Lyons et al. 2011, Seguel et al. in press). The chronic bleeding of these

intestinal wounds and accompanying inflammation elicited by the disruption of the mucosal barrier lead to different degrees of hemorrhagic enteritis, a common consequence of *A. caninum* infections in coyotes (Radomsky 1989), *A. pluridentatum* in young cougars, *Uncinaria sp.* in otariids and *A. lotoris* in raccoons. However, in raccoons these lesions have only been documented with experimental infections leading to burdens not observed in the wild (~ 1500 nematodes) (Balansingam 1968).

The mentioned patterns of lesions in wildlife are similar to that reported in domestic animal and human hookworm infections (Periago and Bethony 2012, Traversa 2012). However, among the high diversity of hookworm species infecting wildlife, there are particular lesion patterns only described in wild host species. These are the cases of peritoneal penetration by *Uncinaria sp.* in pinnipeds, hookworm submucosal infections in large cats, and bile and pancreatic duct hookworm infections of badgers, giraffes and elephants. Complete intestinal penetration by adult hookworms (*Uncinaria sp.*), has been observed in a significant proportion (from 12.5 to 60% of pups found dead) of California sea lions (Spraker et al. 2007), Northern fur seals (Lyons et al. 2011b) and South American fur seals (Seguel et al., in press), leading to peritonitis, septicemia and death. In large felines, the hookworms *Galoncus trudentatus* and *Galoncus perniciosus*, which infect leopards (*Panthera pardus*) and tigers (*Pant hera tigris*), respectively (Khalil, 1922a; Kalaivanan et al., 2015) have the particularity of feeding in the intestinal submucosa and muscularis where they form hemorrhagic nodules, which, once infected with enteric bacteria, can lead to sepsis and death (Khalil 1922a; Kalaivanan et al., 2015). *Tetragomphius melis* and *Tetragomphius arctonycis*, which infect the Japanese (*Meles anakuma*) and Hog (*Arctonyx collaris*) badgers respectively, cause significant inflammation, fibrosis, and "mass-like" lesions in the pancreatic duct (Jansen, 1968; Ashizawa et al., 1976; Matsuda et al.,

2015). Despite significant tissue alterations caused by these mustelid parasites, mortality due to severe infection has not been reported, and the effect of these hookworms at the population level is unknown. *Monodontella giraffae* causes cholangitis and peribiliary fibrosis in giraffes (Bertelsen et al. 2009), and in elephants *Grammocephalus spp* cause severe eosinophilic cholangitis (Allen et al. 1974, Debbie and Clausen 1975, Obanda et al. 2011).

3.1.4 Mortality

Mortality of wild animals due to hookworm infection is in most cases the final outcome of chronic anemia, retarded growth, tissue damage, and secondary bacterial infections. Mortality has been recorded most commonly in canids, felids, and otariids. In southern Texas coyote populations, the effect of *A. caninum* has been tested by experimental infection, where infective doses of over 300 stage 3 larvae/ Kg of *A. caninum* were lethal in pups (Radomsky 1989), and resulted in parasitic loads similar to those reported in naturally infected juvenile coyotes (range 50-150 nematodes) (Thornton and Reardon 1974), suggesting a potential role of *A. caninum* in pup mortality and limiting the expansion of coyote populations. Similarly, in the same region, grey foxes (*Urocyon cinereoargenteus*) were infected with high burdens of *A. caninum*, suggesting some level of population mortality ((Miller and Harkema 1968). In wolves, *A. caninum* and *U. stenocephala* have been associated with pup mortality; however, detailed assessment of parasite effects on mortality through controlled studies have not been performed (Kazacos and Dougherty 1979, Kreeger et al. 1990, Guberti et al. 1993). In Europe, some level of population mortality has been assumed in peri-urban red foxes infected with high burdens of *Uncinaria stenocephala* (Willingham et al. 1996). In Asia, *Arthrostoma miyazakiense* can cause sporadic mortality in raccoon dogs (Sato et al. 2006, Shin et al. 2007). In felines, beside the highly pathogenic *Galoncus spp.* affecting tigers and leopards, probably *A. caninum* and *A.*

pluridentatum cause some level of mortality in bobcats and cougars in the southeastern United States (Mitchel and Beasom 1974, Forrester et al. 1985, Dunmbar et al. 1994). In otariids, *Uncinaria spp*. can cause up to 70% mortality in some colonies of California sea lions (Spraker et al. 2007). In other species, such as New Zealand sea lions, northern fur seal, Australian sea lions and South American fur seals, hookworms cause between 15 to 50% of total pup mortality (Castinel et al. 2007a, Lyons et al 2011a, Seguel et al. 2013, Marcus et al. 2014). In other mammalian groups, reports of hookworms causing mortality are sporadic, as in the case of *Uncinaria sp* nematodes causing the death of a brown bear pup in Turkey (Kilinc et al. 2015), and the role of *Grammocephalus hybridatus* in the death of several young Asian elephants transported to a zoo (Rombolli et al. 1975). Hookworms were suspected to be the cause of death of a maroon langur (*Presbytis rubicunda*) in Asia, but the hookworm genus or species causing death was unknown (Hilser et al. 2014).

3.2 Drivers of hookworm pathologic effects

According to the data retrieved from the reviewed literature and the regression models performed, population level hookworm prevalence is the most significant predictor of the pathogenic effect of hookworms. The role of infection intensity on the severity of hookworm disease is reported in several studies; however, it was not a significant predictor in regression models. This could be due to natural study bias, reporting pathologic effects, as many of them provide good descriptions of mean infection intensity but little or no assessment of tissue damage and other pathologic effects.

There is a wide range of variation in prevalence and mean intensity of hookworm infections among wildlife populations; however, a common pattern of regional and local differences in prevalence is noted, usually attributable to contrasts in local temperature and soil

humidity, which are critical factors for survival of hookworm infective larvae in the soil (Ryan 1976, Yabsley and Noblet 1999, Criado-Fornelio et al. 2000, Gompper et al. 2003, Dybing et al. 2013). Additionally, hookworm species can differ in their resistance to environmental conditions, creating regional patterns of infection. Such is the case of canine hookworms since *A. caninum* is found in higher mean intensities in areas with mild climate like southeastern United States (Miller and Harkema 1968, Mitchel and Beasom 1974, Thornton and Reardon 1974, Schitoskey and Schitoskey 1980, Custer and Pence 1981) while *U. stenocephala* is usually reported in higher prevalence and intensity in canids inhabiting temperate or circumboreal areas (Willingham et al. 1996, Craig and Craig 2005, Reperant et al. 2007, Stuart et al. 2013). An additional factor associated with changes in prevalence and mean intensities is the spatial density of host animals. Higher population density is usually associated with higher prevalence, as this increases the number of infective larvae in the soil (Henke et al. 2002, Lyons et al. 2011a, Seguel et al. submitted). Beside environmental contrasts, intra-host dynamics of hookworm infection are probably important in explaining patterns of prevalence and burden. For instance, canid, felid, ursid and procyonid hookworms establish chronic infections, where significant immunity apparently does not occur, indicating that older animals have higher chances to be in contact with infective stages through their life and harbor higher numbers of parasites (Worley et al. 1976, Yabsley and Noblet 1999, Kresta et al 2009, Liccioli et al. 2012). In some canine populations, however, young animals are the most severely affected with *Ancylostoma* species, probably reflecting the role of lactogenic transmission in the disease dynamics (Custer and Pence 1981). In the case of pinnipeds, infective larvae reach pups only through their mothers' milk and adult hookworms are cleared from the pup's intestine 2 to 6 months after initial infection. This results

on a short life span for adult hookworms in pinnipeds and markedly seasonal prevalence (Lyons et al 2011a, Marcus et al. 2014, Seguel et al. in press, Seguel et al. submitted).

An additional element incorporated in the final model to explain pathologic effect was hookworm length, although the effect was not significant. There is evidence across the reviewed literature suggesting that larger hookworms are potentially more pathogenic, as the case of *Grammocephalus spp* in elephants, which are approximately 35 mm long (Obanda et al. 2011). Something similar occurs with pinniped *Uncinaria spp*., which are larger than their terrestrial relatives (Ramos et al. 2013, Nadler et al. 2014). For other groups of large hookworms, however, such as those within the Bunostominae subfamily, there is little evidence of pathologic effects in their wild ruminant host, although some of those species, such as *Bunostomum phlebotomum, Bunostomum trigonocephalum* and *Gaigeria pachyscelis*, are known to cause anemia, hemorrhagic enteritis and death in domestic ruminants (Hart and Wagner 1971).

The three discussed drivers of hookworm pathogenic effects; prevalence, burden and hookworm length can be considered indicators of hookworm biomass within a population. Since the pathogenic effects described are related to the extraction of host resources by the parasite, it can be inferred that any factor that increases the hookworm biomass within a population will also increase the detrimental effect of these parasites in this group.

Discussion and Conclusions

There are numerous hookworm species described in a wide range of wild mammals. Carnivores are over represented in the literature, and therefore most of the negative effects of hookworm infection have been described in this group. This does not necessarily imply that hookworms are important pathogens only in carnivores; for instance, there is enough evidence to

infer that hookworm infections are significant disease agents in ruminants and primates. These groups, however, are less represented and probably neglected regarding the study of hookworms. This could be in part due to the fact that most wild ruminants and primates live in areas of the planet traditionally underrepresented in terms of parasitology research (Falagas et al. 2006).

Several studies have highlighted the potential of carnivore hookworms to infect multiple species, including humans (Reviewed in Travesa 2012). The literature in wildlife species support these observations, as several human and domestic animal hookworms infect more than 10 different wildlife hosts in a wide range of taxonomic groups. In most cases, these hookworm species are capable of establishing complete life cycles and harm in their "non-native" hosts. This highlights the significance of domestic animal-human-wildlife interface for this disease and the potential for spillover and spillback processes playing an important role in the maintenance of high hookworm burdens in some areas.

A comprehensive understanding of the drivers of hookworm deleterious effects on wildlife hosts is complicated, given the likely strong bias towards the reporting only positive findings. In this sense, the literature available describes in which species and locations hookworms have an effect, but is insufficient to understand why these effects are observed in some species or populations and not in others. Evidence in the literature, however, indicates that hookworm biomass in a population and the host and environmental factors affecting biomass (e.g. environmental temperature, host density), are important in determining the outcome of hookworm infections. It is, however, likely that the importance of these elements change across populations. The role in disease dynamics of other hookworm-related traits, such as genetic variation and virulence factors, is less clear, and studies addressing these aspects are necessary to fully understand drivers of hookworm disease in wildlife.

The hookworms' effects on some populations of canines, felines and eared seals are of special concern, as several species in these families are endangered. Additionally, in all these groups, the dynamics of hookworm infections, and therefore the pathogenic effects, are linked to the density of susceptible animals and environmental variables such as humidity and temperature. These characteristics of wildlife hookworm infections, plus the generalist nature of these nematodes, creates a dynamic scenario where human-related disturbances of wildlife populations and climate change may potentially affect the dynamics and effects of hookworm infections in wildlife.

In the era of molecular pathogenesis, the study of hookworm disease is still very rudimentary in wildlife hosts, despite the fact that these pathogens impact wildlife, domestic animal, and human, health and wildlife conservation. The use of approaches other than opportunistic collection of carcasses, especially experimental and molecular when possible, could substantially improve our understanding of the impact and drivers of hookworm disease in wildlife populations.

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Figures

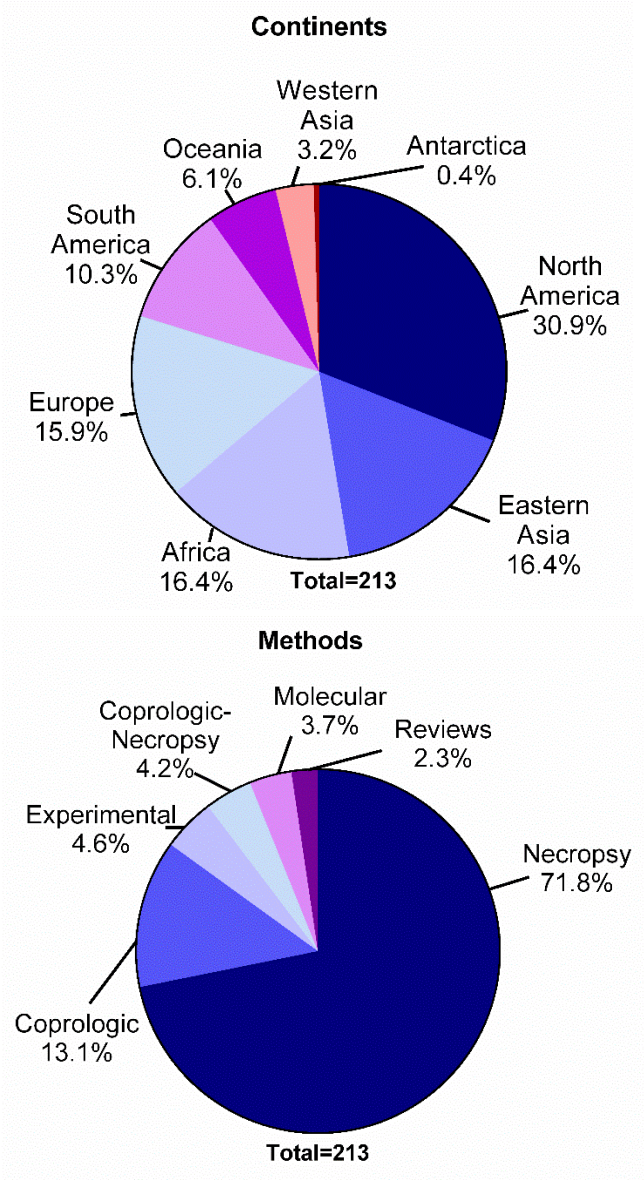
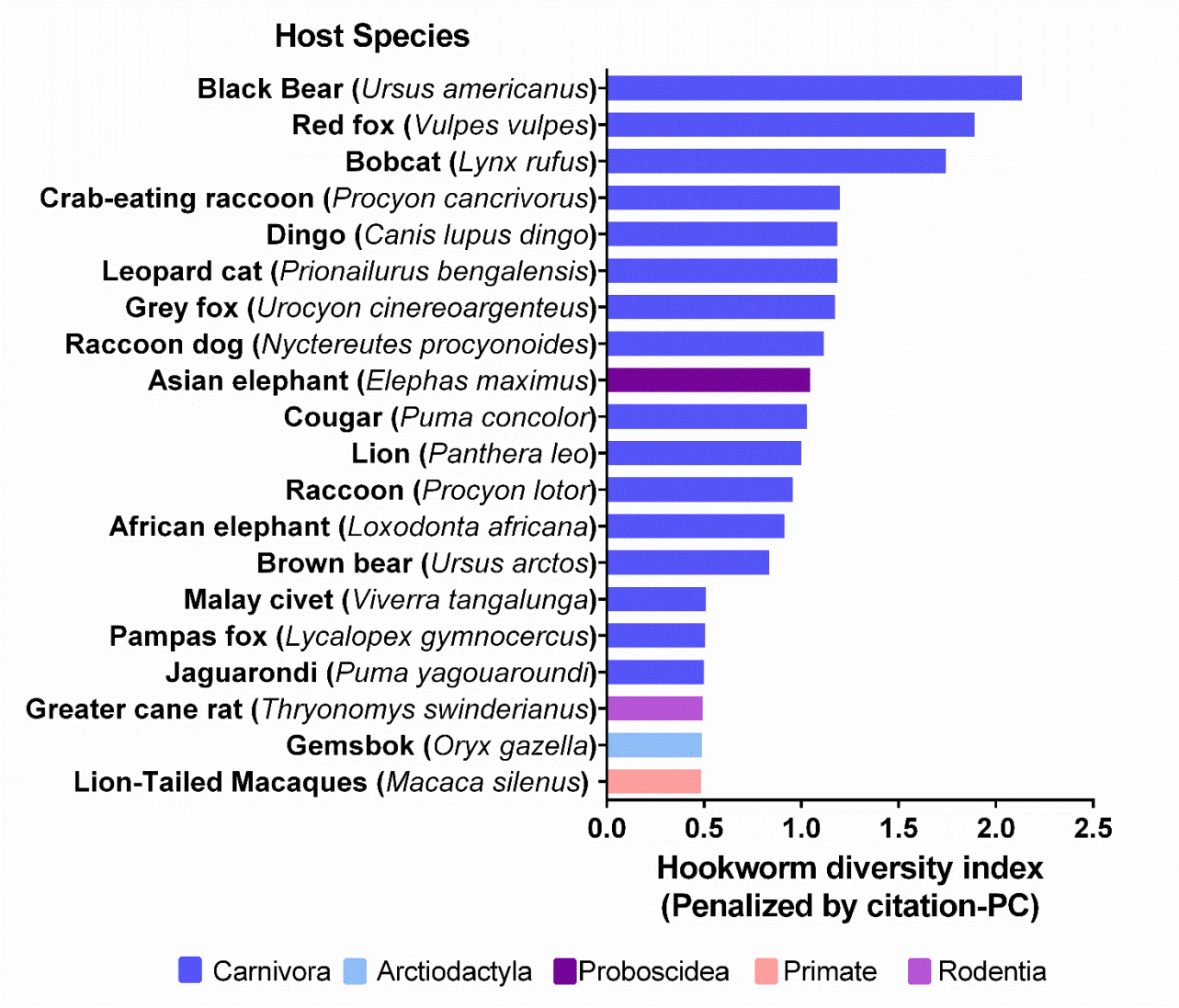


Figure 1.1. Percentage of studies describing hookworm infections in wildlife hosts divided by continent (a) and the main methodology used to collect the data (b).


Host Species
Black Bear (Ursus americanus)
Red fox (Vulpes vulpes)
Bobcat (Lynx rufus)
Crab-eating raccoon (Procyon cancrivorus)
Dingo (Canis lupus dingo)
Leopard cat (Prionailurus bengalensis)
Grey fox (Urocyon cinereoargenteus)
Raccoon dog (Nyctereutes procyonoides)
Asian elephant (Elephas maximus)
Cougar (Puma concolor)
Lion (Panthera leo)
Raccoon (Procyon lotor)
African elephant (Loxodonta africana)
Brown bear (Ursus arctos)
Malay civet (Viverra tangalunga)
Pampas fox (Lycalopex gymnocercus)
Jaguarondi (Puma yagouaroundi)
Greater cane rat (Thryonomys swinderianus)
Gemsbok (Oryx gazella)
Lion-Tailed Macaques (Macaca silenus)
0.0
0.5
1.0
1.5
2.0
2.5
Hookworm diversity index
(Penalized by citation-PC)
Carnivora
Arctiodactyla
Proboscidea
Primate
Rodentia

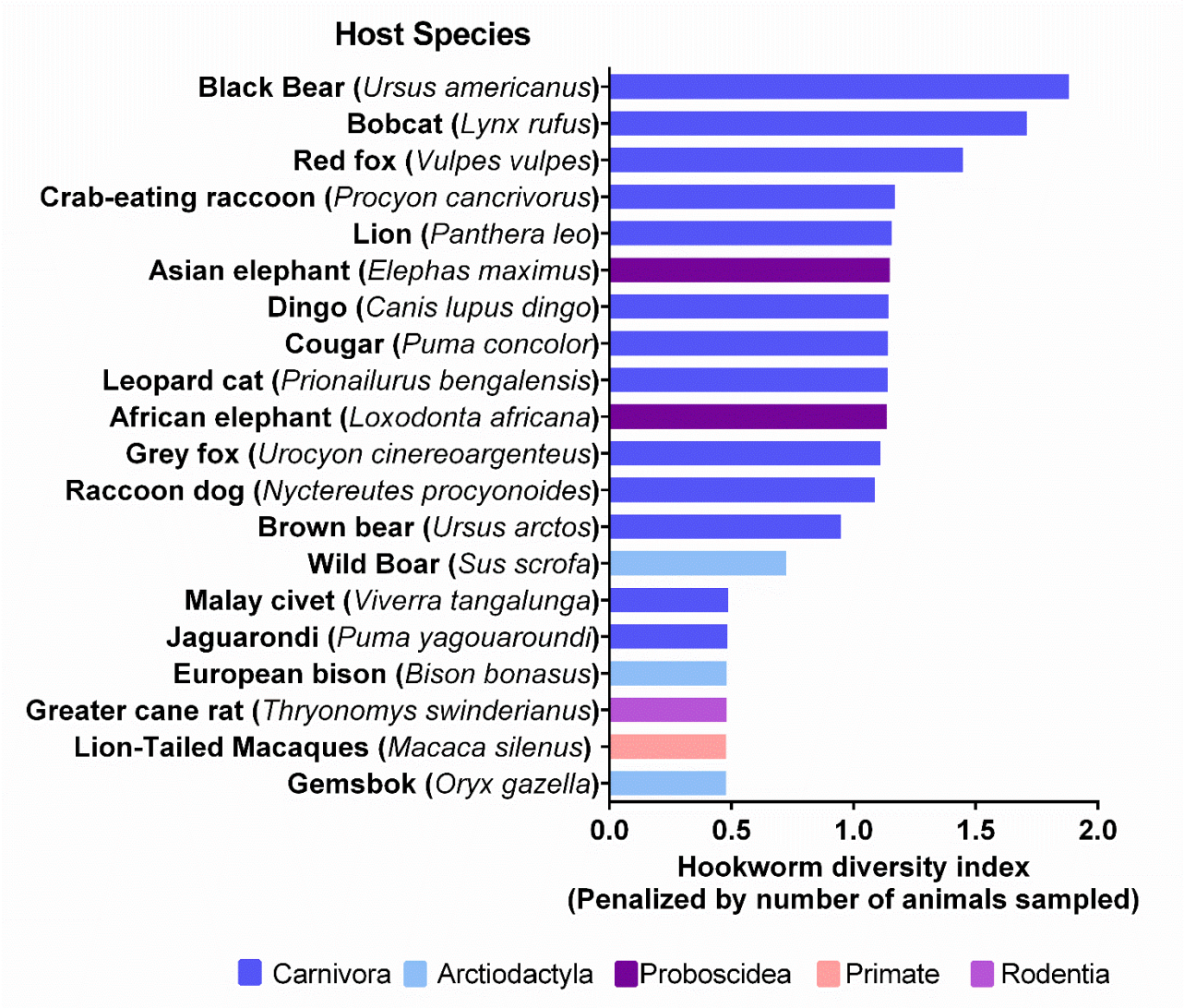
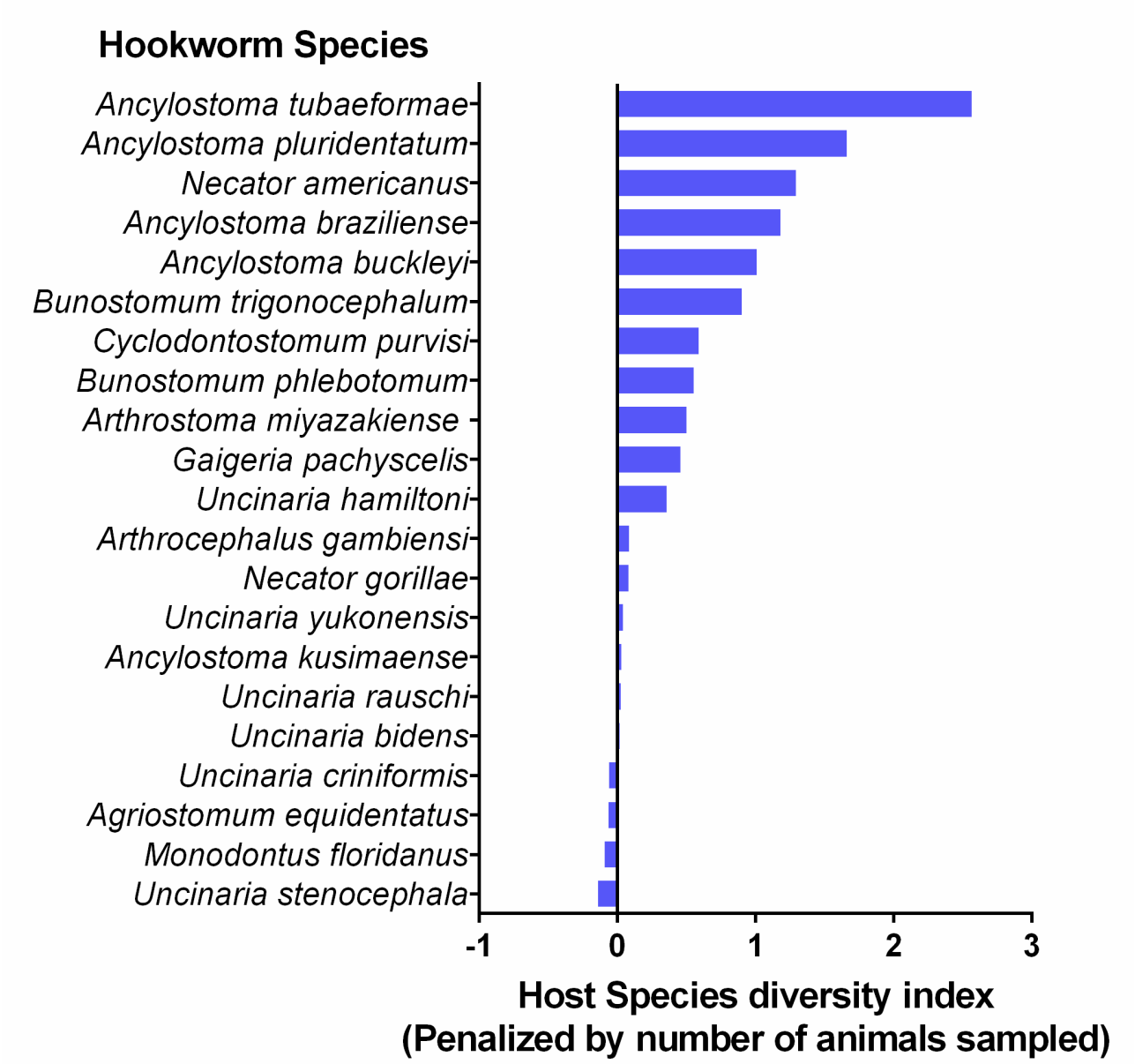


Figure 1.2. Hookworm diversity index penalized by the citation principal component (citation-PC) of the host species (a) and the number of sampled animals of each host species (b). Bar colors indicate represented mammalian orders.


