

Investigation and mitigation of reduced fecundity and pup survival due to infectious disease in the endangered Australian sea lion, *Neophoca cinerea*

A thesis submitted in fulfilment of the requirements for the
degree of Doctor of Philosophy by

Scott A. Lindsay

Sydney School of Veterinary Science, Faculty of Science
The University of Sydney
April 2024



Statement of originality

To the best of my knowledge, the content (including photographs) presented in this thesis is my own work. All information derived from both published and unpublished sources has been acknowledged and referenced within this thesis. I declare that this thesis has not been submitted for any degree or other purpose at this or any other institution.

Scott Lindsay

22 March 2024

Thesis summary

Australia's only endemic pinniped, the Australian sea lion (*Neophoca cinerea*), is endangered and its population is declining. Its failure to recover to pre-sealing abundance contrasts with increasing population trends observed in sympatric otariid species, with high pup mortality additional to low fecundity. The following reporting focuses on the contribution of infectious disease to modulation of population size, and the optimisation of a disease mitigation strategy for the pup cohort.

Chapter one provides background information for the Australian sea lion to contextualise the motivators for this thesis and to underscore the significance of its research findings, including unique aspects of the species' life history, and historical and contemporary threats contributing to its current endangered population status. The chapter focuses on disease threat and known contributors to pup mortality and discusses current understanding of their extent, known knowledge gaps and research priorities outlined in the species' Recovery Plan. Further, it highlights disease reporting in related otariid species as an indication of potential future disease threats posed to the Australian sea lion.

The first two data chapters advance existing knowledge of the substantial contribution of hookworm (*Uncinaria sanguinis*) infection to pup morbidity. **Chapter two** reports findings from a pilot study (n=85) of hookworm treatment at Dangerous Reef, Spencer Gulf, South Australia. It shows similar effectiveness for the novel use of an easily applied topical formulation of the ivermectin anthelmintic, compared to the injected treatment (estimated effectiveness 96.4% and 96.8%, respectively; $p>0.05$). It also provides the first documentation of a growth benefit in Australian sea lion pups following early hookworm elimination, albeit short-term and not achieving statistical significance. This includes a bodyweight specific growth rate [SGR] double that of untreated pups (0.717% [0.472–0.961%] versus 0.342% [0.112–0.573%; $p>0.05$]).

Chapter three reports outcomes from the subsequent large-scale treatment trial (n=322) of this formulation at Seal Bay, Kangaroo Island, South Australia, conducted over consecutive lower (2019, 19.2%) and higher (2020, 28.9%) mortality breeding seasons. It quantifies the significant ($p \leq 0.012$) short-term (one month) haematological changes associated with hookworm infection (exacerbation of neonatal anaemia, severe eosinophilia, a positive acute phase inflammatory response) in the context of neonatal ontogenesis, providing the most detailed assessment to date of the parasite's negative impact on pup health. Further, it provides the first report of significant short- ($p \leq 0.001$) and longer-term ($p \leq 0.012$) treatment-related growth benefits in this species and confirms that optimal outcomes are achieved by preventing patent disease elaboration. Favourable outcomes include substantial 42.1% and 34.2% improvements in standard length and bodyweight SGRs at approximately one month post-treatment. Treatment benefits are observed to be seasonally independent, relevant to the oscillating seasonal pup mortality observed at multiple Australian sea lion breeding colonies.

Chapter four reports 30.4% (95% CI, 13.2–52.9; n=23) adult female Australian sea lion (median age 9.5 years, range 5.5–14.5 years) seroprevalence and high antibody titres (512 to >2048 IU/ml) for *Toxoplasma gondii*, indicating a comparable level of parasite exposure and potential disease risk to that reported in other pinniped populations. An absence of seroconversion in any sampled pup from felid-free and felid-inhabited islands (97.5% CI, 0.0–2.0%; n=184) supports the lack of detectable congenital transmission or early post-natal infection, favouring a hypothesis of predominantly foraging-associated parasite exposure in this species.

Chapter five reports cause-of-mortality and the presence of comorbidity in Australian sea lion pups across five breeding cohorts (n=122) at Seal Bay, identifying an infectious disease (hookworm-associated haemorrhagic enteritis; bacterial infection, mostly *Streptococcus* spp.) contribution in ~38% of deaths. This is additional to confirmation that Australian sea lion pups experience a high level of mortality due to conspecific trauma (41.0%) and starvation (24.6%). The study introduces

population modelling as another tool in the investigation of pup mortality in the species, offering an improved assessment of risk. It predicts a significant ($p < 0.001$) reduction in all-cause-mortality-risk with early hookworm elimination. Relevant to the species' seasonally oscillating pup mortality are predicted associations of summer-autumn season with increased risk of death with trauma (odds ratio [OR] 2.49), consequential bacterial infection (OR 5.97) and hookworm-associated haemorrhagic enteritis (OR 1.61).

Following a discussion of the relevance of thesis outcomes, **Chapter six** proposes management implications relating to potential hookworm mitigation strategies and offers suggestions for the direction of future disease investigation in the Australian sea lion.

Acknowledgements

Completion of this study would not have been possible without the support, encouragement, commitment, generosity, and sacrifice of others. First and foremost, I am deeply indebted to my supervisors, Associate Professors Rachael Gray and Charles Caraguel. Rachael, thank you for introducing me to one of the most captivating and endearing of species, for sharing your extensive knowledge and passion for their welfare, and for your tremendous patience managing a part-time remote student. Charles, thank you for rescuing me from my data overload, for your perseverance instructing me in the basics of Stata, and for continually challenging my reasoning and the current dogma to advance our research outcomes. Many thanks also to Professors Jan Šlapeta and Damien Higgins, for their supervisory input and guidance in the early stages of my research. I'm extremely appreciative for generous funding contributions enabling this research, from The Hermon Slade Foundation; the FS Quiney and BR Richards Bequests, Sydney School of Veterinary Science, The University of Sydney; the School of Animal and Veterinary Sciences, The University of Adelaide; and the Department for Environment and Water (DEW), South Australia. I would also like to extend my sincere thanks to my former colleagues at The University of Adelaide, for their coverage during fieldwork absences and for laboratory assistance, with special thanks to Associate Professor Lucy Woolford, Professor Ryan O'Handley and the staff at the Veterinary Diagnostic Laboratory. Thank you also to the staff at the Veterinary Pathology Diagnostic Service laboratory at The University of Sydney, for their contribution to this research.

An at times challenging fieldwork component to these studies was made more rewarding by the teamwork, dedication, comradery, and humour of Mariel Fulham, Shannon Taylor, and Matthew Gray. Mariel, I'd especially like to acknowledge your commitment to continued sample collection during the pandemic. It was an honour to have shared my first fieldwork experience with Dr Eugene Lyons. Most of this thesis reports research at Seal Bay on Kangaroo Island. I'd like to extend my sincere thanks to the many islanders who welcomed our team and substantially contributed to these

studies. This includes Seal Bay Conservation Park Staff at the Department for Environment and Water, especially Melanie Stonnill, Melanie Stephens, and Tanya Rosewarne, for their friendly, enthusiastic, and knowledgeable contributions to colony visits and sample collection. A special thanks to Janet Simpson, for hosting our research team, providing all manner of logistical support, and thought-provoking contributions to our team conversations. Finally, I'd like to express my deepest gratitude to my family, for their emotional support, interest, and encouragement for the entirety of this journey. Roz, thank you for your enduring support, for your understanding and substantial contribution to managing everything during my absences, and for always being there to offer advice or to provide a sounding board for ideas.

Visiting some incredible Australian sea lion island colonies, living among them, and contributing to their conservation, has been a unique privilege – thank you all for enabling and sharing this experience.

Author contributions

Chapter two is published as:

Lindsay, S.A., Caraguel, C.G.B., and Gray, R. (2021). Topical ivermectin is a highly effective seal 'spot-on': a randomised trial of hookworm and lice treatment in the endangered Australian sea lion (*Neophoca cinerea*). *International Journal for Parasitology: Parasites and Wildlife* 16, 275-284. doi: [10.1016/j.ijppaw.2021.11.002](https://doi.org/10.1016/j.ijppaw.2021.11.002).

Scott Lindsay was the primary author of this chapter and contributed to all aspects of this publication, including conceptualization, data curation, formal analysis (statistical), funding acquisition, investigation (fieldwork; laboratory), methodology, project administration, resources, validation, visualisation, and writing (original draft; review and editing). Charles Caraguel contributed to data curation, formal analysis (statistical), supervision, validation, visualisation, and writing (review and editing). Rachael Gray contributed to conceptualization, funding acquisition, investigation (fieldwork; laboratory), methodology, project administration, resources, supervision, validation, and writing (review and editing). All authors reviewed and approved the final manuscript.

Chapter three is published as:

Lindsay, S.A., Fulham, M., Caraguel, C.G.B., and Gray, R. (2023). Mitigating disease risk in an endangered pinniped: early hookworm elimination optimizes the growth and health of Australian sea lion pups. *Frontiers in Veterinary Science* 10, 1161185. doi: [10.3389/fvets.2023.1161185](https://doi.org/10.3389/fvets.2023.1161185).

Scott Lindsay was the primary author of this chapter and contributed to all aspects of this publication, including conceptualization, data curation, formal analysis (statistical), funding acquisition, investigation (fieldwork; laboratory), methodology, project administration, resources,

validation, visualisation, and writing (original draft; review and editing). Mariel Fulham contributed to data curation, investigation (fieldwork; laboratory), and writing (review and editing). Charles Caraguel contributed to data curation, formal analysis (statistical), supervision, validation, visualisation, and writing (review and editing). Rachael Gray contributed to conceptualization, funding acquisition, investigation (fieldwork; laboratory), methodology, project administration, resources, supervision, validation, and writing (review and editing). All authors reviewed and approved the final manuscript.

Chapter four is published as:

Lindsay, S.A., Caraguel, C.G.B., O’Handley, R., Šlapeta, J., and Gray, R. (2022). *Toxoplasma gondii* seroprevalence in the endangered Australian sea lion (*Neophoca cinerea*). *Frontiers in Marine Science* 9, 965865. [doi: 10.3389/fmars.2022.965865](https://doi.org/10.3389/fmars.2022.965865).

Scott Lindsay was the primary author of this chapter and contributed to all aspects of this publication, including conceptualization, data curation, formal analysis (statistical), funding acquisition, investigation (fieldwork; laboratory), methodology, project administration, resources, validation, visualisation, and writing (original draft; review and editing). Charles Caraguel contributed to formal analysis (statistical), supervision, visualisation, and writing (review and editing). Ryan O’Handley contributed to investigation (laboratory), methodology, resources, validation, and writing (review and editing). Jan Šlapeta contributed to conceptualization, funding acquisition, supervision (supporting), visualisation, and writing (review and editing). Rachael Gray contributed to conceptualization, funding acquisition, investigation (fieldwork), project administration, resources, supervision, and writing (review and editing). All authors reviewed and approved the final manuscript.

Chapter five is submitted as:

Lindsay, S.A., Fulham, M., Stonnill, M., Caraguel, C.G.B., and Gray, R. (2024). Contribution of disease to Australian sea lion (*Neophoca cinerea*) pup mortality at Seal Bay, Kangaroo Island, 2016–2022. *Marine Mammal Science* (submitted 28 March 2024).

Scott Lindsay was the primary author of this chapter and contributed to all aspects of this publication, including conceptualization, data curation, formal analysis (statistical), funding acquisition, investigation (fieldwork; laboratory), methodology, project administration, resources, visualisation, and writing (original draft; review and editing). Mariel Fulham contributed to data curation, investigation (fieldwork; laboratory), and writing (review and editing). Melanie Stonnill contributed to investigation (fieldwork), funding acquisition, and writing (review and editing). Charles Caraguel contributed to data curation, formal analysis (statistical), supervision, visualisation, and writing (review and editing). Rachael Gray contributed to conceptualization, funding acquisition, investigation (fieldwork), methodology, project administration, resources, supervision, visualisation, and writing (review and editing). All authors reviewed and approved the final manuscript.

In chapters three, four and five, the corresponding author (Rachael Gray) has provided permission to include published material in this thesis.

Scott Lindsay

22 March 2024

Attesting author contribution statement

As the primary supervisor for the candidature upon which this thesis is based, as well as the corresponding author for publications included in chapters three, four and five, I can confirm that the authorship attribution statements above are correct.

Rachael Gray

26 March 2024

Lindsay, S.A., and Gray, R. (2021). A novel presentation of tuberculosis with intestinal perforation in a free-ranging Australian sea lion (*Neophoca cinerea*). *Journal of Wildlife Diseases* 57(1), 220–224. [doi: 10.7589/JWD-D-20-00104](https://doi.org/10.7589/JWD-D-20-00104).

Lindsay, S.A., Caraguel, C.G.B., and Gray, R. (2021). Topical ivermectin is a highly effective seal 'spot-on': A randomised trial of hookworm and lice treatment in the endangered Australian sea lion (*Neophoca cinerea*). *International Journal for Parasitology: Parasites and Wildlife* 16, 275–284. [doi: 10.1016/j.ijppaw.2021.11.002](https://doi.org/10.1016/j.ijppaw.2021.11.002).

Lindsay, S.A., Caraguel, C.G.B., O’Handley, R., Šlapeta, J., and Gray, R. (2022). *Toxoplasma gondii* seroprevalence in the endangered Australian sea lion (*Neophoca cinerea*). *Frontiers in Marine Science* 9, 965865. [doi: 10.3389/fmars.2022.965865](https://doi.org/10.3389/fmars.2022.965865).

Lindsay, S.A., Fulham, M., Caraguel, C.G.B., and Gray, R. (2023). Mitigating disease risk in an endangered pinniped: early hookworm elimination optimizes the growth and health of Australian sea lion pups. *Frontiers in Veterinary Science* 10, 1161185. [doi: 10.3389/fvets.2023.1161185](https://doi.org/10.3389/fvets.2023.1161185).

Lindsay, S.A., Fulham, M., Stonnill, M., Caraguel, C.G.B., and Gray, R. (2024). Contribution of disease to Australian sea lion (*Neophoca cinerea*) pup mortality at Seal Bay, Kangaroo Island, 2016–2022. *Marine Mammal Science* (submitted 28 March 2024).

Presentations arising from candidature

Lindsay, S., Higgins, D., Šlapeta, J., Gray, R (2018). Management of endemic hookworm disease in an endangered pinniped, *Neophoca cinerea*. [Oral presentation]. Australian Marine Sciences Association (AMSA) 55th Annual Conference, 1–5 July, Adelaide Convention Centre, Adelaide, South Australia.

Lindsay, S., Higgins, D., Šlapeta, J., Gray, R (2018). Management of endemic hookworm disease in an endangered pinniped, *Neophoca cinerea*. [Oral and poster presentation]. Sydney School of Veterinary Science (SSVS) Annual Postgraduate Conference, 7 November, University of Sydney, Camperdown, New South Wales.

Lindsay, S.A. (2019) Management of endemic hookworm disease in the Australian sea lion. [Oral presentation]. Wildlife Health Network (WHN) South Australia Biannual Meeting, 19 October, Adelaide Zoo, Adelaide, South Australia.

Gray, R., **Lindsay, S.**, Taylor, S., Fulham, M. (2019). Improving sea lion pup survival. [Oral presentation]. Science in the Pub public seminar, July, Ozone Hotel, Kingscote, Kangaroo Island, South Australia.

Lindsay, S., Higgins, D., Šlapeta, J., Gray, R (2020). Quantifying the medium- to long-term benefit of treating hookworm infection in the endangered pinniped, *Neophoca cinerea*. [Online presentation]. Sydney School of Veterinary Science (SSVS) Annual Postgraduate Conference, November, University of Sydney, Camperdown, New South Wales.

Lindsay, S., Caraguel, C., Gray, R (2021). ‘Spot-on’ ivermectin hookworm treatment in the endangered Australian sea lion: final recruitment, high-mortality 2020–2021 summer breeding season at Seal Bay, South Australia. [Online presentation]. Sydney School of Veterinary Science

(SSVS) Annual Postgraduate Conference, 22 November, University of Sydney, Camperdown, New South Wales.

Gray, R, Fulham, M, Wheatley, S, Terkildsen, M, Stonnill, M, Stephens, M, **Lindsay, S** (2022).

Topical ivermectin treatment improves pup survival in endangered Australian sea lions. [Oral presentation]. Australian Marine Sciences Association (AMSA) 58th Annual Conference, 7–11 August, Pullman International, Cairns, Queensland.

Gray, R, Fulham, M, Stonnill, M, Wheatley, S, Terkildsen, M, Caraguel, C, **Lindsay, S** (2024).

Elimination of hookworm infection using a topical ivermectin treatment reduces mortality risk in the endangered Australian sea lion, *Neophoca cinerea*. [Submitted abstract]. 25th Biennial Conference on the Biology of Marine Mammals, 11–15 November, Perth Convention and Exhibition Centre, Perth, Western Australia.

Research supervision arising from candidature

Stathis, K., **Lindsay, S.A.**, Larum, Z., Marcus, A., Terkildsen, M., Gray, R. (2018). Hookworm-associated pathology in neonatal pups of the endangered Australian sea lion, *Neophoca cinerea*. [Co-supervision]. Honours, Animal and Veterinary Bioscience, Sydney School of Veterinary Science, The University of Sydney, Camperdown, New South Wales.

Lindholm, V., Boardman, W., **Lindsay, S.** (2019). Validation of the ADVIA 2120 and Sysmex XT-2100 haematology analysers for differential leukocyte, nRBC, platelet and reticulocyte counts in the Australian sea lion, *Neophoca cinerea*. [Primary supervision]. Clinical Research Project, Doctor of Veterinary Medicine (DVM1), School of Animal and Veterinary Sciences, The University of Adelaide, Roseworthy, South Australia.

Ho, W., O’Handley, R., **Lindsay, S.** (2019). Short-term efficacy analysis of a treatment for hookworm (*Uncinaria sanguinis*) in the Australian sea lion (*Neophoca cinerea*). [Primary supervision]. Clinical Research Project, Doctor of Veterinary Medicine (DVM1), School of Animal and Veterinary Sciences, The University of Adelaide, Roseworthy, South Australia.

Bielby, M., Trott, D.J., Veltman, T., Kidd, S., **Lindsay, S.** (2021). Identification of streptococcal bacteria and documentation of associated disease syndromes in endangered Australian sea lion pups. [Primary supervision]. Clinical Research Project, Doctor of Veterinary Medicine (DVM1), School of Animal and Veterinary Sciences, The University of Adelaide, Roseworthy, South Australia.

Hajicosta, K., Knight, S., Woolford, L., Boardman, W., **Lindsay, S.** (2022). A scoping review of the impacts and risks of climate change on Australian marine mammals. [Primary supervision]. Clinical Research Project, Doctor of Veterinary Medicine (DVM1), School of Animal and Veterinary Sciences, The University of Adelaide, Roseworthy, South Australia.

TABLE OF CONTENTS

STATEMENT OF ORIGINALITY	II
THESIS SUMMARY	III
ACKNOWLEDGEMENTS	VI
AUTHOR CONTRIBUTIONS	VIII
ATTESTING AUTHOR CONTRIBUTION STATEMENT	XI
PEER-REVIEWED PUBLICATIONS ARISING FROM CANDIDATURE	XII
PRESENTATIONS ARISING FROM CANDIDATURE	XIII
RESEARCH SUPERVISION ARISING FROM CANDIDATURE.....	XV
TABLE OF CONTENTS	XVI
THESIS ABSTRACT	1
CHAPTER ONE – INTRODUCTION, LITERATURE REVIEW AND THESIS AIMS	2
1.1. INTRODUCTION TO THE AUSTRALIAN SEA LION	3
1.2. AUSTRALIAN SEA LION POPULATION	4
1.2.1. <i>Colony distribution and species abundance</i>	4
1.2.2. <i>Study colonies</i>	6
1.3. LIFE HISTORY	11
1.3.1. <i>Breeding cycle and life stages</i>	11
1.3.2. <i>Foraging and diet</i>	15
1.4. CONTRIBUTORS TO POPULATION ABUNDANCE	16
1.4.1. <i>Low fecundity and high pup mortality</i>	16
1.4.2. <i>Contemporary threats</i>	17
1.5. DISEASE IN THE AUSTRALIAN SEA LION	21
1.5.1. <i>Current monitoring status and species’ recovery plan guidelines</i>	21
1.5.2. <i>Hookworm disease</i>	23
1.5.3. <i>Toxoplasmosis</i>	28
1.6. PUP MORTALITY	31
1.6.1. <i>Australian sea lion</i>	31
1.6.2. <i>Other otariid species</i>	33
1.7. THESIS OUTLINE AND RESEARCH AIMS	44
1.8. REFERENCES	46
CHAPTER TWO – TOPICAL IVERMECTIN IS A HIGHLY EFFECTIVE SEAL ‘SPOT-ON’: A RANDOMISED TRIAL OF HOOKWORM AND LICE TREATMENT IN THE ENDANGERED AUSTRALIAN SEA LION (NEOPHOCA CINEREA) ..	64
AUTHOR ATTRIBUTION STATEMENT	65
ABSTRACT	66
1. INTRODUCTION	66
2. MATERIALS AND METHODS.....	67
2.1. <i>Study site and population</i>	67
2.2. <i>Study design and sample collection</i>	67
2.4. <i>Hookworm infection assessment</i>	69
2.5. <i>Statistical analyses</i>	69
3. RESULTS	70
3.1. <i>Recruitment and success of random allocation</i>	70
3.2. <i>Treatment effectiveness against hookworm and lice</i>	70
3.3. <i>Treatment impact on blood analyte levels</i>	71
3.4. <i>Treatment impact on growth</i>	71
3.5. <i>Monitoring of pup survival</i>	72
4. DISCUSSION	72
4.1. <i>Treatment effectiveness against hookworm and lice</i>	72
4.2. <i>Treatment impact on haematological parameters</i>	73
4.3. <i>Treatment impact on growth</i>	73

4.4. <i>Monitoring of pup survival</i>	73
REFERENCES	74
GRAPHICAL ABSTRACT	76
SUPPLEMENTARY DATA.....	77
CHAPTER THREE – MITIGATING DISEASE RISK IN AN ENDANGERED PINNIPED: EARLY HOOKWORM ELIMINATION OPTIMIZES THE GROWTH AND HEALTH OF AUSTRALIAN SEA LION PUPS.....	80
AUTHOR ATTRIBUTION STATEMENT	81
ABSTRACT.....	83
1. INTRODUCTION	83
2. MATERIALS AND METHODS.....	84
2.1. <i>Ethics approvals and reporting</i>	84
2.2. <i>Study site and duration</i>	84
2.3. <i>Study design and sample collection</i>	85
2.4. <i>Specimen processing</i>	85
2.5. <i>Statistical analysis</i>	85
2.6. <i>Survival</i>	86
3. RESULTS	86
3.1. <i>Recruitment, success of random allocation and recapture rates</i>	86
3.2. <i>Treatment effect on estimated hookworm prevalence and incidence of clearance</i>	88
3.3. <i>Treatment effect on growth</i>	89
3.4. <i>Treatment effect on hematological parameters</i>	90
4. DISCUSSION	93
4.1. <i>Treatment effect on hookworm prevalence and incidence</i>	94
4.2. <i>Treatment effect on growth</i>	94
4.3. <i>Treatment effect on hematological parameters</i>	94
4.4 CONCLUSION	96
REFERENCES.....	97
SUPPLEMENTARY MATERIAL	99
CHAPTER FOUR – <i>TOXOPLASMA GONDII</i> SEROPREVALENCE IN THE ENDANGERED AUSTRALIAN SEA LION (<i>NEOPHOCA CINEREA</i>)	103
AUTHOR ATTRIBUTION STATEMENT	104
ABSTRACT.....	106
INTRODUCTION.....	107
MATERIALS AND METHODS	108
<i>Study colonies</i>	108
<i>Sample collection and age estimation</i>	108
<i>Serological testing and analysis</i>	109
RESULTS	110
DISCUSSION	111
REFERENCES.....	114
SUPPLEMENTARY MATERIAL	116
CHAPTER FIVE – CONTRIBUTION OF DISEASE TO AUSTRALIAN SEA LION (<i>NEOPHOCA CINEREA</i>) PUP MORTALITY AT SEAL BAY, KANGAROO ISLAND, 2016–2022	118
AUTHOR ATTRIBUTION STATEMENT	119
ABSTRACT.....	122
1 INTRODUCTION.....	123
2 MATERIALS AND METHODS	125
2.1 <i>Ethics approvals and reporting</i>	125
2.2 <i>Study site</i>	126
2.3 <i>Detection of dead pups and data collection</i>	128
2.4 <i>Gross necropsy examination</i>	129
2.5 <i>Pup age at death</i>	130
2.6 <i>Primary cause-of-mortality categorization</i>	131
3 RESULTS	134

3.1 <i>Sampling summary</i>	134
3.2 <i>Mortality demographics and distribution</i>	135
3.3 <i>Primary cause-of-mortality categorization</i>	135
3.4 <i>Temporal and seasonal association with primary cause-of-mortality categorization</i>	146
3.5 <i>Modelling of population risk – seasonal and treatment effects</i>	147
4 DISCUSSION	153
REFERENCES	165
SUPPLEMENTARY MATERIAL	174
CHAPTER SIX – GENERAL DISCUSSION AND CONCLUSIONS	176
6.1 INTRODUCTION	177
6.2 RESEARCH OUTCOMES	178
6.3 MANAGEMENT IMPLICATIONS AND FUTURE DIRECTIONS	184
6.3.1 <i>Hookworm mitigation strategies</i>	185
6.3.2 <i>Future directions</i>	187
6.4 CONCLUSION	190
6.5 REFERENCES	192
APPENDIX – ADDITIONAL PUBLICATION ARISING FROM CANDIDATURE	197
A NOVEL PRESENTATION OF TUBERCULOSIS WITH INTESTINAL PERFORATION IN A FREE-RANGING AUSTRALIAN SEA LION (<i>NEOPHOCA CINEREA</i>)	198
COLOUR FIGURES	203

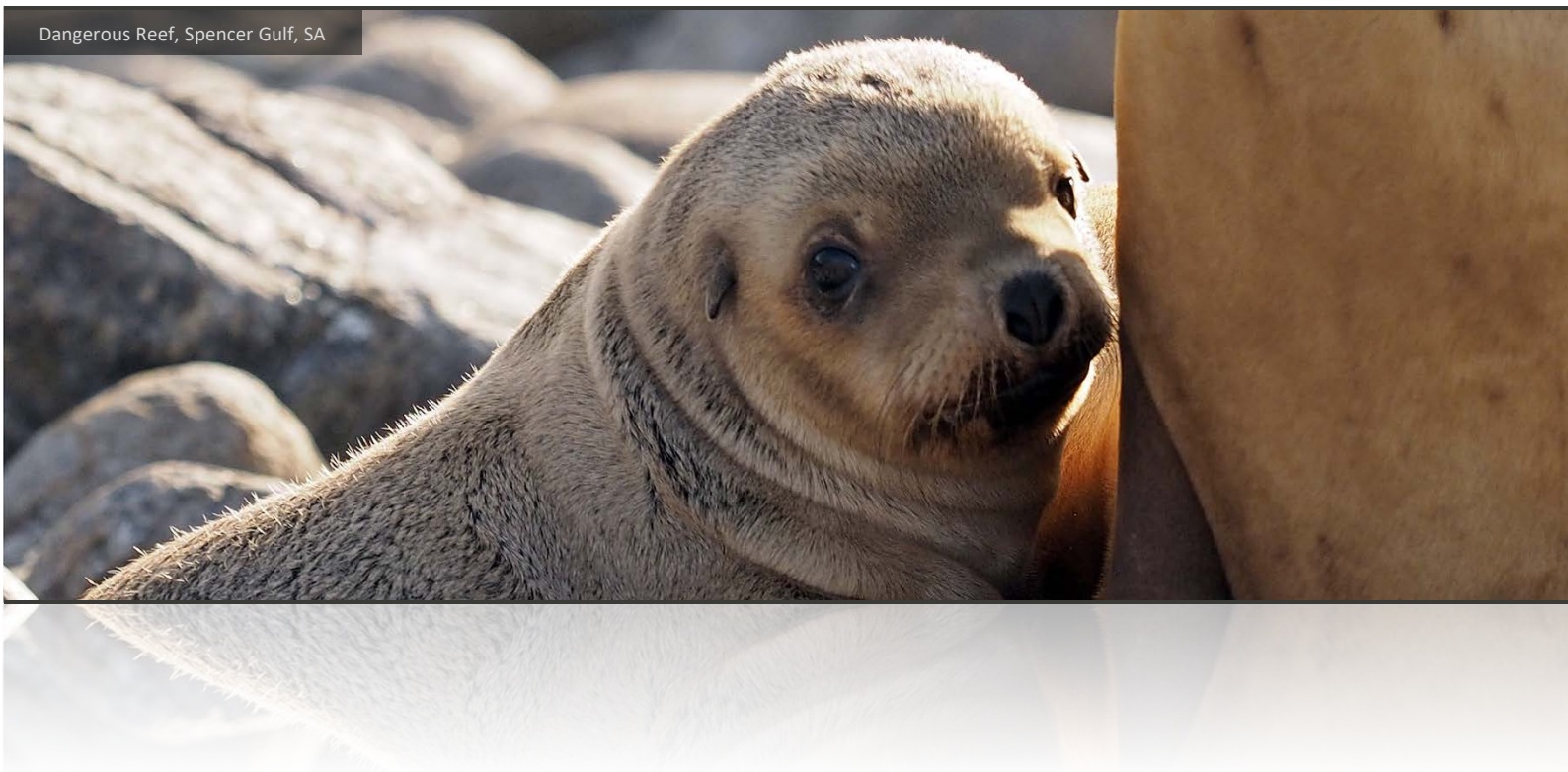
Thesis abstract

Australia's only endemic pinniped, the Australian sea lion (*Neophoca cinerea*), is endangered and declining. Its failure to recover to pre-sealing abundance contrasts with population increases in sympatric otariid species, with high pup mortality additional to low fecundity. This thesis investigates infectious disease contribution to population size modulation, and the optimisation of a disease mitigation strategy. The first two data chapters advance existing knowledge of substantial pup morbidity attributable to hookworm (*Uncinaria sanguinis*) infection. Chapter two reports similar effectiveness for the novel use of an easily applied topical ivermectin formulation to the injected treatment, in a pilot study (n=85) at Dangerous Reef, Spencer Gulf, South Australia. Chapter three reports outcomes from the subsequent two-season treatment trial (n=322) of this formulation at Seal Bay, Kangaroo Island, South Australia. It quantifies significant negative haematological consequences of infection in the context of neonatal ontogenesis, provides the first reporting of significant ($p<0.05$) short- and longer-term treatment-related growth benefits in this species, and demonstrates optimal outcomes by the prevention of patent disease elaboration, while reporting the seasonal independence of treatment benefits. Chapter four reports 30.4% (n=23) seroprevalence and high antibody titres in adult females for *Toxoplasma gondii*, indicating a comparable disease risk to other pinniped populations. An absence of detectable pup seroconversion (n=184) supports the lack of detectable congenital transmission or early post-natal infection, favouring a hypothesis of predominantly foraging-associated parasite exposure. Chapter five reports causation for mortality across five pup cohorts (n=122) at Seal Bay, identifying an infectious disease (hookworm-associated haemorrhagic enteritis; *Streptococcus* spp. infection) contribution in ~38% of deaths and modelling a significant ($p<0.001$) reduction in all-cause mortality risk with early hookworm elimination. Combined, this thesis confirms the substantial contribution of infectious disease to the species low pup survival and defines an effective mitigation strategy to aid species' conservation.

CHAPTER ONE

Introduction, literature review and thesis aims

Dangerous Reef, Spencer Gulf, SA



1.1. Introduction to the Australian sea lion

This thesis documents the study of morbidity and mortality in Australian sea lion (*Neophoca cinerea* Péron, 1816) pups for the purposes of informing future disease investigation and conservation management. The species, Australia's only endemic pinniped (Gales et al., 1992), is classified within the order Carnivora and suborder Pinnipedia. It is one of 14 extant members of the family Otariidae (eared seals), the other two pinniped families being Phocidae (true seals) and Odobenidae (walruses) (Committee on Taxonomy, 2023). The otariids comprise two groups – sea lions and fur seals. The Australian sea lion is one of the eight sea lion taxa (six species and two subspecies, including the extinct Japanese sea lion (*Zalophus japonicus*)), and is the only member of its genus, *Neophoca*. Three of these sea lion taxa, including the Australian sea lion, are currently listed as endangered with a declining population trend by the International Union for the Conservation of Nature (IUCN) (IUCN, 2024) – suggesting the potential for common species' threats.

The Australian sea lion's endangered status was acknowledged by the IUCN in 2008 (Goldsworthy and Gales, 2008) based on a three generational population decline in the range of 50–80% and was retained in a 2015 status update (Goldsworthy, 2015b). In 2020, the species was up-listed to endangered status under the Australian Government's Environment Protection and Biodiversity Conservation (EPBC) Act of 1999 (Threatened Species Scientific Committee, 2020). The Australian sea lion is also protected by state laws: in 2003 under the Western Australian Wildlife Conservation Act 1950 — Wildlife Conservation (Specially Protected) Fauna Notice; and in 2008 under the South Australian National Parks and Wildlife Act 1972 and Fisheries Management Act 2007 (Department of Sustainability Environment Water Population and Communities, 2013a).

Concern for the species survival in the face of current or future challenges is highlighted by modelling indicating that the effective population sizes of colonies in South Australia (SA) and Western Australia (WA) are such that an ongoing loss of genetic heterogeneity is likely, and that the

speed at which this occurs will accelerate as the population continues to decline (Bilgmann et al., 2021). The outcome of a lowered population size with reduced genetic heterogeneity is a decreased ability to adapt to novel challenges (e.g., increased disease threat) (Heard et al., 2013), and consequently a greater extinction risk (Spielman et al., 2004; Frankham, 2005).

1.2. Australian sea lion population

1.2.1. Colony distribution and species abundance

The Australian sea lion population is widely dispersed along approximately 3,500km of Australia's southern and south-western coastlines, from The Pages Islands (35.756°S, 138.300°E) east of Kangaroo Island, SA, to Easter Island (28.468°S, 113.814°E) in the Abrolhos Islands, WA (Gales et al., 1994). Most breeding occurs on offshore islands, with limited mainland breeding. Currently, 80 colonies are recognised as breeding sites, with substantial variation in colony size (Goldsworthy et al., 2021a). Approximately half of breeding sites produce fewer than 14 pups per 18-month breeding cycle, while just four sites have greater than 100 pup births each breeding season. These four SA colonies – The Pages Islands, Seal Bay, Dangerous Reef and Nuyts Reef – are separated by less than 350 nautical miles, and between them contribute almost 40% of the species total pup number (Goldsworthy et al., 2021a).

The most recent assessment of the Australian sea lion population estimated a species-wide abundance of 2,739 pups equating to a population of 10,402 individuals (Goldsworthy et al., 2021a), although the authors suggested this could be an over-estimate given the variation in site growth rates and declining pup production (Goldsworthy et al., 2021a). This present-day abundance and range reflect historical and contemporary impacts on the species' (Goldsworthy et al., 2021a).

Historically, Australian seal species were subjected to uncontrolled commercial harvesting in the late 18th, 19th, and early 20th centuries (Dennis and Shaughnessy, 1996; Ling, 1999; Stuart, 2018), with

some uncertainty of pre-sealing population sizes. In contrast with the Australian sea lion, regional fur seal populations (Australian fur seal (*Arctocephalus pusillus doriferus*) and long-nosed fur seal (*Arctocephalus forsteri*)) have since shown population growth (albeit with a slower recovery and recent decline for the Australian fur seal) (McIntosh et al., 2022) and their conservation status is currently of least concern on the IUCN Red List (Chilvers and Goldsworthy, 2015; Goldsworthy, 2015a). Consequently, the Australian sea lion is Australia's rarest, and one of the world's rarest, otariid species. While it is possible that its population size was historically always comparatively low (Ling, 1999), genetic assessment indicates that the populations in SA and WA have both experienced historical bottlenecks of the order of a 10-fold reduction (Bilgmann et al., 2021). Historical reports also suggest that the species' range once extended further east, to islands at the eastern end of Bass Strait – those in the southern Furneaux and Kent Groups of Islands in Tasmania (Gales et al., 1994; Ling, 1999).

Overall, population distribution and abundance are areas of Australian sea lion research that are comparatively well resourced and increasingly well documented, despite the population's extensive distribution (Goldsworthy, 2020; Goldsworthy et al., 2020; Goldsworthy et al., 2021a; Goldsworthy et al., 2021b). A key outcome of this research is that local factors are an important influence on population dynamics at the individual colony level – intrinsic factors such as foraging strategies and diet (also well researched and reported) (McIntosh et al., 2006; Peters et al., 2015a; Peters et al., 2015b; Lowther and Goldsworthy, 2016; Angelakis et al., 2023) and extrinsic factors such as natural and anthropogenic threats (Goldsworthy et al., 2021a). Across 30 colonies, the median population decline has been 1.2% per year but ranges from a 9.9% decline to 1.7% increase per year for individual colonies (Goldsworthy et al., 2021a). Even low-level pup mortality at any location is of concern for the species' conservation, given that many colonies are very small and hence vulnerable to a range of threatening processes.

1.2.2. Study colonies

Three breeding colonies were studied for this thesis – Seal Bay, Dangerous Reef, and Olive Island. All colonies are in SA, and individually are grouped within the Kangaroo Island, Spencer Gulf and Chain of Bays regions, respectively (Figure 1).

1.2.2.1. Seal Bay Conservation Park, Kangaroo Island

The Seal Bay colony (35.995°S, 137.317°E) is located on the south coast of Kangaroo Island, SA. Of the three study colonies, it is geographically the largest (approximately 3km) and most diverse – a heterogeneous mix of sand and pebble beaches, rocky coastal platforms, limestone promontories and caves, sandy coastal swales covered in coastal spinach (*Tetragonia sp.*), and low coastal scrub comprising coastal saltbush (*Atriplex sp.*) and tea tree (*Melaleuca sp.*) (Figure 2a–f). The colony is the most accessible and is intensively managed by the South Australian Department for Environment and Water (National Parks and Wildlife Service South Australia), including at least twice weekly observation for new births and deaths within the breeding season, microchipping of new-born pups, and monthly colony counts and microchip scanning. Over 12 breeding seasons (2004–2020/21) the colony has experienced a significant ($p=0.008$) decline in pup production of 1.3% per breeding season, with stabilisation in the past five seasons (Goldsworthy et al., 2021b). The estimated three-generation (~42.3 years) decline is 33.9% (Goldsworthy et al., 2021a) and seasonal pup births have ranged from 232 to 268 in the past 10 breeding seasons (2007–2020/21). Seasonal pup mortality over the same period has ranged from 17.9% to 41.8%. While there has been no significant monotonic trend in pup mortality over the past 13 seasons (average 26.9%), it is 1) consistently higher in summer-autumn verses winter-spring breeding seasons (32.8% and 21.4%, respectively), and 2) significantly ($p=0.016$) negatively associated with live pup count (Goldsworthy et al., 2021b). The logical corollary of the latter is that declines in the number of live pups in a season relates to both lower pup production and higher pup mortality. A population viability analysis conducted by

McIntosh (2007) using vital rates from the 2002–2006 breeding cohorts at Seal Bay, suggested that an increase in pup survival of 12.8% was necessary to stabilise the population decline being observed at that time.

1.2.2.2. Dangerous Reef, Sir Joseph Banks Group Conservation Park, Spencer Gulf

The Dangerous Reef colony (34.817°S, 136.217°E) is located in the Spencer Gulf of SA. Colony access and monitoring is logistically challenging, being approximately 17 nautical miles offshore from Port Lincoln. The main site of the colony is a low-lying (maximum 4m above sea level at its centre), shelf-like granite and limestone-capped island within a small reef system, approximately 250–300m long and 100m wide (Marlow, 1975). Its rocky substrate is guano covered, and in contrast to the other two study sites, is devoid of significant sandy substrate, or vegetation to provide shelter from the elements (minimum *Cakile*, *Lepidium* and *Atriplex* spp.) (Marlow, 1975) (Figure 3a–c). The estimated three-generation decline in pup production at this colony is 43.8% (Goldsworthy et al., 2021a). It has the highest population density of the three colonies studied, with an estimated pup production of 382 in the 2019 breeding season (Goldsworthy et al., 2021a).

1.2.2.3. Olive Island Conservation Park, Great Australian Bight

The Olive Island colony (32.717°S, 133.983°E) is off the west coast of the Eyre Peninsula in SA, approximately 14 nautical miles from Streaky Bay. The small island, approximately 720m long and 450m wide, consists of a central raised plateau with a perimeter of variably steep cliffs and caves, surrounded by a rocky shelf-like coastal margin inclusive of a sandy beach (Figure 3d–f). The estimated three-generation decline in pup production at the colony is 68.4%, with an estimated production of 86 pups in 2019 (Goldsworthy et al., 2021a).

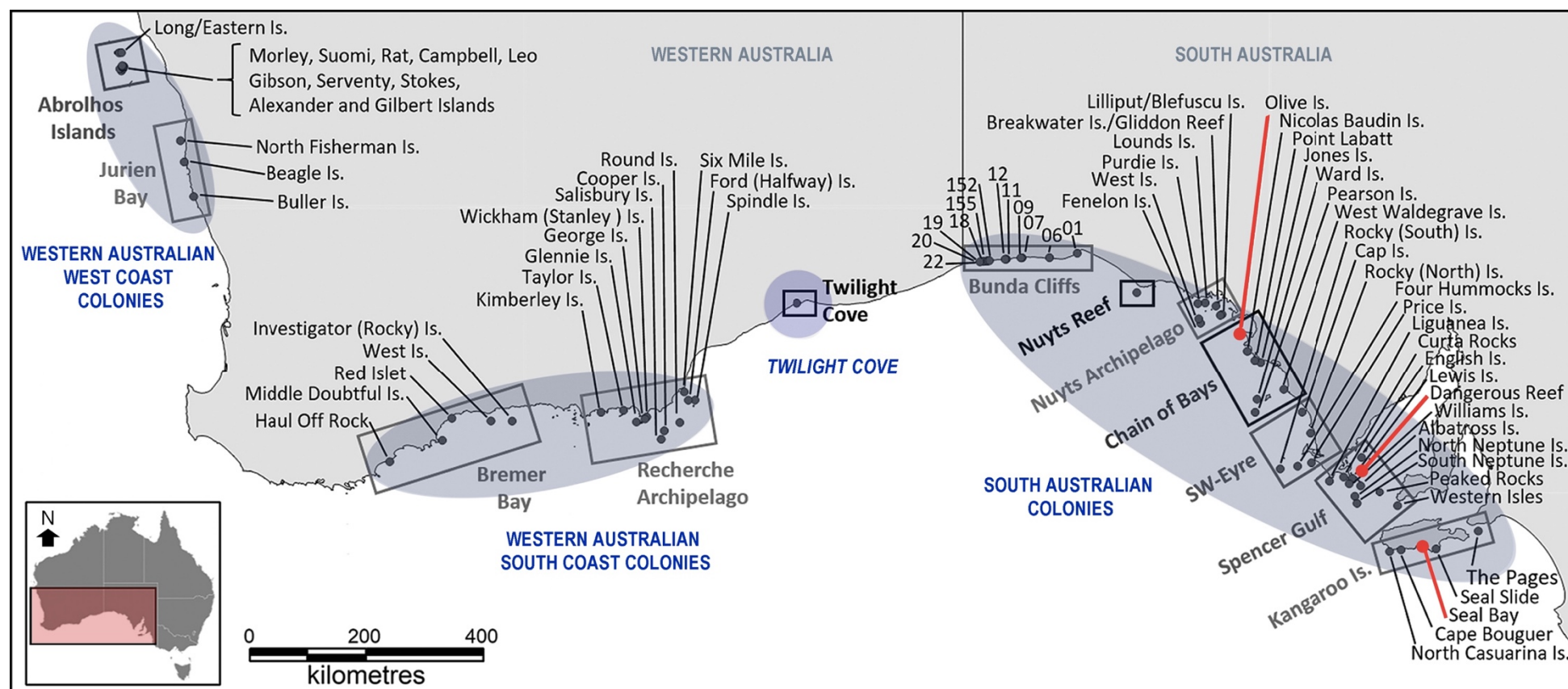


Figure 1. The distribution of the Australian sea lion (*Neophoca cinerea*) by recognised breeding colonies, showing the three major (South Australia, south coast of Western Australia, and west coast of Western Australia) and one minor (Twilight Cove) metapopulations (shaded ellipses). Within each metapopulation, individual colonies are grouped into 12 regions (labelled boxes). Red labels mark the location of the three colonies studied in this thesis (Seal Bay, Dangerous Reef, and Olive Island). Figure adapted from Goldsworthy et al., 2021a.



Figure 2. Seal Bay, Kangaroo Island, South Australia: a) the sandy cove with tea tree-covered dunes of the Eastern Prohibited Area (EPA) 1 (foreground), separated from the sandy Main Beach (middle distance) by a limestone promontory; b) the sandy coastal cove and steep cliffs of EPA2; c) the sandy and rocky beach of the Western Prohibited Area (WPA); d) Pebble Beach and shallow coastal cliffs with caves in the WPA; e) sandy swales covered in coastal spinach (*Tetragonia* sp.) located adjacent to Main Beach; and, f) saltbush (*Atriplex* sp.) and tea tree (*Melaleuca* sp.) coastal scrub inland from the sandy swales of Main Beach.

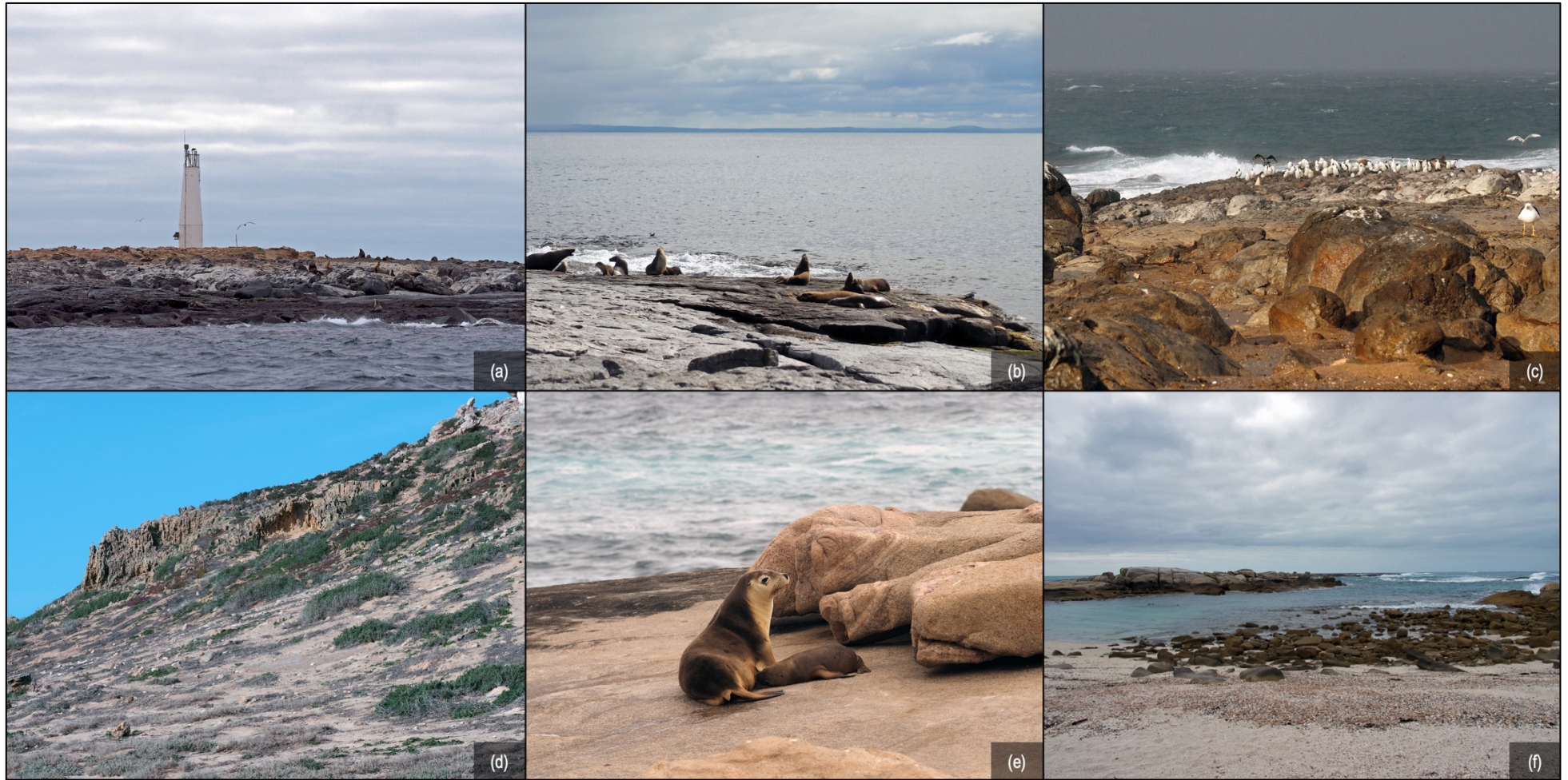


Figure 3. a-c) Dangerous Reef, Spencer Gulf, South Australia: the low-lying and shelf-like granite rock formation is essentially devoid of vegetation and sandy substrate. d-f) Olive Island, South Australia: a small central island plateau with sparse coastal vegetation and variably navigable cliffs, surrounded by eroded rock formations, rock shelves, and a small sandy beach.

1.3. Life history

1.3.1. Breeding cycle and life stages

The breeding cycle of the Australian sea lion is unique among pinniped species, being non-annual and 17–18 months (average 17.6 months) in duration (Ling and Walker, 1976; Higgins, 1993; Shaughnessy et al., 2006). Breeding seasons are protracted, extending from four to 12 months – longer at the larger colonies (Higgins, 1993; Goldsworthy et al., 2021b) – and are asynchronous between colonies, including those within the same region (Kirkwood and Goldsworthy, 2013). Consequently, breeding can occur at any time of the year across the species' range – independent of season (Shaughnessy et al., 2006; Goldsworthy et al., 2014). Reasons for this breeding strategy are not fully understood, with speculation given to adaption to improve foraging success (and therefore breeding outcomes) by a reduction in resource competition in the low density prey environment of the benthos (Gales et al., 1994). Breeding colony asynchrony is thought to persist because of extreme female natal site fidelity (philopatry), resulting in population sub-structuring or stratification (Campbell et al., 2008b; Lowther et al., 2012). Additionally, there is limited male gene flow restricted to a few hundred kilometres (Campbell et al., 2008b; Lowther et al., 2011; Lowther et al., 2012; Ahonen et al., 2016). Consequently, most breeding sites are effectively closed subpopulations, even where distances between adjacent colonies are relatively small (Campbell et al., 2008b; Lowther et al., 2012). Furthermore, consequences of such a breeding strategy and the species' large number of relatively small, widely dispersed colonies are two-fold: 1) fecundity and pup survival become increasingly important modulators of colony size (McIntosh and Kennedy, 2013), and 2) the probability of recolonisation of extinct breeding colonies is reduced. Relevant to the former, is reporting of survival estimates for the various life-stages of the species, showing that as with most slow-living species, apparent yearly survival rates increase with increasing age – 0.30–

0.67 for pups from recruitment to weaning (0.17–1.5 years), 0.89 for juveniles (1.5–3.0 years) and 0.96: 0.89 for female: male adults (3.0–14.0 years) (McIntosh et al., 2013).

The Australian sea lion is a sexually dimorphic species. Adult males weigh 180–250kg and are 1.85–2.25m in length (Kirkwood and Goldsworthy, 2013); adult females weigh 65–100kg and are 1.30–1.85m in length. Sexual maturity for females is four and a half to six years of age, with an average breeding age of 11 years (McIntosh, 2007). Sexual maturity for males is similar, although most males do not commence mate-guarding an adult female until at least 10 years of age (McIntosh, 2007; Goldsworthy et al., 2021b). Females can breed until at least 24 years of age (McIntosh, 2007).

Males are polygynous, sequentially mate-guarding individual females (or small harems at the higher-density colonies) throughout the breeding season (Gales, 2008). Males defend their females from rival males through vocalisation, gesturing and fighting. For mature females the estimated annual pregnancy rate is approximately 70% (Higgins and Gass, 1993). They have the longest placental gestation period of any pinniped species, up to 14 months, with a 4–6 month period of delayed blastocyst implantation following conception (Gales et al., 1997). Australian sea lion females also nurse their pups for an extended 15–18 month period, with weaning approximately a month prior to the birth of a new pup (Higgins and Gass, 1993). In the absence of a new pup (failure to conceive, early pup death, juvenile competition) offspring can be suckled for three or more years (Gales et al., 1997). Females come ashore approximately two days prior to giving birth and are immediately guarded until mating, which occurs about seven days post-partum (Higgins and Gass, 1993). They remain on land with their pup for 7–10 days after giving birth, prior to commencing alternating foraging trips and time ashore attending pups (each approximately 1–2 days) (Higgins and Gass, 1993).

At birth, pups weigh 6.4–7.9kg and are 62–68cm in length (Ling, 2002). New-born pups are recognisable by their dark chocolate brown to black pelage, which lightens through grey to become various shades of brown over 1–2 weeks (Figure 4a–f). Their first moult is generally recognised at 4–

5 months of age (Higgins and Gass, 1993; Dennis and Shaughnessy, 1996), although Marlow (1975) reported moulting commencing from 3–4 months of age at Dangerous Reef (Marlow, 1975). Pups are classified as juveniles at the point of weaning in the following pupping season. Juveniles of both sexes resemble adult females, in having darker dorsal fur (post-moult silver-grey becoming brown) and paler yellow-cream ventral fur (Shaughnessy et al., 2005; Gales, 2008; Goldsworthy et al., 2009a). Older juvenile and subadult males develop dark spots on the chest, which eventually coalesce and create a dark chest. Adult males are dark brown, with a distinguishing blonde cap extending dorsally from the eyebrows to the lower neck.

Pups are predominantly terrestrial bound and remain in the vicinity of their birth colony until the completion of their first moult. However, multiple observations suggest females will introduce pups to tidal pools from as young as eight days of age to cool them in hot weather (Marlow, 1975), and that pups will increasingly explore the pools from 1–3 months of age (especially during summer breeding seasons; personal observation). Subsequently, pups increasingly accompany females on foraging trips. Limited pup dispersal at 4–5 months of age has been documented from Dangerous Reef to nearby English Island (Marlow, 1975), and from Seal Bay to Cape Gantheaume (approximately 20 nautical miles) (Ling and Walker, 1976). Migration and congregation of juvenile males on islands up to 150 nautical miles from breeding sites in non-breeding periods is reported for WA colonies, consistent with their gregarious nature (Gales et al., 1992). Low-level dispersal and life-stage aggregation have potential implications for the transmission of infectious agents between individual animals and colonies.



Figure 4. Life stages of the Australian sea lion: a) new-born black pup (<7 days); b) older brown pup (<1–2 months); c) older pups exploring tidal pools prior to their first moult (2–3 months); d) juvenile, developing brown spots on the ventral neck typical of a male; e) a male mate-guard with a female and new-born black pup; f) a wandering brown pup in the vicinity of a mature bull with characteristic blonde cap.

1.3.2. Foraging and diet

The Australian sea lion is a benthic (ocean floor) forager, predominantly of Australian continental shelf waters, spending approximately a third of its time at or close to the sea floor when foraging (Costa and Gales, 2003). There are temporal (seasonal) and spatial (regional) variabilities in the species' diet, reflecting its extensive range and adaptability within and between colonies in foraging behaviour (Kirkwood and Goldsworthy, 2013). Foraging trips are relatively short – for juveniles, adult females and adult males averaging 1.1, 1.2 and 2.5 days, respectively (Kirkwood and Goldsworthy, 2013). In contrast to males, females forage predominantly in waters surrounding the breeding colony (Lowther et al., 2011), with inshore and offshore foraging ecotypes identified (Lowther and Goldsworthy, 2011). Prey species comprise predominantly the following: 1) cephalopods – octopus (Octopodidae), cuttlefish (Sepiidae), and squid (Loliginidae); 2) benthic or demersal (near the ocean floor) fish, both cartilaginous fish – sharks and rays (Urolophidae, Rhinobatidae) – and ray-finned fish – leatherjackets (Monacanthidae), wrasse (Labridae), flatheads (Platycephalidae), perches (Sebastidae, Serranidae), cods, (Moridae), mullets (Mullidae), and nannygai/redfish (Berycidae), whiting (Sillaginidae), salmon, and eels; and 3) crustaceans – rock lobster, swimming crabs (Gales and Cheal, 1992; McIntosh et al., 2006; Kirkwood and Goldsworthy, 2013; Peters et al., 2015a; Peters et al., 2015b; Berry et al., 2017; Goldsworthy, 2018). Pups learn foraging skills at least eight months before weaning (Lowther and Goldsworthy, 2012; Lowther and Goldsworthy, 2016) and show increasing dive depth and duration from six months of age through to adulthood (Fowler et al., 2006; Fowler et al., 2007).

1.4. Contributors to population abundance

1.4.1. Low fecundity and high pup mortality

The current population status of the Australian sea lion, and its failure to recover in number or geographical range, is partly reflective of its unique life history and resultant low fecundity, as well as the impact of ongoing contemporary threats (Department of Sustainability Environment Water Population and Communities, 2013a). Regarding fecundity, a female Australian sea lion will produce relatively few offspring over her lifetime (Gales and Costa, 1997), a consequence of slow maturity and late first breeding age, a low mean breeding age (McIntosh, 2007), and a long interbreeding interval with extended maternal investment in the care of a single pup (Ling and Walker, 1978; Higgins and Gass, 1993). While the first two factors are also variably reflective of other otariid species, the third is unique (Gales and Costa, 1997). Consequently, the generation length (mean age at which individuals produce offspring) of the Australian sea lion has been calculated as 14.1 years (Goldsworthy et al., 2020).

Additionally, the Australian sea lion experiences a high rate of pup mortality, which varies between colonies and is positively correlated with colony size and density, as it is in other otariid species (Doidge et al., 1984; Harcourt, 1992; Goldsworthy et al., 2009a; Katz et al., 2012). Mortality rates are most frequently reported for the largest colonies, with highest rates for The Pages Islands (55.6% in 1995/1996) (Shaughnessy et al., 2013), Dangerous Reef (44.6% in 2002) (Goldsworthy et al., 2014) and Seal Bay (41.8% in 2011/2012) (Goldsworthy et al., 2019) providing an indication of losses in high mortality seasons. Across seal species, a range of factors have been implicated in density-dependent increases in pup mortality – for example, increased levels of conspecific aggression (male fighting and heightened maternal protectiveness) (Doidge et al., 1984; Harcourt, 1992), decreased pup activity and slower transition to protective (sheltered) colony sites (Nagel et al., 2021), and decreased maternal body condition and provisioning of pups (Doidge et al., 1984; Hammill et al.,

2017). At individual Australian sea lion colonies, mortality also oscillates with breeding season, the reasons for which are unknown. Two negative associations have been reported to date: 1) oceanographic conditions (sea surface temperature) in the maternal foraging range impacting prey species availability and provisioning of pups – i.e., warmer temperatures associated with decreased pup survival (McIntosh et al., 2013); and 2) hookworm (*Uncinaria sanguinis*) infection intensity – i.e., a significant association of higher infection intensity with decreased individual pup body condition and survival (the former also suggesting that hookworm infection could also increase the risk of death from conspecific trauma or other comorbidity) (Marcus et al., 2014a).

Seasonal variation in pup mortality because of factors such as epidemic disease outbreak or variation in weather conditions (e.g., El Niño–Southern Oscillation [ENSO] weather systems), can complicate short-term comparisons between species. For instance, the average seven season (1998/1999–2004/2005) mortality of 14.4% in New Zealand sea lions (*Phocarctos hookeri*) included 22.1%–31.3% mortality during epidemic disease (hypermucoviscous *Klebsiella pneumoniae*) seasons and 10.2% in non-epidemic seasons (Castinel et al., 2007b). More recently, the five season (2013/2014–2017/2018) average mortality was reported as 19.5% as disease had established endemic status (Michael et al., 2019). Indicative average mortality in other species range from 6.0% in the South American fur seal (*Arctocephalus australis gracilis*) over five seasons at Guafo Island, Chile (Seguel et al., 2011; Seguel et al., 2013), to the 15.4% reported for the Steller sea lion (*Eumetopias jubatus*) in the Gulf of Alaska, USA (Maniscalco et al., 2008).

1.4.2. Contemporary threats

The Australian sea lion's life history make it highly vulnerable to extinction in the face of sufficient threat severity (Department of Sustainability Environment Water Population and Communities, 2013a). That is, a consequence of a lower individual lifetime reproductive potential is an increased population vulnerability to minor variation in survival rate (Page et al., 2004; McIntosh et al., 2013).

It is a high-order marine predator and sentinel species, dependent on the marine environment for foraging and coastal habitats for successful reproduction. Consequently, changes in population status may reflect direct (e.g., disease) as well as indirect (e.g., prey species depletion) threats to the species. Furthermore, the cumulative impact of threats (especially anthropogenic threats) will vary in nature and severity across the species' range (Department of Sustainability Environment Water Population and Communities, 2013a). The predominant natural predator of the Australian sea lion is the great white shark (*Carcharodon carcharias*), with potential predation by the orca (*Orcinus orca*) in some regions (Shaughnessy et al., 2007; Ling, 2002).

Contemporary threats to the conservation of the Australian sea lion, suggested actions for species recovery, and recommendations for future research to address species' knowledge gaps, are all well documented (Department of Sustainability Environment Water Population and Communities, 2013a; b; Threatened Species Scientific Committee, 2020). The following subsections will discuss threats from fisheries interaction, the focus of conservation efforts over the past decade, and those with potential to impact pup health and therefore relevant to this thesis.

1.4.2.1. Fisheries interaction and fisheries-related pollution

Interaction with commercial fishing has been suggested to be pose the greatest anthropogenic risk to the Australian sea lion in the recent past (Goldsworthy et al., 2009a; Goldsworthy et al., 2009b; Goldsworthy et al., 2010b; Goldsworthy et al., 2022). This encompasses bycatch, especially interactions with demersal gillnet fisheries (drowning or mortal injury) (Goldsworthy and Page, 2007), and to a lesser extent the rock lobster fisheries (Campbell et al., 2008a), as well as entanglement in fisheries-related marine debris (Page et al., 2004). The considerable research conducted on the interaction of fisheries with the Australian sea lion resulted in quantification of its impact and implementation of mitigation strategies. An Australian Sea Lion Management Strategy for the Gillnet Hook and Trap Fishery in SA commenced in 2010, with permanent closure around Australian sea lion breeding sites and temporary 18 month closures when bycatch exceeds trigger

levels (Australian Fisheries Management Authority, 2010). These strategies have contributed to an estimated 98% reduction in gillnet bycatch-related mortality of Australian sea lions in the decade since their introduction (Goldsworthy et al., 2022). A comparison of pup abundance in the decades before and after implementation of the strategies suggest an association with the arrest of population declines at some impacted breeding colonies in SA (Goldsworthy et al., 2022).

1.4.2.2. Pollution – organic and inorganic

Additional to fisheries-related pollution of the marine environment is that of terrestrial origin, including chemicals and heavy metals, sewerage and biological agents, and enduring man-made materials such as plastics, reaching the oceans by terrestrial run-off and deliberate discharge or dumping. There are numerous reviews in the recent scientific literature reporting exposure, bioaccumulation, and negative health impacts of this pollution in marine mammal species, including endangered species (Deng et al., 2023; Landrigan et al., 2023; Lukić Bilela et al., 2023; Omeyer et al., 2023; Peritore et al., 2023; Schaap et al., 2023; Yesilay et al., 2023). Recent studies in the Australian sea lion have focused on bacterial, heavy metal and persistent organic pollutant exposure and bioaccumulation.

In 2018, the first isolation of an antimicrobial resistant (AMR) *E. coli* bacterium from the Seal Bay colony was reported, as was a significant relationship between human-associated *E. coli* phylotype presence and higher white cell counts at the Dangerous Reef colony, with suggestions that colony approximation to human populations can alter the gut microbiota composition and the risk of pathogen exposure (Fulham et al., 2018). Subsequent studies found a predominance of human-associated *E. coli* phylotypes across eight Australian sea lion, Australian fur seal, and long-nosed fur seal colonies (Fulham et al., 2020), including AMR bacteria in the former two species (Fulham et al., 2022a) – consistent with widespread anthropogenic pollution of the marine environment. Relevant health implications for Australian sea lion pups are the risk of exposure to atypical (pathogenic) bacteria with possible disease elaboration, and the potential for an altered gut microbiome to

negatively impact the development and maintenance of their immune system (with subsequent risk of increased disease susceptibility). Importantly for the current thesis, the same authors also found that topical ivermectin treatment of pup hookworm infection did not significantly alter the gut microbiome composition (Fulham et al., 2022b). A similar finding of widespread contamination of the marine environment, resulting in high-level bioaccumulation in Australian sea lion pups has been documented for the per- and polyfluoroalkyl substances (PFAS) (Taylor et al., 2021) and heavy metals (especially mercury) (Taylor et al., 2022). Once again, potential health implications resulting from toxicity are immunosuppression and increased disease susceptibility. For instance, the possibility that high lead and cadmium levels already potentiate the severity of hookworm-associated anaemia (Taylor et al., 2022). A review of reported contamination of Australian marine fauna suggests that significant knowledge gaps remain, with a key recommendation being a comprehensive pathological assessment of sampled animals to more accurately correlate toxin exposure with disease presence (Jarolimek et al., 2023).

1.4.2.3. Resource availability and climate impact

Prey species abundance and distribution may impact pup morbidity and mortality through altered maternal provisioning, with inter-specific competition one potential factor. While the foraging range of the Australian sea lion overlaps substantially with that of the long-nosed fur seal, interspecies competition is likely to be low based on differing foraging strategies – epipelagic versus benthic, respectively (Goldsworthy et al., 2009a). This contrasts with a currently undetermined potential impact of any further westward extension of the population distribution of the Australian fur seal, which is also a benthic forager with currently limited range overlap with the Australian sea lion (Peter et al., 2010; Department of Sustainability Environment Water Population and Communities, 2013a; Threatened Species Scientific Committee, 2020).

Of greater potential bearing on prey species availability and requiring further study, is climate change impact (Department of Sustainability Environment Water Population and Communities,

2013a; Threatened Species Scientific Committee, 2020). For instance, the effect of changing marine conditions (temperature, acidity) on the extensive sea grass meadows of SA (Shepherd and Robertson, 1989), which likely provide a suitable habitat for many of the prey species of the Australian sea lion (Lowther and Goldsworthy, 2011). A negative correlation between apparent yearly pup survival and foraging range sea surface temperature has previously been reported for the Australian sea lion (McIntosh et al., 2013). More recently, a study of environmental impact on Australian fur seal pup production identified positive correlations with the current-year Southern Oscillation Index (SOI) and Southern Annular Mode (SAM) conditions (Geeson et al., 2022). The Australian sea lion may be less resilient to changing ocean temperatures than the sympatric fur seal species, based on assessment of metabolic rates and comparative physiological flexibility (Ladds et al., 2017). Climate change has already been shown to affect disease elaboration in otariid pups through its impact on maternal provisioning. For the South American fur seal (another otariid in which hookworm disease is a substantial contributor to pup death), in years with higher sea surface temperature there is lower maternal attendance of pups, a less robust pup immune response (necessary for hookworm elimination), and consequently increased hookworm-associated pup mortality (Seguel et al., 2018a). A similarly detailed current analysis of direct and indirect climate impact on the Australian sea lion remains a knowledge gap. Additionally, rising sea levels resulting in inundation of low-lying colonies and increased frequency of extreme weather events (e.g., large swells) are relevant direct threats to pup survival (IPCC, 2014).

1.5. Disease in the Australian sea lion

1.5.1. Current monitoring status and species' recovery plan guidelines

The Australian sea lion is particularly vulnerable to an epidemic disease outbreak, due to a low level of genome-wide heterogeneity (the result of its historical bottleneck) and potential immunological naivety consequential to its geographical isolation and possible limited pathogen exposure

(Bilgmann et al., 2021). Furthermore, compared with some other pinniped species it shows an overall lower allelic diversity in Major Histocompatibility Complex class II genes, which are associated with adaptive immunity and specifically antigen recognition, binding, and presentation (Lau et al., 2015). Modelling of future population declines has identified a large rapid decline, as expected with an epidemic disease outbreak, as posing the greatest threat to the species in terms of in-breeding (Bilgmann et al., 2021). In SA, the modelling suggests disease threat to exceed the cumulative threat from the ongoing loss due to fisheries bycatch and other threats (Bilgmann et al., 2021).

There are currently no active programs for the systematic monitoring of disease presence or for serological surveillance of infectious pathogen exposure. For instance, it is likely that tuberculosis caused by *Mycobacterium pinnipedii* infection results in more deaths than those sporadically reported (Lindsay and Gray, 2021). However, insufficient data due to limited necropsy or serological assessment of adult animals mean that the risk level remains undetermined (Threatened Species Scientific Committee, 2020). The knowledge gap and potential threat to the species from absence of monitoring have been acknowledged in the Issues Paper, which specifically highlights:

- 1) the unknown contribution of disease in limiting Australian sea lion population recovery, and its significant contribution to mortality in other sea lion populations
- 2) evidence of hookworm causing significant mortalities in other sea lion populations
- 3) concern for the risk of mass disease outbreak as reported in other species, and the need for ongoing research.

Consequently, one of the six objectives of the Recovery Plan for the Australian Sea Lion (*Neophoca cinerea*) (Department of Sustainability Environment Water Population and Communities, 2013b) is to “investigate and mitigate other potential threats to Australian sea lion populations, including disease, ...”. Specifically, relevant actions relating to an improved understanding of the threat and

importance of health-related factors to Australian sea lion populations, were listed as undertaking research to:

- 1) better understand pup mortality due to disease and variance between seasons and colonies
- 2) evaluate the effect of providing a broad-spectrum treatment to eliminate parasites, and to assess whether this affects pup mortality.

1.5.2. Hookworm disease

1.5.2.1. Introduction

Hookworms are hematophagous nematodes parasitising the small intestine of many terrestrial and marine animal species. In otariids, hookworms within the genus *Uncinaria* Frölich, 1789 (Nematoda: Ancylostomatidae) (*Uncinaria spp.*) are reported from most species (Nadler et al., 2013). Three hookworm species are currently well described – *Uncinaria lucasi* Stiles, 1901 from the Northern fur seal (*Callorhinus ursinus*) (Olsen and Lyons, 1965), *Uncinaria hamiltoni* Baylis, 1933 from the South American sea lion (*Otaria byronia*) (Baylis, 1933), and *Uncinaria sanguinis* Marcus, 2015 from the Australian sea lion (Marcus et al., 2014b). *Uncinaria lucasi* has been shown to also parasitise the Steller sea lion and *U. hamiltoni* the South American fur seal (Nadler et al., 2013). Furthermore, it is also likely that Australian otariids are infected by the same (or a closely related) species of hookworm (Ramos et al., 2013).

Over the past decade, publications by Marcus et al. have considerably advanced the understanding of hookworm infection and disease in Australian sea lion pups. This includes identifying and describing *U. sanguinis* as a novel hookworm species (Marcus et al., 2014b), determining the epidemiology of infection in Australian sea lion pups (Marcus et al., 2014a), investigating the impact of infection on haematological parameters of health (Marcus et al., 2015a), and describing outcomes from the first ivermectin treatment trial in the species (Marcus et al., 2015b). Key findings (see

below) included a high disease impact within the species, albeit with marked variation in outcomes between individual pups, and a 100% prevalence of pup infection. Combining this information with the likelihood that the *Uncinaria* species infecting Australian otariids is one of only two endemic hookworm species (and therefore that the host-parasite relationship is longstanding) (Ramos et al., 2013), has prompted the suggestion that changing environmental factors could predispose animals to disease (Meza Cerda et al., 2022).

1.5.2.2. Epidemiology

The epidemiology of hookworm infection in otariid species is well reported, initially from the study of *U. lucasi* in Northern fur seals (Lyons, 1964; Olsen and Lyons, 1965; Lyons and Keyes, 1978; Lyons et al., 1997), and subsequently from studies such as those of hookworm infection in the New Zealand (Castinel et al., 2006; Castinel et al., 2007a), South American (Seguel et al., 2017; Seguel et al., 2018a; Seguel et al., 2018b; Seguel et al., 2019) and Australian sea lions (Marcus et al., 2014a; Marcus et al., 2014b; Marcus et al., 2015a). The lifecycle of *U. sanguinis* in the Australian sea lion is shown (Figure 5). At the two most studied colonies of Dangerous Reef, Spencer Gulf and Seal Bay, Kangaroo Island, hookworm infection of pups occurs with 100% prevalence (Marcus et al., 2014a). The single time point of neonatal pup infection is soon after birth, and results from ingestion of infective L3 larvae present in colostrum. Maturation from larval to adult worms occurs in the small intestine with patent egg production apparent from 11 to 14 days of pup age. Adult worms are cleared from the intestine by 2–3 months of pup age, in association with the development of a host immune response and likely altered host intestinal micro-environment hostile to the parasite (Seguel et al., 2019). Eggs shed into the environment can persist for multiple breeding seasons (over-wintering), independent of the colony substrate, and this environmental contamination becomes the source of subsequent infection for animals of all ages. For these animals, infection occurs by percutaneous transmission of infective L3 larvae, with subsequent preferential migration to, and dormancy within, the subcutaneous adipose tissue. Males and non-reproductive females are dead

end hosts with regards to perpetuation of the lifecycle. In contrast, in pregnant females impending birth and lactation result in migration of L3 larvae to mammary tissue and their subsequent presence in colostrum.

1.5.2.3. Clinical disease

The reported pathogenicity of hookworm infection and its contribution to pup mortality varies by otariid species and location, reflecting differing host-parasite-environment interactions. For instance, *U. lucasi* collected from the Steller sea lion are significantly larger than those collected from the Northern fur seal, suggesting growth retardation in the latter species and a more recent host–parasite association (Nadler et al., 2013). Furthermore, hookworm infection is thought to act as a density-dependent moderator of population size in some species (Lyons et al., 2000; Lyons et al., 2001), explaining for example, the disparately high contribution of hookworm infection to Northern fur seal pup death at the high-density colony of San Miguel Island, California (DeLong et al., 2009), compared with the substantially lower contribution at the low-density colony of St Paul Island, Alaska (Spraker and Lander, 2010). Hookworm disease is a substantial contributor to pup morbidity and mortality in Northern fur seal pups at San Miguel Island, USA (DeLong et al., 2009), South American fur seal pups at Guafo Island, Northern Chilean Patagonia, and in Australian sea lion pups at Dangerous Reef and Kangaroo Island, Australia (Marcus et al., 2015b).

