Australian Marine Mammal Centre Grants Program
Final Report

- **Project No**. – 09/17
- **Title -** Prevalence and impact of hookworm infection on Australian sea lion populations
- **Chief Investigator** – Dr Rachael Gray and Dr Damien Higgins
- **Co-Investigators –** Associate Professor Simon Goldsworthy and Emeritus Professor Paul Canfield
- **Organisation** – The University of Sydney

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1. Project Summary

A clear, plain English summary of approximately 500 words outlining the work undertaken and any significant findings (for publication on the Department's web site). Include what was done, why and the key findings resulting in recommendations summarised from the sections below.
The objectives of this study were to determine the prevalence and intensity of hookworm infection due to *Uncinaria* spp., a significant disease entity and contributor to mortality in Australian sea lion, *N. cinerea* pups; to investigate factors predisposing pups to hookworm infection, disease and subsequent mortality; and to investigate the significance of hookworm disease and mortality on *N. cinerea* populations. Sample collection from Australian sea lion pups was undertaken during two breeding seasons at Seal Bay, Kangaroo Island (2010, 2012) and Dangerous Reef, Spencer Gulf (2011, 2013). Key findings of the study highlight the importance of hookworm as a cause of clinical disease, characterised by anaemia and haemorrhagic enteritis in Australian sea lion pups, and its significant role in pup mortality. The prevalence of hookworm infection in both live and dead known-aged *N. cinerea* pups was determined to be 100%; mean hookworm burden in dead pups (infection intensity) was also high, and seasonal variation in mean adult hookworm burden was observed. Mean hookworm eggs per gram (epg) of faeces was also high and significantly correlated with pup age. Methodology was developed to quantify the presence of blood in faecal samples, and preliminary analysis suggests that hookworms, when present in sufficient numbers, cause significant intestinal blood loss due to haemorrhagic enteritis during the prepatent (prior to the shedding of eggs in the faeces) and early stages of infection, which subsequently decreases with pup age. Analysis of blood haematological parameters clearly demonstrates that hookworm causes clinical disease in Australian sea lion pups with anaemia, hypoproteinaemia (low blood protein concentrations) and elevations in white blood

cells indicative of inflammation.

In addition to causing clinical disease, hookworm-associated haemorrhagic enteritis was determined to be an important cause of pup mortality. Based on gross necropsy findings (n=114), cause of mortality was attributed to: trauma (32%), hookworm-associated haemorrhagic enteritis (18%), starvation (16%), still-birth/premature (8%), misadventure/other (2%), and unknown causes (24%). Whilst a single cause of mortality based on gross necropsy findings was assigned for each pup, the high prevalence and intensity of hookworm infection in dead pups and associated pathology provides evidence for the significant contributory role of hookworm disease in increased mortality due to these other causes.

Molecular investigations using polymerase chain reaction (PCR) amplification of DNA extracted from individual hookworms have determined that *N. cinerea* pups sampled at three colonies (Seal Bay, Dangerous Reef, and The Pages Islands) are infected with a single species of *Uncinaria* hookworm. There was no significant evidence for intra-species diversity at the molecular markers examined, and based upon molecular and morphological data, the species of *Uncinaria* infecting *N. cinerea* is closely related to hookworms collected from other southern hemisphere pinniped hosts.

Analysis of major histocompatability complex (MHCII DRB) diversity indicated limited diversity across the colonies, with reduced heterozygosity in the smaller colony of Seal Bay. This suggests even lower diversity is likely at smaller colonies, with likely associated vulnerability to introduced disease. Characterization of MHCII and several tools for immune assessment in this study provides tools to investigate this on a broader scale in future studies.

An anthelmintic (ivermectin) treatment and control study at Dangerous Reef has determined that a single ivermectin dose provides close to 100% efficacy for reducing the prevalence of patent hookworm infection. Whilst significantly higher mean daily growth rates were observed in treated compared to untreated young pups, no statistically significant difference in survival between the treated and untreated pups was observed, likely due to several factors including variation in the age of recruited pups, individual hookworm burden, and stage of hookworm infection.

Based on these findings, we conclude that ivermectin is an effective anthelmintic for the treatment of hookworm infection in Australian sea lion pups. Whilst wide-spread, routine anthelmintic treatment of free-ranging populations is not advocated and is unlikely to be logistically viable, the limited use of targeted anthelmintic treatment may have a role in improving pup survival in the event of concurrent disease threats, subsequent to further research into the efficacy, safety and usefulness of anthelmintic treatment as a management and conservation tool in this threatened pinniped species.

2. The Outcomes and Objectives – Key Findings

List the Project Objectives and address each one, noting the degree to which the objective was achieved through the research and issues that may have hampered its success. Describe the key findings as they relate to the objectives and the management questions identified in the initial application.

1. Determine the prevalence and intensity of hookworm infection and associated

disease and mortality in *N. cinerea* pups.

Overall, Objective 1 has been successfully achieved.

Sample collection was undertaken during the 2010 Seal Bay breeding season during three field trips in July, September/October, and November/December and an extended field trip during the 2012 breeding season (February-June). Final collection of survival data for pups sampled during the 2010 and 2012 breeding seasons will be undertaken during a short field trip in September 2013.

Sample collection was undertaken during the 2011 Dangerous Reef breeding season in May, July, August, and September 2011, and for the 2013 breeding season in January and February.

Objective 1a. Key findings

Prevalence and intensity of hookworm infection: Dead pups

Although high hookworm prevalence was seen, seasonal variation in hookworm prevalence in dead pups was noted with 72% (SB2010, n=26), 80% (SB2012, n=51), 96% (DR2011, n=25), and 71% (DR2013, n=7) prevalence. The prevalence of hookworm infection in a known-aged cohort of dead pups (6 – 62 days; n=21) at Seal Bay during 2012 was 100%.