Hookworm Species
Ancylostoma tubaeformae
Ancylostoma pluridentatum
Necator americanus
Ancylostoma braziliense
Ancylostoma buckleyi
Bunostomum trigonocephalum
Cyclodontostomum purvisi
Bunostomum phlebotomum
Arthrostoma miyazakiense
Gaigeria pachyscelis
Uncinaria hamiltoni
Arthrocephalus gambiensi
Necator gorillae
Uncinaria yukonensis
Ancylostoma kusimaense
Uncinaria rauschi
Uncinaria bidens
Uncinaria criniformis
Agriostomum equidentatus
Monodontus floridanus
Uncinaria stenocephala
-1
0
1
2
3
Host Species diversity index
(Penalized by number of animals sampled)

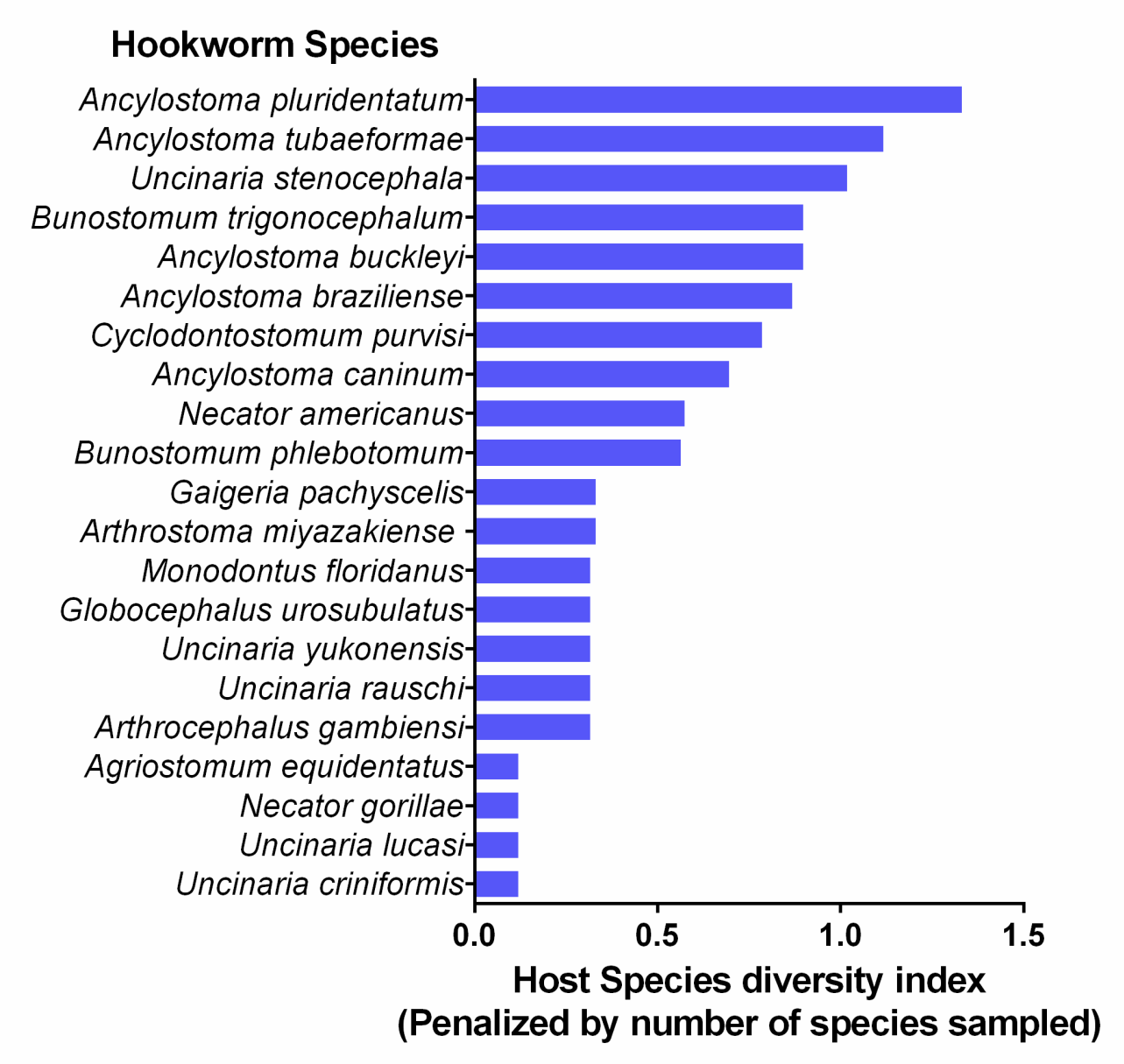


Figure 1.3. Host species diversity index for represented hookworm species, penalized by number of animals sampled (a) and the number of animal species sampled in each study (b).

Tables

Table 1.1. Canine hosts infected with hookworms with the corresponding references.

Host Species	Hookworm species	References
Coyote (*Canis latrans*)	*Ancylostoma caninum*	Erickson 1944, Ameel 1955, Mitchel and Beasom 1974, Thornton and Reardon 1974, Schitoskey 1980, Conti 1984, Pence et al. 1988, Radomsky 1989, Guberti et al. 1993, Henke et al. 2002, Foster et al. 2003, Manning 2007, Liccioli et al. 2012
	Uncinaria stenocephala	Schitoskey 1980, Gompper et al 2003, Bridger et al 2009, Manning 2007, Liccioli et al. 2012, Huffman et al. 2013
Red fox (*Vulpes vulpes)*	*Ancylostoma caninum*	Smith 1943, Ryan 1976, Conti 1984, Dalimi et al. 2006, Alagaili et al. 2011, Ubelaker et al. 2013
	Ancylostoma tubaeformae	Conti 1984
	Ancylostoma kusimaense	Kamiya and Ohbayashi 1975, Sato et al. 1999
	Arthrostoma miyazakiense	Noda and Kudi 1980, Sato et al. 1999
	Uncinaria stenocephala	Erickson 1944, Miller and Harkema 1968, Kamiya and Ohbayashi 1975, Ryan 1976, Hackett and Walters 1980, Loos-Frank and Zeyhle 1982, Willingham et al 1996, Criado-Fornelio et al. 2000, Reperant et al. 2007, Cerbo et al. 2008, Al-Sabi et al. 2013, Dybing et al. 2013, Huffman et al. 2013, Stuart et al. 2013, Lahmar et al. 2014, Razmjoo et al. 2014
	Uncinaria sp.	Borecka et al. 2013
Wolf (*Canis lupus*)	*Ancylostoma caninum*	Kazacos and Dougherty 1979, Kreeger et al 1990, Guberti et al 1993, Torres et al. 1997
	Ancylostoma spp	Borecka et al. 2013
	Uncinaria stenocephala	Erickson 1944, Guberti et al 1993, Torres et al. 1997, Craig and Craig 2005
Crab-eating fox (*Cerdocyon thous*)	*Ancylostoma buckleyi*	Dos Santos et al. 2003
	Uncinaria carinii	Duarte 2016

Arctic fox (*Alopex lagopus*)	*Uncinaria stenocephala*	Aguirre et al. 2000
Darwin's fox (*Pseudalopex fulvipes)*	*Uncinaria stenocephala*	Jimenez et al. 2012
Dingo (*Canis lupus dingo*)	*Ancylostoma caninum*	Smout et al 2013
	Ancylostoma ceylanicum	Smout et al 2013
	Ancylostoma braziliense	Smout et al 2013
Golden jackal (*Canis aureus*)	*Ancylostoma caninum*	Sadighian 1969, Takács et al. 2013, Lahmar et al. 2014
	Uncinaria stenocephala	Sadighian 1969, Takács et al. 2013, Lahmar et al. 2014
Grey fox (*Urocyon cinereoargenteus*)	*Ancylostoma caninum*	Miller and Harkema 1968, Conti 1984, Buechner 1944
	Ancylostoma braziliense	Conti 1984, Buechner 1944
	Ancylostoma tubaeformae	Conti 1984
Pampas fox (*Lycalopex gymnocercus*)	*Ancylostoma buckleyi*	Scioscia et al. 2016
	Uncinaria sp.	Fiorello et al. 2006
Raccoon dog (*Nyctereutes procyonoides*)	*Uncinaria stenocephala*	Al-Sabi et al. 2013
	Ancylostoma kusimaense	Noda and Kudi 1980, Sato et al. 1999, Sato et al. 2006
	Arthrostoma miyazakiense	Noda and Kudi 1980, Sato et al. 1999, Sato et al. 2006, Shin et al. 2007
Red Wolf (*Canis rufus*)	*Ancylostoma caninum*	Custer and Pence 1981, Philips and Scheck 1991
Short-eared fox (*Atelocynus microtis*)	*Ancylostoma buckleyi*	Thatcher 1971
South American grey fox (*Lycalopex griseus*)	*Uncinaria stenocephala*	Alarcon 2005

Swift fox (*Vulpes velox*)	*Uncinaria stenocephala*	Miller et al. 1998

Table 1.2. Feline hosts infected with hookworms and corresponding references.

Host Species	**Hookworm species**	**References**
Bobcat (*Lynx rufus*)	*Ancylostoma caninum*	Miller and Harkema 1968, Little et al. 1971, Mitchel and Beasom 1974, Schitoskey and Linder 1981, McLaughlin et al 1993, Hiestand et al. 2014
	Ancylostoma braziliense	Miller and Harkema 1968, McLaughlin et al 1993
	Ancylostoma tubaeformae	Tiekotter 1985, McLaughlin et al 1993
	Ancylostoma pluridentatum	McLaughlin et al 1993
Iriomote cats (*Prionailurus iriomotensis*)	*Uncinaria maya*	Hasegawa 1989, Yasuda et al. 1994
Bengal tiger (*Panthera tigris*)	*Galoncus perniciosus*	Kalaivanan et al 2015
Leopard *(Panthera pardus*)	*Galoncus tridentatus*	Khalil 1922a, Pythal et al. 1993
Canadian lynx (*Lynx*	*Ancylostoma*	Smith et al. 1986

canadensis)	*caninum*	
	Uncinaria stenocephala	Smith et al. 1986
Cougar (*Puma concolor*)	*Ancylostoma tubaeformae*	Waid and Pence 1988
	Ancylostoma pluridentatum	Forrester et al. 1985, Dunbar et al 1994
	Ancylostoma buckleyi	Thatcher 1971
Geoffroy's cat (*Leopardus geoffroyi*)	*Ancylostoma tubaeformae*	Beldomenico et al. 2005, Fiorello et al. 2006
Iberian Lynx (*Lynx pardinus*)	*Ancylostoma spp*	Vicente et al 2004
	Ancylostoma tubaeformae	Millan and Blasco-Costa 2012
Jaguar (*Felis onca*)	*Ancylostoma pluridentatum*	Thatcher 1971
Jaguarondi (*Puma yagouaroundi*)	*Ancylostoma tubaeformae*	Thatcher 1971
	Ancylostoma pluridentatum	Thatcher 1971
Leopard	*Ancylostoma*	Yasuda et al 1993

cat (*Prionailurus bengalensis*)	*tubaeformae*	
	Arthrostoma hunanensis	Yasuda et al. 1993
	Uncinaria felidis	Yasuda et al. 1993, Yasuda et al. 1994, Shimono et al. 2012
Lion (*Panthera leo*)	*Uncinaria stenocephala*	Smith and Kok 2006
	Ancylostoma braziliense	Smith and Kok 2006
	Ancylostoma paraduodenale	Bjork et al. 2000
	Ancylostoma spp	Muller-Graf 1995, Bjork et al. 2000
Margay cat (*Leopardus wiedii*)	*Ancylostoma pluridentatum*	Thatcher 1971
Ocelot (*Leopardus pardalis*)	*Ancylostoma tubaeformae*	Pence et al 2003, Fiorello et al. 2006
	Ancylostoma pluridentatum	Thatcher 1971
	Uncinaria sp.	Fiorello et al. 2006

Table 1.3. Eared seals (otariids) infected with hookworms and corresponding references.

Host Species	Hookworm species	References
Australian fur seal (*Arctocephalus pusillus doriferus*)	*Uncinaria hamiltoni*	Ramos et al 2013
Australian sea lion (*Neophoca cinerea*)	*Uncinaria sanguinis*	Haynes et al 2014, Marcus et al 2014a, Marcus et al. 2014b, Marcus et al. 2015a, Marcus et al. 2015b
California Sea Lion (*Zalophus californianus*)	*Uncinaria lyonsi*	Lyons et al. 2000, Lyons et al. 2001, Lyons et al. 2005, Acevedo-Whitehouse et al 2006, Spraker et al. 2007, Lyons et al. 2011b, Kuzmina and Kuzmin 2015
Galapagos sea lion (*Zalophus wollebaeki*)	*Uncinaria sp*	Herbert 2014
Juan Fernandez fur seal (*Arctocephalus phillippii*)	*Uncinaria sp*	Sepulveda et al 1998
New Zealand fur seal (*Arctocephalus forsteri*)	*Uncinaria sp*	Ramos et al 2013
New Zealand sea lion (*Phocarctos hookeri*)	*Uncinaria sp*	Castinel et al 2006, Castinel et al 2007a, Castinel et al. 2007b, Acevedo-Whitehouse et al. 2009, Chilvers et al. 2009, Michael et al. 2015
Northern fur seal (*Callorrhinus ursinus*)	*Uncinaria lucasi*	Olsen and Lyons 1965, Lyons et al. 1978, Lyons et al 1997, Lyons et al. 2000, Lyons et al. 2001, Lyons et al 2003, DeLong et al. 2009, Lyons et al 2011a, Lyons et al. 2011b, Lyons et al. 2014
South American fur seal (*Arctocephalus australis*)	*Uncinaria hamiltoni*	Katz et al. 2013, Nadler et al. 2013
	Uncinaria sp	Seguel et al. 2011, Seguel et al. 2013, Seguel et al. in press

South American sea lion (*Otaria flavescens*)	*Uncinaria hamiltoni*	Beron-vera et al 2004
Steller sea lion (*Eumatopias jubatus*)	*Uncinaria lucasi*	Lyons et al 2003, Nadler et al. 2013

Table 1.4. Procyonids infected with hookworms and corresponding references.

Host Species	**Hookworm species**	**References**
Crab-eating raccoon (Procyon cancrivorus)	*Necator urichi*	Cameron 1936
	Uncinaria maxillaris	Vicente et al. 1997
	Uncinaria bidens	Vicente et al. 1997
Coati (Nasua nasua)	*Uncinaria bidens*	Duarte 2016
Raccoon (Procyon lotor)	*Arthrocephalus lotoris*	Dikmans and Goldberg 1949, Jordan and Hayes 1959, Gupta 1961, Balasingam 1964, Snyder and Fitzgerald 1985, Cole and Shoop 1987, Yabsley and Noblet 1999, Ching et al. 2000, Kresta et al 2009
	Ancylostoma spp	Popiolek et al. 2011
	Ancylostoma kusimaense	Matoba et al. 2006, Sato and Suzuki 2006
	Arthrostoma miyazakiense	Sato and Suzuki 2006

Table 1.5. Mustelids infected with hookworms and corresponding references.

Host Species	**Hookworm species**	**References**
Badger (*Meles meles*)	*Uncinaria criniformis*	Loos-Frank and Zeyhle 1982, Magi et al. 1999, Torres et al. 2001, Millan et al. 2004, Rosalino et al. 2006, Cerbo et al. 2008, Stuart et al. 2013

	Tetragomphius procyonis	Son et al. 2009
Hog badger (*Arctonyx collaris*)	*Tetragomphius arctonycis*	Jansen 1968
Japanese badger (*Meles anakuma*)	*Tetragomphius melis*	Ohbayashi et al. 1974, Ashizawa et al. 1976, Matsuda et al. 2015
Pine martens (*Martes martes*)	*Uncinaria criniformis*	Segovia et al. 2007
	Uncinaria sp.	Borecka et al. 2013
	Ancylostoma spp	Borecka et al. 2013

Table 1.6. Bear species (ursids) infected with hookworms and corresponding references.

Host Species	**Hookworm species**	**References**
Black Bear (*Ursus americanus*)	*Ancylostoma caninum*	Foster et al. 2011, Crum et al. 1978
	Ancylostoma tubaeformae	Foster et al. 2011
	Arthrocephalus lotoris	Crum et al. 1978
	Uncinaria rauschi	Olsen 1968, Catalano et al. 2015a, Catalano et al. 2015b
	Uncinaria yukonensis	Frechette and Rau 1977
	Uncinaria sp.	Worley et al 1976
Brown bear (*Ursus arctos*)	*Ancylostoma malayanum*	Asakawa et al. 2006
	Uncinaria rauschi	Olsen 1968
	Uncinaria yukonensis	Choquette et al 1969, Rausch et al. 1979, Catalano et al. 2015a, Catalano et al. 2015b
	Uncinaria sp.	Greer 1972, Worley et al. 1976, Kilinc et al. 2015

Table 1.7. Members of the Mephtididae, Herpestidae, Phocidae, Hyenidae and Viverridae families on which hookworms have been described and the corresponding references.

Host Species	Hookworm species	References
Palawan stink badger (*Mydaus marchei*)	*Arthrostoma vampira*	Schmidtz and Kuntz 1968
Andean hog-nosed skunk (*Conepatus chinga*)	*Ancylostoma conepati*	Ibanez 1968
Skunk (*Mephitis nigra*)	*Arthrocephalus lotoris*	Dikmans and Goldberg 1949
Gambian mongoose (*Mungos gambianus*)	*Arthrocephalus gambiensi*	Ortlepp 1925
Crab-eating mongoose (*Herpestes urva*)	*Arthrocephalus gambiensi*	Myers and Kuntz 1964
Small Asian mongoose (*Herpestes javanicus*)	*Uncinaria sp.*	Ishibashi et al. 2010
Southern elephant seal (*Mirounga leonina*)	*Uncinaria sp*	Ramos et al 2013
Mediterranean monk seal (*Monachus monachus*)	*Uncinaria sp*	Nadler et al. 2013
Malay civet (*Viverra tangalunga*)	*Ancylostoma ceylanicum*	Colon and Patton 2012
	Ancylostoma sp.	Colon and Patton 2012
Spotted hyena (*Crocuta crocuta*)	*Ancylostoma duodenale*	Graber and Blanc 1979
	Ancylostoma spp	Engh et al. 2003

Table 1.8. Hookworm species described in members of the Bovidae family with corresponding references.

Host Species	Hookworm species	References
Kudu (Tragelaphus strepsiceros)	*Agriostomum gorgonis*	Boomker et al. 1989, Van Wyk and Boomker 2011
African buffalo (Syncerus caffer)	*Bunostomum sp.*	Ocaido et al. 2004, Senyael et al. 2013
Blue wildebeest (Connochaetes taurinus)	*Agriostomum gorgonis*	Van Wyk and Boomker 2011
	Gaigeria pachyscelis	Horak et al. 1983
Common tsessebe (Damaliscus lunatus)	*Agriostomum cursoni*	Mönnig 1932
Common reedbuck (Redunca arundinum)	*Gaigeria sp.*	Boomker et al. 1989
Gemsbok (Oryx gazella)	*Agriostomum monnigi*	Ogden 1965
	Agriostomum equidentatus	Fourie et al. 1991
European bison (Bison bonasus)	*Bunostomum phlebotomum*	Karbowiak et al. 2014
	Bunostomum trigonocephalum	Karbowiak et al. 2014
Nyala (Tragelaphus angasii)	*Gaigeria pachyscelis*	Boomker et al. 1991
Impala (Aepyceros melampus)	*Gaigeria pachyscelis*	Anderson 1978
	Bunostomum sp.	Ocaido et al. 2004
Iberian ibex (Capra pyrenaica)	*Bunostomum trigonocephalum*	Perez et al. 1996
Springbok (Antidorcas marsupialis)	*Agriostomum equidentatus*	Young et al. 1973, Horak et al. 1982, De Villiers et al. 1985

Lechwe (Kobus leche)	*Bunostomum sp.*	Phiri et al. 2011
Waterbuck (Kobus ellipsiprymnus)	*Bunostomum sp.*	Ocaido et al. 2004

Table 1.9. Hookworm species described in members of the Bovidae family with corresponding references.

Host Species	Hookworm species	References
Wild Boar (Sus scrofa)	*Globocephalus urosubulatus*	Coombs and Springer 1974, Eslami and Farsad-Hamdi 1992, Rajković-Janje et al. 2002, Fernandez-De-Mera et al. 2004, Foata et al. 2005, Foata et al. 2006, Nanev et al. 2007, Senlik et al. 2011, Gasso et al. 2015
	Globocephalus samoensis	Kagei et al. 1984, Sato et al. 2008, Ahn et al. 2015
	Globocephalus longimucronatus	Kagei et al. 1984, Sato et al. 2008
Bushpig (Potamochoerus porcus)	*Globocephalus versteri*	Van Wyk and Boomker 2011
Pecari (Pecari tajacu)	*Globocephalus urosubulatus*	Romero-Castanon et al. 2008

Table 1.10. Hookworm species described in members of the Cervidae and Giraffidae families with corresponding references.

Host Species	Hookworm species	References
Fallow deer (Dama dama)	*Bunostomum phlebotomum*	Omegaric et al. 2011
	Bunostomum trigonocephalum	Pav et al. 1975
Red deer (Cervus elaphus)	*Bunostomum trigonocephalum*	Zalewska-Schonthaler and Szpakiewicz 1987

Roe deer (Capreolus capreolus)	*Bunostomum trigonocephalum*	Demiaszkiewicz et al. 2002
White-tailed deer (Odocoileus virginianus)	*Monodontus lousianensis*	Chitwood and Jordan 1965
Reeves's muntjac (Muntiacus reevesi)	*Bunostomum phlebotomum*	Myers and Kuntz 1964
Giraffe (Giraffa camelopardalis)	*Monodontella giraffae*	Bertelsen et al 2009, Ming et al 2010

Table 1.11. Primate species infected with hookworms with corresponding references.

Host Species	**Hookworm species**	**References**
Lion-tailed macaques (Macaca silenus)	*Ancylostoma spp*	Hussain et al. 2013
	Bunostomum sp.	Hussain et al. 2013
Moustached guenon (Cercopithecus cephus)	*Ancylostoma spp*	Pourrut et al. 2011
Mona monkey (Cercopithecus mona)	*Ancylostoma spp*	Pourrut et al. 2011
De Brazza's monkey (Cercopithecus neglectus)	*Ancylostoma spp*	Pourrut et al. 2011
Greater spot-nosed monkey (Cercopithecus nictitans)	*Ancylostoma spp*	Pourrut et al. 2011
Crested mona monkey (Cercopithecus pogonias)	*Ancylostoma spp*	Pourrut et al. 2011

Agile mangabey (Cercocebus agilis)	*Ancylostoma spp*	Pourrut et al. 2011
Mantled guereza (Colobus guereza)	*Ancylostoma spp*	Pourrut et al. 2011
Gabon talapoin (Miopithecus ogoouensis)	*Ancylostoma spp*	Pourrut et al. 2011
Brown woolly monkeys (*Lagothrix lagothricha*)	*Ancylostoma sp*	Michaud et al. 2003
Vervet monkey (*Chlorocebus pygerythrus*)	*Necator sp.*	Gillespie et al. 2004
Western lowland gorillas (Gorilla gorilla gorilla)	*Necator americanus*	Hasegawa et al 2014
	Necator gorillae	Noda and Yamada 1964
Chimpanzees (Pan troglodytes)	*Necator americanus*	Hasegawa et al 2014
	Ancylostoma spp	Pourrut et al. 2011
Bald uakari (*Cacajao calvus*)	*Necator americanus*	Michaud et al. 2003
Baboons (Papio hamadryas)	*Necator sp*	Howells et al. 2011
Javan slow loris (Nycticebus javanicus)	*Necator sp*	Albers 2014, Rode-Margono et al. 2015

Table 1.12. Rodent species infected with hookworms with corresponding references.

Host Species	**Hookworm species**	**References**
Australian water rat (Hydromys chrysogaster)	*Uncinaria hydromyidis*	Beveridge 1980, Smales and Cribb 1997

Long-tailed giant rat (Leopoldamys sabanus)	*Cyclodontostomum purvisi*	Balasingam 1963
Müller's giant Sunda rat (Sundamys muelleri)	*Cyclodontostomum purvisi*	Balasingam 1963
Bower's white-toothed rat (Berylmys bowersi)	*Cyclodontostomum purvisi*	Balasingam 1963
Greater cane rat (Thryonomys swinderianus)	*Acheilostoma simpsoni*	Kankam et al. 2009
	Acheilostoma moucheti	Popova 1964
Cotton rat (Sigmodon hispidus)	*Monodontus floridanus*	McIntosh 1935
Round-tailed muskrat (Neofiber alleni)	*Monodontus floridanus*	Forrester et al. 1987
Red-rumped agouti (Dasyprocta leporina)	*Monodontus aguiari*	Travassos 1937
Brazilian spiny rat (Mesomys sp)	*Monodontus rarus*	Travassos 1929

Table 1.13. Mammalian species in the Perissodactyla, Proboscidea, Pholidota, Afrosoricida and Scandentia orders affected by hookworms.