Hookworm disease is the direct and indirect result of adult (and potentially immature larval) parasite feeding within the small intestine. The parasites cause physical injury to the intestinal mucosa (and potentially deeper tunics) using buccal plates, with resultant intestinal haemorrhage likely perpetuated by secretion of anticoagulant proteins (summarised in Loukas et al., 2005). With intense acute disease or chronicity, haemorrhage results in anaemia and protein loss, potentially impacting pup growth. Host nutrient deprivation is further exacerbated by intestinal inflammation, which is likely to reduce the absorptive capacity of the intestinal tract. In the Australian sea lion, the manifestation of hookworm infection ranges from subclinical disease detectable only at necropsy or

by measurement of haematology parameters, to severe, clinically apparent, haemorrhagic enteritis, emaciation, and death. A host eosinophilia-lymphocytosis (Marcus et al., 2015a) and neutrophilia (Seguel et al., 2019) in association with hookworm infection have been described in otariids. Disease contribution to reduced growth and lethargy of pups supports an additional indirect contribution to pup death through an increased risk of conspecific trauma. An increased risk of conspecific trauma attributable to hookworm disease remains to be quantified in the Australian sea lion (McIntosh and Kennedy 2013).

1.5.2.4. Disease mitigation

Hookworm treatment in free-ranging otariid species using the anthelmintic ivermectin are summarised in Table 1. These studies consistently demonstrate it to be highly effective in eliminating adult hookworm infection, with intestinal clearance of adult hookworm by 16 hours post-treatment (DeLong et al., 2009). Ivermectin belongs to the avermectin drug group, macrocyclic lactone derivatives with potent anthelmintic and insecticidal properties (reviewed in Ashour, 2019). It has been widely used in domestic and production animal species in oral, topical, and injectable formulations, although in recent years its use has declined in favour of newer generations of drugs developed to overcome issues such as parasite resistance. Prior to the studies presented in this thesis, all peer-reviewed publications reporting ivermectin use in free-ranging otariid species have used the injectable formulation, commonly delivered as a single subcutaneous injection (200µg/kg).

While the elimination of hookworm infection in otariid pups has consistently demonstrated a reduction in the severity of anaemia and inflammation, and less frequently the mitigation of infection-associated growth retardation, the primary goal of mitigation strategies is enhanced pup survival, particularly in endangered populations or those experiencing high pup mortality. From studies to date, there are two key determinants for achieving this goal: 1) anthelmintic administration to pups as young as possible, and 2) treatment in seasons of relatively high pup mortality. For the first point, treatment impact has generally been optimised by application before

two weeks of age, coinciding with the period prior to development of patent infection (and therefore commencement of adult hookworm feeding activity associated with increasing intestinal damage). The second includes higher than usual mortality caused either by hookworm infection, or by another (usually disease) process. For instance, the treatment of Northern fur seal pups on San Miguel Island, California, at a time when pup mortality approaching 50% was partly attributed to hookworm infection, resulted in a significant reduction in pup mortality (DeLong et al., 2009). Similarly, during the 2002 breeding season at Enderby Island in the sub-Antarctic, hookworm treatment of New Zealand sea lion pups during a period of increased mortality related to an epidemic outbreak of *K. pneumoniae* septicaemia was associated with a significant survival benefit (Castinel et al., 2007a; Chilvers et al., 2009). This, despite many deaths being attributed to septicaemia, suggesting that hookworm-associated morbidity (anaemia, inflammation, haemorrhagic enteritis) may contribute to reduced immune function and increased mortality risk from other stressors (Chilvers et al., 2009). This was also the first study to demonstrate the negative impact on short-term growth rate to result from hookworm infection (and therefore the potential for increased traumatic comorbidity).

The first hookworm treatment trial in the Australian sea lion was conducted at Dangerous Reef, Spencer Gulf during the 2011 and 2013 breeding seasons. Consistent with reporting in other otariids, injected ivermectin at a dose of 200ug/kg was found to be highly effective in eliminating adult hookworms (adjusted effectiveness 97.9%), and consequently reducing the severity of anaemia and eosinophilia experienced by infected pups (Marcus et al., 2015b). The study effectively highlighted the peculiar constraints to optimally treating hookworm infection in Australian sea lion pups – those related to its life history (protective maternal attendance for 1–2 weeks post-partum) and logistics of accessing pups (challenging colony access). Additionally, an historically low level of mortality was reported for the recruited pup cohort. Consequently, while this initial treatment trial didn't demonstrate treatment-related growth or survival benefits, it clearly guided the development of protocols for future mitigation studies. These included investigating the effectiveness of the topical

ivermectin formulation, recruitment of a younger pup cohort, and ideally a larger-scale study with longer-term follow-up at more accessible colony.

1.5.3. Toxoplasmosis

Toxoplasma gondii is a well-studied and ubiquitous protozoan (phylum Apicomplexa) parasite capable of infecting all warm-blooded animal species, including marine mammals (Dubey et al., 2020) and birds (Campbell et al., 2022). Since the first identification of this parasite (*T. gondii* Nicolle and Manceaux, 1908), a considerable body of work has amassed describing its lifecycle, disease elaboration in humans and animals, the diagnosis of infection and its treatment (summarised in (Dubey, 2022). In marine mammals, including otariids, *T. gondii* is an emerging pathogen posing a potential conservation risk to endangered populations (Robinson et al., 2023). To date, the parasite's epidemiology and disease impact are less well-studied in marine versus terrestrial species (summarised in Dubey, 2022 and Dubey et al., 2020), with further research necessary to fully understand mechanisms of parasite transmission, for instance.

Chapter four provides a substantial introduction to, and discussion of, *T. gondii* infection in otariid species – supplementary background information is provided in the following sections. Of relevance to this thesis is the listing of toxoplasmosis as a potential threat to the Australian sea lion population (Threatened Species Scientific Committee, 2020), recent reporting of high-level seroprevalence for *T. gondii* antibodies in sympatric dolphin species (confirming regional runoff pollution) (Alberts et al., 2022) and the absence of serological surveillance for quantification of the species' exposure risk from this parasite – an important knowledge gap.

1.5.3.1. Epidemiology

Current understanding of the *T. gondii* lifecycle indicates that cat species (Felidae) are the only known final (definitive) host, in whom sexual reproduction of the parasite occurs in the intestine

(gametogony) resulting in the passing of unsporulated oocysts (Taylor, 2016). Any mammal (including humans) or bird species is a potential intermediate or incidental host, and in these animals the parasite lifecycle is extra-intestinal. Transmission of infection can occur by any of the three developmental stages of the parasite – tachyzoites (congenital transmission following infection or recrudescence in a pregnant animal), bradyzoites within tissue cysts (prey ingestion) and sporulated (infective) oocysts (food, water, and environmental contamination) (reviewed in (Alvarez Garcia et al., 2021)). Ingestion of sporulated oocysts leads to penetration of the intestinal wall, distribution through blood or lymphatic fluid to tissues, cellular invasion with asexual proliferation of rapidly dividing tachyzoites, and subsequent cell rupture with further cell invasion and tissue destruction (acute toxoplasmosis) (Dubey, 2022). Infection with *T. gondii* induces a strong host inflammatory and innate and adaptive immune response, which limit this proliferative stage and result in the formation of tissue cysts containing the slowly dividing bradyzoite form of the organism (Dubey, 2022). Tissue cysts are frequently observed in muscle (skeletal and cardiac) and neural (including ocular) tissues, reflected by the clinical signs and disease apparent in infected animals.

Most infections of marine mammals likely occur by oocyst ingestion (horizontal transmission) following contamination of the marine environment from terrestrial water run-off, and/or oocyst accumulation in prey species – invertebrates and fish (Dubey et al., 2020). Furthermore, following felid infection oocyst shedding is prolific (millions of organisms), with sporulated oocysts being extremely resilient and potentially maintaining infectiveness for years, including survival in saltwater environments (Lindsay et al., 2003; Lindsay and Dubey, 2009). Both factors potentiate contamination of the marine environment and shorelines, and the exposure of marine species (Pouille et al., 2021).

Relevant information relating to the pathogenesis of infection by vertical transmission and abortion (Dubey, 2022) is firstly, that parasitaemia during pregnancy can cause placentitis and/or congenital infection. In higher-order terrestrial mammals (e.g., humans, sheep) congenital infection results most commonly when maternal exposure occurs during pregnancy. The manifestations of vertical

transmission include early embryonic death and resorption, foetal death and mummification or abortion, stillbirth, neonatal death, and birth of infected individuals that can develop clinical disease in later life. Secondly, while clinical infection is more severe in early gestation (partly related to decreased immune responsiveness of trophoblastic cells), *T. gondii* transmission to the foetus is more efficient in the latter half of gestation (partly related to decreased placental thickness) (reviewed in Blaszkowska and Goralska, 2014). A suggestion that the thickness of the endotheliochorial barrier of pinnipeds is less than that of terrestrial carnivore species with similar placentation (Sinha and Erickson, 1974) could therefore be relevant to the frequency and gestational stage of congenital infection in the former. Thirdly, an absence of antibody production in congenitally infected animals, suggesting immunotolerance, is likely limited to species such as mice and rats (Dubey, 2022). Consequentially, for free-ranging marine mammal species in whom early and mid-gestational abortion is likely undetected, it is possible that reliance on pup seroconversion for the quantification of parasite exposure could provide an underestimate of disease impact (selection and survival bias).

The endangered Hawaiian monk seal (*Neomonachus schauinslandi*) is an example of the potential for *T. gondii* to markedly impact marine species. Strandings of infected seals were recently reported to be 25 times more likely to occur following heavy runoff events in regions overlapping with human (and cat) habitation (Robinson et al., 2023). Additionally, toxoplasmosis is one of the three major causes of death in the Main Hawaiian Island population of this phocid species, with a suggested negative impact on fecundity and pup survival (and therefore moderation of population recovery) from adult female deaths, failure of pregnancies, and neonatal pup mortality secondary to vertical transmission of the parasite (Barbieri et al., 2016; Harting et al., 2020). The impact of the parasite on the Australian sea lion remains to be determined, although it is unlikely to be as extreme based on the species dispersed population.

1.5.3.2. Clinical disease

Most reports of clinical toxoplasmosis in pinniped species describe uncommon sporadic disease in juvenile or adult animals, or document instances of abortion (summarised in (Dubey et al., 2020)). In the California sea lion (*Zalophus californianus*) for example, 12 cases inclusive of three aborted foetuses, were identified from review of 1,152 necropsy records of stranded animals (Carlson-Bremer et al., 2015). Of these, toxoplasmosis was considered the cause of death in five animals, including two foetuses with disseminated disease. Consistent with reporting in other pinnipeds, lesions were reported in brain and meninges, heart, lymph nodes, skeletal muscle, urinary bladder, pancreas, and spleen, with all but one animal having neural lesions. During the period of case detection (1998–2009), antibody seropositivity indicating exposure to *T. gondii* was substantially greater than clinical disease, reported to be 32.9% (Carlson-Bremer et al., 2015; Dubey et al., 2020). Key findings for understanding disease epidemiology were increasing exposure risk with age, (indicating a cumulative risk of horizontal exposure and antibody persistence) and disseminated foetal disease confirming vertical transmission. In the first reported case of toxoplasmosis in an Australian pinniped, a subadult long-nosed fur seal, infection with a type II-like strain resulted in multisystemic disease in an emaciated animal – nonsuppurative meningoencephalitis, uveitis, retrobulbar cellulitis, and myocarditis (Donahoe et al., 2014). As in terrestrial species, disease elaboration can be consequential to comorbidity or immunosuppressive factors. A Mediterranean monk seal pup dying with disseminated disease was found to be co-infected with cetacean morbillivirus, likely resulting in immunosuppression (Petrella et al., 2021).

1.6. Pup mortality

1.6.1. Australian sea lion

Estimated Australian sea lion pup mortality is well quantified for the larger and more closely monitored colonies, such as Seal Bay (Goldsworthy et al., 2010a; Goldsworthy et al., 2014; Goldsworthy et al., 2017; Goldsworthy et al., 2019; Goldsworthy et al., 2021b). However, the causes

of this mortality have not been as well studied in comparison with other otariid species (Spraker and Lander, 2010; Michael et al., 2019), contributing to a substantial knowledge gap. The three publications to date, all with high-level observational methodology, identified conspecific trauma and starvation as important contributors to pup death (summarised in Table 2) (Marlow, 1975; Higgins and Tedman, 1990; McIntosh and Kennedy, 2013). The first, a small scale observational study at the high-density Dangerous Reef colony by Marlow (1975), identified conspecific aggression as the main contributor to pup death – the majority from adult females (including rough handling by young or inexperienced mothers), with a lesser contribution from adult males (including inadvertent trampling during fighting) and juveniles or subadult males (Marlow, 1975). The second, another small scale observation study with limited gross necropsy examination at the lower density Seal Bay colony by Higgins and Tedman (1990), attributed only 19% of deaths to conspecific aggression (suggested to typically relate to misdirected aggression from territorial males (Higgins and Tedman, 1990). The authors attributed 43% of deaths to starvation, resulting from 1) sibling competition from the previous juvenile, 2) the non-return of the female, or 3) separation of the pup and female within the colony. The third study, a larger scale investigation at Seal Bay by McIntosh and Kennedy (2013) again suggested trauma due to conspecific aggression was the major identifiable cause of death (31.6%), with lesser contributions from starvation (10.4%) and still- or premature birth (7.6%) (McIntosh and Kennedy, 2013).

In the latter two studies, a relatively high proportion of deaths remained of undetermined cause (28% and 49%, respectively) – pups in good condition (no apparent weight loss) without any gross evidence of trauma. Authors of both studies implicated disease as a contributor to these deaths of undetermined cause. Consequently, a key suggestion by McIntosh and Kennedy (2013) was that future studies of pup mortality should include investigation of disease contribution – parasitism, for instance (McIntosh and Kennedy, 2013).

Although commonalities for cause of pup death were observed at different colonies, these existing studies highlighted that differences in the proportion of each cause were likely between colonies, as is reported in other otariids (Maloney et al., 2009; Drago et al., 2010; Seguel et al., 2013). For conspecific trauma, they also provided observational data for cause of pup injury. Both adult males and females will defend their territory during breeding seasons, with male defence often particularly aggressive (Marlow, 1972; 1975). It is possible for pups to be injured or killed during this territory defence. Additionally, females can behave aggressively towards pups that are not their own (Gales, 2008), especially during the perinatal period, and inexperienced mothers can fail to bond with their new-born pups and passively or aggressively reject them (leading to starvation and trauma, respectively).

1.6.2. Other otariid species

In other otariids species, trauma and starvation are also frequently reported causes of pup mortality, together with a variably important (e.g., epidemic versus non-epidemic seasons) contribution from disease resulting from parasitic and bacterial infection (summarised in Table 3) (Doidge et al., 1984; Castinel et al., 2007b; Spraker et al., 2007; Spraker and Lander, 2010; Seguel et al., 2013). Reporting from these species also categorised causation and contributing factors for death, with a view to implementation of management strategies to minimise the impact on population dynamics. For example, categorisation as extrinsic (environmental) or intrinsic (demographic) (Seguel et al., 2013), and density-dependent (e.g., trampling or suffocation caused by overcrowding) or density-independent (e.g., weather) (Maniscalco et al., 2008). A repeatable observation across all studies has been the frequent presence of comorbidity – more than one predisposing factor, contributing factor or cause for pup death (Castinel et al., 2007b; Maniscalco et al., 2008; Spraker and Lander, 2010; Seguel et al., 2013; Michael et al., 2019).

For the listed studies, where pup mortality due to trauma was reported, it ranged from 3% in the Steller sea lion (Maniscalco et al., 2008) to 38% in the South American fur seal (Katz et al., 2012). The authors of the latter study observed an intra-seasonal 66% peak in trauma-related mortality, coinciding with weaning of pups at nine months of age. Despite some variability in the reported outcomes for the Australian sea lion, the species likely experiences a comparatively high level of traumatic death among the otariids, and potentially (depending on colony) at the higher levels reported in pinnipeds more generally – for instance, the trauma-related deaths in the order of 50%–70% reported for starving elephant seal pups (*Mirounga angustirostris*) (Le Boeuf and Briggs, 1977). Relevant to inter-colony differences for Australian sea lion pups is the positive correlation of trauma-related death with increasing colony density (Seguel et al., 2013).

Similarly, starvation or emaciation as a cause of death range widely in other otariid species, from 13–15% reported in the New Zealand sea lion (Castinel et al., 2007b; Michael et al., 2019), to 53% reported in the Northern fur seal and 64% suggested in the South American fur seal (Katz et al., 2012). For Australian sea lions, mortality due to starvation was reported at 10.4% (McIntosh and Kennedy, 2013) and 43% (Higgins and Tedman, 1990) indicating current uncertainty and the potential for inter-seasonal influences. Beyond the referenced studies, starvation has been reported to substantially impact other otariids within particular seasons, for example ranging from 13–100% in South American sea lion pups, the high figure associated with lack of provisioning due to an El Niño weather event (Soto et al., 2004).

Substantial disease contributions to otariid pup mortality are infection with the hypermucoviscous strain of *K. pneumoniae* in the New Zealand sea lion population at Enderby Island (60.2%; 2013–2018), and the hookworm-enteritis complex reported in the California sea lion (71.5%; 2002–2003) (Spraker et al., 2007) and South American fur seal (28.4%; 2004–2008) (Seguel et al., 2011; Seguel et al., 2013). In the former, bacterial infection in seasons inclusive of the initial emergent disease epidemic (1998/1999–2004/2005) contributed to 24% of deaths (Castinel et al., 2007b) (later

suggested to be a potential underestimate), increasing to 58–60% in more recent seasons as the disease has become endemic (Roe et al., 2015; Michael et al., 2019). In the most recent reporting, the authors commented that the emergence of this infectious disease highlighted the importance of monitoring for disease and thoroughly investigating the causes of pup disease and death (Michael et al., 2019). Beyond the listed studies are multiple reports of disease identification and serological evidence for bacterial, viral, protozoal, and parasitic pathogen exposure within pups, juveniles and adults of other otariid species, emphasising the importance of monitoring programs (Duarte-Benvenuto et al., 2022).

Additional contributors to pup mortality in other otariids include those resulting from extrinsic factors, such as predation. McIntosh and Kennedy (2013) assigned 1.4% of Australian sea lion pup deaths to possible white shark attack, noting pups are an observed prey for this species and that this was more likely as pups matured and began exploring a wider foraging range (McIntosh and Kennedy, 2013). Such predation would be difficult to detect directly, but possible indirectly as loss of resight of older pups and a failure of juveniles to return in subsequent seasons. In any case, in Australian sea lion pups, predation is likely to be a relatively low cause of death compared with the 25% orca predation of Steller sea lion pups (Maniscalco et al., 2008), and the 62%–68% leopard seal (*Hydrurga leptonyx*) predation of Antarctic fur seal (*Arctocephalus gazella*) pups (Boveng et al., 1998).

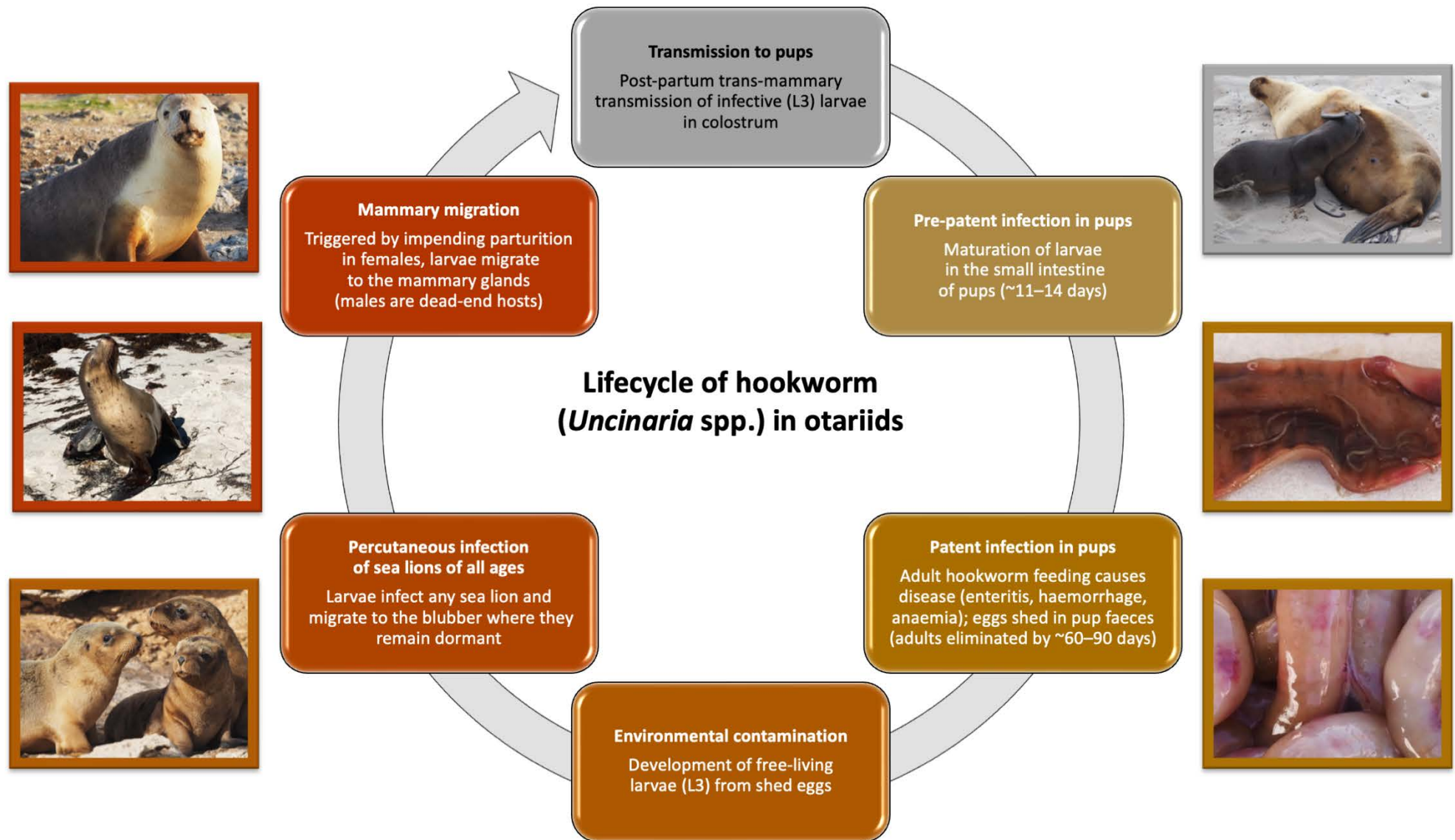


Figure 5. Lifecycle of hookworm (*Uncinaria* spp.) in otariids.



Figure 6. Risk of conspecific competition and trauma, and protective pup sheltering. Examples of conspecific competition risk posed by (a) an unweaned juvenile, and conspecific trauma risk posed by (b) a subadult male and (c) a mate-guarding bull. At the Seal Bay colony pups will shelter beneath the (d) coastal vegetation or (e) between rocks during maternal absence. At the Olive Island colony, some protection from conspecific trauma is offered by (f) coastal boulders and rock crevices.

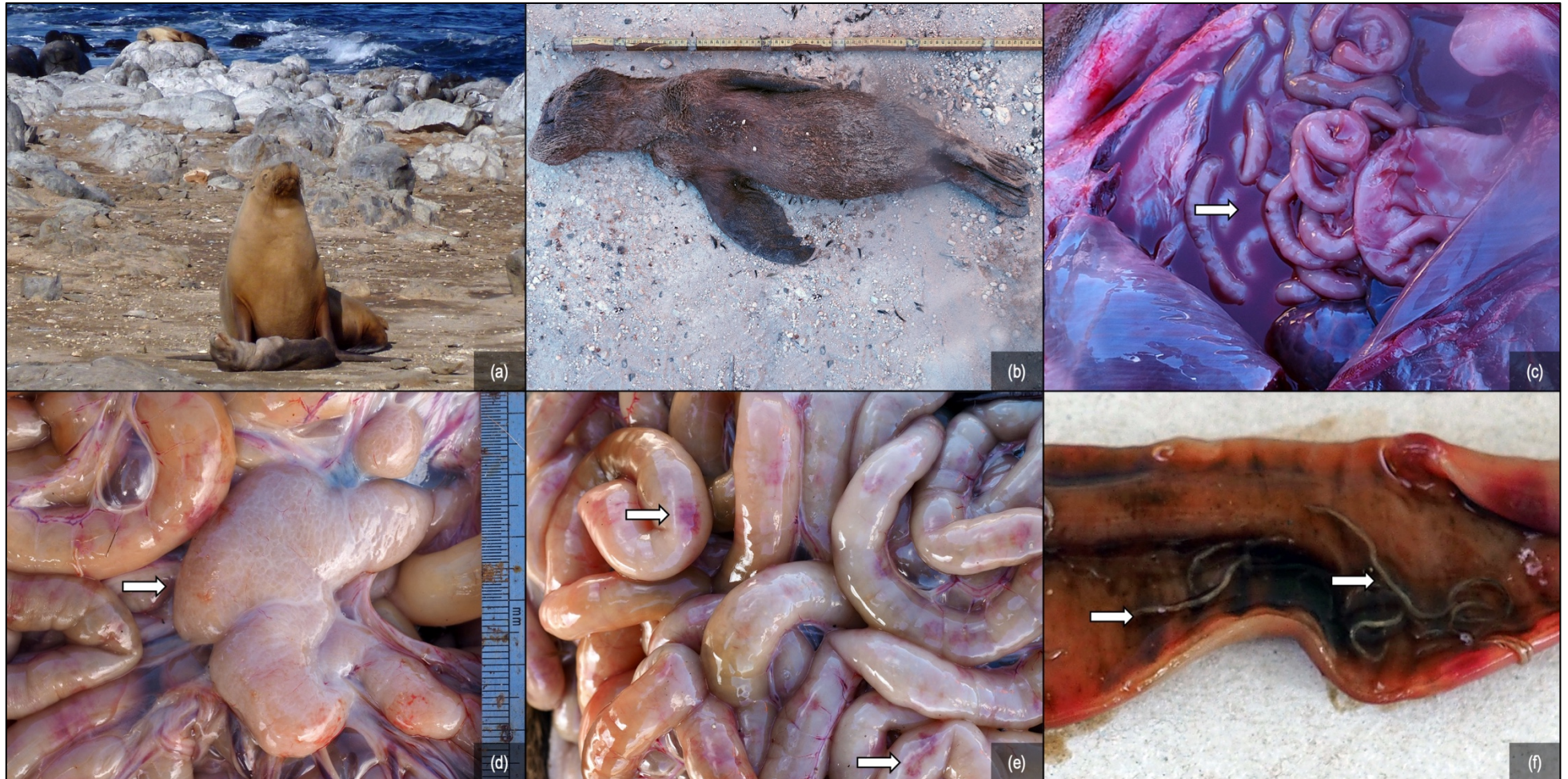


Figure 7. Cause of mortality of Australian sea lion pups: (a) maternal guarding of a stillborn pup; (b) gross appearance of an emaciated pup; (c) septic peritonitis in an older pup following a penetrating bite injury; (d-f) hookworm-associated haemorrhage enteritis, including (d) enlargement of mesenteric lymph nodes, (e) multifocal serosal ecchymoses, and (f) the presence of adult hookworms within an opened intestinal lumen (all marked by white arrows).



Figure 8. Traumatic injury in Australian sea lion pups resulting from conspecific trauma. Sharp and blunt trauma is most frequent in the head and neck region, often associated with extensive haemorrhage and fractures apparent (a-b) on the dorsum or (c) ventrum; (d-e) penetrating bite injuries to the dorsal neck, inflicted by adult conspecific animals; (f) septic inflammation and abscessation following a penetrating bite wound to the shoulder of a pup.

Table 1. Summarised details of hookworm treatment trial publications in otariid species.

Publication	Host species and location [hookworm species]	Pup number	Treatment protocol [pup age at treatment]	Demonstrated treatment effect
Michael et al., 2021	New Zealand sea lion (<i>Phocarctos hookeri</i>), Enderby Island, New Zealand sub-Antarctic; [<i>Uncinaria sp.</i>]	666	200µg/kg, subcutaneous, one dose [2–19 days]	Significantly higher survival
Seguel et al., 2018b	South American fur seal (<i>Arctocephalus australis</i>), Guafo Island, Chile; [<i>Uncinaria hamiltoni</i>]	30	400µg/kg, subcutaneous, one dose [1–7 days]	Significantly higher survival (compared with severely infected pups) Significantly reduced anaemia (compared with severely infected pups) (Anaemia and mortality relate to parasite infection intensity)
Marcus et al., 2015b	Australian sea lion (<i>Neophoca cinerea</i>), Dangerous Reef, Australia; [<i>Uncinaria sanguinis</i>]	64	200µg/kg, subcutaneous, one dose [2–8 weeks]	Significantly reduced growth rate – unexpectedly observed in older pups (standard length ≥67cm at recruitment) Significantly higher erythrocyte and lower eosinophil counts
DeLong et al., 2009	Northern fur seal (<i>Callorhinus ursinus</i>), San Miguel Island, USA; [<i>Uncinaria lucasi</i>]	300	200µg/kg, subcutaneous, one dose [~2 weeks]	Significantly higher growth rate Significantly higher survival Significantly higher hookworm clearance
Chilvers et al., 2009	New Zealand sea lion (<i>Phocarctos hookeri</i>), Enderby Island, New Zealand sub-Antarctic; [<i>Uncinaria sp.</i>]	84	200µg/kg, subcutaneous, three doses [3, 7, 30 days]	Significantly higher growth rate Significantly higher survival – limited to high-mortality season resulting from <i>Klebsiella pneumoniae</i> epidemic
Beekman, 1984	Northern fur seal (<i>Callorhinus ursinus</i>), St. Paul Island, USA; [<i>Uncinaria lucasi</i>]	10	100 and 200µg/kg, subcutaneous, single dose [unreported]	~100% effectiveness for elimination of adult hookworm

Reporting excludes the two publications contained in the present thesis. Pup number reports the combined treatment and control pup number.

Table 2. Summarised details of published mortality studies in the Australian sea lion (*Neophoca cinerea*).

Publication	Location	Breeding seasons	Investigation type	Pup number	Pup age	Mortality categorisation and comment
McIntosh and Kennedy, 2013	Seal Bay Conservation Park, Kangaroo Island, SA	2002/3, 2004, 2005/6	Observation and gross necropsy	128	Young: average male 8.0kg and 75.2cm, average female 7.0kg and 71.3cm	49.0% – undetermined cause (good condition, no injuries) 31.6% – conspecific trauma (more likely in new-born [54%] versus older [32%] pups) 10.4% – emaciation 7.6% – stillbirth or premature birth 1.4% – possible shark attack (no sex ratio bias overall or within each category)
Higgins and Tedman, 1990	Seal Bay Conservation Park, Kangaroo Island, SA	1986/7, 1988	Observational and gross necropsy	21 (necropsy) 116 (observation)	7–57 days known age	43% – starvation, comprising: – 19%: sibling competition from previous juvenile – 14%: death/ non-return of female to colony – 10%: separation of female and pup within colony; 28% – undetermined (no weight loss or injuries) 19% – conspecific trauma (misdirected aggression from territorial males) 10% – drowning related to high-water conditions (attacks witnessed on 5% (6/116) of observed pups)
Marlow, 1975	Dangerous Reef, Spencer Gulf, SA	1967, 1969, 1970	Observational with external examination	20	Not reported	Primary cause of death was conspecific aggression: 20 attacks observed, comprising: – 5 by adult bulls – 4 by juvenile or subadult males – 11 by adult females (occasional trampling by fighting bulls or rough handling by young or inexperienced mothers)

Table 3. Summarised details of comparable published mortality studies in related otariid species.

Publication	Species and location	Breeding seasons	Investigation type	Pup number	Mortality rate	Mortality categorisation and comment
Michael et al., 2019	New Zealand sea lion (<i>Phocartos hookeri</i>), Enderby Island, New Zealand sub-Antarctic	2013/14–2017/18	Gross necropsy Histology Microbiology	284	10.6%*–24.5% (annually) 19.5% (average of the five seasons) *Suggested to be an underestimate due to shorter season length. Ivermectin treatment trial may have influenced mortality in two seasons.	60.2% – <i>Klebsiella pneumoniae</i> infection 14.8% – starvation 9.9% – trauma/ asphyxiation 7.0% – other infections 6.0% – open 1.4% – stillbirth/periparturient death* 0.7% – congenital *Likely underestimated due to sampling time within season. Daily mortality rate significantly higher in late versus early season. Seasonal variations noted.
Roe et al., 2015	New Zealand sea lion (<i>Phocartos hookeri</i>), Enderby Island, New Zealand sub-Antarctic	2006/7–2009/10	Gross necropsy Histology Microbiology	212	Not reported	58% – bacterial infection (94% due to <i>Klebsiella pneumoniae</i>)* *Most deaths occurred in the latter part of the breeding season.
Seguel et al., 2011; Seguel et al., 2013	South American fur seal, (<i>Arctocephalus australis gracilis</i>), Punta Weather, Guafo Island, Chile	2004–2008	Gross necropsy Histology ^a	81	4.2%–9.2% (annually)* 6.0% (average of the five seasons) *Pup mortality to 12 weeks.	28.4% – hookworm enteritis-bacteraemia complex* 23.5% – starvation 19.8% – trauma# 21.0% – drowning# 2.6% – stillbirth/ perinatal mortality 2.5% – open/ other 1.2% – encephalitis 1.2% – pneumonia *Early to mid-season bias; #late season bias (significant interseasonal variability)
Katz et al., 2012	South American fur seal, (<i>Arctocephalus australis</i>), Cabo Polonio, Isla de Lobos, Uruguay	2001–2010 (stranded animals)	Gross necropsy Parasitology	105	Not reported	64% of pups <6 months in poor condition (suggested to indicate a high prevalence of starvation in this cohort) 37.8% - trauma present in pups ≤ 1 month (perinatal) with a second peak, and highest proportion of trauma-related deaths (66.7%) in 9 month old pups (weaning)
Spraker and Lander, 2010	Northern fur seal (<i>Callorhinus ursinus</i>), St Paul Island, Alaska, USA	1986–2006	Gross necropsy Histology Microbiology	2,735	Not reported	53% – starvation 19% – stillbirth/ perinatal mortality* 18% – trauma 7% – open/ miscellaneous

						3% – bacterial infection *Early season bias. Congenital anomalies 1.8%; multifocal necrotizing myopathy and cardiomyopathy 3.4%.
Maniscalco et al., 2008	Steller sea lion (<i>Eumetopias jubatus</i>), Gulf of Alaska, USA	2001–2007	Observational (remote video monitoring)	69	3.8%–27.8% (annually)* 15.4% (average of the seven seasons) *Pup mortality to 2.5 months.	32% – washed away (high surf) 25% – predation 14% – abandonment 14% – undetermined 12% – stillbirth 3% – trauma Substantial inter-year variation noted; mortality age dependent.
Castinel et al., 2007b	New Zealand sea lion (<i>Phocarcos hookeri</i>), Enderby Island, New Zealand sub-Antarctic	1998/9–2004/5	Gross necropsy Histology ^a Microbiology ^a	455	6.4%–31.3% (annually)* 14.4% (average of the seven seasons) *31.3% and 22.1% for epidemic (<i>Klebsiella pneumoniae</i>) years of 2001/2 and 2002/3, respectively, were significantly higher than the 10.2% for remaining years.	35% – trauma 24% – bacterial infection 13% – hookworm 13% – starvation 4% – stillbirth/ perinatal mortality Variation noted between epidemic (<i>Klebsiella pneumoniae</i>) and non-epidemic years.
Spraker et al., 2007	California sea lion (<i>Zalophus californianus</i>), San Miguel Island, California, USA	2002/3	Gross necropsy Histology ^a	208	Not reported	72% – hookworm enteritis-bacteraemia complex 26% – starvation

a: selected cases.

1.7. Thesis outline and research aims

This thesis investigates the contribution of infectious disease to reduced fecundity and pup survival in the endangered Australian sea lion. Specifically, it is a study of morbidity and mortality in Australia sea lion pups and the optimisation of a mitigation strategy for endemic hookworm disease.

Reporting is presented as four research chapters and a general discussion, with an overarching hypothesis that infectious disease is a substantial and modifiable contributor to the species' low fecundity and pup survival.

Chapters two and three report outcomes of endemic hookworm disease mitigation trials, and in doing so, further explore the impact of hookworm disease on pup health. Chapter two reports outcomes of an initial small-scale treatment trial at Dangerous Reef, with the primary aim of assessing the effectiveness of the novel use of a minimally invasive topical formulation of the ivermectin anthelmintic. Secondary aims were to compare the outcomes of topical treatment against the standard injected formulation on pup health – pup growth and haematological parameters of health. The study intended to recruit a younger pup cohort than that for an earlier treatment trial of the injected formulation conducted at the same location. Chapter three reports outcomes of the subsequent large-scale, two-season topical ivermectin treatment trial at Seal Bay, with the primary aims of demonstrating a longer-term growth benefit from early hookworm elimination, and to differentiate the haematological impact of infection from those attributable to neonatal ontogeny. Secondary aims were to optimise the treatment protocol and assess seasonal differences in disease impact or treatment effectiveness.

Chapter four reports the seroprevalence of *T. gondii* antibodies in historical samples from adult female Australian sea lions and unrelated pups from three breeding colonies. The primary study aim was to provide an estimate of the species' exposure, and therefore potential disease risk, from this ubiquitous protozoal parasite. Secondary aims were to add to the current knowledge base regarding

the epidemiology of disease in pinniped species, specifically the route of parasite transmission and temporal data for maternal antibody persistence and transmission to pups.

Chapter five reports the cause of mortality of Australian sea lion pups across five breeding cohorts at Seal Bay Conservation Park. Using observational data, gross and microscopic necropsy, and ancillary microbiological and parasitological assessment, the study's primary aim was to quantify the contribution of infectious disease to pup death. Secondary aims were to model any seasonal differences in causes of mortality and to identify potential mitigation opportunities.

1.8. References

- Ahonen, H., Lowther, A.D., Harcourt, R.G., Goldsworthy, S.D., Charrier, I., and Stow, A.J. (2016). The limits of dispersal: fine scale spatial genetic structure in Australian sea lions. *Frontiers in Marine Science* 3, 65. doi: 10.3389/fmars.2016.00065.
- Alberts, M., Kemper, C., Tomo, I., and O’Handley, R. (2019). *Seroprevalence of Toxoplasma gondii in dead and live stranded dolphins (Tursiops truncatus and Tursiops aduncus) from South Australia*. Doctor of Veterinary Medicine Clinical Research Project dissertation, The University of Adelaide.
- Alvarez Garcia, G., Davidson, R., Jokelainen, P., Klevar, S., Spano, F., and Seeber, F. (2021). Identification of oocyst-driven *Toxoplasma gondii* infections in humans and animals through stage-specific serology-current status and future perspectives. *Microorganisms* 9(11), 2346. doi: 10.3390/microorganisms9112346.
- Angelakis, N., Goldsworthy, S.D., Connell, S.D., and Durante, L.M. (2023). A novel method for identifying fine-scale bottom-use in a benthic-foraging pinniped. *Movement Ecology* 11(1), 34. doi: 10.1186/s40462-023-00386-1.
- Ashour, D.S. (2019). Ivermectin: From theory to clinical application. *International Journal of Antimicrobial Agents* 54(2), 134–142. doi: 10.1016/j.ijantimicag.2019.05.003.
- Australian Fisheries Management Authority (2010). *Australian sea lion management strategy: Southern and Eastern Scalefish and Shark Fishery (SESSF)* [Online]. Commonwealth of Australia. https://fish.gov.au/Archived-Reports/2012/reports/Documents/AFMA_2010b.pdf.
- Barbieri, M.M., Kashinsky, L., Rotstein, D.S., Colegrove, K.M., Haman, K.H., Magargal, S.L., et al. (2016). Protozoal-related mortalities in endangered Hawaiian monk seals *Neomonachus schauinslandi*. *Diseases of Aquatic Organisms* 121(2), 85–95. doi: 10.3354/dao03047.
- Baylis, H. (1933). A new species of the nematode genus *Uncinaria* from a sea lion, with some observations on related species. *Parasitology* 25, 308–316. doi: 10.1017/S0031182000019508.

- Beekman, G.K. (1984). Clinical use of ivermectin against the hookworm (*Uncinaria lucasi*) of Northern seal pups (*Callorhinus ursinus*). *International Association for Aquatic Animal Medicine (IAAAM) Proceedings* 15, 50.
- Berry, T.E., Osterrieder, S.K., Murray, D.C., Coghlan, M.L., Richardson, A.J., Greal, A.K., et al. (2017). DNA metabarcoding for diet analysis and biodiversity: a case study using the endangered Australian sea lion (*Neophoca cinerea*). *Ecology and Evolution* 7(14), 5435–5453. doi: 10.1002/ece3.3123.
- Bilgmann, K., Armansin, N., Ferchaud, A.L., Normandeau, E., Bernatchez, L., Harcourt, R., et al. (2021). Low effective population size in the genetically bottlenecked Australian sea lion is insufficient to maintain genetic variation. *Animal Conservation* 24(5), 847–861. doi: 10.1111/acv.12688.
- Blazkowska, J., and Goralska, K. (2014). Parasites and fungi as a threat for prenatal and postnatal human development. *Annals of Parasitology* 60(4):225-234.
- Boveng, P.L., Hiruki, L.M., Schwartz, M.K., and Bengtson, J.L. (1998). Population growth of Antarctic fur seals: limitation by a top predator, the leopard seal? *Ecology* 79(8), 2863–2877. doi: 10.1890/0012-9658(1998)079[2863:PGOAFS]2.0.CO;2.
- Campbell, K., Paparini, A., Gomez, A.B., Cannell, B., and Stephens, N. (2022). Fatal toxoplasmosis in little penguins (*Eudyptula minor*) from Penguin Island, Western Australia. *International Journal for Parasitology: Parasites and Wildlife* 17, 211–217. doi: 10.1016/j.ijppaw.2022.02.006.
- Campbell, R., Holley, D., Christianopolous, D., Caputi, N., and Gales, N.G. (2008a). Mitigation of incidental mortality of Australian sea lions in the west coast rock lobster fishery. *Endangered Species Research* 5(2–3), 345–358. doi: 10.3354/esr00155.
- Campbell, R.A., Gales, N.J., Lento, G.M., and Baker, C.S. (2008b). Islands in the sea: extreme female natal site fidelity in the Australian sea lion, *Neophoca cinerea*. *Biology Letters* 4(1), 139–142. doi: 10.1098/rsbl.2007.0487.
- Carlson-Bremer, D., Colegrove, K.M., Gulland, F.M., Conrad, P.A., Mazet, J.A., and Johnson, C.K. (2015). Epidemiology and pathology of *Toxoplasma gondii* in free-ranging California sea lions (*Zalophus californianus*). *Journal of Wildlife Diseases* 51(2), 362–373. doi: 10.7589/2014-08-205.

- Castinel, A., Duignan, P.J., Lyons, E.T., Pomroy, W.E., Gibbs, N., Lopez-Villalobos, N., et al. (2007a). Epidemiology of hookworm (*Uncinaria* spp.) infection in New Zealand (Hooker's) sea lion (*Phocarctos hookeri*) pups on Enderby Island, Auckland Islands (New Zealand) during the breeding seasons from 1999/2000 to 2004/2005. *Parasitology Research* 101(1), 53–62. doi: 10.1007/s00436-006-0453-z.
- Castinel, A., Duignan, P.J., Pomroy, W.E., Lopez-Villalobos, N., Gibbs, N.J., Chilvers, B.L., and Wilkinson, I.S. (2007b). Neonatal mortality in New Zealand sea lions (*Phocarctos hookeri*) at Sandy Bay, Enderby Island, Auckland Islands from 1998 to 2005. *Journal of Wildlife Diseases* 43(3), 461–474. doi: 10.7589/0090-3558-43.3.461.
- Castinel, A., Duignan, P.J., Pomroy, W.E., Lyons, E.T., Nadler, S.A., Dailey, M.D., et al. (2006). First report and characterization of adult *Uncinaria* spp. in New Zealand sea lion (*Phocarctos hookeri*) pups from the Auckland Islands, New Zealand. *Parasitology Research* 98(4), 304–309. doi: 10.1007/s00436-005-0069-8.
- Chilvers, B.L., Duignan, P.J., Robertson, B.C., Castinel, A., and Wilkinson, I.S. (2009). Effects of hookworms (*Uncinaria* sp.) on the early growth and survival of New Zealand sea lion (*Phocarctos hookeri*) pups. *Polar Biology* 32(2), 295–302. doi: 10.1007/s00300-008-0559-0.
- Chilvers, B.L. and Goldsworthy, S.D. (2015). *Arctocephalus forsteri*. The IUCN Red List of Threatened Species 2015: e.T41664A45230026. <https://dx.doi.org/10.2305/IUCN.UK.2015-2.RLTS.T41664A45230026.en>. [Accessed on 28 September 2023].
- Committee on Taxonomy (2023). *List of marine mammal species and subspecies*. Society for Marine Mammalogy. <https://marinemammalscience.org/science-and-publications/list-marine-mammal-species-subspecies/> [Accessed 25 September 2023].
- Costa, D.P., and Gales, N.J. (2003). Energetics of a benthic diver: seasonal foraging ecology of the Australian sea lion, *Neophoca cinerea*. *Ecological Monographs* 73(1), 27–43. doi: 10.1890/0012-9615(2003)073[0027:EOabds]2.0.Co;2.
- DeLong, R.L., Orr, A.J., Jenkinson, R.S., and Lyons, E.T. (2009). Treatment of northern fur seal (*Callorhinus ursinus*) pups with ivermectin reduces hookworm-induced mortality. *Marine Mammal Science* 25(4), 944–948. doi: 10.1111/j.1748-7692.2008.00274.x.
- Deng, Y., Wu, J., Chen, J., and Kang, K. (2023). Overview of microplastic pollution and its influence on the health of organisms. *Journal of Environmental Science and Health Part A:*

Toxic/Hazardous Substances and Environmental Engineering 58(4), 412–422. doi: 10.1080/10934529.2023.2190715.

Dennis, T.E., and Shaughnessy, P.D. (1996). Status of the Australian sea lion, *Neophoca cinerea*, in the Great Australian Bight. *Wildlife Research* 23(6), 741–754. doi: Doi 10.1071/Wr9960741.

Department of Sustainability, Environment, Water, Population and Communities (2013a). *Issues paper for the Australian sea lion (Neophoca cinerea)* [Online]. Commonwealth of Australia. <https://www.dcceew.gov.au/sites/default/files/documents/neophoca-cinerea-issues-paper.pdf>.

Department of Sustainability, Environment, Water, Population and Communities (2013b). *Recovery plan for the Australian sea lion (Neophoca cinerea)* [Online]. Commonwealth of Australia. <http://www.environment.gov.au/system/files/resources/1eb9233c-8474-40bb-8566-0ea02bbaa5b3/files/neophoca-cinerea-recovery-plan.pdf>.

Doidge, D.W., Croxall, J.P., and Baker, J.R. (1984). Density-dependent pup mortality in the Antarctic fur seal *Arctocephalus gazellu* at South Georgia. *Journal of Zoology* 202(3), 449–460. doi: <https://doi.org/10.1111/j.1469-7998.1984.tb05095.x>.

Donahoe, S.L., Rose, K., and Slapeta, J. (2014). Multisystemic toxoplasmosis associated with a type II-like *Toxoplasma gondii* strain in a New Zealand fur seal (*Arctocephalus forsteri*) from New South Wales, Australia. *Veterinary Parasitology* 205(1–2), 347–353. doi: 10.1016/j.vetpar.2014.07.022.

Drago, M., Cardona, L., García, N., Ameghino, S., and Aguilar, A. (2010). Influence of colony size on pup fitness and survival in South American sea lions. *Marine Mammal Science* 27, 167–181. doi: 10.1111/j.1748-7692.2010.00402.x.

Duarte-Benvenuto, A., Sacristán, C., Reisfeld, L., Santos-Costa, P.C., Fernandes, N., Ressio, R.A., et al. (2022). Clinico-pathologic findings and pathogen screening in fur seals (*Arctocephalus Australis* and *Arctocephalus tropicalis*) stranded in southeastern Brazil, 2018. *Journal of Wildlife Diseases* 58(1), 86–99. doi: 10.7589/jwd-d-21-00060.

Dubey, J.P. (2022). *Toxoplasmosis of Animals and Humans*. 3rd Ed. Boca Raton, Florida, United States: CRC Press. doi: 10.1201/9781003199373.

- Dubey, J.P., Murata, F.H.A., Cerqueira-Cézar, C.K., Kwok, O.C.H., and Grigg, M.E. (2020). Recent epidemiologic and clinical importance of *Toxoplasma gondii* infections in marine mammals: 2009–2020. *Veterinary Parasitology* 288, 109296. doi: 10.1016/j.vetpar.2020.109296.
- Fowler, S.L., Costa, D.P., Arnould, J.P., Gales, N.J., and Kuhn, C.E. (2006). Ontogeny of diving behaviour in the Australian sea lion: trials of adolescence in a late bloomer. *Journal of Animal Ecology* 75(2), 358–367. doi: 10.1111/j.1365-2656.2006.01055.x.
- Fowler, S.L., Costa, D.P., Arnould, J.P.Y., Gales, N.J., and Burns, J.M. (2007). Ontogeny of oxygen stores and physiological diving capability in Australian sea lions. *Functional Ecology* 21(5), 922–935. doi: 10.1111/j.1365-2435.2007.01295.x.
- Frankham, R. (2005). Genetics and extinction. *Biological Conservation* 126(2), 131–140. doi: 10.1016/j.biocon.2005.05.002.
- Fulham, M., McDougall, F., Power, M., McIntosh, R.R., and Gray, R. (2022a). Carriage of antibiotic resistant bacteria in endangered and declining Australian pinniped pups. *PLoS One* 17(1), e0258978. doi: 10.1371/journal.pone.0258978.
- Fulham, M., Power, M., and Gray, R. (2018). Comparative ecology of *Escherichia coli* in endangered Australian sea lion (*Neophoca cinerea*) pups. *Infection, Genetics and Evolution* 62, 262–269. doi: 10.1016/j.meegid.2018.05.002.
- Fulham, M., Power, M., and Gray, R. (2020). Diversity and distribution of *Escherichia coli* in three species of free-ranging Australian pinniped pups. *Frontiers in Marine Science* 7, 571171. doi: 10.3389/fmars.2020.571171.
- Fulham, M., Power, M., and Gray, R. (2022b). Gut microbiota of endangered Australian sea lion pups is unchanged by topical ivermectin treatment for endemic hookworm infection. *Frontiers in Microbiology* 13, 1048013. doi: 10.3389/fmicb.2022.1048013.
- Gales, N.J. (2008). Australian sea lion: *Neophoca cinerea*. In S. van Dyck and R. Strahan (Eds.), *The Mammals of Australia*, 3rd ed (pp. 715–716). Chatswood, New South Wales: Reed New Holland.
- Gales, N.J., and Cheal, A.J. (1992). Estimating diet composition of the Australian sea lion (*Neophoca cinerea*) from scat analysis: an unreliable technique. *Wildlife Research* 19(4), 447–456. doi: 10.1071/WR9920447.

- Gales, N.J., Cheal, A.J., Pobar, G.J., and Williamson, P. (1992). Breeding biology and movements of Australian sea lions, *Neophoca cinerea*, off the west coast of Western-Australia. *Wildlife Research* 19(4), 405–416. doi: 10.1071/Wr9920405.
- Gales N. J., and Costa D. P. (1997). The Australian sea lion: a review of an unusual life history. In M. A. Hindell and C. Kemper (Eds.), *Marine Mammal Research in the Southern Hemisphere, Volume 1: Status, Ecology and Medicine* (pp. 78–87). Sydney: Surrey Beatty.
- Gales, N.J., Shaughnessy, P.D., and Dennis, T.E. (1994). Distribution, abundance and breeding cycle of the Australian sea lion *Neophoca cinerea* (Mammalia: Pinnipedia). *Journal of Zoology* 234(3), 353–370. doi: 10.1111/j.1469-7998.1994.tb04853.x.
- Gales, N.J., Williamson, P., Higgins, L.V., Blackberry, M.A., and James, I. (1997). Evidence for a prolonged postimplantation period in the Australian sea lion (*Neophoca cinerea*). *Journal of Reproduction and Fertility* 111(2), 159–163. doi: 10.1530/jrf.0.1110159.
- Geeson, J.J., Hobday, A.J., Speakman, C.N., and Arnould, J.P.Y. (2022). Environmental influences on breeding biology and pup production in Australian fur seals. *Royal Society Open Science* 9(4), 211399. doi: 10.1098/rsos.211399.
- Goldsworthy, S. (2020). *Australian sea lion listing assessment. Report to the Department for Environment and Water, Department of Agriculture, Water and the Environment* [Online]. South Australian Research and Development Institute (Aquatic Sciences), Adelaide. SARDI Publication No. F2020/000131-1. SARDI Research Report Series No. 1056.
- Goldsworthy, S. and Gales, N. (IUCN SSC Pinniped Specialist Group) (2008). *Neophoca cinerea*. The IUCN Red List of Threatened Species 2008: e.T14549A4443172. <https://www.iucnredlist.org/species/14549/4443172> [Accessed 1 September 2021].
- Goldsworthy, S.D. (2015a). The IUCN Red List of Threatened Species 2015: e.T2067A66991075. <https://dx.doi.org/10.2305/IUCN.UK.2015-4.RLTS.T2067A66991075.en> [Accessed 27 September 2023].
- Goldsworthy, S.D. (2015b). *Neophoca cinerea*. The IUCN Red List of Threatened Species 2015: e.T14549A45228341. <https://dx.doi.org/10.2305/IUCN.UK.2015-2.RLTS.T14549A45228341.en> [Accessed 1 September 2020].

- Goldsworthy, S.D. (2018). Australian Sea Lion. In B. Würsig, J.G.M. Thewissen and K.M. Kovacs (Eds.), *Encyclopedia of Marine Mammals, 3rd Ed* (pp.44–46). San Diego: Academic Press. doi: 10.1016/B978-0-12-804327-1.00021-2
- Goldsworthy, S.D., Bailleul, F., Shaughnessy, P.D., Stonnill, M., Lashmar, K., and Mackay, A. (2017). *Monitoring of Seal Bay and other pinniped populations on Kangaroo Island: 2016/2017. Report to the Department of Environment, Water and Natural Resources* [Online]. South Australian Research and Development Institute (Aquatic Sciences), Adelaide. SARDI Publication No. F2014/000322-4. SARDI Research Report Series No. 951.
- Goldsworthy, S.D., Hodgson, J., and Holman, D. (2020). *Australian sea lion investigations: 2018–19. Report to the Department for Environment and Water* [Online]. South Australian Research and Development Institute (Aquatic Sciences), Adelaide. SARDI Publication No. F2020/000052-1. SARDI Research Report Series No. 1051.
- Goldsworthy, S.D., Mackay, A.I., Shaughnessy, P.D., Bailleul, F., and McMahon, C.R. (2014). *Maintaining the monitoring of pup production at key Australian sea lion colonies in South Australia (2013/14). Final report to the Australian Marine Mammal Centre* [Online]. South Australian Research and Development Institute (Aquatic Sciences), Adelaide. SARDI Publication No. F2010/000665-4. SARDI Research Report Series No. 818.
- Goldsworthy, S.D., McKenzie, J., Shaughnessy, P.D., McIntosh, R.R., Page, B., and Campbell, R. (2009a). *An update of the report: understanding the impediments to the growth of Australian sea lion populations. Report to the Department of the Environment, Water, Heritage and the Arts* [Online]. South Australian Research and Development Institute (Aquatic Sciences), Adelaide. SARDI Publication No. F2008/00847-1. SARDI Research Report Series No. 356.
- Goldsworthy, S.D., and Page, B. (2007). A risk-assessment approach to evaluating the significance of seal bycatch in two Australian fisheries. *Biological Conservation* 139(3/4), 269–285. doi: 10.1016/j.biocon.2007.07.010.
- Goldsworthy, S.D., Page, B., Hamer, D.J., Lowther, A.D., Shaughnessy, P.D., Hindell, M.A., et al. (2022). Assessment of Australian sea lion bycatch mortality in a gillnet fishery, and implementation and evaluation of an effective mitigation strategy. *Frontiers in Marine Science* 9, 799102. doi: 10.3389/fmars.2022.799102.

- Goldsworthy, S.D., Page, B., and Shaughnessy, P.D. (2010a). *Pup production assessment of the Australian sea lion *Neophoca cinerea* at Dangerous Reef and English Island, South Australia, in the 2009/10 breeding season. Report to the South Australian Department of Environment and Natural Resources Wildlife Conservation Fund* [Online]. South Australian Research and Development Institute (Aquatic Sciences), Adelaide. SARDI Publication No. F2010/000639-1. SARDI Research Report Series No. 480.
- Goldsworthy, S.D., Page, B., Shaughnessy, P.D., Hamer, D.J., Peters, K.D., McIntosh, R.R., et al. (2009b). *Innovative solutions for aquaculture planning and management: addressing seal interactions in the finfish aquaculture industry* [Online]. South Australian Research and Development Institute (Aquatic Sciences), Adelaide. SARDI Research Report Series No. 228.
- Goldsworthy, S.D., Page B, Shaughnessy, P.D and Linnane A (2010). *Mitigating seal interactions in the SRLF and the gillnet sector SESSF in South Australia. Report to the Fisheries Research and Development Institute* [Online]. South Australian Research and Development Institute (Aquatic Sciences), Adelaide. SARDI Publication No. F2009/000613-1. SARDI Research Report Series No. 405.
- Goldsworthy, S.D., Shaughnessy, P.D., Mackay, A.I., Bailleul, F., Holman, D., Lowther, A.D., et al. (2021a). Assessment of the status and trends in abundance of a coastal pinniped, the Australian sea lion *Neophoca cinerea*. *Endangered Species Research* 44, 421–437. doi: 10.3354/esr01118.
- Goldsworthy, S.D., Shaughnessy, P.D., Smart, J., Mackay, A., Bailleul, F., Reinhold, S.-L., et al. (2019). *Monitoring of Seal Bay and other populations on Kangaroo Island: 2017/2018. Report to the Department for Environment and Water* [Online]. South Australian Research and Development Institute (Aquatic Sciences), Adelaide. SARDI Publication No. F2014000322-5. SARDI Research Report Series No. 1018.
- Goldsworthy, S.D., Stonnill, M., and Kirkwood, R. (2021b). *Monitoring of the Australian sea lion population at Seal Bay: 2020/21. Report to the Department for Environment and Water* [Online]. South Australian Research and Development Institute (Aquatic Sciences), Adelaide. SARDI Publication No. F2014/000322-6. SARDI Research Report Series No. 1121.
- Hammill, M.O., Sauvé, C., and Northridge, S. (2017). Growth and condition in harp seals: evidence of density-dependent and density-independent influences. *ICES Journal of Marine Science* 74(5), 1395–1407. doi: 10.1093/icesjms/fsw237.

- Harcourt, R. (1992). Factors affecting early mortality in the South American fur seal (*Arctocephalus australis*) in Peru: density-related effects and predation. *Journal of Zoology* 226(2), 259–270. doi: <https://doi.org/10.1111/j.1469-7998.1992.tb03838.x>.
- Harting, A.L., Barbieri, M.M., Baker, J.D., Mercer, T.A., Johanos, T.C., Robinson, S.J., et al. (2020). Population-level impacts of natural and anthropogenic causes-of-death for Hawaiian monk seals in the main Hawaiian Islands. *Marine Mammal Science* 37(1), 235–250. doi: 10.1111/mms.12742.
- Heard, M.J., Smith, K.F., Ripp, K., Berger, M., Chen, J., Dittmeier, J., et al. (2013). The threat of disease increases as species move toward extinction. *Conservation Biology* 27(6), 1378–1388. doi: 10.1111/cobi.12143.
- Higgins, L.V. (1993). The nonannual, nonseasonal breeding cycle of the Australian sea lion, *Neophoca cinerea*. *Journal of Mammalogy* 74(2), 270–274. doi: 10.2307/1382381.
- Higgins, L.V., and Gass, L. (1993). Birth to weaning: parturition, duration of lactation, and attendance cycles of Australian sea lions (*Neophoca cinerea*). *Canadian Journal of Zoology* 71(10), 2047–2055. doi: 10.1139/z93-290.
- Higgins, L.V., and Tedman, R.A. (1990). Effect of attacks by male Australian sea lions, *Neophoca cinerea*, on mortality of pups. *Journal of Mammalogy* 71(4), 617–619. doi: 10.2307/1381802.
- IPCC (2014). *Climate Change 2014: Synthesis Report* [Online]. Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change [Core Writing Team, R.K. Pachauri and L.A. Meyer (eds.)]. IPCC, Geneva, Switzerland.
- IUCN (2024). *Search for “sea lion”*. The IUCN Red List of Threatened Species. Version 2023-1. <https://www.iucnredlist.org/search?query=sea%20lion&searchType=species> [Accessed 3 April 2024].
- Jarolimek, C.V., King, J.J., Apte, S.C., Hall, J., Gautam, A., Gillmore, M., et al. (2023). A review of inorganic contaminants in Australian marine mammals, birds and turtles. *Environmental Chemistry* 20(4), 147–170. doi: 10.1071/en23057.
- Katz, H., Morgades, D., and Castro-Ramos, M. (2012). Pathological and parasitological findings in South American fur seal pups (*Arctocephalus australis*) in Uruguay. *International Scholarly Research Network (ISRN) Zoology* 2012, 1–7. doi: 10.5402/2012/586079.

- Kirkwood, R., and Goldsworthy, S.D. (2013). *Fur seals and sea lions Australian Natural History Series*. Collingwood, Victoria: CSIRO Publishing.
- Ladds, M.A., Slip, D.J., and Harcourt, R.G. (2017). Intrinsic and extrinsic influences on standard metabolic rates of three species of Australian otariid. *Conservation Physiology* 5(1), cow074. doi: 10.1093/conphys/cow074.
- Landrigan, P.J., Raps, H., Cropper, M., Bald, C., Brunner, M., Canonizado, E.M., et al. (2023). The Minderoo-Monaco Commission on Plastics and Human Health. *Annals of Global Health* 89(1), 23, 1–215. doi: 10.5334/aogh.4056Glob Health 89(1), 23. doi: 10.5334/aogh.4056.
- Lau, Q., Chow, N., Gray, R., Gongora, J., and Higgins, D.P. (2015). Diversity of MHC DQB and DRB genes in the endangered Australian sea lion (*Neophoca cinerea*). *Journal of Heredity* 106(4), 395–402. doi: 10.1093/jhered/esv022.
- Le Boeuf, B.J., and Briggs, K.T. (1977). The cost of living in a seal harem. *Mammalia* 41(2), 167–196. doi: 10.1515/mamm.1977.41.2.167.
- Lindsay, D.S., Collins, M.V., Mitchell, S.M., Cole, R.A., Flick, G.J., Wetch, C.N., et al. (2003). Sporulation and survival of *Toxoplasma gondii* oocysts in seawater. *The Journal of Eukaryotic Microbiology* 50 Suppl, 687–688. doi: 10.1111/j.1550-7408.2003.tb00688.x.
- Lindsay, D.S., and Dubey, J.P. (2009). Long-term survival of *Toxoplasma gondii* sporulated oocysts in seawater. *Journal of Parasitology* 95(4), 1019–1020. doi: 10.1645/GE-1919.1.
- Lindsay, S.A., and Gray, R. (2021). A novel presentation of tuberculosis with intestinal perforation in a free-ranging Australian sea lion (*Neophoca cinerea*). *Journal of Wildlife Diseases* 57(1), 220–224. doi: 10.7589/JWD-D-20-00104.
- Ling, J. (1999). Exploitation of fur seals and sea lions from Australian, New Zealand and adjacent subantarctic islands during the eighteenth, nineteenth and twentieth centuries. *Australian Zoologist* 31(2), 323–350. doi: 10.7882/az.1999.036.
- Ling, J.K. (2002). Australian sea lion. In W.F. Perrin, B. Wursig and J.G.M. Thewissen (Eds.), *Encyclopedia of Marine Mammal* (pp.51–54). San Diego: Academic Press.
- Ling, J.K., and Walker, G.E. (1976). Seal studies in South Australia: progress report for the year 1975. *South Australian Naturalist* 50(4), 59–68.

- Ling, J.K., and Walker, G.E. (1978). An 18-month breeding cycle in the Australian sea lion? *Search* 9(12), 464–465.
- Loukas, A., Constant, S.L., and Bethony, J.M. (2005). Immunobiology of hookworm infection. *FEMS Immunology and Medical Microbiology* 43(2), 115–124. doi: 10.1016/j.femsim.2004.11.006.
- Lowther, A., and Goldsworthy, S. (2012). Head start: Australian sea lion pups gain experience of adult foraging grounds before weaning. *Marine Biology* 159(12), 2687–2696. doi: 10.1007/s00227-012-2026-2.
- Lowther, A.D., and Goldsworthy, S.D. (2011). Detecting alternate foraging ecotypes in Australian sea lion (*Neophoca cinerea*) colonies using stable isotope analysis. *Marine Mammal Science* 27(3), 567–586. doi: 10.1111/j.1748-7692.2010.00425.x.
- Lowther, A.D., and Goldsworthy, S.D. (2016). When were the weaners weaned? Identifying the onset of Australian sea lion nutritional independence. *Journal of Mammalogy* 97(5), 1304–1311. doi: 10.1093/jmammal/gyw106.
- Lowther, A.D., Harcourt, R.G., Goldsworthy, S.D., and Stow, A. (2012). Population structure of adult female Australian sea lions is driven by fine-scale foraging site fidelity. *Animal Behaviour* 83(3), 691–701. doi: 10.1016/j.anbehav.2011.12.015.
- Lowther, A.D., Harcourt, R.G., Hamer, D.J., and Goldsworthy, S.D. (2011). Creatures of habit: foraging habitat fidelity of adult female Australian sea lions. *Marine Ecology Progress Series* 443, 249–263. doi: 10.3354/meps09392.
- Lukić Bilela, L., Matijošytė, I., Krutkevičius, J., Alexandrino, D.A.M., Safarik, I., Burlakovs, J., et al. (2023). Impact of per- and polyfluorinated alkyl substances (PFAS) on the marine environment: Raising awareness, challenges, legislation, and mitigation approaches under the One Health concept. *Marine Pollution Bulletin* 194(Pt A), 115309. doi: 10.1016/j.marpolbul.2023.115309.
- Lyons, E.T. (1964). *Biology of the hookworm, Uncinaria lucasi Stiles, 1901, in the northern fur seal, Catterhinus ursinus Linn, on the Pribilof Islands, Alaska*. Doctor of Philosophy dissertation, Colorado State University, 196324.
- Lyons, E.T., DeLong, R.L., Gulland, F.M., Melin, S.R., Tolliver, S.C., and Spraker, T.R. (2000). Comparative biology of *Uncinaria* spp. in the California sea lion (*Zalophus californianus*) and

- the Northern fur seal (*Callorhinus ursinus*) in California. *Journal of Parasitology* 86(6), 1348–1352. doi: 10.1645/0022-3395(2000)086[1348:CBOUSI]2.0.CO;2.
- Lyons, E.T., DeLong, R.L., Melin, S.R., and Tolliver, S.C. (1997). Uncinariasis in northern fur seal and California sea lion pups from California. *Journal of Wildlife Diseases* 33(4), 848–852. doi: 10.7589/0090-3558-33.4.848.
- Lyons, E.T., and Keyes, M.C. (1978). Observations on the infectivity of parasitic third-stage larvae of *Uncinaria lucasi* Stiles 1901 (Nematoda: Ancylostomatidae) of Northern fur seals, *Callorhinus ursinus* Linn., on St. Paul Island, Alaska. *Journal of Parasitology* 64(3), 454–458.
- Lyons, E.T., Melin, S.R., DeLong, R.L., Orr, A.J., Gulland, F.M., and Tolliver, S.C. (2001). Current prevalence of adult *Uncinaria* spp. in northern fur seal (*Callorhinus ursinus*) and California sea lion (*Zalophus californianus*) pups on San Miguel Island, California, with notes on the biology of these hookworms. *Veterinary Parasitology* 97(4), 309–318. doi: 10.1016/s0304-4017(01)00418-6.
- Maloney, A., Chilvers, B., Haley, M., Muller, C., Roe, W., and Debski, I. (2009). Distribution, pup production and mortality of New Zealand sea lion (*Phocarctos hookeri*) on Campbell Island / Motu Ihupuku, 2008. *New Zealand Journal of Ecology* 33, 97–105.
- Maniscalco, J.M., Calkins, D.G., Parker, P., and Atkinson, S. (2008). Causes and extent of natural mortality among Steller sea lion (*Eumetopias jubatus*) pups. *Aquatic Mammals* 34(3), 277–287. doi: 10.1578/am.34.3.2008.277.
- Marcus, A.D., Higgins, D.P., and Gray, R. (2014a). Epidemiology of hookworm (*Uncinaria sanguinis*) infection in free-ranging Australian sea lion (*Neophoca cinerea*) pups. *Parasitology Research* 113(9), 3341–3353. doi: 10.1007/s00436-014-3997-3.
- Marcus, A.D., Higgins, D.P., and Gray, R. (2015a). Health assessment of free-ranging endangered Australian sea lion (*Neophoca cinerea*) pups: effect of haematophagous parasites on haematological parameters. *Comparative Biochemistry and Physiology, Part A: Molecular and Integrative Physiology* 184, 132–143. doi: 10.1016/j.cbpa.2015.02.017.
- Marcus, A.D., Higgins, D.P., and Gray, R. (2015b). Ivermectin treatment of free-ranging endangered Australian sea lion (*Neophoca cinerea*) pups: effect on hookworm and lice infection status, haematological parameters, growth, and survival. *Parasitology Research* 114(7), 2743–2755. doi: 10.1007/s00436-015-4481-4.

- Marcus, A.D., Higgins, D.P., Slapeta, J., and Gray, R. (2014b). *Uncinaria sanguinis* sp. n. (Nematoda: Ancylostomatidae) from the endangered Australian sea lion, *Neophoca cinerea* (Carnivora: Otariidae). *Folia Parasitologica (Praha)* 61(3), 255–265. doi: 10.14411/fp.2014.037.
- Marlow, B.J. (1972). Pup abduction in the Australian sea lion *Neophoca cinerea*. *Mammalia* 36(1), 161–165.
- Marlow, B.J. (1975). The comparative behaviour of the Australasian sea lions *Neophoca cinerea* and *Phocarctos hookeri* (Pinnipedia: Otariidae). *Mammalia* 39(2), 159–230. doi: 10.1515/mamm.1975.39.2.159.
- McIntosh, R. R. (2007). *Life history and population demographics of the Australian sea lion*. Doctor of Philosophy dissertation, La Trobe University.
https://www.researchgate.net/publication/332343240_life_history_and_population_demographics_of_the_australian_sea_lion
- McIntosh, R.R., Arthur, A.D., Dennis, T., Berris, M., Goldsworthy, S.D., Shaughnessy, P.D., and Teixeira, C.E.P. (2013). Survival estimates for the Australian sea lion: negative correlation of sea surface temperature with cohort survival to weaning. *Marine Mammal Science* 29(1), 84–108. doi: 10.1111/j.1748-7692.2011.00558.x.
- McIntosh, R.R., and Kennedy, C.W. (2013). Morphology, sex ratio and cause of death in Australian sea lion (*Neophoca cinerea*) pups. *Australian Mammalogy* 35(1), 93. doi: 10.1071/am12037.
- McIntosh, R.R., Page, B., and Goldsworthy, S.D. (2006). Dietary analysis of regurgitates and stomach samples from free-living Australian sea lions. *Wildlife Research* 33(8), 661–669. doi: 10.1071/WR06025.
- McIntosh, R.R., Sorrell, K.J., Thalmann, S., Mitchell, A., Gray, R., Schinagl, H., et al. (2022). Sustained reduction in numbers of Australian fur seal pups: implications for future population monitoring. *PLoS One* 17(3), e0265610. doi: 10.1371/journal.pone.0265610.
- Meza Cerda, M.I., Gray, R., Thomson, P.C., Butcher, L., Simpson, K., Cameron, A., et al. (2022). Developing immune profiles of endangered Australian sea lion (*Neophoca cinerea*) pups within the context of endemic hookworm (*Uncinaria sanguinis*) infection. *Frontiers in Veterinary Science* 9, 824584. doi: 10.3389/fvets.2022.824584.

- Michael, S.A., Hayman, D.T.S., Gray, R., and Roe, W.D. (2021). Risk factors for New Zealand sea lion (*Phocarctos hookeri*) pup mortality: ivermectin improves survival for conservation management. *Frontiers in Marine Science* 8. doi: 10.3389/fmars.2021.680678.
- Michael, S.A., Hayman, D.T.S., Gray, R., Zhang, J., Rogers, L., and Roe, W.D. (2019). Pup mortality in New Zealand sea lions (*Phocarctos hookeri*) at Enderby Island, Auckland Islands, 2013–18. *PLoS One* 14(11), e0225461. doi: 10.1371/journal.pone.0225461.
- Nadler, S.A., Lyons, E.T., Pagan, C., Hyman, D., Lewis, E.E., Beckmen, K., et al. (2013). Molecular systematics of pinniped hookworms (Nematoda: Uncinaria): species delimitation, host associations and host-induced morphometric variation. *International Journal for Parasitology* 43(14), 1119–1132. doi: 10.1016/j.ijpara.2013.08.006.
- Nagel, R., Mews, S., Adam, T., Stainfield, C., Fox-Clarke, C., Toscani, C., et al. (2021). Movement patterns and activity levels are shaped by the neonatal environment in Antarctic fur seal pups. *Scientific Reports* 11(1), 14323. doi: 10.1038/s41598-021-93253-1.
- Olsen, O.W., and Lyons, E.T. (1965). Life cycle of *Uncinaria lucasi* Stiles, 1901 (Nematoda: Ancylostomatidae) of fur seals, *Callorhinus ursinus* Linn., on the Pribilof Islands, Alaska. *Journal of Parasitology* 51(5), 689–700. doi: 10.2307/3276141.
- Omeyer, L.C.M., Duncan, E.M., Abreo, N.A.S., Acebes, J.M.V., AngSinco-Jimenez, L.A., Anuar, S.T., et al. (2023). Interactions between marine megafauna and plastic pollution in Southeast Asia. *Science of The Total Environment* 874, 162502. doi: 10.1016/j.scitotenv.2023.162502.
- Page, B., McKenzie, J., McIntosh, R., Baylis, A., Morrissey, A., Calvert, N., et al. (2004). Entanglement of Australian sea lions and New Zealand fur seals in lost fishing gear and other marine debris before and after Government and industry attempts to reduce the problem. *Marine Pollution Bulletin* 49(1–2), 33–42. doi: 10.1016/j.marpolbul.2004.01.006.
- Peritore, A.F., Gugliandolo, E., Cuzzocrea, S., Crupi, R., and Britti, D. (2023). Current review of increasing animal health threat of per- and polyfluoroalkyl substances (PFAS): harms, limitations, and alternatives to manage their toxicity. *International Journal of Molecular Sciences* 24(14). doi: 10.3390/ijms241411707.
- Peter, D.S., Jane, M., Melanie, L.L., Simon, D.G., and Terry, E.D. (2010). Australian fur seals establish haulout sites and a breeding colony in South Australia. *Australian Journal of Zoology* 58(2), 94–103.