Mean hookworm burdens (infection intensity) were 1,378 ± 1,482 (SB2010, n=15), 3,280 ± 2,104 (SB2012, n=42), 3,256 ± 2,381 (DR2011, n=22), and 372 ± 218 (DR2013, n=5) with a significantly higher infection intensity at Dangerous Reef in 2011 compared to 2013 and compared to Seal Bay 2010 ($p < 0.001$). No significant difference in mean hookworm burden was identified between colonies excluding year of sampling ($p = 0.912$).

Patent infections (eggs identified in faeces) were detected in 82% (SB2010, n=17), 54% (SB2012, n=52), 63% (DR2011, n=24) and 100% (DR2013, n=5) of dead pups with identified hookworm infection. Variation in patency may reflect sampling effort or differences in the ages of dead pups between years.

Mean hookworm eggs per gram (EPG) in dead pups ranged from 8,021 ± 10,229 (SB2012, n=26) and 17,710 ± 8,290 (DR2011, n=8), although no significant difference between colonies or years was detected ($p = 0.109$). A significant linear correlation between hookworm EPG and burden was identified at Seal Bay in 2010 (Pearson correlation = 0.934, $p = 0.006$; n=6), however, no significant relationship was identified during the other breeding seasons at either colony.

Prevalence and intensity of hookworm infection: Live pups

The prevalence of infection in unknown pups (faeces collected from the ground) varied from 74% (SB2010, n=35), 90% (SB2012, n=10), and 100% (DR2013, n=2).
Hookworm prevalence in identified live pups was 39% (SB2010, n=102), 78% (SB2012, n=97), 81% (DR2011, n=160), and 98% (DR2013, n=80) and was 100% in known-aged live pups (12 – 65 days; n=47) at Seal Bay during 2012.
Mean hookworm EPG were 7,331 ± 8,455 (SB2012, n=118) and 6,041 ± 7,715 (DR2013, n=68) and data from known-aged pups demonstrates that EPG is significantly related to age ($p = 0.016$).

The finding of 100% prevalence of hookworm infection in a cohort of known-aged pups, in combination with the high prevalence identified in pups of undetermined age, suggests that the incidence of hookworm infection in all pups is 100%. The observed prevalence of less than 100% is likely due to age-related variation in the stage of hookworm infection (prepatent, patent, recovered) at the time of sampling.

Faecal occult blood (FOB)
Quantification of FOB was performed on samples collected from live and dead pups at Seal Bay (2012) and Dangerous Reef (2013). FOB was measured in milligrams of haemoglobin (Hb) per gram of faeces and classified into five groups (negative-large). An association between high FOB values, low EPG and age (young) pups was seen. Preliminary interpretation of this data suggests that hookworms, when present in sufficient numbers, cause significant intestinal blood loss during the prepatent to early stages of infection. During the course of infection, pups demonstrate an initial increase in hookworm EPG, which then decreases with age along with the degree of FOB.

Haematological parameters (live pups)
Blood samples (n=474) were collected from 327 individual pups during the four breeding seasons. Data show that hookworm infection has a significant effect on the haematological parameters of pups, causing a regenerative anaemia associated with hypoproteinaemia and pan-leukocytosis. Statistically significant relationships ($p < 0.01$) for packed cell volume (PCV), RBC count, reticulocyte count, total white blood cell count, all leukocyte counts (excluding basophil counts), and total plasma protein were seen with hookworm status (positive or negative).

Objective 1b. Key findings

Contribution to Mortality
Gross necropsies were performed on pups (n=114) found dead at Seal Bay and Dangerous Reef. One pup from The Pages was also available for necropsy. Tissue samples for histopathology were collected from pups (n=73) for further characterisation of pathological findings and to establish cause of death. Histopathology analysis is ongoing.

The contribution of hookworm infection to pup mortality was estimated with consideration of the morbidity associated with haemorrhagic enteritis and anaemia resulting from faecal blood loss, the gross and histopathological findings from necropsies, and a comparison of experimentally treated 'hookworm-free' pups with saline-treated controls (results of the ivermectin experiments are outlined below in Objective 3a). Based solely on gross necropsy findings, cause of pup mortality (2009-2013) was attributed to: trauma (32%), hookworm-associated haemorrhagic enteritis (18%), starvation (16%), still-birth/premature (8%), misadventure/other (2%), unknown (24%). Whilst a single cause of mortality based on gross necropsy findings was assigned for each pup, the high prevalence and intensity of hookworm in dead pups and associated pathology, provides evidence for the significant contributory role of hookworm disease in increased mortality due to these other causes, particularly trauma and starvation.

2. Investigate what primary factors predispose *N. cinerea* pups to disease and

mortality associated with hookworm.

Objective 2a. Key findings

Species of hookworm causing infection in N. cinerea pups

This objective has been successfully achieved.

i) Morphological features: Comparisons with published morphological measurements of hookworms from Australian fur seals, New Zealand fur seals, New Zealand sea lions, Southern elephant seals and specimens of *U. lucasi* and *U. hamiltoni* demonstrates a wide range of overlapping measurements. Based on the examination of hookworms collected from Australian sea lions at Seal Bay (n=47) and Dangerous Reef (n=27), no distinctive morphological features have been identified to differentiate hookworms collected from Australian sea lions from those infecting these other pinniped hosts.

ii) Molecular features: following morphological examination, DNA was extracted from ethanol-preserved individual hookworms (n=10 from each of Seal Bay and Dangerous Reef and n=1 from The Pages Islands) for PCR amplification of two regions of nuclear ribosomal DNA, the internal transcribed spacers (ITS1 and ITS2) and a partial sequence of the large subunit 28S gene.

Based on these investigations, *N. cinerea* pups are infected with a single species of *Uncinaria* hookworm with no significant evidence for intra-species diversity at the molecular markers examined. Based upon molecular and morphological data, the species of *Uncinaria* infecting *N. cinerea* is closely related to other southern hemisphere otariid *Uncinaria* species.