Host Species	**Hookworm species**	**References**
South American tapir (*Tapirus terrestris*)	*Monodontus nefastus*	Travassos 1937
Malayan tapir (*Tapirus indicus*)	*Brachyclonus indicus*	Khalil 1922b

Black Rhinoceros (*Rhinoceros bicornis*)	*Grammocephalus intermedius*	Neveu-lemaire 1924
African elephant (*Loxodonta africana*)	*Bunostomum brevispiculum*	Monnig 1925
	Bunostomum hamatum	Monnig 1925
	Grammocephalus clathratus	Allen et al. 1974, Obanda et al. 2011
	Grammocephalus sp	Debbie and Clausen 1975
Asian elephant (*Elephas maximus*)	*Grammocephalus hybridatus*	Romboli et al. 1975
Asian elephant (*Elephas maximus)*	*Grammocephalus varedatus*	Van Der Westhuysen 1938
Asian elephant (*Elephas maximus*)	*Bathmostomum sangeri*	Setasuban 1976
Chinese pangolin (*Manis pentadactyla*)	*Necator americanus*	Cameron and Myers 1960, Myers and Kuntz 1964
Indian pangolin (*Manis crassicaudata*)	*Necator americanus*	Mohapatra et al. 2015
Greater hedgehog tenrec (*Setifer setosus*)	Uncinaria bauchoti	Chabaud et al. 1964
Treeshrew (*Tupaia sp*)	Uncinaria olseni	Chabaud et al. 1974

CHAPTER 2

HOOKWORM INFECTION IN SOUTH AMERICAN FUR SEAL (*ARCTOCEPHALUS AUSTRALIS*) PUPS: PATHOLOGY AND FACTORS ASSOCIATED WITH HOST TISSUE DAMAGE AND MORTALITY.[2]

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[2]Seguel M., F. Muñoz, M.J. Navarrete, E. Paredes, E. Howerth, N. Gottdenker. 2017. *Veterinary Pathology*. 54:288-297.

Abstract

Tissues of South American fur seal pups naturally infected with hookworms (*Uncinaria sp)* were examined. Hookworm infection was found in nearly all pups examined (132/140, 94%) and hookworm enteritis with secondary bacteremia was considered the cause of death in 46 (35%) pups. Common findings in these pups included severe hemorrhagic enteritis and numerous (mean intensity=761.8) hookworms in the jejunum. Hookworms were recovered from the abdominal cavity in 12 of 55 pups (22%) examined through peritoneal wash; these pups had an average of 1343.3 intestinal hookworms and marked fibrinohemorrhagic peritonitis. In all pups that died as consequence of hookworm infection, the intestinal villi were short, blunt, fused, and there were variable numbers of free and intrahistiocytic Gram-negative bacteria in submucosal hookworm feeding tracks, mesenteric lymph nodes, spleen, blood vessels and liver sinusoids. Pups that died of causes unrelated to the hookworm infection (trauma) had hookworm feeding tracks confined to the apical portions of the mucosa, and moderate to marked catarrhal eosinophilic enteritis. The number of hookworms was negatively correlated with intestinal villous length and number of leukocytes in the intestine. Pups with hookworm peritoneal penetration had nematodes with little or no blood in the hookworm intestine, suggesting that lack of food for the nematode could be associated with peritoneal penetration. Findings suggest that the initial burden of larval infection, the level of the host tissue response, or a combination of the above determine the number of nematodes in the intestine, the severity of hookworm tissue damage, and pup mortality.

Introduction

Hookworms are highly pathogenic nematodes that parasitize a wide range of mammals including most species of otariid seals (Lyons et al. 2011). In all hosts, adult hookworms live in the small intestine, attach to the intestinal mucosa, and bite small portions of the epithelium and lamina propria, sucking blood from intestinal wounds (Loukas et al. 2010). This feeding behavior leads to chronic mucosal bleeding, in part due to the several anticoagulant proteins that the parasite secretes in the attachment site, resulting in hemorrhagic enteritis and chronic anemia that is characteristic of hookworm infection in most host species (Loukas et al. 2010). Experimental models show that pathogenic effects of hookworm infection depend on the species of hookworm, the host inflammatory response, and the number of nematodes in the intestine (Ishikawa et al. 1994).

Hookworms have been described in 13 of 15 extant otariids and in 3 phocid species (Ramos et al. 2013). However, the taxonomy of marine mammal hookworms is probably incomplete, since to date there are only four fully described species: *Uncinaria hamiltoni* in the South American sea lion (*Otaria flavescens*) (Nadler et al. 2013) *Uncinaria lucasi* in the Northern fur seal (*Callorhinus ursinus*) (Lyons et al. 2011), *Uncinaria lyonsi* in California sea lions (*Zalophus californianus*) (Kuzmina et al. 2015) and *Uncinaria sanguinis* in the Australian sea lion (*Neophoca cinerea*) (Marcus et al. 2014). Although all hookworms described in pinnipeds belong to the genus *Uncinaria*, there are significant morphological and genetic differences among hookworms of different otariids (Nadler et al 2013, Ramos et al. 2013).

The hookworm life cycle is similar in the 3 otariid species that have been investigated (Castinel et al. 2007a, Lyons et al. 2011b, Marcus et al. 2015). Neonates become infected most likely through the colostrum, and the prepatent period is between 12 and 18 days. Pups release

embryonated eggs in the feces that hatch in the soil and larvate to the infective L3 stage which penetrates the skin of most animals in the rookery. These larvae remain in subcutaneous tissues and mammary glands of females until the next reproductive cycle when they are passed to the next generation of pups (Lyons et al. 2011b). Contrary to other species of hookworms, otariid uncinarias apparently do not migrate within the host to reach the intestine and the lactogenic route is the main, and probably the only, form of transmission to the pups (Castinel et al. 2007a, Lyons et al. 1997, 2001, 2011b, Marcus et al. 2014).

Although most otariid species harbor hookworms, they can be an important cause of disease and mortality of pups in populations of Northern fur seals (Lyons et al. 1997, 2001, 2011b), California sea lions (Spraker et al. 2007), New Zealand sea lions (*Phocarctos hookeri*) (Castinel et al. 2007b), Australian sea lions (Marcus et al. 2015) and South American fur seals (*Arctocephalus australis*) (Seguel et al. 2013). The most detailed description of pathological findings and proposed pathogenesis of hookworm disease in marine mammals is of California sea lion pups (Spraker et al. 2007). This study described a hookworm enteritis and bacteremia syndrome as the cause of death of up to 70% of pups on San Miguel Island. This syndrome is characterized by severe hemorrhagic enteritis with presence of numerous free and deeply attached intestinal nematodes associated with multiple 2-4 mm diameter mucosal feeding sites and rare adult nematodes free in the abdominal cavity. Additionally, these pups had secondary colonization of several bacterial species in blood vessels of multiple organs, leading to sepsis and death. A similar syndrome with peritoneal penetration by hookworms has been observed in the same location in Northern fur seal pups (Lyons et al. 2011a). However, in all these populations with high prevalence and mortality from hookworm disease, it is unknown if peritoneal infection is associated with specific nematode characteristics and/or host-specific factors.

In South American fur seals (SAFS), we have previously described the main pathological findings and causes of pup death (Seguel et al. 2011, 2013). In these studies, we registered hookworm-related disease as the cause of up to 50% of the total pup mortality in some breeding seasons. In contrast to our findings, hookworm infection in SAFS rookeries in Uruguay is of low prevalence (< 5%) and has not been implicated in mortality based on gross postmortem examinations (Katz et al. 2012). However, preliminary data indicate that SAFS in Uruguay are infected with *Uncinaria hamiltoni* while SAFS at Guafo Island are infected with a larger species of *Uncinaria sp.* morphologically and molecularly related to the uncinaria species described in Australian fur seals (*Arctocephalus pusillus*) (Ramos et al. 2013) (Seguel et al. unpublished data). The aim of this study was to 1) characterize lesions associated with hookworm infection in SAFS pups and 2) elucidate the role of nematode burden and host tissue response in the development of hookworm disease and peritoneal penetration.

Materials and Methods

a) Animals

Necropsies were performed on 140 SAFS pups that were found dead on Guafo Island, Chilean Patagonia (43° 36'S y 74° 43'W), between December 28th and March 10th during the 2004 to 2008 and 2012 to 2015 breeding seasons. Histopathology was performed in 112 animals, and in 55 of these pups, peritoneal washing and bone marrow evaluation was also performed. These cases were selected based on minimal or mild postmortem autolysis and no signs of scavenging. The sex, rookery sector, total body length and weight was recorded for all but 2 pups. Pups were between 2 to 11 weeks old, based on parturition peak date for the Guafo Island rookery (December 15) (Paves and Schlatter 2008). A body mass index for the pups was

calculated by dividing the weight (in kilograms) by the total length (centimeters) (Seguel et al. 2013). After excluding animals that did not have a peritoneal wash, and those that had incomplete samples for histology, inadequate intestinal fixation or mild to moderate autolysis at histopathology, a total of 45 animals were included in the histologic statistical analyses. To test the significance of tissue changes in animals with different outcomes of hookworm infection, pups were divided in three groups; the first included pups that were considered to have died as consequence of hookworm infection (termed hookworm enteritis with bacteremia, HEB) but without hookworm peritoneal penetration (n=13); a second group included pups with HEB and hookworm peritoneal penetration (n=12); and a third group of pups that died because of South American sea lion attacks, falling off cliffs, being crushed during territorial bull fights or drowning (n=20). This third group, designated as "trauma", also had hookworm infection but the cause of death was not considered to be associated with hookworm infection since their behavior and clinical examination were normal during previous observational or growth rate studies and the incident that caused their death was directly observed during monitoring of the rookery (performed daily for 1 hour AM and 1 hour PM from December 15 through March 15). Six animals without hookworm infection that died of trauma were used as histologic controls but not used for any statistical analyses. Additional causes of death among pups were starvation, bronchopneumonia, *Encephalitozoon cuniculi* disseminated infection and encephalitis of unknown etiology. These pups were not included in the statistical models due to the potential presence of tissue changes caused by the major disease processes that caused the pup's death.

b) Necropsies and histopathology

Complete necropsies, including detailed gastrointestinal tract examination for nematodes, were performed in the field following a previously described protocol (Seguel et al. 2013). Additionally, from 2012 to 2015 breeding seasons, examination of the peritoneal cavity for nematodes was conducted as described in otariid seals. Tissues routinely sampled for histopathology included brain, lung, trachea, heart, spleen, submandibular, prescapular, bronchial, mediastinal and mesenteric lymph nodes, colon, kidney, testis, ovary, adrenal gland, esophagus, stomach, liver, pectoral and masseter skeletal muscles and skin from the pectoral and maxillary regions, and at least 3 cross sections from each small intestinal segment (duodenum, jejunum and ileum). The sections of ileum were systematically sampled at 10 cm cranial to the ileo-cecal junction. Bone marrow was examined in the mainland lab (Universidad Austral de Chile) after formalin fixation and formic acid decalcification of the tibial epiphysis in samples collected between 2012 and 2015. Tissue samples were fixed in 10% neutral buffered formalin, embedded in paraffin wax, sectioned (4 µm) and stained with hematoxylin and eosin (HE). In selected cases, sections were stained with PAS-Alcian blue, Lillie Twort or modified Brown and Brenn methods for Gram stain, Giemsa, acid-Fast, Gomori methenamine and Warthin-Starry silver stains.

Tissue changes associated with hookworm infection were measured in hookworm-infected and hookworm-free pups with complete necropsies (including peritoneal wash) and tissue samples (n=45). In randomly selected HE-stained sections of ileum, the length of 20 villi was measured using a microscope with calibrated reticle eyepiece and following the morphological measurement criteria described. The average villus length was calculated for each animal. The number of cells undergoing mitosis in the base of the villi was counted in ten

random 400X fields. Additional data recorded during the examination of intestine, mesenteric lymph node, spleen and liver included: the number and location of hookworm feeding tracks in 3 intestinal sections, the average number of leukocytes in a 400X field of the intestinal lamina propria counted in 10 random fields, the number of macrophages containing erythrocytes in ten 400X fields of the mesenteric lymph node medullary sinuses (as a measure of the level of erythrophagocytosis), the average number of megakaryocytes associated with erythroid precursors counted in ten 400X fields of the spleen and liver (as a measure of the level of extramedullary hematopoiesis) and the number of crypt abscesses in three sections of ileum.

Differences in erythrocyte consumption in the hookworm intestinal tract were observed microscopically between pups with peritoneal penetration and those without. To test if there were actual differences in the intestinal content of hookworms between pups with different causes of deaths, a group of 30 to 40 hookworms per host were extended in a histology cassette, embedded in 2.5% agarose gel,and fixed in 10% buffered formalin, and two 0.3-cm-thick cross sections were obtained from the mid-portion of the worms. The sections were processed routinely for histopathology, resulting in a 4 μm HE-stained section containing between 40 and 60 cross sections of hookworms at the level of the intestine and reproductive organs. Thirty sections containing segments of the nematodes' intestine were randomly selected and the number of sections that contained at least 1 erythrocyte, and the average number of erythrocytes in the sections were recorded.

c) Parasitology

All nematodes found at necropsy were retrieved, counted based on their location, washed with sea water. Most were placed in 5% formalin for morphological and histological studies, and

a small subset were placed in 70% ethanol for genetic studies. A subset of nematodes from each host were mounted on permanent glass slides and observed microscopically. Standard measurements were recorded and genus-level identification was performed according to the key for the identification of parasitic nematodes.

d) Bacteriology

In cases from 2012 to 2015, samples from the lung, liver, kidney, bronchial and mesenteric lymph nodes, distal jejunum, adrenal gland and brain were collected in sterile plastic bags and stored at -19°C degrees for up to 1 month in the field and later at -80°C in the mainland lab. In cases with gross evidence of peritonitis (peritoneal exudate and/or fibrin), sterile peritoneal swabs were taken. Carcasses with an estimated postmortem interval of less than 6 hours, based on absence of rigor mortis or previous observation of a sick pup found dead hours later, had blood swabs taken from the heart or cranial vena cava. Swabs were placed in a sterile plastic tube with Stuart medium, stored for up to 1 month at -19°C and later at -80°C until bacteriological culture. Frozen tissues were defrosted at room temperature for 12 hours and a sterile section obtained from the center of the sample was incubated in enriched medium at 37°C for 24 hours prior to plating on McConkey and blood agars. Swabs were defrosted at room temperature for 6 hours prior to plating. Isolated bacterial colonies were identified to the genus or species level by Gram stain and biochemical reactions using a BBL Crystal ID systems for enteric non/fermenting and Gram positive bacteria (BD, Sparks, Maryland).

e) Statistical analyses

Statistical analyses and graphics utilized R 3.2.0®, and Graphpad 6® softwares. To test histologic differences between animals that died because of HEB and those that died of trauma, Mann-Whitney tests were performed. Nematode burden and histologic differences between animals that died of trauma, HEB without peritoneal penetration and HEB with peritoneal penetration were assessed by generalized linear models with negative binomial error structure (GLM.NB). Spearman rho correlation tests were performed in a matrix containing all histologic counting data and hookworm burden.

To test which variables were associated with hookworm peritoneal penetration, we used Firth's logistic regression. Based on predictor correlations and histopathologic assessment, the predictor variables in the maximal (global) model were; nematode burden, hookworm sections containing erythrocytes, intestinal villi length, number of leukocytes in the intestinal mucosa and their interactions. The final models were selected based on the significance of the predictors, likelihood ratio and the Akaike information criterion (AIC).

Results

Hookworm infection was found in 132 of 140 necropsied pups (94.2%; 68 males, 64 females). In 46/132 (34.8%) infected animals, the lesions caused by hookworms and secondary bacterial colonization, translocation and likely sepsis were considered as the cause of death. This cause of death was designated as hookworm enteritis and bacteremia (HEB). The gross and histologic lesions observed in SAFS pups infected with *Uncinaria sp.* differed between pups that died due to trauma (n=36) and those that died of HEB (n=46).

Lesions in SAFS Dying of Trauma

In all of the pups that died of trauma, hookworm-associated gross lesions were restricted to the distal jejunum, ileum, and mesenteric lymph nodes. Peyer's patches were prominent and the mesenteric lymph nodes markedly enlarged. There was mild to moderate amount of clear mucus on the jejunal and ileal mucosa admixed with small to moderate numbers (mean intensity = 163, bootstrap 95% confidence interval 102.76 - 240.71) of nematodes that were 1.0 (males) or 1.9 (females) cm in length. In some areas of the ileal mucosa there were a few 0.1-0.2 cm diameter hemorrhages (hookworm attachment sites).

Microscopically, the mid and distal jejunum and ileum were filled with abundant PAS-Alcian blue-positive mucus and degenerate eosinophils. The villi were long and large numbers of eosinophils and fewer lymphocytes and plasma cells expanded the lamina propria. The apical portion of the villi had rare areas with loss of epithelial cells (hookworm feeding sites) and exposure of the lamina propria. These areas were usually surrounded by numerous neutrophils, eosinophils and occasional macrophages. At the base of the villi there were numerous mitotic figures in the intestinal epithelium and large numbers of goblet cells. Peyer's patches were prominent and populated by numerous lymphocytes and plasma cells and fewer macrophages. The mesenteric lymph node cortex was expanded by numerous secondary lymphoid follicles. No bacteria were observed in the lamina propria or mesenteric lymph nodes with Gram, Giemsa, acid fast and silver stains, and no bacteria were isolated from liver, mesenteric lymph node, spleen, brain or blood. The hypercellular bone marrow contained approximately 60% of hematopoietic elements and at least 3 megakaryocytes per 400X section (Table 2.1).

Lesions in SAFS Dying of HEB**.** In pups that died of HEB (n=46), the jejunal and ileal serosa had variable numbers of petechiae and/or ecchymoses covered by fibrin strands. In 12 of

55 pups (21.8%) examined by peritoneal wash from 2012 to 2015, between 1 and 10 intact and degenerate adult hookworms were found in the turbid fluid that occupied the abdominal cavity. Rarely, adjacent to intestinal wall ecchymoses, few adult nematodes were trapped in the mesentery (Figure 2.1). In these animals, segmental hemorrhage thickened the intestinal wall (hookworm intestinal penetration sites) (Figure 2.2). During the 2004-2008 period, there was histologic evidence suggesting intestinal wall penetration by hookworms in at least 3 pups, but these were unconfirmed because peritoneal wash was not performed in this time period. Regardless of peritoneal infection status, the intestinal lumen of pups dying of HEB was filled with bloody, mucoid content and moderate to large numbers (mean intensity = 761.8, bootstrap 95% confidence interval 517.73 - 1170.00) of nematodes that were 1.0 (males) to 1.9 cm (females) in length (Figure 2.3), and sometimes adjacent to 0.2-0.4 cm diameter mucosal erosions (hookworm feeding sites) (Figure 2.4). The mesenteric lymph nodes were markedly enlarged and the spleen congested. Some pups had pale mucous membranes and skeletal muscles, and the blood was thin with little or no coagulation (anemia).

Microscopically, the lumens of the jejunum and ileum contained large amounts of cellular debris admixed with large numbers of bacteria, degenerate erythrocytes and occasional 310 to 510 µm in diameter nematodes characterized by a thin cuticle, polymyarian, platymyarian musculature, a gastrointestinal tract lined by simple columnar epithelium with a prominent brush border and reproductive tract with either numerous spermatids or 100 µm embryonated eggs (Figure 2.5). In some nematodes, the cuticle was lined by numerous small Gram negative bacilli and the gastrointestinal tract brush border was occupied by a few Gram positive or Gram negative bacilli. Intestinal villi were short, blunt and occasionally fused. There were multiple hookworm feeding sites with exposure of the lamina propria, and numerous Gram negative short

bacilli on the apical surface of adjacent epithelial cells. Macrophages, lymphocytes, plasma cells, fewer neutrophils and rare eosinophils mildly expanded the lamina propria of the villi. The crypts were occasionally dilated and filled with cellular debris, degenerate neutrophils and variable numbers of Gram negative short bacilli (Figures 2.6 and 2.7), and/or numerous Gram negative, silver-positive, thin, filamentous bacteria.

In pups with hookworm peritoneal penetration, multiple hookworm feeding tracks expanded the submucosa and muscularis (Figures 2.8). These areas were characterized by a center of lytic necrosis surrounded by numerous macrophages, rare Gram negative short bacilli and profuse hemorrhage. Rare macrophages, lymphocytes, and plasma cells slightly expanded the serosa and mesentery.

Peyer's patches, mesenteric lymph nodes and spleen were depleted of lymphocytes and there was moderate erythrophagocytosis in the mesenteric lymph node. In 18/25 (78%) cases, the subcapsular and medullary sinuses contained a few intrahistiocytic or free colonies of Gram-negative coccobacilli. Similarly, intrahistiocytic and free Gram negative bacilli were observed in blood vessels of the spleen (Figures 2.9), liver and lung. In some pups there was moderate to marked splenic (14/25, 56%) and hepatic (8/25, 32%) extramedullary hematopoiesis. The liver had small to moderate numbers of macrophages, neutrophils and rare lymphocytes within the portal areas and in most pups (21/25, 84%), occasional small random foci of hepatocellular dissociation, microvacuolar degeneration and necrosis. In rare animals (6/25, 24%), the tunica media of hepatic arteries was expanded by numerous neutrophils and macrophages admixed with scant fibrin. In most pups (23/25, 92%), there was mild to moderate interstitial histiocytic pneumonia. The bone marrow was mildly hypercellular with approximately 40% of hematopoietic components and few (less than 1 per 400X field) megakaryocytes. Findings that

occurred with less frequency included moderate purulent conjunctivitis in 6 pups, mild to moderate interstitial histiocytic nephritis in 6 pups, mild to moderate purulent meningitis in 4 animals and fibrinous arthritis in 2 pups.

Bacteriology

Different species of enterobacteria were isolated from the small intestine, mesenteric lymph nodes, spleen, blood, liver and other tissues (Table 2.2). The most commonly isolated species were non-hemolytic *E. coli* and non-hypermucoid *Klebsiella pneumoniae*.

Statistical models and differences between groups.

The number of nematodes was higher in animals that died because of HEB (mean intensity = 761.8, bootstrap 95% confidence interval 517.73 - 1170.00) and in those that had hookworm peritoneal penetration (mean intensity = 1384.33, bootstrap 95% confidence interval 1112.56 - 1843.78) compared to animals that died due to trauma (mean intensity = 163, bootstrap 95% confidence interval 102.76 - 240.71) (GLM.NB, df = 44, $p < 0.0001$) (Figure 2.10). The intestinal villi were longer in animals that died due to trauma, followed by animals with HEB without peritoneal penetration, and then HEB with peritoneal penetration (GLM.NB, df = 44, $p < 0.001$) (Figure 2.11). There were larger numbers of leukocytes in the intestinal mucosa of pups dead due to trauma compared to animals that died because of HEB (GLM.NB, df = 44, $p < 0.001$) (Figure 2.12). There were larger numbers of hookworm sections containing blood (food) in animals dead because of trauma and HEB without peritoneal penetration when compared to pups that died due to HEB with peritoneal penetration (GLM.NB, df = 44, $p < 0.001$) (Figure 2.13). There were no differences in the body mass index, age, number of mitoses at the base of villi and the number of megakaryocytes in the spleen and liver between groups (GLM.NB, df = 44, p values 0.1-0.9).

The number of nematodes was negatively correlated to the intestinal villous length ($r = -0.83$, $p < 0.0001$) and the number of leukocytes in the intestinal lamina propria ($r = -0.61$, $p < 0.0001$), and positively correlated to the number of hookworm feeding tracks ($r = 0.70$, $p < 0.0001$) and the number of abscesses in the intestinal crypts ($r = 0.57$, $p < 0.001$) (Fig. 14). The model for the presence of hookworm peritoneal penetration with the lowest AIC had intestinal villous length as the only predictor (AIC= -23.1, Likelihood ratio=25.1, df=2, $p<0.001$) while the model with the highest likelihood ratio included the interaction between villous length and the number of hookworm sections with erythrocytes as the only predictor (AIC=-22.65, Likelihood ratio=28.66, df=2, $p<0.001$) (Table 2.3).

Discussion

We describe detailed gross and histopathological findings in SAFS infected with *Uncinaria sp*. Hookworms in this fur seal species are highly pathogenic, causing severe intestinal damage, which in a significant number of pups led to bacterial translocation and death. The level of hookworm-induced mortality and severity of lesions we describe are among the greatest reported in otariid populations (Lyons et al. 2011a, Seguel et al. 2013). However, the prevalence of hookworm intestinal penetration in our population during the period 2012-2015 (21.8%) is lower than reported in Northern fur seals in San Miguel Island (61.4%), but higher compared to California sea lions in the same location, where 12.5% of hookworm infected animals have peritoneal penetration (Lyons et al. 2011a).

In pups that died of causes unrelated to hookworm infection (trauma group), there was marked eosinophilic enteritis with large amount of mucus. A similar type of enteritis has been described in rats infected with *Nippostrongylus brasiliensis* (Ogilvie and Jones 1971) and

humans infected with *Necator americanus* (Croese and Speare 2006) or *Ancylostoma caninum* (Walker et al. 1995). Interestingly, this type of inflammatory response has been associated with early hookworm expulsion in the rat-*N. brasiliensis* and human-*N.americanus* experimental models (Ogilvie and Jones 1971, Croese and Speare 2006,). The inflamed and mucinous intestine is in theory a worse environment for hookworm attachment and feeding, and could be one of the reasons for the presence of fewer nematodes in the animals that died of trauma when compared to HEB pups.

The average hookworm burden in pups that died due to HEB (mean intensity = 762 nematodes) is smaller when compared with those reported in necropsied Australian sea lion pups (mean 2138 nematodes) (Marcus et al. 2014), New Zealand sea lions (mean 824) (Castinel et al. 2007a), northern fur seals (means 643, 1200) (Lyons et al. 1997, 2001,2011), and California sea lions (means 612, 1284) (Lyons et al. 1997, 2001,2011). These differences could be due to population-specific factors such as overall prevalence, immunity, genetic susceptibility and rookery substrate, or they could reflect species-specific differences, such as *Uncinaria spp*. sizes. In the aforementioned species the average female length is 10.0 to 13.8 cm (Kuzmina et al. 2015) while in Chilean SAFS population the average female length is 19.5 cm, which, along with *Uncinaria sp*. in Australian fur seals and Southern Elephant seals, are the largest hookworms described in any animal species (Ramos et al. 2013). This could explain why we observe HEB with burdens as low as 270 nematodes.

In the HEB group, most observed lesions have been previously described in California sea lions infected with *Uncinaria lyonsi* (Spraker et al. 2007). However, in contrast to the previous study, we did not find lesions in the large intestine or centrilobular degeneration in the liver due to anemia. In the California sea lion study, anemia was assessed based on gross and

histologic findings, however, in our study, some of the HEB animals were part of a long term health assessment and therefore we were able to confirm anemia through complete blood cell counts compared to this population´s reference ranges one or two weeks prior to the animal´s death (Seguel et al. 2016). However, we did not have a large enough sample size to correlate the severity of anemia with histologic findings. Despite this fact, we did observe pale carcasses and marked EMH in some pups, but the severity of these observations were not different from pups that died due to trauma or HEB, and this was not associated with parasite burden. This could be due to natural bias of the histological count of random sections in a process that is not necessarily homogeneous in the tissues and/or to a true high level of variance in the EMH response among pups. However, in most pups dead due to HEB, there was evidence of decreased hematopoiesis, probably associated with a high parasitic burden and chronic blood loss, characterized by small numbers of erythroid precursors and megakaryocytes in the bone marrow when compared to the highly active hematopoietic response seen in pups dying of trauma.

