The chicken or the egg: is the immune response of South American fur seal pups the cause or the consequence of parasitic burden?

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SUMMARY

In the Northern Chilean Patagonia hookworm disease is the main cause of pup mortality in South American fur seals. Parasitic burden is the main driver of hookworm-related mortality and that animals with heavy hookworm infection have marked disarrangement of their immune system. However it is unknown if the changes in the immune system are due to a reaction to heavy primary infection with hookworms or if the immune system regulates the number of hookworms in the pups. Through an experimental approach we will answer this question, which can have important ecological implications for fur seal populations.

Project Description

General Description: In the Pacific Ocean two major groups of South American fur seals are recognized; the Peruvian fur seal population, composed of approximately 11,000 individuals and a more southern Chilean Patagonia population composed of approximately 20,000 individuals (Venegas et al. 2002). These numbers represent a decline of 57% over the last 20 years in the Chilean population (Paves 2007) and more recent assessments have shown that numbers of fur seals have not increased despite legal protection (Dr Hector Paves, 2015, unpublished data, personal communication). The most important reproductive colony of this species on Chilean waters is located at Guafo Island (43°35´ S 74°42´O) in the Northern Pacific Patagonia. The major cause of SAFS pup mortality at Guafo Island is hookworm disease, a condition caused by the parasite Uncinaria sp., which sucks blood in the intestine causing anemia and predisposing to secondary bacterial infections (Seguel et al. 2011, 2013, accepted for publication). Although the impact of this disease in the Peruvian fur seal population is unknown, this small, isolated and threatened fur seal population is affected by the same Uncinaria sp parasite present at Guafo Island and the range of histological lesions are very similar to those observed in SAFS at Guafo Island (Calderon et al. unpublished data, M. Seguel personal observations).

The Uncinaria sp. parasite infects the pups through the colostrum and reaches adulthood in the pups' intestine in 14-18 days. Hookworm eggs are released with the pups's feces and larvae develop and survive in the rookery substrate, and later penetrate the skin of adult females and stay in their subcutaneous and mammary tissues until females give birth, produce colostrum and infect the next generation of pups (Lyons et al 2011, Seguel et al unpublished data). In previous field work seasons at Guafo Island we have seen that prevalence of hookworm infection in the SAFS pups is 100% and approximately 30% of the pups born each year are severely infected. Within this severely infected group, the survival rate during the first two months of life is 40% while pups with low hookworm burden have a survival rate of over 90%. Other differences between groups of pups with high and low hookworm burdens is their immune response. Pups with severe hookworm infection have decreased numbers of lymphocytes in the blood and total leukocytes in the small intestine (Seguel et al. accepted for publication). In a preliminary field experiment performed in the reproductive season of 2016 we found that animals with severe hookworm infection had mild or no inflammatory response to a phytohemagglutinin (PHA) challenge while animals with mild hookworm infection had a marked inflammatory response. These findings indicate that the immune response is different in animals with different hookworm infection status. However these differences could be the result of two mechanisms:

i) The infective hookworm doses. Since hookworm larvae are transferred to the pups through the colostrum, the number of larvae ingested could be the main driver of the burden. In this case the differences in immune response will be due to the immunomodulatory effect of the parasite on the host. In several animal models it has been shown that hookworms can elicit immunosuppression of the host and therefore the immune response is weaker in animals with heavy infections (Fujiwara et al. 2006).

ii) Primary differences in immune response among pups. In this case the strength of the inflammatory response is the main driver of hookworm burden. In pups with strong immune reaction (due to genetic predisposition or environmental effects on the immune system) the ingested hookworm larvae cannot develop in large numbers and many of the hookworms that do develop in the intestine are expelled shortly after infection, which result in mild clinical infections. This mechanism has been proposed in several laboratory animal models of hookworm infection and also in field and experimental studies conducted in humans, where some individuals are "predisposed" to have a stronger immune reaction to hookworms resulting in mild clinical disease (Loukas et al 2005, Croese and Speare 2006, Fujiwara et al. 2006).

A third and last possibility is that these two mechanisms are operating in this SAFS population.
To know which mechanism operates in SAFSs is important, because in the case of a primary effect of the parasitic burden all the environmental variables that affect the hookworm larval stages in the soil would affect burdens in the pups (e.g. warmer temperatures due to climate change or ENSO events). On the other hand, if the immune response is the main driver of the parasitic burden, and therefore of pup mortality, factors that affect the pup immune system function such as genetic structure of the SAFS population and environmental factors such as nutrition would be more important on modulating SAFS pup mortality.