Objective 2b. Key findings

The effect of season and substrate-type on the recovery of free-living hookworm larval stages was investigated by the collection of sediment samples from Seal Bay (n=23; 2010 and 2012), Seal Slide (n=2; 2010), and Dangerous Reef (n=34; 2011 and 2013). Nematode larvae were recovered from one sample at Seal Slide, 17 samples across both breeding seasons at Seal Bay, and 16 samples (during 2011 only) at Dangerous Reef. Due to logistical difficulties associated with the field work including limited field time and difficulties associated with sample storage and analysis at our remote field sites, insufficient data is available to comprehensively report on the longevity and density of free-living hookworm larvae. However, the successful incubation of hookworm eggs from pup faeces demonstrated that for this species, eggs develop and hatch within 8 – 11 days, and has provided a source of material for future morphological and genomic studies.

Objective 2c. Key findings

This objective has been successfully achieved.

Immune development

In our initial study on immune development in pups, mean percentage of labelled B cells was significantly lower ($p = 0.002$) in the 2011 Dangerous Reef (winter) group compared with the 2010 Dangerous Reef (summer) and the Seal Bay 2010 (spring) groups. Pup standard length was also significantly lower in the 2011 Dangerous Reef group (70.75cm ±1.525) than in the 2010 Reef (80.81cm ± 1.Dangerous 47) and Seal Bay 2010 groups (78.97 ± 1.364). Pearson's correlation coefficient tests indicated a significant positive linear relationship between B cell percentages and standard

length, r (38) = 0.56, $p < 0.001$. These results point to the need to consider age effects in investigating the potential differences in immune development between season or colony. Due to the unreliability of age proxies, such as standard length and weight, discovered in this project, this requires comparisons of rate of immune development of known age pups. As of February 2013 we have serial samples from Seal Bay pups of known age from one breeding season and serial samples of known intervals from ivermectin treated and non-treated pups from Dangerous Reef over two seasons. These samples are currently being analysed by flow cytometry to assess rate of development of B cells and T cell activity (MHCII expression) for comparison between seasons and colonies, MHCII genotype, and in relation to clinical data to assess the role of adaptive immunity in clearance of hookworm infection and the potential for genotype, season or colony to affect immune development and, therefore, persistence of hookworm infection. Additional to the proposed project, RNA samples being extracted as part of the MHCII study will be used for ongoing analysis of immune development using qPCRs developed during this study for a range of immune mediator genes.

Additional findings include identification of additional cross-reactive antibodies for IgE ELISA, and other antibodies to allow confirmation of an unstained population of cells in flow cytometry (Natural Killer NK vs B cells) by labelling of surface IgM or Pax5. Trials of several antibodies over the course of the study have been unsuccessful but we have had recent success with antibodies to all of these in immunohistochemistry, indicating potential for use in ELISA and flow cytometry.

MHCII genotypes
The peptide binding regions of the MHCII genes DQB1 and DRB1 have been described, with 3 and 5 alleles identified, respectively, by a combination of direct sequencing, cloning and sequencing and single conformational polymorphism (SSCP). Low diversity of DQB1 has been described across Dangerous Reef and Seal Bay, with all three alleles shared across both colonies (the first evidence suggesting possibility of male-mediated gene flow between these two colonies), and a higher degree of homozygosity in the Seal Bay colony than Dangerous Reef. The study of diversity of DRB1 on a population level using the proposed method of OSCP has been unsuccessful to date due to difficulties in resolving alleles. We will be resolving this issue using 454 sequencing of cDNA from exons 1-3 in the coming months. Delaying this aspect until this late stage in the project has been necessary to allow careful selection of animals with known clinical and treatment status and immune development rates, to permit efficient use of this more expensive approach.

3. Investigate the implications of hookworm-associated disease as a forcing factor of population demography in *N. cinerea*.

Objective 3a. Key findings
This objective has been successfully achieved.

We investigated the association of hookworm infection with clinical disease and its relationship to pup morbidity and mortality by comparing saline-treated (control) pups with ivermectin-treated pups. Experiments were undertaken at Dangerous Reef during the 2011 and 2013 breeding seasons, representing high and low pup mortality seasons respectively. Pups were randomly allocated to either a treatment or control group based upon their standard length and samples of faeces and blood were collected.

In 2011, pup recruitment (treatment n=77; control n=74) was undertaken during May (n=32), July (n=62), and August (n=57). Resights of recruited pups were undertaken during subsequent field trips with a subset recaptured and sampled during July (n=19), August (n=35), and September (n=10). In 2013, pup recruitment (treatment n=16; control n=16) was undertaken during January (n=32) with resights and recaptures being undertaken during February (n=24).

Ivermectin was significantly associated with a subsequently negative faecal sample, however, efficacy was not 100% with one treated pup in each breeding season with positive hookworm status at the time of re-sampling.

Comparisons of growth rates, haematological parameters, and survival was examined between treatment and control pups for each season. In 2011, mean daily weight gain was significantly greater ($p < 0.05$) for younger pups (< 70cm standard length) receiving ivermectin (n=26) compared to controls (n=23). This observed increase in growth rates following treatment with ivermectin provides evidence that hookworm infection acts to limit and reduce growth rates in young pups. Older pups (≥ 70cm standard length) receiving ivermectin demonstrated significantly lower ($p < 0.01$) mean daily weight gain (n=8) compared to controls (n=7). The reason for this effect on growth rates in older pups is not clear; however based on this finding, only pups < 70cm standard length were recruited for the subsequent 2013 trial. Interestingly, no significant difference in mean daily growth rates ($p = 0.65$) was observed in the 2013 treatment trial.

Analysis of haematological parameters in treated and control pups has provided further evidence that hookworm infection is associated with anaemia in pups, and induces an eosinophilia (increase in eosinophil counts) in pups.

A key outcome of the ivermectin experiments was to investigate the impact of hookworm infection on pup survival. Recruited pups were classified as 'alive', 'dead', or 'not seen'. 'Not seen' pups may have either temporarily or permanently emigrated, or their death may have been unidentified due to the limited field time appropriate for this field site. Temporary emigration was confirmed for several pups resighted on subsequent field trips during 2011.