In our population, as previously described in California sea lions, hookworm-induced host death is most likely produced by a combination of marked anemia, intestinal damage with absorption of endotoxins, and translocation of bacteria into the vascular system and dissemination to major organs. It is likely that these events lead to sepsis with the concurrent immune-metabolic disturbances that define this syndrome (Angus and Van der Poll 2013). Nonetheless, since it is not possible to confirm sepsis by postmortem examination, we preferred to use the term introduced by Spraker et al. of HEB despite the fact that the word bacteremia does not necessarily reflect a pathological state, since it can occur under normal physiological conditions (Angus and Van der Poll 2013).

Bacterial infections found in most pups with HEB played an important role in their death, however the variety and type of species isolated suggests that these bacteria were opportunistic pathogens, probably taking advantage of a disrupted intestinal barrier and a debilitated immune system. On the other hand, some of the isolated bacterial species are usually considered of low pathogenicity and common contaminants of biological samples (*e.g*. *Proteus sp*.). However, the agreement of the species isolated with microscopic morphology, distribution, staining profile, tissue reactions, freshness of the carcasses sampled, negative isolation results on control (trauma) pups and sterile collection of swabs and tissues suggest that these bacteria were likely antemortem invaders of the blood and tissues of the pups.

The parasitic burden was markedly different between trauma and HEB groups, suggesting that the number of nematodes is one of the most significant factors driving hookworm-induced tissue damage and mortality. Additionally, hookworm burden was highly correlated with intestinal villous length, number of intestinal leukocytes, and hookworm feeding tracts. The short villi found in animals with high nematode burdens is probably caused by the "grazing" effect of hookworms on the intestinal villi, because to feed on blood the nematodes have to bite and cut out pieces of the mucosa. Despite the large number of nematodes in pups with HEB, inflammation was minimal. This could be due to a primary lack of strong immune reaction in these pups allowing for the establishment of a larger number of larvae in the intestine. On the other hand, this weak inflammatory response could be the consequence of immunomodulatory hookworm secretion products. Both types of host-pathogen relationship have been shown to be significant in experimental models of hookworm infection and it is very likely that these two mechanisms acted in our studied animals (Ogilvie and Jones 1971, Croese and Speare 2006, Loukas et al. 2005). Another explanation for differences observed in the level of

inflammation between pups with trauma and HEB could be the temporal variation in the process of hookworm disease, with an early strong eosinophilic response that later is overwhelmed, leading to mild inflammation by the time the hookworms kill a pup and we examine the tissues. However, given the marked synchronization of parasite transmission with the host reproductive cycle, we would expect to see differences in the type and level of mucosal inflammation in pups of different ages. This was not the case, and animals that died due to HEB were as young as 21 days old, while some pups dying of trauma with marked eosinophilic enteritis were up to 80 days old, making the temporal variation in the inflammatory process a less likely explanation.

To our knowledge, adult hookworm peritoneal penetration has not been reported in animal groups other than pinnipeds. This peculiarity could be related to the life strategy of marine hookworms, which are highly adapted to the reproductive cycle and lifestyle of their host. In general, pinniped hookworms infect neonates through the colostrum but are expelled from the intestine before their hosts are weaned between 2 to 6 months of age (Castinel et al. 2007b, Lyons et al. 1997, 2001, 2011). This means that they have a short period of time to grow, mate and release eggs. This probably is associated with aggressive feeding behavior to supply their high metabolic demands and could be one of the reasons for the high pathogenicity of hookworms in many pinniped species (Lyons et al. 1997, 2011b, Spraker et al. 2007). Additionally, our histologic and statistical analyses indicate that in pups with hookworm peritoneal penetration, the parasites tend to have little blood in their gastrointestinal tract suggesting that lack of food for the parasite could be one of the factors that make hookworms penetrate deeper into the intestinal wall and reach the peritoneal cavity. Another factor associated with increased likelihood of peritoneal penetration was the average intestinal villous length,

which could indicate that a thinner mucosa is less of a barrier for deeper hookworm penetration or provides lesser possibilities for the nematode to feed.

In this study we have shown that the parasitic burden is one of the main, but probably not the only factor, determining hookworm pathology and mortality. Large numbers of hookworms in the intestine lead to short intestinal villi, which combined with primary or secondary mild intestinal inflammatory response, allow hookworms to feed deep into the mucosa, facilitating disruption of the intestinal barrier and bacterial translocation into the blood. Additionally, in some animals, the large number of nematodes likely depletes the pups' erythrocyte reserves and capacity for intestinal villous regeneration, therefore there are decreased resources available for the parasites. This, plus the proximity to the submucosa and muscularis due to shortened villi, and the mild inflammatory response could be some of the factors contributing to hookworm peritoneal penetration.

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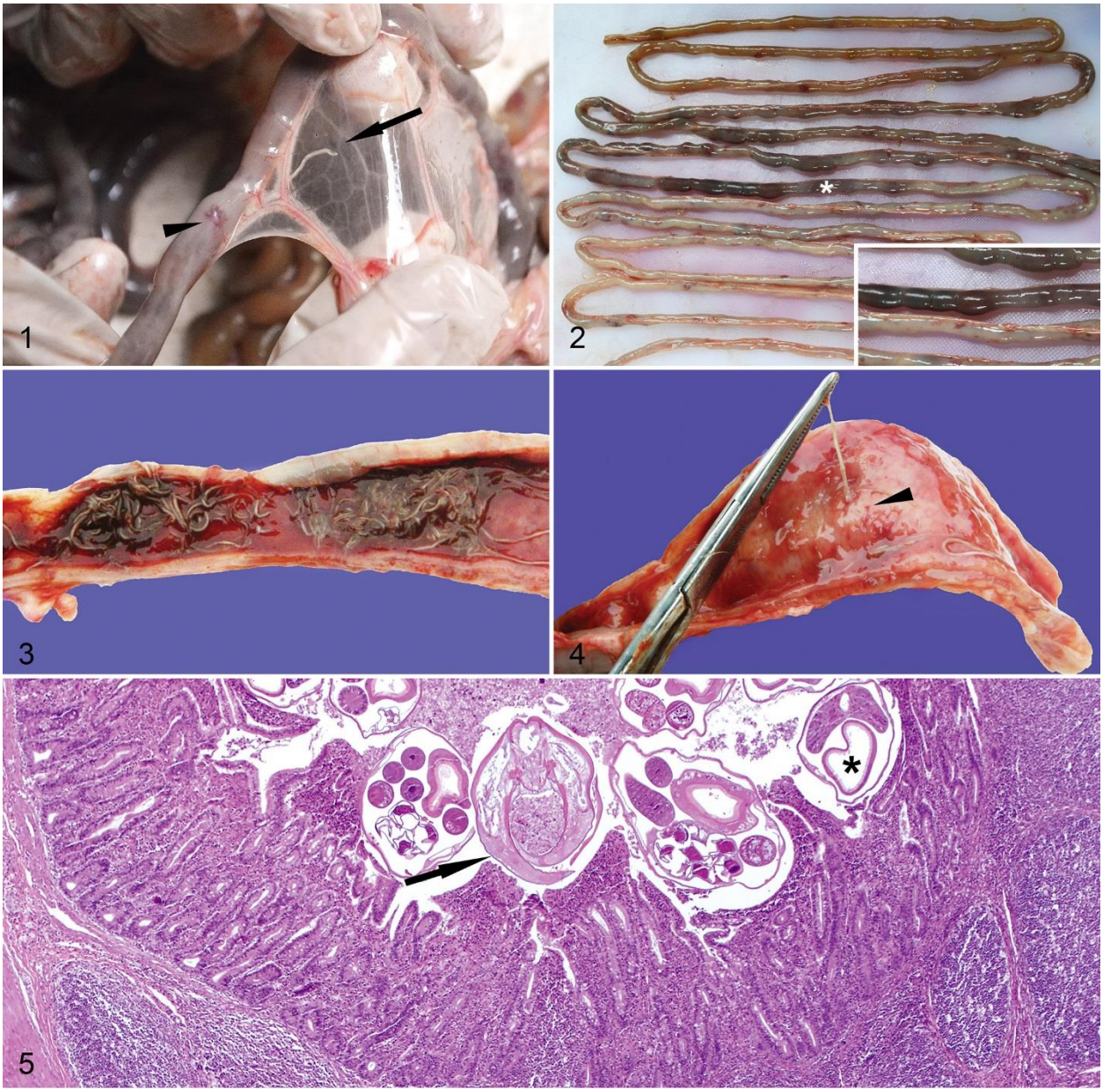
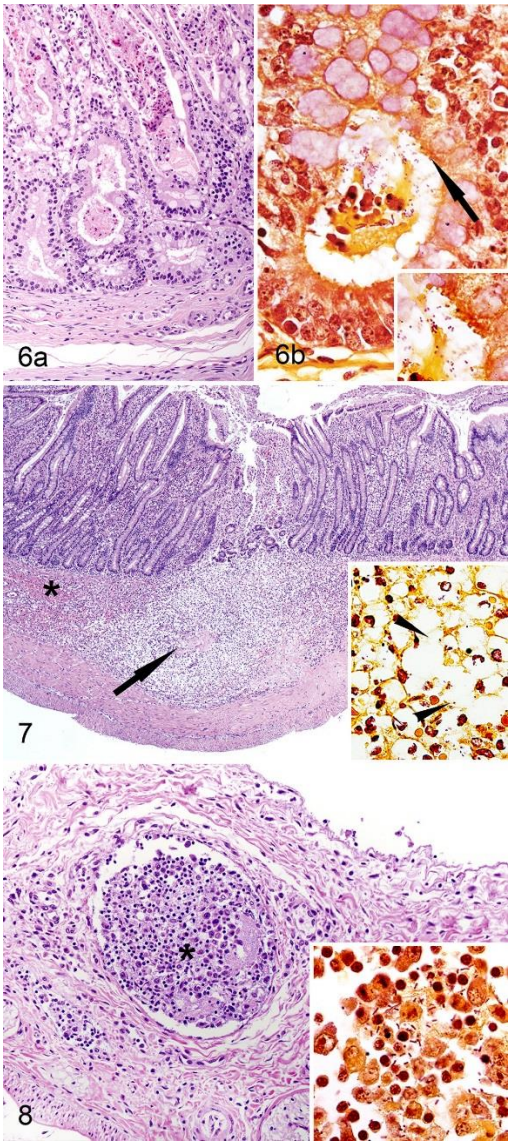
Figures



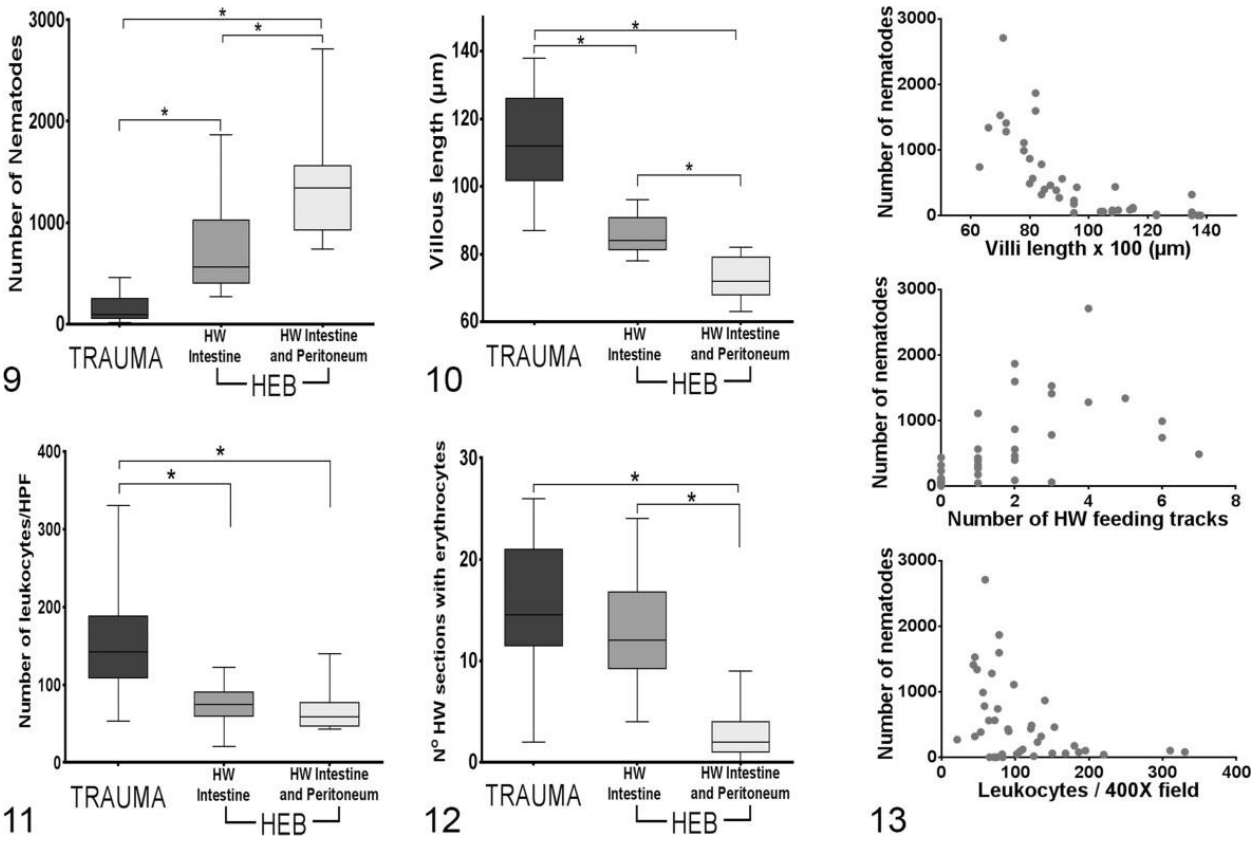
Figures 2.1-2.5. Hookworm enteritis with bacteremia, small intestine, South American fur seal pups. Figure 2.1. A large female hookworm (arrow) is trapped in the mesentery and adjacent to an area of jejunal hemorrhage (arrowhead), a site of presumed hookworm peritoneal penetration. Figure 2.2. Hemorrhage expands the intestinal wall in multiple segments (asterisk). Figure 2.3.

Large numbers of hookworms admixed with bloody intestinal content occupy the lumen of the distal jejunum. Figure 2.4. A female hookworm is deeply attached in the mucosa of the distal jejunum, and there are multiple 2-mm-diameter mucosal erosions (arrowhead; hookworm feeding sites). Figure 2.5. Numerous nematodes in the lumen of the ileum are admixed with degenerate erythrocytes. Note the prominent *Uncinaria sp*. buccal capsule (arrow) and that all nematodes have an empty gastrointestinal tract (asterisk). Intestinal villi are short, blunt, fused. Hematoxylin and eosin.



Figures 2.6–2.8**.** Hookworm enteritis with bacteremia, South American fur seal. Figure 2.6**.** Ileum. Intestinal crypts contain abundant cellular debris. (a) Hematoxylin and eosin (HE). (b) Cellular debris in the crypts is admixed with gram-negative bacilli that sometimes line the apical surface of enterocytes (arrow). Inset: Higher magnification of the bacteria. Gram stain. Figure 2.**7.** Jejunum. Large numbers of macrophages surround a center of lytic necrosis (hookworm feeding track; arrow). There is acute hemorrhage in the adjacent tunica muscularis

(asterisk). HE. Inset: Hookworm feeding track with intrahistiocytic gram-negative bacilli (arrowheads). Modified Brown and Brenn Gram stain. Figure 2.8. Splenic hilum. Numerous macrophages, lymphocytes, and bacteria occupy the blood vessel lumen (asterisk). HE. Inset: Intrahistiocytic and extracellular gram-negative large bacilli. Modified Brown and Brenn Gram stain.



Figures 2.9–2.13. The number of nematodes was highest in pups with hookworm enteritis with bacteremia (HEB) and hookworms (HW) in both the intestine and peritoneum (ie, peritoneal penetration), followed by pups dead due to HEB without peritoneal penetration (HW Intestine; GLM.NB, df = 44, P < .0001). Figure 2.10. Pups that died due to HEB with hookworm peritoneal penetration had shorter villi compared with pups that died due to HEB without

peritoneal penetration and pups that died due to trauma (GLM.NB, df = 44, P < .001). Figure 2.11. The pups that died due to trauma had a larger number of intestinal leukocytes compared with pups that died due to HEB (GLM.NB, df = 44, P < .001). Figure 2.12. In pups that had hookworm peritoneal penetration, the number of hookworms containing blood in their intestinal tract was significantly lower compared with pups without hookworm peritoneal penetration (GLM.NB, df = 44, P < .001). Figure 2.13. Distribution of data points of variables highly correlated with the number of nematodes. Villous length: r = –0.83, P < .0001. Number of hookworm feeding tracts: r = 0.70, P < .0001. Number of leukocytes: r = –0.61, P < .0001.

Tables

Table 2.1. Histopathologic findings in South American fur seal pups infected with hookworms (*Uncinaria sp.*) and death attributed to hookworm enteritis with bacteremia (HEB) and trauma.

Microscopic Lesions per Group	**Severity**			**Total**
	Mild	**Moderate**	**Severe**	
Microscopic Lesions Trauma group (n=20)				
Catarrhal eosinophilic enteritis	12	9	1	20/20 (100%)
Intestinal goblet cell hyperplasia	13	7	0	20/20 (100%)
Peyer´s patch hyperplasia	12	5	2	19/20 (95%)
MLN follicular hyperplasia	10	8	2	20/20 (100%)
Bone marrow erythroid hyperplasia	11	6	0	17/20 (85%)

Hookworm feeding sites	1	3	16	20/20 (100%)
Microscopic Lesions HEB group (n=25)				
Histiocytic and neutrophilic enteritis	4	6	11	21/25 (84%)
Hookworm feeding sites	19	4	2	25/25 (100%)
Intestinal crypt abscesses	3	5	4	12/25 (48%)
Intestinal submucosal hemorrhages	4	5	1	10/25 (40%)
Splenic EMH	9	3	2	14/25 (56%)
Hepatic EMH	3	2	3	8/25 (32%)
Bone marrow myeloid hyperplasia	1	2	15	18/23 (78%)
Histiocytic interstitial pneumonia	3	14	6	23/25 (92%)
Peyer´s patch depletion	19	3	0	22/25 (88%)
MLN lymphoid depletion	14	5	6	25/25 (100%)
Splenic lymphoid depletion	8	7	4	19/25 (76%)
Intravascular or sinusoidal bacteria	2	5	11	18/25 (72%)
Vasculitis	0	2	4	6/25 (24%)
Periportal histiocytic hepatitis	1	3	8	12/25 (48%)
Hepatic random degeneration and necrosis	4	5	14	23/25 (92%)
Interstitial histiocytic nephritis	0	3	3	6/25 (24%)
Purulent meningitis	0	2	4	6/25 (24%)

Purulent conjunctivitis	1	3	2	6/20 (30%)
Fibrinous arthritis	0	1	1	2/16 (13%)

HEB= hookworm enteritis and bacteremia, EMH= extramedullary hematopoiesis, MLN= mesenteric lymph node.

Table 2.2. Bacteria genera and tissues of isolation in 13 South American fur seals dead due to hookworm enteritis and bacteremia.

	Location[a]							
Bacteria Isolated	SI	LN	SP	BL	PC	LU	LI	CJ
Non hemolytic *E. coli*	8	3	5	5	4	3	4	0
Klebsiella pneumoniae	4	2	1	2	3	2	1	0
Vibrio sp.	2	0	0	0	0	0	0	0
Streptococcus sp	0	0	0	0	0	1	0	2
Pseudomona sp.	4	1	2	3	0	0	1	0
Proteus vulgaris	4	2	0	2	1	0	0	0
Salmonella sp.	2	0	0	0	0	0	0	0

[a] SI=Small Intestine, LN=Lymph Node, SP=Spleen, BL=Blood, PC=Peritoneal Cavity, LU=lung, LI=Liver, CJ=Conjunctiva.

Table 2.3. Coefficients, likelihood ratios and Akaike information criteria of selected binomial logistic regression models (Firth´s method) for the presence of peritoneal penetration in South American fur seal pups.

Predictors					Likelihood Ratio	p-value	AIC	Delta AIC
Villi length	HW RBC	Number Nematodes	Leukocytes Mucosa	HW RBC*Villi length				
-0.3526***					25.104	5.43E-07	-23.104	0.000
				0.00306***	28.656	2.64E-06	-22.656	-0.448
-0.14019*	-0.30901*				26.532	1.73E-06	-22.532	-0.572
-0.3209	-0.5613		0.0596		27.557	4.5E-06	-2.558	-1.547
	-0.3643***	0.002*			25.038	3.66E-06	-21.039	-2.066
-0.28818**		0.0008			24.611	4.53E-06	-20.612	-2.493
-0.21323*	-1.4512			0.01388	26.225	8.55E-06	-20.226	-2.879
-0.1004	-0.28115*	0.0007			26.175	8.76E-06	-20.176	-2.929
-0.23432*	-0.45740*	0.002	0.0526*		27.443	1.62E-05	-19.443	-3.661

Significance. codes: p-value < 0.001 = '***', p-value between 0.001-0.01 ='**' , p-value between 0.05 – 0.01 ='*'. Int.= Intercept, AIC=Akaike Information Criteria, HW RBCs = Hookworm sections containing red blood cells.

CHAPTER 3

LIVE FAST AND DIE YOUNG LIFE HISTORY STRATEGY DRIVES HIGH VIRULENCE OF PINNIPED HOOKWORMS.[3]

[3]Seguel M., Muñoz F, Perez-Venegas D, Muller A, Pavés H, Howerth E, Gottdenker N. Submitted to *Parasitology*, 12/31/2017.

Abstract

The impact of parasites on animal populations is often correlated with the level of "harm" or virulence they induce in their host. Therefore, for disease management approaches and assessment of parasite ecosystem effects, it is critical to understand how highly virulent pathogens emerge in nature. However, few studies have addressed this question using natural systems. Here, we describe a marine mammal (*Arctocephalus australis*)-hookworm (*Uncinaria sp.*) system where we propose that parasite adaptation to their host's life history traits has led to maximizing parasite fitness with high levels of virulence. Fur seal pups acquire hookworms during their first days of life through their mothers' colostrum and release hookworm eggs 14-18 days later. Eggs larvate in the rookery soil, and larvae penetrate the skin of adult female's to remain in their blubber until the next reproductive cycle. Larvae do not survive in the soil from one reproductive season to the next, and most adult hookworms are expelled from the pups' intestine 30 to 50 days after initial infection, giving hookworms little time to feed and reproduce in the intestine of fur seal pups. This translates in a lack of restriction in extraction of host resources (blood) and production of eggs despite reaching high intra-host densities, therefore pups with high hookworm burden contribute disproportionally to parasite transmission and fitness. Nevertheless, these pups also suffer the worst consequences of parasitism in terms of severe anemia and up to 60% of hookworm-induced mortality. Marine hookworms exploit a live fast-die young life history strategy, which translates in aggressive feeding behavior and the highest levels of virulence recorded among hookworms. Parasite alternative strategies to maximize fitness such as increased environmental survival of larval stages or avoidance of clearance from the intestine have not been developed, probably related to the marine lifestyle of

the host. We propose that parasite adaptation to host life history traits can result in high levels of virulence.

Introduction

Parasite virulence is traditionally defined as a decrease in host reproductive output or fitness due to parasitism (Schmidt-Hempel 2011, Cressler et al. 2015). The study of parasite virulence has elicited great interest because the effect of parasites in host populations is usually associated with the level of pathogen virulence (Alizon et al. 2009, 2015, Schmidt-Hempel 2011, Hatcher et al. 2012, Cressler et al. 2015). Therefore, determining why and how parasite virulence develops is critical to understanding the emergence of pathogens that can have deleterious effects in animal and human populations.

The adaptive model of virulence or transmission-virulence trade-off hypothesis suggests that virulence arises as an unavoidable consequence of parasite adaptations to maximize its transmission within the host population (Alizon et al. 2009, 2015, Cressler et al. 2015). Under this theoretical framework, the emergence of different levels of virulence is feasible (Alizon et al. 2009, 2015, Schmidt-Hempel 2011, Hatcher et al. 2012, Cressler et al. 2015), but most models and empirical evidence suggest that parasite and host find an equilibrium at intermediate levels of virulence (Day 2001, Fraser et al. 2007, De Roode et al. 2008, Cresslet et al. 2015). Nevertheless, highly virulent pathogens emerge on a regular basis in nature with catastrophic consequences for animal and human populations (Hatcher et al. 2012, Fauci and Morens 2012). The mechanisms leading to the emergence of high virulence in parasites are not well understood, but models and empirical evidence suggest that intra-host competition between parasite species or strains (Mackinnon and Read 1999, Elena 2001, Schmidt-Hempel 2011), vector mediated

transmission (Day 2001, 2002), host immune processes (Andre et al. 2003, Mackinnon and Read 2004) and increased environmental resistance of parasite infective stages can lead to the selection of more virulent pathogens (Walther and Ewald 2004). However, for some mechanisms, such as vector transmission and environmental resistance, empirical evidence of evolution towards increased virulence is contradictory (Schmidt-Hempel 2011, Day 2001, 2002), and for others most of the literature is theoretical with little or no empirical evidence of such processes occurring in natural systems (Alizon and Van Baalen 2005, Read et al. 2015). Therefore, how highly virulent pathogens emerge under natural conditions remain a fundamental question in the study of infectious diseases.