Our hypotheses are:
1) The mild immune response in pups with high parasitic burden is due to a "parasite effect" on the pup's immune system.

2) The mild immune response in pups with high parasitic burden is due to a primary incapability of some pups to mount a strong immune response, allowing the survival of large numbers of hookworms in the pups' intestine.

3) The mild immune response in pups with high parasitic burden is due to a combination of a "parasite effect" on the immune system and primary predisposition of some pups to mount a mild immune response.

The following are the main research goals:

Capture and mark 300 South American fur seal pups.

Treat a subgroup (n=150) of pups with ivermectin at different ages.

Perform a phytohemagglutinin immune challenge in different groups of pups.

RESEARCH PLAN AND METHODS

Field work: The field work of this project will be carried out at Guafo Island (43º36'S and 74º43'W), located in northern Chilean Patagonia. One hundred pups will be captured soon after birth (1 week old) and treated with ivermectin as previously described in Northern fur seals (DeLong et al 2009). This group will be our "control group" as they were not exposed to adult hookworms because the patent period of hookworm infections in SAFS pups is approximately 15 days and ivermectin eliminates all hookworm larvae in the pup's intestine and pups do not get re-infected (based on previous experiments in this rookery). Later, on the second week of January, when pups are approximately 1-month-old, another group of 100 pups will be captured and treated with ivermectin. This group will be the "exposed" group as they were infected with hookworms through the colostrum and artificially cleared the infection with the ivermectin. Finally, a third group of 100 pups will be captured when pups are between 1-week and 1-month-old and not-treated. During each capture all pups will undergo complete clinical examination, coprological exam (to detect hookworm eggs) and we will record all standard body measurements, body weight and sex. All pups will be marked using a hair dye that is lost when they change their fur at 3.5 month old. During all capture procedures blood samples will be taken from the caudal gluteal vein and processed in a field clinical pathology laboratory as previously described (Seguel et al. 2016).
At the end of the reproductive season and hookworm infection period (second week of February) a phytohemagglutinin (PHA) immune challenge will be performed on the pups. Pups from the 3 different

groups will be recaptured (expected recapture success of 40%, based on previous seasons) and challenged with intradermal 0.1 ml of 1 μg/ml PHA as previously described in California sea lions (Vera-Massieu et al 2015). Six hours later, pups will be recaptured and sedated with 0.5 mg/Kg of midazolam. The change in the pelvic flipper skin fold thickness will be recorded for each pup. After application of local flipper anesthesia (lidocaine) a small biopsy will be taken with a 4mm biopsy punch. The biopsy samples will be placed in 10% buffered formalin and processed routinely for histology at the mainland lab.

Mainland lab work: The number of different leukocyte subsets will be determined for each biopsy sample through routine histopathology (total lymphocytes, plasma cells, neutrophils), special histochemical stains (mast cells) and immunohistochemistry (T and B lymphocytes, macrophages).
The total globulins and total IgE and IgG will determined in serum samples of different pup groups through routine chromatographic techniques (globulins) or with dog-adapted ELISAs.

Data Analyses: The immune response variables obtained will be: thickness of flipper skin fold (or level of swelling), number of the different leukocyte types in the biopsy samples, number of the different leukocytes (e.g. neutrophils, lymphocytes) in the peripheral blood and levels of globulins, IgG and IgE. The immune response variables of the three groups of pups will be compared through non-parametric tests for counting data (e.g. GLM with negative binomial distribution) and parametric tests if data is normally distributed and not obtained through counting.

EXPECTED OUTCOME AND DATA INTERPRETATION

Expected outcome if differences on the immune response against hookworm infection are due to a primary predisposition of the severely infected pups to mount a mild immune response against the parasite. The pups that were exposed to the parasite (hookworm exposed and treated) will have the same level of immune response compared to animals with infection (hookworm infected) suggesting that both groups are composed of “infra-responder” animals regardless of current hookworm infection status.

Expected outcome if differences on the immune response against hookworm infection are due to a doses dependent effect of the parasite on the host. In this case there should be a significant difference between animals with different parasitic burden (mild and severe) that carry the parasite (Hookworm infected), while in the groups of pups that were infected with hookworms (exposed) and treated before PHA challenge there should be no difference between pups that previously had mild or severe hookworm (HW) infection. This means that differences on immune response in function of the parasitic load are due to the effect of the parasite on the host and that when the hookworms are eliminated this immunomodulatory effect disappear.