Unexpectedly, no significant differences in mortality or 'not seen' were present between treatment and control groups. Further analysis examining the time to death or 'not seen' during 2011 also demonstrated no significant differences between groups. Variation in the ages of recruited pups, their hookworm burden and stage of infection, together with hypothesised differences in the dynamics of disease between seasons (observed as variable mortality rates and age-at-mortality), may explain the apparent lack of treatment effect on pup survival. Data obtained from known-aged pups at Seal Bay indicates that the maximal treatment effect on hookworm-related mortality would likely be achieved by administering treatment prior to the onset of patent hookworm infection and the appearance of hookworm-associated pathology, occurring within 14 days of parturition.

The Dangerous Reef treatment trials have demonstrated that the use of morphological age-proxies is inadequate for the selection of appropriately-aged pups for hookworm

treatment directed at preventing mortality.

Objective 3b. Key findings

Demographic impact of variable pup survival and recruitment due to hookworm infection

Two treatment and control studies have been conducted at Dangerous Reef during the 2011 and 2013 breeding seasons, for which final sample collection was undertaken in February 2013. Whilst preliminary survival analysis has been undertaken for the first treatment trial conducted in 2011 as detailed above, data analysis is ongoing for the 2011 study. As such, population modelling incorporating hookworm survival data is ongoing and is awaiting analysis of the 2013 treatment and control study.

Objectives 3c. Key findings

Demographic implications of density and environmental dependence of hookworm infection

As noted above for Objective 2b, logistical difficulties associated with the remote nature of the field locations including limited field time and difficulties associated with sample storage and analysis at these field sites, has resulted in insufficient data to comprehensively report on the longevity and density of free-living hookworm larvae in the colony substrate. As such, it is not possible to meaningfully evaluate the impact of population density and environmental dependence of hookworm infection on population demographics. It is proposed that this data be collected as part of a focussed investigation of the hookworm life cycle in this species, which would necessitate extended periods of sampling in individual colonies across a range of environmental conditions/seasons and varying host density.

Based on the approximately 100% incidence of hookworm infection in pups, it appears likely that factors affecting load of hookworm larvae, rather than presence, are of high importance; such as immune or general health status of mothers, or period since last lactation.

Objective 3d. Key findings

Potential efficacy and/or complications of hookworm control in the management of threatened/declining populations.

This objective has been successfully achieved and is also addressed in section 3. Implications for Management.

The treatment and control trial has demonstrated that a single dose of ivermectin is sufficient to eliminate patent hookworm infection in treated pups (excluding n=2 individuals), indicating high therapeutic efficacy for the management of hookworm infection in Australian sea lion pups. In addition, no direct adverse effects were identified with our sampling procedures, including the subcutaneous injection of pups with either the ivermectin treatment or saline control. In the 2011 treatment trial, a significantly lower mean daily weight gain ($p < 0.01$) was observed in treated (n=8) compared to control pups (n=7) in the older cohort of pups (> 70cms standard length), the reason for which is unclear. No difference in mortality was observed between the groups. Based on this finding, a precautionary approach was applied in the second treatment trial in 2013, with only pups < 70cm standard length, in which increased growth rates in ivermectin treated pups was seen in 2011, were treated. However, in

the absence of increased mortality or other adverse impacts occasioned by ivermectin treatment in this older pup cohort, further investigation of this treatment effect on a larger sample size of older pups may be warranted to determine if this is a repeatable finding.

We conclude that ongoing monitoring of key *N. cinerea* populations is essential for the identification of disease and population trends and for the early recognition of threats requiring intervention. Despite the near 100% efficacy of ivermectin treatment in eliminating patent hookworm infection in *N. cinerea* pups, based on the findings of the treatment and control trial, and the logistical constraints that would limit feasibility and successful implementation, the widespread routine treatment of pups for hookworm infection in free-ranging, threatened *N. cinerea* populations is not recommended without further research of seasonal prevalence of hookworm infection across the species range, efficacy, route of administration, and safety of anthelmintic applications, including further investigations on the effect of treatment on pup growth rates. However, whilst we do not consider the widespread routine use of hookworm treatment to be logistically viable in free-ranging *N. cinerea* populations based on our study, the limited use of targeted treatment may serve to improve survival in the event of other disease threats, and may in certain circumstances be considered beneficial for the management and conservation of the species.

3. Implications for Management

What are the key recommendations for management based on the findings.

Hookworm infection and disease is a highly prevalent (100%) endemic disease in Australian sea lion pups and contributes significantly to direct pup mortality (18%). It is also a significant indirect contributor to mortality, particularly in pups dying from trauma and starvation. The discovery of prepatent hookworm infections causing significant faecal blood loss (indicative of haemorrhagic enteritis) is an important finding with implications for conservation management (see below).

Key recommendations for management based on our findings include:

- Ongoing monitoring of key populations is essential for the identification of disease and population trends and the early recognition of threats requiring intervention. Additionally, critical data regarding disease epidemiology may be obtained from marked populations such as the population at Seal Bay.

- The widespread routine treatment of pups for hookworm infection is not recommended. Preliminary analysis of our ivermectin studies did not demonstrate benefit to pup survival and raised important questions regarding the potential for adverse effects on pup development. Whilst it is likely that the cohort of pups enrolled in the survival studies encompassed a wide range of ages, potentially 'hiding' any true treatment effect, the widespread targeted treatment of an appropriate age group (less than 7-14 days of age) is likely to be logistically prohibitive. Consideration must also be given to the potential to impact the pup-maternal bond at such an early age unless a non-invasive method of administration can be developed (we are currently undertaking in-vitro studies for a non-invasive transdermal administration method), the environmental contamination with anthelmintics, and the risk of selection for parasitic resistance.