- Peters, K.J., Ophelkeller, K., Bott, N.J., Deagle, B.E., Jarman, S.N., and Goldsworthy, S.D. (2015a). Fine-scale diet of the Australian sea lion (*Neophoca cinerea*) using DNA-based analysis of faeces. *Marine Ecology* 36(3), 347–367. doi: 10.1111/maec.12145.
- Peters, K.J., Ophelkeller, K., Herdina, Bott, N.J., and Goldsworthy, S.D. (2015b). PCR-based techniques to determine diet of the Australian sea lion (*Neophoca cinerea*): a comparison with morphological analysis. *Marine Ecology* 36(4), 1428–1439. doi: 10.1111/maec.12242.
- Petrella, A., Mazzariol, S., Padalino, I., Di Francesco, G., Casalone, C., Grattarola, C., et al. (2021). Cetacean morbillivirus and *Toxoplasma gondii* co-infection in Mediterranean monk seal pup, Italy. *Emerging Infectious Diseases* 27(4), 1237–1239. doi: 10.3201/eid2704.204131.
- Pouille, M.L., Le Corre, M., Bastien, M., Gedda, E., Feare, C., Jaeger, A., et al. (2021). Exposure of pelagic seabirds to *Toxoplasma gondii* in the Western Indian Ocean points to an open sea dispersal of this terrestrial parasite. *PLoS One* 16(8), e0255664. doi: 10.1371/journal.pone.0255664.
- Ramos, P., Lynch, M., Hu, M., Arnould, J.P., Norman, R., and Beveridge, I. (2013). Morphometric and molecular characterization of the species of *Uncinaria* Frolich, 1789 (Nematoda) parasitic in the Australian fur seal *Arctocephalus pusillus doriferus* (Schreber), with notes on hookworms in three other pinniped hosts. *Systematic Parasitology* 85(1), 65–78. doi: 10.1007/s11230-013-9407-x.
- Robinson, S.J., Amlin, A., and Barbieri, M.M. (2023). Terrestrial pathogen pollutant, *Toxoplasma gondii*, threatens Hawaiian monk seals (*Neomonachus schauinslandi*) following heavy runoff events. *Journal of Wildlife Diseases* 59(1), 1–11. doi: 10.7589/JWD-D-21-00179.
- Roe, W.D., Rogers, L., Pinpimai, K., Dittmer, K., Marshall, J., and Chilvers, B.L. (2015). Septicaemia and meningitis caused by infection of New Zealand sea lion pups with a hypermucoviscous strain of *Klebsiella pneumoniae*. *Veterinary Microbiology* 176(3), 301–308. doi: <https://doi.org/10.1016/j.vetmic.2015.01.019>.
- Schaap, I., Buedenbender, L., Johann, S., Hollert, H., and Dogruer, G. (2023). Impact of chemical pollution on threatened marine mammals: a systematic review. *Journal of Hazardous Materials* 459, 132203. doi: 10.1016/j.jhazmat.2023.132203.

- Seguel, M., Montalva, F., Perez-Venegas, D., Gutierrez, J., Paves, H.J., Muller, A., et al. (2018a). Immune-mediated hookworm clearance and survival of a marine mammal decrease with warmer ocean temperatures. *Elife* 7, e38432. doi: 10.7554/eLife.38432.
- Seguel, M., Munoz, F., Navarrete, M.J., Paredes, E., Howerth, E., and Gottdenker, N. (2017). Hookworm infection in South American fur seal (*Arctocephalus australis*) pups. *Veterinary Pathology* 54(2), 288–297. doi: 10.1177/0300985816677151.
- Seguel, M., Munoz, F., Perez-Venegas, D., Muller, A., Paves, H., Howerth, E., and Gottdenker, N. (2018b). The life history strategy of a fur seal hookworm in relation to pathogenicity and host health status. *International Journal for Parasitology: Parasites and Wildlife* 7(3), 251–260. doi: 10.1016/j.ijppaw.2018.07.003.
- Seguel, M., Paredes, E., Paves, H., Molina, R., Henriquez, F., De Groote, F., and Schlatter, R. (2011). Pathological findings in South American fur seal pups (*Arctocephalus australis gracilis*) found dead at Guafo Island, Chile. *Journal of Comparative Pathology* 145(2–3), 308–317. doi: 10.1016/j.jcpa.2011.01.006.
- Seguel, M., Pavés, H., Paredes, E., and Schlatter, R. (2013). Causes of mortality in South American fur seal pups (*Arctophoca australis gracilis*) at Guafo Island, southern Chile (2004–2008). *Marine Mammal Science* 29(1), 36–47. doi: 10.1111/j.1748-7692.2011.00534.x.
- Seguel, M., Perez-Venegas, D., Gutierrez, J., Crocker, D.E., and DeRango, E.J. (2019). Parasitism elicits a stress response that allocates resources for immune function in South American fur seals (*Arctocephalus australis*). *Physiological and Biochemical Zoology* 92(3), 326–338. doi: 10.1086/702960.
- Shaughnessy, P.D., Dennis, T.E., and Seager, P.G. (2005). Status of Australian sea lions, *Neophoca cinerea*, and New Zealand fur seals, *Arctocephalus forsteri*, on Eyre Peninsula and the far west coast of South Australia. *Wildlife Research* 32(1), 85–101. doi: 10.1071/Wr03068.
- Shaughnessy, P.D., Goldsworthy, S.D., Burch, P., Dennis, T.E., and Cooper, P. (2013). Pup numbers of the Australian sea lion (*Neophoca cinerea*) at The Pages Islands, South Australia, over two decades. *Australian Journal of Zoology* 61(2), 112–118. doi: 10.1071/ZO13015.
- Shaughnessy, P.D., McIntosh, R.R., Goldsworthy, S.D., Dennis, T.E., and Berris, M. (2006). Trends in abundance of Australian sea lions, *Neophoca cinerea*, at Seal Bay, Kangaroo Island, South Australia. In A.W. Trites, S.K. Atkinson, D.P. DeMaster, L.W. Fritz, T.S. Gelatt, L.D. Rea, K.M.

- Wynne (Eds.), *Sea Lions of the World* (pp. 325–351). Fairbanks, USA: University of Alaska Fairbanks.
- Shepherd, S.A., and Robertson, E.L. (1989). Regional studies – seagrasses of South Australia, Western Victoria and Bass Strait. In A.W.D. Larkum, A.J. McComb and S.A. Shepherd (Eds.), *Biology of seagrasses: a treatise on the biology of seagrasses with special reference to the Australian region* (pp. 211–229). Amsterdam: Elsevier.
- Sinha, A. A., and Erickson, A. W. (1974). Ultrastructure of the placenta of Antarctic seals during the first third of pregnancy. *American Journal of Anatomy* 141(2), 263–279. doi: 10.1002/aja.1001410208
- Soto, K.H., Trites, A.W., and Arias-Schreiber, M. (2004). The effects of prey availability on pup mortality and the timing of birth of South American sea lions (*Otaria flavescens*) in Peru. *Journal of Zoology* 264(4), 419–428. doi: 10.1017/S0952836904005965.
- Spielman, D., Brook, B.W., and Frankham, R. (2004). Most species are not driven to extinction before genetic factors impact them. *Proceedings of the National Academy of Sciences of the United States of America* 101(42), 15261–15264. doi: 10.1073/pnas.0403809101.
- Spraker, T.R., DeLong, R.L., Lyons, E.T., and Melin, S.R. (2007). Hookworm enteritis with bacteremia in California sea lion pups on San Miguel Island *Journal of Wildlife Diseases* 43(2), 179–188. doi: 10.7589/0090-3558-43.2.179.
- Spraker, T.R., and Lander, M.E. (2010). Causes of mortality in northern fur seals (*Callorhinus ursinus*), St. Paul Island, Pribilof Islands, Alaska, 1986–2006. *Journal of Wildlife Diseases* 46(2), 450–473. doi: 10.7589/0090-3558-46.2.450.
- Stuart, J.L. (2018). The cruise of the Jane and Emma. In: Warneke, R.M. (Ed.), *Three colonial adventures, Part 3* (pp. 1–77). Melbourne: Renard.
- Taylor, M. A. (2016). Parasites of sheep and goats. In M. A. Taylor, R. L. Coop, and R. L. Wall (Eds.), *Veterinary Parasitology* 4th Ed (pp. 436–523). Chichester, UK: John Wiley and Sons, Ltd.
- Taylor, S., Terkildsen, M., McQuilty, R., Lee, D., Wing-Simpson, A., and Gray, R. (2022). Non-essential heavy metals and protective effects of selenium against mercury toxicity in endangered Australian sea lion (*Neophoca cinerea*) pups with hookworm disease. *Environment International* 169, 107521. doi: 10.1016/j.envint.2022.107521.

Taylor, S., Terkildsen, M., Stevenson, G., de Araujo, J., Yu, C., Yates, A., et al. (2021). Per and polyfluoroalkyl substances (PFAS) at high concentrations in neonatal Australian pinnipeds. *Science of The Total Environment* 786, 147446. doi: 10.1016/j.scitotenv.2021.147446.

Threatened Species Scientific Committee (2020). *Conservation advice. Neophoca cinerea, Australian sea lion*. Department of Agriculture, Water and the Environment, Canberra.
www.environment.gov.au/biodiversity/threatened/species/pubs/22-conservation-advice-23122020.pdf. [Accessed 29 August 2021].

Yesilay, G., Dos Santos, O.A.L., A, B.R., Hazeem, L.J., Backx, B.P., J, J.V., et al. (2023). Impact of pathogenic bacterial communities present in wastewater on aquatic organisms: application of nanomaterials for the removal of these pathogens. *Aquatic Toxicology* 261, 106620. doi: 10.1016/j.aquatox.2023.106620.

CHAPTER TWO

Topical ivermectin is a highly effective seal ‘spot-on’:
A randomised trial of hookworm and lice treatment in the
endangered Australian sea lion (*Neophoca cinerea*)



Dangerous Reef, Spencer Gulf, SA

Author attribution statement

Chapter two is published as:

Lindsay, S.A., Caraguel, C.G.B., and Gray, R. (2021). Topical ivermectin is a highly effective seal 'spot-on': a randomised trial of hookworm and lice treatment in the endangered Australian sea lion (*Neophoca cinerea*). *International Journal for Parasitology: Parasites and Wildlife* 16, 275–284. [doi: 10.1016/j.ijppaw.2021.11.002](https://doi.org/10.1016/j.ijppaw.2021.11.002).

Scott Lindsay was the primary author of this chapter and contributed to all aspects of this publication, including conceptualization, data curation, formal analysis (statistical), funding acquisition, investigation (fieldwork; laboratory), methodology, project administration, resources, validation, visualisation, and writing (original draft; review and editing). Charles Caraguel contributed to data curation, formal analysis (statistical), supervision, validation, visualisation, and writing (review and editing). Rachael Gray contributed to conceptualization, funding acquisition, investigation (fieldwork; laboratory), methodology, project administration, resources, supervision, validation, and writing (review and editing). All authors reviewed and approved the final manuscript.

Scott A. Lindsay

22 March 2024

Charles G.B. Caraguel

02 April 2024

Rachael Gray

26 March 2024



Topical ivermectin is a highly effective seal ‘spot-on’: A randomised trial of hookworm and lice treatment in the endangered Australian sea lion (*Neophoca cinerea*)

Scott A. Lindsay^{a,b,*}, Charles G.B. Caraguel^b, Rachael Gray^a

^a Sydney School of Veterinary Science, The University of Sydney, Camperdown, New South Wales, Australia

^b School of Animal and Veterinary Sciences, The University of Adelaide, Roseworthy, South Australia, Australia

ARTICLE INFO

Keywords:

Australian sea lion
Hookworm
Ivermectin
Lice
Neophoca cinerea
Uncinaria stenocephala

ABSTRACT

The Australian sea lion (*Neophoca cinerea*) is an endangered and declining otariid species, with a high rate of pup mortality associated with endemic hookworm (*Uncinaria stenocephala*) infection a suspected contributor to this decline. Injected ivermectin is an effective treatment for *Uncinaria* sp. in otariids, with optimal outcomes achieved by the early treatment of pups prior to disease development. This randomised controlled trial evaluated the effectiveness of the novel use of a topical ivermectin formulation against hookworm infection and lice (*Antarctophthirus microchiri*) infestation, in comparison with injected ivermectin. During the 2017 breeding season at Dangerous Reef, South Australia, pups ≤ 70 cm in standard length (≤ 2 weeks of age; $n = 85$) were randomised to single dose topical (500 $\mu\text{g/kg}$ spot-on; $n = 27$) or injected (200 $\mu\text{g/kg}$ subcutaneous; $n = 29$) ivermectin treatment groups, or to an untreated control group ($n = 29$). Topical ivermectin was highly effective for *U. stenocephala* elimination, and not significantly different to the injected formulation (estimated effectiveness 96.4% and 96.8%, respectively; $P > 0.05$). Its application resulted in an 81.6% reduction and 62.7% additional clearance for *A. microchiri* infestation by 15–24 days post-treatment, compared with untreated control pups (also not significantly different to injected ivermectin; 83.1% and 59.4%, respectively; $P > 0.05$). Treatment with either ivermectin formulation significantly ameliorated increases in inflammatory markers detected in the blood of untreated control pups – peripheral blood eosinophil counts (persisting to 36–41 days post-recruitment $P < 0.05$) and increased plasma protein concentrations (15–24 days post-recruitment; $P < 0.05$). Further, an initial short-term decrease in body condition in the control group was not observed in either of the treatment groups. This study demonstrates that topical ivermectin is an effective antiparasitic treatment in *N. cinerea*. It offers an alternative administration method for ivermectin delivery to a young pup cohort in this species, and an alternative, minimally invasive management tool for species conservation.

1. Introduction

The Australian sea lion's estimated population of 10,000 animals continues to decline, with the species having been listed as endangered by the International Union for Conservation of Nature since 2008 (Goldsworthy and Gales, 2008) and up-listed from threatened to endangered status under the Australian Government's *Environment Protection and Biodiversity Conservation Act* in 2020 (Threatened Species Scientific Committee, 2020). Contributors to this decline are multifactorial, with suggested anthropogenic factors that include fisheries interaction (by-catch, resource competition) (Hamer et al., 2013), marine debris entanglement (Byard and Machado, 2019; Page et al., 2004),

and pollution (such as human-source microbiota and chemical pollutants) (Fulham et al., 2018; Taylor et al., 2021), being additional to habitat degradation, climate alteration (Kovacs et al., 2012; Schumann et al., 2013) and disease (Lindsay and Gray, 2021; Marcus et al., 2014). Despite ongoing research, the role of known pathogens and their resultant disease in modulating population recovery remains an important knowledge gap for this species (DSEWPC, 2013; Threatened Species Scientific Committee, 2020).

Disease investigation is limited by the species' population structure – currently 80 temporally asynchronous breeding sites (Goldsworthy et al., 2021) dispersed across more than 3000 km of Australia's southern and southwestern coastline from the Houtman Abrolhos (Easter Island):

* Corresponding author. Sydney School of Veterinary Science, The University of Sydney, Camperdown, New South Wales, Australia.
E-mail address: scott.lindsay@adelaide.edu.au (S.A. Lindsay).

<https://doi.org/10.1016/j.ijppaw.2021.11.002>

Received 29 September 2021; Received in revised form 10 November 2021; Accepted 10 November 2021

Available online 23 November 2021

2213-2244/© 2021 The Authors. Published by Elsevier Ltd on behalf of Australian Society for Parasitology. This is an open access article under the CC BY-NC-ND

license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

28.468°S, 113.814°E) to The Pages Islands (35.756°S, 138.300°E) (Gales et al., 1994). Just four of these sites, including Dangerous Reef, Spencer Gulf, exceed 100 births each breeding season and contribute nearly 40% of the species total pup abundance, currently estimated at 2739 individuals (Goldsworthy, 2020; Goldsworthy et al., 2021). This represents a 64% reduction over three generations (Goldsworthy et al., 2021). Limited recruitment to the breeding population from fewer pup births is compounded by a high rate of preweaning pup mortality, which varies substantially by season and between breeding colonies (Goldsworthy et al., 2015). At the extreme of wide-ranging mortality rates, during the highest mortality seasons at each of the three largest breeding colonies pup deaths have reached 40–50%: The Pages Islands (55.6%, 1995–96) (Shaughnessy et al., 2013), Seal Bay (41.8%, 2011–12) (Goldsworthy et al., 2019) and Dangerous Reef (44.6%, 2002) (Goldsworthy et al., 2007). Gross observations have attributed pup death to trauma from conspecific animals (31.6%), emaciation (starvation) (10.4%) and stillbirth or prematurity (7.6%), with cause of death being undetermined in half of the cases (Higgins and Tedman, 1990; McIntosh and Kennedy, 2013). More recent investigations indicate that hookworm (*Uncinaria sanguinis*) infection could contribute to up to 40% of pup deaths (Gray, unpublished), and a direct correlation has been shown between increased hookworm infection intensity and higher seasonal colony pup mortality (Marcus et al., 2014).

Uncinaria sanguinis is a blood-feeding small intestinal nematode with demonstrated 100% prevalence in Australian sea lion pups at Dangerous Reef (Marcus et al., 2014). Pup infection occurs soon after birth by ingestion of infective third-stage larvae in colostrum, and patency is detectable from 11 days of age, with adult hookworms cleared from the intestine by 2–3 months of age (Marcus et al., 2014). Resultant disease ranges from mild, subclinical, and detectable antemortem by changes in blood parameters, to severe haemorrhagic enteritis and emaciation grossly apparent at necropsy (Marcus et al., 2015a). Adult hookworm feeding causes the direct intestinal loss of red blood cells and protein, as well as local intestinal inflammation that reduces intestinal absorptive capacity. Consequently, hookworm infection results in anaemia and hypoproteinaemia and elicits a systemic host inflammatory response – the latter reported as eosinophilic and lymphocytic in the Australian sea lion (Marcus et al., 2015a, b), with an additional neutrophilic component reported in South American fur seals infected with an *Uncinaria* species of hookworm (Seguel et al., 2019).

Ivermectin is a potent broad-spectrum anthelmintic available commercially in oral, topical (pour-on, spot-on) and injectable formulations (Papich, 2016). To date, its off-label use for the treatment of hookworm in free-ranging otariid (fur seal and sea lion) populations has utilised the injected formulation, commonly administered as a single 200 µg/kg subcutaneous dose, with effectiveness approaching or achieving 100% for elimination of adult hookworms from pups in the Northern fur seal (*Callorhinus ursinus*) (Beekman, 1984; DeLong et al., 2009), New Zealand sea lion (*Phocarctos hookeri*) (Castinel et al., 2007a; Chilvers et al., 2009; Michael et al., 2021b), Australian sea lion (Marcus et al., 2015b) and South American fur seal (*Arctocephalus australis*) (Seguel et al., 2019). Optimal treatment outcomes, including a survival benefit, have resulted from the early treatment of young pups in populations experiencing an increased seasonal pup mortality rate – either as a direct result of hookworm infection or from an unrelated comorbidity (Chilvers et al., 2009; DeLong et al., 2009; Lyons et al., 2001; Michael et al., 2021b). In the northern fur seal, treatment of endemic hookworm infection contributing to 50% pup mortality significantly reduced pup deaths, with a survival benefit attributed to the treatment of pups younger than 2 weeks of age (DeLong et al., 2009). In the New Zealand sea lion, early ivermectin treatment also significantly improved pup survival during an epizootic outbreak of *Klebsiella pneumoniae* septicemia (Chilvers et al., 2009) that caused a doubling of the mean seasonal pup mortality rate (Castinel et al., 2007b). The benefit occurred despite more than half of the pup deaths still being attributed to bacterial septicemia (Castinel et al., 2007b). The treatment benefit was not

apparent in the following low mortality seasons (Castinel et al., 2007a; Chilvers et al., 2009), but was again seen subsequent to the bacterial disease becoming endemic within the population (Michael et al., 2021b).

As well as demonstrated effectiveness approaching 100%, the first ivermectin hookworm treatment trial in the Australian sea lion (Dangerous Reef, 2011–13; injected ivermectin, 200 µg/kg sc) showed treatment-related haematological benefits – an increased absolute erythrocyte count and decreased eosinophil count (Marcus et al., 2015b). A lack of survival benefit was partly attributed to the practical limitations of early (<2 weeks old) pup recruitment in this species, including the prolonged 10-day post-natal period of maternal attendance with a male mate-guard (Higgins and Gass, 1993). Consequently, the present study planned for colony visits approximately 2 weeks apart to maximise the opportunity to detect and treat the younger pup cohort. Further, a topical formulation of ivermectin was evaluated as a minimally invasive hookworm treatment option for these pups. Ivermectin was selected in preference to newer generation macrocyclic lactones due to its demonstrated effectiveness and safety in multiple otariid species in the aforementioned trials, and the adequacy of a short-acting product for treating a parasite acquired at a single timepoint without the risk of pup reinfection. The infestation of Australian sea lion pups with sucking lice (*Antarctophthirus microchir*) occurs from conspecific animals at any pup age (McIntosh and Murray, 2007), has a limited pathological impact on the host and is associated with a mild anaemia and hyperproteinemia (Marcus et al., 2015a, b). Evaluation of topical ivermectin effectiveness against lice was included in the present study as an additional comparator with the injected formulation.

The primary objective of the current study was therefore to assess the effectiveness of the topical formulation of ivermectin in treating hookworm and lice parasitism in Australian sea lion pups less than 2 weeks of age. Equivalence to the injected formulation was hypothesised. Intrinsically, the availability of a minimally invasive formulation requiring limited pup handling or operator expertise for application, was anticipated as the next step in the development of a disease management tool for the Australian sea lion.

2. Materials and methods

All samples in this study were collected with approval of the Government of South Australia, Department of Environment and Water (Permit/Licence Number: MR00073-2-R; Scientific Research Permit Number: A25088-12) and the study protocol was approved by The University of Sydney Animal Ethics Committee (Protocol Number 2014/726). Trial reporting was guided by the recommendations in the CONSORT (Consolidated Standards of Reporting Trials) statement (Schulz et al., 2010).

2.1. Study site and population

This study was conducted at Dangerous Reef (34.817°S, 136.217°E), a small reef system of rocky substrate within the Sir Joseph Banks Group Conservation Park in Spencer Gulf, South Australia. The colony produces approximately 400 pups over a 7-month breeding season (Goldsworthy et al., 2007, 2021). Sample collection was undertaken at three colony visits of 4–6 days duration separated by approximately two weeks during the 2017 winter breeding season (19–22 July, 8–13 August and 27–30 August).

2.2. Study design and sample collection

A controlled trial allocated recruited pups to one of three experimental groups – ‘untreated control’, ‘topical ivermectin’, or ‘injected ivermectin’. Randomisation (blocked in groups of 30) was achieved by assigning a random number (Microsoft Excel for Mac 2016) against pup capture number, sorting those numbers by size and then assigning each

sequential group of 10 pups to one of the three trial groups. Treatment allocation was concealed from assessors until preliminary capture data were recorded, and for the entirety of pup recapture and sample processing. Topical ivermectin (IVOMEC® Pour-on for Cattle, 5 mg/ml, Boehringer Ingelheim Animal Health Australia Pty. Ltd., North Ryde NSW) was administered at 500 µg/kg to the skin surface by parting the pelage of the dorsal interscapular region. The injected ivermectin (IVOMEC® Antiparasitic Injection for Cattle, 10 mg/ml, Boehringer Ingelheim Animal Health Australia Pty. Ltd., North Ryde NSW) was administered at 200 µg/kg subcutaneously in the same region. Topical and injected ivermectin treatment of Australian sea lion pups represented off-label use, with due consideration given to potential risks. The topical ivermectin dose rate was chosen based on the generic recommendation for use of this formulation in domestic cattle (Vercruysse and Claerebout, 2014) as was the dose for the injected formulation, the latter dose rate having previously been shown to be effective in the Australian sea lion (Marcus et al., 2015b).

Protocols for pup health assessment, specimen collection and processing followed those of the earlier study at this site (Marcus et al., 2015b); summarised and differing details are as follows. Recruited pups were manually restrained within a purpose-made canvas bag to cover the head and minimise potential capture stress and injury. Initial capture state (sleeping, awake or mobile), sex and standard morphometrics – bodyweight (nearest 0.1 kg), standard length (linear distance between extremities of nose and tail (nearest 0.5 cm), moult status and subjective four-scale body condition assessment (poor, fair-thin, good, excellent) – were recorded. A subsequent physical examination determined the presence of macroscopic lesions such as hair loss, dermatitis, skin ulceration, wounds or swellings. Lice presence and subjective four-scale intensity assessment (0-none, 1-low, 2-medium or 3-high) were evaluated at three body locations – the ventral thorax, ventral abdomen and dorsal lumbosacral area – and later summed to provide a semi-quantitative measure of infestation intensity (lice intensity score; range: 0–9). A faecal sample was collected using a rayon-tipped dry swab (Copan Diagnostics, Murrieta, USA) introduced within a lubricated open-ended polyethylene sheath (modified 1–3 mL transfer pipette, Livingstone International, Sydney, Australia). A blood sample was collected from the brachial vein using a 21-gauge x 1-inch needle and 5 mL syringe and transferred to 1.3 mL EDTA-anticoagulated tubes (Sarstedt, Nümbrecht, Germany). When allocated, treatment was applied just prior to pup release. Respiration rate and effort were assessed at multiple timepoints during restraint and prior to release for signs of distress, and all pups were monitored from a distance for 1–2 min after release for signs of an immediate adverse event.

The inclusion criterion for the study was standard length ≤ 70.0 cm,

used as an age proxy for recruiting pups less than 2 weeks of age (McIntosh and Kennedy, 2013). Only pups likely to meet the size criterion based on remote visual assessment and whose mothers were absent were approached for capture. To enable resight and recapture of recruited pups a unique hair clip and bleach mark were applied to the dorsosacral pelage, permitting remote pup identification from approximately 20–30 m and which remained for the duration of the study (being lost at the first moult occurring 4–5 months of age).

Pups were recruited during the first and second colony visits and opportunistically recaptured during the second and third colony visits (Fig. 1). Consequently, two time periods were available for analysis – period 1 (P1): recruitment-to-first recapture; and period 2 (P2): first recapture-to-second recapture. Given that the low recruitment number and short follow-up period were unlikely to provide relevant survival data, recruited pups found dead were examined using standard gross necropsy technique (degree of decomposition permitting) to exclude treatment-related death, and for assessment of hookworm infection and lice infestation status. Resights of recruited pups unable to be recaptured were also noted.

2.3. Haematological analyses

Anticoagulated blood samples were stored at 4 °C prior to initial processing in a field laboratory within 10 h of collection. Following centrifugation at 13,700g for 120s (StatSpin MP, StatSpin Technologies, Norwood, USA) in microhaematocrit tubes (IRIS Sample Processing, Westwood, USA), the packed cell volume (PCV; L/L) was measured, and total plasma protein (TPP; g/L) was estimated by hand-held refractometer (Reichert TS Meter, Cambridge Instruments, Buffalo, USA). Comment was made of any degree (mild, moderate or marked) of sample haemolysis or lipaemia. Duplicate blood smears were prepared and fixed by immersion in 100% methanol (Chem-Supply Pty Ltd, Port Adelaide, South Australia) for 4 min. A 200 µL aliquot of EDTA anticoagulated blood was preserved with an equal volume of Streck Cell preservative (Streck, Omaha, USA) and stored at 4 °C.

Automated haematological analysis (Sysmex XT-2000iV, Sysmex, Kobe, Japan) at the Veterinary Pathology Diagnostic Service, Sydney School of Veterinary Science, The University of Sydney, was performed within 2–8 days of sample collection to determine the total erythrocyte count (RBC, $\times 10^{12}/L$), routine red cell parameters (haemoglobin [Hb, g/L]; haematocrit [HCT, %]; mean cell volume [MCV, fL]; and, mean cell haemoglobin concentration [MCHC, g/L]), platelet count (PLT, $\times 10^9/L$) and total nucleated cell count (TNCC, $\times 10^9/L$). Methanol-fixed duplicate blood smears were stained at the Veterinary Diagnostic Laboratory, School of Animal and Veterinary Sciences, The University of

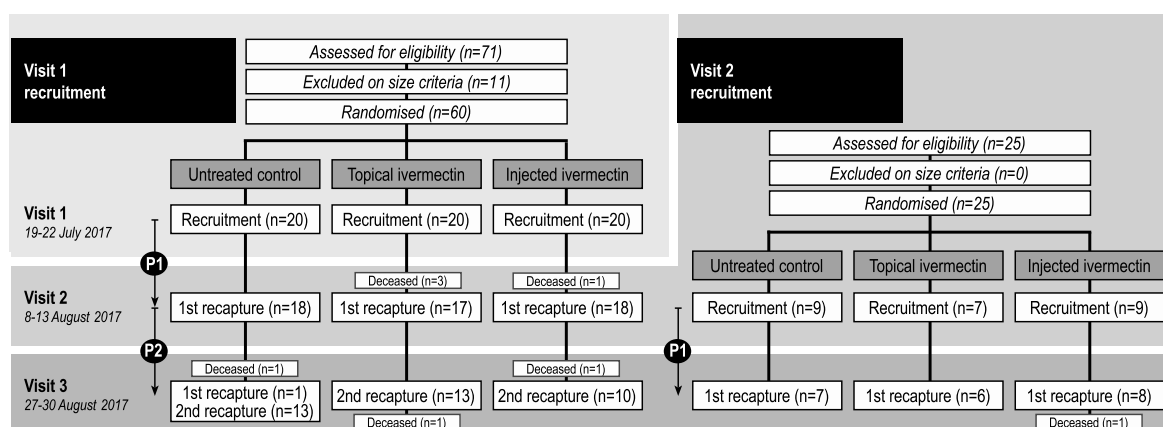


Fig. 1. Flow diagram of the trial course showing pup recruitment and recapture count for the three experimental groups for each of the three colony visits. P1 = time between recruitment and first recapture; P2 = time between first recapture and second recapture. Observation of deceased pups is shown relative to (i.e., before or after) the pup's sampling at that visit.

Adelaide using a Wrights-Giemsa protocol: 0.26% Wrights-Giemsa stain (Kinetik Pty Ltd, Narangba, Queensland) for 2 min; 1:5 ratio of 0.26% Wrights-Giemsa in Sorensen's buffer pH 6.8 (Fronine Pty Ltd, Riverstone, New South Wales) for 6 min; Sorensen's buffer rinse for 5 dips. Stained smears were reviewed (Leica N PLAN 100x/1.25/FN26.5 oil objective) to determine the differential leukocyte count (100 leukocytes were counted for every 10x10⁹/L TNCC) and nucleated red cell (nRBC) count per 100 WBC. The corrected leukocyte count (cWBC, x10⁹/L), and absolute neutrophil, lymphocyte, monocyte and eosinophil counts (x10⁹/L) were subsequently determined for each sample.

2.4. Hookworm infection assessment

Faecal swabs were stored at 4 °C in the field and –20 °C prior to processing within 14 days of collection. Faecal specimens were collected from all captured pups, both at recruitment and recapture, and from all necropsied pups. Patent hookworm infection (positive ≥ one ova) was detected by examining a faecal smear on a glass slide. A single drop of deionised water was used to liberate faecal material from dry swabs. Negative smears were repeated to confirm the absence of any parasite ova. Based on a previous reporting of 100% prevalence of hookworm infection in Australian sea lions at this colony (Marcus et al., 2015b) and the young age of recruited pups, a negative faecal smear at recruitment was interpreted as likely pre-patent infection.

2.5. Statistical analyses

All statistical analyses were performed using the software package Stata version 16.1 (StataCorp LLC., College Station, Texas, USA). Statistical test results were interpreted at the 5% level of significance (type-I-error or α). A Bonferroni correction was used to adjust for multiple comparisons between pairs of experimental groups.

2.5.1. Evaluation of successful random allocation

Distribution of morphometrics and health records at recruitment were compared across the three experimental groups to assess the success of the predetermined random allocation. Pairs of experimental group means were compared using simple linear regression for continuous variables. Frequencies across categorical variables were compared among experimental groups using the Fisher's Exact test.

2.5.2. Standard multifactorial modelling

A standard multifactorial model building process was used across this study unless described otherwise. Using fundamental biological understanding of the investigated parasitoses, a base additive model was set up as the foundation of the model construction including the experimental groups allocation as the primary investigation aim. Even if one or more factors of the base model was not significant all remained in the final model. Then a forward selection process was used to select additional factors, retaining only factors significantly associated to the outcome of interest at the 5% significance level. If some predictors were strongly collinear, the most meaningful factor was retained. Potential first-degree interactions were explored among all possible pairwise combinations of single predictors retained in the model.

2.5.3. Evaluation of treatment effectiveness against hookworm and lice

The therapeutic effectiveness of ivermectin on hookworm was assessed by comparing across experimental groups the (i) apparent prevalence of hookworm at first recapture and (ii) apparent incidence of hookworm clearance during P1 (between recruitment and first recapture). The base additive model for effectiveness against hookworm included by default the duration of the time period in days until first recapture, the pup's standard length at recruitment (proxy of age) and its experimental group allocation. The hookworm prevalence and the incidence of clearance at first recapture were modelled using the Firth's variant (Firth, 1993) of multifactorial logistic regression which is based

on a penalized maximum likelihood estimation to overcome the problem of 'separation' (i.e. covariate factors where all observations are the same, either all positive or all negative, perfectly predict the outcome and become inestimable using the conventional maximum likelihood estimation approach). The model construction followed the process described previously. Marginal estimates of hookworm frequencies (either prevalence or incidence) in each treatment group from the final models were used to calculate the treatment effectiveness as follow:

$$\text{Effectiveness} = \frac{\text{infection frequency}_{\text{Control}} - \text{infection frequency}_{\text{Treatment}}}{\text{infection frequency}_{\text{Control}}} \quad (\text{Eq.1})$$

The boundaries of the 95% CI for the effectiveness were calculated using the lower and upper limits of the 95% CI of the hookworm frequency estimates, respectively.

The effectiveness of ivermectin against lice was assessed by comparing across experimental groups the (i) apparent incidence of lice clearance during P1 in pups infested at recruitment (therapeutic effectiveness), (ii) apparent incidence of lice infestation during P1 and P2 in lice-free pups at recruitment or first recapture, respectively (persistent effectiveness), and (iii) apparent lice prevalence at first and second recapture in any pups (combined therapeutic and persistent effectiveness). All base additive models included the length of the time period in days until recapture, the pup's standard length at recruitment and its experimental group allocation. The base models for therapeutic and combined effectiveness also included the lice intensity score at recruitment. Conventional multifactorial logistic regressions were built following the described standard process. Effectiveness estimates and their 95% CIs were calculated as per those for hookworm (Eq. (1)).

2.5.4. Evaluation of treatment impact on blood analyte levels

The impact of treatment on pup haematological profiles was evaluated by comparing the means of each measured blood analyte across experimental groups at first and second recaptures. For each analyte, the base additive model included the time duration in days until recapture, the pup's standard length at recruitment and its experimental group allocation. Additional factors included sex, analyte measure at recruitment, presence of lice at recruitment and at recapture and presence of hookworm at recapture. All factors were fitted within a multifactorial regression model (full model) and a stepwise backward elimination process was utilised to remove ancillary factors not associated with the outcome or those highly collinear with another predictor to build the final model. A Box-Cox transformation analysis was conducted on each analyte's full model to identify potential transformation of the analyte values to suit assumptions of a linear regression. When the model outcome required transformation, model estimates and their 95% CI boundaries were back-transformed for reporting. Back-transformed means must be interpreted as medians.

2.5.5. Evaluation of treatment impact on growth

The impact of treatment on pup growth was evaluated on the changes in three morphometrics – body mass (kg), standard length (m) and body condition index (BCI). The BCI was calculated using the slope-adjusted ratio index approach (Jakob et al., 1996):

$$\text{Body condition index (BCI)} = \frac{\text{body mass (kg)}}{\text{standard length (m)}^{\text{slope}}} \quad (\text{Eq.2})$$

where the slope is the least-squares estimates from the linear regressing of ln (body mass) against ln (standard length), and ln is the natural log of the data.

To be comparable, the bodyweight, standard length and BCI growth were compared using a daily specific growth rate (SGR) calculated at each study period (P1, P2 and P1+P2) as follows:

$$\text{Specific growth rate (SGR)}_{\text{daily}} = \frac{\ln(\text{morphometric}_{t+1}) - \ln(\text{morphometric}_t)}{\text{time}_{t+1}(\text{day}) - \text{time}_t(\text{day})} \quad (\text{Eq.3})$$

Estimates of SGRs were compared across experimental groups using multifactorial linear regressions. The base additive model for the morphometric SGRs included by default the length of the time period in days, the pup's absolute morphometric measurement at the start of the period (standard length used for BCI) and its experimental group allocation.

3. Results

3.1. Recruitment and success of random allocation

There was no evidence of difference in demographic or health distribution across the three experimental groups, supporting a successful random group allocation (Bonferroni adjusted P -values > 0.05 for Fisher's exact test; similar adjustment applied to P -values for all subsequently reported pair-wise comparisons) (Supplementary Table 1). A total of 96 pups were captured and assessed for eligibility during the first and second visits (71 pups and 25 pups, respectively). Eleven of the pups from the first visit were not recruited as they did not meet the inclusion criterion (≤ 70 cm in standard length). Variations from the inclusion criterion were exclusion of two pups (69 cm and 70 cm and weighing 10.0 kg) in visit 1 and inclusion of a one pup (71 cm and weighing 9.8 kg) in visit 2, due to an early intension to minimise cohort age and a later need to balance cohort numbers, respectively. Overall, 85 pups were recruited and randomly allocated to the untreated control ($n = 29$), topical ivermectin ($n = 27$) and injected ivermectin ($n = 29$) experimental groups (Fig. 1). One pup allocated to the topical ivermectin group was excluded from the treatment impact analysis because it was diagnosed with severe anaemia, considered to be unrelated to hookworm disease, which deteriorated during the trial, and it died shortly after the second recapture. A second pup with severe anaemia allocated to the injected ivermectin group was excluded from treatment impact analysis due to the lack of a second capture event.

Of the 85 pups, $n = 75$ (88.2%) were recaptured once 15–24 days after recruitment (excepting a single pup recruited at visit 1 and recaptured 39 days later at visit 3). Of the $n = 53$ pups recruited during visit 1 and recaptured during visit 2, 36 (67.9%) were recaptured a second time during visit 3, 36–41 days after recruitment. Excluding animals found dead, the recapture rates for first and second recaptures were 91.4% and 70.6%, respectively.

3.2. Treatment effectiveness against hookworm and lice

Compared to the control group, both ivermectin treatment groups demonstrated a similar significant reduction in hookworm prevalence at first recapture (topical 96.4%; injected 96.8%; $P < 0.05$) and additional hookworm clearance during P1 (topical 94.2%; injected 95.5%; $P < 0.05$) (Table 1). There was no significant difference in effectiveness between the two ivermectin treatment groups ($P > 0.05$) for either outcome. None of the factors ancillary to the base additive models (visit, sex, bodyweight or BCI) were associated with the therapy effectiveness against hookworm. Standard length at recruitment (OR = 2.80, $P < 0.001$), P1 duration (OR = 33.0, $P < 0.001$) and their interaction (OR = 0.95, $P < 0.001$) were significantly associated with hookworm prevalence at recapture. However, neither factor explained the incidence of hookworm clearance during P1 ($P = 0.795$, $P = 0.639$, respectively).

Irrespective of infection status at recruitment, patent hookworm infection was not detected in any pup in either of the ivermectin treatment groups at first or second recapture. In the untreated control group, 17/21 (81.0%) of pups with patent hookworm infection at recruitment had patent hookworm infection at first recapture, and 4/5 (80%) of pups without patent hookworm infection at recruitment

Table 1
The estimated prevalence and incidence of hookworm infection and lice infestation at recapture (with 95% confidence interval) in Australian sea lion (*N. cinerea*) pups, and the adjusted effectiveness of topical and injected ivermectin treatments.

Condition of interest	Incidence or prevalence estimate (95%CI)			Treatment effectiveness estimate (95%CI)		
	n	Control		Topical ivermectin	Injected ivermectin	
Hookworm						
Prevalence at first recapture	25	82.7% (69.2%–96.2%)	22	2.9% ^A (0.0%–10.4%)	26	2.6% ^A (0.0%–9.2%)
Incidence of clearance during P1	20	19.5% (2.4%–36.5%)	15	95.3% ^A (83.4%–100.0%)	17	96.4% ^A (86.9%–100.0%)
Lice						
Prevalence at first recapture	25	71.8% (54.5%–89.1%)	22	22.0% ^A (5.2%–37.3%)	26	24.3% ^A (8.0%–40.5%)
Prevalence at second recapture	13	83.8% ^A (56.5%–100.0%)	12	43.1% ^{A,B} (16.0%–70.3%)	10	30.5% ^B (5.4%–55.7%)
Incidence of clearance during P1	14	23.3% (0.1%–46.4%)	14	71.4% ^A (47.7%–95.0%)	17	68.8% ^A (46.0%–91.7%)
Incidence of infestation during P1	11	64.6% (36.9%–92.3%)	8	11.9% ^A (0.0%–34.3%)	9	10.9% ^B (0.0%–31.3%)
Incidence of infestation during P2	4	74.7% ^A (32.1%–100.0%)	10	34.5% ^{A,B} (6.6%–62.4%)	7	11.7% ^B (0.0%–33.8%)

^{A,B}For incidence and prevalence estimates, experimental groups that share the same superscripted letter within the same parameter row are not significantly different at the 5% level after accounting for multiple pairwise comparisons using the Bonferroni adjustment. The incidence of clearance of patent hookworm infection or detectable lice infestation in pups positive at recruitment reflects the therapeutic effectiveness. The incidence of lice infestation in pups without detectable lice infestation at recruitment or first recapture reflects the persistent effectiveness during P1 and P2, respectively. The adjusted effectiveness of each treatment is calculated using the relative risk reduction of each treatment compared with the control group.

developed patent hookworm infection by first recapture.

Both ivermectin treatment groups achieved a significant reduction in lice prevalence at first recapture (topical 69.4%; injected 66.2%) compared to the control group, resulting from a reduction in lice infestation (topical 81.6%; injected 83.3%) and additional lice clearance during P1 (topical 62.7%; injected 59.4%; all $P < 0.05$). There was no significant difference between the two treatment groups ($P > 0.05$) (Table 1). The impact of the treatment on lice during P2 could only be investigated in a subset of the pups recaptured a second time ($n = 35$). While the sample size was too small for sufficient evidence to differentiate between the topical ivermectin treatment and untreated control groups ($P > 0.05$), longer-term effectiveness in the injected ivermectin treatment group during the second period ($P < 0.05$) was seen. Injected ivermectin reduced the lice prevalence at second recapture by 63.5% and the lice infestation during P2 by 84.3%, compared to control pups. There was no significant difference between the two ivermectin treatment groups' longer-term (P2) effectiveness ($P > 0.05$). None of the potential ancillary factors or the factors included in the base additive models were associated with the treatment effectiveness against lice, regardless of outcome or time period.

3.3. Treatment impact on blood analyte levels

A comparison of the model predictions for haematological parameters for each experimental group at first and second recapture are reported in Table 2. Both topical and injected ivermectin groups showed significantly lower total plasma protein concentration compared with the untreated control group at first recapture but not at the second recapture (topical -13.1 g/L, 95%CI: -18.9 – 7.4 and injected -14.8 g/L, 95%CI: -20.5 – 9.2). The total plasma protein concentration at first recapture was also associated with the presence of lice at recruitment ($+5.2$ g/L, 95%CI: 0.9 – 9.5) and hookworm at first recapture (-16.7 g/L, 95%CI: -26.6 – 6.9); however, these effects were no longer evident at second recapture. None of the other base additive model factors were significantly associated with total plasma protein concentration (Supplementary Table 2).

Topical and injected ivermectin treatment groups showed significantly lower absolute eosinophil counts compared with the untreated control group at both the first (topical -0.73×10^9 /L, 95%CI: -0.93 – 0.51 and injected -0.77×10^9 /L, 95%CI: -1.10 – 0.51) and second recaptures (topical -1.05×10^9 /L, 95%CI: -1.93 – 0.50 and injected -1.09×10^9 /L, 95%CI: -2.03 – 0.51) (Table 2), and none of the base additive model factors were significant (Supplementary Table 2).

The injected ivermectin group showed significantly higher red cell parameters (PCV, RBC, Hb and HCT) compared with the control group at the first but not the second recapture (e.g., PCV $+0.052$ L/L, 95%CI: 0.020 – 0.084) (Table 2) and none of the base additive model factors were significant (Supplementary Table 2). While the topical ivermectin group also showed higher red cell parameters compared with the control group at first recapture, the difference between the groups was not significant ($P > 0.05$). Likewise, there was no significant difference in red cell parameters between either of the two ivermectin treatment groups themselves at first recapture. There were no significant differences in red cell parameters between any of the experimental groups by second recapture.

3.4. Treatment impact on growth

Using study pup measurements for BCI calculation, the slope between bodyweight and standard length on the logarithm scale was estimated at approximately 2.5 (slope = 2.52, 95%CI: 2.28–2.76). Regardless of group allocation, pup standard length increased faster during P1 (0.300% per day) than during P2 (0.230% per day), while pup weight increased faster during P2 (0.676% per day) than during P1 (0.572% per day) translating into a relative loss of BCI during P1 (-0.163 per day) and a relative gain during P2 (0.125% per day)

Table 2

The predicted mean or median values (with 95% confidence interval) of haematological parameters of Australian sea lion (*N. cinerea*) pups at first and second recapture.

		Predicted means or medians ^a (95% CI)		
Parameters	n	Control	Topical ivermectin	Injected ivermectin
At first recapture				
Field blood parameters				
PCV (L/L)	58	0.307 ^A (0.288–0.324)	0.332 ^{A,B} (0.313–0.349)	0.359 ^B (0.344–0.372)
Total plasma protein (g/L)	65	85.2 (82.2–88.3)	72.1 ^A (68.2–75.9)	70.4 ^A (66.7–74.0)
Laboratory blood parameters				
RBC (x10 ¹² /L)	48	3.93 ^A (3.71–4.14)	4.03 ^{A,B} (3.79–4.27)	4.33 ^B (4.12–4.54)
Haemoglobin (g/L)	48	111.9 ^A (105.2–118.5)	115.2 ^{A,B} (107.8–122.6)	127.2 ^B (120.6–133.7)
Haematocrit (L/L) ^b	48	0.348 ^A (0.327–0.368)	0.359 ^{A,B} (0.336–0.380)	0.391 ^B (0.373–0.409)
MCV (fL)	48	86.4 ^A (84.2–88.5)	88.3 ^A (85.9–90.7)	89.4 ^A (87.3–91.6)
MCHC (g/L)	48	324.0 ^A (318.3–329.7)	327.5 ^A (321.2–333.8)	327.1 ^A (321.4–332.8)
Platelets (10 ⁹ /L)	59	336.0 ^A (278.2–393.7)	289.4 ^A (227.0–352.0)	297.4 ^A (242.6–352.1)
WBC (10 ⁹ /L)	59	17.9 ^A (14.4–21.9)	13.6 ^A (10.3–17.2)	15.6 ^A (12.4–19.1)
Neutrophils (10 ⁹ /L)	60	11.6 ^A (8.8–14.8)	9.0 ^A (6.4–12.0)	10.3 ^A (7.8–13.2)
Lymphocytes (10 ⁹ /L)	60	4.0 ^A (3.2–4.8)	2.9 ^A (2.2–3.7)	3.7 ^A (3.0–4.5)
Monocytes (10 ⁹ /L)	60	1.1 ^A (0.8–1.4)	1.0 ^A (0.7–1.3)	1.2 ^A (0.9–1.4)
Eosinophils (10 ⁹ /L)	60	0.77 (0.51–1.12)	0.04 ^A (0.00–0.19)	0.00 ^A (0.00–0.02)
nRBC/100WBC ^b	60	0.09 ^A (0.00–1.14)	0.00 ^A (0.00–0.02)	0.02 ^A (0.00–0.24)
At second recapture				
Field blood parameters				
PCV (L/L)	31	0.306 ^A (0.280–0.333)	0.329 ^A (0.297–0.361)	0.328 ^A (0.289–0.366)
Total plasma protein (g/L)	35	75.5 ^A (72.1–78.8)	76.1 ^A (72.5–79.6)	76.4 ^A (72.2–80.7)
Laboratory blood parameters				
RBC (x10 ¹² /L)	26	4.10 ^A (3.66–4.53)	4.18 ^A (3.74–4.61)	4.23 ^A (3.65–4.80)
Haemoglobin (g/L)	26	112.8 ^A (98.3–127.3)	113.6 ^A (98.6–128.6)	119.0 ^A (100.2–137.7)
Haematocrit (L/L)	26	0.332 ^A (0.290–0.374)	0.341 ^A (0.298–0.385)	0.352 ^A (0.298–0.407)
MCV (fL)	26	79.4 ^A (76.3–82.5)	82.6 ^A (79.5–85.8)	81.4 ^A (77.5–85.3)
MCHC (g/L)	33	340.7 ^A (333.2–348.2)	333.6 ^A (326.8–340.4)	329.1 ^A (321.4–336.8)
Platelets (10 ⁹ /L)	33	357.8 ^A (282.6–433.1)	324.9 ^A (244.6–405.1)	351.4 ^A (260.0–442.8)
WBC (10 ⁹ /L)	33	17.2 ^A (13.9–21.3)	15.6 ^A (12.4–19.5)	16.8 ^A (12.9–21.7)
Neutrophils (10 ⁹ /L)	34	11.0 ^A (8.2–13.8)	10.1 ^A (7.2–12.9)	10.4 ^A (7.1–13.8)
Lymphocytes (10 ⁹ /L)	26	4.56 ^A (3.31–6.30)	4.37 ^A (3.15–6.06)	4.7 ^A (3.10–7.12)
Monocytes (10 ⁹ /L) ^b	33	0.871 ^A (0.681–1.114)	0.915 ^A (0.704–1.190)	1.063 ^A (0.788–1.433)
Eosinophils (10 ⁹ /L)	34	1.12 (0.51–2.18)	0.07 ^A (0.01–0.25)	0.03 ^A (0.00–0.15)
nRBC/100WBC ^b	34	0.002 ^A (0.000–1.129)	0.000 ^A (0.000–0.004)	0.000 ^A (0.000–0.044)

RBC: total red blood cell count; MCV: mean cell volume; MCHC: mean cell haemoglobin concentration; WBC: total leukocyte count, corrected for presence of nucleated red blood cells (nRBC).

^{A,B}Experimental groups that share the same superscripted letter within the same parameter row are not significantly different at the 5% level after accounting for multiple pairwise comparisons using the Bonferroni adjustment. Manual PCV

was included in addition to haematocrit to overcome any impact on the latter from the delay to processing.

^a Median reported for back-transformed model estimates.

^b Sub-optimal compliance with linear regression assumptions.

Table 3

The predicted mean or median values (with 95% confidence interval) of daily specific growth rate (SGR) for the bodyweight, standard length and body condition index in Australian sea lion (*N. cinerea*) pups during the first and second treatment trial period.

		Predicted means or medians ^a (95% CI)		
Parameters	n	Control	Topical ivermectin	Injected ivermectin
Recruitment-to-first recapture (P1)				
Bodyweight	73	0.342 ^A	0.717 ^A	0.671 ^A
daily SGR (%)		(0.112–0.573)	(0.472–0.961)	(0.446–0.897)
Standard length	73	0.281 ^A	0.338 ^A	0.307 ^A
daily SGR (%)		(0.229–0.334)	(0.283–0.394)	(0.256–0.358)
Body condition	73	−0.357 ^A	−0.090 ^A	−0.039 ^A
index daily SGR (%)		(−0.573–0.141)	(−0.318–0.138)	(−0.250–0.172)
First recapture-to-second recapture (P2)				
Bodyweight	35	0.619 ^A	0.852 ^A	0.538 ^A
daily SGR (%)		(0.316–0.923)	(0.536–1.167)	(0.199–0.878)
Standard length	35	0.197 ^A	0.286 ^A	0.207 ^A
daily SGR (%)		(0.123–0.271)	(0.209–0.363)	(0.123–0.292)
Body condition	35	0.127 ^A (−0.160–	0.132 ^A	0.113 ^A
index daily SGR (%)		0.415)	(−0.168–0.433)	(−0.217–0.442)

^AExperimental groups that share the same superscripted letter within the same parameter row are not significantly different at the 5% level after accounting for multiple pairwise comparisons using the Bonferroni adjustment.

^a Median reported for back-transformed model estimates.

(Table 3). A comparison of the model predictions for bodyweight, standard length and BCI SGR in each experimental group during P1 and P2 is reported in [Supplementary Table 3](#). During P1, the bodyweight SGR of treated pups (topical and injected) was approximately twice that of the control pups while standard length SGR was equivalent across the three experimental groups. This resulted in a decreased BCI in the control pups but not in treated pups (BCI SGR estimate not significantly different from zero). However, after Bonferroni correction for multiple pairwise comparison, the observed evidence was not sufficiently strong to discriminate between groups ($P > 0.05$).

Within the final bodyweight SGR models, the duration in days of the period (+0.088% per day, 95%CI: 0.025–0.150) and the presence of hookworm at recruitment (+0.338% per day, 95%CI: 0.030–0.646) impacted the bodyweight SGR during P1; while only bodyweight at first recapture (start of P2) was associated with the bodyweight SGR during P2 (−0.135% per day, 95%CI: −0.242–0.028) ([Supplementary Table 3](#)). Standard length at recruitment (−0.058% per day, 95%CI: −0.071–0.044), bodyweight at recruitment (+0.098% per day, 95%CI: 0.065–0.013) and male sex (+0.101% per day, 95%CI: 0.065–0.013) influenced standard length SGR during P1; bodyweight at the first recapture was marginally associated (+0.040% per day, 95%CI: 0.000–0.080) with standard length SGR during P2. Duration in days of the period (+0.084% per day, 95%CI: 0.026–0.143), standard length at recruitment (+0.137% per day, 95%CI: 0.082–0.193), bodyweight at recruitment (−0.325% per day, 95%CI: −0.461–0.189) and the presence of hookworm at recruitment (+0.353% per day, 95%CI: 0.063–0.643) impacted the BCI SGR during P1; both standard length (+0.100% per day, 95%CI: 0.004–0.192) and bodyweight at the first recapture (−0.371% per day, 95%CI: −0.504–0.188) impacted the standard length SGR during P2.

3.5. Monitoring of pup survival

Of the 85 pups recruited, eight were found dead (9.4%: control $n = 1$; topical $n = 4$; injected $n = 3$) during 14 visit days across the 42-day study period, with seven cadavers suitable for necropsy. Based on gross examination, the cause of death was determined to be conspecific trauma in three pups (topical $n = 2$; injected $n = 1$), and starvation with conspecific trauma in two pups (control $n = 1$; injected $n = 1$). The cause of death was undetermined in one injected ivermectin treatment pup. The cause of death during visit three of the topical ivermectin pup excluded from treatment impact analysis was progression of the previously detected severe anaemia. This pup's health status was monitored during all three visits – the cause of the anaemia was undetermined based on gross necropsy review (and additional microscopic tissue specimen review). None of the pups necropsied from any experimental group were determined to have died from hookworm infection, based on the absence of haemorrhagic enteric pathology. At necropsy, all treated pups had a negative faecal hookworm smear, while the untreated control pup had a positive faecal hookworm smear. In addition to these known deaths, four pups (control $n = 1$; topical $n = 2$; injected $n = 1$) were not resighted at subsequent visits.

4. Discussion

This study reports the first use of topical ivermectin treatment for hookworm infection and lice infestation in a free ranging otariid population and found a high level of effectiveness not significantly different to that of the injected ivermectin formulation. There were no treatment related deaths detected with either topical or injected ivermectin administration. The Dangerous Reef colony was an appropriate site for this study, based on adequate pup recruitment and a short-term recapture rate providing statistically significant effectiveness outcomes. Inclusion of two recapture events 15–24 days and 36–41 days post-recruitment provided further short-term temporal detail to the earlier study observations in this species, which had showed an absence of a growth benefit, a trend for decreased total plasma protein concentration and a significant reduction in eosinophil count at 27–67 post-treatment ([Marcus et al., 2015b](#)). Treatment with either formulation in the present study ameliorated a decrease in body condition (based on BCI) and elevation of plasma protein present in untreated pups present 15–24 days post-recruitment, while a beneficial reduction in a systemic eosinophilic inflammatory response persisted to 36–41 days post-recruitment in line with ongoing patent hookworm infection in untreated animals. The topical formulation provided an alternative method of ivermectin administration that was minimally invasive with little requirement for operator expertise. In the field these benefits could translate to a reduction in the restraint time necessary to treat a pup and offer an additional management tool for the early elimination of endemic hookworm infection in Australian sea lion pups.

4.1. Treatment effectiveness against hookworm and lice

In the present study, estimated hookworm prevalence at 15–24 days post-treatment with topical ivermectin treatment (2.9%) did not differ significantly to that of the injected formulation (2.6%), and both were comparable to that reported 27–67 days after injected ivermectin treatment (2.4%) in the previous study at this colony ([Marcus et al., 2015b](#)). Clearance of hookworm within this shorter time period and the absence of re-infection post-treatment were anticipated based on the exclusive lactogenic transmission (in colostrum) of hookworm in otariid species ([Lyons et al., 2011](#); [Olsen and Lyons, 1965](#)), and demonstrated clearance of adult worms from the intestine by 16 h ([DeLong et al., 2009](#)) and 47 h ([Marcus et al., 2015b](#)) after injected ivermectin treatment ([DeLong et al., 2009](#); [Marcus et al., 2015b](#)). The absence of re-infection following elimination of infection was one justification for the use of an older generation, shorter-acting anthelmintic of known

safety profile in the current study. The assumption of prepatent infection in pups with a negative faecal smear at recruitment in our young cohort was supported by 80% ($n = 5$) of such animals in the untreated control group subsequently displaying patent hookworm infection at first recapture. This finding combined with that of 81.0% ($n = 21$) of pups in the untreated control group with patent hookworm infection at recruitment maintaining that status at first recapture, suggested minimal transference of topical ivermectin treatment from conspecific animals.

As an additional comparator against the injected formulation, inclusion of effectiveness against lice achieved the objective of showing no significant differences between the formulations for prevalence, incidence of clearance or incidence of infestation at either of the measured time points. Given possible reinfestation at any age, the lower incidence of infestation during period 2 (from 15–24 days to 36–41 days post-recruitment) for the treatment groups compared with the untreated control group (significance reached only for the injected group) indicated some persistent activity during this period. This complements findings from the earlier site study of injected ivermectin, which showed the persistent treatment benefit for lice prevalence was no longer apparent by 60 days post-treatment (Marcus et al., 2015b).

4.2. Treatment impact on haematological parameters

In the present study hookworm elimination contributed to increases in measures of red cell quantity (PCV, RBC, Hb, HCT) by 15–24 days post treatment (reaching significance only for the injected group), a benefit that did not persist to 36–41 days post-treatment. This short-term benefit with elimination of a *U. sanguinis* explains the absence of a similar treatment-associated difference for PCV in the earlier site study (recaptures 27–67 days post-recruitment), although in that study a significantly higher RBC count was still apparent with treatment (Marcus et al., 2015b). A similar treatment benefit has been documented in the South American fur seal, with higher haemoglobin levels at 3–7 weeks of age compared with hookworm-infected pups (Seguel et al., 2019). Any treatment-associated increase in red cell parameters is superimposed on the rapid physiological decline reported in the first weeks of life in otariid pups, which must be considered when contemplating treatment-related temporal changes during this post-natal period (Michael et al., 2021a; Seguel et al., 2019; Trillmich et al., 2008).

A significantly lower peripheral eosinophil cell count in ivermectin-treated Australian sea lion pups persisting to the second recapture 36–41 days post-treatment is consistent with previous reports in this and other otariid species (Marcus et al., 2015b; Michael et al., 2021a; Montalva et al., 2019; Seguel et al., 2019), and indicates the elimination of parasite-associated eosinophilic tissue inflammation.

Both topical and injected treatment resulted in a significantly lower total plasma protein at 15–24 days post-treatment, a short-term difference that did not persist to 36–41 days post-treatment. This result is contrary to that expected with elimination of a blood-feeding intestinal parasite and with observations in ivermectin-treated New Zealand sea lions (Michael et al., 2021a). A short-term protein elevation in untreated animals resulting from positive acute phase inflammatory response to parasitism (supported by the eosinophilic inflammatory response) and temporal differences in sampling could explain this finding. Significantly higher globulin levels have been reported in South American fur seal pups compared with adult animals, albeit in pups not showing patent hookworm infection, while albumin levels did not differ significantly to those in adult animals (Seguel et al. 2016). Further investigation (serial serum protein analysis) is necessary to further define the cause of the protein elevation observed short-term in untreated Australian sea lions.

4.3. Treatment impact on growth

Although between group differences did not reach significance, pups

treated with either of the ivermectin formulations showed bodyweight specific growth rates approximately double that of the control pups for the period to 15–24 days post-recruitment. This benefit in the treated pup groups ameliorated the decrease in body condition (based on BCI) observed in the untreated control group during this period. The absence of a similar benefit to second recapture at 36–41 days post-treatment indicates that any growth rate benefit from hookworm elimination is short-term in the Australian sea lion, similar to the finding in the previous study at this site (Marcus et al., 2015b). In other otariid species ivermectin hookworm treatment has contributed to significant growth rate improvements measured over various time periods, including in northern fur seal pups (absolute growth rate over 2.5 months) (DeLong et al., 2009), in New Zealand sea lion pups (absolute and relative growth rates over 10–93 days [mean 68 days]) (Chilvers et al., 2009), and in South American fur seal (*Arctocephalus australis*) pups (absolute growth rate over >20 days versus a severely infected pup group) (Montalva et al., 2019). Varying parasite impact on host (parasite species, hookworm infection intensity), differences in methodology for measurement of growth rate and time to resampling may explain these differences (Chilvers et al., 2009; DeLong et al., 2009). In humans, suboptimal nutrition, growth and stunting during gestation and the first 24 months of a child's life are associated with irreversible, life-long detrimental health impacts, including reduced stature (height, body-mass index), decreased learning ability, increased susceptibility to infection and chronic disease, greater mortality risk and reduced off-spring birth-weight (Black et al., 2013; Victora et al., 2008). Not surprisingly, early interventions during this critical period of development to remove disease negatively impacting growth (including hookworm parasitism) (Mofid et al., 2017) have a substantial impact on health outcomes over a life time (Victora et al., 2008). Longer term follow up is necessary to determine if short-term growth rate benefits in ivermectin-treated Australian sea lion pups also translate into the desired long-term goals of improved health and survival benefits.

4.4. Monitoring of pup survival

There were no pup deaths in either of the treatment groups attributed to the treatment interventions. Additionally, the mortality rate for the recruited cohort did not exceed that expected for this population during a higher mortality winter (versus summer) breeding season, during which the incidence of pup mortality averages 37% (observed range 30–45% for the 1996–2005 seasons) (Goldsworthy et al., 2007). Conspecific trauma was a contributing factor in all five pup deaths for which a grossly detectable cause of death was apparent. Dangerous Reef is a high density colony of Australian sea lions (Marcus et al., 2014), a likely contributor to this finding. These results are consistent with recent reporting from the largest controlled trial of injected ivermectin in a free-ranging otariid population (New Zealand sea lions), in which there were no reports of treatment-related adverse events in any pup treated across two breeding seasons (Michael et al., 2021b). Nonetheless, any risk posed by anthelmintic use in otariids requires ongoing review and monitoring, and the small sample size in this study limits recommendations for the more widespread or routine use of topical ivermectin in any of these species.

The present study was not powered or intended to detect a treatment-associated survival benefit. However, this remains the primary objective of any treatment intervention in the Australian sea lion – improved pup survival and a subsequent increase in recruitment to the breeding population necessary to arrest the current declining population trend. Rather, the validation of a minimally invasive disease management tool was intended as a first step to the treatment of a young cohort of Australian sea lion pups, particularly for times when strategic use could counter additional stressors decreasing their survival and that of this endangered species generally (Chilvers, 2015; DeLong et al., 2009; Lyons et al., 2001; Michael et al., 2021b).

To this end, this study confirmed topical ivermectin to be a highly

effective anthelmintic for treatment of endemic hookworm disease in the Australian sea lion, comparable to the injected formulation. The next step in understanding hookworm disease impact on modulating population growth will require a longitudinal study conducted across low and high mortality breeding seasons, at a site free of the logistical constraints of Dangerous Reef. These pertain to easier colony access to increase resighting and mortality investigations, and long-term animal identification (not currently implemented at Dangerous Reef) to permit the collection of resight-recapture data for long-term survival investigations.

Declaration of competing interest

All authors declare that there are no conflicts of interest.

Acknowledgements

This study was funded by The Hermon Slade Foundation (HSF 16-03), and by the FS Quiney and BR Richards bequests (Sydney School of Veterinary Science, The University of Sydney: 2016). The authors thank additional members of the fieldwork team, Shannon Taylor, Mariel Fulham and Matthew Gray, for assistance with sample collection and processing. We greatly appreciate the marine charter and logistics provided by Adam Kemp and associates (Seatec Marine Services Ltd, Port Lincoln, South Australia) and additional logistical assistance provided by Dirk Holman (National Parks and Wildlife Service Division, Department for Environment and Water, Port Lincoln). We also thank Christine Black (Veterinary Pathology Diagnostic Service, The University of Sydney) for provision of laboratory technical services, Michael Terkildsen for his detailed preliminary statistical analysis of the study results, and Jan Slapeta and Damien Higgins from the Sydney School of Veterinary Science, The University of Sydney, for supervisory input.

Appendix A. Supplementary data

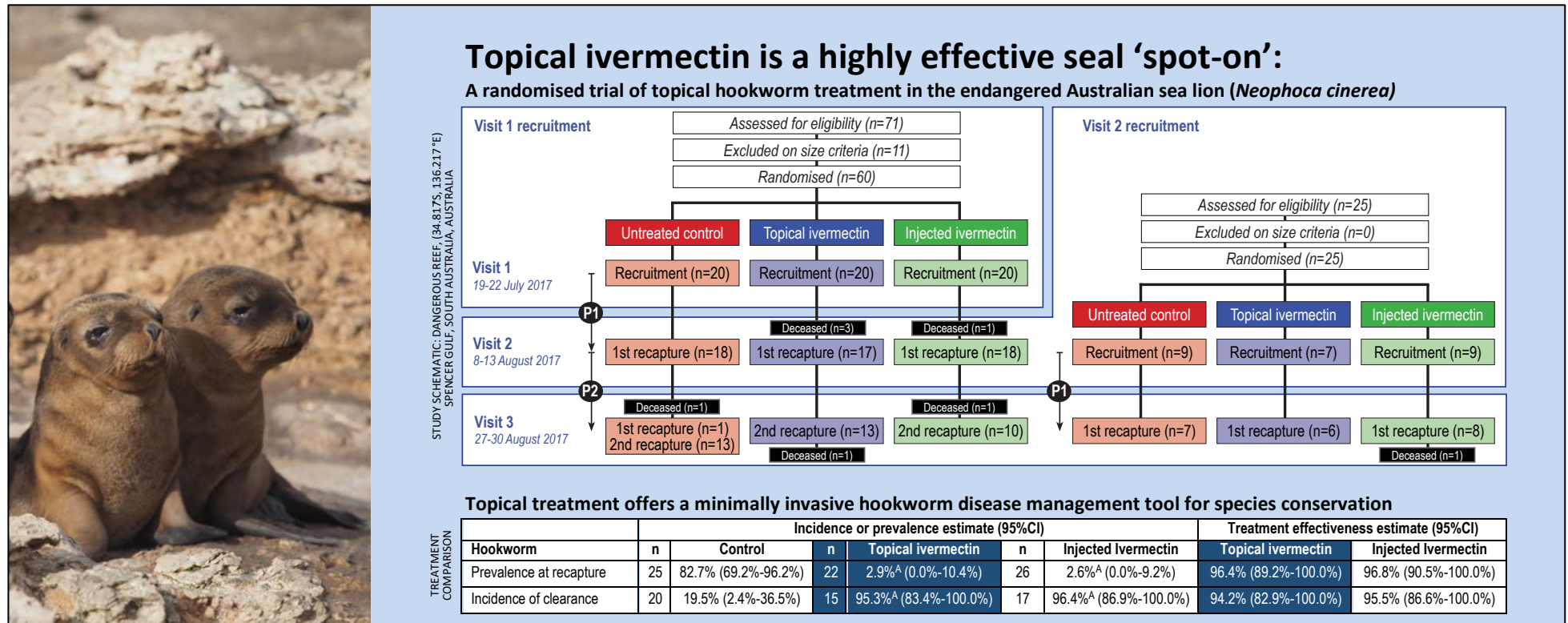
Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijppaw.2021.11.002>.