Expected outcome if differences on the immune response against hookworm infection are due to a combination of the predisposition of the immune system of severely infected pups to react mildly against hookworm and the immunomodulatory effect of the parasite on the host. In this case the immune response of pups that were exposed to large numbers of hookworms (Severe Hookworm infection) and that were treated before PHA challenge will be different from the pups with mild hookworm (Hookworm) and from animals with severe hookworm infection but that are still infected with hookworms at the time of PHA challenge.

IMPORTANCE OF THE STUDY FOR SPECIES BIOLOGY AND CONSERVATION

The fur seal population at Guafo Island is the perfect place to understand how marine mammal populations survive and deal with pathogens as a constant challenge in a changing environment. We pretend to understand the mechanisms that mediate marine mammals´ survival to pathogens by determining if the immune response on its own is a key factor for survival or if the changes in immune response are due to environmental challenges. If the immune response is the main driver of parasitic burden, and therefore pup survival, all the factors that affect the immune system (e.g. pollutants, other infectious diseases) could have a significant population impact. On the other hand if the parasite burden in the host is determined by not-

immune mechanisms (e.g. ingestion of larvae), then environmental factors that influence the survival of lavae in adult females and the environment will be critical for pup survival (e.g. temperature, humidity).

TIMELINE OF THE PROJECT

2016

Dec-January	February	March	April	May	June	December
Field work	X	X				
Laboratory work	X	X	X			
Data analyses and presentation of preliminary results			X	X		
Final report						X

BUDGET

OTHER FUNDS AVAILABLE

The Pathology and Clinical Sciences departments of the College of Veterinary Medicine, Universidad Austral de Chile, will provide funding for the following supplies.

Clinical Pathology supplies…….. $500 (U.S)
Electric generator …… $400 (U.S)
Campsite supplies…… $400 (U.S)
Samples containers …… $200 (U.S)
Fixation solutions …… $100 (U.S)
Legal permits …… $300
Total …… $ 2100 (U.S)

The Society for Marine Mammalogy has granted the following funds

Sea transportation …….. $ 800
Meals …….. $700
Total ……$ 1500

Funds pending (Requested to National Geographic Society and American Association of Zoological Medicine)

Laboratory reagents and kits …….. $ 3000 (U.S)
Field (campsite) supplies …… $ 1000 (U.S)

LEGAL PERMITS

This project will be performed in accordance with current Chilean regulation regarding marine mammals, which include authorization from the Chilean subsecretary of fisheries (Subpesca). This project has been approved by Subpesca and our current permit is valid through March 2017. The animal handling protocols and experiments have been approved by the University of Georgia “Institutional Animal Care and Use Committee (IACUC)”

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Curriculum Vitae

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Education
2012 - Today PhD Candidate, College of Veterinary Medicine, University of Georgia, Athens GA, USA.
2012 - 2015 Anatomic Pathology Residency, College of Veterinary Medicine, University of Georgia, Athens GA, USA.
2003 – 2010 Médico Veterinario (DVM),
Highest Distinction Honors
Universidad Austral de Chile, Valdivia, Chile.
2003 – 2007 Bachelor Degree in Veterinary Sciences (BVSc)
Highest Distinction Honor
Universidad Austral de Chile, Valdivia, Chile.

Board Certification
2015. Anatomic Veterinary Pathology, American College of Veterinary Pathologists (ACVP).

Awards and Scholarships
2016 Morris Animal Foundation Fellowship. Awarded for the project "Host and environmental factors driving hookworm mortality in an isolated fur seal population"
2016 Thesis completion award. Granted by "The Graduate School of the University of Georgia".
2015 University of Georgia Amazing Student Award, profile uploaded to the UGA's website. https://president.uga.edu/amazing/profile/seguel-mauricio/
2014 Outstanding Teaching Assistant Award, awarded by the Office of the Vice President for Instruction, University of Georgia.
2010 Fulbright scholarship. Awarded for PhD studies in United States of America.
2010 Highest Distinction Honor. Awarded to the student with the highest GPA of the 2009 Veterinary Medicine class at the Universidad Austral de Chile.
2008 Chilean-American Veterinary Medical Association award. Granted in recognition to superior scholastics performance and research potential
2007 Merial CO award. Granted to the top student graduated of the Bachelor of Veterinary Sciences at the Universidad Austral de Chile.

Peer Reviewed Publications
Seguel M, Muñoz F, Navarrete MJ, Howerth E, Paredes E, Gottdenker N. Hookworm infection in South American fur seal (Arctocephalus australis) pups. Pathology and factors associated with host tissue damage and mortality. Accepted for publication. Veterinary Pathology.

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