We studied a marine mammal-hookworm system to determine whether parasite adaptations to host life history traits can maximize parasite fitness resulting in high levels of virulence. Hookworms are gastrointestinal blood-feeding parasites of many mammals, including millions of humans (Bartsch et al. 2016). Because infection can result in considerable blood loss, these parasites are often highly pathogenic, with the most virulent being the species infecting pinnipeds (seals, sea lions and fur seals). In these species, infection can cause up to 70% mortality (Spraker et al. 2007, Lyons et al. 2011, Seguel and Gottdenker 2017). One possible explanation for this high mortality is that specific adaptations to host biological rhythms and life history traits drove parasite selection pressure towards heightened virulence. Pinnipeds reproduce on land for a short period of time and spend large periods foraging in the ocean (Paves and Schlatter 2008, Paves et al. 2016). Since all hookworms are soil-transmitted nematodes, marine mammals represent a difficult “niche” to colonize because opportunities for transmission are dramatically reduced. Nevertheless, marine hookworms are very successful, with prevalence close to 100% in many populations (Lyons et al. 2011, Seguel et al. 2013, 2017). These parasites

could have attained these high infection rates by being very efficient at extracting host resources and transforming these resources into infective stages within a short period of time, which, according to the transmission-virulence trade-off hypothesis, would favor increased parasite numbers/infection and thus higher levels of virulence. On the other hand, marine hookworms could have developed alternative routes of transmission or be "less sensitive" to changes in host density by having higher survival of infective stages in the environment, which would increase their chances of transmission. In this case, aggressive hookworm feeding, which negatively impacts the host, would not necessarily pay off in terms of increased parasite fitness.

To test these predictions, we studied and manipulated hookworm (*Uncinaria sp.*) infection in a colony of South American fur seals (SAFS, *Arctocephalus australis*). First, we describe the life cycle of *Uncinaria sp.* in SAFS, showing that transmission is markedly synchronized with the reproductive cycles of the host. Through measurement of egg shedding patterns we demonstrate that contrary to other members of the hookworm family, *Uncinaria sp*. infecting fur seals produce eggs continuously, despite reaching high intra-host density, resulting in anaemia and mortality, our measurements of virulence, that are driven by parasite burden. Based in the parasite life cycle, we estimated the proportion of secondary infections caused by the parasites of each pup (R_0). Parasite fitness is maximized at high levels of virulence because hookworms that produce large number of eggs early in the reproductive season, when host density is the highest, have better chances to find the next host. We believe this life history strategy has allowed pinniped hookworms to succeed in an animal group that spend long time at sea, limiting soil-based transmission. However, the same live fast-die young strategy has led to the highest levels of virulence recorded among hookworms.

Materials and Methods

a) Studies on hookworm transmission and life history traits

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).

During 2014 and 2015, 1 to 7 day-old pups were captured by hand and physically restrained. Age of pups was exactly known or estimated based on previously described methods (supplementary materials and methods) (Seguel et al. 2016). 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. The pups were recaptured and the sampling procedure repeated on each pup every 5 to 10 days during the duration of the study (10 weeks).

Parasitological examination was performed in all fecal samples collected according to a method standardized for this rookery (supplementary materials and methods). Samples from the fecal material and soil were examined microscopically to determine time of hookworm embryo and larvae development. To determine if the fecal egg count was a good estimate of nematode burden, the standarized 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).

A randomly selected group of 30 pups, 10 of them of exact known age, were treated in their first capture with 400 $\mu g*kg^{-1}$ of subcutaneous Ivermectin (antiparasitic drug, control group).

The average number of eggs per hookworm female was determined according to standard methods (Hussey and Barker 1973).

In 2015, milk samples were collected from 15 SAFS during the second week of January. The same year samples of colostrum were opportunistically collected from 3 freshly dead adult female SAFS.

In order to identify hookworm larvae, Baerman tests were performed in milk and colostrum samples, rookery soil from several locations and fresh tissues from dead SAFS adult females, males and pups. The same method was applied to rookery soil stored for 1 or 2 years.

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 shedding of hookworm eggs in a given day was calculated by multiplying the prevalence for that day with the median of the fecal egg count of all pups sampled the same day.

b) Measurements of parasite fitness and virulence

Parasite fitness was assessed by estimating the proportion of secondary infections generated by each infected pup (R_0), which was assumed to be $R_0= \beta S/(\mu+\alpha+\lambda)$, where β is the transmission rate of the parasite, S is the density of susceptible hosts in the population, μ is the host natural death rate, α is the host mortality due to the infection (i.e. the virulence) and λ is the recovery rate. We estimated this parameter in each pup by using the average hookworm egg shedding as a proxy for transmission rate (β), the density (S) of fur seal females in a $40m^2$ area around the pup (previously estimated to be the pup movement range), the mortality in pups treated with ivermectin (hookworm-free) as a measure of background mortality (μ), the probability of mortality (α) due to hookworm disease based on the predicted values for that pup

using a generalized linear model and the recovery rate on each pup (λ). The latter was estimated as the inverse of the infectious period, which was calculated by adding all the days between successive captures that the animals had positive fecal egg counts plus half the number of days between the last positive fecal test and the first negative test. The absolute numbers of each value were transformed to proportions (0 to 1) and used to estimate R_0. Since aspects of the parasite life cycle, such as the proportion of larvae that finally pass from the female's tissues to the milk, were logistically not possible to measure, this approach did not allow to know the number of secondary infections generated by each pup but the proportion of the total transmission that could be attributed to a particular pup. We considered this calculation a good approximation to the real R_0 since epidemiological studies on pinniped hookworm disease and our own results suggest that the presence of larvae in adult female's tissues and transmission through the milk are related to the density of larvae in the soil (*e.g.* more larvae in the soil = more larvae transmitted through the milk) (Lyons et al. 2011).

To know the variation in the number of secondary infections produced by each pup (V), we calculated this index on each pup by multiplying the parasitic load as an estimate for infectiousness, number of female fur seals around a pup as proxy for contact rate, and the infectious period (Vanderwaal and Ezenwa 2016). To estimate the proportion of the new infections that were caused by different groups of pups, we added the total number of estimated secondary infections produced by all pups (based on R_0) and then calculated the proportion of that total attributed to a specific number or group of pups.

Since hookworms are hematophagous nematodes, anemia is one of the hallmarks of infection and a direct measurement of the extraction of resources from the host. Therefore, we used anemia (measured as low hemoglobin (HG) and/or red blood cell (RBC) counts) and

mortality as virulence indices. RBC and HG were measured as previously described (Seguel et al. 2016). Additionally, we used the body mass index (total length/weight) (BMI), total plasmatic protein concentration (TP) and white blood cell counts (WBC) as general measurements of the pups' health. These indicators were measured in each capture 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. 2011, 2017). Mortality rates were calculated by incorporating recapture (or re-sighting) data into survival tables.

c) Data analyses

Exploratory analyses were performed by Spearman rho or Pearson correlations between hookworm and pup variables, and graphing of distributions and data points of all variables in the final data set. The relationship between the number of eggs in feces and number of nematodes in the pup's intestine, the number of 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) and significant linear or polynomial interactions was selected to fit a curve with 95% confidence intervals.

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 larvae load and 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.

To determine the pup health parameters and hookworm traits that influenced pup mortality binomial generalized linear models were fitted using the number of hookworm eggs in feces, BMI, TP, WBC, RBC, HG, sex, year and infectious period as predictors in the global model. Multiple models were run by adding and subtracting variables and their interactions and final model selection was based on AIC, significance of predictors and predictability (mean absolute error). Similar approaches were used to construct and select models (GLMs) with hookworm fitness (R_0), number of hookworm eggs in the feces (negative binomial GLM), HG and infectious period as response.

Posterior to 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 (bloody feces). The mortality rates between pups with mild and severe hookworm infection and the control (ivermectin treated) group were compared with 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. The contribution to the log transformed hookworm fitness indices (R_0 and V) by animals with different hookworm burden, infection outcome and presence or absence of anemia were compared by GLM using the log-transformed fitness values as continuous response variable and the groups of pups with different hookworm burdens or infection outcome as categorical predictors.

To estimate which factor contributed most to the variation in the number of secondary infections (V) we performed principal component analysis using infectious period, parasitic burden and host density as factors of the principal component.

The estimated log transformed R_0 values of each pup were plotted against the level of anemia (transformed HG and RBC values) and probability of mortality for each pup as predicted by the final binomial generalized linear model (Mortality~ parasite burden + HG + RBC + infectious period). Multiple linear and polynomial regression models were fitted and the model with the lowest AIC selected to fit a curve with 95% confidence intervals.

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).

Results

Hookworm and fur seal life history traits.

The proposed hookworm (*Uncinaria sp.*) life cycle in South American fur seals is summarized in Figure 3.1, and is based in several observations. Stage 3 larvae (L3s) were recovered from colostrum samples collected from 3 freshly dead adult female SAFS, 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, suggesting a lack of transplacental transmission. We collected and measured the nematodes from 31 SAFS pups found dead, and intra-host hookworm size was highly homogenous. The difference between the bigger and smaller female or male hookworm within a host was never bigger 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 0.14-0.44 mm, indicating that ingestion of larvae occurs during a short period of time. None of the 10 pups treated with ivermectin one day after birth became re-infected. All these findings indicate that infection occurs through colostrum during the pup's first days of life. Pups released *Uncinaria sp*. embryonated eggs 14 to 18 days after infection (prepatent period), and these eggs larvated at Guafo Island room temperature (12-14 °C) within 24 to 72 hours. L2 development occurred *in ovo* and within 48 to 72 hours L3s were released from the eggs. Recovery of infective L3s was common in the rookery soil in January (> 50 larvae per sample), but only 1 L3s was recovered from all the soil samples left at 12°C for one year (n=10) and no L3s were recovered in any of the samples (n=10) stored in the same conditions for two years or in rookery soil samples collected in early December, indicating low environmental resistance of fur seal hookworm larvae. Between 1 and 25 L3s were recovered from the subcutaneous tissues of pups (15/20), adult females (5/5) and males (1/2), indicating that hookworm L3 penetrate the skin of all age/sex animals in the rookery.

Ninety percent of the sampled pups in 2014 and 2015 shed hookworm eggs (Figure 3.2a). The highest number of eggs were shed on January 7th, when pups are on average 3-weeks-old and there are large numbers of adult, reproductive, females in the rookery (Figure 3.2b), which are the next host in the hookworm life cycle. Most pups cleared hookworm infection between 3 and 6 weeks after initial infection and the infectious period ranged between 9 and 48 days. Infective, sheathed *Uncinaria sp.* L3s were recovered in higher numbers in areas with higher pup density (GLM, df=30, P <0.001) (Figure 3.2c), indicating density dependent transmission of L3s.

Parasites do not experience density dependent declines in growth or egg output

Fecal smear egg counts were a good estimator of the number of intestinal nematodes (third order polynomial regression, adj-r^2= 0.921, P< 0.001) (Figure 3.3a). Hemoglobin concentrations of SAFS pups were significantly and negatively correlated with the number of hookworm eggs in their feces (second order polynomial regression, adj-r^2=0.401, P<0.001) (Figure 3b), suggesting that the parasites deplete hemoglobin in a density dependent manner (additional models outputs in table 3.1). 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 intra-host hookworm density (males, females and total) (linear regression, adj-r^2=-0.03, P=0.621) (Figure 3.3c), indicating a lack of density dependent declines in egg output. The size of females was not correlated with the average number of eggs per female in each host (Spearman-rho, r=0.01, p=0.73).

Virulence is dependent on parasite burden.

Fur seal pups with higher hookworm burdens tend to have longer infectious periods, lower hemoglobin concentrations and a higher probability of mortality (GLM.NB, log-likelihood= -321.8, df=9, P < 0.001). Mortality likelihood increased in pups with lower hemoglobin concentration and RBC counts, longer infectious periods, and higher hookworm burden (GLM Binomial, log-likelihood= -38.083, df=5, P<0.001), although the effect of hookworm burden was not statistically significant (tables 3.2 and 3.3).

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 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)" groups (Log-rank Mantel-Cox test, X^2= 44.43, df=2, P < 0.001) (Figure 4.4a). In the severe infection group, 30 pups died (30/53, 56.6% mortality), and in at least 21 cases they were confirmed to die due to hookworm infection by necropsy and histopathology (21/53, 39.6%). 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-treated group (n=30), 2 animals (6.6%) died due to trauma. The difference in survival between the mild infection and treated groups were 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, df=104, P<0.001). 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<0.001) (Figure 4.4b).

Parasite fitness is maximized at high levels of virulence.

We calculated two indices of parasite fitness. One corresponded to the basic reproductive number $R_{0,}$ which was calculated using a standard equation and indicated the proportion of new (secondary) infections attributed to the population of parasites infecting one pup. The second estimate corresponded to the variation in parasite fitness (V), and was calculated as the product of the infectious period, host density and parasite burden, and was used to estimate which of these three components contributed to most of the parasite fitness. Parasite burden contributed to 53.3% of the overall variance in parasite fitness. Fur seal female density and infectious period

contributed to 36.7% and 9.9% of the variance in parasite fitness respectively (Principal component analysis) (Figure 3.5a-c).

To evaluate the degree to which virulence contributes to parasite fitness, we compared the relative contribution to secondary infections (R_0) of pups that died or survived, and of those with and without anemia. The animals with severe hookworm infection who died (30/149, 20.1%) contributed to 47.3% of the secondary infections (parasite fitness), and pups with the same parasitic burden that survived (n=22/149, 15.4%) contributed to 37.2% of the new infections. The pups with mild hookworm infection (96/149, 64.4% of pups) contributed to 15.5% of the secondary infections (GLM, df=148, $P < 0.001$). Anemic pups (n=24, 21.8%) contributed to 71.4% of the new infections, while non-anemic pups (n=86, 71.4%) contributed to 28.6% of them (GLM, df=146, $P < 0.001$) (Figures 3.4d and 3.4e).

Overall, higher levels of virulence, measured as increased probability of mortality or higher levels of anemia in the host, were associated with increased parasite fitness (Figure 3.5f and 5g). However, the relationship between virulence and the level of mortality was non-linear and almost saturated at mortality levels close to 40% (third order polynomial regression, $r^2 = 0.640$, $P < 0.001$) (Figure 5f). This differed from the relationship between parasite fitness and anemia, which was best explained by a linear relationship (linear regression, adj-$r^2 = 0.275$, $P<0.001$) (Figure 3.5g) (table 3.4).

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 found that fur seal hookworms have overcome this problem through tight synchronization with the host

reproductive cycles. However, the particular reproductive biology of fur seals and early clearance of adult hookworms from the pups' intestine gives the hookworm little time for growth, reproduction, eggs shedding, and development of infective-stage larvae. 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 intra-host density. Therefore, a third of the pups born each year suffer significant levels of anemia and mortality as consequence of hookworm infection, yet these pups contribute to more than 70% of new infections. Therefore, increased parasite virulence always pays off in terms of parasite fitness, favoring selection for high virulence. This parasite strategy probably explains why marine hookworms are the most virulent within this parasitic group (Seguel and Gottdenker 2017), and suggests that parasite adaptation to host biological rhythms and life history traits can lead to the emergence of highly virulent pathogens.

Biological rhythms have been proposed as a significant player in host-parasite relationship, however little evidence exist on their role in the selection of high virulence, although theory suggest that such effect is possible (Martinez-Bakker and Helm 2015). In the fur seal hookworm life history there is remarkable timing in egg shedding, which is probably associated with tight 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 approximately 2 weeks after the peak of births. The tight association of this parasite with the fur seal reproductive cycles is probably one the keys for the parasite's success, as egg shedding occurs when the likelihood of finding a new host (females) is also higher. In addition, 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). These factors would explain why population density was the second most important factor contributing to the variance in the parasite fitness.

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 probably explains why this parasite has not evolved successful mechanisms to avoid clearance. In fur seals, 100% of the adult hookworms are dead within 4 to 6 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 hookworm 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, for which extraction of host resources always pays off in terms of fitness, regardless of the adverse consequences for the host in terms of anemia and mortality.

Although the transmission-virulence trade off hypothesis supposes that parasites can evolve different levels of virulence, most host-parasite systems evolve towards intermediate virulence (Alizon et al. 2009, 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 theory 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 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). However, the same adaptations have favored a host-parasite equilibrium where the most virulent nematodes have greater chances of transmission to the next fur seal generation. This suggests that highly virulent pathogens can emerge under natural conditions as a result of adaptations to their host's life history traits and biological cycles.

Why pinniped hookworms have not exploited other alternatives to increase fitness is not clear but it could be related to the reproductive ecology and habitat of fur seals. For a parasite with indirect transmission, increasing the environmental survival of larval stages in the soil would increase its chances of transmission, however fur seal hookworm larvae do not survive from one reproductive season to the next (Lyons et al. 2011). 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. Another significant component of parasite fitness is the infectious period. The universal

and early clearance of hookworms by fur seals significatively reduces the parasite time to release infectious stages. 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.

As predicted by the transmission-virulence trade-off hypothesis, in this parasite-host system, virulence is coupled to transmission and parasite fitness, which can have important consequences for parasite virulence evolution. This study found that transmission is highly synchronized with the host reproductive cycles, which allowed the parasites to be transmitted to adult females, even though it was at the expense of the damage caused in pups where the parasite reproduces. Successful parasites in this system are those that maximize the output of reproductive stages (eggs) in a short period of time, a situation probably linked to parasite clearance and the presence of a higher density of intermediate hosts in the beginning of the host reproductive season. Alternative mechanisms to increase parasite fitness, such as resistance of free living larvae to environmental conditions and evasion of host clearance, have apparently not been developed. Hookworm and fur seal life history traits are markedly linked, which in the context of the evolutionary advantage of parasites over hosts, could indicate a remarkable adaptation of hookworms to their marine mammal hosts. However, such adaptive traits result in the greatest level of virulence among hookworms, showing that the concept of "parasite adaptation" is probably better explained in terms of the parasite fitness, and that it is not necessarily linked to the host health. We provide novel experimental evidence in a large mammal

system that even under long standing host-parasite relationships, highly virulent pathogens can emerge in nature.

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Figures

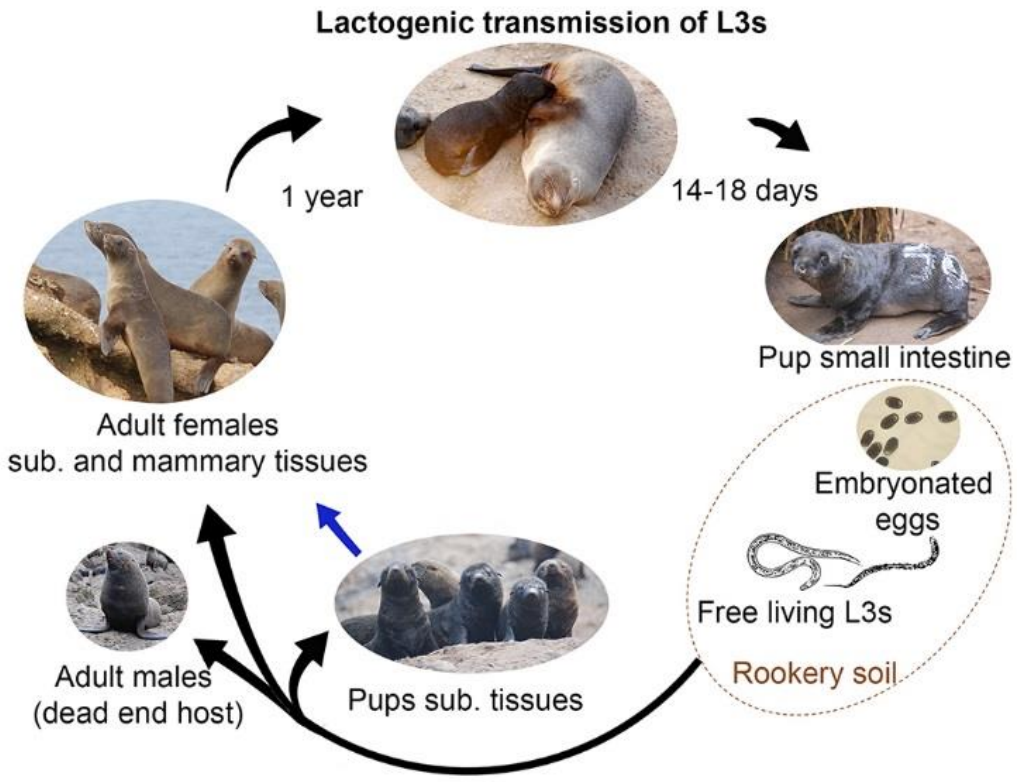

Figure 3.1. Life cycle of *Uncinaria sp*. in South American fur seals (*Arctocephalus australis*). Pups get infected through ingestion of colostrum that contains infective stage 3 larvae (L3s). Within 2-weeks, hookworms reach adulthood in the small intestine and shed embryonated eggs in the pup's feces. 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.

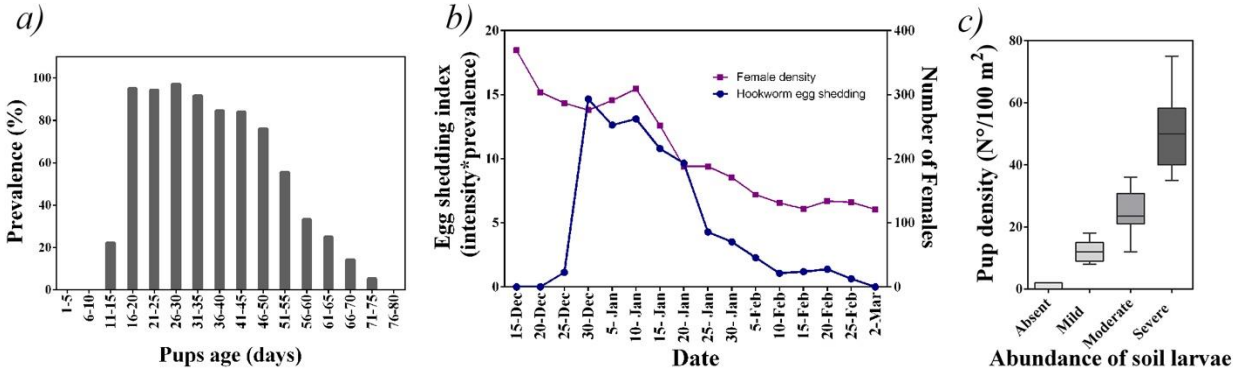

Figure 3.2. Hookworm prevalence, egg shedding and abundance of larvae in the soil are correlated with adult females and pup density. (a) Prevalence reach over 90% when pups are between 20 and 30 days old, then substantially decline and by 75 days-old on average, all pups have cleared hookworm infection. (b) Egg shedding follow a similar curve with the highest number of eggs being shed when the number of 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, df=30, P<0.001).

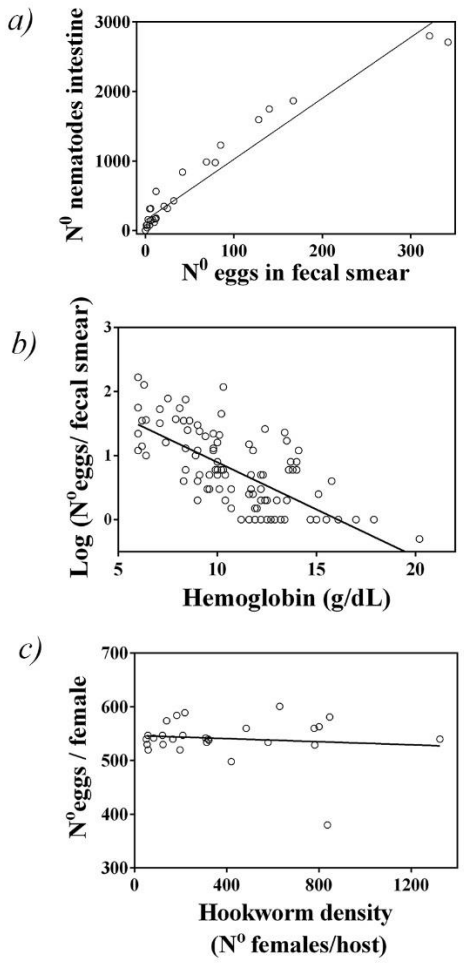

Figure 3.3. Correlations between hookworm burden, egg shedding and extraction of host resources. (a) Hookworm burden is highly correlated with egg shedding in pup's feces (third order polynomial regression, adj-r^2= 0.921, P< 0.001). (b) Hemoglobin concentration decreases as the number of hookworm eggs in pup's feces increase (second order polynomial regression, adj-r^2=0.401, P<0.001), 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, P=0.621), 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).

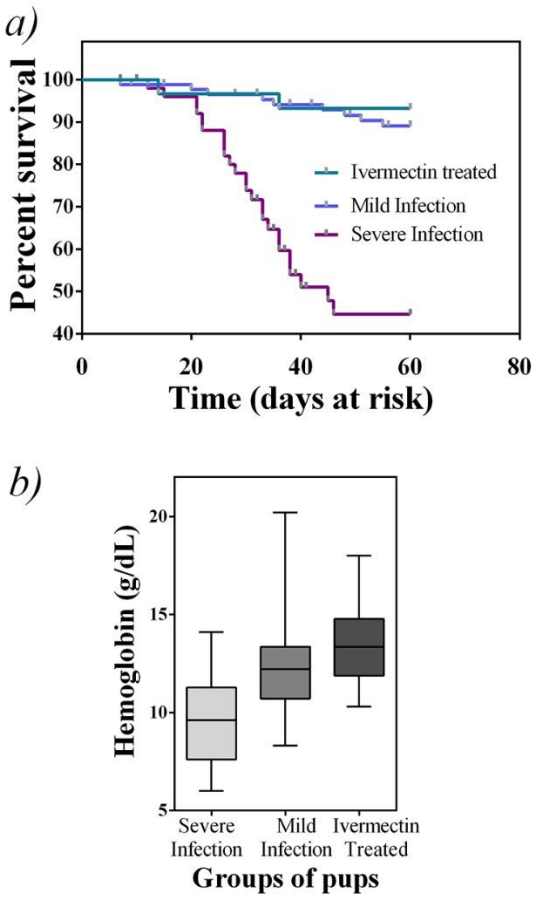

Figure 3.4. Virulence is driven by parasite burden. (a) Survival rates of pups with severe hookworm infection was 44.4%, compared to 90.6% survival of pups with mild hookworm infection and 93.4% survival of pups treated with the antiparasitic ivermectin (Log-rank Mantel-Cox test, X^2= 44.43, df=2, P < 0.001). (b) Hemoglobin concentrations were markedly lower in the group with high parasitic burden (severe infection) (ANOVA, F=31.47, df=2, P<0.001).