References

- Beekman, G.K., 1984. Clinical use of ivermectin against the hookworm (*Uncinaria lucasi*) of northern Fur seal pups (*Callorhinus ursinus*). *Internat. Assoc. Aqua. Med. (IAAAM) Proc.* 15, 50.
- Black, R.E., Victoria, C.G., Walker, S.P., Bhutta, Z.A., Christian, P., de Onis, M., Ezzi, M., Grantham-McGregor, S., Katz, J., Martorell, R., Uauy, R., and the Maternal and Child Nutrition Study Group, 2013. Maternal and child undernutrition and overweight in low-income and middle-income countries. *Lancet* 382, 427–451.
- Byard, R.W., Machado, A., 2019. Characteristic "neck collar" injuries in Australian sea lions (*Neophoca cinerea*) caused by marine debris. *Forensic Sci. Med. Pathol.* 15, 631–634.
- Castinel, A., Duignan, P.J., Lyons, E.T., Pomroy, W.E., Gibbs, N., Lopez-Villalobos, N., Chilvers, B.L., Wilkinson, I.S., 2007a. Epidemiology of hookworm (*Uncinaria* spp.) infection in New Zealand (Hooker's) sea lion (*Phocarcus hookeri*) pups on Enderby Island, Auckland Islands (New Zealand) during the breeding seasons from 1999/2000 to 2004/2005. *Parasitol. Res.* 101, 53–62.
- Castinel, A., Duignan, P.J., Pomroy, W.E., Lopez-Villalobos, N., Gibbs, N.J., Chilvers, B.L., Wilkinson, I.S., 2007b. Neonatal mortality in New Zealand sea lions (*Phocarcus hookeri*) at sandy Bay, Enderby Island, Auckland Islands from 1998 to 2005. *J. Wildl. Dis.* 43, 461–474.
- Chilvers, B.L., 2015. *Phocarcus hookeri*. The IUCN Red List of Threatened Species 2015: e.T17026A1306343. <https://doi.org/10.2305/IUCN.UK.2015-2.RLTS.T17026A1306343.en>. (Accessed 1 September 2021).
- Chilvers, B.L., Duignan, P.J., Robertson, B.C., Castinel, A., Wilkinson, I.S., 2009. Effects of hookworms (*Uncinaria* sp.) on the early growth and survival of New Zealand sea lion (*Phocarcus hookeri*) pups. *Polar Biol.* 32, 295–302.
- DeLong, R.L., Orr, A.J., Jenkinson, R.S., Lyons, E.T., 2009. Treatment of northern Fur seal (*Callorhinus ursinus*) pups with ivermectin reduces hookworm-induced mortality. *Mar. Mamm. Sci.* 25, 944–948.
- Department of Sustainability, Environment, Water, Population and Communities (DSEWPC), 2013. Recovery plan for the Australian sea lion (*Neophoca cinerea*). www.environment.gov.au/coasts/species/seals/index.html.
- Firth, D., 1993. Bias reduction of maximum likelihood estimates. *Biometrika* 80, 27–38.
- Fulham, M., Power, M., Gray, R., 2018. Comparative ecology of *Escherichia coli* in endangered Australian sea lion (*Neophoca cinerea*) pups. *Infect. Genet. Evol.* 62, 262–269.
- Gales, N.J., Shaughnessy, P.D., Dennis, T.E., 1994. Distribution, abundance and breeding cycle of the Australian sea lion *Neophoca cinerea* (Mammalia: pinnipedia). *J. Zool.* 234, 353–370.
- Goldsworthy, S., 2020. Australian Sea Lion Listing Assessment. Report to the Department for Environment and Water, Department of Agriculture, Water and the Environment. South Australian Research and Development Institute (Aquatic Sciences), Adelaide. SARDI Publication No. F2020/000131-1. SARDI Research Report Series No. 1056. 26pp.
- Goldsworthy, S., Gales, N., IUCN SSC Pinniped Specialist Group, 2008. *Neophoca cinerea*. The IUCN Red List of Threatened Species 2008: e.T14549A4443172. (Accessed 1 September 2021).
- Goldsworthy, S.D., Lowther, A.D., Shaughnessy, P.D., McIntosh, R., Page, B., 2007. Assessment of Pup Production and the Maternal Investment Strategies of the Australian Sea Lion *Neophoca cinerea* at Dangerous Reef in the 2006-07 Breeding Season. SARDI Aquatic Sciences Publication, Adelaide, Australia. Number F2007/000929-1. SARDI Research Report Series No. 249.
- Goldsworthy, S.D., Mackay, A.I., Shaughnessy, P.D., Bailleul, F., Holman, D., 2015. Maintaining the Monitoring of Pup Production at Key Australian Sea Lion Colonies in South Australia (2014/15). Final Report to the Australian Marine Mammal Centre. South Australian Research and Development Institute (Aquatic Sciences), Adelaide Australia. SARDI Publication No. F2010/000665-5. SARDI Research Report Series No. 871.
- Goldsworthy, S.D., Shaughnessy, P.D., Mackay, A.I., Bailleul, F., Holman, D., Lowther, A.D., Page, B., Waples, K., Raudino, H., Bryars, S., Anderson, T., 2021. Assessment of the status and trends in abundance of a coastal pinniped, the Australian sea lion *Neophoca cinerea*. *Endanger. Species Res.* 44, 421–437.
- Goldsworthy, S.D., Shaughnessy, P.D., Smart, J., Mackay, A.I., Bailleul, F., Reinhold, S.-L., Stonnill, M., Lashmar, K., 2019. Monitoring of Seal Bay and Other Populations on Kangaroo Island: 2017/2018. Report to the Department for Environment and Water. South Australian Research and Development Institute (Aquatic Sciences), Adelaide. SARDI Publication No. F2014000322-F2014000325. SARDI Research Report Series No. 1018, Adelaide, Australia.
- Hamer, D.J., Goldsworthy, S.D., Costa, D.P., Fowler, S.L., Page, B., Sumner, M.D., 2013. The endangered Australian sea lion extensively overlaps with and regularly becomes by-catch in demersal shark gill-nets in South Australian shelf waters. *Biol. Conserv.* 157, 386–400.
- Higgins, L.V., Gass, L., 1993. Birth to weaning: parturition, duration of lactation, and attendance cycles of Australian sea lions (*Neophoca cinerea*). *Can. J. Zool.* 71, 2047–2055.
- Higgins, L.V., Tedman, R.A., 1990. Effect of attacks by male Australian sea lions, *Neophoca cinerea*, on mortality of pups. *J. Mammal.* 71, 617–619.
- Jakob, E.M., Marshall, S.D., Uetz, G.W., 1996. Estimating fitness: a comparison of body condition indices. *Oikos* 77, 61–67.
- Kovacs, K.M., Aguilar, A., Auriolles, D., Burkanov, V., Campagna, C., Gales, N., Gelatt, T., Goldsworthy, S.D., Goodman, S.J., Hofmeyr, G.J.G., Härkönen, T., Lowry, L., Lydersen, C., Schipper, J., Sipilä, T., Southwell, C., Stuart, S., Thompson, D., Trillmich, F., 2012. Global threats to pinnipeds. *Mar. Mamm. Sci.* 28, 414–436.
- Lindsay, S.A., Gray, R., 2021. A novel presentation of tuberculosis with intestinal perforation in a free-ranging Australian sea lion (*Neophoca cinerea*). *J. Wildl. Dis.* 57, 220–224.
- Lyons, E.T., Melin, S.R., DeLong, R.L., Orr, A.J., Gulland, F.M., Tolliver, S.C., 2001. Current prevalence of adult *Uncinaria* spp. in northern Fur seal (*Callorhinus ursinus*) and California sea lion (*Zalophus californianus*) pups on San Miguel Island, California, with notes on the biology of these hookworms. *Vet. Parasitol.* 97, 309–318.
- Lyons, E.T., Spraker, T.R., De Long, R.L., Ionita, M., Melin, S.R., Nadler, S.A., Tolliver, S.C., 2011. Review of research on hookworms (*Uncinaria lucasi* Stiles, 1901) in northern Fur seals (*Callorhinus ursinus* Linnaeus, 1758). *Parasitol. Res.* 109, 257–265.
- Marcus, A.D., Higgins, D.P., Gray, R., 2014. Epidemiology of hookworm (*Uncinaria sanguinis*) infection in free-ranging Australian sea lion (*Neophoca cinerea*) pups. *Parasitol. Res.* 113, 3341–3353.
- Marcus, A.D., Higgins, D.P., Gray, R., 2015a. Health assessment of free-ranging endangered Australian sea lion (*Neophoca cinerea*) pups: effect of haematophagous parasites on haematological parameters. *Comp. Biochem. Physiol. Mol. Integr. Physiol.* 184, 132–143.
- Marcus, A.D., Higgins, D.P., Gray, R., 2015b. Ivermectin treatment of free-ranging endangered Australian sea lion (*Neophoca cinerea*) pups: effect on hookworm and lice infection status, haematological parameters, growth, and survival. *Parasitol. Res.* 114, 2743–2755.
- McIntosh, R.R., Kennedy, C.W., 2013. Morphology, sex ratio and cause of death in Australian sea lion (*Neophoca cinerea*) pups. *Aust. Mammal.* 35, 93–100.
- McIntosh, R.R., Murray, M.D., 2007. Louse infestations of the Australian sea lion *Neophoca cinerea*. *Aust. Mammal.* 29, 103–106.
- Michael, S., Hayman, D., Gray, R., Roe, W., 2021a. Clinical diagnosis of hypervirulent *Klebsiella pneumoniae* disease in New Zealand sea lion (*Phocarcus hookeri*) pups. *Vet. Microbiol.* (in press).
- Michael, S.A., Hayman, D.T.S., Gray, R., Roe, W.D., 2021b. Risk factors for New Zealand sea lion (*Phocarcus hookeri*) pup mortality: ivermectin improves survival for conservation management. *Front. Mar. Sci.* 8, 680678.
- Mofid, L.S., Casapia, M., Aguilar, E., Silva, H., Montresor, A., Rahme, E., Fraser, W.D., Marquis, G.S., Vercruysse, J., Allen, L.H., Blouin, B., Razuri, H., Pezo, L., Gyorkos, T.W., 2017. A double-blind randomized controlled trial of maternal postpartum

- deworming to improve infant weight gain in the peruvian Amazon. *PLoS Neglected Trop. Dis.* 11, e0005098.
- Montalva, F., Perez-Venegas, D., Gutierrez, J., Seguel, M., 2019. The contrasting hidden consequences of parasitism: effects of a hematophagous nematode (*Uncinaria* sp.) in the development of a marine mammal swimming behavior. *Ecol. Evol.* 9, 3689–3699.
- Olsen, O.W., Lyons, E.T., 1965. Life cycle of *Uncinaria lucasi* Stiles, 1901 (Nematoda: ancylostomatidae) of Fur seals, *Callorhinus ursinus* Linn., on the Pribilof Islands, Alaska. *J. Parasitol.* 51, 689–700.
- Page, B., McKenzie, J., McIntosh, R., Baylis, A., Morrissey, A., Calvert, N., Haase, T., Berris, M., Dowie, D., Shaughnessy, P.D., Goldsworthy, S.D., 2004. Entanglement of Australian sea lions and New Zealand Fur seals in lost fishing gear and other marine debris before and after Government and industry attempts to reduce the problem. *Mar. Pollut. Bull.* 49, 33–42.
- Papich, M.G., 2016. Ivermectin. In: Papich, M.G. (Ed.), *Saunders Handbook of Veterinary Drugs*, fourth ed. W.B. Saunders, St. Louis, pp. 420–423.
- Schulz, K.F., Altman, D.G., Moher, D., 2010. CONSORT 2010 statement: updated guidelines for reporting parallel group randomized trials. *Ann. Intern. Med.* 152, 726–732.
- Schumann, N., Gales, N.J., Harcourt, R.G., Arnould, J.P.Y., 2013. Impacts of climate change on Australian marine mammals. *Aust. J. Zool.* 61, 146–159.
- Seguel, M., Perez-Venegas, D., Gutierrez, J., Crocker, D.E., DeRango, E.J., 2019. Parasitism elicits a stress response that allocates resources for immune function in South American Fur seals (*Arctocephalus australis*). *Physiol. Biochem. Zool.* 92, 326–338.
- Shaughnessy, P.D., Goldsworthy, S.D., Burch, P., Dennis, T.E., 2013. Pup numbers of the Australian sea lion (*Neophoca cinerea*) at the Pages Islands, South Australia, over two decades. *Aust. J. Zool.* 61, 112–118.
- Taylor, S., Terkildsen, M., Stevenson, G., de Araujo, J., Yu, C., Yates, A., McIntosh, R.R., Gray, R., 2021. Per and polyfluoroalkyl substances (PFAS) at high concentrations in neonatal Australian pinnipeds. *Sci. Total Environ.* 786, 147446.
- Threatened Species Scientific Committee, 2020. Conservation Advice. *Neophoca cinerea*. Australian sea lion. Department of Agriculture, Water and the Environment, Canberra. www.environment.gov.au/biodiversity/threatened/species/pubs/22-conservation-advice-23122020.pdf. (Accessed 29 August 2021).
- Trillmich, F., Rea, L., Castellini, M., Wolf, J.B.W., 2008. Age-related changes in hematocrit in the Galápagos sea lion (*Zalophus wollebaeki*) and the Weddell seal (*Leptonychotes weddellii*). *Mar. Mamm. Sci.* 24, 303–314.
- Vercruysse, J., Claerebout, E., 2014. Macrocytic Lactones. *MSD Veterinary Manual*. <https://www.msdvetmanual.com/pharmacology/anthelmintics/macrocytic-lactones>. (Accessed 27 September 2021).
- Victora, C.G., Adair, L., Fall, C., Hallal, P.C., Martorell, R., Richter, L., Sachdev, H.S., for the Maternal and Child Undernutrition Study Group, 2008. Maternal and child undernutrition: consequences for adult health and human capital. *Lancet* 371, 340–357.

Graphical abstract



Supplementary data

Supplementary table 1

Comparison of Australian sea lion pup demographics and health distributions at time of recruitment across the three experimental groups.

Variables	Control (n=29)	Topical ivermectin (n=27)	Injected ivermectin (n=29)	Combined (n=85)
Sex				
Male (%)	13 ^A (44.8%)	11 ^A (70.7%)	7 ^A (24.1%)	31 (36.5%)
Female (%)	16 ^A (55.2%)	16 ^A (59.3%)	22 ^A (75.9%)	54 (63.5%)
Standard length - mean (range) cm	66.6 ^A (59.5-70.0)	65.5 ^A (58.0-70.0)	65.9 ^A (55.0-71.0)	66.0 (55.0-71.0)
Body Weight - mean (range) kg	9.6 ^A (7.6-12.6)	9.1 ^A (5.2-12.4)	9.5 ^A (6.0-12.8)	9.4 (5.2-12.8)
Body condition index - mean (range)	26.5 ^A (19.4-32.3)	25.9 ^A (17.9-31.2)	26.8 ^A (20.6-31.8)	26.4 (17.9-32.3)
Prevalence of patent hookworm - count (%)	23 ^A (79.3%)	17 ^A (63.0%)	19 ^A (65.5%)	59 (69.4%)
Prevalence of lice - count (%)	18 ^A (62.1%)	16 ^A (59.3%)	19 ^A (65.5%)	53 (62.4%)
Lice intensity score - mean (range)	1.79 ^A (0-6)	1.70 ^A (0-7)	1.45 ^A (0-6)	1.65 (0-7)

^AExperimental groups that share the same superscripted letter within the same parameter row were not significantly different at the 5% level after accounting for multiple pairwise comparisons using the Bonferroni correction.

Supplementary table 2

The predicted mean or median values (with 95% confidence interval) of haematological parameters of Australian sea lion (*N. cinerea*) pups at first and second recapture.

Parameters	Predicted means or medians ^a (95% CI)			Model features				Base additive model parameters		Ancillary parameters in final model				
	Control	Topical ivermectin	Injected ivermectin	n	Data and regression adjustments	R ²	adjusted R ²	Standard length at recruitment	Time in day to recapture	Sex	Analyte value at recruitment	Lice present at recruitment	Lice present at recapture	Hookworm present at recapture
At first recapture														
Field blood parameters														
PCV (L/L)	0.307 ^A (0.288-0.324)	0.332 ^{A,B} (0.313-0.349)	0.359 ^B (0.344-0.372)	58	cubed outcome	50.8%	46.0%	0.323	0.873	-	<0.001	-	-	-
Total plasma protein (g/L)	85.2 (82.2-88.3)	72.1 ^A (68.2-75.9)	70.4 ^A (66.7-74.0)	65	robust SE	15.7%	&	0.956	0.616	-	-	0.018	-	0.001
Laboratory blood parameters														
RBC (x10 ¹² /L)	3.93 ^A (3.71-4.14)	4.03 ^{A,B} (3.79-4.27)	4.33 ^B (4.12-4.54)	48	none	45.9%	39.4%	0.4	0.98	-	<0.001	-	-	#
Haemoglobin (g/L)	111.9 ^A (105.2-118.5)	115.2 ^{A,B} (107.8-122.6)	127.2 ^B (120.6-133.7)	48	none	67.0%	63.1%	0.167	0.969	-	<0.001	-	-	#
Haematocrit (L/L) ^b	0.348 ^A (0.327-0.368)	0.359 ^{A,B} (0.336-0.380)	0.391 ^B (0.373-0.409)	48	squared outcome	58.3%	53.3%	0.172	0.48	-	<0.001	-	-	#
MCV (fL)	86.4 ^A (84.2-88.5)	88.3 ^A (85.9-90.7)	89.4 ^A (87.3-91.6)	48	none	65.0%	60.9%	0.231	0.098	-	<0.001	-	-	#
MCHC (g/L)	324.0 ^A (318.3-329.7)	327.5 ^A (321.2-333.8)	327.1 ^A (321.4-332.8)	48	none	39.2%	32.0%	0.804	0.008	-	0.001	-	-	#
Platelets (10 ⁹ /L)	336.0 ^A (278.2-393.7)	289.4 ^A (227.0-352.0)	297.4 ^A (242.6-352.1)	59	none	13.2%	6.7%	0.835	0.014	-	-	-	-	#
WBC (10 ⁹ /L)	17.9 ^A (14.4-21.9)	13.6 ^A (10.3-17.2)	15.6 ^A (12.4-19.1)	59	squared root outcome	7.4%	0.6%	0.317	0.161	-	-	-	-	#
Neutrophils (10 ⁹ /L)	11.6 ^A (8.8-14.8)	9.0 ^A (6.4-12.0)	10.3 ^A (7.8-13.2)	60	squared root outcome	5.0%	-2.0%	0.766	0.243	-	-	-	-	#
Lymphocytes (10 ⁹ /L)	4.0 ^A (3.2-4.8)	2.9 ^A (2.2-3.7)	3.7 ^A (3.0-4.5)	60	squared root outcome	14.9%	8.7%	0.066	0.063	-	-	-	-	#
Monocytes (10 ⁹ /L)	1.1 ^A (0.8-1.4)	1.0 ^A (0.7-1.3)	1.2 ^A (0.9-1.4)	60	squared root outcome	2.3%	-4.8%	0.742	0.517	-	-	-	-	#
Eosinophils (10 ⁹ /L)	0.77 (0.51-1.12)	0.04 ^A (0.00-0.19)	0.00 ^A (0.00-0.02)	60	outcome powered .17, robust SE	39.7%	&	0.178	0.821	-	-	-	-	#
nRBC/100WBC ^b	0.09 ^A (0.00-1.14)	0.00 ^A (0.00-0.02)	0.02 ^A (0.00-0.24)	60	outcome powered .08	14.4%	8.2%	0.853	0.045	-	-	-	-	#
At second recapture														
Field blood parameters														
PCV ^b (L/L)	0.306 ^A (0.280-0.333)	0.329 ^A (0.297-0.361)	0.328 ^A (0.289-0.366)	31	none	45.9%	35.1%	0.683	0.066	-	0.001	-	-	-
Total plasma protein (g/L)	75.5 ^A (72.1-78.8)	76.1 ^A (72.5-79.6)	76.4 ^A (72.2-80.7)	35	none	3.3%	-9.5%	0.798	0.345	-	-	-	-	-
Laboratory blood parameters														
RBC (x10 ¹² /L)	4.10 ^A (3.66-4.53)	4.18 ^A (3.74-4.61)	4.23 ^A (3.65-4.80)	26	none	36.4%	20.5%	0.705	0.333	-	0.005	-	-	-
Haemoglobin (g/L)	112.8 ^A (98.3-127.3)	113.6 ^A (98.6-128.6)	119.0 ^A (100.2-137.7)	26	none	53.0%	41.2%	0.562	0.513	-	<0.001	-	-	-
Haematocrit (L/L)	0.332 ^A (0.290-0.374)	0.341 ^A (0.298-0.385)	0.352 ^A (0.298-0.407)	26	none	49.9%	37.4%	0.506	0.411	-	0.001	-	-	-
MCV (fL)	79.4 ^A (76.3-82.5)	82.6 ^A (79.5-85.8)	81.4 ^A (77.5-85.3)	26	none	69.4%	61.8%	0.474	0.958	-	<0.001	-	-	-
MCHC (g/L)	340.7 ^A (333.2-348.2)	333.6 ^A (326.8-340.4)	329.1 ^A (321.4-336.8)	33	none	22.3%	7.9%	0.385	0.785	-	-	-	-	0.022
Platelets (10 ⁹ /L)	357.8 ^A (282.6-433.1)	324.9 ^A (244.6-405.1)	351.4 ^A (260.0-442.8)	33	none	4.1%	-9.7%	0.591	0.635	-	-	-	-	-
WBC (10 ⁹ /L)	17.2 ^A (13.9-21.3)	15.6 ^A (12.4-19.5)	16.8 ^A (12.9-21.7)	33	natural logged outcome	13.9%	1.6%	0.367	0.054	-	-	-	-	-
Neutrophils (10 ⁹ /L)	11.0 ^A (8.2-13.8)	10.1 ^A (7.2-12.9)	10.4 ^A (7.1-13.8)	34	none	5.1%	-8.1%	0.291	0.435	-	-	-	-	-
Lymphocytes (10 ⁹ /L)	4.56 ^A (3.31-6.30)	4.37 ^A (3.15-6.06)	4.7 ^A (3.10-7.12)	26	natural logged outcome	42.3%	27.9%	0.460	0.313	-	0.025	-	-	-
Monocytes (10 ⁹ /L) ^b	0.871 ^A (0.681-1.114)	0.915 ^A (0.704-1.190)	1.063 ^A (0.788-1.433)	33	natural logged outcome	13.2%	0.8%	0.187	0.376	-	-	-	-	-
Eosinophils (10 ⁹ /L)	1.12 (0.51-2.18)	0.07 ^A (0.01-0.25)	0.03 ^A (0.00-0.15)	34	outcome powered .24	49.7%	42.8%	0.595	0.562	-	-	-	-	-
nRBC/100WBC ^b	0.002 ^A (0.000-1.129)	0.000 ^A (0.000-0.004)	0.000 ^A (0.000-0.044)	34	natural logged outcome	9.8%	-2.7%	0.846	0.385	-	-	-	-	-

RBC: total red blood cell count; MCV: mean cell volume; MCHC: mean cell haemoglobin concentration; WBC: total leukocyte count, corrected for presence of nucleated red blood cells (nRBC). &No adjusted R² available with robust SE. #Omitted due to high collinearity with treatment group allocation.

^{A,B}Experimental groups (columns 1-3) that share the same superscripted letter within the same parameter row are not significantly different at the 5% level after accounting for multiple pairwise comparisons using the Bonferroni adjustment.

Manual PCV was included in addition to haematocrit to overcome any impact on the latter from the delay to processing.

^aMedian reported for back-transformed model estimates.

^bSub-optimal compliance with linear regression assumptions.

Supplementary table 3

Comparison of the experimental groups predicted mean or median values (with 95% confidence interval) of the daily specific growth rate (SGR) for the bodyweight, standard length and body condition index of Australian sea lion (*N. cinerea*) pups during the first and second treatment trial period.

Parameters	Predicted means or medians ^a (95% CI)			Model features				Base additive model parameters		Ancillary parameters in final model			
	Control	Topical ivermectin	Injected ivermectin	n	Data and regression adjustments	R ²	adjusted R ²	Duration in days of period	Morphometric value at start of period	Sex	Alternative morphometric value at start of period	Lice present at start of period	Hookworm present at start of period
Recruitment-to-first recapture (P1)													
Bodyweight daily SGR (%)	0.342 ^A (0.112-0.573)	0.717 ^A (0.472-0.961)	0.671 ^A (0.446-0.897)	73	none	24.3%	18.7%	0.007	0.060	-	-	-	0.032
Standard length daily SGR (%)	0.281 ^A (0.229-0.334)	0.338 ^A (0.283-0.394)	0.307 ^A (0.256-0.358)	73	none	53.3%	49.0%	0.850	<0.001	0.004	<0.001	-	-
Body condition index daily SGR (%)	-0.357 ^A (-0.573--0.141)	-0.090 ^A (-0.318-0.138)	-0.039 ^A (-0.250-0.172)	73	none	37.2%	31.5%	0.005	<0.001 ^b	-	<0.001	-	0.018
First recapture-to-second recapture (P2)													
Bodyweight daily SGR (%)	0.619 ^A (0.316-0.923)	0.852 ^A (0.536-1.167)	0.538 ^A (0.199-0.878)	35	none	23.1%	12.8%	0.688	0.015	-	-	-	-
Standard length daily SGR (%)	0.197 ^A (0.123-0.271)	0.286 ^A (0.209-0.363)	0.207 ^A (0.123-0.292)	35	none	38.9%	28.3%	0.191	0.771	-	0.053	-	-
Body condition index daily SGR (%)	0.127 ^A (-0.160-0.415)	0.132 ^A (-0.168-0.433)	0.113 ^A (-0.217-0.442)	35	none	52.1%	43.9%	0.493	0.042 ^b	-	<0.001	-	-

^AExperimental groups that share the same superscripted letter within the same parameter row are not significantly different at the 5% level after accounting for multiple pairwise comparisons using the Bonferroni adjustment.

^aMedian reported for back transformed model estimates.

^bStandard length was used as the default morphometric for the base additive model of the body condition index.

CHAPTER THREE

Mitigating disease risk in an endangered pinniped: early hookworm elimination optimizes the growth and health of Australian sea lion pups



Seal Bay, Kangaroo Island, SA

Author attribution statement

Chapter three is published as:

Lindsay, S.A., Fulham, M., Caraguel, C.G.B., and Gray, R. (2023). Mitigating disease risk in an endangered pinniped: early hookworm elimination optimizes the growth and health of Australian sea lion pups. *Frontiers in Veterinary Science* 10, 1161185. [doi: 10.3389/fvets.2023.1161185](https://doi.org/10.3389/fvets.2023.1161185).

Scott Lindsay was the primary author of this chapter and contributed to all aspects of this publication, including conceptualization, data curation, formal analysis (statistical), funding acquisition, investigation (fieldwork; laboratory), methodology, project administration, resources, validation, visualisation, and writing (original draft; review and editing). Mariel Fulham contributed to data curation, investigation (fieldwork; laboratory), and writing (review and editing). Charles Caraguel contributed to data curation, formal analysis (statistical), supervision, validation, visualisation, and writing (review and editing). Rachael Gray contributed to conceptualization, funding acquisition, investigation (fieldwork; laboratory), methodology, project administration, resources, supervision, validation, and writing (review and editing). All authors reviewed and approved the final manuscript.

Scott A. Lindsay

22 March 2024

Mariel Fulham

22 March 2024

Charles G.B. Caraguel

02 April 2024

Rachael Gray

26 March 2024



OPEN ACCESS

EDITED BY

Marta Canuti,
University of Milan, Italy

REVIEWED BY

Mauricio Seguel,
University of Georgia, United States
Piyanan Taweethavonsawat,
Chulalongkorn University, Thailand
Maria Cristina Monti,
University of Pavia, Italy

*CORRESPONDENCE

Rachael Gray
✉ rachael.gray@sydney.edu.au

[†]These authors share last authorship

RECEIVED 10 February 2023

ACCEPTED 04 April 2023

PUBLISHED 25 April 2023

CITATION

Lindsay SA, Fulham M, Caraguel CGB and Gray R (2023) Mitigating disease risk in an endangered pinniped: early hookworm elimination optimizes the growth and health of Australian sea lion pups.
Front. Vet. Sci. 10:1161185.
doi: 10.3389/fvets.2023.1161185

COPYRIGHT

© 2023 Lindsay, Fulham, Caraguel and Gray. This is an open-access article distributed under the terms of the [Creative Commons Attribution License \(CC BY\)](#). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Mitigating disease risk in an endangered pinniped: early hookworm elimination optimizes the growth and health of Australian sea lion pups

Scott A. Lindsay^{1,2}, Mariel Fulham¹, Charles G. B. Caraguel^{2†} and Rachael Gray^{1*†}

¹Faculty of Science, Sydney School of Veterinary Science, The University of Sydney, Camperdown, NSW, Australia, ²Faculty of Science, School of Animal and Veterinary Sciences, The University of Adelaide, Roseworthy, SA, Australia

The Australian sea lion (*Neophoca cinerea*) experiences high pup mortality of seasonally alternating severity, partly attributed to endemic hookworm (*Uncinaria sanguinis*) infection. To further explore health outcomes of early hookworm elimination, a treatment trial was conducted at Seal Bay Conservation Park, South Australia, over consecutive lower and higher mortality breeding seasons (2019, 19.2%; 2020–1; 28.9%). Pups ($n = 322$) were stratified into two age cohorts (median 14 d and 24 d recruitment ages) and randomly assigned to treated (topical ivermectin 500 $\mu\text{g/kg}$) or control (untreated) groups. A younger prepatent cohort <14 d old (median 10 d) was identified *a posteriori*. A seasonally independent growth benefit resulted from hookworm elimination across all age cohorts. The greatest relative improvements (bodyweight + 34.2%, standard length + 42.1%; $p \leq 0.001$) occurred in the month post-treatment, in the youngest prepatent cohort. A significant benefit of lesser magnitude (bodyweight + 8.6–11.6%, standard length + 9.5–18.4%; $p \leq 0.033$) persisted up to 3 months across all age cohorts – greatest in the youngest pups. Treatment resulted in immediate improvement in hematological measures of health – decreased anemia and inflammation severity ($p \leq 0.012$). These results enhance our understanding of host–parasite–environment interactions within the context of hematological ontogenesis, confirm the seasonally independent benefits of hookworm disease intervention, and further inform conservation recommendations for this endangered species.

KEYWORDS

Australian sea lion, growth, hematology, hookworm, ivermectin, *Neophoca cinerea*, otariid, *Uncinaria sanguinis*

1. Introduction

The contribution of infectious disease to wildlife species' decline is likely underestimated due to limitations in population monitoring, detection of diseased individuals, and in assigning etiology [reviewed in (1)]. This holds true for pinnipeds (seals and sea lions), which spend a substantial amount of time foraging at sea and haul out on remote coastlines, such that conclusive evidence of infectious disease compromising their populations is often relatively limited (2, 3). For populations exposed to endemic infections there is a ubiquitous risk of a

disease outbreak should additional stressors alter the host-pathogen-environment balance (1, 4, 5). Equally, endemic infection can compromise host resilience against evolving population stressors, such as those attributed to climate change – temperature extremes and altered foraging ranges (1, 6, 7). Consequently, deconstructing factors determining endemic infection elaboration and the success of mitigation strategies, is essential for enhanced conservation medicine (8, 9).

Disease impact on population sustainability is particularly relevant for an endangered and declining (10) species such as the Australian sea lion (*Neophoca cinerea*). The species' ability to maintain its population in the presence of increased disease pressure is potentially limited by (1) the absence of a population buffer – less than 10,000 individuals, and (2) a segmented population structure – 80 dispersed breeding colonies (11), each representing a genetically contracted subpopulation with limited adult female dispersal capacity due to extreme fine-scale foraging and philopatric behavior (12, 13). Further, the species' fecundity is low because of female reproduction commencing at 4.5–6.0 years of age, extended non-annual breeding seasons 17–18-months apart, and high mortality among the 2,700 pups born each breeding season (14–17). Ultimately, pup mortality is the major contributor to individuals not reaching breeding age – a progressive increase in age-specific survival apparent in older cohorts, reaching a plateau in early adulthood (18). A high proportion of pup deaths occur in the first few weeks of life, most often attributed to conspecific trauma, starvation (19), and the direct and indirect impact of endemic hookworm (*Uncinaria sanguinis*) infection (20). Consequently, pup disease and specifically hookworm infection, is listed as one of the key threats to the species' recovery (10).

Uncinaria sanguinis infection occurs in all Australian sea lion pups (20). Their single point of infection is ingestion of infective larvae in colostrum (20) – the milk produced within the first 24–48 h after birth. Detection of parasite ova in pup feces (patency) from 11 to 14 days of age confirms larval maturation to adult hookworms in the intestines and their elimination occurs by 2–3 months of age (post-patency) (20). Hookworm is a compromising agent of Australian sea lion pups, its blood-sucking activity contributing to intestinal loss of red blood cells and protein and to local and systemic inflammation (eosinophilic and lymphocytic) (21, 22). Most pup deaths directly attributable to infection occur from hemorrhagic enteritis in the first few weeks of life, supporting a pathogenic contribution from juvenile hookworms in the prepatent period, as well as from adult hookworms (20, 23). Additionally, infection contributes indirectly to pup deaths from increased conspecific trauma in disease-weakened pups, supported by reporting of infection intensity-dependent mortality (20). Two anti-parasiticide treatment trials at Dangerous Reef, Spencer Gulf, South Australia have studied the short-term health benefits of altering the host-parasite relationship in this species. The first trial successfully used an injected anthelmintic (ivermectin) to eliminate hookworm infection and to reduce the severity of anemia (reduction in red blood cell concentration) and systemic eosinophilic inflammation (24). The second trial demonstrated an equivalent effectiveness of a less invasive topical (“spot-on”) formulation of ivermectin in comparison to the injectable formulation and revealed a treatment benefit for the short-term loss in body condition associated with hookworm infection (21). These studies were precautionary in recruitment size and duration, partly to enable assessment of any adverse effects of ivermectin on the host and partly

due to challenging site logistics. In related otariid (eared seal) species, longer term benefits of early hookworm elimination have included increased pup growth, improved pup health based on the evaluation of hematological parameters, and ultimately increased pup survival – the latter most often following treatment of the youngest pups (<2 weeks old) during breeding seasons of higher than usual mortality (caused either by hookworm or an unrelated comorbidity) (25, 26).

Australian sea lion pup mortality oscillates between breeding seasons (14, 15, 27, 28). At the Seal Bay colony on Kangaroo Island, South Australia, higher pup mortality is observed during summer–autumn (versus winter–spring) breeding seasons (15). Reasons for this variation are not fully understood. Proposed contributions include anomalies in sea surface temperature impacting maternal provisioning of pups (29), direct environmental challenge (temperature and weather extremes), higher population density leading to increased conspecific trauma (27), and endemic or emerging disease (20). Significant ($p < 0.05$) correlations exist between hookworm infection intensity, pup mortality, and season at the Seal Bay colony (20). While this does not necessarily equate to causation, hookworm burden perhaps reflecting another comorbidity or population stressor, it highlights the need to consider seasonal impact in any assessment of hookworm disease outcome or its modulation. In the South American fur seal (*Arctocephalus australis*), seasonal variability in pup ability to mount an immune response to eliminate hookworm infection has been attributed to impacts on maternal provisioning of pups (30).

This study investigated the long-term benefit of topical ivermectin on pup health by conducting a large-scale randomized controlled field trial with extended three-month follow-up period over two consecutive breeding seasons at the Seal Bay colony. In addition to treatment effectiveness in clearing hookworm infection, we investigated the optimization of health benefits reflected in pup growth and hematological profiles, by analyzing outcomes in different age cohorts at treatment delivery.

2. Materials and methods

2.1. Ethics approvals and reporting

All samples were collected with the approval of the South Australian Department of Environment and Water (Permit/License Number: MR00073-2-R; Scientific Research Permit Number: A25088-12) and the trial protocol was approved by The University of Sydney Animal Ethics Committee (Protocol Number 2017/1260). Recommendations in the CONSORT (Consolidated Standards of Reporting Trials) statement were used to guide reporting of the study outcomes (31).

2.2. Study site and duration

The study was conducted at Seal Bay Conservation Park, Kangaroo Island, South Australia (−35.996, 137.327), the third largest breeding colony of Australian sea lions. The long-term trend (12 season average) shows a 1.3% decline in seasonal pup production, with recent stabilization (five seasons) (14). During extended 7–12 month breeding seasons, twice weekly colony monitoring provides an accurate assessment of pup births (accurate individual age for some

pups) and deaths (14), and routine monthly microchip scanning and animal counts provide temporal information at an individual and colony level, respectively. Routine pup microchipping offers permanent and unique animal identification, enabling longitudinal observation of individuals and mother-pup pairing. Indicative intra-seasonal pup mortality incidence over the previous 11 breeding seasons were 21.4% (range 17.9–24.7%) for winter–spring seasons and 32.8% (range 28.0–41.8%) for summer–autumn seasons (14). The present trial was conducted in the lower mortality 2019 breeding season (estimated 239 births, 46 deaths and 19.2% mortality incidence) and the higher mortality 2020–1 summer-autumn breeding season (estimated 242 births, 70 deaths and 28.9% mortality incidence) (14).

2.3. Study design and sample collection

A randomized controlled field-based treatment trial initially recruited young Australian sea lion pups <80 cm in standard length (snout-to-tail-tip length; an age proxy) to one of two age cohorts – a younger <70 cm cohort and an older ≥70 cm cohort. After preliminary analysis of season 2019 data the study design was refined (1) to a 75 cm upper length restriction to limit the recruitment of postpatent pups (>60 d of age), and (2) by increasing the frequency of colony observation to detect pups earlier and thereby maximize recruitment to the younger cohort. Within each age cohort, pups were randomly assigned one of two treatment groups – an untreated control group or a topical anthelmintic treated group. Treatment allocation was concealed, but not blinded, from treatment providers until the time of potential anthelmintic application and blinded from handlers at pup recaptures and from operators during sample processing. Predetermined randomization within each age cohort was achieved as previously described (21), with addition of blocking in groups of four in season 2020–1 to maintain equal pup numbers within each treatment group. Pups were recruited each season at all but the final colony visit and recaptured when possible, up to three times at subsequent monthly visits. To assess treatment impact on the youngest of recruited pups prior to the development of patent infection, a third prepatent age cohort was identified *a posteriori*, which comprised pups <14 d of age with a negative fecal hookworm test at recruitment (hereafter referred to as the <14 d and youngest cohort). No distinct random allocation was applied to this cohort.

Protocols for pup health assessment, sample collection and treatment delivery were conducted as previously described (21). Briefly, pup health and morphometric data included sex, bodyweight, standard length, subjective four-scale body condition score, lice infestation, and a visual lice infestation intensity score (lice score: 0–9). Recorded capture details included fieldtrip order (sequential numbering of colony visits to assess for impact from intraseasonal climatic variation), date, and initial capture state [reported as sleeping, awake or mobile, and subsequently used to assess for variable impact of capture stress on hematological parameters as previously identified (22)]. Pups were individually identified by a unique hair clip and bleach pattern (persisting until the first molt at 4–5 months of age), and by microchipping of pups weighing approximately 10 kg or more (HDX, 23 mm; Allflex Australia Pty Ltd., Capalaba, Qld). Fecal swab and EDTA-anticoagulated peripheral blood samples collected from each pup were chilled to approximately 4°C in the field. Treated pups

received topical ivermectin based on bodyweight (IVOMEC® Pour-on for Cattle, 5 mg/ml, Boehringer Ingelheim Animal Health Australia Pty. Ltd., North Ryde NSW; 500 µg/kg; off-label use). Control pups underwent identical handling and assessment to treated pups, except for anthelmintic treatment.

2.4. Specimen processing

2.4.1. Blood analysis

Packed cell volume (PCV; L/L) and total plasma protein (TPP; g/L) concentration were manually determined on the day of sampling, while automated hematological analysis (Sysmex XT-2000iV, Sysmex, Kobe, Japan) of preserved blood specimens (Streck Cell Preservative; Streck, Omaha, USA) was performed 2–8 days later (21). Automated analysis provided indicators of red blood cell number additional to PCV (red blood cell [RBC; $\times 10^{12}/L$] count and hemoglobin [Hb; g/L] concentration), as well as platelet (PLT; $\times 10^9/L$) and total white blood cell (WBC; $\times 10^9/L$) counts. Duplicate Wrights-Giemsa-stained blood smears prepared the day of sampling were later reviewed for manual differential WBC and nucleated RBC (nRBC) counts. Reticulocyte percentage was determined from new methylene blue-stained blood smear counts (Reticulocyte stain, Sigma-Aldrich, Germany) using a Miller Square (9:1 ratio) eyepiece reticule to count the equivalent of >1,000 RBC. In addition to nRBC, the absolute reticulocyte count for each pup was subsequently calculated (reticulocyte percentage $\times$ RBC count) as a measure of the intensity of RBC regeneration.

2.4.2. Fecal analysis

The presence of a single *U. sanguinis* ovum [elliptical, thin shelled, 110–135 µm $\times$ 55–95 µm; (23)] in any glass slide smear (minimum of two reviewed) prepared from each fecal swab was sufficient for reporting positive patent hookworm infection (21). Hookworm incidence in Seal Bay pups has previously been reported as 100% (22). Consequently, a negative fecal smear at recruitment was interpreted as prepatent infection (with pup age reviewed [<60 days] to exclude the likelihood of postpatent infection), and at any of the recaptures as postpatent infection (with review of subsequent capture results [all negative] to exclude false negative results).

2.5. Statistical analysis

Statistical analysis was performed using version 17.0 of the Stata statistical package (StataCorp LLC., College Station, Texas, United States) and results were considered significant at the 5% level (type-I-error or α). The study utilised standard multifactorial modelling to compare treatment groups, unless otherwise described. A base additive model included by default treatment allocation as the primary factor of interest, season, and a set of biological parameters (outlined below) expected to predict the model outcome (i.e., biological hypothesis). All base additive predictors were retained in the final model, irrespective of significance. Additional ancillary factors were then explored for significance one at the time in addition to the base additive model, and only those significantly associated were considered for inclusion into the final model. For strongly collinear predictors, only the most biologically meaningful factor was

retained. All possible pairwise (first order) interactions were explored among the remaining factors and retained in the final model if significant. Pup age was available for pups with a birth date record that were able to be monitored for identification until the time of recruitment (possible due to limited colony movement of neonatal pups). For pups without a birth date, an estimated age at recruitment was predicted from a multifactorial linear regression model using records from pups of known age (refer to [Supplementary Data S1](#)). Pup age at recaptures was calculated by adding the known period length (days) between captures to the age at recruitment.

2.5.1. Random allocation

To evaluate successful random allocation, pup demographics and hematological parameters at recruitment were compared between the two treatment groups, in the <70 and ≥ 70 cm age cohorts, as well as in the prepatent <14 d old pup cohort which was largely a subset of the <70 cm cohort with fewer individuals in the ≥ 70 cm cohort. For continuous outcomes with no evidence of deviation from normality, group means were reported and compared using the non-paired t-test. When a continuous outcome revealed strong deviation from normality, a non-parametric Wilcoxon rank-sum test was used to test equality of group distribution and medians were reported. For binary outcomes, frequencies within treatment groups were compared using Fisher's exact test.

2.5.2. Effectiveness

Topical ivermectin effectiveness was estimated by comparing either the predicted prevalence of patent hookworm infection at first recapture or the predicted incidence of hookworm clearance in the recruitment-to-first recapture period in both treatment groups, to calculate relative risk reductions (21). The incidence of hookworm clearance was calculated as the complement of the predicted proportion of pups with patent infection at recruitment that remained patent at recapture. Using Firth's variant of the multifactorial logistic regression, the base additive model included treatment group, season, and pup age at first recapture. Ancillary factors assessed for significance were sex; bodyweight, standard length, and body mass index [BMI; included for consistency with previous reporting (21)] at first recapture; first recapture fieldtrip; and the recruitment-to-first recapture period length.

2.5.3. Growth

The treatment effect on pup growth was assessed by calculating a daily specific growth rate (SGR) for bodyweight and standard length, for the periods between each capture and for the period from recruitment to final (third) recapture (21). The base additive model included treatment group, season, pup age and baseline growth parameter result at the start of the period, and the period length. Additional ancillary factors assessed and retained if found to have a significant effect on the daily SGR estimate, were sex; BMI, the alternative growth parameter, fieldtrip, and patent hookworm presence at the start of the period; and lice presence at the start and end of the period.

2.5.4. Hematology

When evaluating treatment effect on hematological parameters, data distribution and presence of potential outliers for each analyte were explored visually by a scatterplot of analyte value against standard length, a histogram of analyte results, and the interquartile

range (IQR) rule. For RBC and platelet results, the latter used three times the IQR to identify extreme outliers. These were subsequently reviewed and excluded if *in vivo* or *in vitro* sample hemolysis artefact was suggested by the reported sample appearance at collection, or by a comparatively large discrepancy between the manually determined PCV (assessed the day of collection) and the automated hematocrit (assessed 2–8 days later). For the WBC parameters, which were less impacted by sample artefact, four times the IQR was used to identify extreme outliers for exclusion. Means for each hematological analyte at each recapture were compared between treatment groups for each of the age cohorts. The base additive model included treatment group, season, pup age at current capture and the analyte measure at the previous capture (for pup recaptures – a baseline analyte level). Additional ancillary factors included sex, the presence of lice, the presence of patent hookworm, standard length, bodyweight, BMI, fieldtrip, and capture state at current capture. To satisfy the assumptions of a linear regression, a Box-Cox analysis was performed on the maximized model to detect the need for outcome value transformation. All transformed model estimates and their associated 95% CI boundaries were back transformed prior to reporting (these estimates should be interpreted as medians).

2.6. Survival

Analysis of potential association of treatment with pup survival and cause of mortality will be reported elsewhere.

3. Results

3.1. Recruitment, success of random allocation and recapture rates

There were 14 dedicated recruitment fieldtrips across seasons 2019 and 2020–1 ($n=6$, 2019; $n=8$, 2020–1), resulting in 399 individual pups being assessed for eligibility ($n=206$, 2019; $n=193$, 2020–1; [Figure 1](#)). Of these, 322 ($n=158$, 2019; $n=164$, 2020–1) pups were recruited based on the pre-defined size criteria – 196 ($n=82$, 2019; $n=114$, 2020–1) into the <70 cm cohort and 126 ($n=76$, 2019; $n=50$, 2020–1) into the ≥ 70 cm cohort. Of these, 99 pups ($n=39$, 2019; $n=60$, 2020–1) were additionally subclassified into the third <14 d cohort. As expected, this age cohort was predominantly a subset of the younger <70 cm cohort ($n=86$, <70 cm; $n=13$, ≥ 70 cm). Sixteen pups ($n=15$, 2019; $n=1$, 2020–1) >75 cm in standard length were recruited prior to implementing the 75 cm upper size restriction and were retained in the analysis. Pup age at recruitment was known for 56.8% ($n=183$) of the pups (38.6% [$n=61$], 2019; 74.4% [$n=122$], 2020–1), with age estimated for the remaining pups – median recruitment ages (and range) for the <14 d, <70 cm and ≥ 70 cm cohorts being 10 d (4–13 d), 14 d (4–33 d), and 24 d (8–63 d), respectively. [Figure 2](#) shows pup age at recruitment and each recapture for each cohort, relative to the anticipated hookworm status in untreated pups – patency developing from 11 to 14 d and infection cleared from 60 to 90 d (20) (refer to [Supplementary Table S1](#) for cohort age values at each recapture). Observed variation from these expectations based on fecal analysis ($n=381$) in untreated control pups of known age were one prepatent pup 18 d old and six patent

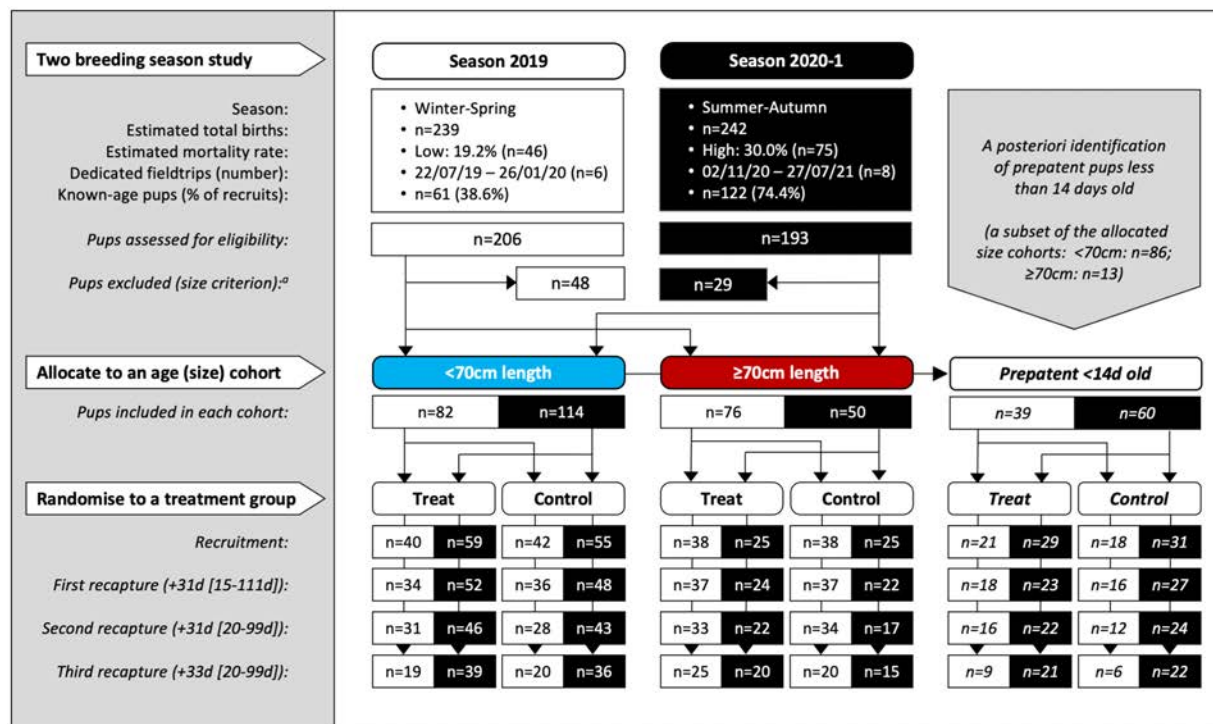


FIGURE 1

Study design for the two-season, randomized and controlled field-based trial of Australian sea lion pups at Seal Bay Conservation Park, Kangaroo Island, South Australia. Pups were recruited in consecutive breeding seasons (at all fieldtrips except the last of each season) and recaptured up to three times at subsequent monthly fieldtrips within the same season. Within two age cohorts (allocated by the standard length proxy to <70 and ≥70cm) pups were randomized to treatment or control groups. A third cohort of prepatent pups <14 d old at recruitment was identified *a posteriori*. The median period length [and range] in days prior to recapture is shown for the combined age cohorts. ^aSixteen pups (n=15, 2019; n=1, 2020–1) >75cm in standard length were recruited prior to implementing the 75 cm upper size restriction and were retained in the analysis.

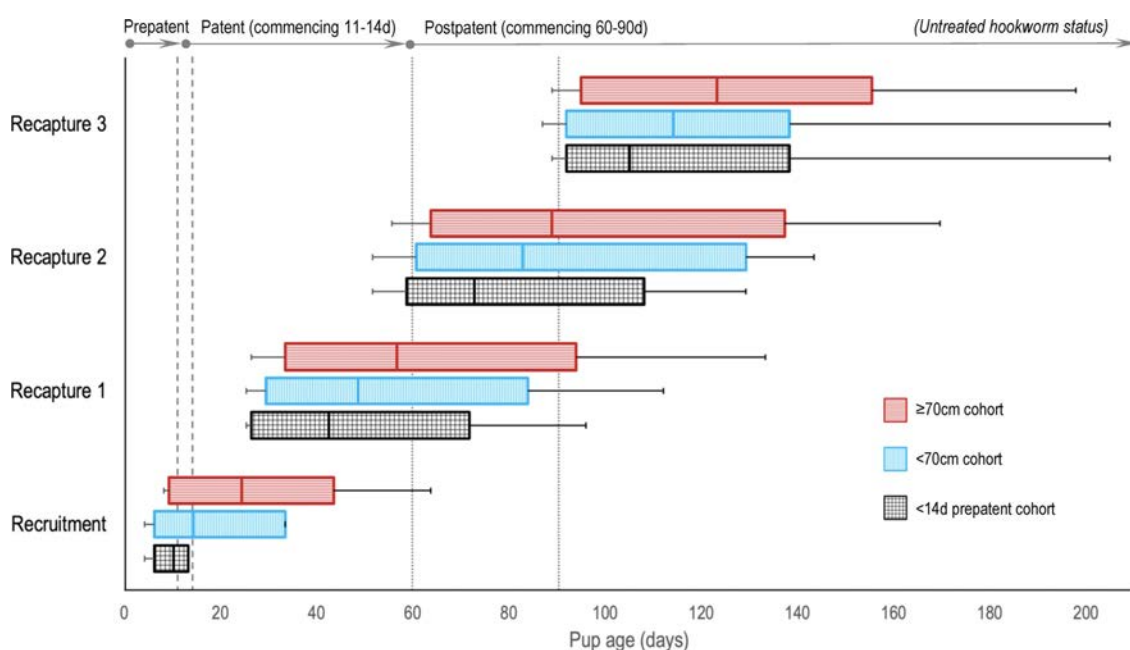


FIGURE 2

A comparison of Australian sea lion pup ages at recruitment and recaptures for the three age cohorts, relative to anticipated hookworm status in untreated pups. Patent hookworm infection develops from approximately 11–14 d of age (patency onset range – indicated by the vertical dashed lines), and infection is naturally cleared from approximately 60–90 d of age (patency termination range – indicated by the vertical dotted lines) (20). Boxes indicate the interquartile range for pup age with the median value marked (most distributions were right skewed), and the horizontal lines represent pup age range (minimum to maximum).

pups aged 8–10 d old at recruitment; two postpatent pups 40 d and 49 d old at recapture 1; six patent pups aged 92–124 d at recapture 2; and five patent pups aged 97–148 d at recapture 3. A total of 162 ($n=78$, 2019; $n=84$, early 2020–1) pups were allocated to receive the topical ivermectin treatment; 160 pups ($n=80$, 2019; $n=80$, early 2020–1) received no treatment.

Pup recruitment season, demographics and parasitic burden data are reported in Table 1, and pup recruitment hematology values are reported in Table 2. At recruitment, there were no significant differences for season, sex, age and size measures, and parasitic burdens, nor for any of the hematological variables, between treatment groups within any of the age cohorts (including within the <14 d cohort; $p \geq 0.05$; Tables 1, 2). This is consistent with successful random allocation at recruitment. Median period length [and range] for the combined age cohorts for recruitment-to-first recapture (31 d [15–111 d]), first recapture-to-second recapture (31 d [20–99 d]), second recapture-to-final recapture (33 d [20–99 d]) and recruitment-to-final recapture (95 d [76–195 d]) periods was similar for each of the individual age cohorts. Excluding dead pups, the proportion of pups with a first, second and third recapture for both seasons combined were 96% ($n=291$), 87% ($n=254$), and 66% ($n=194$), respectively. Where patent hookworm status was determined after death, five of 10 control pups and one of seven treated pups were positive. The positive treated pup (post-mortem smears totaling two hookworm

ova over three slides) was found dead from conspecific trauma 3 days post-recruitment (recruitment smears >30 hookworm ova per slide), with heavy scavenging and marked post-mortem autolysis consistent with death >1–2 days earlier. No deaths in any of the treated pups were attributable to the topical ivermectin application.

3.2. Treatment effect on estimated hookworm prevalence and incidence of clearance

Patent hookworm infection was not detected in any treated pup at any live recapture. Regardless of pup age cohort, topical ivermectin significantly reduced hookworm prevalence at first recapture by at least 98%, the estimated relative risk reduction ($p \leq 0.001$; Table 3). No evidence of interaction between treatment effectiveness (based on estimated hookworm prevalence) and season for any age cohort could be found. However, season 2020–1 had significantly higher predicted hookworm prevalence at first recapture than season 2019 in the <14 d (51.1% [46.7–55.5%] versus 35.1% [23.7–46.4%]; $p=0.043$) and <70 cm (46.4% [42.7–50.1%] versus 35.6% [29.2–42.0%]; $p=0.015$) age cohorts. These findings coincided with younger median pup age [and range] at first recapture for season 2020–1 versus 2019 in the <14 d (39 d [25–95 d] versus 47 d [33–71 d]) and <70 cm (46 d

TABLE 1 Recruitment season, demographics, and health details for treatment groups of Australian sea lion pups for each age cohort.

Variable	Prepatent pups <14 d old ($n=99$)				Pups <70 cm standard length ($n=196$)				Pups ≥ 70 cm standard length ($n=126$)			
	Control		Treatment		Control		Treatment		Control		Treatment	
	n	Estimate	n	Estimate	n	Estimate	n	Estimate	n	Estimate	n	Estimate
Recruitment season												
2019	18	36.7%	21	42.0%	42	43.3%	40	40.4%	38	60.3%	38	60.3%
2020–1	31	63.3%	29	58.0%	55	56.7%	59	59.6%	25	39.7%	25	39.7%
Sex												
Male	24	49.0%	33	66.0%	40	41.2%	47	47.5%	37	58.7%	41	65.1%
Female	25	51.0%	17	34.0%	57	58.8%	52	52.5%	26	41.3%	22	34.9%
Age and size measures												
Age – median (range) days ^{a,b}	49	10 (4–13)	50	10 (5–13)	97	14 (4–33)	99	13 (5–33)	63	24 (9–63)	63	24 (8–47)
Standard length – mean (range) cm	49	66.4 (59.0–73.5)	50	67.1 (61.0–75.0)	97	66.4 (59.0–69.5)	98 ^c	66.2 (59.0–69.5)	63	73.3 (70.0–82.0)	63	72.8 (70.0–78.0)
Body weight – mean (range) kg	49	8.6 (5.6–11.2)	50	8.8 (5.8–11.6)	97	8.7 (5.6–12.8)	99	8.8 (5.6–11.7)	63	11.5 (7.4–17.0)	63	11.6 (9.0–14.6)
Body mass index – mean (range)	49	28.0 (21.4–34.4)	50	27.9 (20.6–36.8)	97	28.4 (21.4–36.8)	98 ^c	29.0 (20.6–38.4)	63	28.3 (20.4–41.9)	63	29.2 (24.3–35.5)
Parasitic burden												
Hookworm prevalence – count (%)	49	0.0 (0.0%)	50	0.0 (0.0%)	97	53 (54.6%)	96 ^c	51 (53.1%)	63	57 (90.5%)	63	54 (85.7%)
Lice prevalence – count (%)	49	13 (26.5%)	50	14 (28.0%)	97	34 (35.1%)	99	35 (35.4%)	63	31 (49.2%)	63	33 (52.4%)
Lice intensity score – median (range) ^{a,d}	49	0 (0–3)	50	0 (0–3)	97	0 (0–4)	99	0 (0–3)	63	0 (0–6)	63	1 (0–6)

Among the prepatent pups <14d old (negative fecal hookworm smears), 86 were part of the <70 cm standard length cohort and 13 were part of the ≥ 70 cm cohort. All within age cohort comparisons were deemed non-significant at the 5% level.

^aStrong evidence of the continuous variable not being normally distributed – treatment group equalities were compared using non-parametric statistical test (Wilcoxon rank-sum test).

^bPredicted age used where birth date was missing.

^cVariation from stated n -values due to absence of parameter assessment at recruitment: standard length (and body condition index) was not determined in one treated pup <70 cm in season 2019; patent hookworm infection was not determined in three treated pups <70 cm (one in season 2019 and two in season 2020–1).

^dLice intensity score is a subjective assessment of lice infestation intensity, ranging from 0 (no lice) to a maximum of 9.

TABLE 2 Recruitment median values (and ranges) of hematological parameters for treatment groups of Australian sea lion pups for each age cohort.

Variable [mean (range)]	Prepatent pups <14 d old (n =99)				Pups <70 cm standard length (n =196)				Pups ≥70 cm standard length (n =126)			
	Control		Treatment		Control		Treatment		Control		Treatment	
	n	Estimate	n	Estimate	n	Estimate	n	Estimate	n	Estimate	n	Estimate
PCV (L/L)	43	0.400 (0.270–0.520)	45	0.400 (0.310–0.520)	89	0.360 (0.210–0.520)	92	0.360 (0.210–0.520)	60	0.345 (0.200–0.440)	58	0.340 (0.190–0.460)
RBC (x10 ¹² /L)	83	3.84 (1.64–5.00)	89	3.74 (1.98–5.52)	83	3.84 (1.64–5.00)	89	3.74 (1.98–5.52)	57	3.70 (2.80–4.78)	55	3.64 (2.94–4.60)
Hemoglobin (g/L)	83	126.0 (70.0–172.0)	89	128.0 (68.0–192.0)	83	126.0 (70.0–172.0)	89	128.0 (68.0–192.0)	57	122.0 (98.0–166.0)	55	118.0 (78.0–152.0)
Reticulocytes (10 ⁹ /L)	79	35.6 (3.9–269.4)	88	28.3 (0.0–234.7)	79	35.6 (3.9–269.4)	88	28.3 (0.0–234.7)	55	62.4 (3.9–218.4)	54	70.3 (17.2–182.7)
nRBC (10 ⁶ /L)	81	55.0 (0.0–1280.9)	86	50.8 (0.0–1212.1)	81	55.0 (0.0–1280.9)	86	50.8 (0.0–1212.1)	57	89.5 (0.0–891.5)	52	136.3 (0.0–1544.0)
Platelets (10 ⁹ /L)	80	351.0 (46.0–854.0)	86	346.0 (80.0–896.0)	80	351.0 (46.0–854.0)	86	346.0 (80.0–896.0)	57	416.0 (116.0–828.0)	54	446.0 (68.0–702.0)
WBC (10 ⁹ /L)	82	11.84 (4.58–25.26)	88	12.16 (5.27–25.00)	82	11.84 (4.58–25.26)	88	12.16 (5.27–25.00)	56	11.71 (5.41–24.89)	54	11.34 (6.93–21.26)
Neutrophils (10 ⁹ /L)	82	6.35 (1.70–20.00)	88	7.10 (1.00–18.7)	82	6.35 (1.70–20.00)	88	7.10 (1.00–18.7)	57	5.90 (1.30–18.3)	54	5.75 (1.80–12.2)
Lymphocytes (10 ⁹ /L)	83	3.20 (1.60–6.40)	89	3.20 (0.70–7.10)	83	3.20 (1.60–6.40)	89	3.20 (0.70–7.10)	57	3.70 (1.40–7.1)	54	3.45 (1.40–6.90)
Monocytes (10 ⁹ /L)	82	0.60 (0.00–1.90)	89	0.60 (0.10–1.90)	82	0.60 (0.00–1.90)	89	0.60 (0.10–1.90)	57	0.60 (0.10–1.70)	53	0.60 (0.10–1.40)
Eosinophils (10 ⁹ /L)	82	0.80 (0.00–4.40)	88	0.80 (0.0–4.20)	82	0.80 (0.00–4.40)	88	0.80 (0.0–4.20)	56	1.3 (0.20–4.80)	52	1.45 (0.40–3.40)
Total plasma protein (g/L)	89	61.0 (42.0–83.0)	91	60.9 (40.0–82.0)	89	60.0 (42.0–83.0)	91	60.0 (40.0–82.0)	60	62.0 (46.0–92.0)	58	63.0 (48.0–92.0)

Among the prepatent pups <14d old (negative fecal hookworm smears), 86 were part of the <70 cm standard length cohort and 13 were part of the ≥70 cm cohort. All within age cohort comparisons were deemed non-significant at the 5% level. There was strong evidence for all variables being non-normally distributed. Median value and range are therefore reported, and treatment group equalities were compared using non-parametric statistical test (Wilcoxon rank-sum test). PCV: packed cell volume; RBC: total red blood cell count; WBC: total white blood cell count; nRBC: nucleated cell blood cell count.

TABLE 3 Adjusted effectiveness of topical ivermectin treatment in Australian sea lion pups for each age cohort using either the hookworm prevalence at first recapture or incidence of hookworm clearance during the recruitment-to-first recapture period.

Recruitment age cohort	Hookworm prevalence or clearance estimate (95%CI) ^e				Effect estimate (95%CI)		
	n	Control	n	Treatment	p-value	Odds ratio	Treatment effect ^a
Hookworm prevalence at first recapture							
Prepatent pups <14d old ^b	43	85.5% (76.0–94.9%)	41	1.4% (0.0–5.2%)	<0.001	0.002 (0.000–0.003)	+98.4% (94.5–100.0%)
Pups <70 cm standard length	83	84.5% ^c (78.0–91.1%)	86	1.2% ^c (0.0–3.5%)	0.001	0.002 (0.000–0.004)	+98.6% (96.1–100.0%)
Pups ≥70 cm standard length	59	81.3% (72.3–90.3%)	61	0.9% (0.0–3.4%)	<0.001	0.002 (0.000–0.004)	+98.9% (96.3–100.0%)
Incidence of hookworm clearance during the recruitment-to-first recapture period ^d							
Pups <70 cm standard length	45	18.4% (9.5–27.2%)	47	99.1% (96.7–100.0%)	<0.001	0.002 (0.000–0.004)	+98.9% (96.4–100.0%)
Pups ≥70 cm standard length	53	19.6% (9.8–29.3%)	52	98.9% (96.1–100.0%)	<0.001	0.003 (0.000–0.004)	+98.7% (95.7–100.0%)

Prepatent pups (<14d old and negative fecal hookworm smears at recruitment) were part of the other two cohorts defined by standard length alone. Age at recruitment (median [range]) for the <14d prepatent, <70 cm standard length and ≥70 cm standard length pup cohorts were 10d [4–13d], 14d [4–33d], and 24d [8–63d], respectively. Recruitment-to-first recapture period length (median [range]) for the same three age cohorts were 32d [16–86d], 31d [15–94d], and 31d [16–111d], respectively.

^aThe adjusted effect estimate was calculated using the relative risk reduction associated with treatment versus control.

^bAs these pups were prepatent at recruitment, the prevalence at first capture corresponds to the incidence of infection until first recapture.

^cThe final model includes a significant interaction between treatment and pup age. The reported estimates correspond to the prevalence of an average aged pup in either treatment group.

^dTreatment effectiveness for the incidence of clearance (recruitment-to-first recapture period) calculation in pups with patent infection at recruitment used the predicted proportion of patent infection at recapture (the complement of clearance from recruitment-to-first recapture).

^eHookworm prevalence and incidence estimates were calculated using Firth's variant of the multifactorial logistic regression. The base additive model included treatment group, season, and pup age at first recapture. Ancillary factors assessed for significance were sex; bodyweight, standard length, and body mass index at first recapture; first recapture fieldtrip; and the recruitment-to-first recapture period length.

[25–103 d] versus 50 d [33–111 d]) cohorts. For the <70 cm cohort, there was a significant interaction between treatment effectiveness and age at first recapture, whereby a significant treatment benefit was no longer supported by 80 d of age at first recapture (coinciding with the natural elimination of hookworm in control pups). In pups with patent hookworm infection at recruitment, treatment also significantly increased hookworm clearance before first recapture by at least 98% ($p < 0.001$; Table 3). As expected, given natural hookworm clearance at 2–3 months of age (20), older age at first recapture was marginally

associated with increased hookworm clearance for the two older <70 cm (OR 1.13 [1.00–1.28]; $p = 0.047$), and ≥70 cm (OR 1.05 [1.00–1.11]; $p = 0.042$) age cohorts.

3.3. Treatment effect on growth

During the recruitment-to-first recapture period, topical ivermectin treatment resulted in higher growth rates based on changes

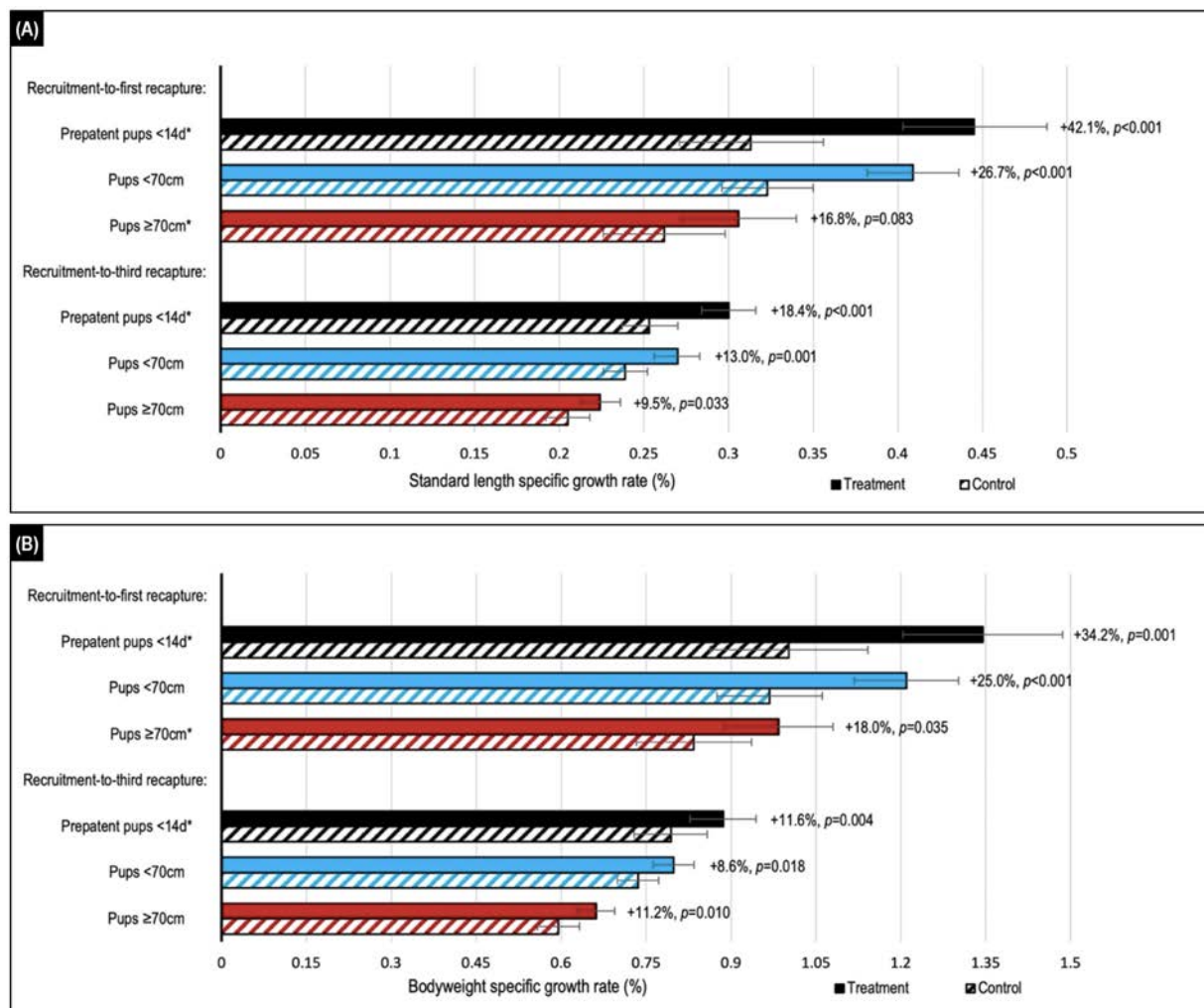


FIGURE 3

Comparison of estimated median values for (A) standard length and (B) bodyweight daily specific growth rates (SGRs) in Australian sea lion pups after either topical ivermectin application and early hookworm elimination (treated), or no treatment (control). Treatment effect (calculated as the relative difference in daily SGR between treatment and control groups) and p -value are shown to the right of the treatment bars. Stratification by age showed the highest relative benefits in the youngest <14d age cohort of prepatent pups. Assessment across the recruitment-to-first recapture (median 31 d, range: 15–111 d) and recruitment-to-third recapture (median 95 d, range: 76–195 d) periods showed the greatest benefits in the month immediately after treatment and persistence of benefits to 3 months post-treatment for all age cohorts. Daily SGRs were calculated as the natural log of each morphometric at the start of the period, minus that at the end of the period, divided by period length in days. Error bars indicate the estimated 95% confidence intervals. *A significant interaction between treatment and follow-up period length in days was apparent, therefore estimates are reported for the daily SGR at the median length of the follow-up period.

in standard length and bodyweight, reaching significance ($p \leq 0.035$) for all age cohort comparisons, excepting standard length in the ≥ 70 cm cohort ($p \leq 0.083$; Figure 3; Supplementary Table S1). The youngest <14 d cohort benefited the most from treatment, with a relative increase in bodyweight and standard length growth of approximately 35–40% compared to the <70 cm (approximately 25%) and ≥ 70 cm (approximately 18%) cohorts for the same period. The treatment benefit decreased after the first recapture, and while mostly trending positive it was usually not significant in the subsequent follow-up periods. An approximately 10–15% overall beneficial impact of treatment persisted for the entire follow-up period (recruitment-to-third recapture) for all age cohorts, with the highest relative benefit seen in the youngest <14 d cohort. There was no significant interaction of season with treatment when assessing growth ($p > 0.05$).

3.4. Treatment effect on hematological parameters

3.4.1. Red blood cell and platelet counts

Comparison of temporal changes in predicted mean (or median) values of hematological parameters likely to be impacted by hookworm disease is shown in Figures 4, 5 (refer to Supplementary Table S2 for individual parameter values). The parameters indicative of red blood cell number, and therefore anemia (PCV, RBC count and Hb concentration; Figures 4A–C), showed similar trends, exemplified by the PCV. At recruitment, PCV was highest for the <14 d cohort and lowest for the ≥ 70 cm cohort. In the <14 d and <70 cm cohorts, levels declined for both treatment groups in the recruitment-to-first recapture period, but to a significantly

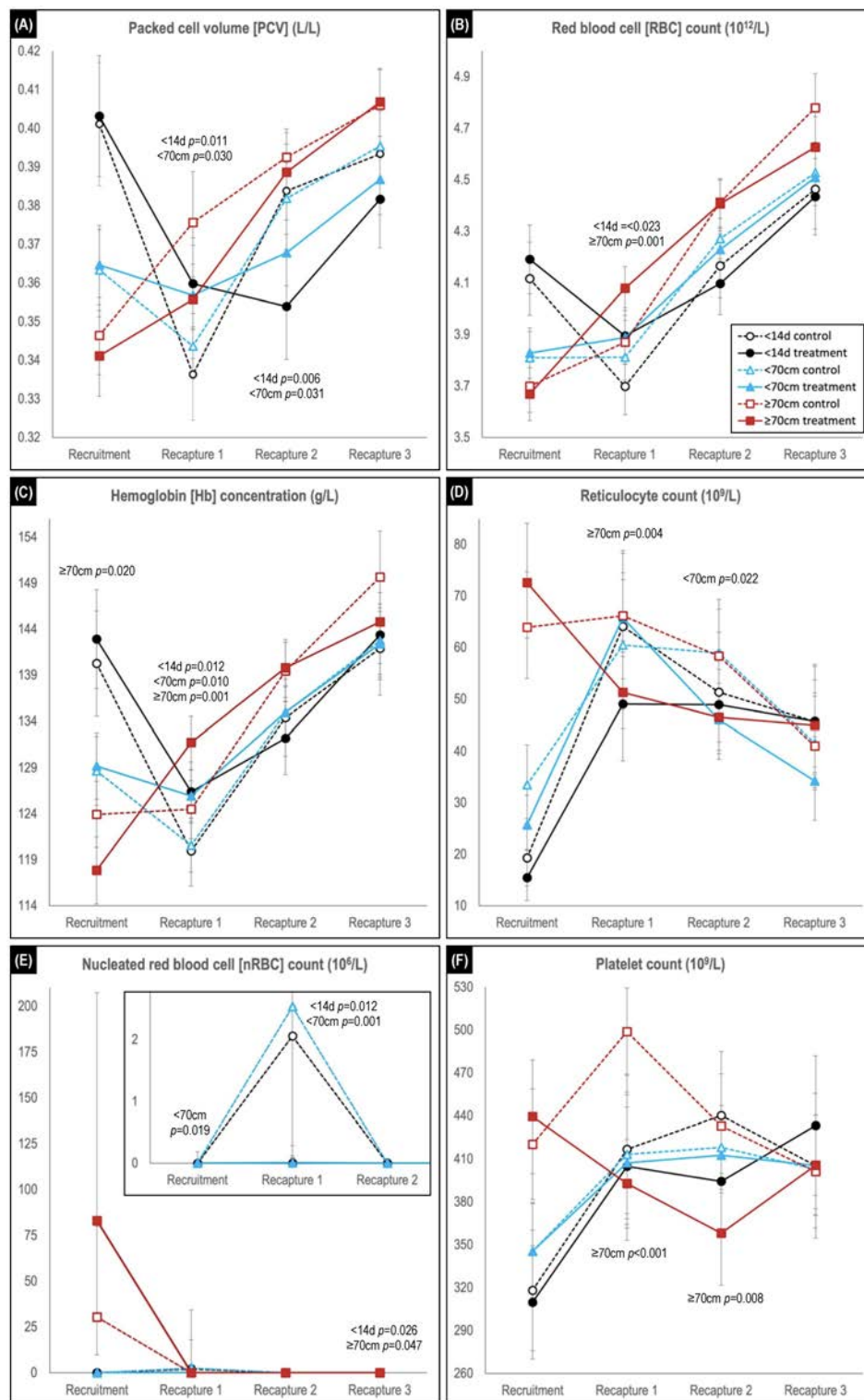


FIGURE 4

Comparison of predicted mean or median* values (and 95% confidence intervals) for (A) packed cell volume, (B) red blood cell count, (C) hemoglobin concentration, (D) reticulocyte count, (E) nucleated red blood cell count, and (F) platelet count in hookworm treated and control groups of Australian sea lion pups. Results are stratified by pup recruitment age (size) cohorts – <14 d prepatent, <70 cm standard length and ≥ 70 cm standard length. Significant between treatment group differences within any of the three age cohorts are listed adjacent to the respective capture. Differences between treatment groups within the same age cohort quantify the impact of hookworm infection, which is superimposed on temporal changes attributable to age-related hematological ontogenesis in maturing pups. The median interval (and range) for the recruitment-to-recapture 1, recapture 1-to-recapture 2, and recapture 2-to-recapture 3 periods for the combined cohorts were 31 d (15–111 d), 31 d (20–99 d), and 33 d (20–99 d), respectively. E shows a magnification of the results for the <14 d and <70 cm cohorts for the first 3 captures. *Transformed model estimates and their associated 95% CI boundaries were back transformed prior to reporting and should be interpreted as medians rather than means (refer to [Supplementary Table S2](#) for specific detail values of transformed models).