- Although we do not consider the widespread routine use of hookworm treatment to be viable logistically, the **limited** use of targeted treatment may have a role in improving survival in the event of other disease threats (for example, in the event of a bacterial epidemic as seen in other pinniped populations) or population endangerment. For this reason, further research regarding seasonal prevalence of hookworm infection across the species range, efficacy, route of administration, and safety of anthelmintic applications is paramount.

4. Other Benefits

How has this project advanced the field of research? (e.g. scientific discoveries, new methodologies)

Several new methodologies have been developed during the course of this project.

Haematological analysis

The determination of haematological parameters, such as total erythrocyte and leukocyte counts, is essential for the interpretation of clinical and immunological measures of health. However, significant limitations in obtaining this data exist for remote field locations where access to laboratory equipment is limited. Total cell counts were obtained for samples collected at Seal Bay using the manual haemocytometer method. Whilst effective, this method is time-consuming and requires microscopic examination of blood samples, which due to logistical constraints, precluded its use at Dangerous Reef. To overcome this limitation, EDTA anti-coagulated whole-blood samples collected from individuals at Dangerous Reef were preserved with Streck Cell Preservative (Streck, Omaha, NE, USA), chilled and then analysed using an automated haematology analyser within nine days of collection. This method has been successfully cross-validated using samples collected at Seal Bay (n=15) and stability experiments have also been conducted with no significant difference ($p = 0.10$) identified in total erythrocyte counts. Whilst differences in total leukocyte counts over time were statistical significant ($p = 0.02$), the variation was not clinically relevant.

To the best of our knowledge, this is the first study to determine haematological parameters using preserved whole blood samples collected from free-ranging wildlife. The methods we employed may be adapted for use in other species, facilitating health and disease investigations previously not logistically possible and can be applied to other remote Australian sea lion colony sample collection.

Faecal occult blood (FOB)

Detecting the presence of blood in faeces is critical to demonstrating the pathology associated with hookworm infection in live and dead pups. Commercially available kits were found to be inappropriate for this project due to the qualitative nature of the tests, the specificity of some kits to human haemoglobin, and the limited faecal sample volumes obtained in the study. The limitations of these kits were overcome by adapting the methodology of Colditz and Jambre (2008) for use with small faecal samples. Faecal samples were stored frozen and defrosted immediately prior to analysis. Samples (ranging from 5mg to 1.3g) were weighed, made up to a total volume of 10ml with saturated NaCl solution. Quantitative hookworm eggs per gram were determined, then an aliquot was serially diluted to 1:2000 and faecal occult blood was quantified using Multistix® 10 SG (Siemens Healthcare Diagnostics,

Bayswater, Victoria, Australia). Validation of this methodology with the novel use of frozen samples is underway.

Colditz, I.G. & Le Jambre, L.F. (2008) Development of a faecal occult blood test to determine the severity of *Haemonchus contortus* infections in sheep. Veterinary Parasitology 153(1-2): 93-99.

Genetics studies
The regions of *N. cinerea* MHCII involved in peptide binding have been characterised in this study, providing the tools for analysis of MHCII diversity in additional, smaller colonies; to assess likely limitations to diversity and associated disease vulnerability of those colonies.

Immune studies
Studies of immune function in free-ranging wildlife are rare due to the strict requirements for sample storage for many of these studies, and the lack of immunological reagents for these genetically divergent species. In this study we have developed a method for collecting and preserving quality samples for flow cytometry in remote field sites, and validated a suite of cross-reactive and novel reagents for flow cytometry and immune gene expression studies in otariids. We expect these to be applicable to a range of species, facilitating future investigations of immune susceptibility or epidemiological surveys in *N. cinerea* and other otariid species.

5. Problems Encountered (if any)

Describe any major problems encountered during the Activity and how they were addressed.

No major problems were encountered during the Activity period. However, it was necessary during the course of these investigations to develop new and innovative methodologies (refer to 4. Other benefits) in order to achieve some of the proposed project outcomes. Whilst this requirement did temporarily delay the progress of some of the proposed activities, their development has provided beneficial outcomes and applications for this and similar projects in free-ranging populations.

We encountered logistic challenges inherent in undertaking an investigation of a highly prevalent, endemic disease in free-ranging Australian sea lion populations. These include:

- Limited field time and logistical issues associated with sample storage and analysis in remote field sites (for example, this impacted on our ability to address Objective 2b: substrate analysis for free-living hookworm stages). A focussed study of hookworm life cycle in this species, including extended periods of sampling in individual colonies across a range of environmental conditions, would be necessary and was determined to be beyond the scope of the current project. It is certainly an area for future research;
- The role of maternal factors such as passive immunity, nutrition, and parity on the prevalence and intensity of pup hookworm infection cannot be controlled nor predicted;
- It was necessary to recruit pups already infected, and affected, by hookworm in our treatment and control study. In the Australian sea lion, naturally-occurring hookworm infection appears to infect 100% of pups; as such, infection-free controls may only be recruited into treatment trials by treatment

with ivermectin. The limitations imposed by the extended breeding season of the species, the limited available time in the field, and our ethical obligation/desire to minimise the impact on individuals and the colonies studied also affected the outcomes of our treatment/control ivermectin study.

- The role of unexpected emigration of pups to neighbouring sites at Dangerous reef- 'Not seen' pups may have either temporarily or permanently emigrated, or their death may have been unidentified, complicating the determination of individual pup survival in the treatment/control ivermectin study. Temporary emigration was confirmed for several pups resighted on subsequent field trips during the 2011 breeding season at Dangerous Reef.

All activities in the Work plan (excluding completion of the demographic analysis of the ivermectin treatment trials, comprehensive substrate analysis, and completion of histopathology of necropsy samples) have been achieved.