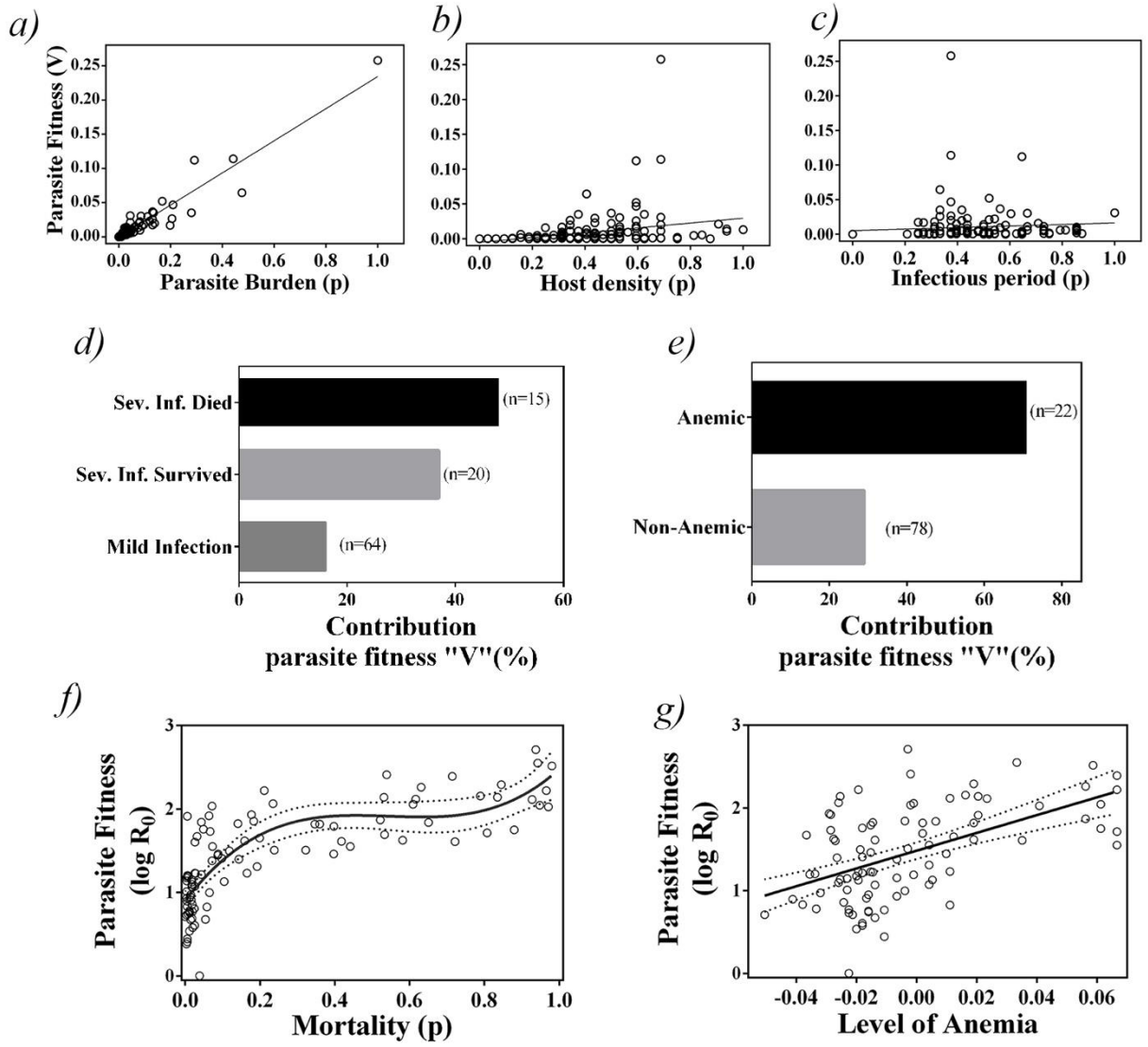

Figure 3.5. Virulence is linked to parasite fitness. (a), (b) and (c) are components of the variation on parasite fitness (V). Most of the variation in the fitness parameter was produced by the parasite burden (a) of each individual, accounting for 53.3% of the variance. Host density (b) contributed to 36.7% of variance while the infectious period had little effect in the overall variation of the fitness index (9.9%) (Principal component analysis). (d) Pups that suffered the worst consequences of parasitism (Sev. Inf. Died), contributed to almost half of the new

infections in the population (c), while animals that suffered severe infection but survived (Sev. Inf. Survived) contributed to 37% of the new infections. Animals with mild infection (Mild Infection) contributed very little to new infections (15.5%) despite being the most numerous group (64% of pups) (GLM, df=144, P<0.001). (e) Pups suffering anemia due to hookworm disease (21.8%) contributed to 71.4% of the new infections, while non-anemic pups (71.4%) contributed to 28.6% of the new infections (GLM, df=148, P <0.001). (f) Parasite fitness (R_0) increases markedly with higher levels of virulence, measured as probability of pup mortality. The solid line represents the best fit model (lowest Akaike Information Criteria) with 95% confidence intervals (dashed lines) (Third order polynomial linear regression, r^2 =0.65, P <0.001). (g) Parasite fitness (R_0) increases markedly in pups with higher levels of virulence, measured as pup's anemia. The 0.0 anemia level represents the anemia threshold (10 g/dL) calculated based on reference values of hemoglobin concentration for this population. The solid line represents the best fit model (lowest Akaike Information Criteria) with 95% confidence intervals (dashed lines) (Multiple linear regression, r^2 = 0.29, P<0.001).

Tables

Table 3.1

Selected models for the curve of parasite burden vs pup and nematode variables

Number of hookworm eggs in feces vs hookworm burden

Model	df	AIC	p-value	Ad-R^2	Observations
Linear	1	0.17053	1.60E-14	0.8499	
Parabolic	2	-14.685	2.20E-16	0.907	
Cubic	3	-19.429	2.20E-16	0.9215	
Quartic	4	-21.734	2.20E-16	0.9287	Fourth term interaction non-significant

Hemoglobin vs number hookworm eggs in feces

Model	df	AIC	p-value	Ad-R^2	Observations
Linear	1	41.7543	6.13E-14	0.3706	
Parabolic	2	36.6677	2.09E-14	0.4011	
Cubic	3	38.1404	1.20E-13	0.3987	Third order interaction not significant

Hookworm female density vs number of eggs per hookworm female

Method	df	AIC	p-value	Ad-R^2	Observations
Linear	1	-132.56	0.6261	-0.03	
Parabolic	2	-131.34	0.6286	-0.0422	
Cubic	3	-129.48	0.7939	-0.0819	

Table 3.2

Coefficients and significance of predictors for hookworm burden in selected negative binomial Generalized Linear Models in South American fur seal pups (*Arctocephalus australis*) infected with hookworms (*Uncinaria sp.*).

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

* Predictors are significant at α=0.05

Table 3.3

Coefficients and significance of predictors of mortality for selected binomial Generalized Linear Models in South American fur seal pups (*Arctocephalus australis*) infected with hookworms (*Uncinaria sp.*)

Predictors														
Hg	WBC	PT	RBC	Hg*RBC	Eggs	BMI	Sex (Male)	Infectious Period	log-lr	X^2	df	p-value	AIC	delta AIC
-0.35*			-6.77E-07*		0.03			5.16*	-34.911	18.8	4	1.29E-07	79.82	
-0.34*	2.39E-05		-7.19E-07*		0.03			5.28*	-34.172	19.5	5	2.19E-07	80.34	0.522
-0.48*			-6.68E-07*					4.73*	-36.429	17.3	3	5.45E-07	80.85	1.028
-0.35*	4.72E-05		-8.23E-07*		0.03	-15.49		6.36*	-33.765	19.9	6	4.57E-07	81.53	1.708
-0.49					0.03			5.08*	-37.109	16.6	3	2.78E-07	82.22	2.398
-0.55			-1.07E-06	4.55E-08	0.02			5.57*	-34.848	18.9	5	1.22E-07	82.77	2.948
-0.59*								4.52*	-38.964	14.7	2	3.75E-07	83.93	4.108
-0.46	4.51E-05	-0.05	-1.04E-06	3.23E-08	0.02	-15.96	-0.15	6.11*	-33.699	20.0	9	7.22E-06	87.39	7.568
-0.54	4.32E-05	-0.07	-1.46E-06		0.03	-16.59	-0.30	6.53*	-33.727	20.0	8	4.42E-07	87.40	7.577

Hg= Hemoglobin, WBC= White blood cell count, PT= Total serum proteins, Eggs= number of hookworm eggs in feces, BMI= body mass index

* Predictors are significant at $\alpha=0.05$

Table 3.4

Selected models for the curve of parasite virulence vs parasite fitness

Mortality vs Parasite Fitness

Model	df	AIC	p-value	Ad-R^2	Observations
Linear	1	-190.219	<0.0001	0.5582	
Parabolic	2	-199.689	<0.0001	0.6021	
Cubic	3	-209.004	<0.0001	0.6409	
Quartic	4	-210.059	<0.0001	0.6481	Fourth term interaction non-significant

Level of Anemia (Hemoglobin) vs Parasite Fitness

Model	df	AIC	p-value	Ad-R^2	Observations
Linear	1	-139.9074	<0.0001	0.2751	
Parabolic	2	-138.6335	<0.0001	0.2728	Second order interaction not significant
Cubic	3	-139.2679	<0.0001	0.285	Second and third order interactions not significant

Level of Anemia (Red blood cell count) vs Parasite Fitness

Method	df	AIC	p-value	Ad-R^2	Observations
Linear	1	-115.7811	0.004499	0.07004	
Parabolic	2	-114.9484	0.01025	0.07136	Second order interaction not significant
Cubic	3	-113.4827	0.022	0.06669	Second and third order interactions not significant

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CHAPTER 4

MATERNAL CARE AFFECTS IMMUNITY AND DISEASE DYNAMICS IN A MARINE MAMMAL.[4]

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[4]Seguel M, F. Montalva, D. Perez-Venegas, J. Gutierrez, H. Paves, A. Muller, C. Valencia, E. Howerth, V. Mendiola, N. Gottdenker. To be submitted to *eLife*

Abstract

Climate models predict that most life in the oceans will be affected by more common and extreme anomalies in sea surface temperature (SST). Marine mammals are very sensitive to changes in ocean conditions, however little is known about how climate change will affect the dynamics of disease and mortality in their populations. The dynamics and mechanisms that drive hookworm disease were studied for 10 years in a population of South American fur seals (*Arctocephalus australis*) at the Chilean Patagonia in order to understand how changes in ocean conditions can affect diseases outcome in these animals. During years with high SST, fur seal females increase their foraging trip lengths and decrease maternal care, suggesting less availability of food. Adult females that provide less maternal care have pups with lower glucose levels and milder T-cell reactivity. These pups fail to clear hookworm infection early in the season and are at higher risk of mortality. Pups with higher levels of maternal attendance have higher glucose levels and produce parasite specific IgG, which along with T-lymphocytes, mast cells and basophils, contribute to nematode clearance from the intestine. In years with high SST, pups have lower levels of glucose, cholesterol and immune parameters associated with hookworm clearance. These changes establish a pattern where increases in SST are associated with higher mean hookworm burden and mortality. This study describes pathophysiological and ecological mechanisms that drive increased mortality of fur seals during years with higher sea surface temperatures, and provides evidence that climate change can affect marine mammal disease dynamics through effects on the immune response to virulent parasites.

Introduction

Changes in ocean temperature, pH, and other physic and chemical properties can alter nutrient exchange cycles and affect the dynamics of phytoplankton, causing a decline in upstream food chain heterotrophic organisms, including key pelagic and mesopelagic fish species (Lewandowska et al. 2014). Current climate models and empirical evidence indicate that warm oceans will become more common and temperature anomalies more extreme (Nagelkerken and Connell 2015). Therefore, it is anticipated that most ocean life will be affected by current changes in global temperatures and high levels of atmospheric carbon dioxide (Nagelkerken and Connell 2015). However, little is known on the adaptability of different species to rapidly changing ocean conditions, and if through compensatory mechanisms marine species will be able to avoid substantial decline in their populations.

Marine mammals are a diverse group of animals highly sensitive to changes in aquatic ecosystems because of their key role as top predators in food webs (Gulland and Hall 2007). The best documented examples of the effects of ocean temperature anomalies on marine mammals occur during ENSO events, in which substantial increase in ocean sea surface temperature (SST) causes a decline in marine mammal prey leading to abortions and up to 100% pup mortality in sea lions (Soto et al. 2004, Fuxing et al. 2017). The effects of ocean temperature anomalies are often dramatic in fur seals and sea lions (family: *Otariidae*) because of the reproductive ecology of these species. Reproductively active otariids congregate during summer months in rookeries in order to give birth and mate (Soto et al. 2006), and during this period, pups obtain energy from their mothers, who alternate periods of pup nursing on land with days of foraging at sea. Therefore, if prey become less available, females switch their diet and/or increase the length of their foraging trips. These changes are usually associated with a decrease in the time females can

spend with their pups (maternal attendance), affecting the transfer of energy to the pups through the milk (Maniscalco et al. 2006). Declines in maternal attendance have been associated with decreased pup growth rates and survival (Maniscalco et al. 2006, Soto et al. 2006), however, it is not well understood if sustained increase in ocean temperature could have more subtle and chronic effects in otariid populations that extend beyond energy balance. Current evidence suggests that at least some significant population fitness components, such as genetic heterozygosity, can change in response to warming ocean conditions. In the South Atlantic, a declining Antarctic fur seal (*Arctocephalus gazella*) population has increased heterozygosity over the last 30 years, probably as an adaptation to decreased food availability due to warming ocean temperatures as part of global climate change (Forcada and Hoffman 2014). However, it is not known if changes in ocean productivity and temperature favor survival of genetically more diverse individuals, and/or if these environmental changes primarily impact how organisms can respond to infectious diseases. In the case of aquatic organisms other than mammals and birds, changes in patterns and severity of diseases as consequence of climate change are well documented (Burge et al. 2014). In most of these cases, changes in disease severity and distribution are associated with warmer or more acid ocean conditions, and therefore affect pathogens transmitted in the water column that are temperature or pH sensitive (Burge et al. 2014). In the case of marine mammals, there is no current evidence of changes in disease dynamics due to climate change or how warming oceans could affect marine mammal health, but preliminary evidence suggests that such effects could exist. California sea lions (*Zalophus californianus*) born in years with warmer SST have signs of nutritional stress and show a weaker immune response when compared to pups born in years with low SST (Banuet-Martinez et al.

2017). However, how these changes in immunity could affect disease dynamics and survival is still unknown.

South American fur seals (*Arctocephalus australis*) are a widespread otariid species in the South Pacific and Atlantic oceans (Rodrigues et al. in press). However, populations in the Chilean Patagonia experienced a decline close to 60% percent 20 years ago and, despite current legal protection, populations have failed to thrive to historical numbers (Seguel and Paves, in review). The main cause of fur seal mortality in Chilean Patagonia is hookworm disease, caused by *Uncinaria sp*., a blood feeding nematode that reaches nearly 100% prevalence and kills up to 20% of the pups born each year on Guafo Island (Seguel et al. 2013, Seguel et al. in review). Hookworms have been described in nearly all otariid species, and their effects are apparently regional, with some populations suffering little mortality while others experience up to 70% of hookworm related mortality during epidemics (Spraker et al. 2007, Seguel and Gottdenker 2017). Despite the fact that some populations are apparently not affected at the population level, the lack of other significant pathogens capable of similar levels of mortality among otariids, make hookworm disease the most significant infectious disease of young fur seals and sea lions (Seguel and Gottdenker 2017). Long term studies in fur seal populations show that hookworm prevalence and mortality varies over time, but the mechanisms driving these patterns are unknown (Lyons et al. 2011).

Otariid hookworm infections are unique compared to hookworms of terrestrial mammals because most infected pups clear intestinal infection a few months after initial exposure to infective larvae (Lyons et al. 2011, Seguel et al. in review). Most terrestrial hookworms establish chronic infections in their host (Seguel and Gottdenker 2017), but for some laboratory animal models of hookworm infection, such as *Nippostrongylus brasilienses* in rodents, hookworms are

naturally cleared from the host gut through an immune mediated mechanism (Cortes et al. 2017). In the case of fur seals, it is possible that a similar process occurs in the intestine, since animals that die due of hookworm infection have morphological evidence of less active inflammation when compared to animals infected with hookworm but that die of unrelated causes (e.g. trauma) (Seguel et al. 2017). Given the known sensitivity of fur seal and sea lion immune system to changes in ocean conditions (Banuet-Martinez et al. 2017), and the potential link between immune response and hookworm disease, it is possible that through an indirect mechanism, ocean temperature and productivity affect disease dynamics in fur seals. This could happen if foraging strategies of females impact the pup's immune response to hookworm infection. In that case, in years with high SST and lower prey availability, females would have to increase their foraging trip length, decreasing maternal attendance and affecting the energy input in their pups. Pups with lower energy balance could be less likely to mount a strong immune response against hookworms and die. If this sequence of changes occur at the Pacific Patagonia during warm ocean conditions, there should be a strong positive correlation between SST and hookworm mortality.

To test the hypothesis that hookworm disease dynamics are correlated with ocean temperature and productivity we examined 10 years of records on hookworm disease and SST in a rookery of fur seals at Guafo Island, Chilean Patagonia. Several controlled field experiments were performed in order to determine the most significant epidemiologic components and pathophysiologic mechanisms that drive inter-seasonal variation in hookworm mortality. The effect of fur seal foraging strategy and maternal attendance on the factors that drive hookworm disease was later assessed by continuous monitoring of the fur seal rookery mother-pup pairs and controlled deworming and immune challenge experiments. Finally, we tested if the metabolic

and immune elements that drive hookworm disease, and that are affected by fur seal foraging strategies, followed the expected pattern across reproductive seasons related to SST.

In this manuscript, we provide novel data on the pathophysiological and ecological mechanisms that drive increased mortality of fur seals during years with higher sea surface temperatures. Additionally, it provides evidence that climate change can affect the immune response and disease dynamics of marine mammals.

Materials and Methods

a) Fur seal population, ocean temperature and primary productivity data

In Austral summers of 2004-2008 and 2012-2017, fresh fur seal pup carcasses were retrieved from the South American fur seal rookery at Guafo Island, Northern Chilean Patagonia (43° 35′ 34.9″ S, 74° 42′ 48.53″ W). Complete necropsies and histopathology were performed on these carcasses as previously described, in order to determine the cause of death of each pup (Seguel et al. 2011, Seguel et al. 2013, Seguel et al. 2017).

During necropsies all parasites were collected and stored in 5% formalin for later counting (Seguel et al. 2017). The median of the number of hookworms per pup in a given year was used as a measurement of hookworm burden for that particular season. The yearly prevalence of hookworm infection was calculated as the total number of pups with hookworms at necropsy divided by the total number of pups necropsied during that season.

Sea surface temperature and chlorophyll-a satellite data was retrieved from NASA earth observation (NEO) website (https://neo.sci.gsfc.nasa.gov). The latitude and longitude to retrieve the chlorophyll-a and temperature data were selected to represent standard points 25 to 50 Km

west, south, north and east of the South American fur seal rookery at Guafo Island. This approach was used to represent all the potential foraging areas of fur seals at Guafo Island.

b) Females foraging trip length and maternal attendance

In 2007 and 2017 observational studies were conducted in previously marked SAFS pups. Pups were observed daily for 1.5 hours in the AM and 1.5 hours in the PM. If a female was with her pup, this was characterized as nursing, and if a female was not present at the rookery and her pup was alone, this was characterized as foraging. In order to confirm mother-pup pairs fidelity 10 adult females and their pups were captured and marked and observed during the study period. All female-pup pairs showed high fidelity and allo-sucking events were not observed. For data analyses, a maternal attendance index was calculated by dividing the number of observations of a pup with its mother by the number of times observed alone. Adult female's foraging trip length was calculated by adding the number of observation periods (12 hours intervals) when the female was not present at the rookery and her pup was alone. Only animals with continuous observations were included in the data series.

c) Fur seal pup health assessment

From 2012 through 2017 South American fur seal pups were captured by hand every 7 to 15 days between December 15th and March 10th. At the first capture, pups were marked with a number in the fur using commercial hair decoloring solution. During each capture procedure, standard length, weight, sex and body condition were recorded. The pup age was calculated based on the peak of parturition for Guafo Island rookery (December 15, Paves et al. 2016) and for a group age was exactly known because their parturition was observed and they were marked

24 hours later. Blood was drawn from the caudal gluteal vein of pups into EDTA, heparin and plain (serum) vacutainer tubes. Plain blood tubes were centrifuged within 1-3 hours post-collection in the field laboratory to obtain serum, which was preserved at -20°C until later long term storage (-80°C) or analyses in the mainland laboratory. Plasma was obtained and stored following similar procedures with heparin non-coagulated blood. During each capture procedure, a rectal swab was collected and stored in Sheather's sucrose for later semi-quantitative determination of hookworm egg burden according to standardized methods for this fur seal population (Seguel et al. submitted). Hookworm burden of pups found dead was determined by collection, sexing and counting of all nematodes present in the small intestine and correlated with egg burden through a fecal swab collected during necropsy (Seguel et al. 2017, Seguel et al. submitted). Using non-coagulated blood, hematocrit, hemoglobin concentration, total red blood cell count (RBC), total white blood cell count (WBC) and differential leukocyte counting were determined for each pup as previously described (Seguel et al. 2016). The total concentration of albumin, globulins, cholesterol, glucose, triglycerides, blood urea nitrogen and creatinine were determined in the mainland lab using serum and previously described methods for this population (Seguel et al. 2016).

d) Immune challenge experiments

In 2016 and 2017, a subset of pups (n=65) was challenged with injection of 0.1 ml of a 1.0 mg * ml $^{-1}$ solution of phytohemagglutinin (PHA) in the interdigital skin of the right posterior flipper (Vera-Massieu et al. 2017). The same volume of a saline solution was injected in the same location of left flipper (control). Swelling was measured in both injection sites 12 hours after challenge and a 4mm punch biopsy was collected following anesthesia with 5% isoflurane.

Biopsy samples were stored in 10% buffered formalin and routinely processed for histopathology. Groups of pups selected for PHA challenge included animals treated at day 1 or 2 of life with 300 µg*Kg^{-1} of ivermectin (antiparasitic drug, Ivomec, Merial ®). Because of the exclusive transmission of fur seal hookworms' through colostrum (Seguel et al. in review), these pups were never exposed to adult hookworms in the intestine or re-infected and were thus categorized as the "non-exposed" group (n=14). The second group consisted of pups infected with hookworms and treated with the same dose of ivermectin 2 weeks prior to PHA challenge (hookworm infected-treated group, n=13). The third group (n=19) was composed of animals that at the time of PHA challenge (first week February) had cleared a previously significant hookworm infection as determined by previous clinical, hematological and coprological analyses. The last group of pups consisted of animals infected with a significant hookworm burden (more than 6 eggs per fecal smear) at the time of PHA challenge.

e) ELISAs

A parasite specific IgG ELISA was developed using whole worm extract as antigen. Fresh hookworms were collected during necropsies at Guafo Island, washed in PBS and frozen at -20^{o}C in the field until transported to the mainland laboratory where they were stored at -80^{o}C. Thawed nematodes were macerated in phosphate buffered saline (PBS) using a glass homogenizer. The macerated nematodes were centrifuged at 15,000 RPM, 4^{o}C for 1 hour. Supernatant was collected, filtered and total protein concentration determined using Bradford, bicinchoninic acid and "NanoDrop®" methods. Extracts were diluted in PBS for a final protein concentration of 1.6 µg/ml. High binding ELISA plates were coated overnight at 4^{o}C using 100 µL per well of diluted (1:100) hookworm extract. Plates were washed with PBS/Tween and

100µL of sample (fur seal serum) diluted in 5% dry milk/PBS were added to each well and incubated for 5 minutes at room temperature. A serial dilution of pooled fur seal serum sample from samples with high absorbance in previous experiments was used to construct a standard curve in each assay. Plates were washed 3 times and 100µL of TMB was added to each well and incubated for 30 min at room temperature. ELISA reaction was stopped using 100µL per well of 1.0 N HCl and the plate was read at 450 nm wave length absorbance. The anti-hookworm IgG concentrations were calculated semi quantitatively by comparing the optic density (OD) of the standard curve with the OD of the samples and reported as arbitrary units (AU). All these reactions were run in duplicate.

f) Special stains and Immunohistochemistry

Sections of small intestine from necropsied pups and the skin sections of pups that underwent PHA immune challenge were routinely processed for histopathology and immunolabelled with antibodies against CD3, Iba1, CD79a, CD21, CD127 (c-kit), MUM1 and IL-4. The details of the antibodies used, retrieval and visualization methods and dilutions are provided in table 4.1. General steps applied to all IHC protocols included deparaffinization of 4 µm tissue sections through immersion in xylene, and rehydration with graduated alcohols, antigen retrieval, quenching of endogenous peroxidase with hydrogen peroxide 3% for 15-20 minutes, incubation with primary antibody, blocking of nonspecific binding sites with a commercial blocking solution (Power Block, DAKO®, Carpinteria, CA, USA), incubation with biotinylated secondary antibody (1:100 dilution, Vector Laboratories, Burlingame, CA) at room temperature for 20-30 minutes and with horseradish peroxidase labeled streptavidin for 15 minutes (Biocare®, Chicago, IL). Antigen antibody complexes were visualized by incubation at room temperature

for 5 minutes with diaminobenzidine (DAB) (Vector Laboratories, Burlingame, CA). Slides were counterstained with hematoxylin, dehydrated and coverslipped. Tissue sections were observed in an optic microscope and representative sections photographed. The number of cells with positive imunolabelling were counted using the digital images with the use of the counting function of Adobe Photoshop®. Sections from small intestine were stained through PAS-Alcian blue reaction to detect the number of goblet cells and amount of mucin produced. Slides were examined and standard sections photograph in order to calculate the amount of mucin present in the intestine. This number was calculated using Adobe Photoshop® selection and calibration tools as the proportion of the total photographed area that stained positive with PAS-Alcian blue.