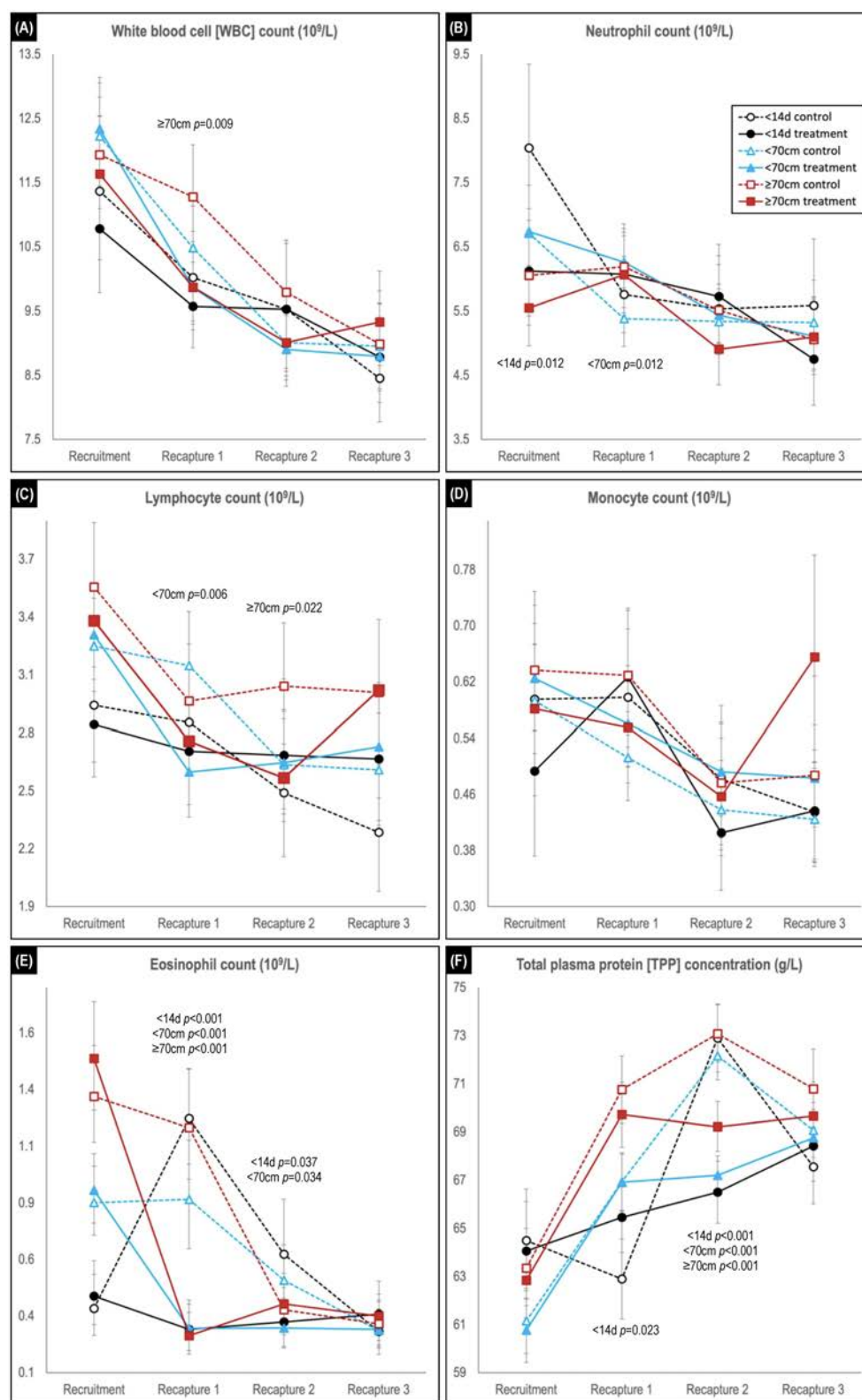


FIGURE 5

Comparison of predicted mean or median* values (and 95% confidence intervals) for (A) white blood cell, (B) neutrophil, (C) lymphocyte, (D) monocyte, and (E) eosinophil counts, and for (F) total plasma protein concentration in hookworm treated and control groups of Australian sea lion pups. Results are stratified by pup recruitment age (size) cohorts – <14 d prepatent, <70 cm standard length, and ≥ 70 cm standard length. Significant between treatment group differences within any of the three age cohorts are listed adjacent to the respective capture. Differences between treatment groups within the same age cohort quantify the impact of hookworm infection, which is superimposed on temporal changes attributable to hematological ontogenesis in maturing pups. The median interval (and range) for the recruitment-to-recapture 1, recapture 1-to-recapture 2, and recapture 2-to-recapture 3 periods for the combined cohorts were 31 d (15–111d), 31 d (20–99 d), and 33 d (20–99 d), respectively. *Transformed model estimates and their associated 95% CI boundaries were back transformed prior to reporting and should be interpreted as medians rather than means (refer to [Supplementary Table S2](#) for specific detail values of transformed models).

greater degree for untreated pups (<14 d cohort $p=0.011$; <70 cm cohort $p=0.030$), followed by increases in the subsequent two periods for these age cohorts (as occurred across all follow-up periods in the ≥ 70 cm cohort). For the <14 d cohort, the relative decline in PCV between recruitment and first recapture in untreated pups was approximately 50% greater than in the treated pups (16.1% vs. 10.8%, respectively). Likewise, untreated pups showed 43% and 25% greater declines in RBC count (10.1% versus 7.1%) and Hb concentration (14.5% and 11.6%), respectively. In the first-to-second capture period, the increase in PCV of the control group exceeded that of the treated group, such that significantly higher values ($p=0.006$ and $p=0.031$, respectively) were apparent for the <14 d and <70 cm cohorts at the end of the period. A similar non-significant trend for higher PCV in the control group of the oldest ≥ 70 cm cohort was also apparent in the recruitment-to-first recapture and first-to-second recapture periods. By trial conclusion, indicators of red cell number were frequently highest in the ≥ 70 cm cohort and lowest in the <14 d cohort. The greater rebound in red cell number in control groups suggested by PCV was less apparent in Hb concentration and RBC count.

Reticulocyte counts at recruitment were lowest for the <14 d cohort and highest for the ≥ 70 cm cohort (Figure 4D). In the recruitment-to-first recapture period, counts subsequently increased for the <14 d and <70 cm cohorts and remained stable or decreased for the ≥ 70 cm cohorts, reaching similar levels across all age cohorts by study conclusion. While counts at first and second recapture were most commonly lower in treated groups, significant between group differences were limited to lower counts in the treated group at first recapture for the ≥ 70 cm cohort ($p=0.004$) and at second recapture for the <70 cm cohort ($p=0.022$). For nRBC count (Figure 4E), counts at first recapture were significantly higher in control versus treated groups for the <14 d ($p=0.012$) and <70 cm ($p=0.001$) cohorts (noting the latter cohort's absence of random allocation at recruitment when using multifactorial modelling rather than simple direct between group comparison). This difference was not apparent for both control and treated pups at recruitment in the ≥ 70 cm cohort.

At recruitment, platelet count was lowest in the <14 d cohort and highest in the ≥ 70 cm cohort (Figure 4F). For the two youngest cohorts (<14 d and <70 cm), increasing counts in the treated groups in the recruitment-to-first recapture period paralleled those in the control group, before plateauing in the first-to-second recapture period – a change less apparent in the respective control groups. In the ≥ 70 cm cohort by contrast, lower platelet counts in the treated pups were significant at the first ($p<0.001$) and second ($p=0.008$) recaptures, prior to approximating those of the control group (and other cohorts) by the study conclusion.

3.4.2. White blood cell counts

Across the study, a trend for decreasing WBC count was observed in all age cohorts, characterized usually by declines in each of the individual leukocytes (Figures 5A–E). Within each age cohort, counts tended to be lower in the treated groups, although significance was apparent only for the ≥ 70 cm cohort at first recapture ($p=0.009$). For the individual leukocytes at recruitment, neutrophil counts were generally highest, and lymphocyte and eosinophil count lowest in the youngest <14 d age cohort, while monocyte counts did not show a clear delineation. For neutrophil, lymphocyte, and monocyte counts, significant between treatment

group differences at recaptures were limited to higher neutrophil count in treated pups of the <70 cm cohort at first recapture ($p=0.0012$), and lower lymphocyte count in treated pups for the <70 cm cohort at first recapture ($p=0.006$) and ≥ 70 cm cohort at second recapture ($p=0.022$). In contrast, in the recruitment-to-first recapture period across all age cohorts, eosinophil counts in treated pups decreased significantly ($p<0.001$) compared with control pups – immediately declining to a level comparable with the eventual control group baseline (Figure 5E). These decreases contrasted with the changes observed in the control groups – a rapid increase in the <14 d cohort, a plateau in the <70 cm cohort and a lesser decline in the ≥ 70 cm cohort. At second recapture, eosinophil levels remained significantly higher in the control groups of the <14 d ($p=0.037$) and <70 cm ($p=0.034$) age cohorts, before declining to comparable levels with the treated animals at third recapture.

3.4.3. Total plasma protein concentration

In the recruitment-to-first recapture period, the treated group TPP concentration increased in parallel with that of the control group for the <70 and ≥ 70 cm cohorts, but not for the <14 d cohort [in which the control group showed a significant initial decline in TPP ($p=0.023$; Figure 5F)]. In the subsequent second-to-third recapture period, a similar (delayed) increase for the younger <14 d cohort's control group was not paralleled by its treated group, and levels also plateaued in the treated groups of the other two age cohorts. Consequently, at second recapture TPP concentration was similar across all three age cohort control groups, and significantly higher than for the respective treated groups ($p<0.001$ for all age cohorts). By study conclusion there were no significant differences between treated and control groups for TPP concentration in any age cohort.

Significant interactions between season and treatment were only seen in a small number of comparisons (5.6%; $n=144$), half of which were at recruitment – WBC, neutrophil and monocyte counts in the <14 d cohort and nRBC count at recruitment in the <70 cm cohort. The other four significant interactions were at recapture (3.7%; $n=108$; post-treatment) – PCV and nRBC count at second recapture for the <14 d cohort; reticulocyte count at second recapture for the <70 cm cohort; and monocyte count at third recapture for the ≥ 70 cm cohort.

4. Discussion

Hookworm infection is an impactful endemic disease of Australian sea lion pups – occurring in all pups, contributing to substantial morbidity (22), and having a significant positive correlation with colony pup mortality based on infection intensity (20). By addressing the logistical limitations of previous hookworm treatment trials in the Australian sea lion, this study suggests a growth benefit following early hookworm elimination and better defines the hematological changes attributable to hookworm infection. Growth benefits were greatest in the month immediately post-treatment, persisted for at least 3 months, and were optimal for pups treated prior to patent infection (11–14 d of age). Similarly, early hookworm elimination resulted in immediate hematological benefits with reduced systemic eosinophilic inflammation and elimination of the decline in RBC count associated with patent infection. Importantly, the benefits of hookworm elimination on growth and health parameters were independent of season.

4.1. Treatment effect on hookworm prevalence and incidence

The estimated effectiveness of topical ivermectin approaching 100% for elimination of hookworm infection in Australian sea lion pups further supports findings of a prior trial of this formulation (21). Treatment was effective as a single application in pups at any age – including those with prepatent disease – and was well tolerated, with no death attributed to its application. The effectiveness of topical ivermectin parallels that of the injected formulation, evaluated in earlier Australian sea lion studies (21, 24) and in studies in other pinniped species (25, 26, 32–34). The present study also demonstrated topical ivermectin's benefits across lower and higher mortality breeding seasons. Seasonal differences in hookworm prevalence (significantly higher in season 2020–1) and incidence of clearance (trending lower in season 2020–1) are explained by the recruitment of a younger cohort in season 2020–1 – more untreated animals still of patent infection age (approximately 11–60 days) at first recapture. Similarly, the natural clearance of hookworm infection in untreated pups from approximately 60–90 days explains the progressive decline of treatment effectiveness with increasing pup age at the post treatment assessment (first recapture).

4.2. Treatment effect on growth

In contrast to previous studies in the Australian sea lion, this larger scale study provided evidence of a significant treatment-related growth benefit that was both immediate and persisted for at least 3 months. Demonstration of a growth benefit following early hookworm elimination in pups is consistent with observations in other seal species, including those in the northern fur seal (*Callorhinus ursinus*) (26), New Zealand sea lion (*Phocarcos hookeri*) (25), and South American fur seal (35). In the earlier treatment trials in Australian sea lion pups, a significant growth benefit was either not apparent (24) or only observed for a short term (15–24 d post-treatment) (21). In a more recent study of New Zealand sea lions, trends for treatment-related improvement in weight and length were also detected, although these differences were not significant (34). Differences between the present study and others may reflect differences in sample size, methodology for assessing growth, species-specific host and parasite attributes, and age of treatment and recapture (21). Multifactorial regression of daily SGR (for two parameters of growth) was favored in the present study to account for the potential effect of pup age at treatment, including baseline morphometrics, as well as follow-up period length.

Key growth-related outcomes unique to this study highlighted optimization of treatment benefit by treating pups prior to infection patency. Notably, even a 4 days increase in median recruitment age and inclusion of pups with patent disease (comparing the <70 cm and <14 d cohorts) decreased treatment benefit due to limiting juvenile (L4 stage larvae) parasite-associated damage to the intestines and subsequent nutrient deprivation, and preventing any disease caused by adult parasites. In comparison, pups in the oldest age cohort (median recruitment age 24 days) had experienced a median of approximately 2 weeks of patent disease prior to treatment. Importantly, this study demonstrated persistence of significant growth benefits beyond the age (approximately 60 days) (20) that untreated pups begin to naturally eliminate hookworm infection – again, even

for the oldest age cohort. This suggests the potential for persistence of a treatment-related growth benefit up to weaning (18 months of age), and possibly into the juvenile and adult life stages. Such an outcome could have substantial implications for longer term health, reproductive fitness, and survival – key objectives of infection control in Australian sea lion pups. Multiple studies have confirmed a significant positive correlation between pup size (weight, body condition) at weaning and post-weaning survival, including in grey (*Halichoerus grypus Fabricius 1791*) (36, 37) and harbour (*Phoca vitulina*) (38) seals to one year of age, and Hawaiian monk (*Monachus schauinslandi*) (39) and Northern fur (*Callorhinus ursinus*) (40) seals to 2 years of age. Additionally, only one treatment comparison in this study demonstrated a higher growth rate in the untreated group (bodyweight in the second recapture-to-third recapture period for the <14 d cohort; $p=0.029$), providing little support for compensatory growth (41) in untreated pups following natural hookworm elimination during the 3 month follow-up period. Using the commencement of hookworm elimination from 60 d of age, the median (range) length of the postpatent period monitored in the present study was up to 44 d (26–143 d), 53 d (26–143 d), and 62 d (28–136 d) for the <14 d, <70 cm, and ≥ 70 cm age cohorts, respectively – presumed sufficient for detection of compensatory growth given a significant treatment-related benefit from early hookworm elimination was apparent within a month. Ongoing monitoring of growth rates in Australian sea lions becomes logistically challenging with increasing age. However, the philopatric nature of the species means that persistent treatment-related growth and health benefits manifesting as increased longer-term survival could be assessed at the closely monitored Seal Bay colony.

4.3. Treatment effect on hematological parameters

Previous studies have developed hematological reference values for Australian sea lion pups with patent and post-patent hookworm infection (22), and defined the impact of patent infection by comparison with disease-free (anthelmintic treated) individuals (21). Based on these prior studies, regenerative anemia, hypoproteinemia or hyperproteinemia, and lymphocytic and/or eosinophilic inflammation have been associated with patent hookworm infection. In the present study, the extended 3 months follow-up period, stratification into three recruitment (and therefore treatment) age cohorts, inclusion of infection-free treated and control groups, and multifactorial modelling to account for the impact of non-treatment factors, all served to further explore the pathological impact of hookworm infection from physiological changes attributable to hematological ontogenesis.

4.3.1. Red blood cell and platelet counts

Detection of higher RBC count in younger pups, followed by an early post-natal decline, is consistent with previous observations in the Australian sea lion and other otariid species (22, 42, 43). Similar change is apparent in terrestrial mammals and attributed to a physiological decline associated with the rapid transition from a low (placental-based) to high (lung-based) oxygen environment at birth (44, 45). Mechanisms include decreased RBC production following the negative feedback from higher tissue oxygen saturation on erythropoietin (RBC stimulatory factor) production, a shortened

lifespan of fetal and neonatal RBCs versus adult RBCs, and the dilutionary effect of expanded blood volume associated with rapid perinatal growth [reviewed in (46, 47)]. The present study demonstrates an additional significant decline in RBC count in untreated versus treated pups (25–50%, depending on RBC parameter), quantifying the pathological contribution directly attributable to hookworm infection. Subsequent increases in RBC count in treated pups suggests the restoration of erythropoietin stimulation of RBC production in response to the physiological decline in hemoglobin levels (46). Untreated pups demonstrate a significantly greater (second recapture PCV: $p = 0.006$ and $p = 0.001$ for <14 d and <70 cm cohorts, respectively) regenerative response from a comparatively more severe anemia resulting from the additional pathological contribution of hookworm infection. Higher reticulocyte and nRBC counts in the untreated pups at the time of the RBC nadir support an increased level of erythropoietin-driven RBC production. It remains to be determined if this rebound increase in RBC production is consequential to host–parasite co-evolution and clinically advantageous to pups – for instance, providing a survival benefit during this period. Given that pup mortality occurs mostly in the first month of life (19), which coincides with the initial significant RBC decline apparent in hookworm-infected pups, it is more likely that improvement in pup health resulting from early hookworm elimination will translate to improved pup survival.

Highest platelet counts at recruitment apparent in the older versus younger pups, and a treatment-independent increase in the first month post-recruitment in the younger pups, suggest initial platelet increases are driven by ontogenesis. The subsequent stabilization of platelet counts in younger treated pups to levels approximating those at the study conclusion, differed from further mild increases in the control group. Additionally, treated pups in the oldest ≥ 70 cm cohort showed significantly lower platelet counts compared with untreated pups for two months post-treatment. Both observations suggest a pathological contribution of hookworm to a relative platelet increase (thrombocytosis) during the latter patent and early post-patent period. Potential contributors to a thrombopoietin-driven reactive thrombocytosis include (1) a direct response to ongoing platelet utilization related to hookworm feeding and intestinal hemorrhage, and (2) an indirect response driven by other inflammatory cytokines released in response to hookworm-associated intestinal inflammation. The latter is supported by a temporal relationship with higher eosinophil and lymphocyte counts in the untreated pups at first and second recapture. These results are consistent with the role of platelets in chronic intestinal mucosal inflammation and coagulation [reviewed in (48)]. Further, they add to the outcomes of an earlier study in the Australian sea lion, in which platelets trended lower in treated versus control pups at 15–24 d post treatment and were similar by 36–41 d (21).

4.3.2. White blood cell counts

Consistent with findings in the Steller sea lion (*Eumetopias jubatus*) in the first month of life (49) and contrasting those in the South American fur seals in the first 2 months of life (42), WBC count in the Australian sea lion pups decreased in the first 3–4 months of life, independent of hookworm infection status. Decreasing counts attributable to ontogeny were typically characterized by a decrease in each of the individual WBCs – apart from the marked increase in eosinophil count attributed to the onset, and persisting for the duration of, patent hookworm infection. The close temporal

relationship between patent hookworm infection and systemic eosinophilic inflammation, is previously reported in treatment trials in otariid species (21, 24, 50). Treatment-related changes in the remaining WBCs were less significant, excepting the frequently higher lymphocyte counts in the untreated pups, also reported in those earlier studies. Significant between group differences in lymphocyte count coincided with mid-to late patent hookworm infection, indicating antigenic stimulation and a host immune response. This has been confirmed in an immunological study of hookworm infected Australian sea lions, showing a positive association of increasing pup age with B-lymphocyte count and immunoglobulin G (IgG; antibody) concentration across the period of hookworm patency (51). While there is the potential for early hookworm elimination to negatively impact lymphocyte ontogenesis, comparable lymphocyte counts in treated and control pups of the oldest two age cohorts by the study conclusion suggest any impact is likely to be short term.

4.3.3. Total plasma protein concentration

Results of this study, particularly for TPP concentration, highlight the importance of including age and other contributing factors in modelling of disease or treatment impact on pup hematological parameters – an outcome also evident in a recent disease investigation in the New Zealand sea lion (50). The declining TPP concentration in the recruitment-to-first recapture period in untreated pups of the youngest <14 d cohort contrasts with a mild increase in treated pups of that cohort, and more rapid increases in treated and untreated pups of the older two age cohorts. Treatment-independent increases in the older cohorts favor a physiological (ontogeny-related) increase, with a significant superimposed impact from hookworm infection in the youngest cohort (median [range] age at first recapture of 42 d [25–95 d] – mid-patency; time post-recruitment 32 d [16–86 d]). Lower TPP levels have previously been reported in Australian sea lion pups with patent, versus pre- or postpatent hookworm infection, and reasonably attributed to intestinal hemorrhage (in association with lower RBC counts) caused by the blood-sucking nature of the intestinal parasite (22). Lower TPP has also been reported with hookworm-infected (versus treated) New Zealand sea lion pups, albeit with an important age-related influence (50). In contrast, the two previous treatment trials in the Australian sea lion showed either significantly higher TPP concentrations in the untreated pups at 15–24 d (but not at 36–41 d) post-treatment (21), or a non-significant trend for the same outcome at 27–67 d post-treatment (24). These outcomes were suggested as reflective of positive acute phase inflammatory responses or the influence of unrelated factors (e.g., lice infestation). In the present study, a longer follow-up period identified significantly higher TPP concentrations ($p < 0.001$) in untreated pups of all three age cohorts at second recapture (combined median time [range] post recruitment 63 d [41–147 d]; ages 72 d [51–128 d], 82 d [51–142], and 88 d [55–168 d] for the <14 d, <70 cm, and ≥ 70 cm cohorts, respectively – all approximating the age of natural hookworm elimination). The above mentioned study of the immune response to hookworm infection in the Australian sea lion provides some explanation for the apparent discrepancies and suggests an inflammatory contribution to increased TPP concentration in untreated pups (51). In that study, the start of the patent period was marked by a peak in IL-6 (a pro-inflammatory cytokine) gene expression indicating early stage inflammation, followed initially by an increase in positive acute phase proteins (proteins increasing early in the inflammatory process), and then by

the increase in B-lymphocyte count and IgG production. Observations were consistent with an antihelminth inflammatory response, peaking at the time of natural hookworm elimination. Superimposed on this inflammatory contribution to TPP concentration was an age-related increase in albumin concentration, reaching a maximum in the postpatent period (51). This followed an initial decline in the very early stages of the inflammatory response (confirming albumin as a negative acute phase protein in the Australian sea lion). In the present study, therefore, is the potential for (1) the initial reduction in the TPP concentration in the youngest <14 d prepatent cohort to reflect intestinal protein loss and a negative acute phase response to the commencement of patent disease, and (2) the comparative plateauing observed in treatment groups at the time of natural hookworm elimination in untreated animals to indicate amelioration of the hookworm association inflammatory response. A reduction in TPP concentrations in untreated groups of all cohorts to levels comparable with those in the treated groups indicates an end to the hookworm-associated inflammatory response by the study conclusion – consistent with observations in the eosinophil count.

The few hematological parameters of health where a seasonal interaction with treatment outcome was detected statistically, were inconsistent across cohorts and not considered clinically relevant. Consequently, independent of whether treatment was administered in a lower or higher mortality season, early hookworm elimination provided immediate improvements in hematological analytes frequently impacted by hookworm disease. Ongoing monitoring of these individuals will indicate whether these improvements in health will translate into improved pup survival. The absence of significant differences in the hematological parameters of treated and untreated pups by study conclusion (nRBC count the exception, likely impacted by factors unrelated to hookworm disease) highlights the absence of any positive or negative longer term hematological alterations consequential to anthelmintic treatment in this species.

4.4. Conclusion

This study indicates that optimal growth and health outcomes from early hookworm elimination in the Australian sea lion are attained by treatment of pups in the prepatent period (first 2 weeks of age) and achievable in both lower and higher mortality breeding seasons. Intervention in pups of this age was readily achievable using an easily applied topical anthelmintic and offers a critical opportunity to mitigate the negative health impacts of an endemic disease contributing to substantial morbidity and mortality in this species. At an individual level, hookworm elimination improved pup health and therefore animal welfare. At a population level, the goal of intervention is to contribute to sustaining or increasing population size – addressing the high pup mortality in this species by improving overall pup health. Measuring the impact of hookworm infection to pup mortality and improving pup survival following early hookworm elimination are therefore two important next steps in the study of the endangered Australian sea lion.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by the University of Sydney Animal Ethics Committee (Protocol Number 2017/1260), The University of Sydney, Australia.

Author contributions

RG and SL contributed to conception and design of the study. MF, RG, and SL collected and processed samples. SL performed the blood film reviews. SL and RG organized the database. SL and CC performed the statistical analysis. SL wrote the first draft of the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This study was partly funded by The Hermon Slade Foundation (HSF 16-03), the FS Quiney and BR Richards Bequests (Sydney School of Veterinary Science, The University of Sydney: 2016), and the Department for Environment and Water, South Australia (DEW).

Acknowledgments

The authors thank Shannon Taylor, Matthew Gray, Clarence Kennedy, Tanya Rosewarne and the staff of the Department for Environment and Water (DEW), Seal Bay, Kangaroo Island (Melanie Stonnill, Melanie Stephens, and team) and the many volunteers for their fieldwork assistance. We also thank Janet Simpson for her logistical support. We appreciate the laboratory processing provided by staff of VPDS (University of Sydney) and VDL (University of Adelaide). Microchips referenced in this study were donated by Sure Petcare.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fvets.2023.1161185/full#supplementary-material>

References

- Russell RE, DiRenzo GV, Szymanski JA, Alger KE, Grant EHC. Principles and mechanisms of wildlife population persistence in the face of disease. *Front Ecol Evol.* (2020) 8:569016. doi: 10.3389/fevo.2020.569016
- O'Byrne AM, Lambourn DM, Rejmanek D, Haman K, O'Byrne M, Van Wormer E, et al. *Sarcocystis neurona* transmission from opossums to marine mammals in the Pacific northwest. *EcoHealth.* (2021) 18:84–94. doi: 10.1007/s10393-021-01536-w
- Roe WD, Rogers L, Pinpimai K, Dittmer K, Marshall J, Chilvers BL. Septicaemia and meningitis caused by infection of New Zealand Sea lion pups with a hypermucoviscous strain of *Klebsiella pneumoniae*. *Vet Microbiol.* (2015) 176:301–8. doi: 10.1016/j.vetmic.2015.01.019
- McDonald JL, Bailey T, Delahay RJ, McDonald RA, Smith GC, Hodgson DJ. Demographic buffering and compensatory recruitment promotes the persistence of disease in a wildlife population. *Ecol Lett.* (2016) 19:443–9. doi: 10.1111/ele.12578
- Shapiro K, Conrad PA, Mazet JA, Wallender WW, Miller WA, Largier JL. Effect of estuarine wetland degradation on transport of *Toxoplasma gondii* surrogates from land to sea. *Appl Environ Microbiol.* (2010) 76:6821–8. doi: 10.1128/AEM.01435-10
- Kovacs KM, Aguilar A, Auriolles D, Burkanov V, Campagna C, Gales N, et al. Global threats to pinnipeds. *Mar Mamm Sci.* (2012) 28:414–36. doi: 10.1111/j.1748-7692.2011.00479.x
- McLean LJ, George S, Ierodiaconou D, Kirkwood RJ, Arnould JPY. Impact of rising sea levels on Australian fur seals. *PeerJ.* (2018) 6:e5786. doi: 10.7717/peerj.5786
- McDonald JL, Robertson A, Silk MJ. Wildlife disease ecology from the individual to the population: insights from a long-term study of a naturally infected European badger population. *J Anim Ecol.* (2018) 87:101–12. doi: 10.1111/1365-2656.12743
- Restif O, Hayman DT, Pulliam JR, Plowright RK, George DB, Luis AD, et al. Model-guided fieldwork: practical guidelines for multidisciplinary research on wildlife ecological and epidemiological dynamics. *Ecol Lett.* (2012) 15:1083–94. doi: 10.1111/j.1461-0248.2012.01836.x
- Threatened Species Scientific Committee (2020). Conservation advice. Neophoca cinerea, Australian sea lion. Department of Agriculture, water and the environment, Canberra. [online]. Available at: www.environment.gov.au/biodiversity/threatened/species/pubs/22-conservation-advice-23122020.pdf [Accessed August 29, 2021].
- Goldsworthy SD, Shaughnessy PD, Mackay AI, Bailleul F, Holman D, Lowther AD, et al. Assessment of the status and trends in abundance of a coastal pinniped, the Australian sea lion *Neophoca cinerea*. *Endanger Species Res.* (2021) 44:421–37. doi: 10.3354/esr01118
- Campbell RA, Gales NJ, Lento GM, Baker CS. Islands in the sea: extreme female natal site fidelity in the Australian sea lion, *Neophoca cinerea*. *Biol Lett.* (2008) 4:139–42. doi: 10.1098/rsbl.2007.0487
- Lowther AD, Harcourt RG, Goldsworthy SD, Stow A. Population structure of adult female Australian sea lions is driven by fine-scale foraging site fidelity. *Anim Behav.* (2012) 83:691–701. doi: 10.1016/j.anbehav.2011.12.015
- Goldsworthy S, Stonnill M, Kirkwood R. (2021). Monitoring of the Australian sea lion population at Seal Bay: 2020/21. Report to the Department for Environment and Water. South Australian Research and Development Institute (aquatic sciences), Adelaide. SARDI publication no. F2014/000322-6. SARDI Research Report Series No. 1121. 30.
- Goldsworthy S.D., Lowther A.D., Shaughnessy P.D., McIntosh R., Page B. (2007). Assessment of pup production and the maternal investment strategies of the Australian sea lion *Neophoca cinerea* at dangerous reef in the 2006–07 breeding season. Report to the Department for the Environment & heritage, wildlife conservation fund, South Australia. South Australian Research and Development Institute (aquatic sciences), Adelaide. SARDI Publication No. F2007/000929-1. SARDI Research Report Series No. 249. pp. 58.
- Shaughnessy PD, Goldsworthy SD, Burch P, Dennis TE, Cooper P. Pup numbers of the Australian sea lion (*Neophoca cinerea*) at the PAGES Islands, South Australia, over two decades. *Aust J Zool.* (2013) 61:112–8. doi: 10.1071/ZO13015
- Trillmich F, Limberger D. Drastic effects of El Nino on Galapagos pinnipeds. *Oecologia.* (1985) 67:19–22. doi: 10.1007/BF00378445
- McIntosh R.R. (2007). Life history and population demographics of the Australian sea lion. Doctor of Philosophy, La Trobe University, Melbourne, Victoria.
- McIntosh RR, Kennedy CW. Morphology, sex ratio and cause of death in Australian sea lion (*Neophoca cinerea*) pups. *Aust Mammal.* (2013) 35:93. doi: 10.1071/AM12037
- Marcus AD, Higgins DP, Gray R. Epidemiology of hookworm (*Uncinaria stenocephala*) infection in free-ranging Australian sea lion (*Neophoca cinerea*) pups. *Parasitol Res.* (2014) 113:3341–53. doi: 10.1007/s00436-014-3997-3
- Lindsay SA, Caraguel CGB, Gray R. Topical ivermectin is a highly effective seal 'spot-on': a randomised trial of hookworm and lice treatment in the endangered Australian sea lion (*Neophoca cinerea*). *Int J Parasitol Parasites Wildl.* (2021) 16:275–84. doi: 10.1016/j.jippaw.2021.11.002
- Marcus AD, Higgins DP, Gray R. Health assessment of free-ranging endangered Australian sea lion (*Neophoca cinerea*) pups: effect of haematophagous parasites on haematological parameters. *Comp Biochem Physiol A Mol Integr Physiol.* (2015) 184:132–43. doi: 10.1016/j.cbpa.2015.02.017
- Marcus AD, Higgins DP, Slapeta J, Gray R. *Uncinaria stenocephala* sp. n. (Nematoda: Ancylostomatidae) from the endangered Australian sea lion, *Neophoca cinerea* (Carnivora: Otariidae). *Folia Parasitol.* (2014) 61:255–65. doi: 10.14411/fp.2014.037
- Marcus AD, Higgins DP, Gray R. Ivermectin treatment of free-ranging endangered Australian sea lion (*Neophoca cinerea*) pups: effect on hookworm and lice infection status, haematological parameters, growth, and survival. *Parasitol Res.* (2015) 114:2743–55. doi: 10.1007/s00436-015-4481-4
- Chilvers B, Duignan P, Robertson B, Castinel A, Wilkinson I. Effects of hookworms (*Uncinaria* sp.) on the early growth and survival of New Zealand Sea lion (*Phocarcos hookeri*) pups. *Polar Biol.* (2009) 32:295–302. doi: 10.1007/s00300-008-0559-0
- DeLong RL, Orr AJ, Jenkinson RS, Lyons ET. Treatment of Northern fur seal (*Callorhinus ursinus*) pups with ivermectin reduces hookworm-induced mortality. *Mar Mamm Sci.* (2009) 25:944–8. doi: 10.1111/j.1748-7692.2008.00274.x
- Gales NJ, Cheal AJ, Pobar GJ, Williamson P. Breeding biology and movements of Australian sea lions, *Neophoca cinerea*, off the west coast of Western Australia. *Wildl Res.* (1992) 19:405–16. doi: 10.1071/Wr9920405
- Shaughnessy PD, McIntosh RR, Goldsworthy SD, Dennis TE, Berris M. Trends in abundance of Australian sea lions, *Neophoca cinerea*, at Seal Bay, Kangaroo Island, South Australia. In *Sea Lions of the World*. Eds. AW. Trites, SK. Atkinson, DP. DeMaster, LW. Fritz, TS. Gelatt, LD. Rea, and KM. Wynne, *Sea Lions of the World* (2006) (pp 325–351). University of Alaska Fairbanks.
- McIntosh RR, Arthur AD, Dennis T, Berris M, Goldsworthy SD, Shaughnessy PD, et al. Survival estimates for the Australian sea lion: negative correlation of sea surface temperature with cohort survival to weaning. *Mar Mamm Sci.* (2013) 29:84–108. doi: 10.1111/j.1748-7692.2011.00558.x
- Seguel M, Montalva F, Perez-Venegas D, Gutierrez J, Paves HJ, Muller A, et al. Immune-mediated hookworm clearance and survival of a marine mammal decrease with warmer ocean temperatures. *elife.* (2018) 7:e38432. doi: 10.7554/eLife.38432
- Schulz KE, Altman DG, Moher D. CONSORT 2010 statement: updated guidelines for reporting parallel group randomized trials. *Ann Intern Med.* (2010) 152:726–32. doi: 10.7326/0003-4819-152-11-201006010-00232
- Beekman GK. Clinical use of ivermectin against the hookworm (*Uncinaria lucasi*) of Northern sea pups (*Callorhinus ursinus*). *Internat Assoc Aqua An Med Proc.* (1984) 15:50.
- Castinel A, Duignan PJ, Lyons ET, Pomroy WE, Gibbs N, Lopez-Villalobos N, et al. Epidemiology of hookworm (*Uncinaria* spp.) infection in New Zealand (Hooker's) sea lion (*Phocarcos hookeri*) pups on Enderby Island, Auckland Islands (New Zealand) during the breeding seasons from 1999/2000 to 2004/2005. *Parasitol Res.* (2007) 101:53–62. doi: 10.1007/s00436-006-0453-z
- Michael SA, Hayman DTS, Gray R, Roe WD. Risk factors for New Zealand Sea lion (*Phocarcos hookeri*) pup mortality: Ivermectin improves survival for conservation management. *Front Mar Sci.* (2021) 8:680678. doi: 10.3389/fmars.2021.680678
- Montalva F, Perez-Venegas D, Gutierrez J, Seguel M. The contrasting hidden consequences of parasitism: effects of a hematophagous nematode (*Uncinaria* sp.) in the development of a marine mammal swimming behavior. *Ecol Evol.* (2019) 9:3689–99. doi: 10.1002/ece3.4914
- Hall A, McConnell B, Barker R. The effect of total immunoglobulin levels, mass and condition on the first-year survival of Grey seal pups. *Funct Ecol.* (2002) 16:462–74. doi: 10.1046/j.1365-2435.2002.00649.x
- Hall AJ, McConnell BJ, Barker RJ. Factors affecting first-year survival in grey seals and their implications for life history strategy. *J Anim Ecol.* (2001) 70:138–49. doi: 10.1111/j.1365-2656.2001.00468.x
- Harding K, Fujiwara M, Axberg Y, Harkonen T. Mass-dependent energetics and survival in harbour seal pups. *Funct Ecol.* (2005) 19:129–35. doi: 10.1111/j.0269-8463.2005.00945.x
- Craig MP, Ragen TJ. Body size, survival, and decline of juvenile hawaiian monk seals, *Monachus schauinslandi*. *Mar Mamm Sci.* (1999) 15:786–809. doi: 10.1111/j.1748-7692.1999.tb00843.x
- Baker J, Fowler C. Pup weight and survival of Northern fur seals *Callorhinus ursinus*. *J Zool.* (2009) 227:231–8. doi: 10.1111/j.1469-7998.1992.tb04819.x
- Hector KL, Nakagawa S. Quantitative analysis of compensatory and catch-up growth in diverse taxa. *J Anim Ecol.* (2012) 81:583–93. doi: 10.1111/j.1365-2656.2011.01942.x
- Seguel M, Munoz F, Keenan A, Perez-Venegas DJ, DeRango E, Paves H, et al. Hematology, serum chemistry, and early hematologic changes in free-ranging South American fur seals (*Arctophoca australis*) at Guafu Island, Chilean Patagonia. *J Wildl Dis.* (2016) 52:663–8. doi: 10.7589/2015-11-293
- Trillmich F, Rea L, Castellini M, Wolf JBW. Age-related changes in hematocrit in the Galapagos Sea lion (*Zalophus wollebaeki*) and the Weddell seal (*Leptonychotes weddellii*). *Mar Mamm Sci.* (2008) 24:303–14. doi: 10.1111/j.1748-7692.2007.00177.x
- Ree IMC, Lopriore E. Updates in neonatal hematology: causes, risk factors, and management of anemia and thrombocytopenia. *Hematol Oncol Clin North Am.* (2019) 33:521–32. doi: 10.1016/j.hoc.2019.01.013

45. Rortveit R, Sævik BK, Eggertsdottir AV, Skancke E, Lingaas F, Thoresen SI, et al. Age-related changes in hematologic and serum biochemical variables in dogs aged 16–60 days. *Vet Clin Pathol.* (2015) 44:47–57. doi: 10.1111/vcp.12220
46. O'Brien RT, Pearson HA. Physiologic anemia of the newborn infant. *J Pediatr.* (1971) 79:132–8. doi: 10.1016/S0022-3476(71)80076-8
47. Widness JA. Pathophysiology of anemia during the neonatal period, including anemia of prematurity. *NeoReviews.* (2008) 9:e520;–e525. doi: 10.1542/neo.9-11-e520
48. Matowicka-Karna J. Markers of inflammation, activation of blood platelets and coagulation disorders in inflammatory bowel diseases. *Postepy Hig Med Dosw.* (2016) 70:305–12. doi: 10.5604/17322693.1199305
49. Keogh MJ, Maniscalco JM, Atkinson S. Steller sea lion (*Eumetopias jubatus*) pups undergo a decrease in circulating white blood cells and the ability of T cells to proliferate during early postnatal development. *Vet Immunol Immunopathol.* (2010) 137:298–304. doi: 10.1016/j.vetimm.2010.05.008
50. Michael SA, Hayman DTS, Gray R, Roe WD. Clinical parameters of hypervirulent *Klebsiella pneumoniae* disease and ivermectin treatment in New Zealand Sea lion (*Phocartos hookeri*) pups. *PLoS One.* (2022) 17:e0264582. doi: 10.1371/journal.pone.0264582
51. Meza Cerda MI, Gray R, Thomson PC, Butcher L, Simpson K, Cameron A, et al. Developing immune profiles of endangered Australian sea lion (*Neophoca cinerea*) pups within the context of endemic hookworm (*Uncinaria stenocephala*) infection. *Front Vet Sci.* (2022) 9:824584. doi: 10.3389/fvets.2022.824584

Supplementary Material

Mitigating disease risk in an endangered pinniped: early hookworm elimination optimizes the growth and health of Australian sea lion pups

Scott A. Lindsay, Mariel Fulham, Charles G.B. Caraguel, and Rachael Gray*

* Correspondence: Rachael Gray: rachael.gray@sydney.edu.au

1 SUPPLEMENTARY DATA S1 Estimation of pup age

To predict the age at recruitment for pups of unknown birth date, a multifactorial linear regression was built using other available parameters from pups of known age. Predicted age was therefore specific to the pup cohort (and site and seasons) in the present study, intentionally optimised for use only in the present study.

1.1 Method

For model building, the base additive model included pup sex and season, and ancillary factors were standard length, bodyweight, presence of lice and lice score, presence of patent hookworm infection, subjective body condition score, and body mass index (BMI). For consistency with a previous study, BMI was calculated using a similar method for investigation as an independent variable in modelling of other parameters (Lindsay et al., 2021). Ancillary factors were only retained, independent of significance, where they contributed to maximising the predictive value of the regression. A Box-Cox analysis was performed to explore the potential need of model outcome transformation to suit the assumptions of model's residuals normal distribution and homoscedasticity. Outliers at the extremes of recruitment age were assessed for negatively impacting the predictive value of the regression.

1.2 Results

When predicting age at recruitment, bodyweight was highly colinear with standard length ($r=0.833$) and was favored in the model building. The best model for age prediction ($r^2=0.63$) was:

$$Age^{recruitment} = e^{1.932 + 0.088 \cdot BW - 0.011 \cdot BMI - 0.029 \cdot Male - 0.131 \cdot Season2020 + 0.630 \cdot hookworm - [0.026 - 0.920] \cdot BCS + [0.064 - 0.537] \cdot licescore}$$

where BW =bodyweight, BMI =body mass index, $Male=2$ for males and 1 for females, $Season2020=1$ if recruitment was season 2020-1 and 0 if season 2019, $hookworm=1$ if positive and 0 if negative, BCS =body condition score, and $licescore$ =lice score, all covariates being

measured at time of recruitment. Six known-age pups were excluded from the model estimation – three young outliers reported to be <4d old at recruitment were realistically 2-3 days older (the interval between two colony observations for new births), and three were extreme outliers (visual assessment using scatterplot) for standard length and/or bodyweight when comparing either parameter with age (one of whom was also postpatent rather than prepatent and negatively impacting the model – both testing negative for hookworm). Age at recruitment was natural log transformed to comply with the model residuals assumptions.

2 SUPPLEMENTARY TABLE S1 Comparison of predicted mean values (and 95% confidence intervals) of bodyweight and standard length daily specific growth rates (SGRs) for treatment and control groups across each age cohort of Australian sea lion pups

Predicted means (95% CI) for daily SGR (%)						
Recruitment age cohort [median pup age at end of period (range)]	n	Control	n	Treatment	Effect ^a	p-value
Recruitment-to-first recapture						
Prepatent pups <14d [42d (25-95d)]						
Bodyweight (%) ^b	43	1.002 (0.863-1.142)	41	1.345 (1.204-1.486)	+34.2%	0.001
Standard length (%) ^b	43	0.313 (0.271-0.356)	41	0.445 (0.403-0.488)	+42.1%	<0.001
Pups <70cm [48d (25-111d)]						
Bodyweight (%)	84	0.968 (0.875-1.061)	86	1.210 (1.118-1.302)	+25.0%	<0.001
Standard length (%)	83	0.323 (0.296-0.350)	85	0.409 (0.382-0.436)	+26.7%	<0.001
Pups ≥70cm [56d (26-132d)]						
Bodyweight (%) ^b	59	0.834 (0.733-0.936)	61	0.984 (0.888-1.080)	+18.0%	0.035
Standard length (%) ^b	58	0.262 (0.226-0.298)	61	0.306 (0.272-0.340)	+16.8%	ns
First recapture-to-second recapture						
Prepatent pups <14d [72d (51-128d)]						
Bodyweight (%)	36	0.659 (0.555-0.763)	38	0.778 (0.677-0.879)	+18.1%	ns
Standard length (%) ^c	36	0.158 (0.116-0.199)	38	0.285 (0.245-0.324)	+80.3	0.011
Pups <70cm [82d (51-142d)]						
Bodyweight (%)	71	0.683 (0.604-0.763)	77	0.719 (0.642-0.795)	+5.2%	ns
Standard length (%)	71	0.206 (0.179-0.232)	77	0.234 (0.208-0.259)	+13.5%	ns
Pups ≥70cm [88d (55-168d)]						
Bodyweight (%)	51	0.488 (0.398-0.577)	55	0.592 (0.506-0.677)	+21.3%	ns
Standard length (%)	50	0.169 (0.137-0.200)	55	0.195 (0.166-0.225)	+16.0	ns
Second recapture-to-third recapture						
Prepatent pups <14d [104d (86-203d)]						
Bodyweight (%)	28	0.617 (0.513-0.720)	30	0.434 (0.335-0.532)	-29.7%	0.029
Standard length (%)	28	0.173 (0.129-0.217)	30	0.213 (0.171-0.255)	+23.2%	ns
Pups <70cm [113d (86-203d)]						
Bodyweight (%)	56	0.420 (0.339-0.501)	58	0.520 (0.440-0.599)	+23.8%	ns
Standard length (%)	56	0.158 (0.132-0.184)	58	0.177 (0.151-0.203)	+11.8%	ns
Pups ≥70cm [122d (88-196d)]						
Bodyweight (%) ^b	35	0.445 (0.360-0.531)	45	0.521 (0.446-0.596)	+17.2%	ns
Standard length (%) ^b	35	0.187 (0.154-0.221)	45	0.201 (0.171-0.231)	+7.2%	ns
Recruitment-to-third recapture – study duration						
Prepatent pups <14d [104d (86-203d)]						
Bodyweight (%)	28	0.794 (0.729-0.858)	30	0.886 (0.828-0.944)	+11.4%	0.004
Standard length (%)	28	0.253 (0.237-0.270)	30	0.300 (0.284-0.316)	+18.4%	<0.001
Pups <70cm [113d (86-203d)]						
Bodyweight (%)	56	0.736 (0.699-0.772)	58	0.799 (0.763-0.835)	+8.6%	0.018
Standard length (%)	56	0.239 (0.226-0.252)	58	0.270 (0.257-0.283)	+13.0%	<0.001
Pups ≥70cm [122d (88-196d)]						
Bodyweight (%)	35	0.595 (0.558-0.632)	45	0.662 (0.629-0.694)	+11.2%	0.010
Standard length (%)	35	0.205 (0.192-0.218)	45	0.224 (0.213-0.236)	+9.5%	0.033

^a Effect represents the relative difference (as a percentage) in the SGR of the treatment cohort relative to control cohort.

^b The final model includes a significant interaction between treatment and follow-up period length in days. The reported estimates correspond to the SGR for each treatment group at the median of follow-up period length.

^c The final model includes a significant interaction between treatment and pup age in days at the start of the period. The reported estimates correspond to the SGR for each treatment group at the median pup age.

ns: not significant.

3 SUPPLEMENTARY TABLE S2 Comparison of predicted mean or median values (and 95% confidence intervals) of hematological parameters for treatment and control groups, across all age cohorts and for all captures of Australian sea lion pups.
PCV: packed cell volume; RBC: total red blood cell count; WBC: total white blood cell count; nRBC: nucleated cell blood cell count

	Recruitment					First recapture					Second recapture					Third recapture				
	n	Control	n	Treatment	<i>p</i> -value	n	Control	n	Treatment	<i>p</i> -value	n	Control	n	Treatment	<i>p</i> -value	n	Control	n	Treatment	<i>p</i> -value
Prepatent pups <14d old																				
PCV (L/L)	43	0.401 (0.385-0.417)	45	0.403 (0.388-0.419)	ns	36	0.336 (0.324-0.348)	36	0.360 (0.348-0.372)	0.011	33	0.384 (0.369-0.399) ^d	39	0.354 (0.340-0.368) ^b	0.006	27	0.393 (0.381-0.406) ^a	31	0.382 (0.369-0.394) ^a	ns
Total plasma protein (g/L)	42	64.5 (62.4-66.6) ^a	45	64.1 (62.1-66.1) ^a	ns	36	62.9 (61.2-64.6) ^c	36	65.5 (64.0-66.9) ^c	0.023	33	72.9 (71.5-74.3)	39	66.5 (65.2-67.8)	<0.001	27	67.6 (66.0-69.2) ^a	31	68.4 (67.0-69.9) ^a	ns
RBC (x1012/L)	40	4.12 (3.97-4.26)	45	4.19 (4.06-4.32)	ns	34	3.70 (3.59-3.81)	34	3.89 (3.78-4.00)	0.016	33	4.17 (4.04-4.29)	36	4.10 (3.98-4.21)	ns	27	4.46 (4.31-4.62)	29	4.43 (4.29-4.58)	ns
Haemoglobin (g/L)	40	140.3 (134.6-146.0)	45	142.9 (137.5-148.3)	ns	34	120.0 (116.2-123.5) ^a	34	126.4 (123.0-129.6) ^a	0.012	33	134.4 (130.3-138.6) ^d	36	132.2 (128.3-136.1) ^b	ns	27	141.9 (136.8-146.8) ^a	29	143.4 (138.5-148) ^a	ns
Platelets (109/L)	38	318.0 (275.9-360.2)	43	309.7 (270.1-349.3)	ns	32	416.7 (364.2-469.2)	33	404.8 (353.1-456.5)	ns	32	440.3 (399.7-485.1) ^a	36	394.3 (360.0-431.9) ^a	ns	27	405.3 (354.7-455.8)	29	433.2 (384.5-482.0)	ns
WBC (109/L)	39	11.36 (10.30-12.54) ^{ab}	44	10.78 (9.78-11.87) ^{ab}	ns	34	10.02 (9.34-10.74) ^{ad}	33	9.57 (8.93-10.26) ^{ad}	ns	33	9.53 (8.56-10.60) ^a	36	9.53 (8.60-10.55) ^a	ns	27	8.45 (7.77-9.26) ^a	29	8.78 (8.08-9.62) ^a	ns
Neutrophils (109/L)	39	8.04 (6.91-9.35) ^{ab}	44	6.12 (5.28-7.09) ^{ab}	0.012	34	5.76 (5.17-6.35)	33	6.07 (5.47-6.67)	ns	33	5.54 (4.82-6.36) ^a	36	5.72 (5.01-6.54) ^a	ns	27	5.58 (4.71-6.62) ^a	29	4.75 (4.03-5.60) ^a	ns
Lymphocytes (109/L)	40	2.94 (2.65-3.27) ^a	44	2.84 (2.57-3.14) ^a	ns	34	2.85 (2.58-3.13)	33	2.71 (2.43-2.98)	ns	33	2.49 (2.16-2.87) ^a	36	2.68 (2.34-3.08) ^a	ns	27	2.28 (1.98-2.64) ^a	29	2.67 (2.32-3.06) ^a	ns
Monocytes (109/L)	40	0.59 (0.46-0.75) ^{ab}	44	0.49 (0.37-0.63) ^{as}	ns	34	0.60 (0.50-0.70)	33	0.63 (0.53-0.73)	ns	33	0.48 (0.39-0.59) ^a	36	0.41 (0.32-0.50) ^a	ns	27	0.43 (0.36-0.51)	29	0.44 (0.37-0.51)	ns
Eosinophils (109/L)	40	0.38 (0.26-0.53) ^a	44	0.44 (0.31-0.59) ^a	ns	34	1.22 (1.02-1.44) ^a	33	0.29 (0.20-0.40) ^a	<0.001	33	0.62 (0.43-0.87) ^a	36	0.32 (0.21-0.48) ^a	0.037	27	0.28 (0.18-0.41) ^a	29	0.36 (0.24-0.50) ^a	ns
Reticulocytes (109/L)	39	19.3 (13.9-25.9) ^{ac}	43	15.5 (11.0-20.9) ^{ac}	ns	32	64.1 (51.3-78.3) ^a	33	49.1 (38.1-61.4) ^a	ns	33	51.3 (41.8-63.0) ^a	36	48.9 (40.2-59.5) ^a	ns	27	45.7 (35.4-56.70) ^a	29	45.7 (35.9-56.40) ^a	ns
nRBC (10 ⁶ /L)	40	0.000 (0.000-0.000) ^a	44	0.000 (0.000-0.000) ^a	ns	33	2.056 (0.077-34.396) ^a	33	0.003 (0.000-0.125) ^a	0.012	33	0.000 (0.000-0.048) ^{ab}	36	0.000 (0.000-0.076) ^{as}	ns	27	0.000 (0.000-0.000) ^a	29	0.000 (0.000-0.000) ^a	0.026
Pups <70cm standard length																				
PCV (L/L)	89	0.363 (0.353-0.374)	92	0.365 (0.354-0.375)	ns	72	0.344 (0.335-0.352)	78	0.357 (0.349-0.365)	0.03	64	0.382 (0.373-0.391)	76	0.368 (0.359-0.376)	0.031	53	0.395 (0.386-0.405) ^a	58	0.387 (0.378-0.396) ^a	ns
Total plasma protein (g/L)	89	61.2 (59.8-62.5)	91	60.8 (59.4-62.1)	ns	72	66.9 (65.7-68.1)	77	66.9 (65.7-68.1)	ns	64	72.2 (71.2-73.2) ^a	76	67.2 (66.4-68.0) ^a	<0.001	53	69.1 (67.9-70.2) ^a	58	68.8 (67.7-69.8) ^a	ns
RBC (x1012/L)	83	3.81 (3.71-3.91)	89	3.83 (3.73-3.93)	ns	69	3.81 (3.72-3.90)	74	3.89 (3.80-3.97)	ns	63	4.27 (4.19-4.35)	72	4.23 (4.16-4.30)	ns	52	4.53 (4.42-4.63) ^a	54	4.51 (4.40-4.61) ^a	ns
Haemoglobin (g/L)	83	128.7 (124.9-132.4)	89	129.2 (125.6-132.8)	ns	69	120.6 (117.7-123.5) ^d	74	126 (123.1-128.8) ^d	0.01	63	134.9 (132.0-137.9) ^d	72	135.0 (132.2-137.7) ^b	ns	52	142.7 (139.1-146.3) ^a	54	142.4 (138.8-145.9) ^a	ns
Platelets (109/L)	80	345.3 (312.1-379.7) ^a	86	345.5 (313.4-378.6) ^a	ns	64	413.2 (371.8-454.7)	72	407.2 (368.2-446.2)	ns	60	418.0 (388.9-447.1)	73	412.6 (386.3-439.0)	ns	51	402.9 (371.3-433.3) ^a	55	405.4 (375.1-434.6) ^a	ns
WBC (109/L)	82	12.22 (11.45-13.05) ^a	88	12.33 (11.58-13.14) ^a	ns	69	10.48 (9.87-11.13) ^a	72	9.86 (9.30-10.46) ^a	ns	63	9.01 (8.49-9.59) ^a	72	8.90 (8.43-9.43) ^a	ns	52	8.95 (8.38-9.61) ^a	55	8.79 (8.26-9.41) ^a	ns
Neutrophils (109/L)	82	6.71 (6.04-7.46) ^a	88	6.73 (6.08-7.46) ^a	ns	69	5.38 (4.95-5.86) ^{ac}	72	6.26 (5.77-6.78) ^{ac}	0.012	63	5.34 (4.87-5.84) ^a	72	5.44 (5.00-5.92) ^a	ns	52	5.32 (4.74-5.98) ^a	55	5.11 (4.56-5.72) ^a	ns
Lymphocytes (109/L)	83	3.25 (3.01-3.5) ^a	89	3.31 (3.08-3.55) ^a	ns	69	3.15 (2.88-3.43) ^a	74	2.60 (2.37-2.84) ^a	0.006	63	2.64 (2.38-2.92) ^a	73	2.65 (2.41-2.91) ^a	ns	51	2.61 (2.35-2.90) ^a	55	2.73 (2.46-3.02) ^a	ns
Monocytes (109/L)	82	0.59 (0.52-0.67) ^a	89	0.62 (0.55-0.70) ^a	ns	68	0.51 (0.45-0.58) ^a	74	0.56 (0.50-0.63) ^a	ns	63	0.44 (0.37-0.51) ^a	73	0.49 (0.43-0.56) ^a	ns	52	0.42 (0.36-0.50) ^a	55	0.48 (0.41-0.56) ^a	ns
Eosinophils (109/L)	82	0.85 (0.71-1.01) ^a	88	0.91 (0.76-1.07) ^a	ns	69	0.86 (0.65-1.13) ^{ad}	73	0.30 (0.20-0.42) ^{ad}	<0.001	63	0.51 (0.38-0.66) ^a	73	0.30 (0.22-0.40) ^a	0.034	52	0.29 (0.21-0.38) ^a	55	0.29 (0.22-0.38) ^a	ns
Reticulocytes (109/L)	79	33.5 (26.9-41.2) ^{ac}	88	25.7 (20.8-31.5) ^{ac}	ns	65	60.5 (48.0-74.5) ^a	74	65.8 (53.9-78.9) ^a	ns	63	58.9 (50.9-67.5) ^a	73	46.0 (39.5-53.1) ^a	0.022	52	41.6 (32.9-51.2) ^{ab}	55	34.2 (26.6-42.8) ^{ab}	ns
nRBC (10 ⁶ /L)	81	0.006 (0-0.188) ^{ab}	86	0.000 (0.000-0.001) ^{ab}	0.019	66	2.531 (0.287-18.01) ^a	71	0.008 (0.000-0.094) ^a	0.001	63	0.000 (0.000-0.000) ^{ac}	73	0.000 (0.000-0.000) ^{ac}	ns	52	0.000 (0.000-0.000) ^a	55	0.000 (0.000-0.000) ^a	ns
Pups ≥70cm standard length																				
PCV (L/L)	60	0.346 (0.336-0.356) ^a	58	0.341 (0.331-0.351) ^a	ns	54	0.376 (0.362-0.389) ^{ad}	56	0.356 (0.341-2.802) ^{ad}	ns	50	0.393 (0.385-0.400) ^a	54	0.389 (0.381-0.396) ^a	ns	35	0.406 (0.396-0.416) ^a	45	0.407 (0.398-0.415) ^a	ns
Total plasma protein (g/L)	60	63.3 (61.8-65.0) ^a	58	62.8 (61.3-64.5) ^a	ns	54	70.8 (69.4-72.2) ^d	56	69.7 (68.4-71.1) ^d	ns	50	73.1 (71.9-74.3) ^{ad}	53	69.2 (68.2-70.3) ^{ab}	<0.001	35	70.8 (69.2-72.4) ^a	44	69.7 (68.3-71.1) ^a	ns
RBC (x1012/L)	57	3.70 (3.60-3.80)	55	3.67 (3.57-3.77)	ns	51	3.87 (3.79-3.95)	51	4.08 (3.99-4.16)	0.001	50	4.41 (4.32-4.50) ^a	50	4.41 (4.31-4.50) ^a	ns	33	4.78 (4.65-4.91)	41	4.63 (4.51-4.75)	ns
Haemoglobin (g/L)	57	123.9 (120.4-127.5)	55	117.8 (114.2-121.5)	0.020	51	124.5 (121.3-127.6) ^a	51	131.7 (128.8-134.6) ^a	0.001	50	139.4 (136.2-142.6) ^a	51	139.8 (136.6-142.9) ^a	ns	34	149.7 (144.6-154.7)	42	144.8 (140.3-149.3)	ns
Platelets (109/L)	57	420.2 (381.7-458.8)	54	439.5 (399.9-479.1)	ns	51	498.9 (468.3-529.5)	50	392.7 (361.8-423.6)	<0.001	50	433.0 (396.6-469.3)	50	358.2 (321.8-394.5)	0.008	33	401.2 (361.7-440.6)	41	405.5 (370.3-440.7)	ns
WBC (109/L)	56	11.93 (11.09-12.83) ^a	54	11.63 (10.80-12.53) ^a	ns	50	11.28 (10.52-12.09) ^a	50	9.87 (9.21-10.58) ^a	0.009	50	9.79 (9.04-10.60) ^a	51	9.01 (8.33-9.75) ^a	ns	34	8.99 (8.29-9.81) ^a	42	9.33 (8.65-10.12) ^a	ns
Neutrophils (109/L)	57	6.05 (5.42-6.75) ^a	54	5.55 (4.96-6.21) ^a	ns	51	6.19 (5.56-6.85) ^a	50	6.06 (5.43-6.73) ^a	ns	50	5.51 (4.89-6.22) ^a	51	4.90 (4.35-5.53) ^a	ns	34	5.05 (4.51-5.70) ^a	42	5.09 (4.59-5.68) ^a	ns
Lymphocytes (109/L)	57	3.56 (3.25-3.89) ^a	54	3.38 (3.08-3.71) ^a	ns	50	2.96 (2.69-3.26) ^a	50	2.76 (2.51-3.03) ^a	ns	49	3.04 (2.74-3.37) ^a	51	2.57 (2.32-2.84) ^a	0.022	34	3.01 (2.67-3.39) ^a	42	3.02 (2.72-3.36) ^a	ns
Monocytes (109/L)	57	0.64 (0.55-0.73) ^a	53	0.58 (0.50-0.67) ^a	ns	51	0.63 (0.54-0.72) ^a	49	0.56 (0.48-0.64) ^a	ns	50	0.48 (0.40-0.56) ^a	51	0.46 (0.38-0.54) ^a	ns	34	0.49 (0.36-0.63) ^{as}	42	0.66 (0.52-0.80) ^{as}	ns
Eosinophils (109/L)	56	1.32 (1.12-1.54) ^a	52	1.49 (1.26-1.74) ^a	ns	50	1.18 (0.95-1.44) ^a	48	0.26 (0.18-0.37) ^a	<0.001	50	0.38 (0.27-0.52) ^{ac}	51	0.40 (0.29-0.54) ^{ac}	ns	34	0.32 (0.22-0.42) ^a	42	0.35 (0.26-0.45) ^a	ns
Reticulocytes (109/L)	55	63.9 (54.0-74.7) ^a	54	72.6 (61.9-84.1) ^a	ns	49	66.1 (59.1-73.2)	50	51.3 (44.3-58.3)	0.004	50	58.3 (48.6-69.4) ^a	51	46.5 (38.4-55.7) ^a	ns	33	41.0 (32.5-50.6) ^a	42	45.0 (36.9-53.8) ^a	ns
nRBC (10 ⁶ /L)	57	30.422 (9.814-80.005) ^{ac}	52	83.021 (28.713-207.422) ^{ac}	ns	51	0.001 (0-0.044) ^a	49	0.000 (0.000-0.018) ^a	ns	50	0.000 (0.000-0.000) ^a	50	0.000 (0.000-0.000) ^a	ns	34	0.000 (0.000-0.000) ^a	41	0.000 (0.000-0.000) ^a	0.047

^a Estimates should be interpreted as medians rather than means as transformed model estimates and their associated 95% CI boundaries were back transformed prior to reporting.

^b Interaction of season with treatment effect in final model.

^c Interaction of pup age at capture with treatment effect in final model.

^d Interaction of baseline hematological parameter with treatment effect in final model.

^e Interaction of other parameter with treatment effect in final model.

References

Lindsay, S.A., Caraguel, C.G.B., and Gray, R. (2021). Topical ivermectin is a highly effective seal 'spot-on': A randomised trial of hookworm and lice treatment in the endangered Australian sea lion (*Neophoca cinerea*). *Int J Parasitol Parasites Wildl* 16, 275-284. doi: 10.1016/j.ijppaw.2021.11.002.

CHAPTER FOUR

Toxoplasma gondii seroprevalence in the endangered Australian sea lion (*Neophoca cinerea*)



Olive Island, via Streaky Bay, SA

Author attribution statement

Chapter four is published as:

Lindsay, S.A., Caraguel, C.G.B., O’Handley, R., Šlapeta, J., and Gray, R. (2022). *Toxoplasma gondii* seroprevalence in the endangered Australian sea lion (*Neophoca cinerea*). *Frontiers in Marine Science* 9, 965865. [doi: 10.3389/fmars.2022.965865](https://doi.org/10.3389/fmars.2022.965865).

Scott Lindsay was the primary author of this chapter and contributed to all aspects of this publication, including conceptualization, data curation, formal analysis (statistical), funding acquisition, investigation (fieldwork; laboratory), methodology, project administration, resources, validation, visualisation, and writing (original draft; review and editing). Charles Caraguel contributed to formal analysis (statistical), supervision, visualisation, and writing (review and editing). Ryan O’Handley contributed to investigation (laboratory), methodology, resources, validation, and writing (review and editing). Jan Šlapeta contributed to conceptualization, funding acquisition, supervision (supporting), visualisation, and writing (review and editing). Rachael Gray contributed to conceptualization, funding acquisition, investigation (fieldwork), project administration, resources, supervision, and writing (review and editing). All authors reviewed and approved the final manuscript.

Scott A. Lindsay

22 March 2024

Charles G.B. Caraguel

02 April 2024

Ryan O’Handley

02 April 2024

Jan Šlapeta

25 March 2024

Rachael Gray

26 March 2024



OPEN ACCESS

EDITED BY

Eric N. Villegas,
United States Environmental
Protection Agency,
United States

REVIEWED BY

Raquel Xavier,
Centro de Investigacao em
Biodiversidade e Recursos Geneticos
(CIBIO-InBIO), Portugal
Jitender Dubey,
Agricultural Research Service (USDA),
United States

*CORRESPONDENCE

Rachael Gray
rachael.gray@sydney.edu.au

SPECIALTY SECTION

This article was submitted to
Marine Megafauna,
a section of the journal
Frontiers in Marine Science

RECEIVED 10 June 2022

ACCEPTED 16 August 2022

PUBLISHED 05 September 2022

CITATION

Lindsay SA, Caraguel CGB,
O'Handley R, Šlapeta J and Gray R
(2022) *Toxoplasma gondii*
seroprevalence in the endangered
Australian sea lion (*Neophoca cinerea*).
Front. Mar. Sci. 9:965865.
doi: 10.3389/fmars.2022.965865

COPYRIGHT

© 2022 Lindsay, Caraguel, O'Handley,
Šlapeta and Gray. This is an open-
access article distributed under the
terms of the [Creative Commons
Attribution License \(CC BY\)](https://creativecommons.org/licenses/by/4.0/). The use,
distribution or reproduction in other
forums is permitted, provided the
original author(s) and the copyright
owner(s) are credited and that the
original publication in this journal is
cited, in accordance with accepted
academic practice. No use,
distribution or reproduction is
permitted which does not comply with
these terms.

Toxoplasma gondii seroprevalence in the endangered Australian sea lion (*Neophoca cinerea*)

Scott A. Lindsay¹, Charles G. B. Caraguel², Ryan O'Handley²,
Jan Šlapeta¹ and Rachael Gray^{1*}

¹Sydney School of Veterinary Science, Faculty of Science, The University of Sydney, Camperdown, NSW, Australia, ²School of Animal and Veterinary Sciences, Faculty of Science, The University of Adelaide, Roseworthy, SA, Australia

Toxoplasma gondii is a ubiquitous parasite increasingly detected in marine mammals and suspected to contribute to limited recovery of endangered populations. This study reports on the exposure of the Australian sea lion (*Neophoca cinerea*) to this protozoon using archived adult and pup sera from three island colonies in South Australia. Modified agglutination testing (MAT) detected a seroprevalence of 30.4% (95% CI 13.2–52.9; n = 23) and high antibody titers (512 to > 2048 IU/ml) in adult females (median age 9.5 y, range 5.5–14.5 y) at Dangerous Reef, a felid-free island. Antibodies weren't detected in any surveyed pup (97.5% CI 0.0–2.0%, n = 184) at two felid-free islands (Dangerous Reef: n = 21; Olive Island: n = 65), nor at Kangaroo Island (n = 98), which has a high-density feral cat population. Kangaroo Island pups of known age were 7–104 d old, while standard length and bodyweight comparison suggested younger and older pup cohorts at Dangerous Reef and Olive Island, respectively. This study provides the first quantification of disease risk in this endangered species from *T. gondii* exposure. The absence of detectable pup seroconversion supports the lack of detectable congenital transmission, maternal antibody persistence or early post-natal infection in the sampled cohort yet to commence foraging. An extended serosurvey of *N. cinerea* colonies is recommended to confirm the hypothesis of predominantly forage-associated exposure to *T. gondii* in this species. Findings have implications for parasitic disease risk in wildlife inhabiting Australia's islands and for the feral cat control program on Kangaroo Island.

KEYWORDS

antibody, Australian sea lion, *Neophoca cinerea*, seal, pinniped, serology, *toxoplasma gondii*

Introduction

Toxoplasma gondii is an emerging pathogen in marine mammals, with substantial worldwide seroprevalence in pinniped (seal and sea lion), cetacean (dolphin and whale) and mustelid (sea otter) species, (reviewed in [Dubey et al., 2020](#)) and documented mortalities in critically endangered populations ([Roe et al., 2013](#)). Understanding of the parasite's potential to modulate marine mammal population size and geographical range comes from surveys of European and North American cetaceans and marine mustelids ([Ahmadpour et al., 2022](#)). In the threatened southern sea otter (*Enhydra lutris nereis*), for instance, *T. gondii*-associated encephalitis was considered a primary or contributing cause of more than a quarter of deaths (1998-2001: $n = 105$), predominantly in adults ([Kreuder et al., 2003](#)). In one spatial cluster, it was the primary cause of half of all deaths ($n = 16$). A longer-term study showed nearly two-thirds of sea otters (1998-2012: $n = 542$) were infected with *T. gondii* and the parasite contributed to at least one in ten deaths, while infectious disease more generally was the leading cause of death in this threatened species ([Miller et al., 2020](#)).

In pinnipeds, and specifically otariids (eared seals), *T. gondii* infection and clinical toxoplasmosis are reported in free-ranging populations worldwide ([Jensen et al., 2012](#); [Carlson-Bremer et al., 2015](#); [Sepulveda et al., 2015](#); [Michael et al., 2016](#)). Dependent on serological method and selected cut-off value, seroprevalence can exceed 50% ([Jensen et al., 2012](#)). In comparison, surveying for *T. gondii* exposure in Australian otariid species is limited to Australian fur seals (*Arctocephalus pusillus doriferus*) in Bass Strait, where in 2007-2008 an absence of *T. gondii* antibodies were detected in adult females of unspecified age ($n = 104$) ([Lynch et al., 2011](#)). Reporting of sporadic cases of disseminated toxoplasmosis is also rare: the most recent in Australia was a subadult male long-nosed fur seal (*Arctocephalus forsteri*) that hauled out on the east coast in 2012 ([Donahoe et al., 2014](#)). For the endangered Australian sea lion (*Neophoca cinerea*), toxoplasmosis is listed as a potential threat to the species' recovery ([Threatened Species Scientific Committee, 2020](#)), based on observations in other marine mammals and documented disease susceptibility ([Kabay, 1996](#)). To date there has not been any serological survey for *T. gondii* (or other infectious pathogen) exposure reported in this declining species. This is of particular importance given the species' relatively low genetic diversity potentially associated with an increased susceptibility to infectious disease ([Bilgmann et al., 2021](#)), and a vulnerable population structure of approximately 80 effectively closed sub-populations resulting from extreme female natal site fidelity ([Campbell et al., 2008](#)). Consequently, disease-induced mortality outbreaks pose the

greatest risk to the species' resilience based on modelling of future population decline ([Bilgmann et al., 2021](#)).

Domestic (*Felis catus*) and wild (including feral) felids are the only definitive hosts of *T. gondii*, an intracellular and zoonotic apicomplexan protozoan parasite capable of infecting all warm-blooded animals. Across these species, the mammals and birds, parasite transmission is related either to foraging – ingestion of oocysts in contaminated food or water, or of tissue cysts in prey species – or to transplacental transmission from recently infected mother to offspring ([Dubey, 2022](#)). In the marine mammals, the former can contribute directly to illness or death from localized or disseminated infection, reported for instance in the California sea lion (*Zalophus californianus*) ([Carlson-Bremer et al., 2015](#)), Mediterranean monk seal (*Monachus monachus*) ([Mazzariol et al., 2021](#)) and multiple other seal species ([van de Velde et al., 2016](#); [Reisfeld et al., 2019](#)). Additionally, infection can increase the risk of death from other causes such as predation and conspecific trauma, as observed in the southern sea otter ([Kreuder et al., 2003](#)) and New Zealand sea lion (*Phocarcos hookeri*) ([Roe et al., 2017](#)). Transplacental transmission, placental inflammation and disseminated congenital fetal infection, resulting in early or late-term abortion or stillbirth have also been documented in multiple species, among them Risso's dolphin (*Grampus griseus*) ([Resendes et al., 2002](#)), the California sea lion ([Carlson-Bremer et al., 2015](#)) and the southern sea otter ([Shapiro et al., 2016](#)).

The infection of marine mammals indicates contamination of coastal and marine environments with oocysts shed in cat feces, by terrestrial water run-off or from ocean sewage discharge ([Miller et al., 2002](#); [Fayer et al., 2004](#)) ([Figure 1](#)). For species frequenting coastal habitats, such as pinnipeds, infection could also result from direct contamination of haul out sites by cat feces. In any case, infection is most likely to result from oocyst (versus tissue cyst) ingestion, given the cold-blooded nature of their prey species (e.g., fish and marine invertebrates) precludes susceptibility to active *T. gondii* infection. Oocysts are environmentally resistant, capable of sporulation (becoming infectious) and temperature-dependent maintenance of infectivity in sea water for at least 6-24 months ([Lindsay et al., 2003](#); [Lindsay and Dubey, 2009](#)). Increased exposure of marine mammals is likely facilitated by parasite carriage and maintenance of viability in cold-blooded prey species acting as phoretic hosts – including filter-feeding bivalve mollusks (e.g., blue (*Mytilus edulis*) ([Bigot-Clivot et al., 2022](#)) and green-lipped (*Perna canaliculus*) mussels ([Coupe et al., 2018](#))) and fish species ([Marino et al., 2019](#)), the latter already a suggested source of infection for coastal and pelagic seabirds ([Pouille et al., 2021](#); [Campbell et al., 2022](#)). Independent of prey species, the initial entry of the parasite into the marine food chain is likely enabled by aggregation in gelatinous marine polymers and by adhesion

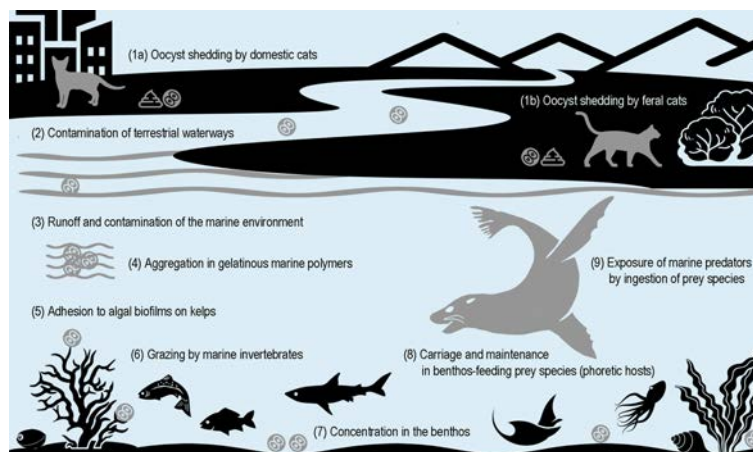


FIGURE 1
Proposed pathways for exposure of marine mammals to *Toxoplasma gondii* oocysts shed by terrestrial-bound definitive felid hosts.

to algal biofilms on kelps grazed by marine invertebrates (Shapiro et al., 2014).

The Australian sea lion inhabits the shores and islands of Australia's southern and south-western coast (Gales et al., 1994), much of which is arid coastline of relatively low human population density. Australia's abundant feral cat population, estimated to be 2.1–6.3 million animals dispersed across 99.8% of the continent (including the arid regions) (Legge et al., 2017) is a likely source of environmental contamination by *T. gondii* oocysts. Furthermore, while some Australian sea lion colonies exist on felid-free offshore islands (e.g., Olive Island and Dangerous Reef, South Australia), others (e.g., Seal Bay, Kangaroo Island, South Australia) are exposed to a feral cat density estimated to be 10 times greater than that of the adjacent mainland (Taggart et al., 2019a). For colonies on feral cat-inhabited islands there are potential health implications for pups (and adults) from increased exposure risk, given pups are mainly terrestrial until their first molt at 4–5 months of age, and are not weaned until 15–18 months of age. However, pups commence exploration of the adult foraging range by 6 months of age and obtain hunting skills at least 8 months prior to weaning (Lowther and Goldsworthy, 2012) at which age, their foraging-associated exposure risk (ingestion to *T. gondii* oocysts present in prey species) is likely to be similar to that of an adult animal.