The only delay in achieving the expected outcomes for the project is in the submission of publications for peer review. The strength of much of the data collected for this project is the analysis and interpretation of a seasonal comparison of data collected from Seal Bay and Dangerous Reef. The final sample collection for the project was completed in February 2013 during the breeding season at Dangerous Reef. The majority of sample analysis has now been completed for this field season and data analysis is underway. To ensure that submitted manuscripts include data sets from all four seasons under investigation, there has been a necessary delay in the completion and submission of the manuscripts for peer review. A timeline for completion is provided in the section entitled 'Planned Publications'.

All other project outcomes (and many additional outcomes) have been achieved within the proposed project time frame.

6. Communication

How will results be communicated to management?

Results of this project have already been communicated to management via public seminars on Kangaroo Island (as detailed below).

- Dr Rachael Gray: Investigating the health status of sea lions on Kangaroo Island: Hookworm-associated disease and mortality in the Australian sea lion, *Neophoca cinerea*. *DENR Seminar Series, Kingscote Kangaroo Island, December 2010*
- Dr Damien Higgins: Disease susceptibility in the Australian Sea lion. *DENR Seminar Series, Kingscote Kangaroo Island, December 2010*
- Dr Rachael Gray Current research at Seal Bay: Health and disease investigations in the Australian sea lion. *DEH public seminar series, Kingscote, Kangaroo Island, June 2009.*

Future plan for communication of results to management:

- A comprehensive summary of the project's results, including implications and outcomes for management will be provided;
- At the earliest opportunity, a seminar will be provided to staff and management on Kangaroo Island to provide an opportunity for the formal presentation of results and informal discussion of the project's outcomes

including a suggested plan for ongoing research;
- All peer-reviewed publications will be forwarded to management and interested staff (particularly those directly involved in field support of the project on Kangaroo Island).

Stakeholder engagement feedback (plain English for feedback to stakeholders)

Based on the communication plan included in our application, we intend to provide feedback on the outcomes of this study to relevant stakeholders as detailed below.

1. **Scientific community:** our results have been communicated to the scientific community at both national and international scientific conferences (refer to 6. Presentations). Three abstracts were submitted for peer review in May 2013 for inclusion at the Biennial Conference on the Biology of Marine Mammals. As detailed below in 6. Planned Publications, we will also engage the scientific community through the publication of peer-reviewed publications in appropriate international scientific journals.
2. **State and Commonwealth Government Agencies involved in the management of the species (e.g. Department for Environment and Heritage, South Australia):** as detailed above, we have already engaged management via public seminars for DENWR, South Australia (previously DEH/ DENR), throughout the course of the project. In addition, we have formulated a plan for further engagement of this stakeholder (see above), and believe that the findings of this study can be used to inform local, state and national management and conservation policy development for the species. In addition, regular written updates on the project were provided to Seal Bay management and staff on the outcomes of the project.
3. **General Public:** as noted for 2. above, we have engaged the general public on Kangaroo Island via public seminars for DENWR, South Australia, throughout the course of the project. In addition, project summaries have been provided on an annual basis for dissemination to commercial tour operators at Seal Bay, Kangaroo Island at the request of management. In addition, as part of our communication of results to management, we propose an additional seminar which will be open to the general public to provide for formal presentation of results as well as informal discussion of the project's outcomes including a suggested plan for ongoing research. These forums will enable us to promote greater awareness of the causes and impact of mortality on the recovery of the species.
4. **Undergraduate students enrolled in veterinary, animal science, and science degrees:** this stakeholder has been engaged throughout the duration of the project via face to face communication in lectures, seminars, and conference presentations. In addition, detailed below are eight undergraduate honour's projects spanning both the veterinary and animal and veterinary bioscience degrees that have arisen from this study. These forums have allowed us to emphasise the importance and need for research for conservation and management of wild populations. The completion of the eight honours projects has also allowed us to engage in training future wildlife scientists in marine mammal research. Lastly, the publication of our results in peer-reviewed journals will also enhance the engagement of this stakeholder.

Students supported (if any)

- **Doctor of Philosophy (PhD)**
  Alan Marcus (Proposed thesis submission May 2014)
  Prevalence and impact of hookworm infection on Australian sea lion populations
- **Bachelor of Science (Veterinary) BSc (Vet) Honours**
  - Natalie Chow (2010) Investigation into MHC class II diversity in two populations of the Australian sea lion (*Neophoca cinerea*).
  - Laura Schmertmann (2010) Investigating aspects of health and disease in Australian sea lion, *Neophoca cinerea*, pups: Haematology and Protein Analysis.
  - Zoe Larum (2010) Histological and immunohistochemical investigations of selected tissues of the Australian sea lion, *Neophoca cinerea*, with a focus on hookworm disease.
  - Loreena Butcher (2011) Immunological development of Australian sea lion pups: Analysis of lymphocyte subsets, and MHC and cytokine expression.
  - Sy Woon (2012) Transdermal penetration of avermectins in Australian sea lion, *Neophoca cinerea*, pups.
- **Animal and Veterinary Bioscience Honours**
  - Tessa Wilkin (2009) Characterization of the MHCII of the Australian sea lion (*Neophoca cinerea*).
  - Elyssa Payne (2011) Primer design for MHCII DRB exon 2 of the Australian sea lion (*Neophoca cinerea*).
  - Benjamin Haynes (2013-current student) Investigating genetic variation of the hookworm species (*Uncinaria* spp.) infecting Australian sea lion pups, *Neophoca cinerea*, sampled at three allopatric colonies in South Australia.

PhD Theses and dissertations (if any)

Alan Marcus (Proposed submission May 2014) Prevalence and impact of hookworm infection on Australian sea lion populations

Publications (other than theses and dissertations)

Final sample collection for the project was undertaken at Dangerous Reef in February 2013 and whilst the majority of sample analysis has been completed for this field season, the analysis and processing of some samples is ongoing. Given that the interpretation of our data is significantly enhanced by a seasonal comparison of the data collected from the two breeding seasons at Seal Bay and Dangerous Reef, proposed publications have been delayed to facilitate the inclusion of data from all four sampling seasons.

In addition, the submission time frames for publication detailed below take in to consideration the proposed thesis submission date (May 2014) of the PhD student supported by the project.