In order to detect the site of binding of anti-hookworm IgG in the body of nematodes formalin-fixed hookworms obtained during necropsies of SAFS pups were routinely processed for histopathology as previously described (Seguel et al. 2017). Antigen retrieval was performed in citrate pH 6.0 for 10 minutes at 120°C. Blocking of nonspecific binding sites was done by incubation with 10% dry milk/PBS for 20 minutes and quenching of endogenous peroxidase by incubation with hydrogen peroxidase 3% for 30 minutes. After 3 washes with PBS slides were incubated for 1 hour at room temperature with SAFS pup serum that had the highest (strongly positive) or lowest (negative) absorbance during ELISA experiments, diluted (1:50) in 3% bovine serum albumin. After 3 washes with dilution buffer TWEEN® (DBT) slides were incubated with biotinylated protein-A (1:1000 dilution) (Vector laboratories, Burlingame, CA) for 20 minutes at room temperature to detect IgG. Slides were washed 3 times with DBT and incubated with streptavidin horseradish peroxidase for 15 minutes at room temperature. Antigen antibody complexes were visualized by incubation with DAB for 5 minutes at room temperature. Slides were counterstained with hematoxylin, dehydrated and coverslip.

g) Data analyses

To assess a potential correlation between SST, Chlorophyll-a and hookworm related variables in the fur seal population, linear and polynomial models were run between hookworm variables and SST and Chlorophyll-a data at the different geographical points and using the average of those measurements for the months of December through March (fur seal reproductive season). Models to describe the correlation between two variables were selected based on the lowest AICc and high R^2 values for that particular relationship.

To identify the factors that affected hookworm mortality, logistic models were fitted using Firth's penalization method due to partial separation of data points in binomial GLMs. Predictors of mortality tested in different models included the average serum or plasmatic concentrations of albumin, globulins, cholesterol, glucose, triglycerides, average blood hemoglobin concentration, the maternal attendance index, pup growth rate, hookworm infectious period, the average and highest hookworm burden detected in the pup and the average number of peripheral blood lymphocytes, neutrophils, eosinophils, basophils and macrophages. Selected models were ranked based on Akaike's information criteria and statistical inference was performed based on the coefficients and p-values of top ranked models.

Based in the recapture data, pups were assigned to different phases of the hookworm infection. These included; prepatent period, which corresponded to the phase when a pup had fresh or recently dried umbilical cord and was negative for hookworms at coprological examination; the patent period, which corresponded to the capture when pups had hookworm eggs in their feces; the clearance period, which corresponded to the capture when pups had a significant decline (more than 50%) in their hookworm burden according to their coprological

exam and compared to the previous captures (Seguel et al. in press); and post-clearance period, which corresponded to the captures when pups with previously positive coprological exam had no hookworm eggs in their feces. The immunological parameters obtained through complete CBCs, serum chemistry and ELISAs were compared at these different stages through repeated measures ANOVA or Friedman tests according to type (counting or continuous) and distribution of data (normal vs skewed). Additionally, the mean of immunological, metabolic and maternal care parameters at each infection stages were compared to the mean of the immunological parameters of age matched control pups and pups that died due to hookworm disease through one-way ANOVAs or Kruskal-Wallis tests. Control pups corresponded to animals that never presented patent hookworm infection because they were treated with ivermectin during their first 5 days of life.

Animals found dead were assumed to be pups undergoing hookworm clearance based on previous clinical data on that particular pup and/or findings at necropsy. These pups usually died due to drowning or trauma. Pups were assumed to die because of hookworm enteritis and bacteremia according to previously established criteria to diagnose this condition (Seguel et al. 2017). The number of immune cells and amount of mucin in these two groups were compared through Kruskall-Wallis and Dunn's multiple comparison tests. The level of swelling in pups undergoing PHA immune challenge were compared among groups through one-way ANOVA and Tukey's multiple comparison test. The number of leukocytes in the PHA injection site were compared through Kruskall-Wallis test and Dunn's multiple comparison test.

In order to determine the factors that affected the level of T-lymphocytes response in the pups challenged with PHA, GLMs with negative binomial distribution were fitted using hookworm burden, number of leukocytes in peripheral blood (basophils, lymphocytes, etc.),

growth rate, body mass index, sex, experimental group and concentration of glucose, cholesterol and hemoglobin as predictors of the number of CD3 positive cells in skin biopsies in the global model. Selected models were ranked based on AIC and statistical inference made based on top ranked models (delta AIC >7.0).

Results

a) Hookworm prevalence, mortality and burden increase with sea surface temperature and lower chlorophyll-a concentrations

The yearly hookworm prevalence ranged from 81% to 100%, the median hookworm burden ranged from 210 to 940 nematodes and hookworm mortality ranged from 13% to 50% of all pups found dead (Table 4.2). Hookworm prevalence, burden and mortality tended to be higher in years with higher SST (linear regression, Ad-R^2=0.50-0.86, P<0.01) but this trend was not significant for prevalence (linear regression, Ad-R^2=0.29, P=0.061). Hookworm prevalence, burden and mortality were higher in years with lower chlorophyll-a concentrations (polynomial regression, Ad-R^2=0.46-0.70, P<0.05), but the trend for hookworm burden was not significant (polynomial regression, Ad-R^2=0.29, P=0.12). The relationship between hookworm epidemiological variables and chlorophyll-a concentrations was not linear because of an anomaly in 2015, when high chlorophyll-a concentration did not translate in a significant decrease on hookworm prevalence, burden or mortality (Figure 4.1).

b) Hookworm clearance dominates infection dynamics and affects pup survival

The lifecycle of hookworm (*Uncinaria sp*.) in South American fur seals at Guafo Island has been described elsewhere (Seguel et al. in review). Based in the life cycle and coprological

analyses in each pup recapture, the hookworm life span in the pup's intestine ranged from 20 to 70 days and the infectious period between 5 and 50 days, however in 90% of the pups the number of days a pup released hookworm eggs (the infectious period) ranged from 14-46 days with a mean of 29 days. The infectious period, along with the average plasma concentration of glucose, were the factors that most consistently predicted mortality in the survival models assessed (Table 4.3). Pups that experienced earlier hookworm clearance and that had on average higher concentration of glucose had a higher chance of surviving hookworm infection (Firth's logistic regression, likelihood ratio=45.69, df=78, $P<0.001$).

c) Hookworm clearance is immune mediated

During the hookworm patent and clearance period, fur seal pups that eliminated the nematodes and survived infection experienced a significant increase in the number of peripheral blood lymphocytes and basophils when compared to age matched controls and to the pups that died due to hookworm infection (Dunn's multiple comparison test, mean rank differences=24.2-30.2, $P<0.001$) (Figure 4.2). The number of neutrophils in peripheral blood were similar in the three groups (Kruskal-Wallis statistic=6.82, P=0.236), whereas monocytes and eosinophils were higher in animals infected with hookworms during the clearance period when compared to controls (Mann-Whitney U=192.0-312.0, P=0.0138-0.019). Pups that cleared the infection developed medium to high levels of parasite specific IgG, whereas the level of these antibodies was significantly lower in pups that died due to hookworm infection and almost non-existent in the control group (Dunn's multiple comparison test, mean rank differences=28.4-41.2, $P<0.0001$). There was moderate to marked immunolabelling of the hookworm intestinal brush border using the serum from pups with moderate to high levels of parasite specific IgG (23-100

arbitrary units) (Figure 2), suggesting that antibodies bind proteins contained in the hookworm intestine. There was no immunolabelling in hookworms intestine using the serum from pups with low levels of antibodies (4-10 arbitrary units) in ELISAs or that were never exposed to adult hookworms in the intestine (controls).

The small intestine mucosa and submucosa of pups undergoing hookworm clearance contained larger numbers of T-lymphocytes, plasma cells and mast cells than the intestinal mucosa of pups that died due to hookworm infection or pups never infected with adult intestinal *Uncinaria sp.* (Kruskall-Wallis statistic= 30.78, P<0.001). Similarly, there was more mucus in the mucosa of pups undergoing clearance and more leukocytes expressing IL-4 when compared to controls and pups with hookworm enteritis and bacteremia (Kruskall-Wallis statistic= 23.4-28.2, P-values<0.0001). This latter group, however, had larger numbers of macrophages in the intestinal mucosa and submucosa compared to pups never infected with hookworms and pups clearing hookworm infection (Kruskall-Wallis statistic=29.61, P<0.001) (Figure 4.3).

Pups never infected with adult hookworms had the lowest levels of skin swelling and recruitment of T-lymphocytes, macrophages and neutrophils in response to the PHA immune challenge, whereas pups that have cleared hookworm infection at the time of the challenge had the highest level of swelling and leukocytes recruitment (One-way ANOVA, F=8.690, P<0.001, Kruskall-Wallis statistic=49.74, P<0.001). Pups infected with hookworms at the time of challenge had lower levels of swelling and T-lymphocyte, neutrophil and macrophages recruitment compared to pups that cleared hookworm infection but higher swelling and numbers of leukocytes at site of injection compared to pups never exposed to adult hookworms (Dunn's multiple comparison test, mean rank differences 13.32-26.65, P-values<0.001) (Figure 4.4).

d) Maternal attendance affects fur seal pup energy balance, immune response and hookworm clearance.

The duration of fur seal adult female foraging trips was correlated with levels of maternal attendance (linear regression, R^2=0.41, P=0.0018). In 2007, a year with SST below Guafo Island historical average, foraging trip duration was shorter than 2017, a year with SST slightly above the average. Additionally, pups born in 2007 experienced higher levels of maternal attendance and faster growth rates when compared to pups born in 2017 (Figure 4.5). In 2017, pups with higher levels of maternal attendance had shorter infectious periods (experience earlier hookworm clearance), higher levels of parasite specific IgG and faster growth rates (GLM, df=76, P<0.001). During the same reproductive season, pups that cleared hookworm infection and survived had significatively faster growth rates and higher levels of maternal attendance, glycaemia and cholesterol when compared to pups that died due to hookworm disease (Kruskal-Wallis tests statistic=45-98, P-values <0.0001-0.02) (Figure 4.6). Overall, maternal attendance, the number of peripheral blood lymphocytes, parasite specific IgG, glucose, and cholesterol are higher in years with increased ocean productivity (low sea surface temperature and high concentration of chlorophyll-a) (Figure 4.6). Similarly, the hookworm infectious period is shorter in years with high ocean productivity (GLM, X^2=6.95, df=1, P=0.00036). Along with hookworm infection status (clearance, infected, never infected, treated), higher maternal attendance and glucose blood concentration, and faster growth rates were associated with a more intense recruitment of T-lymphocytes during PHA immune challenges (GLM with negative binomial distribution, log-likelihood= -308.393, df=54, P<0.001) (Table 4).

Discussion

Hookworm disease is the main cause of pup mortality in South American fur seals in Chilean Patagonia. However, the extent of this mortality varies significatively among seasons, variations that follow a similar pattern to changes in sea surface temperature and indices of ocean productivity such as chlorophyll-a. Since fur seal pups do not forage in the sea and depend completely on maternal care for early growth, adult females are a critical link between hookworm infection dynamics and oceanographic variables. Hookworm transmission to fur seal pups is exclusively through their mother's colostrum and occurs only during the first 1-5 days of life (Lyons et al 2011, Seguel et al. in review). Therefore, fur seal pup hookworm burden and mortality should be driven by transfer of larvae through their mother's milk or by intra pup hookworm dynamics. Although it is likely that there are differences among adult female fur seals in the concentration of larvae in their colostrum, this variation likely does not completely explain the observed patterns of hookworm mortality. Although only pups with high hookworm burdens will suffer the worst consequences of parasitism (Seguel et al. 2017), many pups with high hookworm burden survive infection due to well-developed immune mediated hookworm clearance. Therefore, in most models explaining mortality, it is not hookworm burden but the infectious period, the length of hookworm infection, which is the best predictor of mortality. This suggests that maternal foraging ecology affects hookworm dynamics probably due to indirect effects on their pup's health.

The current study shows, that in fur seals, hookworm clearance is an immune mediated process critical for pup survival. Hookworm immune clearance in fur seal pups is similar to animal laboratory models of hookworm infection such as *Nippostrongylus brazilienses* in rodents. In these host species *N. brazilienses* clearance from the intestine is related to recruitment

of T-lymphocytes, IL-4 production and switching of plasma cells into IgA or IgE type cells. IL-4 also promotes the recruitment of mast cells and production of IL-13 and peptides that cause goblet cell hyperplasia, and increase mucus production and gut motility (Cortes et al. 2017). These changes in the intestinal mucosa create a hostile environment for hookworm attachment and feeding, leading to clearance from the intestine (Ohnmacht and Voehringer 2010). In this study, similar changes were observed in the intestinal mucosa of pups undergoing clearance, although the role of IgA and IgE in the intestine could not be clearly determined due to failure to label these immunoglobulins using anti-dog reagents. However, a key role was identified for a parasite specific IgG, which is produced only in pups exposed to adult hookworms in the intestine. This immunoglobulin binds the intestinal brush border of the hookworm, an anatomical location where hookworms contain several digestive and heme-detoxifying enzymes that are crucial for the nematode blood digestion and survival (Williamson et al. 2003, Wei et al. 2016). In fur seals, it is possible that parasite specific IgG reaches the nematode intestine with each blood meal, impairing the nematode blood digestion and favoring clearance. A similar mechanism has been experimentally induced in dogs and humans through a hookworm vaccine that is currently undergoing clinical trials in people with promising preliminary results (Hotez et al. 2016, Diemert et al. 2017). Although this mechanism of vaccine does not avoid infection, prevents the worst consequence of hookworm infection in humans; chronic anemia (Diemert et al. 2017), in a similar manner to how fur seal pups with higher levels of protective antibodies avoid the worst consequences of fur seal hookworm infection; prolonged disease and death.

Fur seal pups that experience early hookworm clearance exhibit a more active immune system. In response to a lectin-based immune challenge these pups recruit larger numbers of T-lymphocytes, macrophages and neutrophils when compared to pups with late hookworm

clearance. These results are within expected limits since hookworm clearance is immune mediated, therefore it is predictable that pups with a more responsive immune system will clear hookworms earlier. However, the finding of mildest inflammatory response in animals never exposed to adult hookworms is unexpected as in most mammalian systems hookworms are strongly immunomodulatory, usually suppressing Th2 and Th1 immune responses (Maizels and McSorley 2016, Cortes et al. 2017). In the fur seal system, however, hookworms apparently stimulate the immune system and favor a stronger immune response to a non-specific stimulus. The lack of strong parasite-mediated immunosuppression in fur seals could be the result of parasite adaptation to the population dynamics of fur seals. These marine hookworms attain high levels of transmission, not by prolonging their stay in the host intestine but by increasing the rate of host resource extraction and egg production (Seguel et al. in review). Therefore, it is possible that fur seal hookworms have not developed strong immunosuppressive mechanisms.

Fur seal and sea lion maternal attendance patterns can be affected by several factors, including prey availability, maternal experience and body condition (Francis et al. 1998, Georges and Guinet 2000, Arnould and Hindell 2001, Soto et al. 2006). In South American fur seals on Guafo Island, maternal attendance was higher in a year with lower sea surface temperature and higher chlorophyll-a concentrations, suggesting that in this species, as in most otariids, ocean productivity and prey availability affect maternal attendance patterns (Soto et al. 2006, Jeanniard-Du-dot et al. 2017). Otariid mothers compensate for the decline of prey species in the environment by increasing the length of their foraging trip in order to obtain the energy necessary to produce enough milk for their pup (Soto et al. 2006). However, this result in a decrease in the attendance or time spent with their pup onshore (Francis et al. 1998, Soto et al. 2006, Jeanniard-Du-dot et al. 2017). These changes explain the variation on foraging and

maternal attendance behavior observed between seasons with low and high ocean productivity in otariids of this and other studies (Soto et al. 2006). However, factors such as maternal experience and body condition usually explain intra-seasonal variation in foraging trips length and maternal attendance patterns (Maniscalco et al. 2006, Jeanniard-Du-dot et al. 2017). Older females tend to give birth to heavier pups and have increased levels of maternal attendance (Francis et al. 1988, George and Guinet 2000). These females probably have more experience and better foraging strategies than young females, which translates in higher efficiency of energy transfer to their pups (Arnould and Hindell 2001, Maniscalco et al. 2006). These differences between females could explain the intra-seasonal variation in maternal attendance observed in the South American fur seals of this study. Although capture of each female was not possible to assess the effect of female age/size, body condition and metabolic status on maternal attendance, it was evident that maternal attendance explained a large proportion of the variation in pup growth rate and metabolic state. Pups with higher levels of glucose were more likely to clear and survive hookworm infection. Since control pups had glucose levels slightly lower to infected pups that survived, it is more likely that glucose levels are a cause rather than a consequence of infection outcome. It is well-establish that T-cell dependent immune mechanisms are regulated by glucose metabolism and that hypoglicaemic states decrease the reactivity of T-cells (Palmer et al. 2015). Therefore is likely that in years with low ocean productivity, fur seal pups experience nutritional stress, which translates in a less active T-cell dependent immune response undermining their defense mechanisms to hookworm infection. The finding that besides experimental groups, the level of T-cell recruitment in pups challenged with PHA was largely dependent on mean blood glucose values supports this hypothesis, and along with the described changes in foraging

behavior and maternal care during years with high SST establish the necessary link to explain the correlation between oceanographic environmental variables and hookworm disease dynamics.

In the Chilean Patagonia, during years with high SST, ocean productivity decreases, forcing adult female fur seals to increase their foraging trip length and decrease their levels of maternal attendance. Pups receiving less maternal care had reduced growth rates and early signs of undernutrition. Additionally, these pups' immune system is hyporeactive compromising their ability to mount an effective immune response against hookworms in order to expel the parasite from the gut. These pups, with longer hookworm infection periods, usually die as consequence of hookworm disease establishing a pattern where hookworm disease severity and mortality are correlated to indices of oceanographic environmental conditions such as sea surface temperature. The sensitivity of otariid hookworm disease to increases in ocean temperature present a scenario where global climate change will likely increase the extent and severity of a disease present in most fur seal and sea lion populations.

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Figures

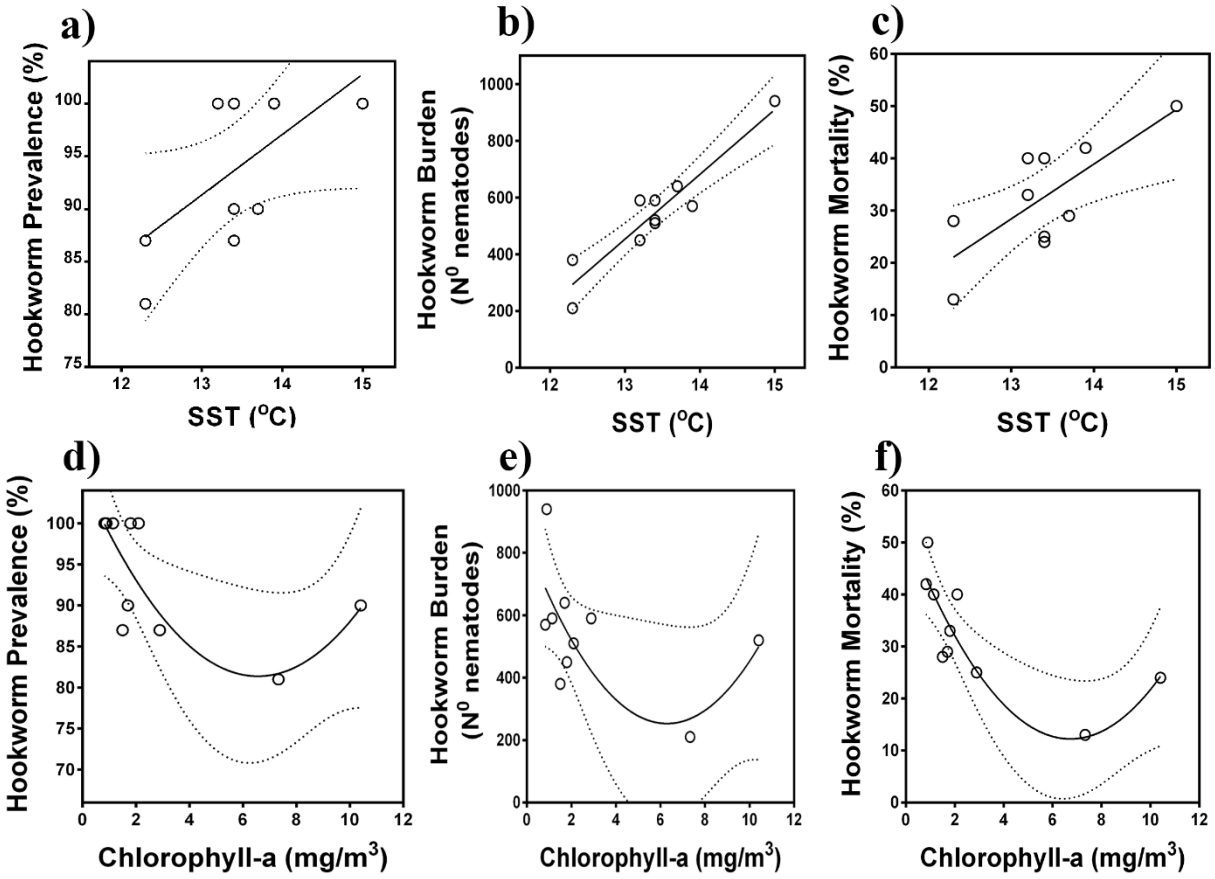

Figure 4.1. Correlation between oceanographic parameters (sea surface temperature and chlorophyll-a) and hookworm disease dynamics in South American fur seals (*Arctocephalus australis*) at the Chilean Patagonia. Hookworm prevalence (a), burden (b) and mortality (c) increase in years with warmer sea surface temperature (Linear regressions. Hookworm prevalence (a), Ad-R^2=0.29, P=0.064. Hookworm burden (b), Ad-R^2=0.86, P<0.001. Hookworm mortality (c), Ad-R^2=0.56, P=0.016). Hookworm prevalence (d), burden (e), and mortality (f) decrease in some years with higher primary productivity (Second order polynomial regressions. Hookworm prevalence (d), Ad-R^2=0.46, P=0.046. Hookworm burden (b), Ad-R^2=0.29, P<0.123.

Hookworm mortality (c), Ad-R^2=0.70, P=0.005). Dashed lines represent 95% confidence intervals.

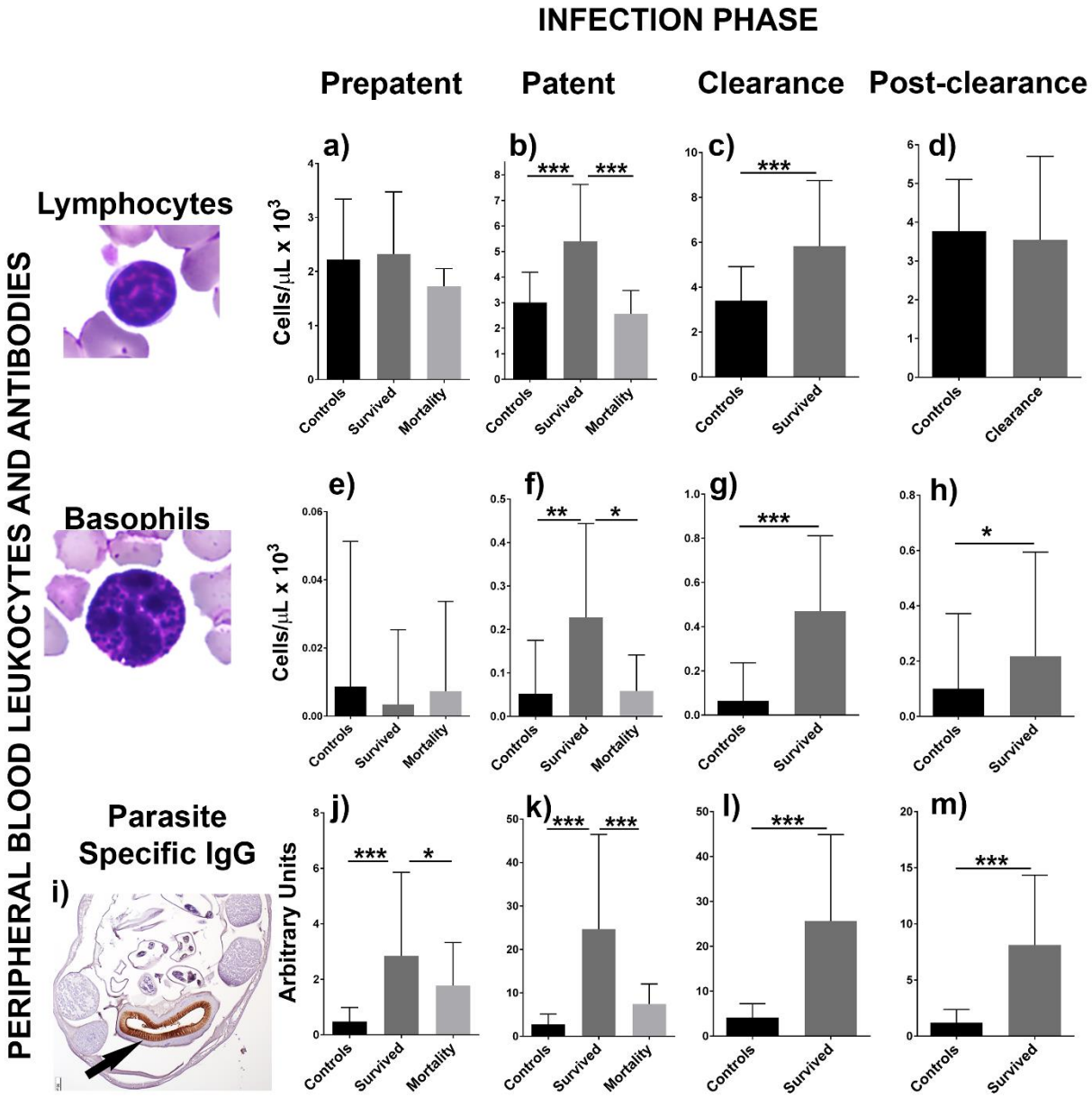

Figure 4.2. Changes in peripheral blood leukocytes and parasite specific IgG antibodies during different phases of hookworm infection in pups that survived and died due to hookworm

infection, and compared to age matched controls (animals treated with ivermectin). (a-d) Pups that survive hookworm infection have higher numbers of lymphocytes during the patent and clearance infection phases when compared to pups that died due to hookworm infection or age matched controls (Kruskall-Wallis statistic= 28.25, P<0.001, Mann-Whitney U=312.0, P=0.013). (e-h) Pups that clear and survive hookworm infection have markedly higher numbers of basophils during the patent (f, Kruskall-Wallis statistic= 15.21, P<0.001), clearance (g, Mann-Whitney U=68.0, P<0.0001) and post-clearance (h, Mann-Whitney U=268.0, P=0.016) infection phases. (i) Fur seal pups that clear hookworm infection produce parasite specific IgG that binds the intestinal brush border of the fur seal hookworms (*Uncinaria sp*.) (arrow). (j-m) Fur seal pups that clear hookworm infection produce large amount of parasite specific IgG during the prepatent (j, Kruskall-Wallis statistic= 17.64, P<0.001), patent (k, Kruskall-Wallis statistic= 46.79, P<0.0001), clearance (l, Mann-Whitney U=268.0, P=0.016) and post-clearance (m, Mann-Whitney U=268.0, P=0.016) infection phases. Asterisk indicate groups are statistically different at alpha=0.05. P-values code: *0.01<0.05, **0.001<0.01, ***<0.001.