This study aimed to provide the first estimate of *T. gondii* exposure in *N. cinerea*, and to infer route of infection in neonatal animals. We hypothesized that adult seroprevalence would parallel rates reported in other otariids, and that seroconversion would not be detected in young, pre-weaned pups yet to commence foraging. The latter investigation would also enable assessment of the persistence of any transplacental or colostrum-derived maternal antibodies.

Materials and methods

Study colonies

Archived sera from adult female Australian sea lions and pups were collected from Dangerous Reef (−34.817, 136.217), a small, uninhabited, and felid-free rocky island in the Spencer Gulf, South Australia (Figure 2), located 17.5 km from Lincoln National Park on the east coast of the Eyre Peninsula and 33 km from the coastal town of Port Lincoln (population 16,400). Pups were also sampled on Olive Island (−32.717, 133.983), a small, uninhabited, and felid-free island located 5 km from Cape Bauer on the west coast of the Eyre Peninsula and 25 km from the small regional coastal town of Streaky Bay (population 1,400), and at Seal Bay (−35.995, 137.317) on the south coast of Kangaroo Island, Australia's third largest (4,405 km²) inhabited island (population 4,700). Kangaroo Island, located 13.5 km from the southern tip of the Fleurieu Peninsula and 112 km southwest of the state capital city of Adelaide (regional population 1,359,760), has a high-density feral cat population (Taggart et al., 2019a).

Sample collection and age estimation

Australian sea lion pup samples were collected with approval of South Australian Department of Environment and Water (Permit/License Number: MR00073-2-R; Scientific Research Permit Number: A25088-12) and the study protocol was approved by The University of Sydney Animal Ethics Committee (Protocol Number 2014/726). Adult Australian sea lion samples were collected with approval of the South Australian Department for Environment and Heritage (DEH) (Permit Number U24630) and the study protocol was approved

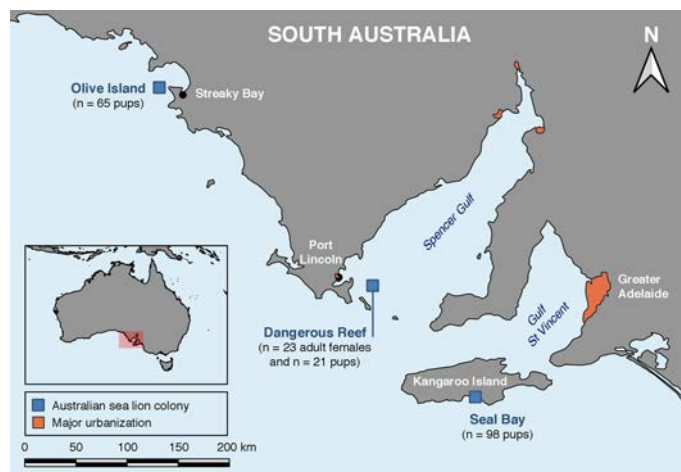


FIGURE 2

Location of the three Australian sea lion (*Neophoca cinerea*) colonies sampled for the serological survey of *T. gondii* antibody presence in adult females and pups.

by La Trobe University Animal Ethics Committee (Permit AEC00/42(L)). Adult southern sea lion samples were collected with approval of the Falkland Islands Government (Research Permit R16/2012). Adult female samples ($n = 23$) from Dangerous Reef were collected from 17 September to 21 November 2003, for a demographic study of the female breeding population (McIntosh, 2007). All but one female had recently given birth (pup age range 4–60 d). Dental aging was available for 20 animals (median age 9.5 y, range 5.5–14.5 y). Additional morphometric data were bodyweight (median 84.5 kg, range 65.5–103.5 kg) and standard length (median 159 cm, range 142–169 cm). Blood samples were collected into clot activation tubes and the separated serum stored in liquid nitrogen (-196°C) in the field before longer-term freezing (-80°C). Pup samples were collected as previously described (Lindsay et al., 2021), for health monitoring and hookworm disease investigations. Pup morphometric data included bodyweight (kg) and linear standard length (cm), both proxies for pup age. At Seal Bay, known pup age based on observation of birth or detection of the pup as a newborn, was recorded when available and repeat sampling was performed on a subset of pups. Known-age pups provided a reference to determine indicative pup ages for other cohorts. Simple linear regressions of the age proxies of standard length and bodyweight were made between six cohorts, one each from Dangerous Reef and Olive Island, and four from Seal Bay (initial and repeat samples from pups of known age, initial and repeat samples from pups of unknown age). A Bonferroni correction was used to adjust for multiple comparisons between pairs of the above cohorts.

Sample collection dates for Dangerous Reef ($n = 21$), Olive Island ($n = 65$) and Seal Bay ($n = 126$) were 21 July to 29 August 2017, 15 to 19 September 2017, and 5 March to 8 May 2018,

respectively. Serum was separated within 12 h of blood collection after centrifuging plain blood tubes for 2 min at 13,700 g (StatSpin MP, StatSpin Technologies, Norwood, USA). Samples were stored in the field in liquid nitrogen (Dangerous Reef and Olive Island) or frozen at -20°C (Seal Bay), before longer term freezing at -80°C .

Serological testing and analysis

A commercially available modified agglutination test (MAT) (Toxo-Screen DA[®], Biomerieux, Marcy-l'Étoile, France) was used to screen sera for *T. gondii* tachyzoite immunoglobulin G (IgG) antibodies at 1:40 and 1:4,000 dilutions, as per manufacturer directions. Samples (freeze-thaw cycles: adults (1), pups (≤ 3)) were analyzed between 11 July and 27 September 2018 at the Veterinary Diagnostic Laboratory, The University of Adelaide. Non-specific agglutination was suppressed by including 2-mercaptoethanol (0.2 mol/L) in the dilution buffer. Manufacturer supplied positive and negative goat control sera and phosphate-buffered saline control were included with each MAT test plate. Quantitative assessment of antibody titer was determined for positive sera using sequential doubling of sample dilution (from a 1:40 baseline and commencing at 1:640). Antibody titer was converted to IU/ml as per the manufacturer's instructions (equivalent to one-tenth of the reciprocal of the highest dilution giving a positive reaction – a higher titer indicating a higher level of antibody).

When seropositive adult females were detected, the association of demographic factors (age, bodyweight, and standard length) with seropositivity was explored using simple logistic regressions. Binomial exact 95% confidence intervals were reported for estimated seroprevalences in this study and

calculated for comparative studies using published data. Seroprevalence analyses were conducted using version 17.0 of the Stata software package (StataCorp LLC., College Station, Texas, USA). For pup groups that tested all seronegative, a one-sided 97.5% confidence interval was reported. For these cohorts, the survey sensitivity (S_{Se}) was calculated *post-hoc* according to the achieved sample size using the online AusVet EpiTools calculator (Sergeant, 2018a). Assumptions were representative sampling, 100% diagnostic test sensitivity and specificity, as well as a 5% design seroprevalence (minimum assumed seroprevalence in an exposed animal group). The population size was considered finite according to referenced pup production estimates for each colony. Calculation of the S_{Se} used a modified hypergeometric exact method and was then used to estimate the probability of pup population freedom from *T. gondii* exposure (Sergeant, 2018b). Assumptions for probability of freedom estimations were 100% diagnostic (and therefore survey) specificity, as well as a non-informative 50% pre-survey probability of freedom from exposure.

Results

Seven adult female Australian sea lion serum samples (n = 23) collected from Dangerous Reef were seropositive for *T. gondii* (30.4%; 95% CI 13.2–52.9%). All animals screening positive were positive at the 1:4,000 dilution and the result was confirmed in all cases by subsequent titration. Antibody titers of 512 (n = 3), 1024 (n = 2) and >2048 IU/ml (n = 2) were

determined. There was no significant association between age (OR 0.95 [0.67–1.36]), bodyweight (OR 0.96 [0.88–1.05]) or standard length (OR 1.06 [0.92–1.23]), and *T. gondii* seropositivity.

None of the Australian sea lion pup serum samples (n = 212 samples from 184 animals) collected from Dangerous Reef, Olive Island (both felid-free), and Kangaroo Island (felid-inhabited), were seropositive (0.0%; 97.5% CI 0.0–2.0% for pooled initial sampling; Table 1). None of the pups sampled from any colony was likely to have commenced foraging, based on subjective assessment of age (R. Gray, pers comm). One-third (n = 33) of the 98 pups initially sampled from Seal Bay were of known age (median age 26 d, age range 7–73 d). From these 98 pups there were 28 pups subsequently resampled approximately four weeks later, and half (n = 15) of these pups were of known age (median age 62 d, age range 34–104 d). Multiple pair-wise comparisons of age proxies (bodyweight and standard length; Table 1) found that using either parameter: 1. at initial sampling of both Seal Bay cohorts, the pups of unknown age were significantly larger (older) than the pups of known age (Bonferroni corrected *P* < 0.05; Supplementary Figure 1); 2. at their initial sampling, the Seal Bay pups of unknown age did not differ significantly from the Seal Bay pups of known age at their subsequent resampling (*P* > 0.05); 3. the Dangerous Reef pups did not differ significantly from the Seal Bay pups of known age at their initial sampling (Bonferroni corrected *P* > 0.05); 4. the Olive Island pups did not differ significantly from the Seal Bay pups of known age at their subsequent resampling (*P* > 0.05).

TABLE 1 Results of a serosurvey of Australian sea lion (*Neophoca cinerea*) pups for *T. gondii* antibodies sampled at three South Australian colonies, using a modified agglutination test (MAT).

Capture order	Location (population size)	Sampling period	Pups sampled	Bodyweight (kg) Median (range)	Standard length (cm) Median (range)	Seroprevalence (97.5% CI) [#]	Survey sensitivity	Probability of freedom
First	Seal Bay (232 pups) ¹	5 March–14 April 2018	98	11.4 (6.6–24.0)	73.0 (61.0–96.0)	0.0% (0–3.6%)	99.9%	99.9%
	Olive Island (101 pups) ²	15 September–19 September 2017	65	15.6 (7.4–26.0)	80.0 (67.0–94.0)	0.0% (0–5.5%)	99.8%	99.8%
	Dangerous Reef (338 pups) ²	21 July–29 August 2017	21	10.0 (6.4–11.9)	68.5 (62.0–77.0)	0.0% (0–16.1%)	66.4%	74.8%
	Combined (671 pups)	21 July 2017–14 April 2018	184	12.6 (6.4–26.0)	75.0 (61.0–96.0)	0.0% (0–2.0%)	100.0%	100.0%
Second	Seal Bay (232 pups) ¹	7 April–8 May 2018	28	13.4 (8.4–19.0)	76.3 (70.0–88.0)	0.0% (0–12.3%)	78.6%	82.4%

CI, confidence interval. [#]One-sided, 97.5% confidence interval report following 100% negative results. ¹2018 estimate based on cumulative births (Goldsworthy et al., 2019). ²Calculated as the mean of the estimated pup production reported for seasons prior to and following the sampling season (Goldsworthy et al., 2020).

The probabilities of detecting at least one seropositive pup if the pup cohort had been exposed to *T. gondii* are reported in Table 1. The resultant probabilities of the seasonal pup population freedom from *T. gondii* exposure at Dangerous Reef, Olive Island and Seal Bay (initial and subsequent sampling) were 74.8%, 99.8%, 99.9% and 82.4%, respectively. Pooling all first capture negative results provided 100% probability of the combined pre-weaned seasonal pup population (indicative age range 7–104 d) freedom from *T. gondii* exposure.

Discussion

Disease surveillance in an extensively distributed otariid population such as the Australian sea lion is logistically challenging. Sampling of subadult and adult animals is difficult, requiring either chemical restraint with its safety risks or the unlikely sampling of recently deceased individuals. Access to archived sera from 23 adult females sampled in 2003 was a unique opportunity to explore *T. gondii* exposure in a mature cohort. Evidence of *T. gondii* seroconversion in approximately a third of adult females suggests that, even 20 years ago in a felid-free island colony (Dangerous Reef), this cohort's exposure to *T. gondii* was frequent although not systematic. Adapting calculations reported by Taggart et al. (2020), the prevalence of prey carrying *T. gondii* could be as low as 0.001% to achieve an adult female seal seroprevalence of 30.4% (assuming a median cumulative exposure time of 9.0 y and a conservative daily consumption of 10 prey: Supplementary data). That equates to one in every 100,000 prey carrying *T. gondii*, recognizing that higher prevalence is likely to be clustered in areas where contamination of the marine environment may be higher. By comparison, none of the colony's pups ($n = 21$) sampled 14 years later showed evidence of *T. gondii* exposure. Ideally, paired mother and pup serology would have been analyzed, however, this was not logistically or ethically feasible. Cumulatively across the three surveyed colonies ($n = 184$) the available serological evidence provided us with approximately absolute confidence (rounded to 100%) that no pups from these colonies had seroconverted and therefore were yet to be exposed to *T. gondii*.

The estimated 30.4% seroprevalence in adult Australian sea lions approximates that reported in other otariids, including the 32.9% (binomial exact 95% CI 30.6–35.2%; $n = 1,630$) reported in free-ranging California sea lions stranding along the central California coast between 1998 and 2009 (Carlson-Bremer et al., 2015; Dubey et al., 2020). That assessment, using a 1:40 cut-off and indirect fluorescent antibody methodology was reduced to 2.8% (binomial exact 95% CI 2.1–3.7%) at a cut-off titer of 1:640, determined from confirmed cases (Carlson-Bremer et al., 2015). A similar cut-off more specific for diseased (versus infected) animals remains to be determined for the Australian sea lion. In a regional context, the

seroprevalence of antibodies to *T. gondii* in New Zealand sea lions using latex agglutination testing and a 1:32 cut-off was found to be 20.0% (binomial exact 95% CI 2.5–55.6%; $n = 10$) on the Otago Peninsula of the New Zealand South Island, and 8.3% (binomial exact 95% CI 0.2–38.5%; $n = 12$) on nearby Stewart Island (Michael et al., 2016) – both islands having a feral cat population (Roberts et al., 2021). In comparison, anti-*T. gondii* antibodies were not detected in animals from the remote and felid-free Enderby Island in the subantarctic (one-sided 97.5% CI 0.0–12.3%; $n = 28$) (Michael et al., 2016) – despite the presence of a feral cat population on the nearby main island of the Auckland Islands (Roberts et al., 2021). The latter finding is consistent with the negative results (one-sided 97.5% CI 0.0–3.5%; $n = 104$) of the 2007–2008 survey of the Australian fur seal colony (adult females only) on Kanowna Island (–39.1547, 146.3108) in northern Bass Strait (Lynch et al., 2011), and the low 5.3% (binomial exact 95% CI 0.1–26.0%; $n = 19$) seroprevalence in a 2014 survey of the southern (South American) sea lion colony (*Otaria flavescens*) (adults of both sexes) inhabiting Big Shag Island (–51.39493, –58.31644) on the north coast of the East Falkland Island (Lindsay and Gray, unpublished data). Both surveys used the same MAT methodology as the present study (with a higher 1:64 cutoff in the fur seal survey).

Differences in prevalence by location, particularly for island colonies, likely reflect varying degrees of seal exposure to *T. gondii* through prey – a function of parasite prevalence in prey and the quantity of prey consumed. The latter should result in greater cumulative exposure risk with increasing age, which has been documented in multiple pinnipeds (Lambourn et al., 2001; Carlson-Bremer et al., 2015). The absence of an age correlation with seropositivity in the present study is likely due to the small sample size and relatively narrow age range (median age 9.5 y, range 5.5–14.5 y). The parasite prevalence in prey is likely to vary with the species of prey. The Australian sea lion is principally a benthic (ocean floor) forager of Australian continental shelf waters, its temporally and spatially variable diet including many species of cephalopods (e.g., octopus, cuttlefish and squid), benthic or demersal (near the ocean floor) cartilaginous (e.g., sharks and rays) and ray-finned fish (e.g., leatherjackets, salmon and eels), and crustaceans (e.g., rock lobster and swimming crabs) (Peters et al., 2015; Berry et al., 2017). *Toxoplasma gondii* DNA has been detected in demersal and benthopelagic fish species elsewhere in the world, a Mediterranean basin study finding it in both piscivorous (diet predominantly fish) and omnivorous (diet predominantly small benthic invertebrates or zooplankton) species (Marino et al., 2019). The detection of *T. gondii* DNA in intestinal and gill samples supports ingestion (gut transit) and filter-feeding, respectively, as mechanisms for acquisition and carriage of the parasite. Increased exposure of benthic fish species to the parasite may result from the concentration of oocysts in the benthos, suggested to be facilitated by marine invertebrates (Aguirre et al., 2019). The

benthic foraging behavior of the Australian sea lion is therefore one explanation for its level of exposure to *T. gondii*.

Variation in *T. gondii* prevalence in prey is also likely impacted by other factors determining their exposure to the parasite – for instance, regional cat density (reflecting the degree of urbanization for domestic cats and the population density of feral cats), proximity to terrestrial water run-off (e.g., river delta or estuary), factors determining the volume of terrestrial water run-off (e.g., seasonal rainfall and storm events) and ocean currents (given the organism's prolonged persistence in the marine environment). Unlike males, the foraging range for adult female Australian sea lions is focused on the waters surrounding breeding colonies (Lowther et al., 2011), with inshore and offshore foraging ecotypes identified (Lowther and Goldsworthy, 2011). The waters around Dangerous Reef are impacted by The South Australian current, the easterly flowing current sweeping past some of Australia's less populous and arid southern coastline (Australian Government Bureau of Meteorology, 2022), and by tidal flows exiting the Spencer Gulf and potentially the smaller adjacent and more populous Gulf St Vincent (Australia's Integrated Marine Observing System (IMOS), 2021). A high rate of domestic cat ownership among a predominantly coastally distributed population is therefore a possible source of oocyst contamination of the marine environment around the felid-free island of Dangerous Reef.

Some of Australia's other coastal islands, such as Kangaroo Island, (Taggart et al., 2019a; Algar et al., 2020) have a high feral cat population with a high seroprevalence of *T. gondii* (87–89%) (O'Callaghan et al., 2005). Evidence for these animals substantially contributing to biological environmental contamination comes from the high prevalence of cat-borne parasitic diseases (toxoplasmosis and sarcocystosis) in the island's domestic sheep flocks (Taggart et al., 2019b; Lanyon and O'Handley, 2020). Consequently, a serosurvey of pups from the Seal Bay colony on Kangaroo Island was included in the present study to better understand any direct health implications for the colony's pup population, and as a comparator to the serosurvey results from the two felid-free islands. The absence of exposure evidence for the pup population (and the positive serosurvey of adults from a regional felid-free island) suggest survey of the juvenile and adult cohorts (ideally including mother-pup pairings) is necessary to better understand the age-related level of *T. gondii* exposure in this colony. This could guide risk assessment for epizootic disease outbreak should other factors like comorbidity alter the existing host-parasite balance and inform the scope and effectiveness of the feral cat eradication program recently implemented on the island (Commonwealth of Australia, 2015; Landscape South Australia, 2022). Furthermore, comparison of *T. gondii* genotype from clinical toxoplasmosis cases in seals with that in feral (and domestic) cats may add further to our understanding of disease epidemiology.

While acknowledging the potential for selection and survival bias, an absence of pup seropositivity in the present study excludes detection of early postnatal infection in pre-weaned pups yet to commence prey ingestion, the transplacental vertical transmission of infection from mother to pup, and the transplacental or colostrum transfer of maternal IgG antibodies (or their lack of persistence to sampling age). For seal pups exposed to *T. gondii* oocysts in the postnatal period, seroconversion with MAT-detectable antibodies occurs within three weeks (Gajadhar et al., 2004). The seropositivity of approximately 8–12 m old California sea lion pups (weaning between 6–11 m of age) was attributed to commencement of foraging trips and their initial ingestion of prey (Carlson-Bremer et al., 2015). The transplacental vertical transmission of *T. gondii* to an early or late-stage fetus is less commonly reported but has been detected in marine mammals such as Risso's dolphin (Resendes et al., 2002) and the California sea lion (Carlson-Bremer et al., 2015).

The adaptation of neonates to the extrauterine environment and their protection from infectious agents is variably reliant on the passive transfer of maternal antibodies across the placenta *in utero*, or in colostrum soon after birth (reviewed in Chucuri et al., 2010). Pinnipeds have an endotheliochorial placenta (Sinha and Erickson, 1974), and typically only 5–10% of maternal IgG antibodies undergo transfer to the fetus in placentas of this type (Tizard, 2018). Acquisition of passive immunity in pinniped pups is likely to be highly dependent on adequate ingestion and absorption of colostrum. The exact duration of persistence of maternal antibodies in pinniped pups is not known, however it is likely to be short, perhaps a few weeks (Harder et al., 1993; Puryear et al., 2021). *Toxoplasma gondii* seropositivity has been detected in a 14 d old grey seal (*Halichoerus grypus*) pup along with seronegativity in two 18–19 d old pups, all three belonging to seropositive females (Measures et al., 2004). Failure to detect maternal antibodies in pups in the present study may therefore reflect limited sampling of pups in the first two weeks of life – partly attributed to protective maternal (and aggressive male mate-guard) attendance for the first 9–10 days after birth. The importance of sampling age is also supported by a seroprevalence study of bearded seals in Norway, which included 65 pre-weaned pups, all of whom tested negative, while two-thirds ($n = 15$) of adults tested positive (Jensen et al., 2009). This included six seropositive females, all nursing seronegative pups of unspecified age. Recommendations for subsequent *T. gondii* serosurveys of Australian sea lion pups include sampling of stillbirths and perinatal deaths less than two weeks old, and histologic and immunohistochemical examination of placental tissues followed by PCR confirmation of the presence of *T. gondii* DNA. Detection of recent births and collection of well-preserved placental tissues is only feasible at a closely monitored colony such as Seal Bay. The number of observed stillborn animals at this colony is historically low, while perinatal death within the first two

weeks is a more common occurrence, often the result of conspecific trauma, endemic hookworm infection or starvation (R. Gray, pers comm).

Storage time of the adult sera is a potential limitation of the present study, although results are unlikely to have been impacted given their storage at -80°C . Reliable serological measurement of anti-toxoplasma antibodies in human sera has been observed for at least six years in samples stored at -20°C (Dard et al., 2017). A lack of validation in pinniped species of the MAT test is an additional study limitation, although it has been the most used method for serological surveys in these species over the past decade (Dubey et al., 2020), at cut-off values ranging from 1:25 to 1:65. The cut-off value of 1:40 for the present study was selected to best align with previous investigations in these species. MAT has not been validated in other marine mammals either, although in northern sea otters (*Enhydra lutris kenyoni*) for instance, 95% ($n = 20$) of *T. gondii* isolates in a study of that species were from animals testing seropositive by MAT (Verma et al., 2018). This included one animal seropositive at 1:100 dilution. Comparatively, all seropositive animals in the present study had high titers – positive at $>1:4,000$ dilution. The MAT methodology has been suggested to overestimate seroprevalence in the presence of sample hemolysis or a high serum lipid content, reducing specificity (Blanchet et al., 2014; van de Velde et al., 2016). Of note was the absence of any false positive results for the pup population, in which grossly detectable lipemia is frequently observed following recent feeding.

The endangered Australian sea lion population has failed to recover to 18th century pre-sealing abundance, despite its protected status. Given the contribution of *T. gondii* to limiting population recovery in other marine mammals and the role of high order predators as sentinels for marine contamination (Bossart, 2011), ongoing serological surveillance is recommended with expansion to include other infectious diseases likely to compromise population resilience.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal studies were reviewed and approved by The University of Sydney (Pups - Protocol Number 2014/726) and La Trobe University Animal Ethics Committees (Adults - Permit AEC00/42(L)).

Author contributions

SL and RG designed (with additional input from JS) and executed the study and collected pup samples. SL performed the laboratory testing (with additional input from RO), curated the data, and wrote the initial manuscript draft. SL and CC analyzed the data. All authors contributed to the manuscript and approved the submitted version.

Funding

This study was supported in part by The Hermon Slade Foundation (HSF 16-03), and by the FS Quiney and BR Richards bequests (Sydney School of Veterinary Science, The University of Sydney: 2016).

Acknowledgments

The authors greatly appreciate the provision of adult Australian sea lion serum samples and morphometric data by Rebecca McIntosh (Phillip Island Nature Parks, Cowes, Victoria, Australia) and Simon Goldsworthy (South Australian Research and Development Institute, West Beach, South Australia, Australia). We also appreciate the provision of adult southern sea lion serum samples by Alastair Baylis (South Atlantic Environmental Research Institute, Stanley, Falkland Islands). We thank Mariel Fulham, Shannon Taylor, and Matthew Gray for their assistance with sample collection from Australian sea lion pups. We also thank the staff of the South Australian Department for Environment and Water (DEW), Seal Bay, Kangaroo Island, for their logistical support and fieldwork assistance.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

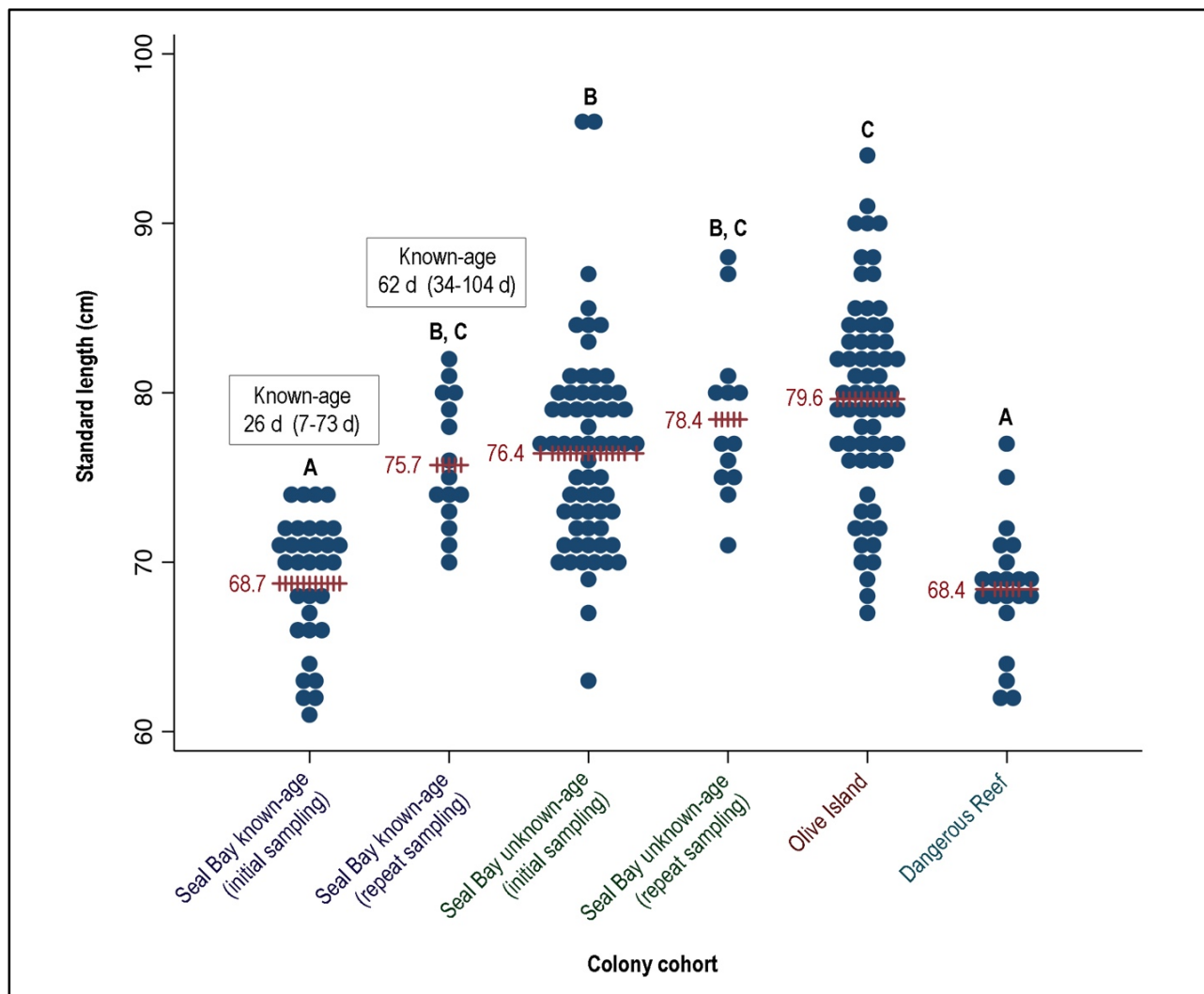
The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2022.965865/full#supplementary-material>

References

- Aguirre, A. A., Longcore, T., Barbieri, M., Dabritz, H., Hill, D., Klein, P. N., et al. (2019). The one health approach to toxoplasmosis: epidemiology, control, and prevention strategies. *Ecohealth* 16, 378–390. doi: 10.1007/s10393-019-01405-7
- Ahmadpour, E., Rahimi, M. T., Ghoghghi, A., Rezaei, F., Hatam-Nahavandi, K., Oliveira, S. M. R., et al. (2022). *Toxoplasma gondii* infection in marine animal species, as a potential source of food contamination: a systematic review and meta-analysis. *Acta Parasitol* 67, 592–605. doi: 10.1007/s11686-021-00507-z
- Algar, D., Johnston, M., Tiller, C., Onus, M., Fletcher, J., Desmond, G., et al. (2020). Feral cat eradication on Dirk Hartog island, Western Australia. *Biol. Invasions* 22, 1037–1054. doi: 10.1007/s10530-019-02154-y
- Australian Government Bureau of Meteorology (2022) *Forecast help*. Available at: <http://www.bom.gov.au/oceanography/forecasts/forecast-help.shtml> (Accessed 23 March 2022). Australian Government.
- Australia's Integrated Marine Observing System (IMOS) (2021) *Ocean current: tides*. Available at: http://oceancurrent.imos.org.au/tides/SA_spd/2021/2021122100.html (Accessed 24 March 2022).
- Berry, T. E., Osterrieder, S. K., Murray, D. C., Coghlan, M. L., Richardson, A. J., Grealy, A. K., et al. (2017). DNA Metabarcoding for diet analysis and biodiversity: A case study using the endangered Australian sea lion (*Neophoca cinerea*). *Ecol. Evol.* 7 (14), 5435–5453. doi: 10.1002/ece3.3123
- Bigot-Clivot, A., La Carbona, S., Cazeaux, C., Durand, L., Géba, E., Le Foll, F., et al. (2022). Blue mussel (*Mytilus edulis*) - a bioindicator of marine water contamination by protozoa: laboratory and *in situ* approaches. *J. Appl. Microbiol.* 132, 736–746. doi: 10.1111/jam.15185
- Bilgmann, K., Armansin, N., Ferchaud, A. L., Normandeau, E., Bernatchez, L., Harcourt, R., et al. (2021). Low effective population size in the genetically bottlenecked Australian sea lion is insufficient to maintain genetic variation. *Anim. Conserv.* 24, 847–861. doi: 10.1111/acv.12688
- Blanchet, M. A., Godfroid, J., Breines, E. M., Heide-Jørgensen, M. P., Nielsen, N. H., Hasselmeier, L., et al. (2014). West Greenland Harbour porpoises assayed for antibodies against *Toxoplasma gondii*: false positives with the direct agglutination method. *Dis. Aquat. Organ* 108, 181–186. doi: 10.3354/dao02715
- Bossart, G. D. (2011). Marine mammals as sentinel species for oceans and human health. *Vet. Pathol.* 48, 676–690. doi: 10.1177/0300985810388525
- Campbell, R. A., Gales, N. J., Lento, G. M., and Baker, C. S. (2008). Islands in the sea: extreme female natal site fidelity in the Australian sea lion, *Neophoca cinerea*. *Biol. Lett.* 4, 139–142. doi: 10.1098/rsbl.2007.0487
- Campbell, K., Papparini, A., Gomez, A. B., Cannell, B., and Stephens, N. (2022). Fatal toxoplasmosis in little penguins (*Eudyptula minor*) from penguin island, Western Australia. *Int. J. Parasitol. Parasites Wildl.* 17, 211–217. doi: 10.1016/j.ijppaw.2022.02.006
- Carlson-Bremer, D., Colegrove, K. M., Gulland, F. M., Conrad, P. A., Mazet, J. A., and Johnson, C. K. (2015). Epidemiology and pathology of *Toxoplasma gondii* in free-ranging California sea lions (*Zalophus californianus*). *J. Wildl. Dis.* 51, 362–373. doi: 10.7589/2014-08-205
- Chucuri, T. M., Monteiro, J. M., Lima, A. R., Salvadori, M. L., Kfoury, J. R., and Miglino, M. A. (2010). A review of immune transfer by the placenta. *J. Reprod. Immunol.* 87 (1–2), 14–20. doi: 10.1016/j.jri.2010.08.062
- Commonwealth of Australia (2015) *Threat abatement plan for predation by feral cats, commonwealth of Australia*. Available at: <https://www.dcccew.gov.au/environment/biodiversity/threatened/publications/tap/threat-abatement-plan-feral-cats> (Accessed 21 April 2022).
- Coupe, A., Howe, L., Burrows, E., Sine, A., Pita, A., Velathanthiri, N., et al. (2018). First report of *Toxoplasma gondii* sporulated oocysts and *Giardia duodenalis* in commercial green-lipped mussels (*Perna canaliculus*) in new Zealand. *Parasitol. Res.* 117, 1453–1463. doi: 10.1007/s00436-018-5832-8
- Dard, C., Bailly, S., Drouet, T., Fricker-Hidalgo, H., Brenier-Pinchart, M. P., and Pelloux, H. (2017). Long-term sera storage does not significantly modify the interpretation of toxoplasmosis serologies. *J. Microbiol. Methods* 134, 38–45. doi: 10.1016/j.mimet.2017.01.003
- Donahoe, S. L., Rose, K., and Slapeta, J. (2014). Multisystemic toxoplasmosis associated with a type II-like *Toxoplasma gondii* strain in a New Zealand fur seal (*Arctocephalus forsteri*) from New South Wales, Australia. *Vet. Parasitol.* 205, 347–353. doi: 10.1016/j.vetpar.2014.07.022
- Dubey, J. P. (2022). *Toxoplasmosis of animals and humans* (Boca Raton, United States: CRC Press).
- Dubey, J. P., Murata, F. H. A., Cerqueira-Cézar, C. K., Kwok, O. C. H., and Grigg, M. E. (2020). Recent epidemiologic and clinical importance of *Toxoplasma gondii* infections in marine mammals: 2009–2020. *Veterinary Parasitol.* 288, 109296. doi: 10.1016/j.vetpar.2020.109296
- Fayer, R., Dubey, J. P., and Lindsay, D. S. (2004). Zoonotic protozoa: from land to sea. *Trends Parasitol.* 20, 531–536. doi: 10.1016/j.pt.2004.08.008
- Gajadhar, A. A., Measures, L., Forbes, L. B., Kapel, C., and Dubey, J. P. (2004). Experimental *Toxoplasma gondii* infection in grey seals (*Halichoerus grypus*). *J. Parasitol.* 90, 255–259. doi: 10.1645/GE-144R
- Gales, N. J., Shaughnessy, P. D., and Dennis, T. E. (1994). Distribution, abundance and breeding cycle of the Australian sea lion *Neophoca cinerea* (Mammalia: Pinnipedia). *J. Zoology* 234, 353–370. doi: 10.1111/j.1469-7998.1994.tb04853.x
- Goldsworthy, S. D., Hodgson, J., and Holman, D. (2020). *Australian Sea lion investigations: 2018–19* (Adelaide: South Australian Research and Development Institute (Aquatic Sciences). SARDI Publication No. F2020/000052-1. SARDI Research Report Series No. 1051.
- Goldsworthy, S. D., Shaughnessy, P. D., Smart, J., Mackay, A., Bailleul, F., Reinhold, S.-L., et al. (2019). *Monitoring of Seal Bay and other populations on Kangaroo Island: 2017/2018* (Adelaide: Report to the Department for Environment and Water. South Australian Research and Development Institute (Aquatic Sciences), 59pp. SARDI Publication No. F2014000322-5. SARDI Research Report Series No. 1018.
- Harder, T. C., Stede, M., Willhaus, T., Schwarz, J., Heidemann, G., and Liess, B. (1993). Morbillivirus antibodies of maternal origin in harbour seal pups (*Phoca vitulina*). *Vet. Rec.* 132, 632–633. doi: 10.1136/vr.132.25.632
- Jensen, S. K., Aars, J., Lydersen, C., Kovacs, K. M., and Åsbakk, K. (2009). The prevalence of *Toxoplasma gondii* in polar bears and their marine mammal prey: evidence for a marine transmission pathway? *Polar. Biol.* 33, 599–606. doi: 10.1007/s00300-009-0735-x
- Jensen, S. K., Nymo, I. H., Forcada, J., Godfroid, J., and Hall, A. (2012). Prevalence of *Toxoplasma gondii* antibodies in pinnipeds from Antarctica. *Vet. Rec.* 171, 249. doi: 10.1136/vr.100848
- Kabay, M. J. (1996). Toxoplasmosis in a sea lion (*Neophoca cinerea*). Available at: https://aapspectranet.animalhealthaustralia.com.au/wp-content/uploads/2019/04/43_march1996.pdf [Accessed 25 August 2022]. Australian Society for Veterinary Pathology.
- Kreuder, C., Miller, M. A., Jessup, D. A., Lowenstine, L. J., Harris, M. D., Ames, J. A., et al. (2003). Patterns of mortality in southern sea otters (*Enhydra lutris nereis*) from 1998–2001. *J. Wildl. Dis.* 39, 495–509. doi: 10.7589/0090-3558-39.3.495
- Lambourn, D. M., Jeffries, S. J., and Dubey, J. P. (2001). Seroprevalence of *Toxoplasma gondii* in harbor seals (*Phoca vitulina*) in southern Puget Sound, Washington. *J. Parasitol.* 87, 1196–1197. doi: 10.1645/0022-3395(2001)087[1196: Sotghj]2.0.CO;2
- Landscape South Australia (2022) *Kangaroo Island feral cat eradication program*. Available at: <https://www.landscape.sa.gov.au/ki/pest-management/kangaroo-island-feral-cat-eradication-program> (Accessed 9 March 2022).
- Lanyon, S. R., and O'Handley, R. M. (2020). Relationship between *Toxoplasma gondii* seroprevalence and lamb marking in South Australian sheep flocks. *Aust. Vet. J.* 98, 525–528. doi: 10.1111/avj.13004
- Legge, S., Murphy, B. P., McGregor, H., Woinarski, J. C. Z., Augusteyn, J., Ballard, G., et al. (2017). Enumerating a continental-scale threat: how many feral cats are in Australia? *Biol. Conserv.* 206, 293–303. doi: 10.1016/j.biocon.2016.11.032
- Lindsay, S. A., Caraguel, C. G. B., and Gray, R. (2021). Topical ivermectin is a highly effective seal 'spot-on': A randomised trial of hookworm and lice treatment in the endangered Australian sea lion (*Neophoca cinerea*). *Int. J. Parasitol. Parasites Wildl.* 16, 275–284. doi: 10.1016/j.ijppaw.2021.11.002
- Lindsay, D. S., Collins, M. V., Mitchell, S. M., Cole, R. A., Flick, G. J., Wetch, C. N., et al. (2003). Sporulation and survival of *Toxoplasma gondii* oocysts in seawater. *J. Eukaryot. Microbiol.* 50 Suppl, 687–688. doi: 10.1111/j.1550-7408.2003.tb00688.x
- Lindsay, D. S., and Dubey, J. P. (2009). Long-term survival of *Toxoplasma gondii* sporulated oocysts in seawater. *J. Parasitol.* 95 (4), 1019–1020. doi: 10.1645/GE-1919.1
- Lowther, A. D., and Goldsworthy, S. D. (2011). Detecting alternate foraging ecotypes in Australian sea lion (*Neophoca cinerea*) colonies using stable isotope analysis. *Mar. Mammal. Sci.* 27, 567–586. doi: 10.1111/j.1748-7692.2010.00425.x
- Lowther, A., and Goldsworthy, S. (2012). Head start: Australian sea lion pups gain experience of adult foraging grounds before weaning. *Mar. Biol.* 159, 2687–2696. doi: 10.1007/s00227-012-2026-2
- Lowther, A. D., Harcourt, R. G., Hamer, D. J., and Goldsworthy, S. D. (2011). Creatures of habit: foraging habitat fidelity of adult female Australian sea lions. *Mar. Ecol. Prog. Ser.* 443, 249–263. doi: 10.3354/meps09392

- Lynch, M., Nielsen, O., Duignan, P. J., Kirkwood, R., Hoskins, A., and Arnould, J. P. (2011). Serologic survey for potential pathogens and assessment of disease risk in Australian fur seals. *J. Wildl. Dis.* 47, 555–565. doi: 10.7589/0090-3558-47.3.555
- Marino, A. M. F., Giunta, R. P., Salvaggio, A., Castello, A., Alfonzetti, T., Barbagallo, A., et al. (2019). *Toxoplasma gondii* in edible fishes captured in the Mediterranean basin. *Zoonoses Public Health* 66, 826–834. doi: 10.1111/zph.12630
- Mazzariol, S., Centelleghre, C., Petrella, A., Marcer, F., Beverelli, M., Di Francesco, C. E., et al. (2021). Atypical toxoplasmosis in a Mediterranean monk seal (*Monachus monachus*) pup. *J. Comp. Pathol.* 184, 65–71. doi: 10.1016/j.jcpa.2021.02.005
- McIntosh, R. R. (2007). *Life history and population demographics of the Australian sea lion* (Melbourne (Vic: La Trobe University).
- Measures, L. N., Dubey, J. P., Labelle, P., and Martineau, D. (2004). Seroprevalence of *Toxoplasma gondii* in Canadian pinnipeds. *J. Wildl. Dis.* 40, 294–300. doi: 10.7589/0090-3558-40.2.294
- Michael, S. A., Howe, L., Chilvers, B. L., Morel, P., and Roe, W. D. (2016). Seroprevalence of *Toxoplasma gondii* in mainland and sub-Antarctic New Zealand sea lion (*Phocarcos hookeri*) populations. *N Z Vet. J.* 64, 293–297. doi: 10.1080/00480169.2016.1191974
- Miller, M. A., Gardner, I. A., Kreuder, C., Paradies, D. M., Worcester, K. R., Jessup, D. A., et al. (2002). Coastal freshwater runoff is a risk factor for *Toxoplasma gondii* infection of southern sea otters (*Enhydra lutris nereis*). *Int. J. Parasitol.* 32 (8), 997–1006. doi: 10.1016/s0020-7519(02)00069-3
- Miller, M. A., Moriarty, M. E., Henkel, L., Tinker, M. T., Burgess, T. L., Batac, F. I., et al. (2020). Predators, disease, and environmental change in the nearshore ecosystem: mortality in southern sea otters (*Enhydra lutris nereis*) from 1998–2012. *Front. Mar. Sci.* 7. doi: 10.3389/fmars.2020.00582
- O'Callaghan, M., Reddin, J., and Lehmann, D. (2005). Helminth and protozoan parasites of feral cats from Kangaroo Island. *Trans. R. Soc. South Australia Incorporated* 129, 81–83.
- Poulle, M.-L., Le Corre, M., Bastien, M., Gedda, E., Feare, C., Jaeger, A., et al. (2021). Exposure of pelagic seabirds to *Toxoplasma gondii* in the Western Indian ocean points to an open sea dispersal of this terrestrial parasite. *PLoS One* 16 (8), e0255664. doi: 10.1371/journal.pone.0255664
- Peters, K. J., Ophelkeller, K., Bott, N. J., Deagle, B. E., Jarman, S. N., and Goldsworthy, S. D. (2015). Fine-scale diet of the Australian sea lion (*Neophoca cinerea*) using DNA-based analysis of faeces. *Mar. Ecol.* 36, 347–367. doi: 10.1111/maec.12145
- Puryear, W., Sawatzki, K., Bogomolni, A., Hill, N., Foss, A., Stokholm, I., et al. (2021). Longitudinal analysis of pinnipeds in the northwest Atlantic provides insights on endemic circulation of phocine distemper virus. *Proc. Biol. Sci.* 288, 20211841. doi: 10.1098/rspb.2021.1841
- Reisfeld, L., Sacristán, C., Ferreira Machado, E., Sánchez-Sarmiento, A. M., Costa-Silva, S., Ewbank, A. C., et al. (2019). Toxoplasmosis and sarcocystis spp. infection in wild pinnipeds of the Brazilian coast. *Dis. Aquat. Organ* 136, 235–241. doi: 10.3354/dao03410
- Resendes, A. R., Almeria, S., Dubey, J. P., Obón, E., Juan-Sallés, C., Degollada, E., et al. (2002). Disseminated toxoplasmosis in a Mediterranean pregnant Risso's dolphin (*Grampus griseus*) with transplacental fetal infection. *J. Parasitol.* 88, 1029–1032. doi: 10.1645/0022-3395(2002)088[1029:Dtiamp]2.0.Co;2
- Roberts, J. O., Jones, H. F. E., and Roe, W. D. (2021). The effects of *Toxoplasma gondii* on New Zealand wildlife: implications for conservation and management. *Pac. Conserv. Biol.* 27, 208–220. doi: 10.1071/pc20051
- Roe, W. D., Howe, L., Baker, E. J., Burrows, L., and Hunter, S. A. (2013). An atypical genotype of *Toxoplasma gondii* as a cause of mortality in Hector's dolphins (*Cephalorhynchus hectori*). *Vet. Parasitol.* 192, 67–74. doi: 10.1016/j.vetpar.2012.11.001
- Roe, W. D., Michael, S., Fyfe, J., Burrows, E., Hunter, S. A., and Howe, L. (2017). First report of systemic toxoplasmosis in a New Zealand sea lion (*Phocarcos hookeri*). *N Z Vet. J.* 65, 46–50. doi: 10.1080/00480169.2016.1230526
- Sepulveda, M. A., Seguel, M., Alvarado-Rybak, M., Verdugo, C., Munoz-Zanzi, C., and Tamayo, R. (2015). Postmortem findings in four South American sea lions (*Otaria byronia*) from an urban colony in Valdivia, Chile. *J. Wildl. Dis.* 51 (1), 279–282. doi: 10.7589/2013-07-161
- Sergeant, E. S. G. (2018a) *Epitools epidemiological calculators: population or cluster level sensitivity*. Available at: <https://epitools.ausvet.com.au/populationse> (Accessed 8 April 2022). Ausvet.
- Sergeant, E. S. G. (2018b) *Epitools epidemiological calculators: confidence of freedom following negative surveillance*. Available at: <https://epitools.ausvet.com.au/pfree> (Accessed 8 April 2022). Ausvet.
- Shapiro, K., Krusor, C., Mazzillo, F. F., Conrad, P. A., Largier, J. L., Mazet, J. A., et al. (2014). Aquatic polymers can drive pathogen transmission in coastal ecosystems. *Proc. Biol. Sci.* 281 (1795), 20141287. doi: 10.1098/rspb.2014.1287
- Shapiro, K., Miller, M. A., Packham, A. E., Aguilar, B., Conrad, P. A., Vanwormer, E., et al. (2016). Dual congenital transmission of *Toxoplasma gondii* and *Sarcocystis neurona* in a late-term aborted pup from a chronically infected southern sea otter (*Enhydra lutris nereis*). *Parasitology* 143, 276–288. doi: 10.1017/S0031182015001377
- Sinha, A. A., and Erickson, A. W. (1974). Ultrastructure of the placenta of Antarctic seals during the first third of pregnancy. *Am. J. Anat.* 141, 263–279. doi: 10.1002/aja.1001410208
- Taggart, P. L., Caraguel, C. G. B., and McAllister, M. M. (2020). Fractional seroprevalence rates in common prey species can cause more than half of feral cats to be exposed to *Toxoplasma gondii* annually. *Vet. Parasitol.* 288, 109306. doi: 10.1016/j.vetpar.2020.109306
- Taggart, P. L., Fancourt, B., Bengsen, A., Peacock, D., Hodgins, P., Read, J., et al. (2019a). Evidence of significantly higher island feral cat abundance compared with the adjacent mainland. *Wildl. Res.* 46, 378–385. doi: 10.1071/WR18118
- Taggart, P. L., Stevenson, M. A., Firestone, S. M., McAllister, M. M., and Caraguel, C. G. B. (2019b). Spatial analysis of a cat-borne disease reveals that soil pH and clay content are risk factors for sarcocystosis in sheep. *Front. Vet. Sci.* 6. doi: 10.3389/fvets.2019.00127
- Threatened Species Scientific Committee (2020) *Conservation advice. Neophoca cinerea, Australian sea lion*. Department of Agriculture, Water and the Environment, Canberra. Available at: www.environment.gov.au/biodiversity/threatened/species/pubs/22-conservation-advice-23122020.pdf (Accessed 29 August 2021).
- Tizard, I. R. (2018). "Immunity in the fetus and newborn," in *Veterinary Immunology*. tenth edition (St. Louis, Missouri: Elsevier), 247–260.
- van de Velde, N., Devleeschauwer, B., Leopold, M., Begeman, L., Ijsseldijk, L., Hiemstra, S., et al. (2016). *Toxoplasma gondii* in stranded marine mammals from the North Sea and Eastern Atlantic Ocean: findings and diagnostic difficulties. *Veterinary Parasitol.* 230, 25–32. doi: 10.1016/j.vetpar.2016.10.021
- Verma, S. K., Knowles, S., Cerqueira-Cezar, C. K., Kwok, O. C., Jiang, T., Su, C., et al. (2018). An update on *Toxoplasma gondii* infections in northern sea otters (*Enhydra lutris kenyoni*) from Washington state, USA. *Vet. Parasitol.* 258, 133–137. doi: 10.1016/j.vetpar.2018.05.011

SUPPLEMENTARY FIGURE 1 | Comparison of the standard length (age proxy) of Australian sea lion (*Neophoca cinerea*) pups sampled at three island colonies in South Australia, including the initial and repeat sampling of known-age and unknown-age pups from Seal Bay, Kangaroo Island, as well as the single sampling of pups from Olive Island and Dangerous Reef.



Median standard length values are indicated by the red crosshatch and text. Colony cohorts that share the same bolded letter (A, B, C) are not significantly different at the 5% level after accounting for multiple pairwise comparisons using the Bonferroni adjustment. Median known age (and age range) is provided for the Seal Bay colony.

SUPPLEMENTARY DATA 1 | Estimation of the *Toxoplasma gondii* seroprevalence in prey species necessary to achieve a seroprevalence of 30.4% in adult female Australian sea lions (*Neophoca cinerea*) at Dangerous Reef, South Australia.

A. Model structure from Taggart *et al.* (Taggart *et al.*, 2020):

$$\text{Exposure probability}_{\text{predator}} = 1 - (1 - \text{Seroprevalence}_{\text{prey}})^{(\text{DC} \times 365.25)} \quad (1)$$

Where:

Exposure probability_{predator} = the annual probability of a predator to consume at least one *T. gondii* infected prey (or the proportion of the predator population that would be expected to be exposed to *T. gondii* at least once annually)

Seroprevalence_{prey} = the average seroprevalence of *T. gondii* in the prey species

DC = the average daily count of prey individuals consumed by a predator and 365.25 is the number of days in a year.

B. Adapting to the Australian sea lion and solving for prey seroprevalence:

$$\begin{aligned} \text{Exposure probability}_{\text{seal}} &= 1 - (1 - \text{Seroprevalence}_{\text{prey}})^{(\text{DC} \times \text{Time at risk})} \\ \text{Seroprevalence}_{\text{prey}} &= 1 - (1 - \text{Exposure probability}_{\text{seal}})^{1/(\text{DC} \times \text{Time at risk})} \\ &= 1 - (1 - 0.304)^{1/(10 \times 365.25 \times 9)} \\ &= 0.00001 \text{ (0.001\%)} \end{aligned} \quad (2)$$

Where:

Exposure probability_{seal} = 0.304 – from the present study finding of 30.4% seroprevalence in adult females

Time at risk = 365.25 * 9.0 years – cumulative exposure using the 9.5 y median adult female age from the present study (and excluding 6 months prior to commencement of early foraging attempts)

DC = 10 – an estimate of the number of prey animals consumed per day, derived from reporting of a 48 day feeding trial of a non-lactating captive Australian sea lion that was fed 6.0 kg of prey per day (Peters *et al.*, 2015a), and the estimated individual weight of one of the prey species fed (using tommy rough (*Arripis georgianus*) estimated at 440 g/fish = 13.6 prey/d) (Bray, 2020). This is a conservative estimate, acknowledging the wide array of prey identified for the seal species (Peters *et al.*, 2015b) and that lactating females spend approximately two-thirds of their time at sea foraging (versus onshore nursing pups) (Lowther and Goldsworthy, 2011).

References

- Bray, D.J. 2020, *Arripis georgianus* in Fishes of Australia. <https://fishesofaustralia.net.au/home/species/405> [Accessed 19 May 2022].
- Lowther, A.D., and Goldsworthy, S.D. (2011). Maternal strategies of the Australian sea lion (*Neophoca cinerea*) at Dangerous Reef, South Australia. *Australian Journal of Zoology* 59(1), 54-62. doi: 10.1071/Zo11025.
- Peters, K.J., Ophelkeller, K., Bott, N.J., Deagle, B.E., Jarman, S.N., and Goldsworthy, S.D. (2015a). Fine-scale diet of the Australian sea lion (*Neophoca cinerea*) using DNA-based analysis of faeces. *Marine Ecology* 36(3), 347-367. doi: 10.1111/maec.12145.
- Peters, K.J., Ophelkeller, K., Herdina, Bott, N.J., and Goldsworthy, S.D. (2015b). PCR-based techniques to determine diet of the Australian sea lion (*Neophoca cinerea*): a comparison with morphological analysis. *Marine Ecology* 36(4), 1428-1439. doi: 10.1111/maec.12242.
- Taggart, P.L., Caraguel, C.G.B., and McAllister, M.M. (2020). Fractional seroprevalence rates in common prey species can cause more than half of feral cats to be exposed to *Toxoplasma gondii* annually. *Vet Parasitol* 288, 109306. doi: 10.1016/j.vetpar.2020.109306.

CHAPTER FIVE

Contribution of disease to Australian sea lion (*Neophoca cinerea*) pup mortality at Seal Bay, Kangaroo Island, 2016–2022



Author attribution statement

Chapter five is submitted as:

Lindsay, S.A., Fulham, M., Stonnill, M., Caraguel, C.G.B., and Gray, R. (2024). Contribution of disease to Australian sea lion (*Neophoca cinerea*) pup mortality at Seal Bay, Kangaroo Island, 2016–2022. *Marine Mammal Science* (submitted 28 March 2024).

Scott Lindsay was the primary author of this chapter and contributed to all aspects of this publication, including conceptualization, data curation, formal analysis (statistical), funding acquisition, investigation (fieldwork; laboratory), methodology, project administration, resources, visualisation, and writing (original draft; review and editing). Mariel Fulham contributed to data curation, investigation (fieldwork; laboratory), and writing (review and editing). Melanie Stonnill contributed to investigation (fieldwork), funding acquisition, and writing (review and editing). Charles Caraguel contributed to data curation, formal analysis (statistical), supervision, visualisation, and writing (review and editing). Rachael Gray contributed to conceptualization, funding acquisition, investigation (fieldwork), methodology, project administration, resources, supervision, visualisation, and writing (review and editing). All authors reviewed and approved the final manuscript.

Scott A. Lindsay

22 March 2024

Mariel Fulham

22 March 2024

Melanie Stonnill

25 March 2024

Charles G.B. Caraguel

28 March 2024

Rachael Gray

26 March 2024

Contribution of disease to Australian sea lion (*Neophoca cinerea*) pup mortality at Seal Bay, Kangaroo Island, 2016–2022

Scott A. Lindsay¹ | Mariel Fulham¹ | Melanie Stonnill² | Charles G. B. Caraguel^{3†} | Rachael Gray^{1†}

¹Sydney School of Veterinary Science, Faculty of Science, The University of Sydney, Camperdown, New South Wales, Australia. ²National Parks and Wildlife Service, Department for Environment and Water, Seal Bay Conservation Park, South Australia, Australia. ³School of Animal and Veterinary Sciences, Faculty of Science, The University of Adelaide, Roseworthy, South Australia, Australia.

†These authors share last authorship.

Correspondence

Rachael Gray, Sydney School of Veterinary Science, Faculty of Science, The University of Sydney, Camperdown, New South Wales 2006, Australia.

Email: rachael.gray@sydney.edu.au

Abstract

The endangered Australian sea lion (*Neophoca cinerea*) population is declining, and its conservation is threatened by the species' low fecundity and high pup mortality. This study details reasons for pup death and quantifies the impacts of disease and comorbidity on mortality. Of 284 dead pups reported from five breeding seasons (2016–2022) at the Seal Bay colony, Kangaroo Island, gross necropsy was conducted on 122, with fecal analysis for hookworm (*Uncinaria sanguinis*) detection, tissue histology and bacterial culture performed on subsets. Determinable primary causes-of-mortality were conspecific trauma (+/– comorbidity, 41.0%), starvation (24.6%) and infectious disease (hookworm-associated hemorrhagic enteritis [HAHE] +/- bacterial infection, 19.7%). Allowing for comorbidity, 37.7% of pups died with an infectious disease, including 44.0% of deaths with trauma and 41.5% with starvation. In higher mortality summer-autumn breeding seasons, pups were at greater risk of trauma ($p = .001$) and subsequent bacterial infection ($p < .001$). Parasiticide treatment significantly reduced HAHE-associated ($p = .005$) and overall mortality ($p < .001$) risk, suggesting hookworm infection increases pup susceptibility to other causes of mortality. Although the exact causal pathways for this treatment-related overall mortality benefit remain to be confirmed, intervention appears highly impactful and could benefit species conservation efforts.

KEYWORDS

Australian sea lion, conspecific trauma, disease, hookworm-associated hemorrhagic enteritis, bacterial infection, mortality, myiasis, *Neophoca cinerea*, *Streptococcus phocae*, *Uncinaria sanguinis*

1 | INTRODUCTION

The estimated population of endangered Australian sea lions (*Neophoca cinerea*) is less than 10,000 individuals (Goldsworthy et al., 2021a) and is dispersed across an historically truncated range along Australia's southern and western coasts (Ling, 1999). Each of the species' 80 breeding colonies is a relatively closed sub-population, which is largely a consequence of female fine-scale foraging site fidelity and philopatry that limit inter-colony migration (Campbell et al., 2008; Lowther et al., 2012). Consequently, pup birth and survival are important modulators of population size at the colony level (McIntosh & Kennedy, 2013) and conservation at the species level. A three-generational (~42 years) mean annual decline in pup births of 2.0%, has ranged from a 9.9% decline to a 1.7% increase for individual colonies (Goldsworthy et al., 2021a).

An early mortality survey (1967, 1969, and 1970 breeding seasons) at the high population density Dangerous Reef colony (34.817°S, 136.217°E), Spencer Gulf, South Australia, reported trauma from conspecific aggression as the most common finding in dead pups (Marlow, 1975). In contrast, a first survey at the Seal Bay Conservation Park colony (35.995°S, 137.317°E), Kangaroo Island, South Australia (the site of the current study), found starvation to be the most common cause of pup death during the 1986/1987 and 1988 breeding seasons (Higgins & Tedman, 1990). A comparatively lower incidence of trauma at this colony was attributed to its lower population density. In the absence of a detectable loss of body condition or external injury, the cause of death remained undetermined for more than a quarter of the pups examined. A subsequent mortality study at the Seal Bay colony (2002/2003, 2004 and 2005/2006 breeding seasons) reaffirmed conspecific trauma as the most common cause of pup death (McIntosh & Kennedy, 2013). This was despite an 11.1% decline in the population number (and therefore colony density) in the intervening period (Goldsworthy et al., 2008). No seasonal variation in the cause of mortality was apparent, although deceased pups were

smaller (and therefore likely to be younger) in the summer-autumn (versus winter-spring) breeding seasons. A cause of death was not determined for half of the pups examined.

Female Australian sea lions provide 15–18 months of care for their pups, which are weaned in the month prior to the next birth (Higgins & Gass, 1993). Females arrive onshore two days prior to parturition and are mate-guarded by a territorial male until mating at one week postpartum. They remain with their pups for the first 7–10 days of their life. Females then alternate pup feeding with foraging trips, during which time the newborn pups are unprotected from conspecific aggression (each 1–2 days duration) (Higgins & Gass, 1993). An inherent challenge to the study of Australian sea lion pups is the species' 17–18-month non-annual and aseasonal breeding cycle (Higgins 1993), which is asynchronous between colonies (Gales et al., 1994) and extends for 7–12 months at the larger colonies (Goldsworthy et al., 2021b). Additionally, most breeding colonies are remotely located on offshore islands with limited access.

The Seal Bay colony of Australian sea lions is the third largest and most closely monitored due to its relatively good accessibility. A 1.3% seasonal (0.9% annual) decline in pup births over 12 breeding seasons (2004–2020/2021) has stabilized at 232–242 births for the past five of those breeding seasons (Goldsworthy et al., 2021b). Despite the absence of an obvious monotonic trend over this period, pup mortality has been highly variable (17.9% to 41.8%). It has also been consistently higher in summer-autumn versus winter-spring breeding seasons, averaging 32.8% and 21.4%, respectively (Goldsworthy et al., 2021b). Seasonal anomalies in the sea surface temperature impacting prey-species availability for maternal provisioning of pups has previously been suggested as one reason for this seasonal variation (McIntosh et al., 2013). Endemic hookworm (*Uncinaria sanguinis*) infection of Australian sea lion pups has more recently been suggested as an additional cause (Marcus et al., 2014a).

All pups born at the Seal Bay colony are infected post-partum with hookworm, a hematophagic intestinal parasite transmitted by the ingestion of infective larvae present in colostrum (Marcus et

al., 2014a). Studies published since the mortality surveys of the colony have identified hookworm as an important agent of disease in Australian sea lion pups younger than 2–3 months of age (Marcus et al., 2014a; Marcus et al., 2015a; Marcus et al., 2015b; Lindsay et al., 2021; Lindsay et al., 2023). Infection resulted in substantial pup morbidity (increased severity of post-natal anemia and systemic inflammation) and growth retardation (Lindsay et al., 2023). Higher infection intensity contributed to hookworm-associated hemorrhagic enteritis (HAHE) and was positively associated with higher colony pup mortality as well as reduced individual body condition (Marcus et al., 2014a). The latter suggested that hookworm infection could also increase pup vulnerability to death from comorbidities such as conspecific trauma (Marcus et al., 2014a).

This mortality study aimed to quantify the contribution of parasitic and bacterial infectious diseases to Australian sea lion pup mortality. Guided by recommendations from the previous mortality surveys in this species (McIntosh & Kennedy, 2013) and reports in other otariid species (Michael et al., 2019; Seguel et al., 2013), it would incorporate histologic, parasitological, and microbiological examinations whenever possible. It also aimed to determine the contribution of infectious disease comorbidity to pup death from non-infectious causes. Reporting across summer-autumn and winter-spring breeding seasons aimed to detect additional contributors to the seasonal oscillation in pup mortality.

2 | MATERIALS AND METHODS

2.1 | Ethics approvals and reporting

Sampling was approved by the Department for Environment and Water (DEW) South Australia (Permit/License Number: MR00073-2-R; Scientific Research Permit Numbers: A25088-14 to A25088-

20). Study protocols were approved by The University of Sydney Animal Ethics Committee (Protocol Numbers 2014/726, 2017/1260 and 2021/1992).

2.2 | Study site

The study was conducted at the Seal Bay colony of Australian sea lions over five breeding seasons. Pups born in each breeding season were subsequently referred to as that season's 'breeding cohort'. Winter-spring breeding cohorts were comprised of pups born in breeding seasons 2016, 2019 and 2022, and summer-autumn breeding cohorts of those born in seasons 2017/2018 and 2020/2021. Estimates of total pup births and deaths for each breeding cohort were provided by cumulative counts from colony surveys conducted throughout the breeding season (Goldsworthy et al., 2021b). In the absence of recording within the colony, temperature data from the closest weather station of Parndana (19.7km inland; 35.79°S, 137.25°E, elevation 165 m) provided an approximation for the daily variation in weather conditions experienced within the colony (Australian Government Bureau of Meteorology, 2023). The approximate geographical location of dead pups within the 2 km colony was reported relative to two promontories and regional landmarks. To assess for an association of habitat with pup mortality, like habitats were grouped into four study areas (Figure 1). These comprised the three major colony divisions of the Eastern Prohibited Area (EPA), Western Prohibited Area (including the West End and West End Cove locations; WPA) and central Main Beach. The latter was subdivided into the beach and the coastal dunes/scrub (including the Road Reserve locations) areas.

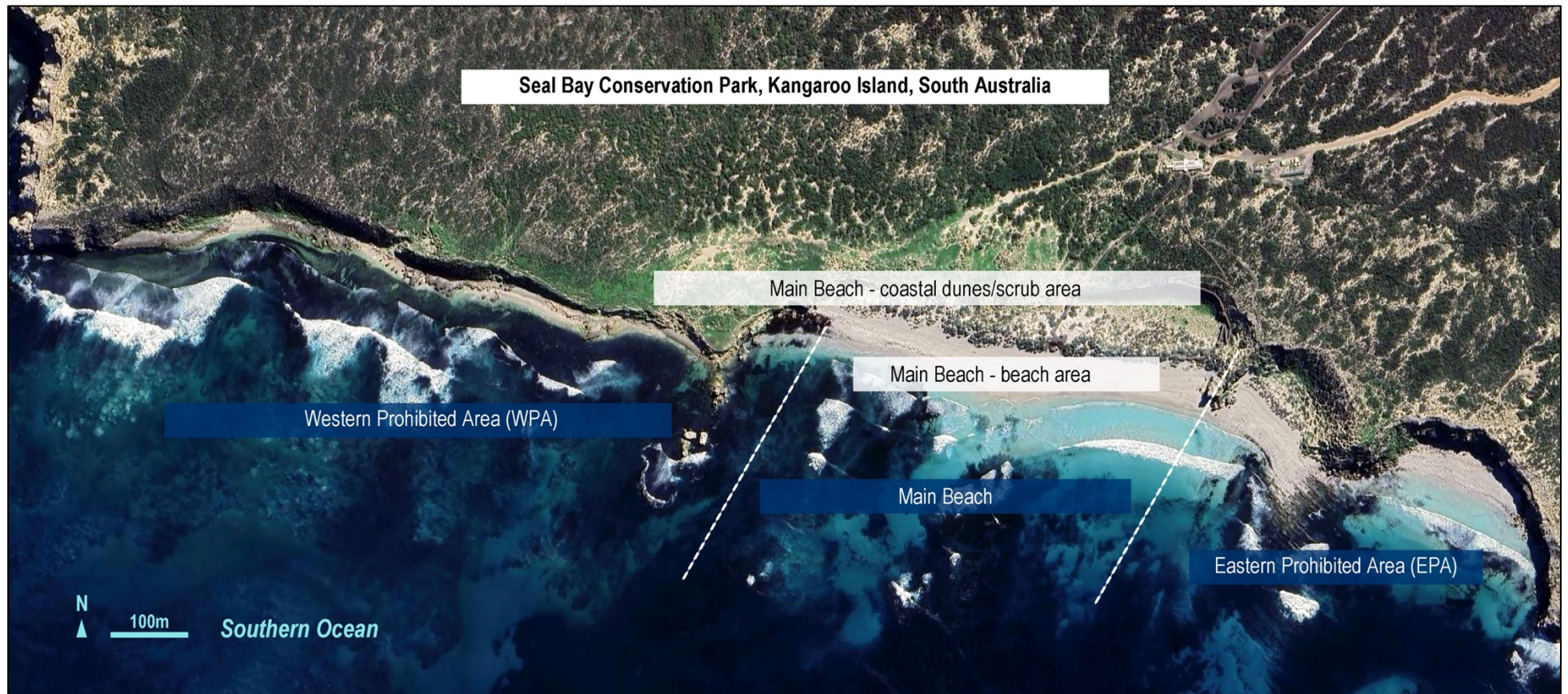


FIGURE 1 The study-defined geographical areas within the Seal Bay Conservation Park breeding colony of Australian sea lions. The Main Beach division was subdivided into beach and coastal dunes/scrub areas. Image adapted from Google Maps (Google, 2023).

2.3 | Detection of dead pups and data collection

To minimize disruption to the breeding population, all colony surveys were restricted to a single walk through in any one day. For the duration of each 8–12-month breeding season, surveys were conducted twice weekly by Seal Bay Conservation Park staff. Deceased pups were only removed from the colony for necropsy where the degree of decomposition was not already advanced. This equated to a good to fair carcass condition (classification code 2–3) (Geraci et al., 2005). Dead pups were concealed in a plastic bag for carriage from the colony to a storage facility 300m away. Carcasses were frozen at -20°C for later necropsy at this site by the study investigators. Additionally, coinciding with periods of peak breeding activity, colony surveys were conducted daily by the study investigators during fieldtrip visits for monitoring of colony health (Table 1.). Dead pups removed from the colony during these visits were necropsied as soon as practicable (<24 hours). Duplication of reporting was avoided by daily briefings with Conservation Park staff. All carcasses were buried off-site following necropsy.

Information recorded at the time of carcass detection were a unique identifier (sequential numbering of the death for each breeding cohort), the date the pup was found dead, its location within the colony, the presence or absence of conspecific adults and their identity if known from microchip data, the presence and nature of any external injuries, and evidence of recent birth (e.g., the presence of a fresh placenta or blood). Where the pup identity was known by its location (newborn pups move little from the birth site in their first 2–4 weeks) (Marlow, 1975) or scanning of an existing microchip, previous observational, capture, and/or resight data were available. This could have included an observed or estimated birth date (and therefore pup age), the frequency of maternal presence and pup suckling, observed traumatic events or injuries, pup attempts to suckle non-maternal females, and the persistent presence of the previous pup (juvenile) or male mate-guard. Additionally, maternal parity and pupping history were available for the subset of pups born

to microchipped females. For pups previously captured as part of concurrent studies, historical information were standard morphometrics (for assessment of weight loss or gain in the intervening period), hematological data, fecal smear assessment, and the presence of external injury or hemorrhagic diarrhea. For the 2019 and 2020/2021 breeding cohorts, a concurrent hookworm treatment trial (a single topical off-label application of ivermectin dosed at 500 µg/kg bodyweight; IVOMEC® Pour-on for Cattle, 5 mg/ml, Boehringer Ingelheim Animal Health Australia Pty. Ltd., North Ryde NSW) was conducted at the colony (Lindsay et al., 2023). Treatment status and date of anthelmintic application were known for pups recruited to that study. Treated pups were retained in mortality reporting, with treatment status accounted for in statistical analysis when determining morbidity and mortality risk.

An observational database of information for all deceased pups was maintained by one study author (MS). This included details for pups that were not recruited to the mortality study, and a reason for this outcome (advanced autolysis, guarding by an adult animal, washed away, or unspecified).

2.4 | Gross necropsy examination

Data recorded at the time of necropsy were carcass state (fresh or frozen-thawed), degree of decomposition (mild, moderate, or marked, based on increasing severity of tissue softening, discoloration, and/or putrefaction), body weight (kg), standard length (the straight line distance between nose and tail extremities; cm), a subjective four-scale body condition score (poor-emaciated, fair-thin, good, excellent), sex, molt state (pups molt at approximately 3–5 months of age) (Goldsworthy, 2018; Marlow, 1975), the presence of fly larvae (and their size and location) and available observational or historical information. Pup necropsy followed a standard technique: external examination, noting the extent and nature of any injuries; measurement of ventral mid-sternal blubber thickness (mm); ventral exposure of the thoracic and abdominal cavities, noting any effusions and the *in situ* appearance of organs; removal of thoracic and abdominal organs for

detailed examination, including opening of the gastrointestinal tract for comment on content (ingesta, hookworm burden, hemorrhage) and presence of serosal hemorrhage; incision of multiple joints; and, opening of the calvarium for examination of the brain and cervical spinal cord. Note was also made of lymph node enlargement, and more detailed comment provided for abnormal tissues.

Where the state of preservation was adequate (good carcass condition), tissue samples were preserved in 10% neutral buffered formalin for histologic examination. This included samples from all thoracic and abdominal viscera, endocrine glands, skin, skeletal muscle, mesenteric lymph nodes, and any grossly abnormal tissues. Fixed tissues were later paraffin embedded and standard 5 µm hematoxylin and eosin-stained sections prepared (Veterinary Pathology Diagnostic Services, The University of Sydney and Veterinary Diagnostic Laboratory, The University of Adelaide).

When colonic or rectal content was present (e.g., not scavenged), a swab was obtained to determine hookworm patency status. From the subset of necropsied animals in good carcass condition, a swab (Copan Diagnostics Inc., USA) of cavitory effusions or wound exudates was obtained for culture when bacterial infection was suspected. All swabs were frozen at –20°C until processed. Fecal swabs were reviewed for the presence of hookworm ova as previously described (Lindsay et al., 2023).

Microbiological swabs were cultured aerobically and anaerobically (Veterinary Diagnostic Laboratory, The University of Adelaide), with pure or predominant colonies subsequently analyzed for bacterial identification to species level using a matrix-assisted laser desorption/ionization and time-of-flight mass spectrometer (MALDI-TOF; Australian Centre for Antimicrobial Resistance Ecology (ACARE), The University of Adelaide).

2.5 | Pup age at death

Pups were assigned to one of five age classes by their known or predicted age antemortem, or the following postmortem findings:

- 1) *Perinatal (inclusive of stillborn pups and neonates 1–2 days old)* – dark brown to black pups (the lanugo color present for the first week after birth) (Goldsworthy, 2018; Marlow, 1975) with meconium present in the large intestine and an attached fresh umbilical cord. In the absence of an observed birth, stillbirth was indicated by atelectatic (non-aerated) lungs, which failed to float in formalin, and the absence of gastric content (not suckled). Pups in this age class are negative for patent hookworm infection (either not infected or prepatent).
- 2) *1–2 weeks* – brown to black pups that lack perinatal age class findings (i.e., atelectatic lungs, presence of a fresh umbilical cord or meconium) and are negative for patent hookworm infection.
- 3) *3–4 weeks* – variable shades of brown pups that are typically < 75 cm in standard length and are positive for patent hookworm infection.
- 4) *1–2 months* – variable shades of brown pups that are typically ≥ 75 cm in standard length and are positive for patent hookworm infection.
- 5) *> 2–3 months* – lighter shades of brown pups that are ≥ 75 cm (and often ≥ 80 cm) in standard length and are negative for patent hookworm infection (postpatent). Pups this age have often commenced or completed their first molt.

2.6 | Primary cause-of-mortality categorization

The minimum requirement for study inclusion was the gross necropsy examination of a dead pup sufficient for assessing the cause of death. Based on available observational, historical, necropsy and ancillary testing data, dead pups were assigned to one of four primary cause-of-mortality categories by author consensus (SL, RG, MF), as follows:

- 1) *Trauma* – the presence of external and/or internal injury of sufficient severity to cause death, with or without a comorbidity (i.e., infectious disease or starvation). Trauma was subcategorized by type (sharp: penetrating bite injuries; blunt: no detectable skin

penetration) and by location (head and neck; body – thoracic/shoulder; body – abdominal/pelvic; limbs). Drowning was included in this category and defined as a pup with sea water and foreign material in the small airways and hyperinflated lungs (Michael et al., 2019).