Planned publications

- *Manuscript submission: Validation of faecal egg counts and PCV/ faecal occult blood as an indicator of intestinal hookworm burdens in N. cinerea*

Samples for haematological, faecal egg count and faecal occult blood, and intestinal worm burden data have been collected for two breeding seasons each at Seal Bay and Dangerous Reef. Initial technical difficulties with the determination of faecal occult blood have been overcome (refer to 4. Other Benefits), with data available for one season at each colony. The validation of this method is scheduled for completion in August 2013. Laboratory processing of haematological, faecal, and intestinal samples

has been completed and statistical analysis of results is underway. The preparation and submission of a manuscript is scheduled for completion in January 2014.

- *Manuscript submission: MHCII diversity in N. cinerea*

Results are complete for one gene (DQB). These and partial results for the other gene (DRB) formed the basis of a BSc (Vet) thesis, and are ready for publication, but we have elected to hold back results for DQB until results for DRB are available, to permit publication of a more comprehensive paper. DRB has been technically problematic. Clinical data and samples have been collected and prepared and are awaiting 454 sequencing of MHCII DRB in June/July 2013 and as such, this publication is scheduled for submission in December 2013. Results will compare MHCII diversity between Dangerous Reef and Seal Bay, and examine data for associations between alleles or heterozygosity, and clinical changes (anaemia) or rate of immune development (see study below).

- *Manuscript submission: Factors affecting immunological evaluation of N. cinerea*

Preliminary results have formed the basis for a BSc (Vet) thesis and have been presented as a poster at the international conference of the Wildlife Disease Association in Lyon, France in July 2012. These results indicate a high likelihood of development-related effects on the adaptive immune capacity of pups. Journal publication of these findings has been postponed pending results of flow cytometry analysis from longitudinal sampling of known-age pups at Seal Bay and samples from the final field season at Dangerous Reef (completed in February 2013). Sample analysis is currently underway.

The samples collected will enable us to examine the rate of immune development of known age pups at Seal Bay and compare this with the rate of development in Dangerous Reef pups in winter and summer seasons, and with and without ivermectin treatment.

The publication of these results is scheduled for submission by December 2013.

- *Manuscript submission: Speciation of hookworm infecting N. cinerea*

Hookworms were collected and preserved from pups found dead at Seal Bay, Dangerous Reef, and The Pages Islands. Morphological examination has been undertaken and standard measurements recorded, facilitating the comparison of *N. cinerea* hookworm specimens with other pinniped hookworms. No distinctive morphological features have been indentified in *N. cinerea* hookworms and specimens appear morphologically identical across the three colonies sampled. Molecular analysis was undertaken to address the question of hookworm speciation within and between *N. cinerea* colonies and other pinniped hosts. Sequencing of two regions of nuclear ribosomal DNA has been completed and the results demonstrate that a single species of *Uncinaria* hookworm infects *N. cinerea* pups. The phylogenetic relationships to other pinniped hookworms has been investigated with results demonstrating close evolutionary relationships between southern hemisphere otariid *Uncinaria*. The preparation and submission of a manuscript is scheduled for completion in October 2013.

- *Manuscript submission: Effect of climate (season) and substrate on free-living larval stages of hookworm in N. cinerea colonies*

Sediment samples were collected from Seal Bay, Seal Slide, and Dangerous Reef

during four breeding seasons (2010 – 2013). Whilst larvae have been recovered from a majority of samples, their identification as hookworm larvae has been hampered by a lack of distinctive morphological features. The successful hatching of hookworm eggs has however provided technical data and a source of material for future genomic studies. Additionally, the limited availability of equipment in the field for sample analysis precluded the collection of comphrehensive data across the wide range of environments present within each colony. Data collected will be incorporated into an epidemiological manuscript detailing the intensity and longevity of hookworm infection in pups.

- *Manuscript submission: Prevalence and intensity of hookworm infection in N. cinerea populations*

Sample collection for the determination of prevalence and intensity of hookworm infection in *N. cinerea* pups has been completed for two breeding seasons each at Seal Bay and Dangerous Reef. Laboratory processing of faecal and intestinal samples has been completed and preliminary statistical analysis has been completed. Significantly, our data demonstrates that the prevalence of hookworm infection is 100% at these two colonies. Intensity varies between individual pups and potentially across colonies and years. Further analysis of results incorporating factors such as pup age, location within colony, maternal age, and time of year, is ongoing. The preparation and submission of a manuscript is scheduled for completion in December 2013.

- *Manuscript submission: Factors affecting hookworm infection and mortality in N. cinerea*

The collection of clinical, faecal, haematological, and necropsy data has been completed for two breeding seasons at Seal Bay and Dangerous Reef. Processing of faecal and haematological samples has been completed. Short-to-medium term survival data has been collected for pups recruited at Seal Bay during 2010 and Dangerous Reef during 2011 and 2013. The accessibility of the Seal Bay colony and the application of permanent identification facilitates the acquisition of longer-term survival data than is possible for the Dangerous Reef colony. As such, additional resight data for pups recruited at Seal Bay during the 2010 and 2012 breeding seasons is scheduled for collection in September 2013. Analysis will incorporate survival data and the relative importance of host-pathogen-envinronmental factors for short-term and longer-term survival will be determined. This analysis is scheduled for late 2013/early 2014. Manuscript preparation and submission is scheduled for completion in March 2014.

- *Manuscript submission: Histopathology findings and cause of mortality in N. cinerea pups*

Gross necropsies have been conducted on a significant number of pups found dead at Seal Bay and Dangerous Reef during 2010 – 2013. Preliminary data regarding cause of mortality has been collated. The processing and examination of preserved tissues is ongoing and is expected to continue throughout 2013 due to the large number of samples collected. An analysis of the gross and histopathological data is expected to be undertaken during early 2014 with preparation and manuscript submission planned for May 2014.