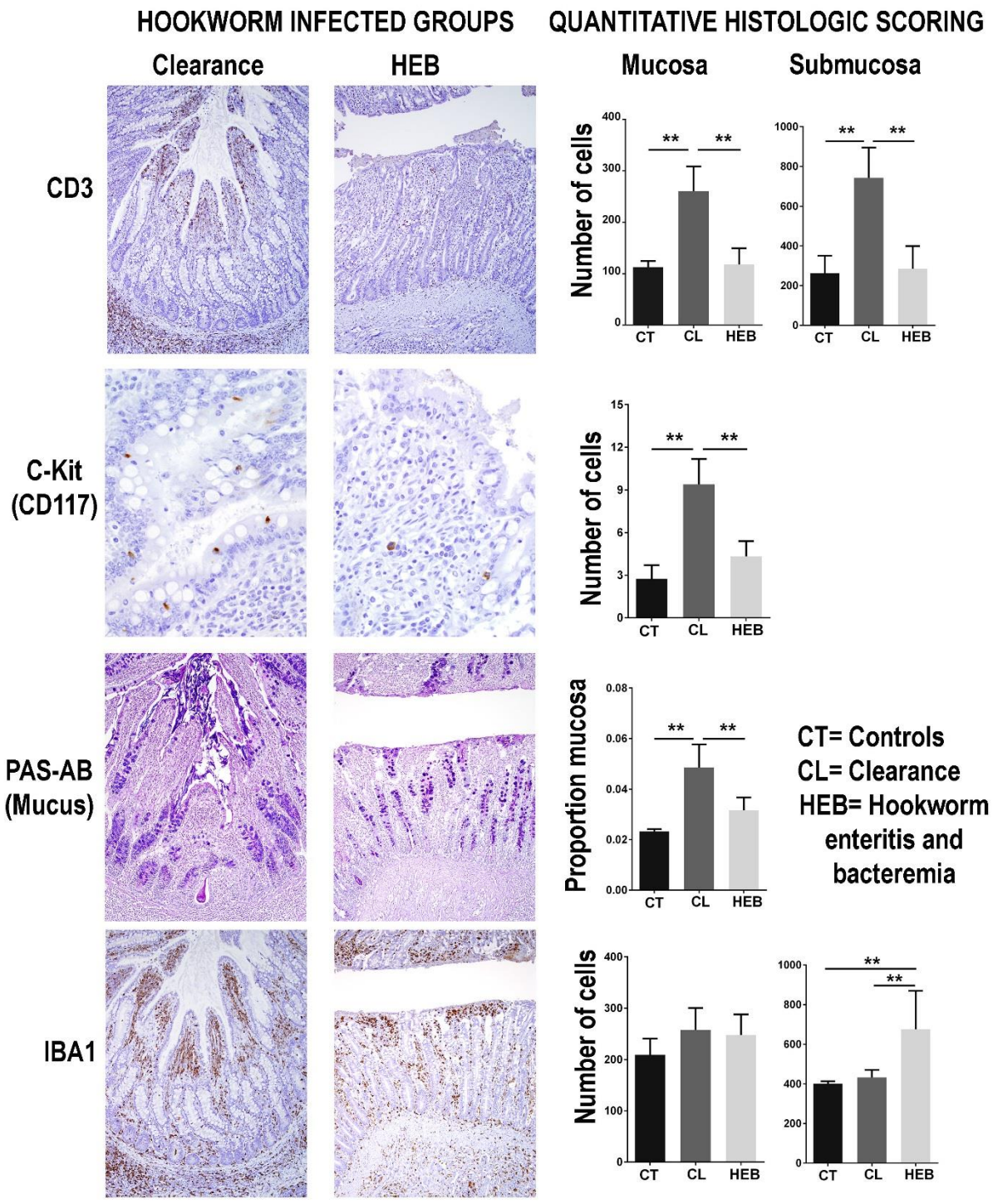

Figure 4.3. Intestinal immune response of South American fur seals (*Arctocephalus australis*) infected with hookworms (*Uncinaria sp.*). During the clearance process, fur seal pups recruit

numerous T-lymphocytes (CD3 stain) (Kruskal-Wallis statistic=30.78, P<0.0001), plasma cells (not shown) (Kruskal-Wallis statistic=28.18, P<0.001), mast cells (C-kit stain) (Kruskal-Wallis statistic=32.75, P<0.0001), IL-4 producing leukocytes (not shown) and large amount of mucus in the jejunum mucosa. Pups that die due to hookworm enteritis and bacteremia (HEB) have lower numbers or proportion of these immune components but higher numbers of macrophages (IBA1 stain) in the jejunum submucosa (Kruskal-Wallis statistic=29.61, P<0.0001). Asterisks indicate groups are statistically different at alpha=0.05 (Dunn's multiple comparison tests). P-values code: *0.01<0.05, **0.001<0.01, ***<0.001.

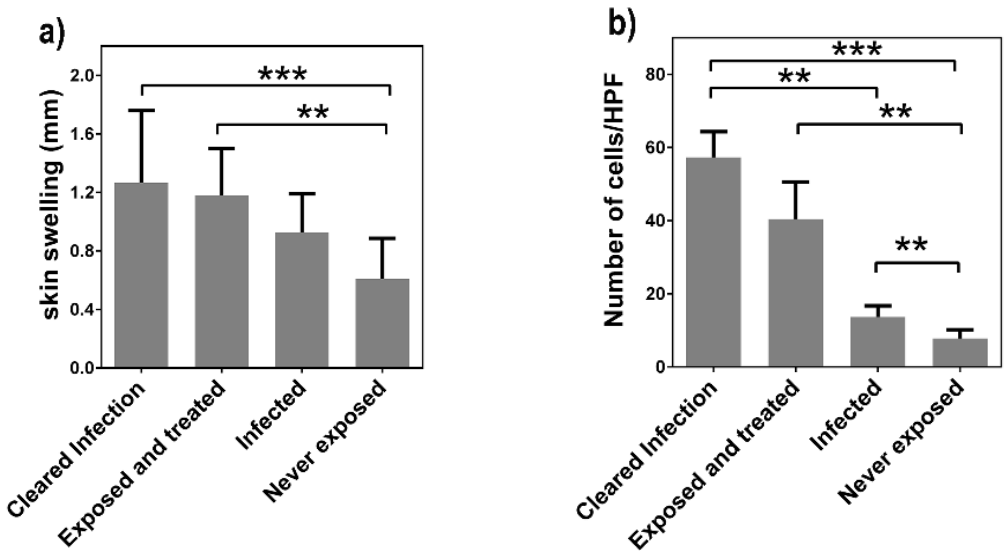

Figure 4.4. Differences in the level of skin swelling (a) and T-lymphocyte recruitment (b) in South American fur seal pups with different hookworm infection status challenged with intradermal phytohemagglutinin (PHA). Pups that clear hookworm infection early in the reproductive season, and therefore were not hookworm infected at the time of challenge (cleared infection), had the highest level of skin swelling (a) (One-way ANOVA, F=8.69, P<0.0001) and

CD3+ lymphocytes recruitment at PHA injection site (Kruskal-Wallis Statistic=49.74, P<0.0001). Pups never exposed to adult hookworms in the intestine had the lowest level of skin swelling and T-lymphocytes recruitment, whereas pups that experienced delayed hookworm clearance and were infected with hookworms at the time of the PHA challenge, experienced intermediate levels of inflammation. Asterisks indicate groups are statistically different at alpha=0.05 (Dunn's or Tukey's multiple comparison tests). P-values code: *0.01<0.05, **0.001<0.01, ***<0.001.

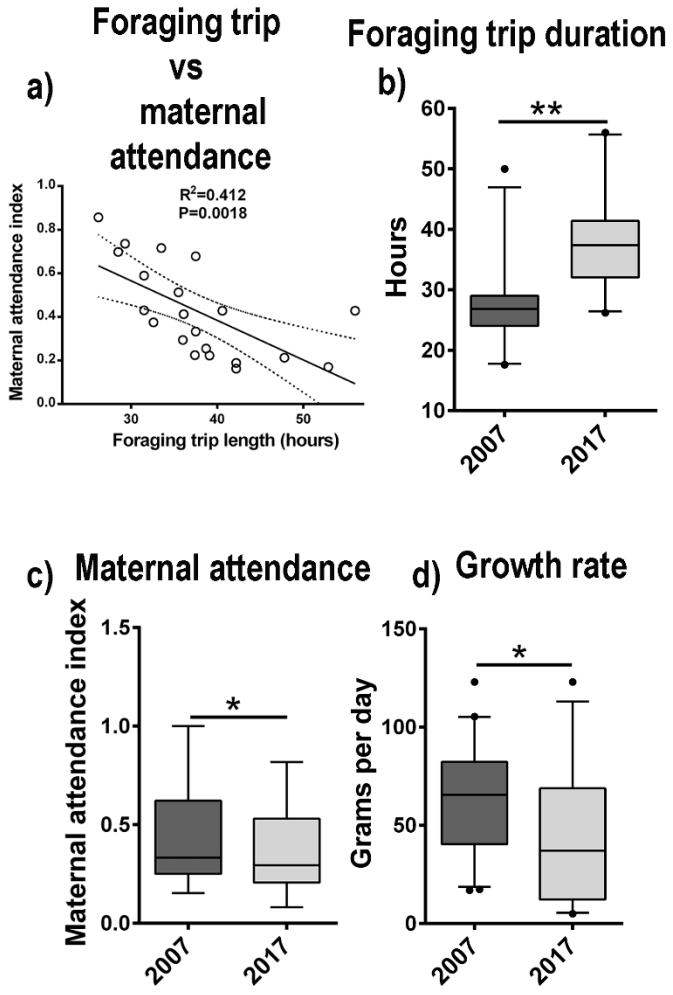

Figure 4.5. South American fur seals foraging behavior and maternal care patterns differ between seasons. (a) The level of maternal attendance decrease as foraging trips become longer (linear regression, R^2=0.412, P=0.016. Dashed lines represent 95% confidence intervals). (b) In a year with sea surface temperature (SST) bellow the historic Guafo Island average (2007), fur seal females foraging trips are shorter when compared to the mean foraging trip duration during a year with SST temperature above the historical average (2017) (unpaired T-test, T=5.133, df=42, P<0.0001). Additionally, maternal attendance index (c) and pup growth rate (d) in 2007 were higher than attendance and growth rates in 2017 (maternal attendance index: unpaired T-test, t=2.060, df=244, P=0.04; growth rate: unpaired T-test, T=2.85, df=66, P=0.0058)

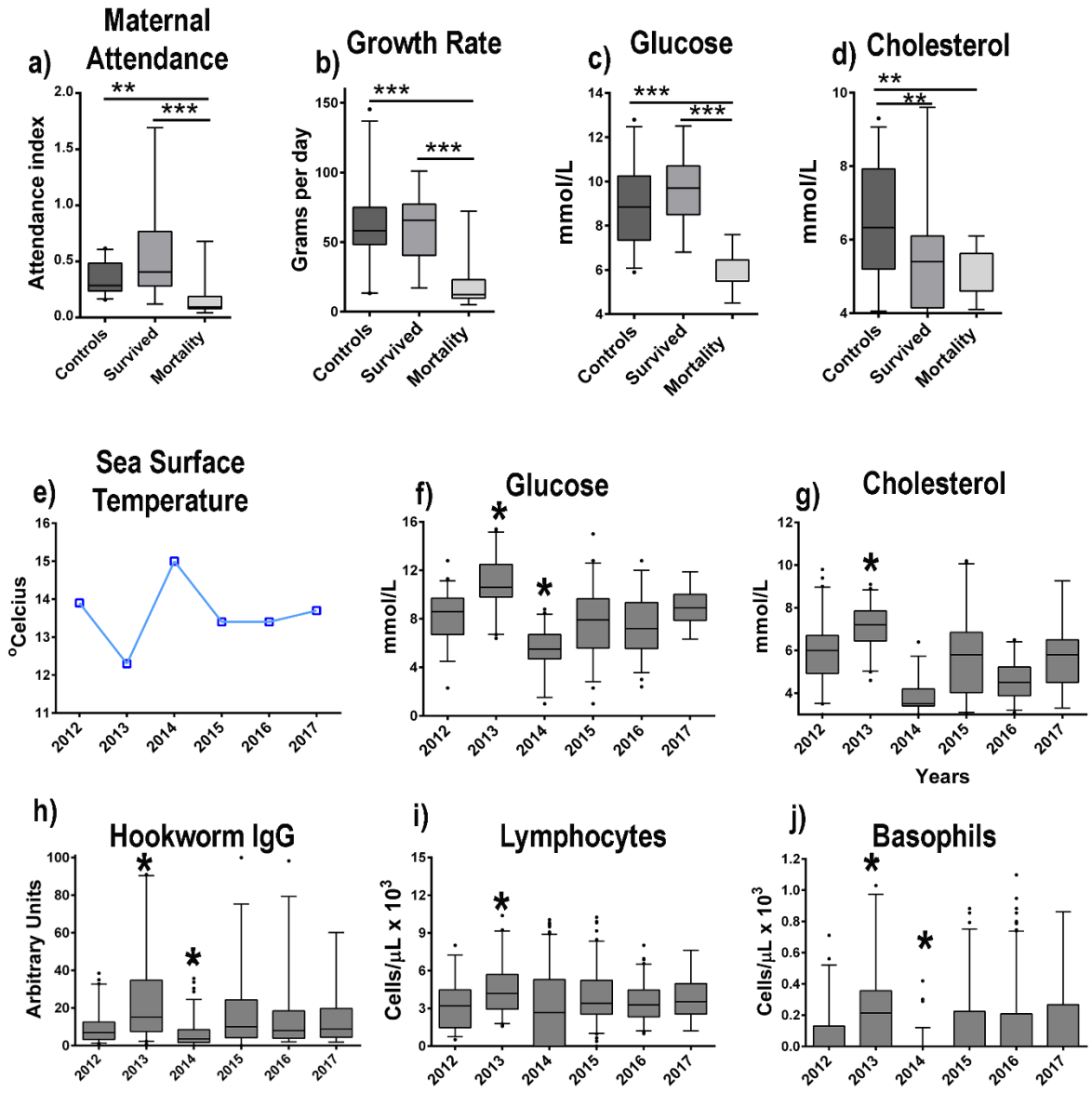

Figure 4.6. Differences in maternal attendance and energetic balance in South American fur seal pups with different hookworm infection outcomes and across 6 different reproductive seasons at Guafo Island. During 2017, pups that cleared and survived hookworm infection had higher levels of maternal attendance (a) (Kruskal-Wallis statistic= 13.74, P=0.001. Dunn's multiple comparison tests, mean rank diff.=27.3-38.4, P-values<0.0001-0.0054), faster growth rate (b)

(Kruskal-Wallis statistic= 22.87, P<0.0001. Dunn's multiple comparison tests, mean rank diff.=27.3-38.4, P-values<0.0001-0.0001), and higher glycaemia (c) (One-way ANOVA. F= 31.16, P<0.0001. Tukey's multiple comparison tests, mean diff.=3.01-3.71, P-values<0.0001) than pups that died due to hookworm (mortality). The cholesterol blood concentrations (d) were similar between animals that survived or succumbed to hookworm disease but these two groups had on average lower cholesterol levels than pups never exposed to adult hookworms (Kruskal-Wallis statistic= 13.74, P=0.001. Dunn's multiple comparison tests, mean rank diff.=18.78-24.4, P-values=0.0038-0.0053). Between 2012 and 2017 the mean values of glucose (f), cholesterol (g), parasite specific IgG (h), peripheral blood lymphocytes (i) and basophils (j) followed an inverse pattern with mean sea surface temperature (SST) at Guafo Island. In 2013, a year with low SST, pups had on average higher levels of glucose, cholesterol, parasite specific IgG, lymphocytes and basophils when compared to the mean values of other reproductive seasons. In 2014, a year with the highest mean SST over the last 15 years at Guafo Island, fur seal pups had the lowest mean values of these metabolic and immune parameters (f-j, asterisk indicate mean is significatively different from means of other seasons, Kruskal-Wallis with Dunn's multiple comparison tests. 2014: Kruskal-Wallis statistic =73.2-114.6, mean rank diff.= -230.83, -83.4, P-values <0.0001-0.017. 2013: Kruskal-Wallis statistic =73.2-114.6, mean rank diff.= 63.98-203.8, P-values <0.0001-0.023).

Tables

Table 4.1. Detail of sources, clone, retrieval methods and dilution of primary antibodies used for immunohistochemistry

Antibody	Source	Antibody Clone, host species, antigen	Antigen Retrieval Method	Primary Antibody dilution	Visualization Method
CD3	Dako[a]	Monoclonal, mouse, Anti-human	Citrate	1:1000	DAB
CD21	Cell Marque[b]	Monoclonal, mouse, Anti-human	Reveal	1:50	DAB
Iba-1	WAKO[c]	Polyclonal, Rabbit, Anti-human	Citrate	1:8000	DAB
Mum1	BioCare[d]	Monoclonal, Rabbit, Anti-human	Citrate	1:50	DAB
C-kit (CD117)	Cell Marque[b]	Monoclonal, Rabbit, Anti-human	Citrate	RTU	DAB
IL-4	Mybiosource[e]	Polyclonal, Rabbit, Anti-dog	Citrate	1:8000	DAB

[a]Dako= Agilent Technologies®, Santa Clara, CA, USA. [b]Cell Marque= Cell Marque biologicals, San Ramon, California USA. [c]WAKO= Wako Chemicals®, Richmond, VA, USA. [d]BioCare= Biocare Medical®, Pacheco, CA, USA. MyBiosource= Mybiosource Inc., San Diego, California, USA. RTU= Ready to use antibody (no dilution). DAB=diaminobenzidine.

Table 4.2. Hookworm prevalence, median hookworm burden, hookworm mortality and mean concentration of Chlorophyll-a and sea surface temperature during 10 South American fur seal reproductive seasons at Guafo Island.

Year	Hookworm prevalence (%)	Hookworm burden (number nematodes)	Hookworm Mortality (%)	Chlorophyll-a (mg/m^3)	Sea Surface Temperature (^oC)
2005	100	450	33	1.8	13.2
2006	100	590	40	1.14	13.2
2007	81	210	13	7.33	12.3
2008	100	510	40	2.1	13.4
2012	100	570	42	0.83	13.9
2013	87	380	28	1.5	12.3
2014	100	940	50	0.89	15.0
2015	90	520	24	10.41	13.4
2016	87	590	25	2.89	13.4
2017	90	640	29	1.7	13.7

Table 4.3. Coefficients and significance of predictors for hookworm related mortality in South American fur seal pups. Firth's penalized logistic regression.

Predictors												Likelihood ratio	p-value	AIC	delta AIC
At.	GR	Inf.Per.	Hg	Chol	TG	Gluc.	Alb.	Glob	Lym.	IgG	At.:GR				
		0.090***				-1.396***			-0.0006***			45.6945	6.59E-10	-39.695	0
		0.0832***				-1.631***						42.8881	4.86E-10	-38.888	0.80643
6.760*	-0.113*	0.1585**				-1.190*			-0.0008*			48.792	2.45E-09	-38.792	0.90252
	-0.030	0.079*				-1.032*			-0.0006*			45.6343	2.93E-09	-37.634	2.06026
		0.0925***				-1.449***			-0.0005***		0.01***	44.5931	4.83E-09	-36.593	3.10145
4.691*	-0.071*	0.108**			1.3	-1.132**			-0.0006*			46.3127	2.57E-08	-34.313	5.38183
4.481	-0.073	0.066	-0.442		1.239	-0.744			-0.002	0.07		47.7231	1.12E-07	-31.723	7.97144
5.577	-0.07	0.1076*	-0.299		1.654*	-1.333*	0.26		-0.001*	-0.52		47.7815	2.81E-07	-29.782	9.913
3.906	-0.0605	0.09**	-0.06		1.287	-1.137**			-0.0008	0.03		44.3494	4.89E-07	-28.349	11.34515
5.53*	-0.0494*	0.065	-0.41	0.311	1.08	-1.325	0.32	0.63	-0.001*			47.3795	8.05E-07	-27.379	12.31505

*p-values <0.05-0.01, ** pvalues <0.01-0.001, *** p-values <0.001

At= Maternal attendance, GR=Growth Rate, Inf.Per= Infectious period, Hg= Hemoglobin, Chol=Cholesterol, Gluc=Glucose, Alb=Albumin, Glob=Globulins, Lym=lymphocytes, IgG=Parasite specific IgG

AIC= Akaike's information criteria

Table 4.4. Coefficients and significance of selected predictors for the number of CD3 lymphocytes in skin biopsies of South American fur seal pups (*Arctocephalus australis*) exposed to phytohemagglutinin immune challenge. Generalized linear models with negative binomial distribution. Models are ranked based on AIC.

Predictors											log-likelihood	AIC	Delta AIC
Lym.	Baso.	HW Burden	Non Infection	Infected	exposed	Gluc.	Chol.	BMI	GR	MA			
			-1.25***	-1.01***	-0.22**	0.067***		-2.68*	0.007**	0.18**	-308.393	326.2	0
			-1.81***	-1.36***	-0.33**	0.053***		-2.52			-323.664	337.66	11.46
			-1.80***	-1.34***	-0.29**	0.049**					-327.195	339.2	13
		0.003246	-1.82***	-1.34***	-0.28**	0.04*					-325.697	339.7	13.5
0.0061	-0.040	0.002613	-1.83***	-1.35***	-0.28*	0.03					-325.331	343.33	17.13
0.006	-0.052	0.002645	-1.810684***	-1.34***	-0.28*	0.03	-0.02				-323.711	343.71	17.51
5.57E-03	-9.10E-02	2.38E-03	-1.81E+00***	-1.37E+00*	-3.29E-01	3.72E-02	-2.79E-02				-320.675	346.67	20.47

*p-values <0.05-0.01, ** pvalues <0.01-0.001, *** p-values <0.001. Lym=lymphocytes, Baso=Basophils, Eos=Eosinophils, HW= hookworm, Gluc= Glucose, Chol=Cholesterol, HG= Hemoglobin, BMI= Body Mass Index, GR=Growth Rate, MA=Maternal Attendance. AIC= Akaike's information criteria.

CHAPTER 5

CONCLUDING REMARKS

There are numerous hookworm species described in a wide range of wild mammals, however some taxonomic groups, such as carnivores, are overrepresented in the literature. This probably represents study effort bias rather than a true higher diversity in carnivores. In a similar manner, the state of the knowledge of the impact of hookworms on wildlife populations is biased by study effort, and for most wildlife populations, understanding of the impact and drivers of hookworm disease is very poor. However, the scarce literature on the causes of the detrimental effects of hookworms on wildlife species suggests a dynamic scenario where human-related disturbances of wildlife populations and climate change may potentially affect the dynamics and effects of hookworm infections in wildlife.

The present dissertation fills part of these knowledge gaps by describing not only the most significant effects of hookworm infection in fur seals, but by determining the factors that drive the observed adverse effects of these nematodes in the host population.

In South American fur seals, the particular adaptations of hookworms to the marine lifestyle of the host has led to lack of avoidance of immune clearance and a short life span of the nematode in the pup's intestine. Therefore, the parasite has a short time to reproduce and release eggs before the next host in the reproductive cycle (females) depart from the rookery. Therefore, fur seal hookworms feed at high rates causing substantial damage to the intestinal mucosa and leading to marked anemia and mortality. This parasite strategy has important consequences for the evolution of virulence in this hookworm species, because an increase in damage to the host

always pays off in terms of parasite fitness, therefore natural selection favor those nematodes expressing higher virulence. This probably explains why marine hookworms are the most pathogenic (virulent) within this parasite group. Additionally, the parasite life history strategy, and its high sensitivity to immune mediated clearance, favors a very dynamic scenario where any factor affecting fur seal pups immunocompetence could have substantial consequences on the impact of hookworm disease. In the case of sea surface temperature (SST), the strong body of knowledge about otariids foraging ecology and the observed patterns in the present study indicate that an increase in ocean temperature will lead to an increase in the time spent by females in the ocean (foraging), therefore, decreasing the time to nurse their pups. These changes were clearly associated with lower energetic balance in fur seal pups at Guafo Island, which could explain in part the weaker immune response of these pups and the delayed hookworm clearance and higher chances of mortality. However, additional roles of maternal attendance in the immune competence such as behavioral and stress related effects cannot be discarded and should be addressed by future investigations.

The fur seal-hookworm system has proved to be a valuable tool to understand important mechanisms that drive disease dynamics in nature. Questions such as the origins and consequences of virulence and the effects of climate change on disease severity can be explored in more detail in this system, which allows for manipulation of the infection and testing of hypotheses. Future efforts directed to establish long lasting monitoring programs in similar systems could be a valuable tool to understand disease processes in the context of environmental change.