- 2) *Infectious disease* – the presence of at least one of two disease processes of sufficient severity to cause death, in the absence of conspecific trauma.
 - a. *Hookworm-associated hemorrhagic enteritis* (HAHE) – defined as a hookworm burden of moderate to high severity and the presence of small intestinal hemorrhage – either hemorrhagic intestinal content or multifocal serosal hemorrhage (petechiae or ecchymoses). Hookworm burden was classified as mild, moderate, or marked based on (1) the number of hookworms seen in a 100mm segment of opened small intestine ($\geq$ five segments examined: worm count approximating <20 , 20–99, and ≥ 100 , respectively); (2) the maximum count of ova in a fecal smear ($\geq$ three smears examined: <10 , 11–19, and ≥ 20 ova, respectively); and/or, (3) the severity of enteritis in histologic sections of small intestine (reported by an ACVP Board-certified veterinary pathologist [SL] as mild, moderate or marked, respectively).
 - b. *Bacterial infection* – at least one focus of inflammation suggestive of bacterial infection (e.g., an abscess, wound or body cavity exudate). An exudate (inflammatory effusion) was assumed by a gross appearance consistent with suppurative (neutrophilic) +/- fibrinous inflammation (i.e., viscous, turbid, flocculent, yellow to red, and variably malodorous). Focal or multifocal inflammation remote from a site of traumatic injury suggested septicemia. Confirmation of an etiologic agent was possible for the subset of carcasses in a sufficient state of preservation for positive bacterial culture +/- microscopic tissue examination.

- 3) *Starvation* – a poor-emaciated or fair-thin body condition, in the absence of traumatic injury or infectious disease sufficient to have caused death. This assessment was supported by a blubber thickness < 3 mm in pups older than perinatal age, the absence of recent feeding (empty stomach, absent or minimal intestinal content) and/or observed lack of maternal care (sighted alone or calling for extended periods, often more than a week; attempting to suckle non-maternal animals).
- 4) *Undetermined* – insufficient findings for categorization into one of the above determinable causes of mortality. This category was subcategorized by age, into perinatal (stillbirths and neonates) and older than perinatal.

These four primary cause-of-mortality categories were mutually exclusive. To fully capture their contribution to pup death, traumatic injury, infectious disease (HAHE, bacterial infection) and starvation were also reported as comorbidities whenever present. This subcategorization was listed for the three determinable primary cause-of-mortality categories and was not mutually exclusive.

2.7 | Statistical analysis

The Stata statistical package (StataCorp LLC., College Station, Texas, United States: version 17.0) was used for statistical analysis.

2.7.1 | Descriptive reporting of necropsy outcomes

To assess for a relationship between primary cause-of-mortality categorization and the timing of a pup's death relative to that of others within its breeding cohort (and to standardize for the species' non-annual breeding cycle), the date of its death was compared with the breeding cohort's median pupping date. Categories were then compared for the combined five breeding cohorts, and separately for the summer-autumn and winter-spring breeding cohorts. Further, primary cause-of-mortality categories were compared by calendar month to detect any weather association, independent of breeding cohort. Comparison of categorical variables and primary cause-of-mortality

were made using Fisher's Exact test with significance at the 5% level (type-I-error or α). Continuous variables with apparent deviation from normality were compared using a non-parametric Kruskal-Wallis equality-of-populations rank test.

2.7.2 | Modelling of population risk – seasonal and treatment effects

The total counts of pup births and deaths for all breeding cohorts, and the number of pups treated for hookworm infection in the 2019 and 2020/2021 breeding cohorts, were known. Assuming that necropsied pups were representative of all dead pups within their breeding cohort (inclusive of those not recruited to the study), the proportions for each comorbidity were extrapolated to this population. Mortality and morbidity incidence risks were then predicted for the population of pups born across the combined five breeding cohorts, using mixed-effects logistic regressions. All regressions (except that for HAHE) included season (summer-autumn versus winter-spring) and hookworm treatment (present versus absent) as fixed effects and breeding cohort as a random effect. Additional biologically relevant fixed effects were HAHE for modelling of trauma and starvation risk, and starvation and trauma for modelling of bacterial infection risk. Firth's variant of the multifactorial logistic regression was used to explore treatment effect on HAHE, with season and breeding cohort as fixed effects.

3 | RESULTS

3.1 | Sampling summary

A summary of total pup birth and death counts, and gross necropsy (+/- tissue histology) number for the five breeding cohorts is reported in Table 1. Overall mortality for the 1,212 recorded pup births was 23.4% ($n = 284$). The average mortality for the three winter-spring and two summer-autumn

breeding cohorts sampled were 18.8% (139/738) and 30.6% (145/474), respectively. Gross necropsy was performed on 122 (43.0%) and histology on 45 (15.8%) of these pups. Fecal swabs were available from 73 (59.8%) of the pups necropsied, and 15 swabs for bacterial culture were obtained from 11 (47.8%) of the 23 pups with potential bacterial infection based on gross necropsy findings. Approximately a third of carcasses were necropsied within 24 hours of removal from the colony, and the remaining two-thirds were necropsied after freezing and thawing. Maternal parity was available for 30 (24.6%) of the necropsied pups, indicating a median of second parity (range first to fifth parity), and a predominance of first and second parity females ($n = 19$, 63.3%).

3.2 | Mortality demographics and distribution

There were 64 (54.2%) male and 54 (45.8%) female pups necropsied (sex was unreported for four pups). Across all necropsied pups, the median (and range) body weight and standard length were 6.8 kg (2.3–20.2 kg) and 70.0 cm (51.0–102.0 cm), respectively. Measurements for males were 7.4 kg (3.3–20.2 kg) and 72.5 cm (57.0–90.0 cm), and for females were 6.3 kg (2.3–17.9 kg) and 67 cm (51.0–102 cm). Further, 19 (15.6%) pups were classed as perinatal aged, 38 (31.2%) as 1–2 weeks old, 39 (32.0%) as 3–4 weeks old, 14 (11.5%) as 1–2 months old, and 12 (9.8%) as > 2–3 months old. Approximately a third of the removed pups were located in each of the study-defined geographical colony divisions of EPA, WPA and Main Beach (Figure 1). Of the 41 dead pups removed from Main Beach, 25 (61.0%) were located in the dunes/scrub area and the remainder in the beach area.

3.3 | Primary cause-of-mortality categorization

A primary cause-of-mortality was determined for 104 (85.2%) of the necropsied pups – trauma for 50 (41.0%), starvation for 30 (24.6%), and an infectious disease for 24 (19.7%) (Table 2). For 18 (14.8%) of the necropsied pups the primary cause-of-mortality was undetermined. There was a significant association of primary cause-of-mortality with pup age class at death ($p < .001$). This

result remained significant ($p = .009$) after exclusion of the perinatal age class from the analysis to assess for an association in the older age classes (i.e., removing any impact from the large contribution of perinatal aged pups to the undetermined category). The primary cause-of-mortality categorization for each age class is shown in Figure 2. It highlights the persistent risk of trauma across all age classes, but greatest in pups less than a month of age; a bimodal peak of starvation in pups 1–2 weeks or > 2–3 months of age; the concentration of infection in pups beyond perinatal age, but younger than 2–3 months of age; and the high proportion of deaths of undetermined cause in the perinatal age class.

3.3.1 | Trauma

Trauma accounted for 50 (41.0%) of the pup deaths with inclusion of pups with a comorbidity ($n = 22$) or evidence of drowning ($n = 1$). That is, a comorbidity was detected in nearly half of the pups dying from trauma. Pre-existing HAHE, or bacterial infection as sequelae to injury, were reported in nine (18.0%) and 17 (34.0%) of these pups, respectively. Conspecific interaction was suspected in all cases of trauma. This included a death from blunt trauma due to a cliff fall that resulted from male aggression. For traumatic deaths, there was no significant association with age class ($p = .268$), persisting ($p = .704$) after exclusion of the perinatal age class. When reported, traumatic injury was most often subcategorized as sharp ($n = 33$, 71.7%; Figure 3a) rather than blunt ($n = 13$, 28.3%). Further, traumatic injury was most frequently detected in the head and neck region of pups ($n = 30$, 65.2%), rather than the abdomen and pelvis ($n = 23$, 50.0%), thorax and shoulder ($n = 15$, 32.6%), and/or one or more of the limbs ($n = 7$, 15.2%). Typical of sharp trauma with per-acute death were catastrophic head injuries resulting from bites from adult animals (Figure 3a,b). Less commonly, per-acute death due to blunt force trauma resulted from rupture of abdominal viscera (liver or spleen) and abdominal hemorrhage. Crushing or blunt impact (likely rock impact) injury to the skull and spinal cord were also detected. A single 3–4 week old pup in good condition that was recovered from the water was assessed as drowning.

TABLE 1 Summary of pup births, deaths and necropsy number for five breeding cohorts (2016–2022) at the Seal Bay colony of Australia sea lions, Kangaroo Island, South Australia.

Breeding cohort	Season type	Breeding duration	Fieldtrip visits	Total pup births	Total pup deaths	Total mortality	Gross necropsies	Proportion necropsied	Tissue histology
2016	Winter-spring	8 months	2 (13 days)	240	43	17.9%	15	34.9%	1
2017/2018	Summer-autumn	10 months	3 (19 days)	232	75	32.3%	29	38.7%	14
2019	Winter-spring	12 months	6 (55 days)	239	46	19.2%	25	54.3%	9
2020/2021	Summer-autumn	12 months	8 (77 days)	242	70	28.9%	40	57.1%	16
2022	Winter-spring	12 months	2 (12 days)	259	50	19.3%	13	26.0%	5
Cumulated		54 months	21 (176 days)	1212	284	23.4%	122	43.0%	45

Breeding duration is the time between first and last observed pup births. Fieldtrip visits (and days within the colony) refers to visits by the study investigators. Gross necropsy and tissue histology refers to the number of deaths investigated by each method. Pup data for seasons 2016–2020/2021 are summarized in Goldsworthy et al., 2021a.

TABLE 2 Primary cause-of-mortality and comorbidities for Australian sea lion pups necropsied during winter-spring (2016, 2019 and 2022 breeding cohorts) and summer-autumn (2017/2018 2020/2021 breeding cohorts) seasons at Seal Bay, Kangaroo Island, South Australia.

PRIMARY CAUSE-OF-MORTALITY and comorbidities	Winter-spring		BREEDING SEASON Summer-autumn		Combined	
	Count	Proportion	Count	Proportion	Count	Proportion
TRAUMA						
Trauma only (total)	10	18.9%	17	24.6%	27	22.1%
+ bacterial infection	3	5.7%	10	14.5%	13	10.7%
+ HAHE	3	5.7%	2	2.9%	5	4.1%
+ HAHE + bacterial infection	1	1.9%	3	4.4%	4	3.3%
Trauma + infection (total)	7	13.2%	15	21.7%	22	18.0%
Drowning	1	1.9%	0	0.0%	1	0.8%
All trauma	18	34.0%	32	46.4%	50	41.0%
STARVATION						
Starvation only (total)	15	28.3%	15	21.7%	30	24.6%
+ HAHE	9	17.0%	4	5.8 %	13	10.7%
+ bacterial infection	1	1.9%	0	0.0%	1	0.8%
+ HAHE + bacterial infection	0	0.0	2	2.9%	2	1.6%
+ trauma	1	1.9%	0	0.0%	1	0.8%
+ trauma + HAHE	1	1.9%	0	0.0%	1	0.8%
+ trauma + bacterial infection	1	1.9%	2	2.9%	3	2.5%
+ trauma + bacterial infection + HAHE	1	1.9%	1	1.4%	2	1.6%
Starvation + comorbidity (total)	14	26.4%	9	13.0%	23	18.9%
All starvation	29	54.7%	24	34.8%	53	43.4%
INFECTIOUS DISEASE						
HAHE only	11	20.8%	8	11.6%	19	15.6%
Bacterial infection only	1	1.9%	1	1.5%	2	1.6%
HAHE and bacterial infection only	0	0.0%	3	4.4%	3	2.5%
Infectious disease only (total)	12	22.6%	12	17.4%	24	19.7%
HAHE + trauma	3	5.7%	2	2.9%	5	4.1%
Bacterial infection + trauma	3	5.7%	10	14.5%	13	10.7%
HAHE + bacterial infection + trauma	1	1.9%	3	4.4%	4	3.3%
Infectious disease + trauma (total)	7	13.2%	15	21.7%	22	18.0%
All infectious disease	19	35.8%	27	39.1%	46	37.7%
UNDETERMINED						
Stillbirth	4	7.5%	2	2.9%	6	4.9%
Neonatal death	2	3.8%	3	4.3%	5	4.1%
Undetermined: perinatal (total)	6	11.3%	5	7.3%	11	9.0%
Undetermined: older than perinatal (total)	2	3.8%	5	7.3%	7	5.7%
All undetermined	8	15.1%	10	14.5%	18	14.8%
Total (of the lightly shaded rows)	53	100.0%	69	100.0%	122	100.0%

The four lightly shaded rows are mutually exclusive and correspond with the number of pups reported for the four defined primary cause-of-mortality categories. Undetermined deaths are subcategorized by pup age: pups dying in the perinatal period (includes pups stillborn and neonatal pups dying in the first 1–2 days of life), and pups dying beyond the perinatal period. HAHE: hookworm-associated hemorrhagic enteritis.

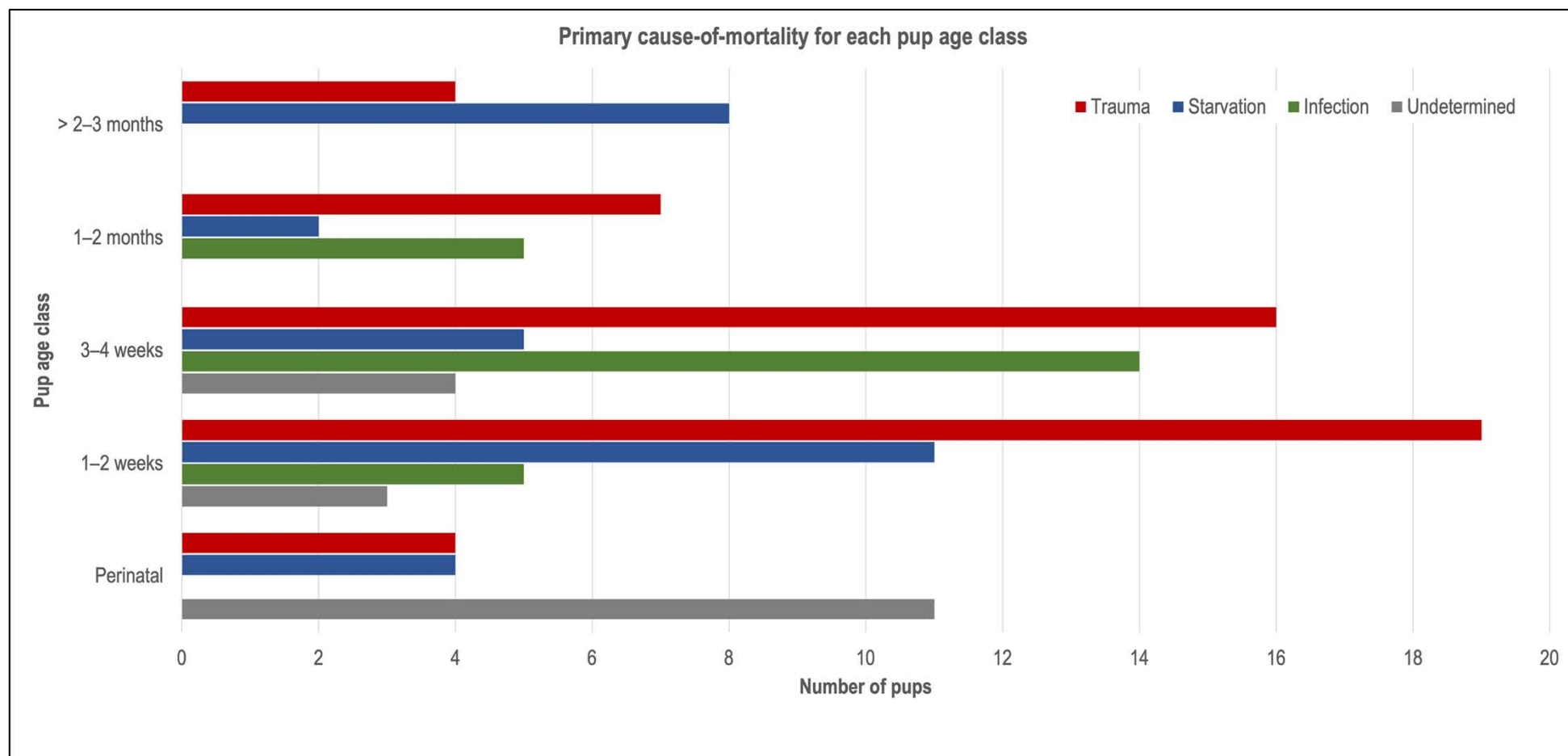


FIGURE 2 Counts of Australian sea lion pups assigned to each primary cause-of-mortality category within each of the study age classes, for the 2016–2022 breeding cohorts at Seal Bay, Kangaroo Island, South Australia.

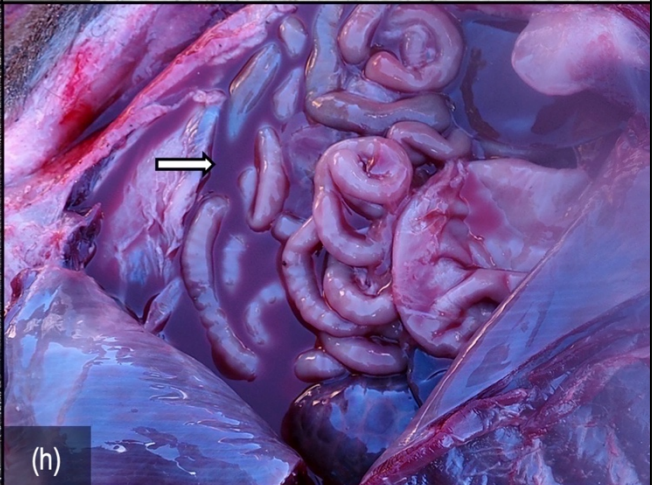
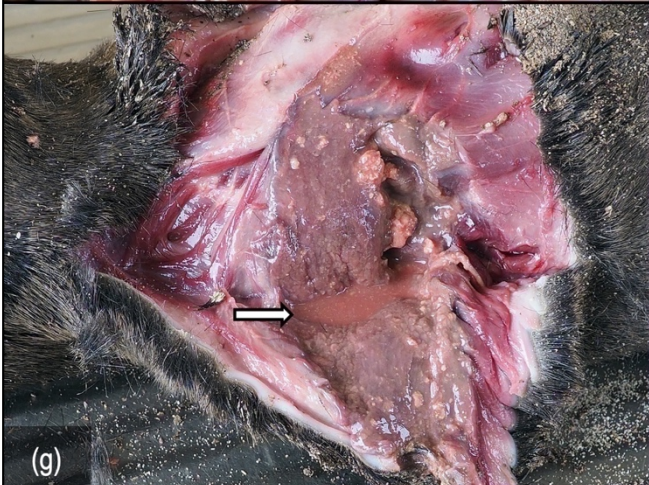
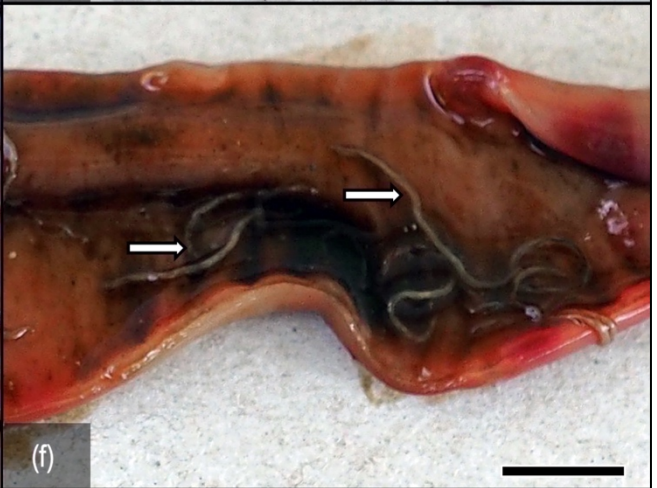
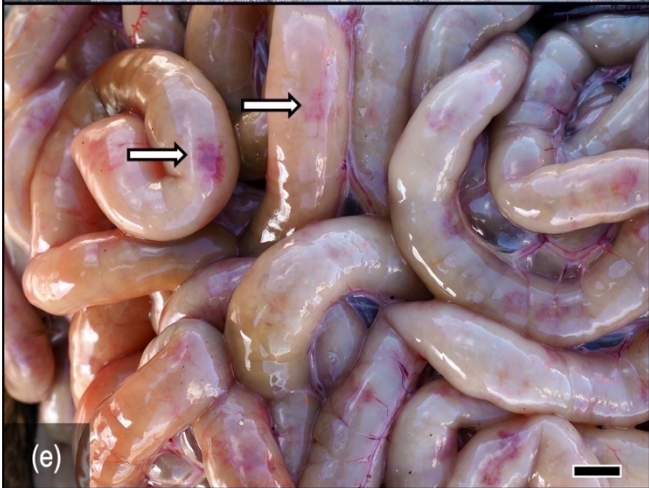


FIGURE 3 Findings in Australian sea lion pups. Conspecific trauma: (a) Bite wounds on the dorsal cervical region of a pup; (b) Catastrophic head injury, including extensive hemorrhage and multiple fractures. Starvation: (c) An emaciated pup with the characteristic appearance of a disproportionately large head and limbs; (d) Juvenile competition as a potential contributor to starvation of a neonatal pup. Hookworm-associated hemorrhagic enteritis: (e) Parasite feeding resulting in multifocal serosal petechiae and ecchymoses (arrows); (f) Adult hookworms (arrows) in an opened segment of small intestine (jejunum) with little red-brown intestinal content. Traumatic wounds with secondary bacterial infection in pups with concurrent hookworm-associated hemorrhagic enteritis (not shown): (g) Penetrating bite injury and scapular fracture in a 3–4-week-old female pup with secondary bacterial infection (pure growth of *Arcanobacterium phocae*), abscessation with a flocculent and turbid exudate, and regional lymph node enlargement. Histologically, severe necrotizing and suppurative cellulitis and myositis were evident; (h) Multifocal penetrating bite injuries in an older male pup with secondary bacterial infection (pure growth of *Streptococcus phocae*) and a large volume serosanguinous, turbid peritoneal exudate. Wound myiasis (not shown) was suspected to have occurred antemortem. Black bars approximate 10 mm.

3.3.2 | Starvation

Starvation (Figure 3c) was the primary cause-of-mortality in 30 (24.6%) of the pup deaths. Across all categories of mortality, including trauma and infectious disease, starvation as a comorbidity was reported in 53 (43.4%) of the pups. There was no significant difference between age classes ($p = .152$), persisting ($p = .580$) with exclusion of the perinatal age class. In starving pups, sand was often present in the stomach (and less commonly the intestinal tract). In older pups the stomach often contained multiple small stones exceeding the gastrolith number observed in well-nourished pups of similar age that were necropsied in this study. Histologic evidence of mild to moderate gastritis was also noted in the older starving pups. Sternal blubber depth in pups beyond the perinatal period was significantly ($p = .007$) different between the four primary cause-of-mortality categories, with the median (and range) for trauma, starvation, infectious disease, and undetermined deaths being 6 mm (0–30 mm), 0 mm (0–2 mm), 0 mm (0–8 mm), and 10 mm (3–13 mm), respectively.

The observational database of all deaths ($n=284$) across the five breeding seasons recorded five cases (1.8%) of an unweaned juvenile competing with (and/or injuring) a newborn pup, two cases (0.7%) of maternal rejection or aggression towards a newborn pup, two cases (0.7%) of a pup allosuckle (suckling of an unrelated pup) or potential twin birth (0.7%), and one case (0.4%) of death of the female within the colony.

3.3.3 | Infectious disease

Infectious disease in the absence of conspecific trauma accounted for 24 (19.7%) of the pup deaths. This comprised 19 (15.6%) attributed to HAHE (Figures 3e,f), two (1.6%) to bacterial infection (Figures 3g,h), and three (2.5%) to co-infection. Including deaths with traumatic injury, there were 46 (37.7%) pups that died with an infectious disease. There were 108 pups of known hookworm infection status. Of these, 46 (42.6%) were positive and 62 (57.4%) were negative (48 were not infected or pre-patent, eight were post-patent and six had been treated). Of the 31 (25.4%) pups

dying with HAHE, 22 (71.0%) died of infectious disease alone (HAHE +/- bacterial infection) and the remaining nine (29.0%) died with traumatic comorbidity. Two of the three pups with HAHE and bacterial infection were aged 1–2 months (late patency) and found to have hookworm-associated jejunal perforations and fibrinous and suppurative peritonitis. Mixed enteric bacteria were cultured from peritoneal exudates in both pups (Supplementary Table 1). The remaining pup was aged 3–4 weeks (early patency) and died with an umbilical abscess. Histologic examination showed marked neutrophilic inflammation and innumerable coccoid bacteria. Histologic review of intestinal specimens from pups with HAHE found moderate to marked enteritis, characterized by mucosal erosion, ulceration, eosinophilic and neutrophilic inflammation, and hemorrhage. Multifocal hookworm feeding tracts extended into the submucosa to the level of the muscular tunics (and to the serosa in the cases of intestinal perforation). There was a significant association of pups dying with HAHE and age class (highest in the 3–4 week and 1–2 month age classes; $p < .001$), reflecting the expected higher disease incidence in pups of patent infection age.

There were 22 (18.0%) pups dying with bacterial infection. In 17 of these pups (77.3%) infection was associated with a traumatic injury. Findings for three of the five pups without a traumatic comorbidity are described above. The remaining two pups were found to have cellulitis and myositis in the umbilical region (tissue necrosis and neutrophilic inflammation confirmed for the single case available for histologic review). The first of these two pups experienced maternal abandonment in the first week of life, and the second (aged 3–4 weeks) was found to have delayed umbilical closure and secondary regional myiasis. Of the 10 pups with positive culture results (summarized in Supplementary Table 1), eight were associated with bite wounds from conspecific animals. A *Streptococcus* spp. (most often *S. phocae*) was cultured from seven of these animals. The pure growth of *S. phocae* from blood and liver samples from one such pup, and from a pleural exudate of another pup with multi-cavitary exudates of similar appearance, suggested septicemia as a sequela to traumatic injury.

Fly larvae were noted at necropsy in 49 (40.2%) of the pup carcasses. Most were assessed as post-mortem infestations, based on small larval size and predisposition for moist sites (orifices, eyes, and wound effusions). In nine (7.4%) pups, larval infestation was assessed as occurring antemortem. This was based on histologic tissue review demonstrating tissue inflammation and hemorrhage, selective wound presence, large larval size relative to post-mortem interval, and/or larger larval size within a wound compared with other body sites. Antemortem myiasis was significantly ($p = .004$) associated with the month of pup death. There were seven cases clustered in February and March of summer-autumn seasons, and two cases in September of a winter-spring season. Additionally, antemortem myiasis was significantly ($p < .001$) associated with pup death with bacterial infection. This included eight pups with traumatic comorbidity, and the one pup with umbilical cellulitis described above.

3.3.4 Undetermined deaths

Perinatal-aged pups

There were 11 (9.0%) deaths of undetermined cause in perinatal-aged pups. Of these, six (4.9%) were stillborn and five (4.1%) were neonates. A body weight < 4.5 kg suggested that at least three of these 11 pups were premature or underweight births. No tissue abnormalities were detected from the single stillborn pup reviewed by histologic examination. Those detected in the single neonate (prematurity suggested by size: standard length 51.0cm; weight 2.3kg with partial intestinal scavenging) examined histologically were limited to the lungs – mild pulmonary oedema and partial atelectasis. Neither pulmonary nor placental inflammation were apparent. Six of seven perinatal-aged pups with known maternal parity (and dying of determinable or undeterminable cause) were born to first ($n = 4$) or second ($n = 2$) parity females.

Older pups

The cause of mortality was undetermined for seven (5.7%) pups older than perinatal age. In all instances, this categorization was attributed to a degree of autolysis that limited gross examination findings and prevented sampling for histologic tissue review or bacterial culture. All seven pups were young (three < 1–2 weeks old and four 3–4 weeks old). For pups older than perinatal age, there was a significant ($p = .013$) association between primary cause-of-mortality category and the maximum temperature of the day prior to death. For four of the seven pups this exceeded 36.0 °C (range 36.1–41.5 °C). For comparison, there were four pups with a determinable cause of mortality also found dead following days with an ambient temperature exceeding 36.0 °C. Three of these pups died with an infectious disease (two with bacterial infection secondary to conspecific trauma and one with HAHE) and one due to conspecific trauma.

3.4 | Temporal and seasonal association with primary cause-of-mortality categorization

3.4.1 Temporal distribution of pup death within the breeding season

Figure 4 shows the temporal distribution of pup deaths for each primary cause-of-mortality category (and for the categories combined), reported separately for summer-autumn and winter-spring breeding seasons. Across the five breeding cohorts the median date of death from any cause was eight days (relative to the median pupping date). The median dates for trauma, starvation and infectious disease categories were eight days, 17 days and eight days, respectively. Further, there was no significant difference ($p = .080$) between the four individual cause-of-mortality categories. When seasons were considered separately, the median dates for trauma, starvation and infectious disease categories for summer-autumn and winter-spring breeding seasons were 10 and four days, 49 and four days, and 11 and –1 days, respectively. That is, death from starvation was skewed to seven weeks later in the season in summer-autumn seasons, in contrast to starvation in winter-spring seasons or to death from trauma or infectious disease in either season. Consequently, between category differences were significant for the summer-autumn ($p = .035$) but not winter-

spring ($p = .948$) seasons. There was also a significant difference ($p < .001$, persisting with exclusion of the perinatal age class) in the median date of death between age classes. Expectedly, median date of death within a breeding season generally increased with increasing pup age class. Median dates for perinatal, 1–2 week, 3–4 week, 1–2 month and > 2–3 month-aged pups for both seasons combined were –61 days, –10 days, 11 days, 14 days and 131 days, respectively. Those for summer-autumn and winter-spring seasons were –70 and –21 days, –9 and –10 days, 12 and 10 days, 34 and –1 days, and 134 and 121 days, respectively.

3.4.2 Seasonal differences in necropsied pup morphometrics and location

There were no apparent differences between summer-autumn and winter-spring seasons for pup age class, sex, bodyweight, standard length, sternal blubber depth, or maternal parity. A difference in pup location was apparent for pups found in the Main Beach division. A higher proportion of pups were located on the beach (versus dunes/scrub) area during the summer-autumn (14/25, 56.0%) versus winter-spring seasons (2/16, 12.5%).

3.5 Modelling of population risk – seasonal and treatment effects

Table 3 presents predicted seasonal and treatment effects on overall and comorbidity-specific mortality incidence for pups born across the five breeding cohorts. When comparing seasons, a significantly ($p < .001$) higher incidence of death from any cause was predicted for the summer-autumn versus winter-spring seasons. This was associated with a significantly higher predicted incidence of death with trauma ($p = .001$) or an infectious disease – either bacterial infection ($p < .001$) or HAHE ($p = .048$)

Exploration of treatment effect predicted a significant 61.2% reduction in the risk of death from any cause ($p < .001$). This was associated with an 83.8% reduction in the risk of death with HAHE ($p = .005$). When modelling was restricted to the breeding cohorts exposed to hookworm treatment

(seasons 2019 and 2020/2021), a predicted 43.8% beneficial effect of treatment on death from trauma was strengthened but remained insignificant (7.2%, 95% CI [3.3%, 11.2%] vs. 12.8%, 95% CI [9.2%, 16.5%], $p = .065$).

Exploration of pup comorbidity predicted that for pups with HAHE, there was a significant increase in the risk of dying with conspecific trauma (29.4%, 95% CI [18.9%, 40.0%] vs. 8.3%, 95% CI [6.2%, 10.4%], $p = .001$) or starvation (57.9%, 95% CI [46.7%, 69.1%] vs. 7.2%, 95% CI [5.7%, 8.7%], $p = .001$). Furthermore, the presence of trauma was a significant predictor of death with bacterial infection (24.5%, 95% CI [18.2%, 30.9%] vs. 1.1%, 95% CI [0.5%, 1.8%], $p = .001$). A visual summary of significant associations based on population risk modelling is shown in Figure 5.

TABLE 3 Overall and comorbidity-specific mortality incidence [and 95% CI] predicted between seasons or treatment allocation using final multifactorial mixed-effects logistic regressions in the Australian sea lion pup population at the Seal Bay colony, for the 2016–2022 breeding cohorts.

	Season comparison			Treatment comparison		
	Summer-autumn	Winter-spring	<i>p</i> -value	Untreated	Treated	<i>p</i> -value
Any death	32.1% [28.0%, 36.3%]	18.9% [16.1%, 21.7%]	< .001	26.3% [23.7%, 29.0%]	10.2% [5.9%, 14.6%]	< .001
Death with trauma	14.5% [10.0%, 19.1%]	6.6% [4.4%, 8.9%]	.001	10.2% [7.8%, 12.6%]	7.1% [3.0%, 11.2%]	.225
Death with starvation	10.1% [7.7%, 12.6%]	10.6% [8.6%, 12.6%]	.760	10.6% [8.9%, 12.3%]	8.9% [4.6%, 13.2%]	.448
Death with HAHE	8.3% [5.8%, 10.9%]	5.4% [3.8%, 7.0%]	.048	7.4% [5.8%, 9.0%]	1.2% [0.0%, 2.9%]	.005
Death with bacterial infection	6.2% [4.6%, 7.8%]	1.9% [1.0%, 2.8%]	< .001	4.1% [3.2%, 5.1%]	3.9% [1.4%, 6.4%]	.878

HAHE: hookworm-associated hemorrhagic enteritis.

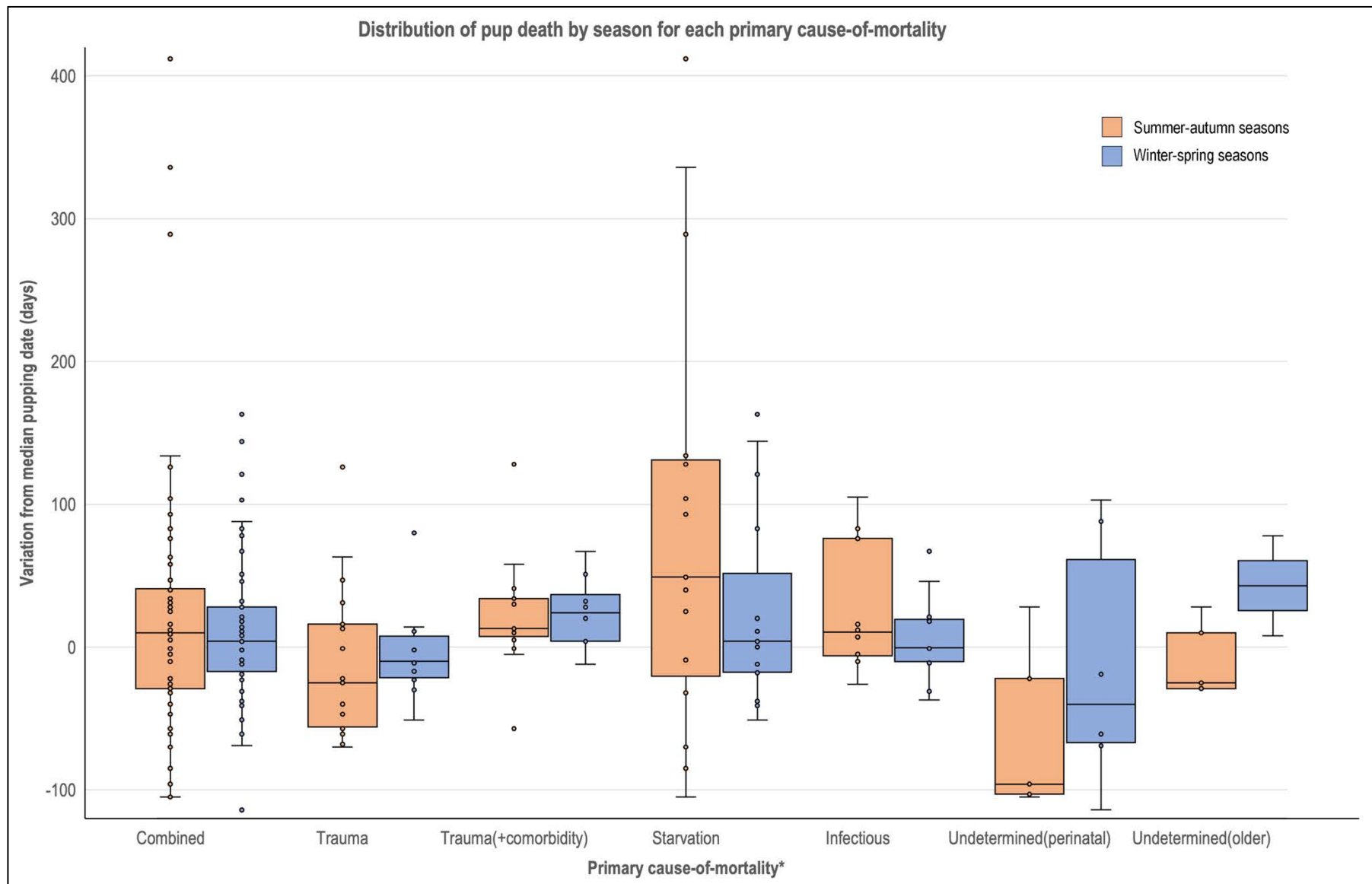


FIGURE 4 The distribution of primary cause-of-mortality for the 2016–2022 breeding cohorts of Australian sea lions at Seal Bay, Kangaroo Island. Results are shown separately for the combined summer-autumn (orange boxes) and winter-spring (blue boxes) breeding seasons. The vertical axis shows the variation in days from the median pupping date. The horizontal axis shows the combined (overall) and individual primary cause-of-mortality categories. *Note, that the trauma category is reported separately for pups with and without infectious comorbidity, and undetermined death is reported separately for perinatal and older pups. Boxes indicate the interquartile day range (calculated inclusive of the median) with the median values marked, and the vertical lines represent the day range from earliest to latest day, excluding outliers.

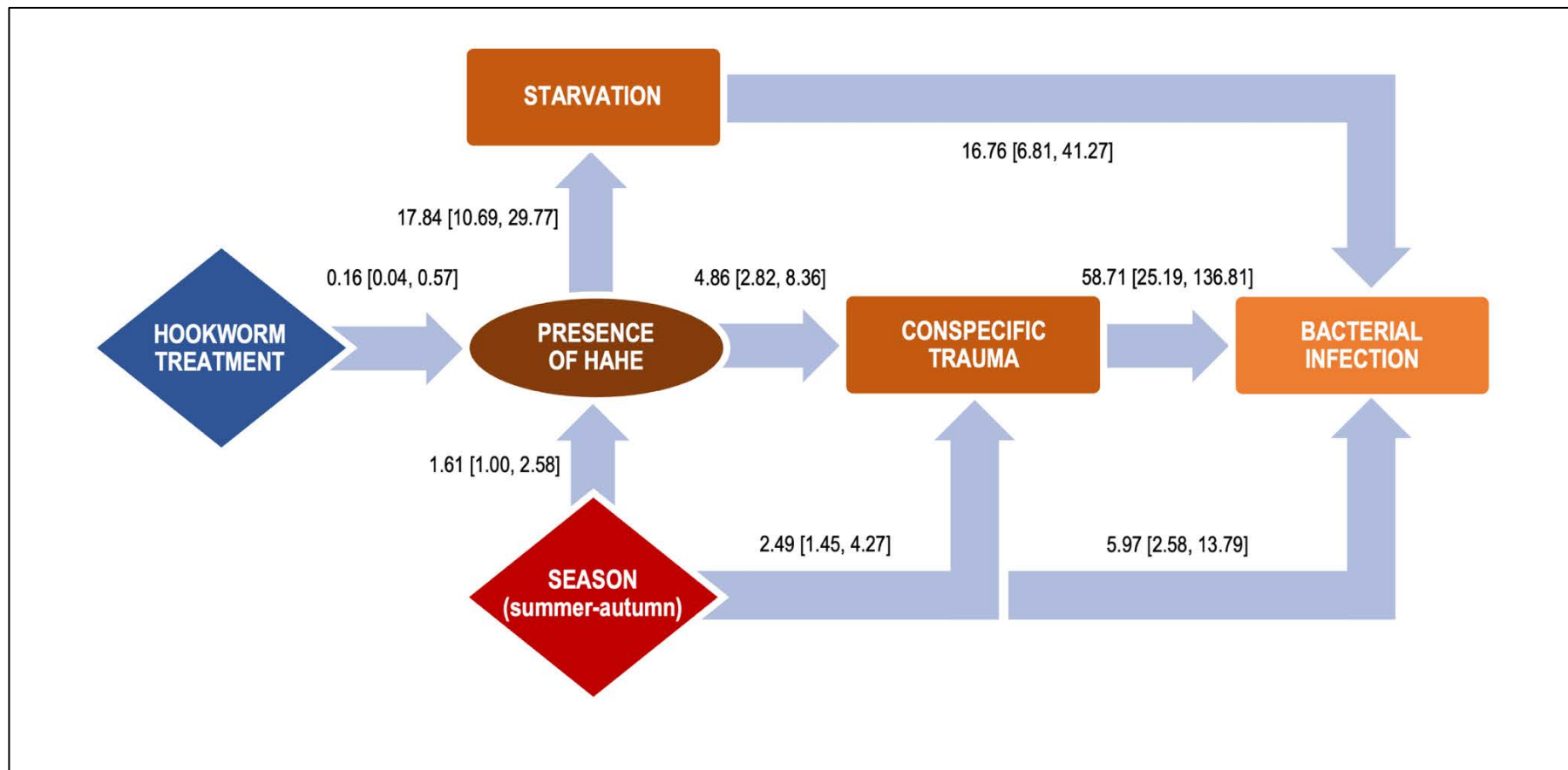


FIGURE 5 Modelling of population risk: significant associations between season (summer-autumn), application of hookworm treatment and a biologically appropriate sequence of necropsy findings. Numerical values represent odds ratios [and 95% CI]. For instance, the likelihood of a dead pup having experienced conspecific trauma or being assessed as starving are approximately five and 18 times greater, respectively, when that pup was assessed as having hookworm-associated hemorrhagic enteritis (HAHE).

4 | DISCUSSION

This study reports a primary cause-of-mortality categorization and comorbidity presence for Australian sea lion pups dying during five breeding seasons at the Seal Bay Conservation Park colony. Consistent with earlier reports, trauma and starvation were major contributors to pup death. Infectious disease was identified as an additional substantial contributor to pup death, and one explanation for the previously large number (28%–49%) of pup deaths of undetermined cause in this species (Higgins & Tedman, 1990; McIntosh & Kennedy, 2013). Further, this study introduces population modelling to predict that 1) trauma (+/– bacterial infection) and hookworm-associated hemorrhagic enteritis are contributors to the higher pup mortality observed in summer-autumn breeding seasons, and 2) early hookworm elimination reduces not just the risk of death from hemorrhagic enteritis, but overall pup mortality risk.

The four primary cause-of-mortality categories used in this study aided differentiation of the cause of pup death despite limited data availability beyond gross necropsy findings. Additionally, they enabled comparison with reported outcomes from earlier mortality observations in the Australian sea lion (Higgins & Tedman, 1990; Marlow, 1975; McIntosh & Kennedy, 2013) and in other otariid species (Castinel et al., 2007; Michael et al., 2019; Seguel et al., 2013; Spraker & Lander, 2010). The separate reporting of comorbidities provided an increased understanding of their individual (and interrelated) contributions to pup mortality. This is potentially relevant in terms of mitigation strategies for a species that experiences such a high degree of conspecific trauma (McIntosh & Kennedy, 2013). Furthermore, the present study has introduced a practical method for classifying pup age in Australian sea lion pups. Using the predictable age of patent hookworm infection overcame reliance on the use of standard length alone, which proved a less robust measurement post-mortem.

In comparison with reporting in other otariid species, the 41.0% of trauma-related deaths in this study exceeds the reported 19.8% in the South American fur seal (*Arctocephalus australis*) (Seguel et al., 2013), 18.0% in the Northern fur seal (*Callorhinus ursinus*) (Spraker & Lander, 2010), and 9.9% in the New Zealand sea lion (*Phocarcos hookeri*) (Michael et al., 2019). The 22.1% of traumatic deaths in the absence of a comorbidity more closely approximates those studies and the 19% reported by Higgins and Tedman (1990), based on their two-season study of 116 Australian sea lion pups and examination of 21 dead pups. The authors suggested their figure may have been an underestimate given that not all observed attacks resulted in external injury. This is supported by the multiple blunt trauma-related deaths detected in the present study. The 41.0% of traumatic deaths with inclusion of animals with an infectious comorbidity exceeds the 31.6% reported from the three-season study of 128 dead pups by McIntosh and Kennedy (2013). That study included gross necropsy for detection of internal injury (McIntosh & Kennedy, 2013). Neither study separately categorized death from trauma with secondary bacterial infection. In doing so, the present report suggests that infection after an initial injury contributes to approximately a third of traumatic pup deaths. This highlights a potential point of intervention should intensive population management be considered necessary for future species conservation. Septicemia following traumatic injury was suspected from gross necropsy to have contributed to the death of multiple pups with bacterial infection. However, confirmation was limited to the smaller proportion of cases with supportive gross, histologic and culture results. In this respect, future mortality investigations will benefit from an increased frequency of sampling from well preserved carcasses.

Previous reporting has identified non-maternal adult females as causing traumatic injury to pups during the perinatal period (Marlow, 1975). However, attacks by adult males are particularly severe in nature, frequently cause per-acute death from direct bite injury and are attributed to misplaced aggression and inadvertent injury during mating or fighting (Marlow, 1975). Current findings support both observations. Firstly, by identifying injury from conspecific trauma as predominantly sharp

injury to the head and neck. Secondly, by showing an alignment of the median date of pup death from trauma with the week following the median pupping date, despite the species' long breeding season duration. This coincides with the period of peak mating and adult aggression. In otariids with shorter breeding seasons, trauma-related deaths also cluster around periods of increased male fighting (Castinel et al., 2007) and protective perinatal maternal aggression (Katz et al., 2012).

McIntosh and Kennedy (2013) determined that most pup death occurred in the first month after birth, and that traumatic death was more likely in newborn pups. The present study found almost 80% of traumatized pups were less than one month of age, however, proportionately most were beyond the perinatal period but still less than two weeks of age. This overlaps the period of male mate-guarding of post-parturient females. Further, the latter part of this period coincides with the first postnatal maternal foraging excursion and newborn pup unattendance. Pups of this age have not yet developed maternal vocal recognition (Pitcher et al., 2009). Compared with older pups, they are therefore at an increased risk of injury when mistakingly approaching non-maternal females.

In comparison, death by starvation (without comorbidity) was spread more widely throughout the breeding period, particularly during the summer-autumn seasons. The 24.6% of deaths from starvation is mid-way between the 10.4% and 43.0% reported in the previous colony studies (Higgins & Tedman, 1990; McIntosh & Kennedy, 2013). The observational nature of the earlier of those two studies enabled starvation to be attributed to sibling competition from the previous juvenile (19%), death or non-return of the female from foraging (14%) or separation of the pup from the female within the colony (10%) (Higgins & Tedman, 1990). In comparison, observational database reporting of all deaths across five seasons in the present study indicates that pup starvation most frequently resulted from failure of the female to return from foraging. The presence of an unweaned juvenile, maternal rejection of a newborn pup, an allosuckle (Pitcher et al., 2011) or twin birth (Ling, 1986), or death of the female within the colony (combined $n = 10/284$, 3.5%) were comparatively lesser

contributors to pup starvation. A similar contribution of starvation to pup death was reported in the South American fur seal (23.5%) (Seguel et al., 2013), with substantially higher and lower contributions in the Northern fur seal (53%) (Spraker & Lander, 2010) and New Zealand sea lion (14.8%) (Michael et al., 2019), respectively. The authors speculated the causes for the higher figure were likely multifactorial, potentially related to maternal provisioning and environment impact (Spraker & Lander, 2010); the lower figure reflecting a substantially greater impact from infectious disease (*Klebsiella pneumoniae*) as an alternative cause of death in the study seasons (Michael et al., 2019).

The contribution of HAHE to a quarter of pup deaths (including those with trauma) potentially underestimates disease impact. This assessment is based on the strict definition for hookworm-associated disease used in this study and the increasing difficulty in detecting intestinal hemorrhage with advancing carcass decomposition (relative to detection of traumatic injury, or measurement of blubber depth supportive of starvation). Both risk excluding hookworm infection as a potential contributor to death. Furthermore, as well as the nearly 30% of pups with HAHE dying from trauma, more than half satisfied the study definition for starvation as a comorbidity. These represent cryptic contributions of HAHE to mortality in the absence of assessment for (or failure to detect) hemorrhagic enteritis. This may partly explain modelling of a 60% reduction in the estimated risk of all-cause mortality for pups treated with an anthelmintic, which suggests a treatment benefit beyond that attributable to prevention of HAHE alone. Further study is necessary to confirm if hookworm treatment can specifically reduce the risk of death due to trauma or starvation, for instance. Restricting modelling to the 2019 and 2020/2021 treated cohorts strengthened the likelihood of a beneficial treatment effect for these comorbidities, although results did not achieve significance.

Previous modelling has suggested that helminths have maximal host impact at intermediate pathogenicity (McCallum, 1994). The impact of hookworm infection on Australian sea lion pup morbidity and mortality is considered substantial (Marcus et al., 2014a). In comparison, hookworm (*Uncinaria*) infection impact in other otariids is variable. For instance, in the Australian fur seal (*Arctocephalus pusillus doriferus*), comparatively low hookworm infection intensity is not associated with pup mortality (Ramos, 2013). Similarly, in the New Zealand sea lion no deaths were directly attributed to hookworm infection in the most recent mortality study of that species (Michael et al., 2019). All deaths of parasitized pups were attributable to a more severe disease process (commonly *Klebsiella pneumoniae* septicemia). Subsequent reporting of a significant survival benefit (and trend for reduced death from *K. pneumoniae* infection) in hookworm-treated pups, was partly attributed to disruption of a parasite-bacteria synergism (Michael et al., 2021). This suggests that across other otariid species, the extent and nature of parasitic and bacterial infection interaction, is also yet to be fully determined.

Five (16.1%) of the 31 pups dying with HAHE in this study had prepatent hookworm infection. This confirms previous reporting that juvenile and adult hookworms share a similar functional capacity to cause intestinal disease in the Australian sea lion (Marcus et al., 2014b). This observation also emphasizes the importance of treating hookworm infection as early as logistically possible if implementing a mitigation strategy (Chilvers et al., 2009; DeLong et al., 2009; Lindsay et al., 2023). The use of a topical anthelmintic formulation may be beneficial in this respect, given the minimal restraint necessary for its application (Lindsay et al., 2023). For the two cases with full thickness hookworm penetration of the intestinal wall, the isolation of mixed enteric bacteria from abdominal effusions more closely resembles the hookworm enteritis with bacteremia (HEB) syndrome reported in California sea lion (*Zalophus californianus*) (Spraker et al., 2007) and South American fur seal pups (Seguel et al., 2017). This also contrasts with the frequent monoculture of *K. pneumoniae* in New Zealand sea lion pups with hookworm infection (Michael et al., 2021). Additional histologic review of

well-preserved tissue specimens is necessary to further explore the prevalence of bacterial infection secondary to hookworm-associated intestinal damage in the Australian sea lion. In the present study however, this was more commonly a sequela to conspecific trauma rather than hookworm infection.

Rather than include a separate age-related mortality category, the present study categorized stillbirth and neonatal deaths as being of undetermined cause when trauma, starvation, or infection could not be detected. It highlights an opportunity to improve future studies of reproductive failure (abortion, still or premature birth, neonatal death) in the Australian sea lion by incorporating molecular, serological and toxicological testing. For instance, to detect bacterial (e.g., *Coxiella burnetii*, *Brucella* spp., *Chlamydia* spp., *Mycoplasma* spp.), viral (morbilliviruses), and protozoal (e.g., *Toxoplasma gondii*) infectious agents, or non-infectious contributors to reproductive failure (Carlson-Bremer et al., 2015; Conway et al., 2022; Esquible et al., 2019; Goldstein et al., 2009; Lynch et al., 2011). Of current regional relevance is the identification of a novel genotype of *Coxiella burnetii* in the partly sympatric Australian fur seal (Gardner et al., 2023a; Gardner et al., 2023b). Furthermore, current baseline data for the Australian sea lion includes the reporting of maternal *T. gondii* seropositivity at high levels comparable to other otariids (Lindsay et al., 2022), the detection of maternally acquired anthropogenic toxins (per and polyfluoroalkyl substances [PFAS]) in pups (Taylor et al., 2021), and the demonstration of antimicrobial resistant bacteria in the gut microbiome of pups (Fulham et al., 2022). In the present study, limited availability of well-preserved tissues from perinatal-aged pups (partly attributed to the species maternal guarding of dead neonates) is unlikely to have detected infectious disease present at low prevalence.

Subcategorization of undetermined deaths by age did permit comparison with perinatal death in other studies. The 9.0% contribution of perinatal death (approximately half each stillbirth and neonatal death) was higher than expected and may reflect some selection (recruitment) bias. In comparison, observational database reporting of a dead newborn pup (including those not recruited

to this study) suggested a figure of 6.7% across the five breeding cohorts. This more closely approximates the 7.6% of still or premature births previously reported (McIntosh & Kennedy, 2013). Across otariid species, the figure ranges widely, likely reflective of species-specific (genetic and environmental) factors and potentially sampling methodology. Lower figures are reported in the New Zealand sea lion (1.4–6.0%) (Michael et al., 2016; Michael et al., 2019) and South American fur seal (2.5%) (Seguel et al., 2013), with higher numbers reported in the Steller sea lion (*Eumetopias jubatus*) (11.6%) (Maniscalco et al., 2008) and Northern fur seal (19%) (Spraker & Lander, 2010). In the latter, perinatal mortality was reported more commonly early in the season (as it was in the present study) and highlighted the risk of under-reporting in temporally limited investigations. Congenital anomalies are a notable absence in the present study and one potential contributor to perinatal death.

The reported association of high ambient temperature and an undetermined cause of pup death is explained by the rapid post-mortem decomposition expected in these cases. Further study is necessary to determine the degree to which weather extremes contribute to pup death more generally, particularly for pups with existing health compromise. Such investigations would be advanced by multi-site in-colony weather recording for assessment of risk. The clustering in warmer months of pup deaths with premortem myiasis is also expected, given high ambient temperature and humidity are associated with increased activity and lifecycle progression for the Australian sheep blowfly (*Lucilia cuprina*). This is an important primary facultative agent of myiasis in domestic species (mainly sheep) and is also capable of infecting wildlife species (Taylor, 2016). That seven of nine cases were in summer-autumn seasons offers another potential contributor to the higher mortality observed in these seasons. Observations in Australian sea lion pups in the present study are consistent with the pathogenesis of disease in domestic species (Taylor, 2016).

The bacterial isolates from this study are consistent with those reported in other otariid species. More generally, *Streptococcus* species are emerging bacterial pathogens of marine mammals, based on increasingly common isolation from diseased individuals (Akineden et al., 2005). *Streptococcus phocae* was first named as a new beta-hemolytic species by Skaar et al. (1994) from tissues sampled during a 1988 morbillivirus epizootic among harbor seals (*Phoca vitulina*) in northwestern Europe. It has since been isolated from marine mammal species worldwide (Ross et al., 2023), including otariid species such as the Cape fur seal (*Arctocephalus pusillus pusillus*) (Henton et al., 1999), California sea lion, Steller sea lion (*Taurisano et al., 2018*), and New Zealand sea lion (Michael et al., 2019). The bacterium is likely a commensal of the oropharynx, upper respiratory tract and lower urogenital tract, and an opportunistic pathogen contributing to local (abscess, cellulitis, meningitis, bronchopneumonia) and systemic (septicemia) inflammatory disease in debilitated or immunocompromised individuals. Concern for disease elaboration in Australian sea lion pups relates to the existing compromising health impacts of endemic hookworm disease (Lindsay et al., 2023), disease vulnerability from high levels of immune-modulating (mercury) and low levels of immune-enhancing (selenium) elements (Taylor et al., 2022), and the species high level of conspecific trauma. In the present study, all five pups from whom *S. phocae* was isolated had evidence of conspecific trauma, consistent with entry through damaged skin or mucosal. Additional vector transmission (e.g., naso-pulmonary mites) (Pesapane et al., 2022) is less likely but can't be completely excluded, given mite detection in a small proportion of examined pups in this study. Pertinent to the considerable contribution of hypermucoviscous *K. pneumoniae* infection to New Zealand sea lion pup mortality (Michael et al., 2019), the present study detected no instances of septic arthritis, and rare instances of meningitis were attributable to bacterial entry secondary to traumatic injury.

Population modelling of season effect predicted more than double the number of traumatic deaths in summer-autumn versus winter-spring seasons (14.5% vs. 6.6%, $p = .001$). Contributing to this difference, and further supporting trauma as an additional factor in the colonies' oscillating seasonal

variation in mortality, was the predicted three-fold increased risk of death with bacterial infection in summer-autumn seasons (modelled prevalence 6.2% vs. 1.9%, $p < .001$). Given that adult aggression and pup mortality are density-dependent in Australian sea lion colonies (Goldsworthy et al., 2009; Harcourt, 1992), seasonal variation in intra-colony population density offers one explanation for these predictions. This is supported by the observation of a seven-fold higher proportion of necropsied pups being located in the beach area of Main Beach (narrow, no protective vegetation, few rock crevices or caves), versus the less densely populated dunes/scrub area, during summer-autumn seasons. In comparison with other colony areas such as EPA, the total number of dead pups recruited from the beach area of Main Beach is low. However, for other areas with variable habitat the distinction between beach and scrub areas is less distinct (and therefore not reported) or pup movement between the two is more frequent. Intra-colony movement related to the weather is previously reported (Sterling, 1972). The overall seasonal (weather) effect on pup location, population density and trauma risk, could therefore be greater than that reported for the Main Beach areas. Reporting of GPS coordinates of pup location and an estimated time of death is suggested to improve future mortality surveys.

Obtaining well preserved carcasses to enable all available diagnostic testing for a more detailed assessment of the cause of death was the major limitation of reporting for this study. Where the reason for non-recruitment of a dead pup was provided ($n = 84$), observational database reporting detailed advanced decomposition as the reason in nearly 75% of cases. Further, a little more than a third of the 122 cases examined grossly were suitable for tissue sampling for histologic review, and of these, three quarters demonstrated moderate to marked tissue autolysis or freeze artefact at the microscopic level, limiting interpretation. Additional resourcing and funding would be required to overcome some of these limitations in future investigations. Mortality studies and disease investigation at other colonies (i.e., higher population density colonies) are also necessary to

determine the degree to which the results of the current study can be translated to other breeding sites.

This study provides current baseline reporting of Australian sea lion pup mortality and reports a considerable contribution from infectious disease. Future disease impact and outcomes of mitigation strategies can be measured against these findings. The Australian sea lion is particularly vulnerable to an epidemic disease outbreak due to its population size and structure (Bilgmann et al., 2021). Such an event (for instance, an outbreak of highly pathogenic avian influenza A (H5N1) (Adlhoch et al., 2023)) poses the greatest potential threat to the species conservation (Bilgmann et al., 2021). Consequently, suggestions for future species investigation remain prioritizing monitoring for pathogen exposure and disease risk assessment (Heard et al., 2013; Lindsay et al., 2022; Lindsay & Gray, 2021).

ACKNOWLEDGMENTS

The authors thank Seal Bay Conservation Staff, Department for Environment and Water (DEW), Seal Bay, Kangaroo Island, for their considerable assistance with pup observation and carcass removal and storage. We also thank the many additional fieldwork volunteers, including Matthew Gray, Clarence Kennedy, Tanya Rosewarne, and Melanie Stephens. We especially thank Shannon Taylor for her considerable fieldwork and necropsy contributions during the 2019 breeding season. We are grateful for the logistical support provided by Janet Simpson, and for the laboratory processing provided by staff of Veterinary Diagnostic Laboratory (VDL) and Australian Centre for Antimicrobial Resistance Ecology (ACARE) at The University of Adelaide and Veterinary Pathology Diagnostic Service (VPDS) at The University of Sydney.

AUTHOR CONTRIBUTIONS

Scott A. Lindsay: Conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; project administration; resources; visualization; writing – original draft; writing – review and editing. **Mariel Fulham:** Investigation; project administration; resources; writing – review and editing. **Melanie Stonnill:** Funding acquisition; investigation; resources; writing – review and editing. **Charles G. B. Caraguel:** Formal analysis; supervision; visualization; writing – review and editing (shared last authorship). **Rachael Gray:** Conceptualization; funding acquisition; investigation; methodology; project administration; resources; supervision; writing – review and editing (shared last authorship).

FUNDING

Financial support for this study was provided by The Hermon Slade Foundation (HSF 16-03), FS Quiney and BR Richards Bequests from the Sydney School of Veterinary Science (The University of Sydney; 2016), the Department for Environment and Water (DEW), South Australia (2019 and

2020/2021), and Research Start-up Funds and a Collaboration Grant from the School of Animal and Veterinary Sciences (The University of Adelaide; 2016 and 2021).

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

REFERENCES

- Adlhoch, C., Fusaro, A., Gonzales, J.L., Kuiken, T., Mirinavičiūtė, G., Niqueux, É., Staubach, C., Terregino, C., Baldinelli, F., Rusinà, A., and Kohnle, L. (2023). Avian influenza overview June–September 2023. *European Food Safety Authority Journal* 21(10), 8328. doi: 10.2903/j.efsa.2023.8328
- Akineden, O., Hassan, A., Alber, J., El-Sayed, A., Estoepangestie, A. T., Lammler, C., Weiss, R., & Siebert, U. (2005). Phenotypic and genotypic properties of *Streptococcus equi subsp. zooepidemicus* isolated from harbor seals (*Phoca vitulina*) from the German North Sea during the phocine distemper outbreak in 2002. *Veterinary Microbiology*, 110(1-2), 147-152. <https://doi.org/10.1016/j.vetmic.2005.06.010>
- Australian Government Bureau of Meteorology (9 August 2023). *Climate Data Online*. <http://www.bom.gov.au/climate/data/>
- Bilgmann, K., Armansin, N., Ferchaud, A. L., Normandeau, E., Bernatchez, L., Harcourt, R., Ahonen, H., Lowther, A., Goldsworthy, S. D., & Stow, A. (2021). Low effective population size in the genetically bottlenecked Australian sea lion is insufficient to maintain genetic variation. *Animal Conservation*, 24(5), 847-861. <https://doi.org/10.1111/acv.12688>
- Campbell, R. A., Gales, N. J., Lento, G. M., & Baker, C. S. (2008). Islands in the sea: extreme female natal site fidelity in the Australian sea lion, *Neophoca cinerea*. *Biology Letters*, 4, 139-142. <https://doi.org/10.1098/rsbl.2007.0487>
- Carlson-Bremer, D., Colegrove, K. M., Gulland, F. M., Conrad, P. A., Mazet, J. A., & Johnson, C. K. (2015). Epidemiology and pathology of *Toxoplasma gondii* in free-ranging California sea lions (*Zalophus californianus*). *Journal of Wildlife Diseases*, 51(2), 362-373. <https://doi.org/10.7589/2014-08-205>
- Castinel, A., Duignan, P. J., Pomroy, W. E., Lopez-Villalobos, N., Gibbs, N. J., Chilvers, B. L., & Wilkinson, I. S. (2007). Neonatal mortality in New Zealand sea lions (*Phocarctos hookeri*) at

- Sandy Bay, Enderby Island, Auckland Islands from 1998 to 2005. *Journal of Wildlife Diseases*, 43(3), 461-474. <https://doi.org/10.7589/0090-3558-43.3.461>
- Chilvers, B. L., Duignan, P. J., Robertson, B. C., Castinel, A., & Wilkinson, I. S. (2009). Effects of hookworms (*Uncinaria* sp.) on the early growth and survival of New Zealand sea lion (*Phocarctos hookeri*) pups. *Polar Biology*, 32, 295–302. <https://doi.org/10.1007/s00300-008-0559-0>
- Conway, R., Duncan, C., Foster, R. A., Kersh, G. J., Raverty, S., Gelatt, T., & Frank, C. (2022). Histologic lesions in placentas of northern fur seals (*Callorhinus Ursinus*) from a population with high placental prevalence of *Coxiella burnetii*. *Journal of Wildlife Diseases*, 58(2), 333-340. <https://doi.org/10.7589/JWD-D-21-00037>
- Delong, R. L., Orr, A. J., Jenkinson, R. S., & Lyons, E. T. (2009). Treatment of northern fur seal (*Callorhinus ursinus*) pups with ivermectin reduces hookworm-induced mortality. *Marine Mammal Science*, 25(4), 944-948. <https://doi.org/10.1111/j.1748-7692.2008.00274.x>
- Esquible, J. A., Burek-Huntington, K., Atkinson, S., Klink, A. C., Bortz, E., Goldstein, T. A., Beckmen, K., Pabilonia, K., & Tiller, R. (2019). Pathological findings and survey for pathogens associated with reproductive failure in perinatal Steller sea lions *Eumetopias jubatus*. *Diseases of Aquatic Organisms*, 137, 131-144. <https://doi.org/10.3354/dao03421>
- Fulham, M., McDougall, F., Power, M., McIntosh, R. R., & Gray, R. (2022). Carriage of antibiotic resistant bacteria in endangered and declining Australian pinniped pups. *PLoS One*, 17(1), e0258978. <https://doi.org/10.1371/journal.pone.0258978>
- Gales, N. J., Shaughnessy, P. D., & Dennis, T. E. (1994). Distribution, abundance and breeding cycle of the Australian sea lion *Neophoca cinerea* (Mammalia: Pinnipedia). *Journal of Zoology, London*, 234(3), 353-370. <https://doi.org/10.1111/j.1469-7998.1994.tb04853.x>
- Gardner, B. R., Bachmann, N. L., Polkinghorne, A., Hufschmid, J., Tadepalli, M., Marenda, M., Graves, S., Arnould, J. P. Y., & Stenos, J. (2023a). A novel marine mammal *Coxiella burnetii*-genome

sequencing identifies a new genotype with potential virulence. *Pathogens*, 12, 893.

<https://doi.org/10.3390/pathogens12070893>

Gardner, B. R., Hufschmid, J., Stenos, J., Tadepalli, M., Sutton, G., Fromant, A., Eizenberg, Y., Geeson, J. J., & Arnould, J. P. Y. (2023b). Pacific gulls (*Larus pacificus*) as potential vectors of *Coxiella burnetii* in an Australian fur seal breeding colony. *Pathogens* 12, 122.

<https://doi.org/10.3390/pathogens12010122>

Geraci, J. R., Lounsbury, V. J., & Yates, N. (2005). *Marine Mammals Ashore: A Field Guide for Strandings*, Second Edition (pp. 177-178). Baltimore: National Aquarium in Baltimore.

Goldstein, T., Zabka, T. S., Delong, R. L., Wheeler, E. A., Ylitalo, G., Bargu, S., Silver, M., Leighfield, T., Van Dolah, F., Langlois, G., Sidor, I., Dunn, J. L., & Gulland, F. M. (2009). The role of domoic acid in abortion and premature parturition of California sea lions (*Zalophus californianus*) on San Miguel Island, California. *Journal of Wildlife Diseases*, 45(1), 91-108.

<https://doi.org/10.7589/0090-3558-45.1.91>

Goldsworthy, S. D. (2018). *Australian sea lion: Neophoca cinerea*. In B. Würsig, J. G. M. Thewissen and K. M. Kovacs (Eds), *Encyclopedia of Marine Mammals*, 3rd Ed. (pp 44-46). Academic Press. <https://www.sciencedirect.com/science/article/pii/B9780128043271000522>

Goldsworthy, Shaughnessy, P. D., McIntosh, R. R., Kennedy, C., Simpson, J., & Page, B. (2008).

Australian sea lion populations at Seal Bay and the Seal Slide (Kangaroo Island): continuation of the monitoring program. Report to the Department for Environment and Heritage Wildlife Conservation Fund Project No. 3723. South Australia Research and Development Institute, Adelaide. SARDI Publication No. F2008/000645-1. SARDI Research Report Series No. 293. 53pp.

Goldsworthy, S. D., Mckenzie, J., Shaughnessy, P. D., McIntosh, R. R., Page, B., & Campbell, R. (2009). An update of the report: understanding the impediments to the growth of Australian sea lion populations. Report to the Department of the Environment, Water, Heritage and the Arts. South Australia Research and Development Institute, Adelaide. SARDI Publication No.