- *Manuscript submission: Implications of hookworm infection on demography of N. cinerea populations*

We have investigated the implications of hookworm infection on the population demography of *N. cinerea* by obtaining data regarding the epidemiology of hookworm infection in pups at Seal Bay and Dangerous Reef and via the treatment and control studies at Dangerous Reef. The collection and processing of samples and data from these experiments has been completed and statistical analysis of the results is ongoing. Determining the impact of infection on population demography requires information regarding the estimated contribution to mortality which will be obtained from the examination and analysis of histopathology, detailed above. The results of the treatment and control studies will be reported in a separate manuscript scheduled for submission in March 2014.

Presentations

2013

Abstracts submitted for peer-review to *20th Biennial Conference on the Biology of Marine Mammals, December 2013, Dunedin, NZ.*

- Gray R, Schmertmann L, Marcus A, Terkildsen, M. Serum haptoglobin and plasma fibrinogen as acute inflammatory markers in hookworm disease in endangered Australian sea lion populations.
- Marcus A, Higgins D, Slapeta J, Gray R. Haematophagous hookworm (*Uncinaria* sp.) associated with high pup mortality in Australian sea lions (*Neophoca cinerea*).
- Haynes B, Marcus A, Slapeta J, Gray R. Barcoding hookworms *Uncinaria* sp. from Australian Sea Lions, *Neophoca cinerea*, using the mitochondrial cytochrome oxidase I gene.

An abstract will also be submitted to the Wildlife Disease Association Conference Australasian section Grampians, Victoria, 29th September - 4th October 2013.

2012

- Higgins DP, Lau Q, Butcher L and Gray R. Use of immunophenotyping to assess the health of two vulnerable endemic Australian species: the koala (*Phascolarctos cinerius*) and the Australian sea lion (*Neophoca cinerea*). *61st International conference of the Wildlife Disease Association, July 2012, Lyon, France*. Poster.
- Marcus AD, Higgins DP, Gray R Ivermectin treatment of free-ranging Australian sea lion pups: effect on hookworm infection status, pup growth and survival and haematological parameters. *Wildlife Disease Association – Australasian Section Conference, North Stradbroke Island, Australia, September 2012.*Oral.
- Marcus AD, Higgins DP, Gray R (2012) Ivermectin treatment of free-ranging Australian sea lion pups. *Faculty of Veterinary Science Postgraduate Conference, The University of Sydney, Sydney, Australia, November 2012.*Oral.

2011

- Gray R, Larum Z, Schmertmann L, Higgins D. Clinical disease and pathological findings associated with hookworm infection in threatened Australian sea lion populations. *19th Biennial Conference on the Biology of Marine Mammals 27th November-2nd December 2011, Tampa, Florida, USA.* Poster.

7. Project Outputs

A list of the actual outputs of the research including milestones, progress reports and data products such as models etc. *as per original application	Proposed date of completion	Actual date of completion

Conference presentation: Wildlife Disease Association (Catlins, NZ). Preliminary data on hookworm associated mortality in *N. cinerea*	12/2009	12/2009
Journal article submission: Factors affecting immunological evaluation of *N. cinerea*	12/2010	12/2013
Journal article submission: Speciation of hookworm infecting *N. cinerea*	04/2011	10/2013
Journal article submission: MHCII diversity in *N. cinerea*	06/2011	12/2013
Present prevalence and intensity data to date at relevant national conference (Wildlife Disease Association)	Approx. 09/2011 Replaced with presentation at 61st International conference of WDA, July 2012, Lyon, France.	07/2012
Present results to date at the Conference on Biology of Marine Mammals	Approx. 12/2011	12/2011 Tampa Florida
Journal article submission: Validation of faecal egg counts and PCV/ faecal occult blood as an indicator of intestinal hookworm burdens in *N. cinerea*	02/2012	01/2014
Data on prevalence of hookworm infection and hookworm associated mortality for population modelling	Throughout; finalise for all sampling 06/2013	05/2013 (see this report)
Present hookworm associated clinical disease and mortality findings to date at relevant national conference (Wildlife Disease Association)	Approx. 09/2012	09/2012 North Stradbroke Island
One PhD graduate and two honours students trained in marine mammal health and parasitology investigations, immunogenetics, immunology, pathology, and a range of other field, clinical and laboratory methods. Importantly, this will be in the context of an integrated study in wildlife health investigations, giving their technical expertise relevance to a range of national and internationally important wildlife and resource management issues	03/2013	PhD thesis submission: May 2014 Refer to 6. Communication for completion times of eight honours projects
Finalise manuscripts for submission: • Effect of climate (season) and substrate on free-living larval stages of hookworm in *N. cinerea* colonies; • Prevalence and intensity of hookworm infection in *N. cinerea* populations; • Factors affecting hookworm infection and associated mortality in *N. cinerea*; • Histopathology findings and cause of mortality in *N. cinerea* pups;	09/2013	 12/2013 (data to be incorporated into paper below) 12/2013 03/2014 05/2014

• Implications of hookworm infection on demography of *N. cinerea* populations. Journals to include: Journal of Wildlife Diseases, Marine Mammal Science, Journal of Parasitology, Parasitology Research, Endangered Species Research		03/2014
Participation in focal group discussions with Department for Environment and Heritage (DEH), South Australia staff at both a local and state level, and contributions to local, state and national management and conservation policy development for the species including providing advice for rapid response to disease epidemics and large-scale mortality events	Throughout and ongoing	24.05.2013 and ongoing
Support of undergraduate teaching in the field of wildlife health and disease investigations	Throughout and ongoing	24.05.2013 and ongoing
Present results at the Conference on Biology of Marine Mammals	Approx. 12/2013	12/2013
Progress Report 1 (Milestone 2)	30.07.2010	30.07.2010
Progress Report 2 (Milestone 3)	29.07.2011	29.07.2011
Progress Report 3 (Milestone 4)	28.07.2012	28.07.2012
Final Report (Milestone 5)	24.05.2013	24.05.2013