F2008/00847-1. SARDI Research Report Series No. 356. 171pp.

https://pir.sa.gov.au/__data/assets/pdf_file/0009/231795/No_356_Update_to_the_report_AS�_impediments_to_recovery.pdf

Goldsworthy, S. D., Shaughnessy, P. D., Mackay, A. I., Bailleul, F., Holman, D., Lowther, A. D., Page, B., Waples, K., Raudino, H., Bryars, S., & Anderson, T. (2021a). Assessment of the status and trends in abundance of a coastal pinniped, the Australian sea lion *Neophoca cinerea*. *Endangered Species Research*, 44, 421-437. <https://doi.org/10.3354/esr01118>

Goldsworthy, S. D., Stonnill, M., & Kirkwood, R. (2021b). Monitoring of the Australian sea lion population at Seal Bay: 2020/21. Report to the Department for Environment and Water. South Australian Research and Development Institute (Aquatic Sciences), Adelaide. SARDI Publication No. F2014/000322-6. SARDI Research Report Series No. 1121. 30pp. https://www.pir.sa.gov.au/__data/assets/pdf_file/0007/402649/seal-bay-2020-21-sea-lion-monitoring.pdf

Google (16 November 2023). *Google Maps*. <https://www.google.com.au/maps/@-35.9952452,137.3147768,1390m/data=!3m1!1e3?entry=ttu>

Harcourt, R. (1992). Factors affecting early mortality in the South American fur seal (*Arctocephalus australis*) in Peru: density-related effects and predation. *Journal of Zoology, London*, 226(2), 259-270. <https://doi.org/https://doi.org/10.1111/j.1469-7998.1992.tb03838.x>

Heard, M. J., Smith, K. F., Ripp, K., Berger, M., Chen, J., Dittmeier, J., Goter, M., Mcgarvey, S. T., & Ryan, E. (2013). The threat of disease increases as species move toward extinction. *Conservation Biology*, 27(6), 1378-1388. <https://doi.org/10.1111/cobi.12143>

Henton, M. M., Zapke, O., & Basson, P. A. (1999). *Streptococcus phocae* infections associated with starvation in Cape fur seals. *Journal of the South African Veterinary Association*, 70(2), 98-99. <https://doi.org/10.4102/jsava.v70i2.763>

- Higgins, L. V., & Gass, L. (1993). Birth to weaning: parturition, duration of lactation, and attendance cycles of Australian sea lions (*Neophoca cinerea*). *Canadian Journal of Zoology* 71(10), 2047-2055. <https://doi.org/10.1139/z93-290>
- Higgins, L. V., & Tedman, R. A. (1990). Effect of attacks by male Australian sea lions, *Neophoca cinerea*, on mortality of pups. *Journal of Mammalogy*, 71(4), 617-619. <https://doi.org/10.2307/1381802>
- Katz, H., Morgades, D., & Castro-Ramos, M. (2012). Pathological and parasitological findings in South American fur seal pups (*Arctocephalus australis*) in Uruguay. *International Scholarly Research Network Zoology*, 2012, 1-7. <https://doi.org/10.5402/2012/586079>
- Lindsay, S. A., Caraguel, C. G. B., & Gray, R. (2021). Topical ivermectin is a highly effective seal 'spot-on': A randomised trial of hookworm and lice treatment in the endangered Australian sea lion (*Neophoca cinerea*). *International Journal for Parasitology: Parasites and Wildlife*, 16, 275-284. <https://doi.org/10.1016/j.ijppaw.2021.11.002>
- Lindsay, S. A., Caraguel, C. G. B., O'handley, R., Šlapeta, J., & Gray, R. (2022). *Toxoplasma gondii* seroprevalence in the endangered Australian sea lion (*Neophoca cinerea*). *Frontiers in Marine Science*, 9, 965865. <https://doi.org/10.3389/fmars.2022.965865>
- Lindsay, S. A., Fulham, M., Caraguel, C. G. B., & Gray, R. (2023). Mitigating disease risk in an endangered pinniped: early hookworm elimination optimizes the growth and health of Australian sea lion pups. *Frontiers in Veterinary Science*, 10, 1161185. <https://doi.org/10.3389/fvets.2023.1161185>
- Lindsay, S. A., & Gray, R. (2021). A novel presentation of tuberculosis with intestinal perforation in a free-ranging Australian sea lion (*Neophoca cinerea*). *Journal of Wildlife Diseases*, 57(1), 220-224. <https://doi.org/10.7589/JWD-D-20-00104>
- Ling, J. (1999). Exploitation of fur seals and sea lions from Australian, New Zealand and adjacent subantarctic islands during the eighteenth, nineteenth and twentieth centuries. *Australian Zoologist*, 31(2), 323-350. <https://doi.org/10.7882/az.1999.036>

- Ling, J. K. (1986). Twin fetuses from an Australian sea lion *Neophoca cinerea* Carnivora otariidae. *Australian Mammalogy*, 9(1), 59-60. <https://doi.org/10.1071/AM86008>
- Lowther, A. D., Harcourt, R. G., Goldsworthy, S. D., & Stow, A. (2012). Population structure of adult female Australian sea lions is driven by fine-scale foraging site fidelity. *Animal Behaviour*, 83(3), 691-701. <https://doi.org/10.1016/j.anbehav.2011.12.015>
- Lynch, M., Taylor, T. K., Duignan, P. J., Swingler, J., Marends, M., Arnould, J. P., & Kirkwood, R. (2011). Mycoplasmas in Australian fur seals (*Arctocephalus pusillus doriferus*): identification and association with abortion. *Journal of Veterinary Diagnostic Investigation*, 23(6) 1123-1130. <https://doi.org/10.1177/1040638711425699>
- Maniscalco, J. M., Calkins, D. G., Parker, P., & Atkinson, S. (2008). Causes and extent of natural mortality among Steller sea lion (*Eumetopias jubatus*) pups. *Aquatic Mammals*, 34(3), 277-287. <https://doi.org/10.1578/am.34.3.2008.277>
- Marcus, A. D., Higgins, D. P., & Gray, R. (2014a). Epidemiology of hookworm (*Uncinaria sanguinis*) infection in free-ranging Australian sea lion (*Neophoca cinerea*) pups. *Parasitology Research*, 113(9), 3341-3353. <https://doi.org/10.1007/s00436-014-3997-3>
- Marcus, A. D., Higgins, D. P., & Gray, R. (2015a). Health assessment of free-ranging endangered Australian sea lion (*Neophoca cinerea*) pups: effect of haematophagous parasites on haematological parameters. *Comparative Biochemistry and Physiology, Part A*, 184, 132-143. <https://doi.org/10.1016/j.cbpa.2015.02.017>
- Marcus, A. D., Higgins, D. P., & Gray, R. (2015b). Ivermectin treatment of free-ranging endangered Australian sea lion (*Neophoca cinerea*) pups: effect on hookworm and lice infection status, haematological parameters, growth, and survival. *Parasitology Research*, 114(7), 2743-2755. <https://doi.org/10.1007/s00436-015-4481-4>
- Marcus, A. D., Higgins, D. P., Slapeta, J., & Gray, R. (2014b). *Uncinaria sanguinis* sp. n. (Nematoda: Ancylostomatidae) from the endangered Australian sea lion, *Neophoca cinerea* (Carnivora: Otariidae). *Folia Parasitologica*, 61(3), 255-265. <https://doi.org/10.14411/fp.2014.037>

- Marlow, B. J. (1975). The comparative behaviour of the Australasian sea lions *Neophoca cinerea* and *Phocarctos hookeri* (Pinnipedia : Otariidae). *Mammalia*, 39(2), 159-230.
<https://doi.org/10.1515/mamm.1975.39.2.159>
- McCallum, H. (1994). Quantifying the impact of disease on threatened species. *Pacific Conservation Biology*, 1(2), 107-117. <https://doi.org/https://doi.org/10.1071/PC940107>
- McIntosh, R. R., Arthur, A. D., Dennis, T., Berris, M., Goldsworthy, S. D., Shaughnessy, P. D., & Teixeira, C. E. P. (2013). Survival estimates for the Australian sea lion: negative correlation of sea surface temperature with cohort survival to weaning. *Marine Mammal Science* 29(1), 84-108. <https://doi.org/10.1111/j.1748-7692.2011.00558.x>
- McIntosh, R. R., & Kennedy, C. W. (2013). Morphology, sex ratio and cause of death in Australian sea lion (*Neophoca cinerea*) pups. *Australian Mammalogy*, 35(1), 93-100.
<https://doi.org/10.1071/am12037>
- Michael, S. A., Chilvers, B. L., Hunter, S. A., Duignan, P., & Roe, W. (2016). Pathology and epidemiology of stillbirth in New Zealand sea lions (*Phocarctos hookeri*) from Enderby Island, Auckland Islands, 1998-2012. *Veterinary Pathology*, 53(6), 1241-1247.
<https://doi.org/10.1177/0300985816638723>
- Michael, S. A., Hayman, D. T. S., Gray, R., & Roe, W. D. (2021). Risk factors for New Zealand sea lion (*Phocarctos hookeri*) pup mortality: ivermectin improves survival for conservation management. *Frontiers in Marine Science*, 8, 680678.
<https://doi.org/10.3389/fmars.2021.680678>
- Michael, S. A., Hayman, D. T. S., Gray, R., Zhang, J., Rogers, L., & Roe, W. D. (2019). Pup mortality in New Zealand sea lions (*Phocarctos hookeri*) at Enderby Island, Auckland Islands, 2013-18. *PLoS One*, 14(11), e0225461. <https://doi.org/10.1371/journal.pone.0225461>
- Pesapane, R., Chaves, A., Foley, J., Javeed, N., Barnum, S., Greenwald, K., Dodd, E., Fontaine, C., Duignan, P., Murray, M., & Miller, M. (2022). Nasopulmonary mites (Acari: Halarachnidae) as

- potential vectors of bacterial pathogens, including *Streptococcus phocae*, in marine mammals. *PLoS One*, 17(6), e0270009. <https://doi.org/10.1371/journal.pone.0270009>
- Pitcher, B. J., Ahonen, H., Charrier, I., & Harcourt, R. G. (2011). Allosuckling behavior in the Australian sea lion (*Neophoca cinerea*): an updated understanding. *Marine Mammal Science*, 27(4), 881-888. <https://doi.org/10.1111/j.1748-7692.2010.00440.x>
- Pitcher, B. J., Ahonen, H., Harcourt, R. G., & Charrier, I. (2009). Delayed onset of vocal recognition in Australian sea lion pups (*Neophoca cinerea*). *Naturwissenschaften*, 96(8), 901-909. <https://doi.org/10.1007/s00114-009-0546-5>
- Ramos, P. W. (2013). Investigation of hookworm (*Uncinaria* sp.) parasites in free-ranging Australian fur seals (*Arctocephalus pusillus doriferus*). Masters Dissertation, University of Melbourne. <https://cat.lib.unimelb.edu.au/record=b5090604>
- Ross, K., Le-Bert, C. R., Goe, A., Meegan, J., Johnson, S., McClain, A., Linnehan, B., Lutmerding, E., Gomez, F., Smith, C. R., Ivancic, M., Terio, K., Colegrove, K., & Jensen, E. (2023). Superficial cervical lymphadenitis associated with *Streptococcus phocae* in five common bottlenose dolphins (*Tursiops truncatus*): a case series. *Journal of Zoo and Wildlife Medicine*, 54(1), 192-201. <https://doi.org/10.1638/2022-0014>
- Seguel, M., Munoz, F., Navarrete, M. J., Paredes, E., Howerth, E., & Gottdenker, N. (2017). Hookworm infection in South American fur seal (*Arctocephalus australis*) pups. *Veterinary Pathology*, 54(2), 288-297. <https://doi.org/10.1177/0300985816677151>
- Seguel, M., Pavés, H., Paredes, E., & Schlatter, R. (2013). Causes of mortality in South American fur seal pups (*Arctophoca australis gracilis*) at Guafo Island, southern Chile (2004–2008). *Marine Mammal Science*, 29(1), 36-47. <https://doi.org/10.1111/j.1748-7692.2011.00534.x>
- Skaar, I., Gaustad, P., Tonjum, T., Holm, B., & Stenwig, H. (1994). *Streptococcus phocae* sp. nov., a new species isolated from clinical specimens from seals. *International Journal of Systematic Bacteriology*, 44(4), 646-650. <https://doi.org/10.1099/00207713-44-4-646>

- Spraker, T. R., Delong, R. L., Lyons, E. T., & Melin, S. R. (2007). Hookworm enteritis with bacteremia in California sea lion pups on San Miguel Island. *Journal of Wildlife Diseases*, 43(2), 179-188. <https://doi.org/10.7589/0090-3558-43.2.179>
- Spraker, T. R., & Lander, M. E. (2010). Causes of mortality in northern fur seals (*Callorhinus ursinus*), St. Paul Island, Pribilof Islands, Alaska, 1986-2006. *Journal of Wildlife Diseases*, 46(2), 450-473. <https://doi.org/10.7589/0090-3558-46.2.450>
- Sterling, I. (1972). Observations on the Australian sea lion, *Neophoca cinerea* (Peron). *Australian Journal of Zoology*, 20, 271-279.
- Taurisano, N. D., Butler, B. P., Stone, D., Hariharan, H., Fields, P. J., Ferguson, H. W., Haulena, M., Cotrell, P., Nielsen, O., & Raverty, S. (2018). *Streptococcus phocae* in marine mammals of northeastern Pacific and Arctic Canada: a retrospective analysis of 85 postmortem investigations. *Journal of Wildlife Diseases*, 54(1), 101-111. <https://doi.org/10.7589/2016-09-208>
- Taylor, M. A. (2016). *Parasites of sheep and goats*. In M. A. Taylor, R. L. Coop, R. L. Wall (Eds), *Veterinary Parasitology* 4th Ed (pp. 436-523). John Wiley and Sons, Ltd.
- Taylor, S., Terkildsen, M., Mcquilty, R., Lee, D., Wing-Simpson, A., & Gray, R. (2022). Non-essential heavy metals and protective effects of selenium against mercury toxicity in endangered Australian sea lion (*Neophoca cinerea*) pups with hookworm disease. *Environment International*, 169, 107521. <https://doi.org/10.1016/j.envint.2022.107521>
- Taylor, S., Terkildsen, M., Stevenson, G., De Araujo, J., Yu, C., Yates, A., McIntosh, R. R., & Gray, R. (2021). Per and polyfluoroalkyl substances (PFAS) at high concentrations in neonatal Australian pinnipeds. *Science of the Total Environment*, 786, 147446. <https://doi.org/10.1016/j.scitotenv.2021.147446>

Supplementary material

SUPPLEMENTARY TABLE 1 Summarized bacterial culture results from deceased Australian sea lion pups, Seal Bay, Kangaroo Island, 2016–2022.

Date of death	Sex	Age	Hookworm (burden) & HAHE status	Trauma status (type)	Necropsy findings	Culture site	Culture result
18/02/2018	M	3–4 weeks	Positive (high) Present	Present (sharp)	Skin wound + myiasis Peritonitis	Peritoneal effusion	<i>Streptococcus phocae</i> (p)
17/03/2018	F	3–4 weeks	Positive (unspecified) Present	Present (sharp)	Skin wound + cellulitis + myositis	Skin wound	<i>Arcanobacterium phocae</i> (p)
19/03/2018	M	1–3 months	Positive (high) Present	Present (sharp)	Skin wound + cellulitis + myositis Peritonitis	Peritoneal effusion	<i>Streptococcus phocae</i> (two isolates) (m)
01/05/2018	F	1–3 months	Positive (high) Present	Absent	Hookworm intestinal wall perforation Peritonitis	Peritoneal effusion	<i>Escherichia coli</i> , other enteric bacteria (m)
14/12/2020	F	Perinatal	Negative – prepatent Absent	Present (unspecified)	Peritonitis	Peritoneal effusion	<i>Streptococcus phocae</i> , <i>Streptococcus canis</i> (m)
20/02/2021	M	1–3 months	Positive (high) Absent	Present (sharp)	Skin wound + myiasis	Peripheral blood Liver Spleen	<i>Streptococcus phocae</i> (p) <i>Streptococcus phocae</i> (p) No growth aerobically or anaerobically
21/02/2021	F	> 2–3 months	Negative – postpatent Absent	Present (sharp and blunt)*	Focal hepatitis Hepatic rupture	Liver Peritoneal effusion (hemorrhage)	<i>Streptococcus canis</i> , <i>Arcanobacterium phocae</i> (m) No growth aerobically or anaerobically

22/02/2021	M	1–3 months	Negative – treated Absent	Present (sharp)	Skin wound + myiasis Abscessation + cellulitis Pleuritis, pericarditis and peritonitis	Skin wound Pleural effusion	<i>Streptococcus canis</i> , <i>Streptococcus halichoeri</i> , <i>Arcanobacterium phocae</i> (m) <i>Streptococcus phocae</i> (p)
11/03/2021	F	1–3 months	Positive (high) Absent	Present (sharp)	Skin wound + myiasis	Skin wound	<i>Escherichia coli</i> , <i>Enterococcus faecalis</i> , <i>Streptococcus canis</i> (m)
03/05/2021	F	1–3 months	Positive (high) Present	Absent	Hookworm intestinal wall perforation Peritonitis	Peritoneal effusion	<i>Escherichia coli</i> , <i>Enterococcus faecalis</i> , other enteric bacteria (m)
15/06/2021	M	< 1–2 weeks	Negative – prepatent Absent	Present (blunt)	Hepatic and splenic rupture Hemorrhage (abdominal)	Peritoneal effusion (hemorrhage)	No growth aerobically or anaerobically

HAHE: hookworm-associated hemorrhagic enteritis; M: male; F: female; m: mixed growth; p: pure growth. *Healing penetrating skin wounds were detected at pup capture 5 days prior to death (potential bacterial entry portal), and per-acute death from blunt trauma was detected at necropsy.

CHAPTER SIX

General discussion and conclusion



6.1 Introduction

Over the past two decades considerable resources have been directed to assessing and monitoring the population distribution and abundance of the Australian sea lion. Findings indicate that the species' population continues to decline, despite acknowledgement of its endangered status (Goldsworthy and Gales, 2008; Threatened Species Scientific Committee, 2020), and protection by Federal and State Government legislation (Department of Sustainability Environment Water Population and Communities, 2013a). Reasons for this population decline are likely multifactorial, based on reporting in the Australian sea lion and other pinniped species – anthropogenic (fisheries interaction, resource competition, pollution), environmental (habitat degradation, toxicity, extreme weather events, ocean acidification, marine heat waves) and disease-related (parasitic, bacterial, and viral pathogens) (Department of Sustainability Environment Water Population and Communities, 2013a).

Fisheries interaction is a substantial and widely recognised anthropogenic threat to pinniped species. Reporting of its impact on Australian sea lion abundance culminated in an assessment that incidental catch mortality was unsustainable in South Australia (Goldsworthy et al., 2010). The identified threat was immediately mitigated by the Australian Sea Lion Management Strategy (Australian Fisheries Management Authority, 2010), resulting in up to 98% reduction in incidental catch mortality within a decade and potential contribution to stabilization of pup abundance at some impacted breeding colonies (Goldsworthy et al., 2022). This exemplar for threat identification, quantification, and mitigation contrasts with the status assigned to infectious disease risk. The species vulnerability to a disease epidemic (low fecundity, population stratification, genetic bottleneck), the significant impact of disease on other endangered otariid populations, and the existing compromising effects of endemic hookworm infection and pollution exposure, are all well documented (Department of Sustainability Environment Water Population and Communities, 2013a; b; Threatened Species Scientific Committee, 2020).

This research aimed to advance current understanding of infectious disease impact on the Australian sea lion, to further identify species' risk, and to offer an effective and practically implementable mitigation strategy. In doing so, its central hypothesis that infectious disease is a substantial and modifiable contributor to low fecundity or high pup mortality in the Australian sea lion has been established. It provides a solid evidence base for future research and justifies a recommendation for the development of a systematic and proactive monitoring program to determine the species exposure risk and for the early detection of emerging disease threat.

6.2 Research outcomes

Chapter two reported the first use of a topical formulation of the ivermectin anthelmintic for the treatment of hookworm infection in a free ranging otariid population, with inclusion of the injected formulation as a treatment comparator of known effectiveness and tolerability. This 2017 randomised controlled trial (n=85) found no significant difference in the estimated effectiveness of the topical and injected formulations (96.4% and 96.8%, respectively; $p>0.05$), and provided the first documentation in the Australian sea lion that early hookworm elimination could provide a growth benefit – evidenced as amelioration of a short-term (~2–3 weeks) decline in body condition observed in the control group (daily specific growth rates [SGRs] for treated and control groups: –0.090% [–0.318–0.138%] and –0.357% [–0.573 – –0.141%]). The study protocol was guided by outcomes of the first (injectable) anthelmintic treatment trial within the species (Marcus et al., 2015b) and by instituting fortnightly colony visits, overcame a reported limitation in recruitment of pups less than two weeks of age. Trialling a topical anthelmintic was also consequential to a recommendation from that study, as a method more likely to enable treatment of pups during the period of postnatal maternal guarding, and thereby preventing patent disease development. This initial trial alleviated concern for transference risk intrinsic to the use of a topical formulation – the hydrophobic nature of the formulation and of the pup's pelage, resulting in the coat surface

remaining free from anthelmintic after correct application directly to the skin surface. Subsequent detection of infection becoming patent and its persistence in untreated individuals within gregarious pup groups confirmed minimal transference.

Three unexpected outcomes were partly attributable to the study's precautionous recruitment size and logistically constrained protocol. Firstly, treated pups demonstrated bodyweight SGRs more than double that of untreated pups in the short-term (0.717% [0.472–0.961%] and 0.342% [0.112–0.573%; $p>0.05$]), although this benefit didn't achieve statistical significance and its magnitude didn't persist to the medium-term (~5–6 weeks). Secondly, treatment-related (versus control) short- (and medium-) term reductions in the severity of hookworm-associated eosinophilia (0.04 [0.00–0.19] and 0.77 [0.51–1.12] $\times 10^9/L$; $p<0.05$) and anaemia (PCV 0.332 [0.313–0.349] and 0.307 [0.288–0.324] L/L ; $p>0.05$) paralleled existing studies in otariids (Marcus et al., 2015b; Michael et al., 2021); however, a significantly lower total plasma protein concentration in the short-term (72.1 [68.2–75.9] and 85.2 [82.2–88.3] g/L ; $p<0.05$) was contrary to expectation based on those studies, and did not persist to the medium-term. This was subsequently hypothesised to be a positive acute phase inflammatory response to hookworm infection. Thirdly, it was apparent that hookworm- and treatment-related effects on blood parameters in younger pups were superimposed on substantial changes attributable to neonatal ontogeny. Despite the logistical challenges posed by the site, study results provided sufficient confidence to undertake a large-scale treatment trial using the topical ivermectin formulation.

Chapter three reported outcomes from the subsequent two-season (2019 winter-spring and 2020/2021 summer-autumn) study at Seal Bay, unencumbered by the logistical constraints of Dangerous Reef. Incorporating stratification by pup age (median 14 day and 24 day recruitment ages) and later hookworm patency status (median 10 day recruitment age), greater recruitment number ($n=322$), and longer-term follow-up (three monthly recaptures) contributed to achieving all the study's aims related to early hookworm elimination – demonstration of an immediate and

enduring growth benefit, quantification of health benefits reported as haematological changes distinguishable from those due to neonatal ontogeny, confirmation of the importance of preventing patent disease development, and demonstration of the treatment's seasonal independence.

The use of statistical modelling to account for confounding factors (pup age, treatment status, breeding cohort/season) provided the clearest indication to date of the impact of hookworm infection on pup health, enabling a greater understanding of the host-parasite-environment balance in an otariid species. For instance, it resolved conflicting reports of infection impact on total plasma protein concentration (Marcus et al., 2015b; Lindsay et al., 2021; Michael et al., 2021) and validated findings of an earlier immunological study (Meza Cerda et al., 2022) – a significant short-term (median 31 days) decline following the onset of disease patency in untreated versus treated pups (62.9 [61.2–64.6] and 65.5 [64.0–66.9] g/L; $p=0.023$) explainable by parasite haematophagy and a negative acute phase response (albumin), and a significant elevation (72.9 [71.5–74.3] and 66.5 [65.2–67.8] g/L; $p<0.001$) coinciding with natural hookworm elimination (median 62 days) supportive of a positive acute phase inflammatory response (anti-helminth immunoglobulin production). Further, comparison of total plasma protein concentration (and the other analytes) across treatment groups for the three separately reported age cohorts offered a clear indication of the negative haematological consequences of delaying treatment until after the onset of patent infection, and of changes attributable to ontogeny. Findings will guide future studies by further underlining the necessity to define the ontogeny impact when using haematological data to assess the outcome of disease manipulation in neonatal animals.

Demonstration of a short-term treatment-related growth benefit was anticipated and aligned with benefits reported in studies of other otariids (Chilvers et al., 2009; DeLong et al., 2009; Montalva et al., 2019). Less expected, and reported as improvements in standard length and bodyweight SGRs, were 1) the sizable magnitude of the benefit when preventing the development of patent infection (42.1% and 34.2% at approximately one month; $p\leq 0.001$); 2) the decline in the size of this growth

benefit attributable to just a median four day delay in treating infection and commencement of disease patency (26.7% and 25.0% at approximately one month; $p < 0.001$), and 3) the persistence of a significant benefit beyond the age (median of three months) of natural hookworm clearance (18.4% and 11.6%; $p \leq 0.004$) for pups treated in the prepatent period, and even for pups that had endured a median of two weeks of patent disease prior to treatment (9.5% and 11.2%; $p \leq 0.033$). Beyond quantifying the short- and long-term growth benefit from preventing infection patency and highlighting the study's success in recruiting a young pup cohort, these findings auger well for a treatment-related survival benefit in pups and juveniles – a fundamental objective for species' conservation. The Dangerous Reef pilot study was not powered to assess for a treatment-related survival benefit. This is an assessable outcome of the Seal Bay study (results to be reported elsewhere); however, modelling of treatment effect (reported in **chapter five**) found a reduced risk of pup mortality and bodes well for demonstration of a significant survival benefit in future reporting.

The **fourth chapter** reported the first assessment of *Toxoplasma gondii* seroprevalence in the Australian sea lion. Results confirmed the hypothesis that the species' exposure risk to this ubiquitous protozoan parasite and emerging marine mammal pathogen was comparable to that of other pinniped species – 30.4% (95% CI 13.2– 52.9; $n=23$) seroprevalence and high antibody titres (512 to > 2048 IU/ml) in archived adult female samples (median age 9.5 years, range 5.5–14.5 years) collected from the felid-free Dangerous Reef colony. It served to demonstrate that despite their geographical remoteness, marine species do not exist in isolation, with the potential for similar infectious disease risk, notwithstanding regional factors. *Toxoplasma gondii* was chosen for assessment over other pathogens because of its ubiquity, identified moderation of population recovery in an endangered marine mammal species (Roe et al., 2013), and regionally high definitive felid host (feral cat) presence (Legge et al., 2017). The availability of adult serum samples was the major limitation to this survey, a major hindrance to disease investigation in the species more generally (see below).

Despite the 30.4% adult female seroprevalence, an absence of detectable *T. gondii* antibodies (97.5% CI 0.0–2.0%, n=184; probability of freedom ~100.0%) in pups yet to commence foraging (including 98 pups from the cat-inhabited Seal Bay colony), supported existing hypotheses of predominantly foraging-related (versus vertical) pathogen transmission in marine species and short-term maternal antibody persistence in pups.

Chapter five reported the primary cause-of-mortality in pups (n=122) across five breeding cohorts (2016–2022) at the Seal Bay colony. The periodic re-assessment of mortality causality every 10–15 years has been suggested, based on disease emergence, improved diagnostics, and the dynamic nature of mortality patterns in wildlife populations (Miller et al., 2020) – this timeframe incidentally approximating mortality reporting in Australian sea lion pups. Consistent with earlier studies (Marlow, 1975; Higgins and Tedman, 1990; McIntosh and Kennedy, 2013) were substantial contributions to primary cause-of-mortality from conspecific trauma (41.0%) and starvation (24.6%), and the novel reporting of a substantial disease (hookworm-associated haemorrhagic enteritis [HAHE] +/- septicaemia) contribution (19.7%). Inclusion of faecal analysis, tissue histology and bacterial culture to observational and gross necropsy data overcame an identified limitation of an earlier mortality study (McIntosh and Kennedy, 2013), reducing the proportion of deaths of undetermined cause from 49% to 15% – many likely assigned to the infectious disease category. The additional separate reporting of comorbidity presence (trauma, starvation, HAHE, septicaemia) enabled a greater appreciation of the contribution of each to pup death. When allowing for the presence of comorbidity, infectious disease was shown to be present in 37.7% of the pups examined, including 44.0% of deaths attributed to trauma and 41.5% of pups assessed as starving at the time of death. The study protocol, including the use of hookworm status to define age class and the separate classification of mortality and comorbidity, offers a template for future mortality reporting in the species. The use of a few, well-defined and comparatively clearly differentiable primary cause-of-mortality categories, countered the difficulty in defining cause of death in a species where rapid autolysis due to environmental conditions is a substantial limitation.

The study's most substantial addition to mortality reporting in the Australian sea lion was inclusion of epidemiological modelling to evaluate treatment and season effects. Study outcomes offer the potential to better guide future management decisions for the species, compared with those available from reporting of descriptive outcomes. For instance, endemic hookworm disease was a substantial observed contributor to pup mortality (25.5%, inclusive of 7.4% in which it was a comorbidity in traumatic death), validating findings of earlier studies reporting negative health consequences of infection (Marcus et al., 2014; 2015a; b; Lindsay et al., 2021; Lindsay et al., 2023) (**chapters two and three**). However, population modelling enabled the prediction of a significant risk reduction for mortality from any cause following its treatment (treated versus untreated: 10.2% [5.9–14.6%] and 26.3% [23.7–29.0%], respectively; $p < 0.001$), incentivising further research of the topical anthelmintic as a conservation management tool. The included visual summary of significant relationships between season, hookworm treatment, and a biologically relevant sequence of the reported comorbidities, builds on an existing understanding of the importance of host-pathogen-environment balance as it relates to hookworm infection. At its centre was the presence of hookworm-associated haemorrhagic enteritis: ameliorated by hookworm treatment (OR 0.16), more likely to be present in summer-autumn seasons (OR 1.61) and increasing the risk of pups dying from trauma (OR 4.86) or with starvation (OR 17.84). It provides explanation for a treatment-related reduction in risk of death beyond that directly attributable to parasitism, an outcome also observed in the New Zealand sea lion (Michael et al., 2021).

Further study of risk relatedness is necessary to better understand the biological reasons for these significant associations, and to identify additional intervention points for lowering mortality risk. Some, such as a higher incidence of wound myiasis contributing to pup death in warmer months are readily explainable, and potentially immediately rectifiable; others, such as the suggested intra-colony shifts in population density increasing trauma risk in summer-autumn seasons (OR 2.49) will require the inclusion of more detailed mortality reporting (e.g., location coordinates and estimated time of death) and risk factor analysis, in future mortality studies. The risk of pups dying with

septicaemia was greater in summer-autumn seasons (OR 5.97), in starving animals (OR 16.76), and especially following conspecific trauma (OR 58.71), with the frequent identification of *Streptococcus* spp. (particularly *S. phocae*) analogous with reporting of these emerging pathogens in other marine mammal species. The study therefore also builds on a growing database of common bacterial isolates, disease presentations and predisposing factors in these species, adding to existing understanding of disease (infection) epidemiology and potentially guiding investigation of future minimisation strategies (e.g., reducing conspecific trauma risk in Australian sea lion pups).

6.3 Management implications and future directions

Understanding the causes of mortality and the extent to which each impacts different age classes is critical to species management, and especially conservation of endangered populations (Maniscalco et al., 2008). This research confirms the substantial contribution of infectious disease to Australian sea lion pup morbidity and mortality, and it is therefore another recognised contributor to the species relatively high pup mortality and current population status. Pup growth retardation, haematological health impacts (**chapters two and three**), and increased mortality risk (**chapter five**) attributable to the development of patent hookworm infection, as well as high level exposure to a ubiquitous protozoan parasite (**chapter four**) and presence of multiple emerging bacterial pathogens (**chapter five**) within the species, are all consistent with reporting in other otariid and marine mammal species. Consequently, disease impact in other otariids is a credible guide for its potential to modulate the Australian sea lion population, and prompt for policy decision-making. The 1997–1998 bacterial epidemic resulting in the death of more than 50% of that season’s New Zealand sea lion pups at the Auckland Islands (Baker, 1999), highlighted the importance of baseline data and impelled a directed longitudinal survey of neonatal mortality at the colony (Castinel et al., 2007). This thesis adds to baseline mortality reporting and suggests the merit of establishing a similar

systematic sampling and reporting program for disease detection and ongoing risk assessment for the Australian sea lion.

6.3.1 Hookworm mitigation strategies

Ongoing data collection and reporting are needed to confirm an observed survival benefit from early hookworm elimination in the Australian sea lion, although this seems likely to be the case based on positive outcomes of risk modelling in the species (**chapter five**) and an observed reduction in pup mortality in other otariid species experiencing elevated levels of disease-related pup death (DeLong et al., 2009; Michael et al., 2021). Further, the Seal Bay study (**chapter three**) demonstrated that the large-scale treatment of hookworm (50% of captured individuals) using a topical anthelmintic is achievable, efficient, well tolerated, highly effective, and substantially mitigates the negative health consequences of parasite infection when optimally applied to prevent patent disease development, independent of breeding season.

To assist the species' conservation, a broader topical anthelmintic treatment program in Australian sea lion pups is therefore feasible, albeit not without risk, and with considerable logistical, financial and time costs. Such an undertaking therefore requires careful rationalisation. Risks include those intrinsic to widespread ivermectin anthelmintic use – eco-toxicological effects on the marine environment (Halley et al., 1989; Bai and Ogbourne, 2016; Mesa et al., 2017; Vokral et al., 2019) and the development of parasite resistance, for instance. Risk of the former for the topical formulation likely parallels that of the injected formulation (used at a lower dose rate), given that much of the parent drug is excreted in faeces unchanged (Halley et al., 1989). Dose volume minimisation is prudent in any case, further rationalising the treatment of pups as early (and small) as possible, rather than for instance, considering alternative protocols such as application to pre-parturient females. Additional to dose volume minimisation and the potential for inaccurate dosing in the absence of animal weight determination, treatment of females is logistically less feasible – for

instance, due to the narrow time window for detecting impending birth in individuals that arrive ashore shortly before pupping (and in whom resting somatic hookworm larvae are reactivated and susceptible to anthelmintic treatment) (Stoye, 1992; Kramer et al., 2009), the risk of physical injury to animals and humans from approaching a mate-guarded female, and the potential to disrupt male-female or new-born pup-female bonding. Minimising the risk of anthelmintic resistance developing is dependent on maintaining adequate parasite refugia (parasites not exposed to anthelmintic) – achievable by limiting the proportion of the pup population treated. Other risks are less apparent, such as treatment impact on the composition and diversity of the gut microbiome and resultant alteration of pup immune system development – previously explored in the Australian sea lion (Fulham et al., 2022).

A significant survival benefit from ivermectin use has most frequently been demonstrated for otariid populations experiencing an elevated level of pup mortality due to infectious disease (Chilvers et al., 2009; DeLong et al., 2009; Michael et al., 2021). Consequently, the large-scale (80% of pups) treatment of New Zealand sea lion pups at two colonies has previously been proposed – 60% of the species' pup deaths attributed to endemic bacterial infection (indicative 2013–2018 mortality 10.6%–24.5%) (Michael, 2022). In comparison, Australian sea lion pup mortality at a colony such as Seal Bay is high (17.9%–41.8%) with no apparent trend for elevation, a lesser contribution of infectious disease to mortality, but consistently higher summer-autumn versus winter-spring breeding seasons deaths (averaging 32.8% and 21.4%, respectively) (Goldsworthy et al., 2021). A measured, next-step approach for ongoing assessment of treatment benefit and effectiveness in the species at the Seal Bay colony, could therefore include the following:

- 1) Application of topical ivermectin in 50%–75% of Australian sea lion pups, limited to the higher mortality summer-autumn seasons, with stringent ongoing monitoring for treatment effectiveness and impact – a minimum of evaluating pup morphometrics (weight and standard length measurement; body condition index calculation), faecal analysis for

hookworm infection status, and assessment of haematological indicators of pup health (packed cell volume and total plasma protein concentration).

- 2) A selection bias for treatment of young pups, ideally those with prepatent infection, or at least most likely to be of young neonatal age (e.g., small size [$<70\text{cm}$ in standard length], dark coat colour, first detected post-parturient maternal absence).

Beyond these two key recommendations could be disease triaging – comparatively limited additional opportunistic treatment of rare individuals most likely to benefit from anthelmintic application – pups detected in poor or declining condition despite observed maternal attendance, with evidence of bloody diarrhoea, and/or with traumatic injury $\pm$ myiasis.

As outlined, suggesting implementation of any hookworm intervention-related management strategy must be contingent on continued robust scientific monitoring of anthelmintic use for effectiveness, risk assessment and beneficial outcome. Additional to pup assessment at the time of treatment, such monitoring would necessitate pup re-evaluation at predetermined intervals post-treatment – for instance, at one month for hookworm status and haematological indicators of pup health, and at the conclusion of the breeding season for survival status.

6.3.2 Future directions

A repeatable constraint to disease investigation in the Australian sea lion is the limited access to biological samples, particularly from juvenile and adult animals. This is partly consequential to the population's dispersed geographical range and relatively limited human interaction, and partly due to limited resourcing of sample collection. While ongoing disease monitoring and the investigation of mortality above baseline is imperative across the species range, a feasible approach is to focus resources on the four breeding sites contributing approximately 40% of the species' pup production. Reliance on anecdotal and *ad hoc* disease detection is likely to underestimate its prevalence and impact, due to the difficulty of detecting mortality in a marine species (in individuals older than

terrestrial-bound pups), and in assigning causation in the presence of comorbidity or multiple contributing factors (summarised in (Russell et al., 2020)). More desirable is ongoing systematic monitoring (including disease outbreak surveillance) and actively maximising current sample collection opportunities. For instance, a requirement incorporated into the current Scientific Permit system for collegial sample collection from any juvenile or adult individual restrained or anaesthetised for research or treatment purposes. Tissue and fluid specimens (blood, faeces/ urine, hair/ whiskers etc.) could be submitted to a centralised location and subjected to a routine suite of tests, prior to preservation and cataloguing for future retrospective analysis. This monitoring would complement additional invasive opportunistic sampling and reporting of deceased individuals, ideally examined promptly after detection. Similar systems exist for other threatened marine mammal species, including that implemented by the California Department of Fish and Wildlife in 1998 for the southern sea otter (*Enhydra lutris nereis*) (Miller et al., 2020). The resultant robust and longitudinal dataset provides a means for identification of threats to population recovery.

In the context of many marine mammals being keystone and sentinel species, such monitoring also has important regional and global one-health, public health, and food security (fisheries) considerations. Of current relevance is the global monitoring of these species for exposure to Influenza A virus (IAV), including for highly pathogenic avian influenza (HPAI) A (H5N1). Beyond supporting infection and replication of avian sourced viruses, pinnipeds could provide a host for virus genetic reassortment and the development of highly pathogenic and mammalian-adapted variants (summarised in Runstadler and Puryear, 2020). Elsewhere, the potential for marine mammals to be an important wild reservoir of IAV (Adlhoch et al., 2023) is partly driving a shift away from the limited opportunistic historical testing for identification of causation in unusual mortality events (Lair et al., 2024), towards active serosurveillance. Similar monitoring of the Australian sea lion (and sympatric pinniped species) seems prudent.

Current limitations to the investigation of disease threat in the Australian sea lion are shared across marine mammal species. Compared with their terrestrial equivalents, management of infectious disease threat in marine species is frequently insufficient or ineffective, due to intrinsic differences between the two (Glidden et al., 2022). Consequently, many broad recommendations for strategies to manage disease in the marine ecosystem (summarised in (Glidden et al., 2022), are applicable to future research suggestions related to this thesis. Many are ultimately dependent on prioritisation of disease research in the Australian sea lion through increased study resourcing.

- 1) Incorporation of disease risk, outcomes from scientifically rigorous studies, and epidemiological modelling in all future population risk assessments.
- 2) Increasing sample availability to improve disease monitoring and surveillance:
 - a) Optimising carcass preservation, through more rapid detection, collection and sampling of deceased individuals, and reduced reliance on post-mortem freezing (use of short-term carcass refrigeration, for instance)
 - b) Protocols for obtaining a standard sample set at the time of carcass collection (e.g., faeces, bacterial swabs, blood, exudates, hair/whiskers, tissue (e.g., toe biopsy).
- 3) Increasing use of advanced disease diagnostic tools to optimise assessment of the species' risk from endemic and emerging infectious disease threats (e.g., molecular testing), and concurrently screening for multiple pathogens forecast by infectious disease detection in other species – bacterial (e.g., *Coxiella burnetii*, *Brucella* spp., *Chlamydia* spp., *Mycoplasma* spp.) and viral (morbilliviruses), as well as protozoal agents.
- 4) Ongoing inclusion of environmental factors (e.g., climate change, ecosystem pollution and health) in disease and mortality investigations, to increase understanding of interactions and identify intervention and mitigation opportunities. Specifically, investigating disease association with host-compromising exposure to toxins (e.g., domoic acid, heavy metals, PFAS), biological marine pollution, altered climatic conditions (e.g., increasing ocean temperatures and acidification, habitat degradation), prey species abundance.

Recommendations for the future study of toxoplasmosis in the Australian sea lion relate to the hypothesis that disease is sporadic, with parasite infection contributing little to the species low fecundity and pup survival. For instance, additional testing to assess for, and differentiate, *in utero* infection (vertical transmission +/- placentitis) and post-partum infection (horizontal transmission):

- 1) Comparison of early or mid-gestational pregnancy rates (determined endocrinologically following the diapause) and birth rates, to detect embryonic/ foetal loss (Gibbens et al., 2010). Ideally this would be performed in conjunction with maternal serological assessment for *T. gondii* exposure status. Such a comparison would have substantial logistical (and potentially ethical) considerations – for instance, the requirement for general anaesthesia to collect blood samples. However, the future validation of less invasive methods for determining pregnancy status (e.g., faecal proteins as pregnancy biomarkers, as explored by Curry et al. (2022)) may increase the feasibility of such a study.
- 2) Histological, immunohistochemical, molecular and serological testing of placental tissue and tissue from aborted, stillborn, premature or weak newborn pups (+ maternal serological assessment) – for detection of parasites and disease compromising pup viability; comparison of parasite genotype with those isolated from regional definitive hosts to inform colony risk assessment.
- 3) Serological testing of older pup, juvenile and young adult cohorts (especially breeding females and their pups) – to further investigate disease epidemiology (i.e., to specifically refine temporal information for parasite exposure/transmission and its relatedness to parasite and antibody transmission).

6.4 Conclusion

This research achieved its intended aim of investigating the contribution of infectious disease to fecundity and pup survival in the Australian sea lion. The central hypothesis was that disease

contribution is substantial and modifiable. This study 1) further defined the negative health impacts of hookworm infection in pups, reflected in significant short- and long-term growth retardation and alterations in haematological parameters (separately defined from changes related to ontogenesis); 2) confirmed that a topical anthelmintic is a highly effective treatment of endemic hookworm infection, optimally administered to prevent patent disease development; 3) quantified the exposure risk of the species to a ubiquitous protozoan parasite, and in doing so highlighted that exposure to other known marine mammal pathogens remains a key knowledge gap; and 4) quantified the contribution of disease to pup mortality (directly and as a comorbidity in pups dying from non-infectious causes), and modelled the potential to reduced pup mortality risk by the early elimination of hookworm infection. Results therefore confirm the central hypothesis. Beneficial outcomes are provision of a current baseline of disease impact on pup morbidity and mortality, and of a mitigation strategy to maximise outcomes from hookworm treatment if deemed appropriate for species conservation management. Future investigation is necessary to progress the systematic monitoring of pathogen exposure and disease risk, for the early detection of increased disease threat. Furthermore, studies to advance understanding of all host, pathogen, and environmental factors contributing to disease elaboration will offer new opportunities for disease intervention and species' conservation.

6.5 References

- Adlhoch, C., Fusaro, A., Gonzales, J. L., Kuiken, T., Mirinavičiūtė, G., Niqueux, É., et al. (2023). Avian influenza overview June–September 2023. *European Food Safety Authority Journal* 21(10), 8328. doi: 10.2903/j.efsa.2023.8328
- Australian Fisheries Management Authority (2010). *Australian sea lion management strategy: Southern and Eastern Scalefish and Shark Fishery (SESSF)* [Online]. Commonwealth of Australia. https://fish.gov.au/Archived-Reports/2012/reports/Documents/AFMA_2010b.pdf.
- Bai, S.H., and Ogbourne, S. (2016). Eco-toxicological effects of the avermectin family with a focus on abamectin and ivermectin. *Chemosphere* 154, 204–214. doi: 10.1016/j.chemosphere.2016.03.113
- Baker, A. (1999). *Unusual mortality of the New Zealand sea lion, Phocarcos hookeri, Auckland Islands, January–February 1998: report of a workshop held 8–9 June 1998, Wellington, and a contingency plan for future events* [Online]. Department of Conservation *Te Papa Atawhai*. <https://www.doc.govt.nz/Documents/science-and-technical/Sealion.pdf>.
- Castinel, A., Duignan, P.J., Pomroy, W.E., Lopez-Villalobos, N., Gibbs, N.J., Chilvers, B.L., and Wilkinson, I.S. (2007). Neonatal mortality in New Zealand sea lions (*Phocarcos hookeri*) at Sandy Bay, Enderby Island, Auckland Islands from 1998 to 2005. *Journal of Wildlife Diseases* 43(3), 461–474. doi: 10.7589/0090-3558-43.3.461.
- Chilvers, B.L., Duignan, P.J., Robertson, B.C., Castinel, A., and Wilkinson, I.S. (2009). Effects of hookworms (*Uncinaria* sp.) on the early growth and survival of New Zealand sea lion (*Phocarcos hookeri*) pups. *Polar Biology* 32, 295–302. doi: 10.1007/s00300-008-0559-0.
- Curry, E., Philpott, M. E., Wojtusik, J., Haffey, W. D., Wyder, M. A., Greis, K. D., and Roth, T. L. (2022). Label-free quantification (LFQ) of fecal proteins for potential pregnancy detection in polar bears. *Life (Basel)* 12, 796. doi.org/10.3390/life12060796
- DeLong, R.L., Orr, A.J., Jenkinson, R.S., and Lyons, E.T. (2009). Treatment of Northern fur seal (*Callorhinus ursinus*) pups with ivermectin reduces hookworm-induced mortality. *Marine Mammal Science* 25(4), 944–948. doi: 10.1111/j.1748-7692.2008.00274.x.

- Department of Sustainability, Environment, Water, Population and Communities (2013a). *Issues paper for the Australian sea lion (Neophoca cinerea)* [Online]. Commonwealth of Australia. <https://www.dcceew.gov.au/sites/default/files/documents/neophoca-cinerea-issues-paper.pdf>.
- Department of Sustainability, Environment, Water, Population and Communities (2013b). *Recovery plan for the Australian sea lion (Neophoca cinerea)* [Online]. Commonwealth of Australia. <http://www.environment.gov.au/system/files/resources/1eb9233c-8474-40bb-8566-0ea02bbaa5b3/files/neophoca-cinerea-recovery-plan.pdf>.
- Fulham, M., Power, M., and Gray, R. (2022). Gut microbiota of endangered Australian sea lion pups is unchanged by topical ivermectin treatment for endemic hookworm infection. *Frontiers in Microbiology* 13. doi: 10.3389/fmicb.2022.1048013.
- Gibbens, J., Parry, L.J., and Arnould, J.P.Y. (2010). Influences on fecundity in Australian fur seals (*Arctocephalus pusillus doriferus*). *Journal of Mammalogy* 91(2), 510–518. doi: 10.1644/08-mamm-a-377.1.
- Glidden, C.K., Field, L.C., Bachhuber, S., Hennessey, S.M., Cates, R., Cohen, L., et al. (2022). Strategies for managing marine disease. *Ecological Applications* 32(7), e2643. doi: 10.1002/eap.2643.
- Goldsworthy, S. & Gales, N. (IUCN SSC Pinniped Specialist Group) (2008). *Neophoca cinerea*. The IUCN Red List of Threatened Species 2008: e.T14549A4443172. <https://www.iucnredlist.org/species/14549/4443172> [Accessed 1 September 2021].
- Goldsworthy, S.D., Page, B., Hamer, D.J., Lowther, A.D., Shaughnessy, P.D., Hindell, M.A., et al. (2022). Assessment of Australian sea lion bycatch mortality in a gillnet fishery, and implementation and evaluation of an effective mitigation strategy. *Frontiers in Marine Science* 9, 799102. doi: 10.3389/fmars.2022.799102.
- Goldsworthy, S.D., Page B, Shaughnessy, P.D and Linnane A (2010). *Mitigating seal interactions in the SRLF and the gillnet sector SESSF in South Australia. Report to the Fisheries Research and Development Institute* [Online]. South Australian Research and Development Institute (Aquatic Sciences), Adelaide. SARDI Publication No. F2009/000613-1. SARDI Research Report Series No. 405.
- Goldsworthy, S.D., Stonnill, M., and Kirkwood, R. (2021). *Monitoring of the Australian sea lion population at Seal Bay: 2020/21. Report to the Department for Environment and Water*

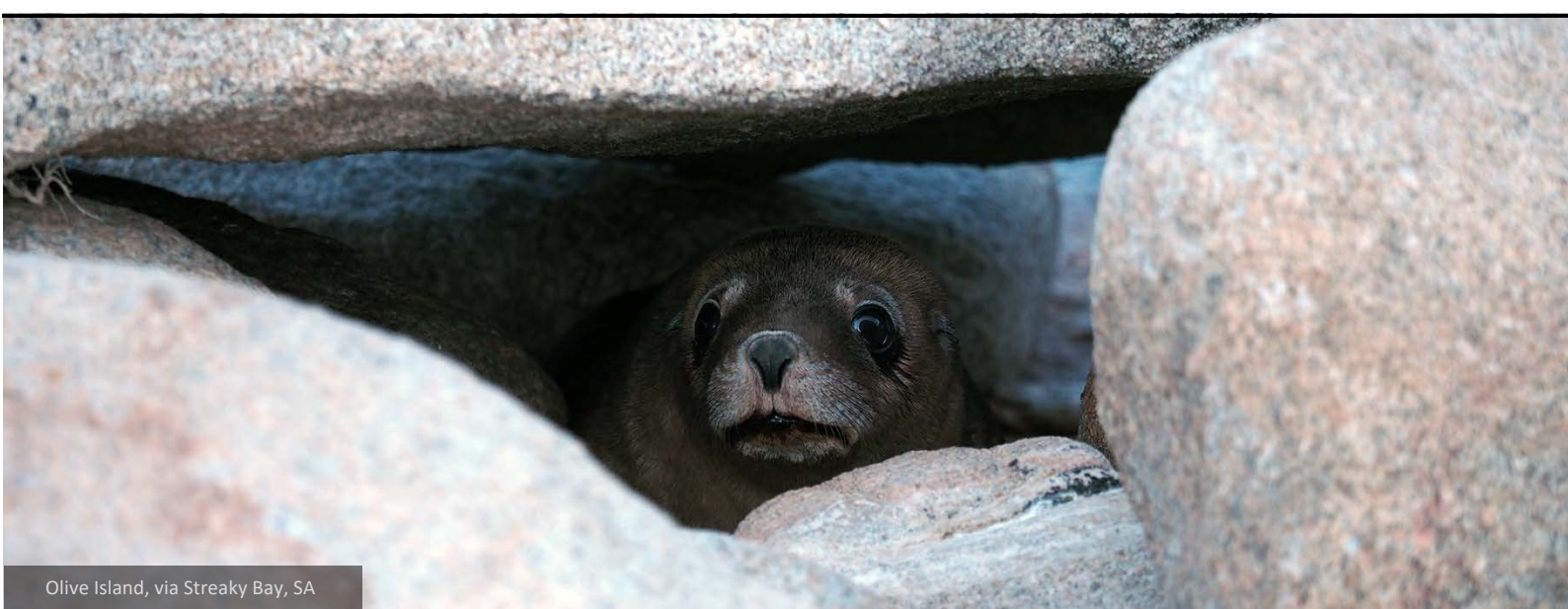
- [Online]. South Australian Research and Development Institute (Aquatic Sciences), Adelaide. SARDI Publication No. F2014/000322-6. SARDI Research Report Series No. 1121.
- Halley, B.A., Jacob, T.A., and Lu, A.Y.H. (1989). The environmental impact of the use of ivermectin: environmental effects and fate. *Chemosphere* 18(7), 1543–1563. doi: 10.1016/0045-6535(89)90045-3.
- Higgins, L.V., and Tedman, R.A. (1990). Effect of attacks by male Australian sea lions, *Neophoca cinerea*, on mortality of pups. *Journal of Mammalogy* 71(4), 617–619. doi: 10.2307/1381802.
- Kramer, F., Epe, C., and Mencke, N. (2009). Investigations into the prevention of neonatal *Ancylostoma caninum* infections in puppies by application of imidacloprid 10% plus moxidectin 2.5% topical solution to the pregnant dog. *Zoonoses and Public Health* 56(1), 34–40. doi: 10.1111/j.1863-2378.2008.01181.x.
- Lair, S., Quesnel, L., Signore, A. V., Delnatte, P., Embury-Hyatt, C., Nadeau, M.-S., et al. (2024). Outbreak of Highly Pathogenic Avian Influenza A (H5N1) virus in seals, St. Lawrence Estuary, Quebec, Canada. *Emerging Infectious Diseases* 30(6), 1133–1143. doi: 10.3201/eid3006.231033
- Legge, S., Murphy, B.P., McGregor, H., Woinarski, J.C.Z., Augusteyn, J., Ballard, G., et al. (2017). Enumerating a continental-scale threat: how many feral cats are in Australia? *Biological Conservation* 206, 293–303. doi: 10.1016/j.biocon.2016.11.032.
- Lindsay, S.A., Caraguel, C.G.B., and Gray, R. (2021). Topical ivermectin is a highly effective seal 'spot-on': a randomised trial of hookworm and lice treatment in the endangered Australian sea lion (*Neophoca cinerea*). *International Journal for Parasitology: Parasites and Wildlife* 16, 275–284. doi: 10.1016/j.ijppaw.2021.11.002.
- Lindsay, S.A., Fulham, M., Caraguel, C.G.B., and Gray, R. (2023). Mitigating disease risk in an endangered pinniped: early hookworm elimination optimizes the growth and health of Australian sea lion pups. *Frontiers in Veterinary Science* 10, 1161185. doi: 10.3389/fvets.2023.1161185.
- Maniscalco, J.M., Calkins, D.G., Parker, P., and Atkinson, S. (2008). Causes and extent of natural mortality among Steller sea lion (*Eumetopias jubatus*) pups. *Aquatic Mammals* 34(3), 277–287. doi: 10.1578/am.34.3.2008.277.

- Marcus, A.D., Higgins, D.P., and Gray, R. (2014). Epidemiology of hookworm (*Uncinaria sanguinis*) infection in free-ranging Australian sea lion (*Neophoca cinerea*) pups. *Parasitology Research* 113(9), 3341–3353. doi: 10.1007/s00436-014-3997-3.
- Marcus, A.D., Higgins, D.P., and Gray, R. (2015a). Health assessment of free-ranging endangered Australian sea lion (*Neophoca cinerea*) pups: effect of haematophagous parasites on haematological parameters. *Comparative Biochemistry and Physiology, Part A: Molecular and Integrative Physiology* 184, 132–143. doi: 10.1016/j.cbpa.2015.02.017.
- Marcus, A.D., Higgins, D.P., and Gray, R. (2015b). Ivermectin treatment of free-ranging endangered Australian sea lion (*Neophoca cinerea*) pups: effect on hookworm and lice infection status, haematological parameters, growth, and survival. *Parasitology Research* 114(7), 2743–2755. doi: 10.1007/s00436-015-4481-4.
- Marlow, B. J. (1975). The comparative behaviour of the Australasian sea lions *Neophoca cinerea* and *Phocarctos hookeri* (Pinnipedia: Otariidae). *Mammalia*, 39(2), 159–230. doi: 10.1515/mamm.1975.39.2.159.
- McIntosh, R.R., and Kennedy, C.W. (2013). Morphology, sex ratio and cause of death in Australian sea lion (*Neophoca cinerea*) pups. *Australian Mammalogy* 35(1), 93. doi: 10.1071/am12037.
- Mesa, L.M., Lindt, I., Negro, L., Gutierrez, M.F., Mayora, G., Montalto, L., et al. (2017). Aquatic toxicity of ivermectin in cattle dung assessed using microcosms. *Ecotoxicology and Environmental Safety* 144, 422–429. doi: 10.1016/j.ecoenv.2017.06.016.
- Meza Cerda, M.I., Gray, R., Thomson, P.C., Butcher, L., Simpson, K., Cameron, A., et al. (2022). Developing immune profiles of endangered Australian sea lion (*Neophoca cinerea*) pups within the context of endemic hookworm (*Uncinaria sanguinis*) infection. *Frontiers in Veterinary Science* 9, 824584. doi: 10.3389/fvets.2022.824584.
- Michael, S.A. (2022). *Causes and mitigation of pup mortality in New Zealand sea lions (Phocarctos hookeri)*. Doctor of Philosophy dissertation, The University of Sydney. <https://ses.library.usyd.edu.au/handle/2123/28192>.
- Michael, S.A., Hayman, D.T.S., Gray, R., and Roe, W.D. (2021). Risk factors for New Zealand sea lion (*Phocarctos hookeri*) pup mortality: ivermectin improves survival for conservation management. *Frontiers in Marine Science* 8, 680678. doi: 10.3389/fmars.2021.680678.

- Miller, M.A., Moriarty, M.E., Henkel, L., Tinker, M.T., Burgess, T.L., Batac, F.I., et al. (2020). Predators, disease, and environmental change in the nearshore ecosystem: mortality in southern sea otters (*Enhydra lutris nereis*) from 1998–2012. *Frontiers in Marine Science* 7, 582. doi: 10.3389/fmars.2020.00582.
- Montalva, F., Perez-Venegas, D., Gutierrez, J., and Seguel, M. (2019). The contrasting hidden consequences of parasitism: effects of a hematophagous nematode (*Uncinaria* sp.) in the development of a marine mammal swimming behavior. *Ecology and Evolution* 9(7), 3689–3699. doi: 10.1002/ece3.4914.
- Roe, W.D., Howe, L., Baker, E.J., Burrows, L., and Hunter, S.A. (2013). An atypical genotype of *Toxoplasma gondii* as a cause of mortality in Hector's dolphins (*Cephalorhynchus hectori*). *Veterinary Parasitology* 192(1–3), 67–74. doi: 10.1016/j.vetpar.2012.11.001.
- Runstadler, J.A., and Puryear, W. (2020). A Brief Introduction to Influenza A Virus in Marine Mammals. In E. Spackman (Ed.), *Animal Influenza Virus. Methods in Molecular Biology*, Vol 2123. New York, NY: Springer US. doi.org/10.1007/978-1-0716-0346-8_33
- Stoye, M. (1992). Biology, pathogenicity, diagnosis and control of *Ancylostoma caninum*. *German Veterinary Weekly: Scientific Journal for Veterinary Medicine* [German] 99(8), 315–321.
- Threatened Species Scientific Committee (2020). *Conservation advice. Neophoca cinerea, Australian sea lion*. Department of Agriculture, Water and the Environment, Canberra. [Online]. www.environment.gov.au/biodiversity/threatened/species/pubs/22-conservation-advice-23122020.pdf.
- Vokral, I., Michaela, S., Radka, P., Jiri, L., Lukas, P., Dominika, S., et al. (2019). Ivermectin environmental impact: excretion profile in sheep and phytotoxic effect in *Sinapis alba*. *Ecotoxicology and Environmental Safety* 169, 944–949. doi: 10.1016/j.ecoenv.2018.11.097

APPENDIX

Additional publication arising from candidature



Olive Island, via Streaky Bay, SA

A Novel Presentation of Tuberculosis with Intestinal Perforation in a Free-ranging Australian Sea Lion (*Neophoca cinerea*)

Scott A. Lindsay^{1,2,3} and Rachael Gray² ¹School of Animal and Veterinary Sciences, Leske Building, The University of Adelaide, Mudla Wirra Road, Roseworthy, South Australia 5371, Australia; ²Sydney School of Veterinary Science, McMaster Building B14, The University of Sydney, Camperdown, New South Wales 2006, Australia; ³Corresponding author (email: scott.lindsay@adelaide.edu.au)

ABSTRACT: We detail a novel presentation of tuberculosis associated with intestinal perforation in an endangered Australian sea lion (*Neophoca cinerea*) from South Australian waters and confirm the presence of this disease in the region of highest pup production. In February 2017, a 3-yr-old juvenile male died shortly after hauling out at the Kingscote beach on Kangaroo Island. On postmortem examination, we found a mid-jejunal intestinal perforation and partial obstruction (from a strangulating fibrous and granulomatous mesenteric mass), a marked multicentric abdominal fibrosing granulomatous lymphadenitis, and a large volume serosanguinous peritoneal effusion. Acid-fast bacteria were detected postmortem in cytologic preparations of the mesenteric lymph node and in histologic sections of jejunum and the encircling mass. Mycobacterial infection was confirmed by positive culture after 3 wk. Molecular typing using mycobacterial interspersed repetitive-unit-variable-number tandem-repeat typing with 12-locus analysis identified *Mycobacterium pinnipedii*. This case highlights the need for vigilance of zoonotic disease risk when handling pinnipeds, including in the absence of specific respiratory signs or grossly apparent pulmonary pathology. Increased serologic population surveillance is recommended to assess the species' risk from this and other endemic diseases, especially given its endangered status.

Key words: Australian sea lion, intestinal perforation, *Mycobacterium pinnipedii*, *Neophoca cinerea*, peritonitis, pinniped, tuberculosis.

Tuberculosis in pinnipeds is typically a pulmonary, pleural, or multicentric granulomatous disease resulting from infection with *Mycobacterium pinnipedii*, one of 11 highly genetically related and variably human- or animal-adapted members of the *Mycobacterium tuberculosis* complex (Cousins et al. 1993; Silva-Pereira et al. 2019). Infection with *M. pinnipedii* has been reported sporadically in free-ranging fur seals and sea lions (otariids) of the southern hemisphere, with increasingly

frequent documentation of cases in captive pinnipeds worldwide. This disease in free-ranging animals has been reported in Australasia in the Australian sea lion (*Neophoca cinerea*), Australian fur seal (*Arctocephalus pusillus doriferus*; Cousins et al. 1993; Woods et al. 1995), New Zealand sea lion (*Phocartos hookeri*), and New Zealand fur seal (*Arctocephalus forsteri*; Roe et al. 2006, 2019) and in South America in the South American sea lion (*Otaria flavescens*) and South American fur seal (*Arctocephalus australis*; Romano et al. 1995; Bernardelli et al. 1996). Disease has also been reported in the subantarctic fur seal (*Arctocephalus tropicalis*; Bastida et al. 1999).

Reporting of tuberculosis in captive Australian sea lions is limited to that of four deaths in 1985–86 described in a case series in a mixed pinniped population housed at a Western Australian marine park (Forshaw and Phelps 1991). Three animals had been collected from the Archipelago of Recherche in southern Western Australia (34°39'S, 122°27'E) and the fourth from closer to the marine park (31°33'S, 115°41'E). To date, bacteriologically confirmed tuberculosis in free-ranging Australian sea lions involves two subadult males found dead on beaches on the south coast of Western Australia (Albany: 35°01'S, 117°53'E; Bremer Bay: 34°24'S, 119°23'E) in 1990–91 (Cousins et al. 1993). Reports of tuberculosis in free-ranging pinnipeds in South Australian waters is limited to that of a subadult male Australian fur seal found dead at Beachport (37°29'S, 140°01'E; Boardman et al. 2014).

The Australian sea lion is endemic to Australian waters and ranges from the Houtman Abrolhos on the west coast of Western Australia to the Pages Islands in South

Australia (Goldsworthy et al. 2009). The species is listed as endangered with a decreasing population trend by the International Union for Conservation of Nature (Goldsworthy 2015). Common to other pinnipeds of small population size, the Australian sea lion is at increased risk of significant population decline from epizootic disease (Lynch et al. 2011). Furthermore, population recovery from the effects of disease would be limited by the species' low fecundity (including a unique 18-mo breeding cycle) and extreme female natal site fidelity dispersed across 76 asynchronous breeding sites (Goldsworthy et al. 2009). The majority (86%) of pup births occur in South Australia, with just five large breeding colonies, including Seal Bay, Kangaroo Island, contributing more than half of the state's pup production (Goldsworthy et al. 2009; Goldsworthy 2015).

On 1 February 2017, a juvenile male Australian sea lion died shortly after hauling out at a public beach at Kingscote (35°39'S, 137°38'E), Kangaroo Island, South Australia. Preliminary assessment was of an animal in fair to thin body condition with no external wounds. Microchip scanning confirmed its birth was at Seal Bay Conservation Park (35°58'S, 137°21'E), Kangaroo Island, and provided a known age of 3 yr. The cadaver was preserved by freezing at -20 C within hours of death for later postmortem examination.

Standard morphometric postmortem data were a weight of 33.3 kg, a standard length (nose to tail) of 122.3 cm, and an axillary girth of 78.0 cm. External examination confirmed the earlier assessment of body condition and found below average muscling and moderate abdominal distension. Internal examination revealed a large volume (exceeding 2 L) of serosanguinous and slightly turbid peritoneal effusion and the absence of subcutaneous blubber or visceral adipose tissue. Mid-jejunum, the intestine was completely encircled by a 120-mm-diameter spherical mesenteric mass with fibrous adhesions to the stomach and spleen (Fig. 1). The 0.60-kg mass was smooth surfaced with fibrin tags, and its cut surface was fibrous, friable, and patchy red-green to yellow. The entrapped



FIGURE 1. Entrapment of jejunum (white arrow-head) by a smooth-surfaced and friable fibrous mesenteric mass in a free-ranging Australian sea lion (*Neophoca cinerea*) secondary to *Mycobacterium pinnipedii* infection. An absence of mesenteric adipose tissue (black arrowhead) and large volume (2-L) peritoneal effusion (black arrow) were also noted. Bar=15 mm.

jejunal lumen remained patent but nonexpansile and constricted to 25% of the adjacent organ diameter. A 12-mm-diameter wall perforation communicated with a shallow 30-mm-diameter cavity within the encircling mass.

There was marked generalized abdominal lymphadenomegaly (gastric: 50×25 mm; mesenteric chain: 160×28 mm, 181×43 mm, and 81×25 mm); the cut surfaces of lymph nodes were pale yellow, nodular, and fibrous (Fig. 2). Extra-abdominal findings were limited to mild tracheobronchial lymphadenomegaly with normal cortical-medullary differentiation; localized grossly detectable pulmonary lesions were not apparent. Ziehl-Neelsen-stained cytologic specimens of a mesenteric lymph node reviewed at necropsy contained



FIGURE 2. Marked mesenteric lymphadenomegaly from fibrosing granulomatous lymphadenitis in a free-ranging Australian sea lion (*Neophoca cinerea*) secondary to *Mycobacterium pinnipedii* infection. Bar=10 mm.

large numbers of acid-fast bacilli (AFBs), presumed to be *Mycobacterium* spp., supporting a preliminary diagnosis of tuberculosis.

Histologic interpretation (limited by substantial freezing and autolysis artifacts) included fibrosing granulomatous jejunitis surrounding the jejunal perforation and marked necrotizing and fibrosing granulomatous peritonitis in the surrounding mass. Multicentric necrotizing and fibrosing granulomatous lymphadenitis of the abdominal lymph nodes was severe. The tracheobronchial lymph node showed reactive hyperplasia. Additionally, mild multifocal nodular change, probably fibrosis and necrosis, was apparent within this lymph node and sampled lung. Microscopic pulmonary and thoracic lymph node involvement in mycobacterial disease was therefore suspected but remained inconclusive.

In histologic sections, AFBs were detected in low number in the mesenteric lymph node, as rare organisms within the mesenteric mass, and as rare organisms within chronically inflamed jejunal submucosa at and adjacent to the perforation site. Organisms were not detected in thoracic tissues.

Mycobacterium tuberculosis complex, subsequently identified as *M. pinnipedii*, was isolated from a mesenteric lymph node sample. The pathogen was identified by the

Xpert MTB/RIF DNA assay (Cepheid, Sunnyvale, California, USA) culture was positive after 3-wk use of the Bactec MGIT 960 automated broth-based system (Becton Dickinson, Cockeysville, Maryland, USA), and the isolate grew on Lowenstein Jensen media (Biomérieux Australia Pty. Ltd., Baulkham Hills, New South Wales, Australia) containing glycerol and pyruvate (SA Pathology, Adelaide, South Australia, Australia). Nitrate reduction testing was not performed on the isolate, which was susceptible to streptomycin, isoniazid, rifampicin, ethambutol, and pyrazinamide. The isolate was positive for the MPT64 antigen by a rapid immunochromatographic identification test (SD Bioline TB Ag MPT64, Alere, Waltham, Massachusetts, USA). Molecular typing to *M. pinnipedii* used mycobacterial interspersed repetitive-unit-variable number tandem repeat (MIRU-VNTR) typing with 12 loci, followed by analysis on the MIRU-VNTR plus website (Allix-Béguec et al. 2008); 12-locus MIRU analysis was conducted (LabPlus, Auckland City Hospital, New Zealand) and compared with two profiles from otariids supplied from the Victorian Infectious Diseases Reference Laboratory (Melbourne, Australia).

While suspicion of microscopic pathology within the thorax existed in the present case, gross lesions were not seen, which is uncommon but not unprecedented in tuberculous disease in pinnipeds. In the Western Australian case series, nodular pulmonary and pleural disease predominated in the six culture-positive animals, although lesions were limited to the liver and its draining hepatic lymph node in one of these animals (Forshaw and Phelps 1991). In our case, detection of intralesional AFBs in the intestine, together with limited extra-abdominal disease, suggested primary alimentary infection with ingestion as the route of infection and a potential for fecal shedding, as previously asserted (Forshaw and Phelps 1991). More commonly, pulmonary disease in pinnipeds results from inhalation, with subsequent bacterial shedding in airway secretions (Cousins et al. 1993). In aquarium and rehabilitation facilities, mucosal secretions, feces, and urine

have been suggested as potential sources of zoonotic infection (Thompson et al. 1993; Waltzek et al. 2012).

Microchipping data confirmed the subject of this report as a 3-yr-old (juvenile) Seal Bay born male sea lion; its young age was consistent with previous reporting of tuberculosis in pinnipeds. In the Western Australian cases of tuberculosis in Australian sea lions, the free-ranging animals were subadult males (approximately 7–10 yr old), with the captive animals 5–14 yr old and three of the latter four animals being 5 yr old or younger. Even for relatively gregarious pinniped species, disease transmission is more likely to occur during animal congregation observed during the breeding season. Preferential involvement of young males, as observed in reports from the Australian sea lion, has been attributed to a tendency for this age and sex cohort to congregate before maturation to breeding age (Cousins et al. 1993).

A recent report of disseminated tuberculous disease in a 3-yr-old New Zealand sea lion described multifocal granulomatous enteritis and bronchopneumonia, with AFBs detected in intestine and lung (Chatterton et al. 2020). By comparison, gross enteric disease in our case was limited to one site, speculatively beginning as focal granulomatous enteritis (supported by detection of intralesional AFBs) with subsequent perforation and mesenteric reaction contributing to the gross observations. It is also possible that the suspected small foci of pulmonary pathology represented a comparatively early stage of subsequent disease within the lung.

As with other *M. tuberculosis* complex members, *M. pinnipedii* infection can spill over into nonpinniped mammalian species, most significantly to humans working with infected marine mammals in zoologic collections and marine parks (Thompson et al. 1993). Less well considered than this occupational disease risk to individuals working with captive animals is the risk to individuals briefly or infrequent exposed to infection. At risk individuals include those involved in animal rescue or washed-up carcass disposal, those working at rehabilitation centers, and those

involved in disease diagnosis. The young age of infected animals and clinical signs that can be absent, nonspecific, or difficult to detect warrant consideration of zoonotic risk when handling any pinniped (Forshaw and Phelps 1991). Reviews of antemortem testing for confirmation of tuberculosis in captive animals and in quarantine surveillance settings outline the specificity and sensitivity limitations of currently available serologic and intradermal tests (Jurczynski et al. 2012; Chatterton et al. 2020).

Individuals hauled out or washed up on the coast provide a biased sample but remain population sentinels for endemic diseases such as tuberculosis. It remains to be determined if the rare reports of tuberculosis in the Australian sea lion reflect a low level of observation and testing or a genuinely low prevalence. A review of tuberculosis in native New Zealand marine mammals found that despite an overall low prevalence (1.6%), the disease was a common cause of mortality in New Zealand sea lions on both the mainland (25%) and Enderby Island (14.6%; Roe et al. 2019). Furthermore, a comparative genomic study of *M. pinnipedii* in the South American sea lion has sequenced two isolates from different organs in the same individual, consistent with mixed-strain infection (Silva-Pereira et al. 2019). This was suggested to indicate an infection that is highly endemic within the population. Large scale opportunistic sampling and serologic testing is needed to define tuberculosis prevalence in the Australian sea lion, similar to that previously conducted in the Australian fur seal (Lynch et al. 2011).

Our report documented a novel presentation for *M. pinnipedii* infection in a marine mammal. It confirmed the presence of this disease in a juvenile male Australian sea lion of known age in South Australian waters and added to the limited reporting of disease in free-ranging animals of this and other otariid species. We recommend increased serologic surveillance as a means of determining population risk from this and other endemic diseases. Furthermore, it highlights the need for constant vigilance of zoonotic disease risk by any person in close contact with free-

ranging pinnipeds of juvenile age or older, independent of presenting signs.

The authors thank Melanie Stonnill, Anthony Maguire, and Michelle Nairn (Department of Environment and Water, South Australia) and David Stemmer (South Australian Museum, South Australia) for collection, storage, and transport of the cadaver. We greatly appreciate the description of laboratory methodology provided by Lisa Shephard from the Mycobacterium Reference Laboratory, SA Pathology.

LITERATURE CITED

- Allix-Beguec C, Harmsen D, Weniger T, Supply P, Niemann S. 2008. Evaluation and strategy for use of MIRU-VNTRplus, a multifunctional database for online analysis of genotyping data and phylogenetic identification of *Mycobacterium tuberculosis* complex isolates. *J Clin Microbiol* 46:2692–2699.
- Bastida R, Loureiro J, Quse V, Bernardelli A, Rodriguez D, Costa E. 1999. Tuberculosis in a wild subantarctic fur seal from Argentina. *J Wildl Dis* 35:796–798.
- Bernardelli A, Bastida R, Loureiro J, Michelis H, Romano MI, Cataldi A, Costa E. 1996. Tuberculosis in sea lions and fur seals from the south-western Atlantic coast. *Rev Sci Tech* 15:985–1005.
- Boardman WSJ, Shephard L, Bastian I, Globan M, Fyfe JAM, Cousins DV, Machado A, Woolford L. 2014. *Mycobacterium pinnipedii* tuberculosis in a free-ranging Australian fur seal (*Arctocephalus pusillus doriferus*) in South Australia. *J Zoo Wildl Med* 45: 970–972.
- Chatterton J, Pas A, Alexander S, Leech M, Uddstrom L, Harvey C, Masters N, Dennison S, Roe WD. 2020. Mycobacterial disease and subsequent diagnostic investigations in a group of captive pinnipeds in New Zealand. *J Zoo Wildl Med* 51:177–187.
- Cousins DV, Williams SN, Reuter R, Forshaw D, Chadwick B, Coughran D, Collins P, Gales N. 1993. Tuberculosis in wild seals and characterisation of the seal bacillus. *Aust Vet J* 70:92–97.
- Forshaw D, Phelps GR. 1991. Tuberculosis in a captive colony of pinnipeds. *J Wildl Dis* 27:288–295.
- Goldsworthy SD. 2015. *Neophoca cinerea*. In: *The International Union for Conservation of Nature red list of threatened species 2015*. e.T14549A45228341. <http://dx.doi.org/10.2305/IUCN.UK.2015-2.RLTS.T14549A45228341.en>. Accessed May 2020.
- Goldsworthy SD, McKenzie J, Shaughnessy PD, McIntosh RR, Page B, Campbell R. 2009. An update of the report: Understanding the impediments to the growth of Australian sea lion populations. SARDI Publication F2008/00847-1. *SARDI Research Report Series No. 356*. South Australia Research and Development Institute, Adelaide, Australia.
- Jurczynski K, Lyashchenko KP, Scharpegge J, Fluegger M, Lacave G, Moser I, Tortschanoff S, Greenwald R. 2012. Use of multiple diagnostic tests to detect *Mycobacterium pinnipedii* infections in a large group of South American sea lions (*Otaria flavescens*). *Aquat Mamm* 38:43–55.
- Lynch M, Nielsen O, Duignan PJ, Kirkwood R, Hoskins A, Arnould JPY. 2011. Serologic survey for potential pathogens and assessment of disease risk in Australian fur seals. *J Wildl Dis* 47:555–565.
- Roe WD, Duignan PJ, Meynier L, Lisle GD, Cousins DV. 2006. Tuberculosis in a New Zealand (Hooker's) sea lion. *N Z Vet J* 54:51.
- Roe WD, Lenting B, Kokosinska A, Hunter S, Duignan PJ, Gartrell B, Rogers L, Collins DM, De Lisle GW, Gedye K, et al. 2019. Pathology and molecular epidemiology of *Mycobacterium pinnipedii* tuberculosis in native New Zealand marine mammals. *PLoS One* 14:e0212363.
- Romano MI, Alito A, Bigi F, Fisanotti JC, Cataldi A. 1995. Genetic characterization of mycobacteria from South American wild seals. *Vet Microbiol* 47:89–98.
- Silva-Pereira TT, Ikuta CY, Zimpel CK, Camargo NCS, De Souza Filho AF, Ferreira Neto JS, Heinemann MB, Guimarães AMS. 2019. Genome sequencing of *Mycobacterium pinnipedii* strains: Genetic characterization and evidence of superinfection in a South American sea lion (*Otaria flavescens*). *BMC Genomics* 20:1030.
- Thompson PJ, Cousins DV, Gow BL, Collins DM, Williamson BH, Dagnia HT. 1993. Seals, seal trainers, and mycobacterial infection. *Am Rev Respir Dis Crit Care* 147:164–167.
- Waltzek TB, Cortés-Hinojosa G, Wellehan JFX Jr, Gray GC. 2012. Marine mammal zoonoses: A review of disease manifestations. *Zoonoses Public Health* 59: 521–535.
- Woods R, Cousins DV, Kirkwood R, Obendorf DL. 1995. Tuberculosis in a wild Australian fur seal (*Arctocephalus pusillus doriferus*) from Tasmania. *J Wildl Dis* 31:83–86.

Submitted for publication 11 June 2020.

Accepted 30 July 2020.



FIGURE 1. Entrapment of jejunum (white arrowhead) by a smooth-surfaced and friable fibrous mesenteric mass in a free-ranging Australian sea lion (*Neophoca cinerea*) secondary to *Mycobacterium pinnipedii* infection. An absence of mesenteric adipose tissue (black arrowhead) and large volume (2-L) peritoneal effusion (black arrow) were also noted. Bar = 15 mm.

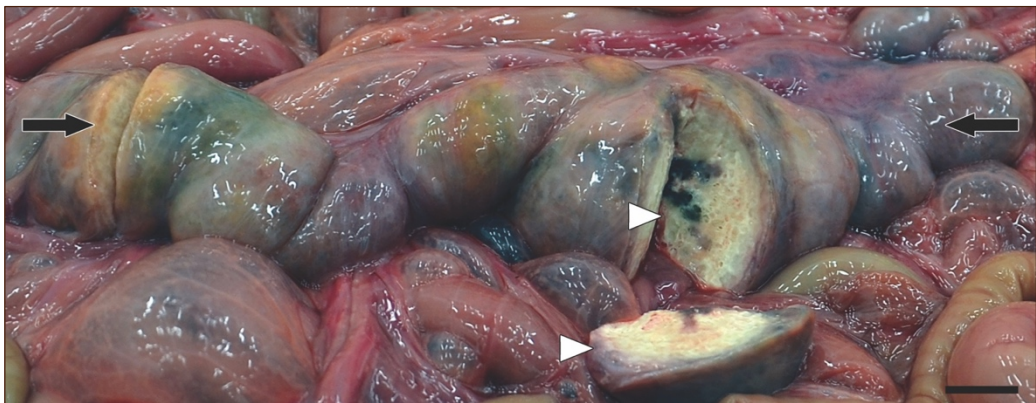


FIGURE 2. Marked mesenteric lymphadenomegaly (black arrows) from fibrosing granulomatous lymphadenitis (white arrow heads) in a free-ranging Australian sea lion (*Neophoca cinerea*) secondary to *Mycobacterium pinnipedii* infection. Bar = 10 mm.

END THESIS

Dangerous Reef, Spencer Gulf, SA

